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Single Molecule Fluorescence of Native and Refolded Peridinin–Chlorophyll–Protein Complexes

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Abstract Single molecule spectroscopy was applied to study the optical properties of native and refolded peridinin–chlorophyll–protein (PCP) complexes. The native system is a trimer with six chlorophyll *a* (Chl *a*) molecules, while the refolded one contains two Chl *a* and resembles structurally and spectroscopically the PCP monomer. The fluorescence emission of single PCP complexes strongly broadens with increasing excitation power. Simultaneously, the distribution of fluorescence maximum frequencies is also broadened. These spectral changes are attributed to photoinduced conformational changes of the protein that influence the fluorescence of embedded chromophores. Comparison of fluorescence intensities measured for PCP complexes in two different solvents indicates that the native PCP trimers are preserved in EDTA Tris buffer, while in PVA polymer matrix only monomers are stable.

Keywords Single molecule spectroscopy · Peridinin–chlorophyll–protein · Protein dynamics · Reconstitution

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Introduction

Proteins are extremely complex systems characterized by rugged potential energy landscapes with multiple barriers separating potential energy valleys associated with different conformational substates [1]. Transitions between protein substates are usually temperature driven but can also be photo-induced through the thermal energy released via radiationless deactivation channels after absorption of light. Pigment–protein complexes, such as light-harvesting units responsible for collecting light energy in photosynthesis, are excellent systems for studying protein dynamics. They are often composed of a protein matrix embedding carotenoid and chlorophyll (Chl) pigments [2]. In particular Chl molecules that exhibit relatively strong fluorescence emission can be considered "natural" reporters of local protein surroundings.

Intrinsic inhomogeneities of photophysical parameters of chromophores, such as fluorescence quantum yield and/or emission frequency, diminish the amount of information available in ensemble experiments. With help of single-molecule spectroscopy [3], which removes ensemble averaging and exploits statistical information obtained for many individual objects, both protein-pigment as well as pigment–pigment interactions in light-harvesting systems can be studied in much greater detail. Experimental studies carried out recently on bacterial light-harvesting complex 2 (LH2) have shown that protein conformational motions can strongly modulate fluorescence spectra of embedded chromophores [4, 5]. However, the excitonic character of LH2 emission, which is due to strongly coupled 18 bacterio-Chl molecules, makes the theoretical modelling quite complex [6] and depending on many arbitrary parameters.

In this work we apply single molecule spectroscopy to gain insight into pigment–pigment and protein–pigment

interactions in a much simpler light-harvesting complex, peridinin–chlorophyll–protein (PCP) from the dinoflagellate, *Amphidinium carterae.* The crystallographic structure of native PCP has been resolved to 2 Å resolution by Hofmann et al. [7]. The native PCP complex (that is also believed to be the in vivo form) is a trimer with each of the three monomeric subunits containing two chlorophyll *a* (Chl *a*) and eight peridinin (Per) molecules embedded in a hydrophobic cavity formed by the protein. The pigments are closely packed and organized into two almost symmetric clusters with Pers in van der Waals contact to the tetrapyrrole ring of Chl *a*. The center-to-center distance between the Chls *a* in the monomer is 17.4 Å. As demonstrated by fluorescence anisotropy [8] and single molecule spectroscopy experiments [9, 10], in contrast to LH2 complex, the fluorescing Chls are weakly interacting through Förster energy transfer [11] and do not form an exciton. The N-terminal domain protein of PCP (N-PCP) can be reconstituted with various Chl mixtures [12, 13], opening a way to control both the pigment composition and the intra-Chl energy transfer [14–16]. The complexes refolded with Chl *a* ((Chl *a*)$_2$-N-PCP) exhibit similar spectroscopic properties to monomers of native PCP [10].

The results obtained for both native PCP and (Chl *a*)$_2$-N-PCP show that the spectral properties of Chl molecules embedded in protein depend dramatically upon the excitation power. For both systems we observe a large increase of the fluorescence linewidth and broadening of the distribution of emission maxima with increasing excitation power. We attribute these changes to photoinduced structural dynamics of the protein. The analysis of fluorescence intensity and polarization traces measured for individual complexes in Tris EDTA buffer and PVA polymer matrix indicates that the trimers of native PCP are stable in Tris EDTA, while in the polymer matrix only monomers are present.

Materials and methods

Native and reconstituted complexes

Native PCP was isolated from *A. carterae*, and N-PCP was produced in *Escherichia coli*, and purified according to Hofmann et al. [7]. The reconstitution of N-PCP with Chl *a* followed the protocol of Miller et al. [12]. First, 625 µl N-terminal domain apoprotein of PCP (2 µM) was combined with 225 µl Tris buffer (25 mM, pH 7.6) containing KCl (10 mM). After the addition of Per (12 µmol) and Chl *a* (3 µmol) in 150 µl ethanol, the mixture was incubated at 4 °C for 48 h. 1.5 µmol of Chl *a* pigment was used. The crude reconstitution mixture was first purified on a small Sephadex G-25 (PD-10) column equilibrated with Tris buffer (5 mM, pH 7.6) containing KCl (2 mM), then on a column of diethylaminoethyl Trisacryl (Sigma, Darmstadt, Germany) using an NaCl gradient; the complexes elute with 0.1 M NaCl. The final product was desalted on Sephadex G-25 (PD-10; Biosciences, Uppsala, Sweden) and equilibrated with Tris buffer (5 mM, pH 7.6). The concentration of Chl pigments in the final reconstitution products was determined as 0.56 µM using absorbance values at the maximum of Q_X absorption of Chl *a* and the respective extinction coefficients [13].

Single molecule fluorescence

Single-molecule experiments were carried out at room temperature in Tris EDTA buffer (Fluka 93302, pH=7.4) or in 2% polyvinyl alcohol (PVA, Mowiol 40-88, Sigma-Aldrich, MW ~127,000). To obtain concentrations suitable for single-molecule studies the stock solutions of (Chl *a*)$_2$-

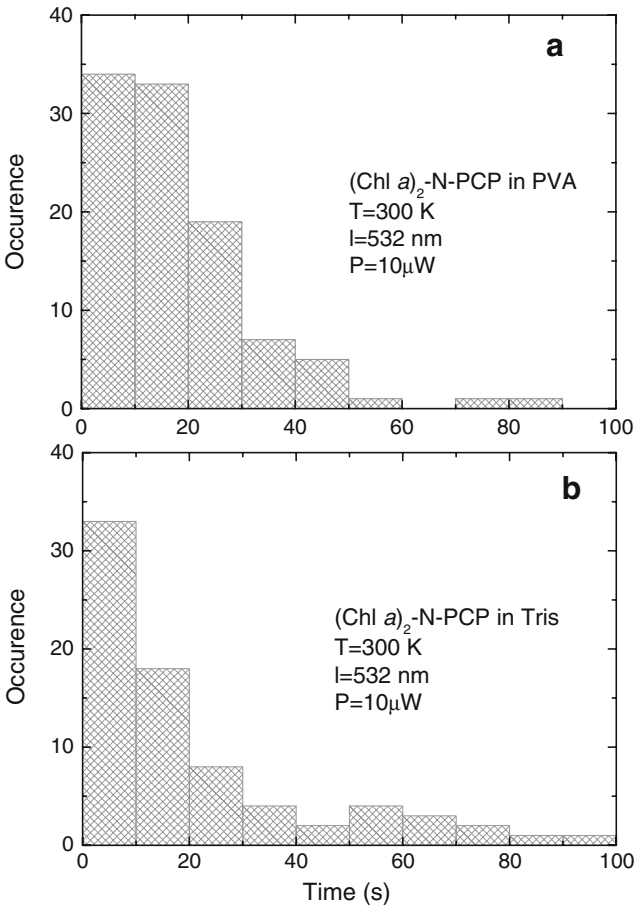

Fig. 1 Comparison of survival times measured for single reconstituted (Chl *a*)$_2$-N-PCP complexes in two different solvents: **a** EDTA Tris buffer and **b** PVA polymer matrix. The excitation power in both experiments was 10 µW

N-PCP and native PCP were diluted to pM concentrations in the respective solvent. The 20 μl of the solution was dispersed on a clean coverslip, which was then glued to a cover glass to prevent the sample from drying and rapid oxidation. Fluorescence of single PCP complexes was excited with a continuous wave Nd:YAG laser (λ= 532 nm), and the spectra were collected using a modified scanning confocal microscope (Zeiss LSM 410; Jena, Germany) equipped with a high numerical aperture oil-immersion objective (Zeiss 60× 1.4 NA). Since the excitation wavelength corresponds to the Per absorption, in this approach all Chls *a* are simultaneously and independently excited via efficient energy transfer from the Pers [2, 10]. The fluorescence emission of Chl *a* was filtered out using a dichroic mirror (575LP dichroic, AHF Analysentechnik, Postfach, Germany) and a band-pass filter (HQ655/150M, Chroma Technologies, Rockingham, VT). The signal was dispersed with an Amici prism and detected using a back-illuminated charge-coupled device camera (EEV 1300/100-EMB-chip; Princeton Instruments, Trenton, NJ). The spectral resolution of the setup was ~1.5 nm. Fluorescence spectra of single complexes were measured continuously with an acquisition time of 0.3 s, until complete photo-bleaching.

Polarization-resolved single molecule spectroscopy

Polarization-resolved single-molecule experiments were performed on a setup based on an Eclipse TE200 (Nikon) epifluorescence microscope with a high numerical aperture oil-immersion objective (Nikon Plan Apo 100/1.40 N.A. Oil). The molecules were excited at 532 nm with a diode-pumped solid state laser (Samba, Cobolt AB, Stockholm, Sweden), and the fluorescence was detected with a back-illuminated EM-CCD camera in frame transfer mode (Andor iXon DV897, 512×512 pixels). Incident laser light was blocked by a dichroic mirror (640 nm cutoff, AHF Analysentechnik, Postfach, Germany) and a bandpass filter (670/10, Chroma Technologies, Rockingham, VT). Excitation power was ~1 mW before the microscope objective. A Wallaston prism was attached in front of the CCD camera to split the signal into two parallel and perpendicular polarization channels that were imaged onto separated regions of the CCD chip.

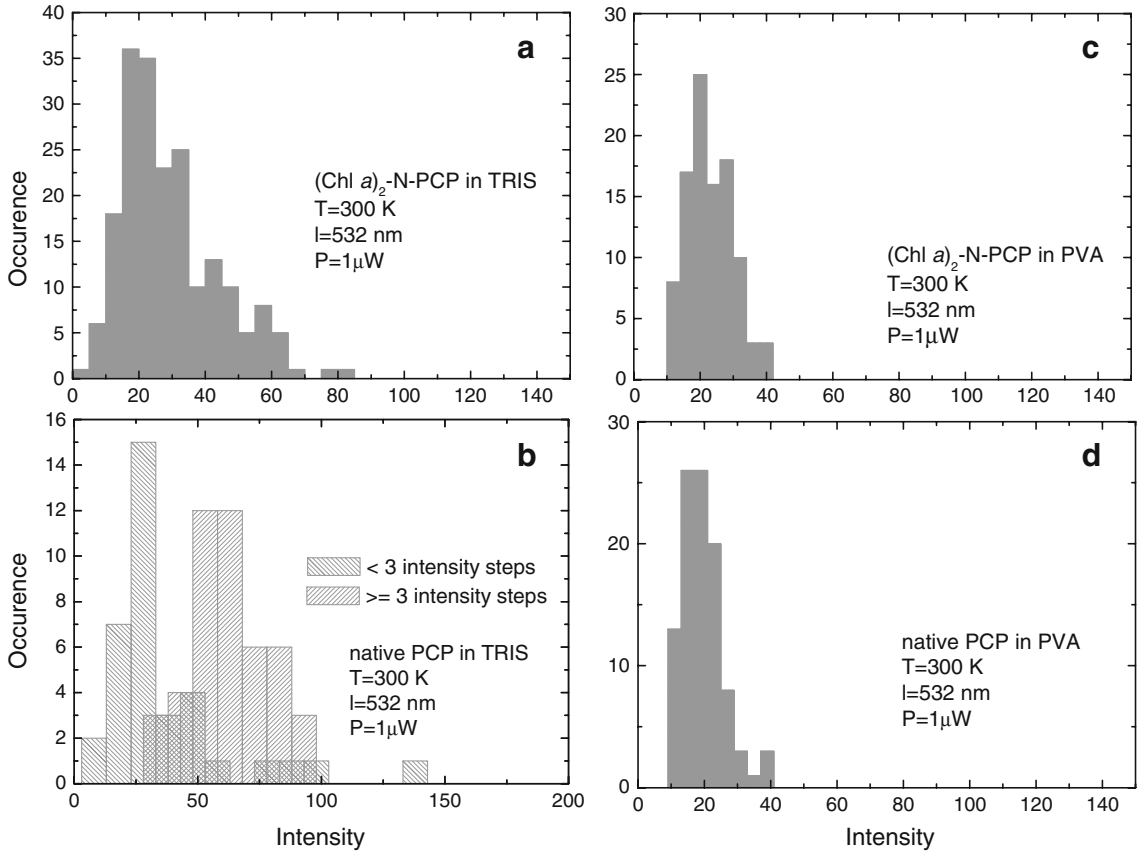

Fig. 2 Histograms of fluorescence emission intensities for **a** (Chl *a*)$_2$-N-PCP and **b** native PCP in Tris buffer and **c** (Chl *a*)$_2$-N-PCP and **d** native PCP in PVA. In the case of native PCP in Tris buffer the subsets of complexes showing <3 (*right shading*) and ≥3 (*left shading*) intensity steps are distinguished

Results and discussion

It has been shown previously [10] that the fluorescence of both native and reconstituted PCP complexes in Tris EDTA buffer is quite stable and features almost no blinking. Therefore, the fluorescence trajectories can be frequently followed for tens of seconds. A qualitatively similar result is obtained for PCP complexes in PVA matrix. In Fig. 1 we compare the photobleaching time measured for native PCP complexes in PVA (Fig. 1a) and Tris EDTA (Fig. 1b). The excitation wavelength of 532 nm and the laser power of 10 μW were used in both measurements. Average survival times measured for PCP complexes in Tris EDTA and PVA matrixes are 22±3 and 26±3 s, respectively. The slight increase of the survival times for polymer matrix, observed also for reconstituted (Chl *a*)$_2$-N-PCP complexes, may be due to better protection against oxygen, which is the major quencher of the Chl fluorescence in light-harvesting systems [17].

The native PCP complex contains three times as many Chls as the reconstituted (Chl *a*)$_2$-N-PCP. Since all these Chls are chemically identical the average fluorescence intensity measured for single native complexes should be larger than for (Chl *a*)$_2$-N-PCP. In Fig. 2 we display histograms of maximum fluorescence intensities collected for over hundred single (a) (Chl *a*)$_2$-N-PCP and (b) native PCP complexes in Tris EDTA buffer. All the spectra were taken with 0.3 s acquisition time. In the case of (Chl *a*)$_2$-N-PCP complexes the distribution features a single relatively broad peak extending slightly toward higher intensities. In contrast, the distribution obtained for native PCP shows two clear peaks of the fluorescence intensity. The lower peak coincides with the one observed for (Chl *a*)$_2$-N-PCP and it is due to PCP monomers. The second one appears at about 2.5 times higher intensity. We attribute this maximum to the fluorescence of PCP complexes, which maintain the trimeric form that contains 6 Chl *a* molecules, three times more that PCP monomers. This result suggests that in Tris EDTA buffer both monomeric and trimeric PCP complexes seem to be stable and coexist in the mixture [7, 8]. The peak intensity of the native PCP complexes is not three times higher as compared to the signal measured for the monomers due to fixed geometry of the trimer [7], that results in unequal contributions of each of the Chl *a* molecules to the total fluorescence intensity measured for the complex. Analogous measurements performed for the reconstituted and native PCP complexes in PVA matrix are displayed in Fig. 2c and d, respectively. In a clear contrast to the results obtained in Tris EDTA buffer, in both cases the maximum fluorescence intensity features only a single peak at the intensity corresponding to monomeric PCP. This suggests that while in Tris EDTA buffer there is equilibrium between monomers and trimers of PCP, in the polymer PVA matrix only monomers are stable.

Supporting evidence for the presence of trimeric PCP complexes in Tris EDTA buffer can be obtained from an analysis of fluorescence intensity steps measured for individual complexes [10]. The photobleaching of reconstituted (Chl *a*)$_2$-N-PCP, which contains two Chl *a* molecules, occurs in two steps that are due to subsequent bleaching of the two Chls within the monomer [10]. Remarkably, the bleaching of the first Chl *a* affects neither the fluorescence intensity nor the emission energy of the remaining Chl, which points towards very weak dipole–dipole coupling between the two Chls *a*. The implication of the weak coupling between Chls is that by exciting a PCP complex into Per absorption one can independently monitor the fluorescence of every Chl present in the complex. In contrast to reconstituted (Chl *a*)$_2$-N-PCP, the fluorescence trajectories collected for native PCP featured frequently more that two steps. Indeed, in many cases the photobleaching occurs in the sequence of four to six intensity steps [10]. We use the information about the intensity steps measured for native PCP complexes and correlate it with

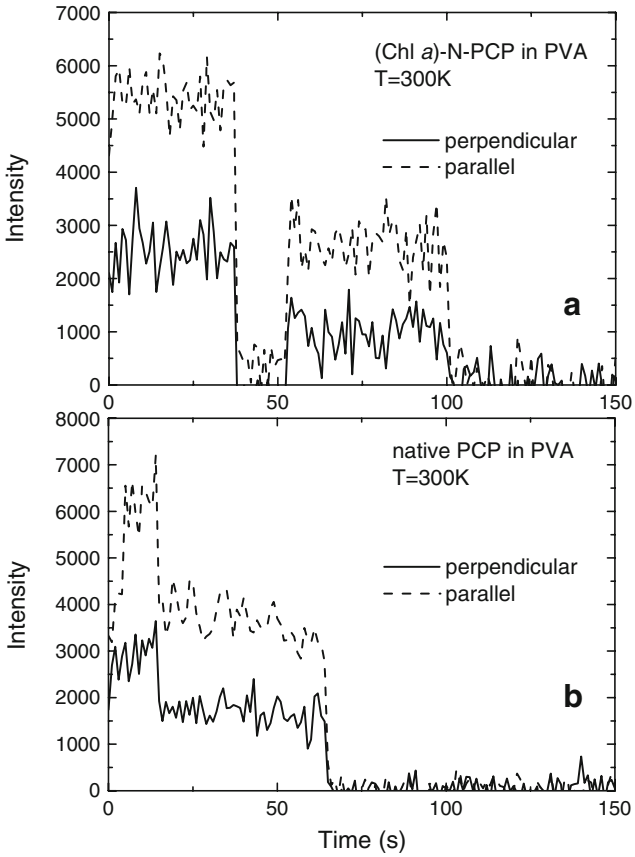

Fig. 3 Polarization-resolved fluorescence trajectories measured for **a** (Chl *a*)$_2$-N-PCP and **b** native PCP in PVA matrix. *Solid* and *dashed lines* correspond to two orthogonal polarizations

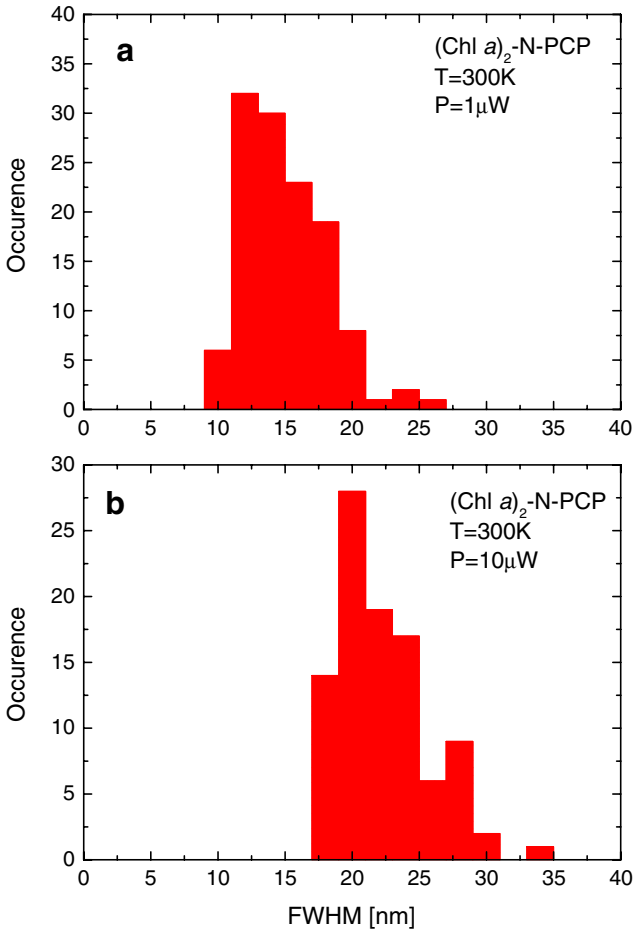

Fig. 4 Distribution of fluorescence linewidths measured for single reconstituted (Chl a)$_2$-N-PCP complexes with excitation powers of **a** 1 μW and **b** 10 μW

the total intensity of the fluorescence, as displayed in Fig. 2b. The PCP complexes showing less than three intensity steps (attributed to monomers) are marked with right shading, while complexes showing more or equal to three intensity steps (attributed to trimers) are marked with left shading. As can be seen, there is a clear correlation between the total intensity and the number of bleaching steps observed for the native PCP complexes. Namely, the complexes featuring three intensity steps or more are grouped around high intensity maximum seen in Fig. 2b. The distinct separation between the two distributions indicates that both monomeric and trimeric PCP complexes are present in the sample prepared with Tris EDTA buffer. Since no trimeric PCP was present in the PVA matrix, the stability of trimers depends on the environment. Ultracentrifugation experiments carried out by Hofmann et al. [7] have shown that the equilibrium between monomers and trimers depends on the protein concentration: at low concentrations monomers are more stable, whereas higher concentrations favor trimers. Moreover, it has been postulated that slightly acidic surroundings are preferable for forming PCP trimers. The results obtained for PCP complexes in PVA matrix are in good agreement with these conclusions. The combination of a neutral environment and low protein concentration (typically a few pM) changes the equilibrium such that only monomers are stable. However, in the case of Tris EDTA buffer, at slightly basic conditions (pH=7.4), PCP trimers can be stabilized even at low concentrations.

Additional evidence supporting the presence of monomers in PVA can be obtained from polarization-resolved measurements, where both the intensity and the polarization are monitored as a function of time. In Fig. 3 we show polarization dependent time traces of fluorescence intensities obtained for (a) (Chl a)$_2$-N-PCP in PVA and (b) native PCP in PVA in two orthogonal polarizations. In both cases, a two-step bleaching behaviour is observed [10], demonstrating the presence of two emitting Chl a molecules. A polarization trace where the emission of individual chromophores can be clearly identified indicates that the molecules are immobilized in the PVA matrix and thus each Chl in PCP can be represented by its electric dipole

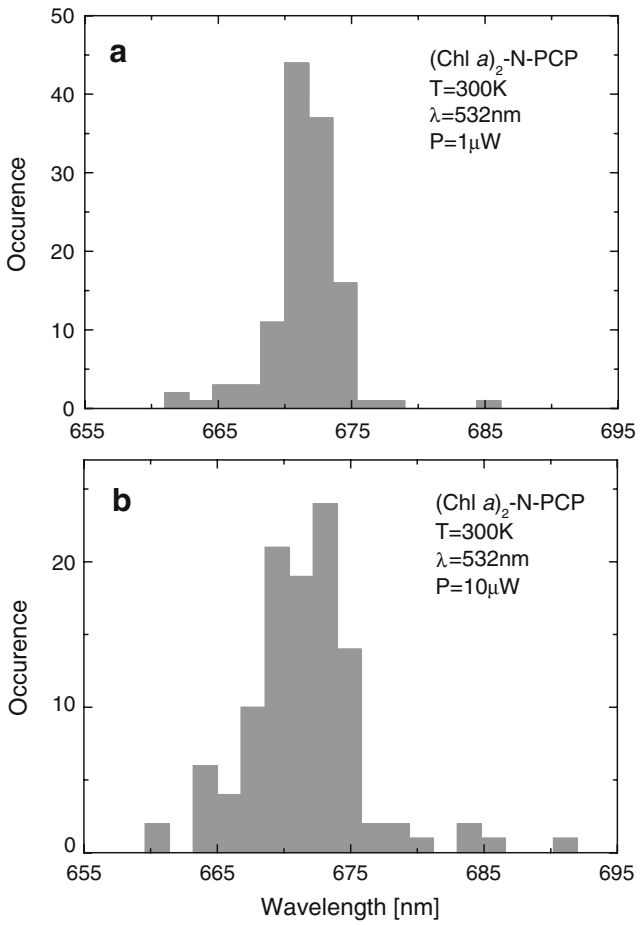

Fig. 5 Fluorescence energy distributions measured for single (Chl a)$_2$-N-PCP with excitation powers of **a** 1 μW and **b** 10 μW

with the overall emission properties defined by their superposition. It is important to note that for all native PCP complexes in PVA matrix the fluorescence, and thus polarization, decays in two steps which confirms that under these conditions only PCP monomers are stable.

The relatively simple structure of the PCP renders this light-harvesting complex an interesting system for studying protein–pigment interactions by monitoring the fluorescence of weakly interacting Chl *a* molecules. The spectral changes of fluorescence linewidth and emission energy observed in such a system can be attributed to photo-induced changes in protein conformational dynamics. Previous experiments carried out on LH2 have shown that such changes should depend on the excitation power [5, 6]. In Fig. 4 we show the distribution of fluorescence linewidths measured for single (Chl $a)_2$-N-PCP complexes at two different excitation powers of 1 μW and 10 μW. The experiment was done in Tris EDTA buffer with the acquisition time of 0.3 s. Although for both excitation powers the distribution has a comparable width, the average value of the linewidth is much larger at higher laser power (21 nm at 10 μW vs 12 nm at 1 μW). We observe similar behavior for native PCP complexes, where the average values of the fluorescence linewidth of 23 and 14 nm have been measured at 10 μW and 1 μW, respectively (not shown). The slightly higher linewidths in the case of native PCP complexes can be due to the presence of a larger number of Chl *a* molecules, which would result in stronger inhomogeneous broadening of the emission. The increase of the fluorescence linewidth with the excitation power is accompanied by a strong broadening of the distribution of fluorescence maximum energies measured for individual PCP complexes. The result is displayed in Fig. 5 where we compare the fluorescence maximum energies measured for many single (Chl $a)_2$-N-PCP complexes at the excitation powers of (a) 1 μW and (b) 10 μW. The average wavelength of about 672 nm does not change with the laser intensity. Remarkably, the histogram obtained for the laser power of 1 μW is rather narrow (SD=2 nm) with the width of the distribution increasing up to 4.4 nm for the molecules excited with 10 μW. The broadening is more prominent towards lower wavelengths, similar to results obtained for LH2 complex [6].

The increase of the fluorescence linewidth and broadening of the maximum emission energy distribution upon raising the excitation power is a signature of photo-induced spectral dynamics of the protein. The conformational motions of the protein in PCP that can alter the spectral properties of Chls *a* have been recently considered theoretically [18] using molecular dynamics simulations. Structural changes of the protein may induce significant fluctuations of the absorption of the Chl molecules. In the fluorescence experiment, continuous irradiation by the laser drives the molecules into conformational substates that are not accessible under ambient conditions or at low-intensity laser exposure. Higher excitation powers could induce larger and more frequent structural motions. We note that the broadening of the fluorescence emission points towards dynamics that occurs on a time scale much shorter that the acquisition time used in the experiment (0.3 s). On the other hand, the increase of energy distribution for high excitation power is an indication for structural changes that take place on a time scale comparable with the acquisition time and hence can be monitored by collecting sequences of fluorescence spectra [9].

Summary

We demonstrate that upon increasing excitation power the fluorescence of single peridinin–chlorophyll–protein complexes exhibits signatures of photoinduced conformational dynamics of the protein. Strong increase of the average linewidth of the emission is attributed to structural changes that occur on a timescale much shorter that the subsecond acquisition time. On the other hand, the broadening of the fluorescence maximum frequency distribution is due to structural changes taking place on a timescale comparable to the acquisition time. Comparison of single molecule spectroscopy results obtained for PCP complexes in two different solvents indicates that PCP trimers are stable in Tris EDTA buffer, in PVA polymer matrix they dissociate and only monomers can be detected.

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