Energy Transfer in Reconstituted Peridinin-Chlorophyll-Protein Complexes: Ensemble and Single-Molecule Spectroscopy Studies

Sebastian Mackowski,* Stephan Wörmke,* Tatas H. P. Brotosudarmo,*† Christophe Jung,* Roger G. Hiller,‡ Hugo Scheer,† and Christoph Bräuchle*

*Department of Chemistry and Biochemistry and Center for Nanoscience, Ludwig Maximilian University, D-81377 Munich, Germany; †Department of Biology 1, Ludwig Maximilian University, D-80638 Munich, Germany; and ‡Biology Department, Division of Environmental and Life Sciences, Macquarie University, North Ryde, NSW 2109, Australia

ABSTRACT We combine ensemble and single-molecule spectroscopy to gain insight into the energy transfer between chlorophylls (Chls) in peridinin-chlorophyll-protein (PCP) complexes reconstituted with Chl *a*, Chl *b*, as well as both Chl *a* and Chl *b*. The main focus is the heterochlorophyllous system (Chl *a*/*b*-N-PCP), and reference information essential to interpret experimental observations is obtained from homochlorophyllous complexes. Energy transfer between Chls in Chl *a*/*b*-N-PCP takes place from Chl *b* to Chl *a* and also from Chl *a* to Chl *b* with comparable Förster energy transfer rates of 0.0324 and 0.0215 ps^{-1}, respectively. Monte Carlo simulations yield the ratio of 39:61 for the excitation distribution between Chl *a* and Chl *b*, which is larger than the equilibrium distribution of 34:66. An average Chl *a*/Chl *b* fluorescence intensity ratio of 66:34 is measured, however, for single Chl *a*/*b*-N-PCP complexes excited into the peridinin (Per) absorption. This difference is attributed to almost three times more efficient energy transfer from Per to Chl *a* than to Chl *b*. The results indicate also that due to bilateral energy transfer, the Chl system equilibrates only partially during the excited state lifetimes.

INTRODUCTION

High-resolution structures of chlorophyll (Chl)- and carotenoid-containing photosynthetic antenna complexes (1–9) have triggered numerous spectroscopic studies aimed at a detailed description of energy transfer processes and photosynthetic light-harvesting strategies (10,11). They were helped by biochemical manipulation through mutations (12–14) or reconstitutions with different pigments (15–18) that provide means to change the structural and/or spectral properties and thus determine exact functions of the components comprising these complexes.

The water-soluble peridinin-chlorophyll a-protein (PCP) from the dinoflagellate *Amphidinium carterae* is a relatively simple light-harvesting system (19). The native building block is a monomer containing two Chl *a* and eight peridinin (Per) molecules arranged into two almost identical clusters. With a center to center distance of 1.7 nm, the Chls are only weakly coupled (20). Therefore, the fluorescence of PCP has no excitonic character, and for individual complexes the emission of each of the Chls could be observed (21). Coupling between the two Chls *a* has been satisfactorily described in terms of Förster energy transfer, with a transfer rate of 0.082 ps^{-1} (22).

The N-terminal domain of PCP, viz. N-PCP, constitutes one-half of the monomer with the amino acid sequence described previously in detail by Hofmann et al. (19). It can be reconstituted with Per and various Chls (23,24), and the resulting chromoproteins dimerize to complexes whose structures and, in the case of $(Chl\ a)_2$-N-PCP, spectroscopic properties are nearly identical to those of the native PCP monomer. Also, the energy transfer between Per and various Chls in reconstituted N-PCP samples is as efficient (>90%) (25) as in the native PCP (26–29). Time-resolved absorption spectroscopy of homochlorophyllous N-PCP reconstitutions showed that the energy transfer from Per to Chl *a* is ~2.7 times more efficient than to Chl *b* and that this process can be satisfactorily described using the Förster formalism of energy transfer (30). Heterochlorophyllous N-PCP complexes can be obtained by reconstitution with mixtures of Chls (23,24). Since N-PCP monomers containing two different Chl molecules are characterized by relatively narrow fluorescence and absorption spectra, they represent a very attractive and simple biological system for studying energy transfer processes. An inherent difficulty of this system is, however, the separation from homochlorophyllous complexes that are obtained simultaneously by the reconstitution procedure (23). This can be solved using single-molecule spectroscopy techniques, which, on the one hand, offer the way to distinguish between the structurally different complexes and on the other hand have been widely applied for studying photophysics and energy transfer processes in other light-harvesting systems (31–37).

In this work we combine ensemble with single-molecule spectroscopy to study PCP complexes reconstituted with Chl *a* ($(Chl\ a)_2$-N-PCP), with Chl *b* ($(Chl\ b)_2$-N-PCP), as well as with both Chl *a* and Chl *b*. Fluorescence excitation spectra of the N-PCP reconstituted with both Chl *a* and Chl *b* reveal that the energy transfer occurs bilaterally between Chl *a*

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Address reprint requests to Christoph Bräuchle, Dept. of Chemistry and Biochemistry and Center for Nanoscience (CeNS), Ludwig Maximilian University, Butenandtstrasse 11, D-81377 Munich, Germany. E-mail: christoph.braeuchle@cup.uni-muenchen.de.

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and Chl *b* with comparable efficiencies. However, any quantitative analysis is impossible due to the presence of homochlorophyllous ((Chl *a*)$_2$-N-PCP and (Chl *b*)$_2$-N-PCP)) species in the solution (21,24). On the other hand, single-molecule spectroscopy provides a way to distinguish between homo- and heterochlorophyllous complexes in the mixture and allows us to resolve contributions to the fluorescence intensity originating from individual Chls comprising the heterochlorophyllous Chl *a*/*b*-N-PCP complex. The average ratio of Chl *a*/Chl *b* intensity for single Chl *a*/*b*-N-PCP complexes is found to be 1.9 $\pm$ 0.2. This finding is reproduced with Monte Carlo simulations performed with the assumption of 2.96 times more efficient energy transfer from Per to Chl *a* than from Per to Chl *b*, which agrees quite well with the ratio of 2.7 measured recently for pure homochlorophyllous N-PCP samples (30). On the other hand, for the fully equilibrated Chl *a*-Chl *b* system the efficiency ratio of 3.7 is obtained, which strongly suggests that the system of two Chl molecules in the Chl *a*/*b*-N-PCP complex does not reach the equilibrium during the excited state lifetimes.

MATERIALS AND METHODS

N-terminal domain protein of native PCP from *A. carterae* was produced in *Escherichia coli* and purified according to Hofmann et al. (19). Three samples were prepared following the reconstitution protocol of Miller et al. (23): PCP reconstituted with Chl *a*, with Chl *b*, as well as with Chl *a* and Chl *b*. 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* (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 Chl pigment was used. The crude reconstitution mixtures were 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 using absorbance values at the maximum of Q_X absorption of Chl *a* and Chl *b* and the respective extinction coefficients (24). The values of 0.56 μM/l and 0.72 μM/l for (Chl *a*)$_2$-N-PCP and (Chl *b*)$_2$-N-PCP preparations have been obtained, whereas for N-PCP reconstituted with both Chl *a* and Chl *b* sample, the concentrations of Chl *a* and Chl *b* were 2.4 μM/l and 3.2 μM/l, respectively. The difference in the concentration of Chl *a* and Chl *b* points toward higher affinities for the Chl *b* (24). The incorporation efficiency for various Chl molecules in N-PCP is currently being investigated in detail by analytical methods.

Ensemble fluorescence excitation and emission measurements were performed with an F900 fluorimeter (Edinburgh Analytical Instruments, Livingston, UK). Emission spectra of all reconstituted complexes were obtained with the excitation wavelength of 532 nm, which corresponds to the Per absorption and which was later used in single-molecule spectroscopy experiments. For the excitation spectra, the detection energies were fixed at the maximum of the Chl emission, and the excitation wavelengths were scanned from 600 to 700 nm. The same setup was used to measure fluorescence decay times for (Chl *a*)$_2$-N-PCP and (Chl *b*)$_2$-N-PCP preparations by time-correlated single-photon counting. Samples were excited at 500 nm by an nF900 nanosecond flash-lamp (pulse width $<$0.7 ns) with a repetition rate of 20 kHz. The signal was detected with a multichannel analyzer, so that the histogram of time delays between ''start'' and ''stop'' events was obtained. Decay times were obtained by deconvoluting the data and the instrument response function (typically 2 ns) using the software of Edinburgh Instruments. The time resolution of the setup was $\sim$100 ps. The fluorescence quantum yields (Φ) of Chl *a* and Chl *b* in PCP were determined by comparing their fluorescence with that of Cy 5 dye, which has a known value of $\Phi = 28\%$, and its fluorescence maximum around 670 nm nicely corresponds to the spectral range of the Chl emission.

Single-molecule experiments were carried out at room temperature in Tris-EDTA buffer (pH = 7.4). To obtain concentrations suitable for single-molecule studies the stock solutions of (Chl *a*)$_2$-N-PCP and (Chl *b*)$_2$-N-PCP preparations were diluted by five orders of magnitude in the same buffer: the resulting concentrations were 5.6 pM/l and 7.2 pM/l, respectively. The stock solution of N-PCP reconstituted with both Chl *a* and Chl *b* was diluted to the concentration of 2.4 pM/l and 3.2 pM/l for Chl *a* and Chl *b* pigments, respectively. Then 20 μl of the solution was dispersed on a 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 neodymium-doped yttrium aluminum garnet laser ($\lambda = 532$ nm, 3 or 5 μW of excitation power), 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 40$\times$ 1.3 numerical aperture oil). Since the excitation wavelength corresponds to the Per absorption, in this approach both Chl *a* and Chl *b* in the Chl *a*/*b*-N-PCP complex are simultaneously and independently excited via efficient energy transfer from the Pers (37). The fluorescence emission of Chl *a* and Chl *b* was isolated by a dichroic mirror (575LP dichroic, AHF Analysentechnik, Postfach, Germany) and a band-pass filter (HQ655/150M, Chroma Technologies, Rockingham, VT), 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 was $\sim$1.5 nm. Fluorescence spectra of a single complex were measured continuously with an acquisition time of 0.3 s, until complete photobleaching.

RESULTS

Ensemble spectroscopy of reconstituted PCP complexes

In Fig. 1 we show the arrangement of Per and Chl molecules in the complexes. Red and blue represent Chl *a* and Chl *b*, respectively, and orange corresponds to Per. The structures were obtained by replacing Chl *a* with Chl *b* and assuming that the binding geometry of Chl *b* is identical to that of the native pigment. Ensemble absorption and circular dichroism (CD) characteristics of the complexes obtained by reconstituting N-PCP apoprotein with several Chl mixtures have been recently described and analyzed in detail (23,24). A strong CD signal observed around the Soret region (around 450 nm) has been attributed to very strong excitonic interactions between Per and Soret bands of the Chls that lead to pronounced energy transfer. It has been shown that the absorption spectrum of the N-PCP sample reconstituted with both Chl *a* and Chl *b* cannot be constructed as a simple superposition of the absorption spectra measured for (Chl *a*)$_2$-N-PCP and (Chl *b*)$_2$-N-PCP, most probably due to interaction between Chl *a* and Chl *b* (24).

Fluorescence spectra of all three systems studied in this work are presented in Fig. 2. The fluorescence spectrum of (Chl *a*)$_2$-N-PCP (Fig. 1 *a*) features a single line at 673 nm originating from the S_1-S_0 transition of Chl *a*. This emission is characterized by a monoexponential decay ($\tau = 3.7$ ns),

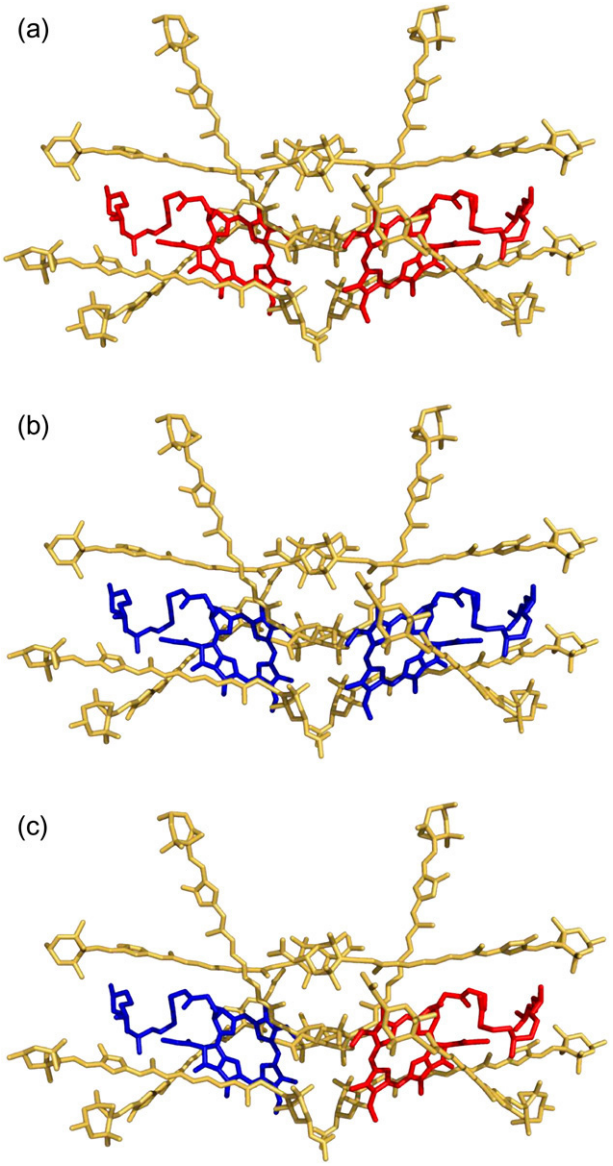

FIGURE 1 Pigment arrangement in the N-PCP reconstituted with (*a*) Chl *a*, (*b*) Chl *b*, and (*c*) both Chl *a* and Chl *b*. The sample reconstituted with both Chl *a* and Chl *b* contains all three types of complexes. Red and blue colors correspond to Chl *a* and Chl *b*, and Pers are colored in orange. The protein scaffold has been omitted for clarity.

which is shorter than the fluorescence decay time of Chl *a* emission in the native PCP complex ($\tau = 4.2$ ns (22)). This difference is most probably due to the different protein surroundings; the reconstituted PCP does not include the C-terminal domain of the native system (19). Compared to (Chl *a*)$_2$-N-PCP, the fluorescence emission of (Chl *b*)$_2$-N-PCP (Fig. 2 *a*) is blue shifted by ~20 nm (~400 cm^{-1}) to 652 nm, and the lifetime of the monoexponential decay is shortened to $\tau = 1.4$ ns. The fluorescence lifetimes obtained for both Chl pigments embedded in N-PCP are thus significantly shorter than in ether, where $\tau = 5.1$ ns and 3.9 ns were measured, respectively, for Chl *a* and Chl *b* (38).

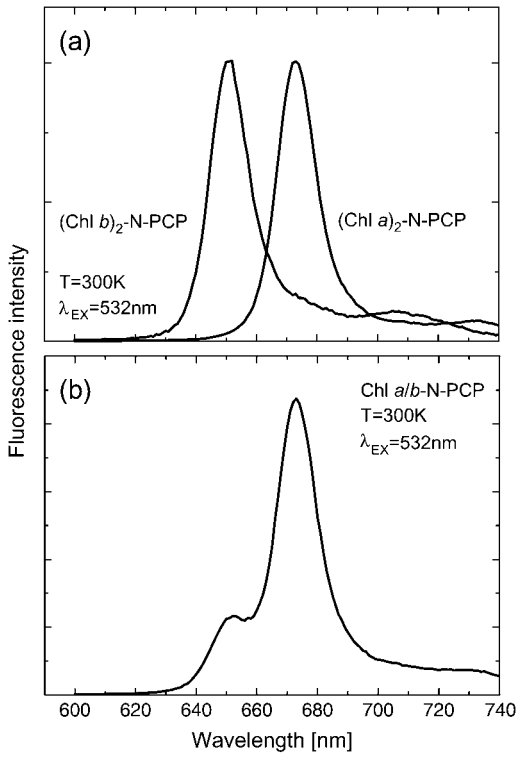

FIGURE 2 Normalized room temperature fluorescence spectra of (*a*) (Chl *a*)$_2$-N-PCP and (Chl *b*)$_2$-N-PCP, and (*b*) the N-PCP reconstituted with both Chl *a* and Chl *b*. The excitation wavelength was 532 nm.

Besides fluorescence lifetimes, fluorescence quantum yield values (Φ) are also required for estimating the rates of Förster energy transfer between the Chl molecules (39). We determined these values by comparing the fluorescence intensity measured for (Chl *a*)$_2$-N-PCP and (Chl *b*)$_2$-N-PCP with the intensity obtained for Cy 5 dye, characterized by $\Phi = 28\%$. The resulting quantum yields of Chl *a* and Chl *b* in N-PCP were $24\% \pm 2\%$ and $11\% \pm 1.5\%$, respectively.

The ensemble fluorescence spectrum of the N-PCP reconstituted with both Chl *a* and Chl *b* shows two emission lines corresponding to the two Chl pigments (Fig. 2 *b*). The fluorescence excitation spectra measured for this sample ($\lambda_{em} =$ 668 nm for Chl *a*, 647 nm for Chl *b*) are compared in Fig. 3 with analogous data obtained for the homochlorophyllous complexes. The excitation spectra of the latter (*dashed lines*) feature exclusively direct Chl *a* or Chl *b* resonances. In contrast, for the N-PCP reconstituted with both Chl *a* and Chl *b*, the Chl *a* fluorescence is strongly enhanced when Chl *b* is excited at 650 nm (*solid line* in Fig. 3 *a*). Similarly, the excitation spectrum detected at the Chl *b* emission (647 nm) features a pronounced resonance at the excitation energy matching the Chl *a* absorption at 670 nm (*solid line* Fig. 3 *b*).

This observation proves not only the presence of heterochlorophyllous complexes containing two different Chl molecules but also the occurrence of energy transfer at the Q_Y level between the two Chls. Notably, the energy transfer

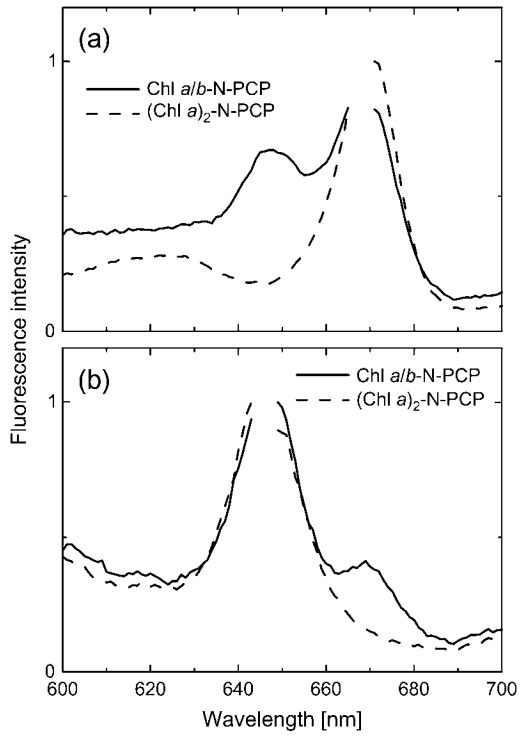

FIGURE 3 Room temperature fluorescence excitation spectra of reconstituted PCP samples. (*a*) N-PCP reconstituted with both Chl *a* and Chl *b* (*solid line*) and (Chl *a*)$_2$-N-PCP (*dashed line*) detected at 668 nm, and (*b*) N-PCP reconstituted with both Chl *a* and Chl *b* (*solid line*) and (Chl *b*)$_2$-N-PCP (*dashed line*) detected at 647 nm.

takes place not only from Chl *b* to Chl *a* but also in the energetically less preferable direction, from Chl *a* to Chl *b*. As shown below, this behavior can be satisfactorily described in terms of Förster energy transfer. However, due to the preparation method, the N-PCP reconstituted with both Chl *a* and Chl *b* contains three different types of complexes: homochlorophyllous ones, containing either two Chl *a* or two Chl *b*, or heterochlorophyllous ones (Chl *a*/*b*-N-PCP), with two different Chl molecules in the monomer. Therefore, the experiment carried out on the ensemble level could provide only limited information about processes such as energy transfer or pigment interaction in the heterochlorophyllous complex. A more precise description can be obtained using single-molecule spectroscopy, which—due to pronounced spectral differences—allows us to clearly distinguish between homo- and heterochlorophyllous complexes in the sample (24).

The ability to reconstitute PCP apoprotein with two spectrally distinguishable Chl molecules provides a way to demonstrate experimentally the influence of coupling between pigments (mainly Per) within two clusters of the monomer. Although in the case of homochlorophyllous complexes the excitation and absorption spectra are almost identical (25), the excitation spectrum of the N-PCP reconstituted with both Chl *a* and Chl *b* measured at the Chl *a* emission differs substantially from the absorption spectrum (24). To display the difference we compare the ratio of the absorption spectra measured for (Chl *a*)$_2$-N-PCP and the N-PCP reconstituted with both Chl *a* and Chl *b* with the ratio of excitation spectra measured for these two systems detected at 670 nm (Fig. 4). As can be seen, above 530 nm the curves are similar, whereas they deviate considerably at shorter wavelengths. This indicates that there is an equally good energy transfer from Per to both Chls only at $\lambda > 530$ nm, whereas this is not true at higher excitation energies. The largest difference appears at around 455 nm, which corresponds to the Soret band of Chl *b*. This observation confirms the findings of molecular dynamics simulation (40), where the coupling between the pigments of native PCP has been calculated.

The strong excitonic interaction (on the order of 100 cm^{-1}) occurs only for Per with excited state energies larger than 18,700 cm^{-1} (535 nm); this spectral region is characterized also by very strong interactions between Per and the Soret bands of Chl *a*. The coupling is significantly weaker ($\sim$10 cm^{-1}) for the two Per molecules in each cluster with excited state energies below 18,700 cm^{-1}. One may therefore expect that at lower excitation energies, the two clusters are, within a good approximation, independent of each other. A similar conclusion can also be drawn from the previous single-molecule experiments on native PCP and (Chl *a*)$_2$-N-PCP, where up to six and up to two steps, respectively, have been observed in the fluorescence intensity traces (21); they are attributed to sequential bleaching of the six and two Chl *a*, respectively, in the complexes. We note that single-molecule experiments described in the following were carried out using excitation wavelength of 532 nm, that is, the PCP complexes were excited below the threshold value. Therefore, each of the two Chl within the monomer is excited predominantly through Per molecules that are in the same cluster, and the coupling between the clusters can be neglected.

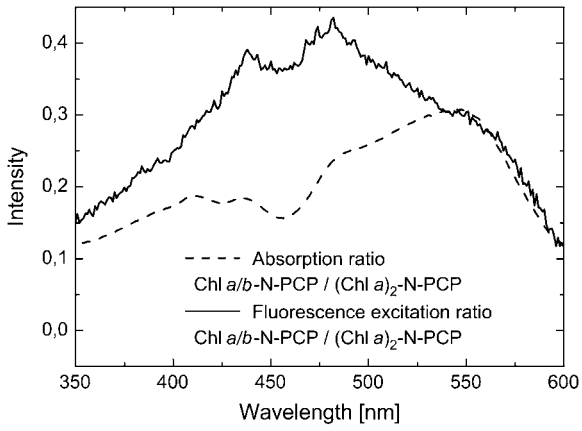

FIGURE 4 Comparison of absorption (*dashed line*) and fluorescence excitation (*solid line*) spectra of the N-PCP reconstituted with both Chl *a* and Chl *b* divided by respective spectra measured for (Chl *a*)$_2$-N-PCP.

Single-molecule spectroscopy

Previous single-molecule studies have shown that the fluorescence of PCP complexes reconstituted with Chl *a* is quite stable and can be observed for tens of seconds (21). Moreover, the photobleaching of these complexes occurs in two distinguishable steps indicating the presence of two Chl *a* molecules in a single complex. This observation agrees with the recently determined structure of PCP reconstituted with Chl *a*, which is equivalent to that of the monomer of native PCP (T. Schulte, R. G. Hiller, and E. Hofmann, unpublished results). It has also been shown that the bleaching of the first Chl does not affect the intensity of the emission attributed to the second one. Consecutive bleaching of the two Chls within the monomer has now also been measured for (Chl *b*)$_2$-N-PCP (Fig. 5 *b*) and Chl *a*/*b*-N-PCP (Fig. 5 *c*). Solid lines are the (early) spectra where both of the Chl are emitting, the dashed lines are emissions of the Chl remaining after photobleaching of the first one, and thin black lines represent the background measured after the second emitting Chl had bleached away too.

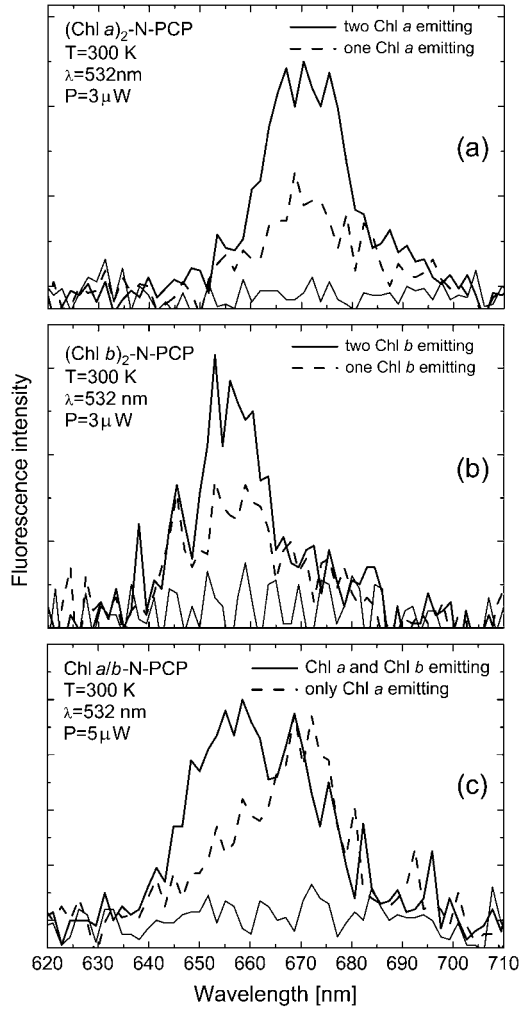

FIGURE 5 Single-molecule spectra measured at room temperature for (*a*) (Chl *a*)$_2$-N-PCP, (*b*) (Chl *b*)$_2$-N-PCP, and (*c*) Chl *a*/*b*-N-PCP. The emission of both Chl molecules in the complex is shown by solid lines, and dashed lines are the spectra where only one Chl contributes to the fluorescence emission. The excitation power was 3 μW and 5 μW for homo- and heterochlorophyllous samples, respectively.

These results demonstrate that in the Chl *a*/*b*-N-PCP complex, one can independently monitor the fluorescence of the two different Chls within the monomer, analogous to the homochlorophyllous complexes. In this regard, single-molecule experiments are extraordinarily valuable, since both of the Chls are simultaneously excited and probed in a single experiment. Yet another important advantage of investigating individual Chl *a*/*b*-N-PCP complexes is that we are able to readily distinguish homochlorophyllous complexes containing either two Chl *a* or two Chl *b* from heterochlorophyllous ones containing one Chl *a* and one Chl *b*. When discussing heterochlorophyllous Chl *a*/*b*-N-PCP in the following, only those complexes are considered that exhibit two energetically separated emission lines attributable to Chl *a* and Chl *b* emissions.

We first analyze the energy distributions of Chl emissions measured for all three types of complexes. The distributions obtained for over 120 homochlorophyllous (Chl *a*)$_2$-N-PCP (*right-hand side shading*) and (Chl *b*)$_2$-N-PCP (*left-hand side shading*) complexes (Fig. 6 *a*) correspond well to the ensemble fluorescence spectra (Fig. 2 *a*). Indeed, the maxima of the distributions observed around 672 nm for (Chl *a*)$_2$-N-PCP and 653 nm for (Chl *b*)$_2$-N-PCP agree with the fluorescence maxima of ensemble emissions (673 nm and 652 nm, respectively). In the heterochlorophyllous Chl *a*/*b*-N-PCP complexes, we take advantage of the sequential bleaching of the two Chls in the complex to determine their emission energies. The result obtained for over 100 heterochlorophyllous complexes showing both Chl *a* and Chl *b* emission is displayed in Fig. 6 *b*. The distributions of maximum emission wavelengths measured for Chl *a*/*b*-N-PCP are directly attributable to either Chl *a* or Chl *b*, thereby supporting our method of distinguishing heterochlorophyllous complexes from the homochlorophyllous ones in this sample. This correspondence validates the procedure and provides a solid basis for further analyzing the fluorescence properties of reconstituted PCP complexes.

The advantage of independently monitoring the fluorescence of the two Chl molecules within the complex is having a way to estimate the individual contributions to the emission intensity. The analysis for (Chl *a*)$_2$-N-PCP complexes that show two-step photobleaching is shown in Fig. 7. The histograms displayed in Fig. 7 *a* correspond to fluorescence intensities measured for both (*upper panel*) and only one (*lower panel*) emitting Chl *a* molecule(s). Next, by dividing the two values measured for each complex studied, we obtain a distribution of intensity ratios, as displayed in Fig. 7 *b*. Apparently, the ratio is $\sim 1 \pm 0.1$ for the majority of (Chl *a*)$_2$-N-PCP complexes, which indicates that the contributions of the two Chl *a* molecules to the fluorescence are almost

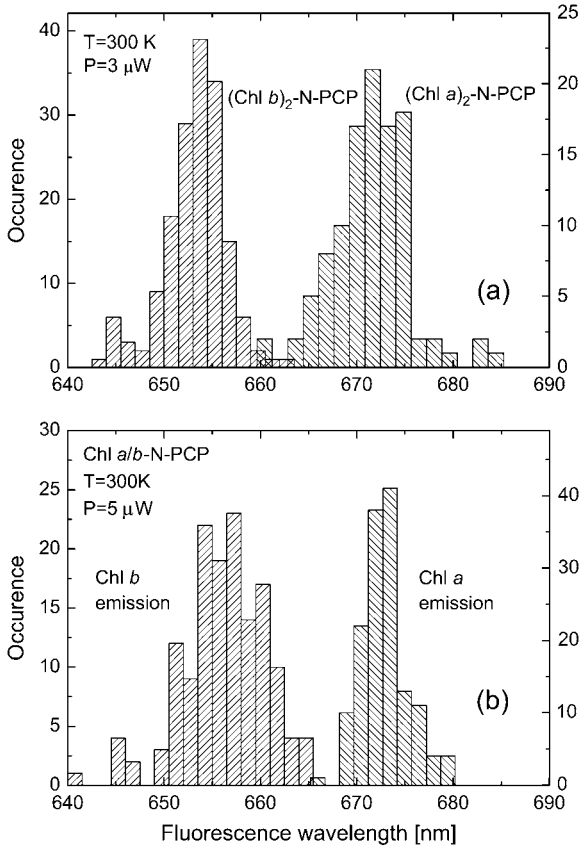

FIGURE 6 Distributions of emission wavelength measured for over 120 complexes of (*a*) homochlorophyllous (Chl $a)_2$-N-PCP (*right-hand side shading*) and (Chl $b)_2$-N-PCP (*left-hand side shading*) samples, and (*b*) heterochlorophyllous Chl *a*/*b*-N-PCP sample for complexes showing both Chl *a* and Chl *b* emissions. The values were extracted using subsequent fluorescence bleaching of the two energetically distinguishable Chl molecules. The excitation power was 3 μW and 5 μW for homo- and heterochlorophyllous samples, respectively.

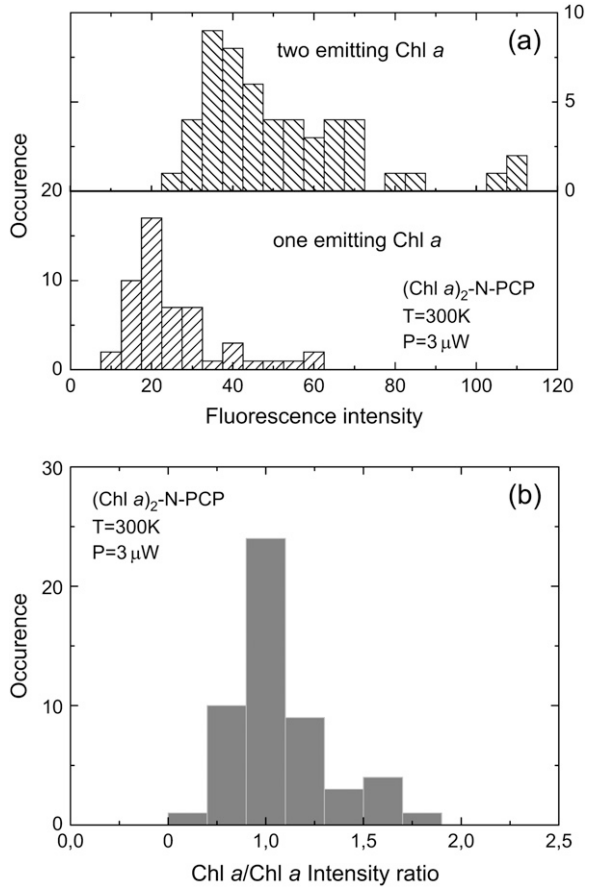

FIGURE 7 (*a*) Distributions of fluorescence intensities measured for (Chl $a)_2$-N-PCP. Upper panel (*lower panel*) displays the intensity of two (one) emitting Chl *a*. (*b*) Experimentally determined distribution of the intensity ratio between emissions of two Chl *a* and of one Chl *a* in the complexes at room temperature. The excitation power was 3 μW.

identical. Moreover, such a narrow spread of intensity ratios suggests that most of the complexes attach similarly—as far as the orientation of the transition dipole moments is concerned—to the coverslip. Similar behavior has also been observed for PCP complexes reconstituted with Chl *b*. Since the protein used in the reconstitutions with various Chl mixtures is the same, it is reasonable to assume that the complexes reconstituted with both Chl *a* and Chl *b* would also bind to the coverslip surface in a similar manner.

The results obtained for (Chl $a)_2$-N-PCP serve as a reference to the data measured for single Chl *a*/*b*-N-PCP heterochlorophyllous complexes. In the Chl *a*/*b*-N-PCP complexes, the intensities of the Chl *b* and Chl *a* emissions can be analyzed separately (Fig. 8 *a*). The intensity of the Chl *a* emission (*upper panel*) is in most cases higher than that of Chl *b* (*lower panel*), which is in qualitative agreement with the observation for homochlorophyllous complexes. However, in the case of the heterochlorophyllous system, one can directly compare the intensities obtained for Chl *a* and Chl *b* emissions in the same complex. The distribution of the Chl *a*/Chl *b* ratios measured for over 100 individual Chl *a*/*b*-N-PCP complexes is shown in Fig. 8 *b*.

For most of the complexes the ratio falls in the range between 1.4 and 1.7, with the average value equal to 1.9 $\pm$ 0.2. This value is larger than the ratio of 1.6 $\pm$ 0.4 between average intensities measured for single (Chl $a)_2$-N-PCP and (Chl $b)_2$-N-PCP complexes that implies the redistribution of the excitation between Chl *a* and Chl *b* due to the energy transfer. It is however important to note that the results displayed in Fig. 8 were obtained for complexes containing both Chl *a* and Chl *b*; therefore they are more accurate. As can be seen, the Chl *a*/Chl *b* intensity ratio measured for Chl *a*/*b*-N-PCP complexes features quite broad distribution. We tried to correlate the intensity ratio with the energy splitting between the emission lines attributed to Chl *a* and Chl *b* in a given complex. However, no correlation behavior was observed. This suggests that the fluorescence intensity of the two Chls within the complex not only depends on the energy separation between Chl *a* and Chl *b* emissions but also must

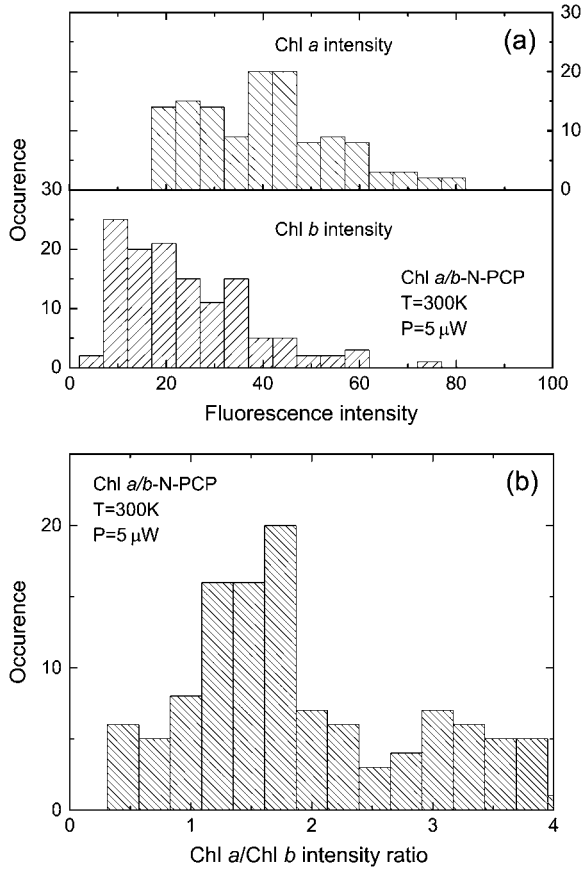

FIGURE 8 (*a*) Distributions of fluorescence intensity measured for Chl *a* (*upper panel*) and Chl *b* (*lower panel*) in Chl *a*/*b*-N-PCP complexes. (*b*) Distribution of the Chl *a*/Chl *b* fluorescence intensity ratio obtained for the same complexes. The excitation power was 5 μW.

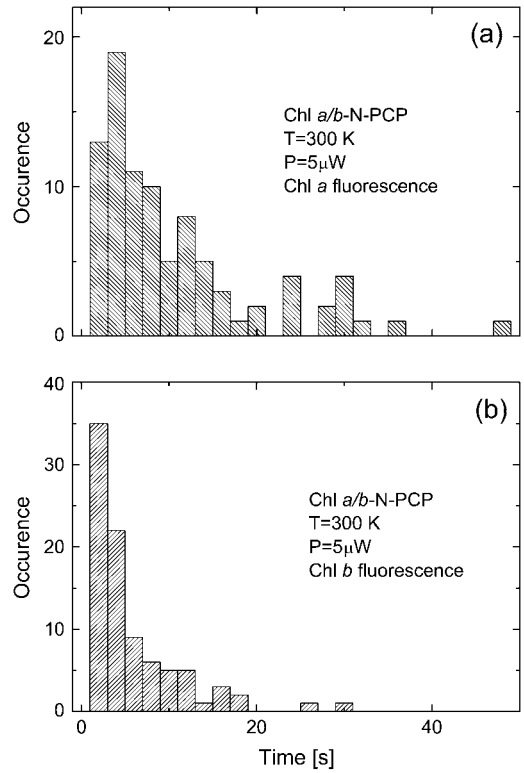

FIGURE 9 Survival times measured for single Chl *a*/*b*-N-PCP complexes. (*a*) Chl *a* fluorescence, and (*b*) Chl *b* fluorescence.

be a more complicated function of the fluorescence lifetime, quantum yield, and/or relative orientation of the Chl molecules in the complex.

Finally, we would like to comment on the average number of photons emitted by Chl *a* and Chl *b* pigments in homo- and heterochlorophyllous PCP complexes. To estimate these numbers for homochlorophyllous PCP complexes, we use distributions of survival times together with average fluorescence intensities measured for these complexes. Generally, the fluorescence of Chl *b* can be observed over much shorter times than that of Chl *a*: average survival times measured for $(\text{Chl } a)_2$-N-PCP and $(\text{Chl } b)_2$-N-PCP are equal to 7.4 ± 0.7 and 3 ± 0.5 s, respectively. A qualitatively similar behavior is seen for the heterochlorophyllous Chl *a*/*b*-N-PCP sample (Fig. 9), with average survival times estimated to be 11 ± 0.9 and 5.4 ± 0.5 s for Chl *a* and Chl *b*, respectively. However, since the experiments were performed at slightly different excitation powers, we multiply average values of survival times by average intensities of Chl *a* and Chl *b* emissions measured for respective reconstituted homochlorophyllous complexes. Estimated in this way, the ratio of the number of photons emitted by Chl *a* and Chl *b* in homochlorophyllous and heterochlorophyllous PCP complexes is equal to 3.2 and 3.4, respectively. The insignificant difference indicates that the photophysics of either of the Chl pigments is very similar in homo- and heterochlorophyllous complexes.

Modeling

To interpret the single-molecule results, we use formalism of Förster energy transfer between the Q_Y bands of the two Chls, which are separated by $R = 1.7$ nm (19). This approach has been recently applied to analyze fluorescence anisotropy measurements performed for native PCP (22). In this description, the rate of energy transfer between donor and acceptor is given by (39):

$$k_T(r) = \frac{9000(\ln 10)\kappa^2\Phi_D}{128\pi^5 N_A n^4 \tau_D R^6}\int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda,$$

where κ is an orientation factor, Φ_D and τ_D are the fluorescence quantum yield and the fluorescence lifetime of the donor, n is the refractive index of the solvent, and R is the distance between the donor and acceptor. The integral describes the overlap between the absorption spectrum of the donor and the fluorescence spectrum of the acceptor.

The Förster energy transfer rates for Chl *a* and Chl *b* were calculated using fluorescence spectra (in the wavelength

scale) of (Chl a)$_2$-N-PCP and (Chl b)$_2$-N-PCP normalized to unity area, and the absorption spectra of these two complexes normalized to their respective extinction coefficients. Fluorescence lifetimes of 3.7 and 1.4 ns were measured for (Chl a)$_2$- and (Chl b)$_2$-N-PCP, respectively (see above). Fluorescence quantum yields of $\Phi = 0.24$ and 0.11 were used for Chl a and Chl b, respectively. In our opinion it is essential to use absorption and emission spectra, as well as fluorescence lifetimes and quantum yields that were measured experimentally for Chl embedded within the protein environment, instead of data obtained for Chl in organic solvents, to minimize any potential influence of the different environments (41). The binding of Chl b to N-PCP apoprotein is assumed to be identical to that of Chl a, which implies the orientation factor to be the same for (Chl a)$_2$-N-PCP and (Chl b)$_2$-N-PCP, viz. $\kappa = -0.394$ (22). The rate calculated for the Chl b to Chl a transfer is $k_T = (31\ \text{ps})^{-1}$, whereas that for the uphill Chl a to Chl b transfer is $k_T = (47\ \text{ps})^{-1}$. The same order of magnitude for both transfer rates is due to the comparably short fluorescence lifetime and smaller quantum yield of Chl b, which both compensate for the difference in spectral overlaps. This estimation, which neglects possible changes of the orientation of transition dipole moments between Chl a and Chl b, explains qualitatively the results of ensemble fluorescence excitation spectroscopy presented in Fig. 3. Any quantitative description of ensemble fluorescence data is hardly possible as it would require precise knowledge of the incorporation rates of Chl a and Chl b into the N-terminal domain of PCP as well as the percentages of each kind of the complexes (homochlorophyllous and heterochlorophyllous) in the reconstitution product.

The energy transfer rates calculated above are important parameters that allow us to simulate the dynamics of the weakly interacting Chl a-Chl b system using the Monte Carlo procedure applied previously for studying energy transfer in phycoerythrocyanin (32). A total of 10^6 random realizations of initial excitation, placed on either of the Chls, yield the percentage of events where the fluorescence emission comes from Chl a or Chl b. We note that the model uses experimentally measured fluorescence lifetimes and calculated Förster energy transfer rates. The result shows that, regardless of whether initial excitation is placed on Chl a or Chl b, the emission occurs more frequently from Chl b, with a Chl a/Chl b ratio of 0.64. This ratio is considerably higher than the one calculated assuming complete thermal equilibration between the two Chls; in this case one obtains a ratio of 0.51. The discrepancy points toward the importance of excitation dynamics in determining the relative fluorescence of the Chls in the heterochlorophyllous complex. Moreover, the calculated values are considerably different from the experimentally observed ratio of 1.9.

The ratio of Chl a/Chl b fluorescence intensity measured for single heterochlorophyllous Chl a/b-N-PCP complexes depends not upon Förster energy transfer between the Chl pigments alone, but also on the efficiency of energy transfer from Per to the respective Chl. Recent time-resolved absorption experiments carried out on the ensembles of homochlorophyllous (Chl a)$_2$-N-PCP and (Chl b)$_2$-N-PCP complexes have shown that when exciting at 530 nm the energy transfer from Per to Chl a is 2.7 times faster than the energy transfer from Per to Chl b (30). The difference in energy transfer rates (3.55 ps vs. 9.4 ps for Chl a and Chl b, respectively) has been qualitatively attributed to the fact that the absorption of Chl b is located on the short wavelength tail of the Per fluorescence, whereas the Chl a absorption is near the maximum. All the relevant energy transfer pathways and characteristic times for the complex reconstituted with both Chl a and Chl b are displayed in Fig. 10. The numbering of Per pigments in each cluster follows Carbonera et al. (40) and the times describing the energy transfer between Per and each of the Chl molecules are taken from Polìvka et al. (30). The parameters of the energy transfer between the Chl have been calculated using Förster formalism, whereas the fluorescence decay times of the Chl a and Chl b have been measured experimentally for homochlorophyllous N-PCP reconstitutions.

Using the average Chl a/Chl b fluorescence intensity ratio of 1.9 obtained by single-molecule spectroscopy and the calculated excitation distributions, we can estimate the ratios of energy transfer efficiency in the heterochlorophyllous Chl a/b-N-PCP complexes. They are particularly attractive for such a comparison because the fluorescence properties of the two Chl molecules with quite different spectral properties, such as absorption maximum, fluorescence lifetime, and quantum yield, can be measured simultaneously in the single-molecule experiment. The excitation distribution between Chl a and Chl b calculated for the fully equilibrated Chl a-Chl b system is equal to 0.51, which implies a 3.7 times more efficient energy transfer from Per to Chl a than

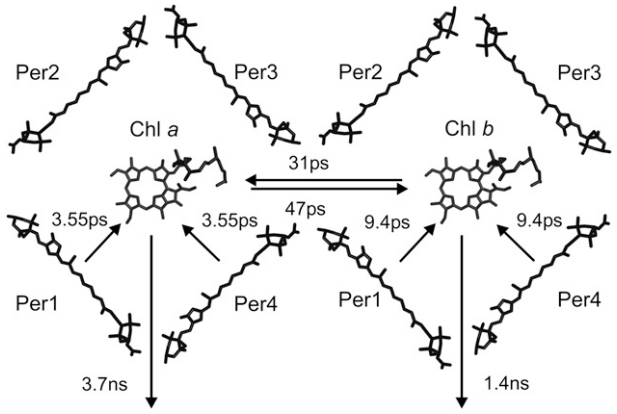

FIGURE 10 Schematic diagram of energy transfer pathways for a single Chl a/b-N-PCP complex excited at 532 nm. The numbering of Per follows the nomenclature of (40). The two Per (1 and 4) excited in each of the clusters transfer the excitation to Chl a (*left*) and Chl b (*right*) and after bilateral energy transfer between the Chl the recombination takes place. All the characteristic times are given.

from Per to Chl *b*. However, when using the excitation distribution between Chl *a* and Chl *b* equal to 0.64, as calculated by the Monte Carlo simulations, the energy transfer from Per to Chl *a* is 2.96 times more efficient than from Per to Chl *b*. The agreement between the value of 2.7 measured by Polivka et al. (30) and the one calculated with parameters derived from the Monte Carlo simulations suggests that the Chl *a*-Chl *b* system in Chl *a*/*b*-N-PCP complexes does not reach thermal equilibrium within the excited state lifetime of the Chls.

CONCLUSION

Ensemble fluorescence measurements combined with single-molecule spectroscopy enabled us to coherently describe energy transfer processes between Per and Chl molecules as well as between two Chl molecules in reconstituted N-PCP. We find that the energy transfer between Chl *a* and Chl *b* in heterochlorophyllous Chl *a*/*b*-N-PCP occurs in both directions with nearly similar efficiency. The transfer rates calculated within the Förster model are $(31\ \text{ps})^{-1}$ and $(47\ \text{ps})^{-1}$ for the Chl *b* to Chl *a* and the Chl *a* to Chl *b* energy transfer, respectively. The excitation dynamics in this system can be described quantitatively using the results of single-molecule spectroscopy, which provides an excellent way to distinguish between homo- and heterochlorophyllous complexes in the reconstitution mixture. Analysis of fluorescence spectra of individual Chl *a*/*b*-N-PCP complexes yields an average ratio of Chl *a*/Ch *b* fluorescence intensity of 1.9. This finding is well reproduced with Monte Carlo simulations performed with the assumption that the energy transfer from Per to Chl *a* is 2.96 times more efficient than from Per to Chl *b*, which agrees with the efficiency ratio of 2.7 measured recently for homochlorophyllous PCP samples containing either Chl *a* or Chl *b*. The discrepancy with the efficiency ratio of 3.7 calculated for the fully equilibrated Chl *a*-Chl *b* system suggests that the two Chl molecules in the Chl *a*/*b*-N-PCP complex do not reach the equilibrium during their excited state lifetimes.

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