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Peridinin–chlorophyll–protein reconstituted with chlorophyll mixtures: Preparation, bulk and single molecule spectroscopy

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Abstract Reconstitution of the 16 kDa N-terminal domain of the peridinin–chlorophyll–protein, N-PCP, with mixtures of chlorophyll *a* (Chl *a*) and Chl *b*, resulted in 32 kDa complexes containing two pigment clusters, each bound to one N-PCP. Besides homo-chlorophyllous complexes, hetero-chlorophyllous ones were obtained that contain Chl *a* in one pigment cluster, and Chl *b* in the other. Binding of Chl *b* is stronger than that of the native pigment, Chl *a*. Energy transfer from Chl *b* to Chl *a* is efficient, but there are only weak interactions between the two pigments. Individual homo- and hetero-chlorophyllous complexes were investigated by single molecule spectroscopy using excitation into the peridinin absorption band and scanning of the Chl fluorescence, the latter show frequently well resolved emissions of the two pigments.
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1. Introduction

Peridinin–chlorophyll–proteins (PCP) are water-soluble light-harvesting complexes from dinoflagellates [1]. The native complex from *Amphidinium* (*A.*) *carterae* is a homotrimer. The boat-shaped, α-helical protomer is the 32 kDa large PCP (l-PCP). It has an approximate C_2-symmetry containing two clusters of pigments, each consisting of one chlorophyll *a* (Chl *a*) that is nested between two pairs of peridinins (Per) [2]. The pigments in each cluster are in Van der Waals contact. There is also contact between Per molecules from the two clusters, while the center-to-center distance of the two Chl is 17.4 Å. Small PCP (s-PCP) of half the size (16 kDa) of the *A. carterae* complex which contain a single Per_4–Chl *a* cluster, has been found in several species including *Heterocapsa pygmaea* [3]. These s-PCP dimerize, resulting in a similar topology as the monomeric protomers from *A. carterae* [4]. An analogous aggregate is also formed by the 16 kDa N-terminal domain of PCP from *A. carterae* (N-PCP) reconstituted with Chl *a* and Per [5][1].

Tight clustering, an exceptionally high carotenoid-Chl ratio, and the presence of a highly modified carotenoid, Per, with an unusually long-lived S_1-state [6] render the PCPs a challenge to understand pigment interactions and excitation energy transfer (EET). Pairing of Per and a close spacing of the pigments had been inferred already in 1976 from circular dichroism (CD) spectra of PCP [7]. Moderately strong excitonic coupling among the pigments within a cluster was also supported by simulations of the CD and absorption spectra, which were based on the X-ray structure [8]. However, the details are still controversial and may require contributions from inter-cluster interactions. Rapid fluorescence depolarization (7 ps) has been assigned to intra-monomer EET between the Chls, a much slower component (350 ps) to Förster type inter-monomer EET transfer in PCP trimers from *A. carterae* [9]. EET from Per to Chl has been studied using N-PCP that have been reconstituted with different Chls [10], the results support theoretical predictions [11] of a major contribution of the relatively long-lived S_1-state of Per [6,12,13] as donor to the Chls. The contributions of the different Per excited states have been reviewed [14]; the S_1-state is stabilized by symmetry breaking and by intra-Per charge-transfer [6,13,15].

The high carotenoid content and relatively small Chl–Chl interactions also render PCP a candidate to study EET between two Chls in a structurally well-known situation that is relatively simple as compared to other photosynthetic systems. In this context, it is desirable to study PCP containing two different, spectrally separated Chls [5]. Here, we report on such complexes prepared by reconstitution of N-PCP from

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Abbreviations: A., *Amphidinium*; CD, circular dichroism; Chl, chlorophyll; EET, excitation energy transfer; l-PCP, large (32 kDa PCP); N-PCP, N-terminal 16 kDa-domain of PCP; PCP, peridinin–chlorophyll–protein; Per, peridinin; s-PCP, small (16 kDa) PCP; SMS, single molecule spectroscopy

[1] The term 'monomer' is used here in a topological context, referring to l-PCP from *A. carterae* from which the X-ray structure has been solved. l-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 this l-PCP monomer, the 16 kDa s-PCP discovered subsequently, as well as N-PCP generated from the *A. carterae* protein, are topologically "halfmers": they carry only a single Chl/Per_4 cluster, and dimerize to species that are homologous to the l-PCP monomer.

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A. carterae with Chl *a* and Chl *b*. The procedure yields dimeric aggregates that correspond topologically to l-PCP monomers[1], and are mixtures with respect to their pigment composition: besides homo-chlorophyllous Chl *a*/Chl *a* and Chl *b*/Chl *b* complexes, the preparation yields hetero-chlorophyllous complexes containing Chl *a* in one cluster and Chl *b* in the other. While these complexes could not be separated by chromatography, identification of the hetero-chlorophyllous complexes and their investigation was possible by difference spectroscopy, and by single molecule spectroscopy (SMS).

2. Materials and methods

2.1. Materials

Trimeric PCP was isolated from *A. carterae* [2]. The N-terminal domain of the apoprotein (N-PCP) was obtained as published [5]. Per was extracted from *A. carterae* as published [6]. Chl *a* was extracted from spray-dried *Spirulina geitleri*, Chl *b* from the Chl *a*/*b* mixture extracted from frozen spinach, and purified over DEAE cellulose [16].

2.2. Reconstitution

Reconstitution followed the protocol of [5]. 625 μl apo-N-PCP (20 μM) in tricine buffer (50 mM, pH 8.0) were combined with 225 μl tricine buffer (50 mM, pH 7.6) containing KCl (10 mM). An ethanolic solution (150 μl) of Per (80 μM) and Chl *a* (or *b*) (20 μM) was added, the mixture incubated at 4 °C for 48 h, and the crude reconstitution product purified over a small DEAE Tris–acryl column (5 × 25 mm, tricine buffer (5 mM, pH 7.6, 2 mM KCl), NaCl step gradient 100, 200 and 500 mM). Fractions containing the reconstituted complex (100 mM NaCl) were dialyzed against the starting buffer.

2.3. Spectroscopy

Absorption spectra were recorded with a Lamda 2 spectrophotometer (Perkin–Elmer), bulk fluorescence excitation and emission spectra with a model LS55 spectrofluorimeter (Perkin–Elmer), and circular dichroism spectra with a model J810 spectropolarimeter (Jasco). All measurements were done at room temperature. Data were transferred to Origin 6.0 (Microcal) for further analysis. Chl *a* and Chl *b* extinction coefficients in the reconstitution system were determined relative to the known ones in acetone [17]. The respective pigment was dissolved in acetone, and an absorption spectrum recorded. The solvent was then evaporated in a stream of Ar, the pigment re-dissolved in the same vol-

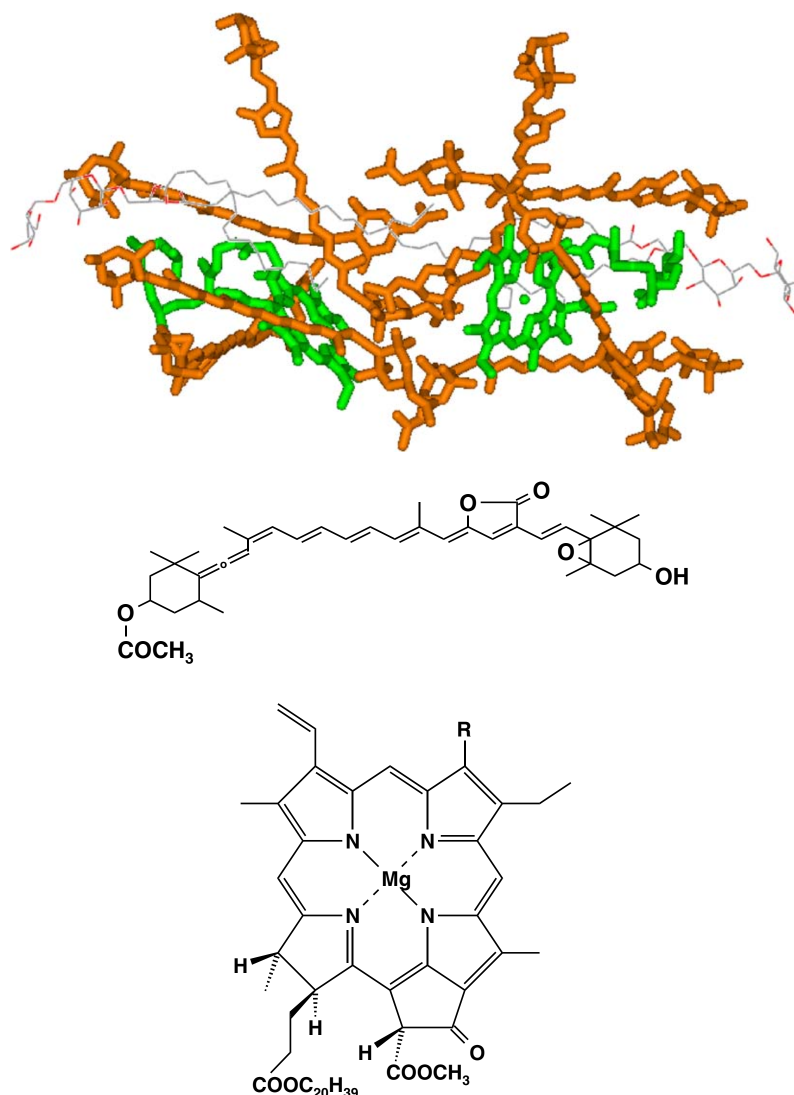

Fig. 1. Molecular structures of the cofactors of the PCP complex. Top: Per and Chl *a* in the two pigment clusters of a PCP monomer from *A. carterae* ([2], pdb entry 1PPR) are shown as orange and green bold stick models, the two lipids as wire models, using DS ViewerPro V5 (Accelrys). The center-to-center (Mg–Mg) distance of the Chls is 17.3 Å. Chemical structures of Per (center) and of Chl *a* (R = CH_3) and Chl *b* (R = CHO) (bottom).

ume of the buffer system by first adding the appropriate amount of ethanol and the aqueous components, and then the absorption was recorded again (see Fig. 1).

Single molecule spectra were obtained using a modified scanning confocal microscope (ZEISS LSM 410). Fluorescence spectra of single immobilized complexes were recorded after excitation into the Per absorption (532 nm). Fluorescence emission in the spectral range from 575 to 725 nm was dispersed with a Amici-prism and projected onto a CCD camera (Princeton Instruments). Typical integration times were 0.3 s with a spectral resolution of 1.5 nm. Further details will be described in a paper dedicated to single molecule spectroscopy of PCP [24].

3. Results and discussion

Reconstitution [10] of N-PCP from *A. carterae* with Chl *a* (1.1 moles/mole protein) and Per (4.4 moles/mole protein) results in hetero-chlorophyllous complexes as evidenced by their optical spectra (see below). Quantitatively, the pigment ratios in the reconstituted and purified complexes were determined spectroscopically [18], after dilution into acetone to a 20:80 water/acetone ratio. The Chl *a*/*b* ratio is decreased from 1:1 in the reconstitution mixture to 43/57 in the purified complex, indicating a slight preference for the non-natural pigment, viz. Chl *b*. Chls with C=O – groups at C-3 and C-7 have been bound before to N-PCP [5]. In the X-ray structure, the region around C-7[1] carrying the carbonyl-oxygen in Chl *b*, has two hydrophilic groups nearby that could assist binding: a ring-N of His66 (3.8 Å) and a backbone C=O of Ala-63 (5.1 Å) [2]. Binding of Chl *b* is, however, even possible in relatively hydrophobic environments [19,20].

Absorption and circular dichroism. The homo-chlorophyllous N-PCP complex containing only Chl *a* has absorption and CD spectra (Fig. 2B) that are similar to those of native l-PCP from the same organism (Fig. 2A), the spectra of the latter are identical to the ones of PCP reported before [8]. However, the negative CD-band of Per peaking around 530 nm is slightly red-shifted and broadened in Chl *a*–N-PCP as compared to native l-PCP. The CD-spectrum of both complexes is dominated by an intense, s-shaped band system peaking at 530 (−) and ∼445 (+), with the zero-crossing near the absorption maximum of peridinin. Superimposed on the positive lobe of this signal is a much smaller and narrower, s-shaped band system that is centered near the absorption maximum of Chl *a*. Its negative lobe shows as a dip in the intense positive Per band,

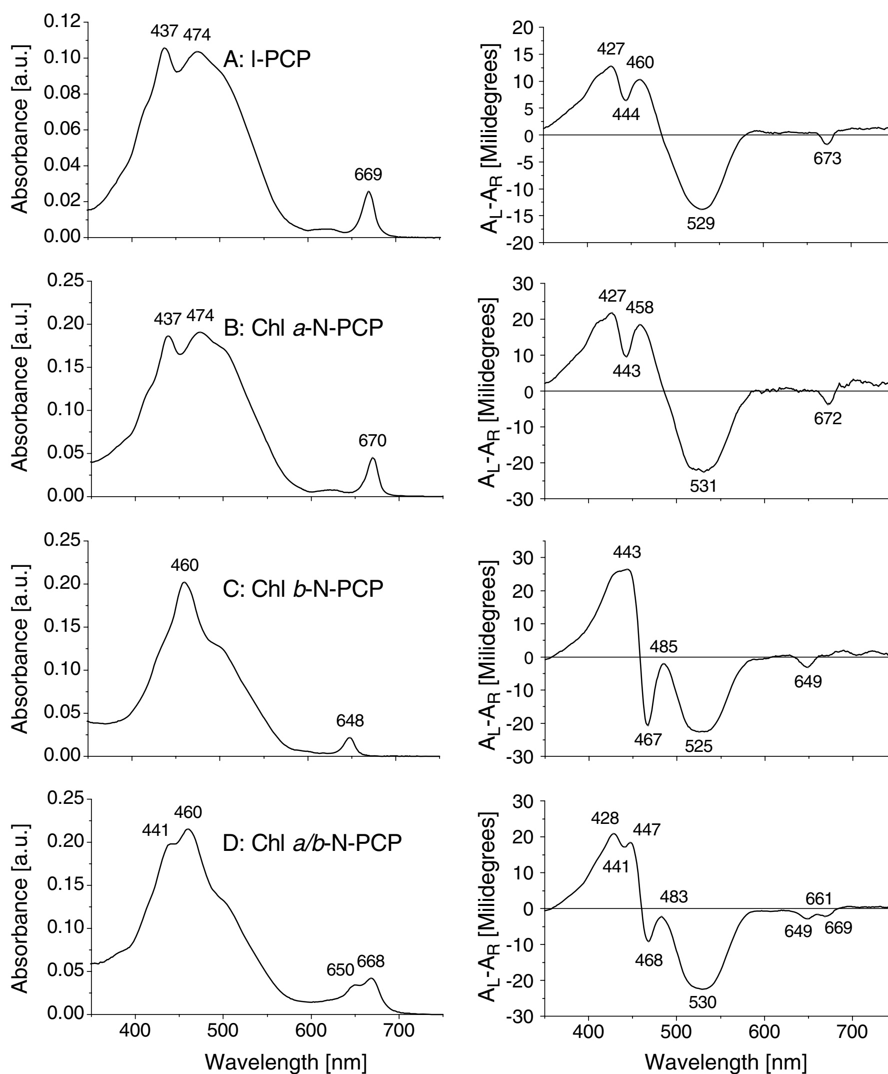

Fig. 2. Absorption (left) and circular dichroism spectra (right) of (A) native PCP, and of RPCP reconstituted with Chl *a* (B); Chl *b* (C) and a 1:1 mixture of Chl *a* and Chl *b* (D).

that is located ~7 nm to the red of the Chl *a* absorption maximum (437 nm). The positive lobe is discernible as a distinct peak in the largely unstructured Per band, located 10 nm to the blue of the Chl *a* absorption maximum. There is no such splitting for the Q_Y-band of Chl *a* at ~672 nm. The major band of Chl *a* in the Soret-region is the B_x-band [21,22]. Since the orientation factors are similar for the x- and y-polarized transitions, the lack of a split Q_Y CD-signal indicates that the split Soret CD-signal is not due to excitonic interactions among the Chls of adjacent pigment clusters, but results mainly from coupling with Per within a single cluster [4,8].

Reconstitution with Chl *b* instead of Chl *a* yields a complex with a blue-shifted Q_y band of the Chl and a red-shifted Soret band (Fig. 2C), these shifts are similar to those of Chl *a* vs. Chl *b* in acetone. The red-shifted (460 nm) and more pronounced Soret band reflects the larger extinction coefficient of Chl *b* as compared to Chl *a* [23]. Accordingly, the absorption of the Chl *b* complex is dominated by the latter (460 nm). The s-shaped CD signal in the Soret region is also much more prominent than in the Chl *a*-containing complex, as shown already by Miller et al. [5]. The zero-crossing of the intense, s-shaped feature is close to the Chl *b* absorption maximum. The Q_y-band shows, like in the Chl *a* complex, no splitting; this further supports [4,8] that excitonic interactions of the Chls occur mainly with Per.

The spectrum of the hetero-chlorophyllous complex (Fig. 2D) is superficially a mixture of the ones of the homo-chlorophyllous complexes. However, a closer inspection reveals differences, that are likely due to Chl *a*/Chl *b* interactions. (i) The Chl *b*-related Q_y-CD band at 649 nm is stronger than that of Chl *a* at 669 nm, while based on the homo-chlorophyllous complexes (Fig. 2, see also [5]) and the Chl *a*/*b*-ratio of 43/57 (see above), at most equal intensities are expected. (ii) The red absorption and CD-bands of Chl *a* are blue-shifted by 2 nm in the Chl *a*/*b* complex as compared to the Chl *a* – only sample (Fig. 2A). (iii) Subtraction of the absorption spectra of the homo-chlorophyllous Chl *a*–N-PCP or Chl *b*–N-PCP complex from that of the hetero-chlorophyllous complex always gave additional bands (Fig. 3). It should be noted that the situation could be further complicated by the possibility that there are mono-chlorophyllous complexes, in which one binding site is empty, and by the presence of "half-mers"[1] carrying only a single Chl/Per_4 cluster. However, these are unlikely based on the tight pigment binding and dimerization of individual N-PCP, and were not considered here. Fluorescence spectroscopy of bulk samples gave likewise inconclusive results (data not shown).

The emission spectra of the hetero-chlorophyllous complex show one major peak at 673 nm and a smaller one at 652 nm, corresponding to emissions from Chl *a* and Chl *b*, respectively (Fig. 4). The excitation spectrum for the shorter-wavelength emission shows, accordingly, mainly contributions of Per and Chl *b*. The excitation spectrum for the long-wavelength peak has contributions from Per ($\lambda_{max} \approx 505$ nm), Chl *b* ($\lambda_{max} = 463$ nm) and Chl *a* ($\lambda_{max} = 437$ nm). This clearly indicates inter-cluster energy transfer from Chl *b* to Chl *a* in the hetero-chlorophyllous N-PCP complex. Assuming that the pigment geometry is similar to that in the l-PCP monomer, and the separation of the two emission bands corresponds to the equilibrium energy difference between Chl *a* and Chl *b*; relative contributions of 6.1:1 can be estimated for the emissions of Chls *a* and *b*, respectively. This value was estimated in the following way: Boltzmann equilibrium ($n_{\text{Chl}\,a}/n_{\text{Chl}\,b} = \exp(-\Delta E/kT)$ between the two emittors (673 and 651 nm, respectively) results, at a temperature of 298 K, in relative populations of 10.1:1. Since the fluorescence results from a mixture of species consisting of the homo-chlorophyllous complexes, (Chl $a)_2$–N-PCP and (Chl $b)_2$–N-PCP, and the hetero-chlorophyllous complex, Chl *a*/*b*–N-PCP, contributions from the three components have to be summed. Their relative populations were estimated assuming a random distribution of Chls *a* and *b* in the experimentally determined pigment ratio of 43:57 (see above), resulting in relative populations of 18.5%, 32.5% and 49%. Finally, the emission of Chl *b* was corrected by the relative fluorescence yield of 0.45 [5] of Chl *b*–N-PCP compared to Chl *a*–N-PCP. The experimentally obtained Chl *a*/*b* emission ratio, determined from the data in Fig. 4 by

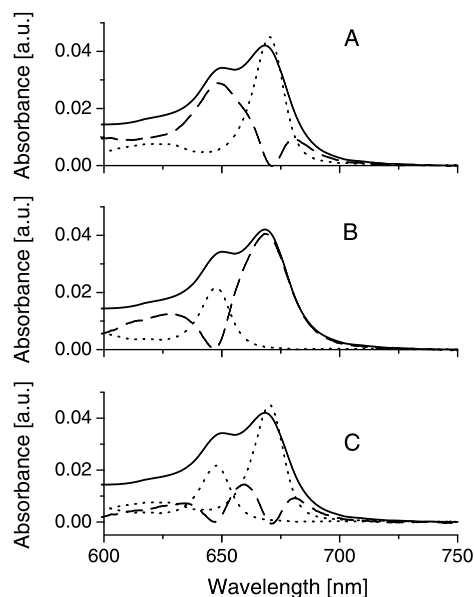

Fig. 3. Comparison of homo- and hetero-chlorophyllous N-PCP complexes. Absorption spectra of Chl *a*/*b*–N-PCP (—) and absorption differences (- - - -) with the spectra of Chl *a*–N-PCP (A), Chl *b*–N-PCP (B) and the sum of Chl *a*–N-PCP and Chl *b*–N-PCP (C). The subtracted homo-chlorophyllous spectra are shown as dotted lines, they were scaled to give minimum deviation in the region of the respective Chls.

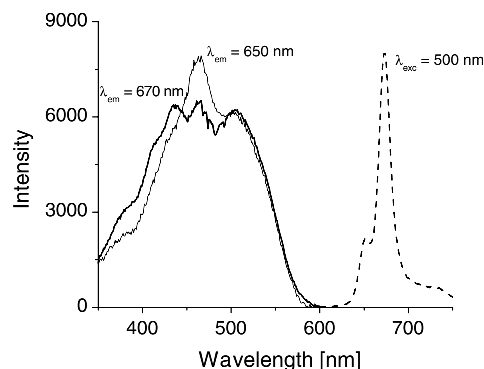

Fig. 4. Fluorescence of reconstituted Chl *a*/*b*–N-PCP complex; emission ($\lambda_{exc} = 500$ nm, – – – – –) and excitation spectra ($\lambda_{em} = 650$ nm, ———; $\lambda_{em} = 670$ nm, ———). Excitation spectra were normalized to the Per maximum near 505 nm.

Gaussian deconvolution, is 4.7. This is somewhat lower than the estimated theoretical value of 6.1, but indicates nearly complete equilibration between the two Chls in the Chl *a*/*b*-complex. A more detailed analysis is difficult, however, considering the aforementioned interactions among the Chls, whose contributions are difficult to estimate, and the assumptions used in the analysis.

The Chl *a* emission of the hetero-chlorophyllous complex (671 nm) is red-shifted by 2 nm compared to the Chl *a* – only complex. Minor but reproducible shifts are also seen in the Soret-region. Likewise there were minor shifts in the Chl *b*-bands when comparing the hetero-chlorophyllous complex with that of the one containing only Chl *b*. All these shifts point to small, but distinct Chl–Chl interactions between the Chls in the two clusters.

Proof that hetero-chlorophyllous complexes are indeed formed can be obtained by investigations of single complexes. Therefore we recorded room temperature spectra of individual complexes obtained from the N-PCP reconstitution with Chls *a* and *b* [24]. After excitation at (532 nm) where absorption is dominated (>90%) by Per, the detected fluorescence of the Chls ($\lambda > 600$ nm) originates predominantly from energy transfer. Several hundred molecules of all three reconstituted complexes have been investigated in this fashion. Most of them can be grouped by their emission spectra into three types of complexes. In the case of homo-chlorophyllous complexes, a single line was observed, as displayed in the first and second row in Fig. 5. The complexes containing only Chl *a* show narrow emission lines centered around 670 nm, while the fluorescence emission measured for those containing only Chl *b* is

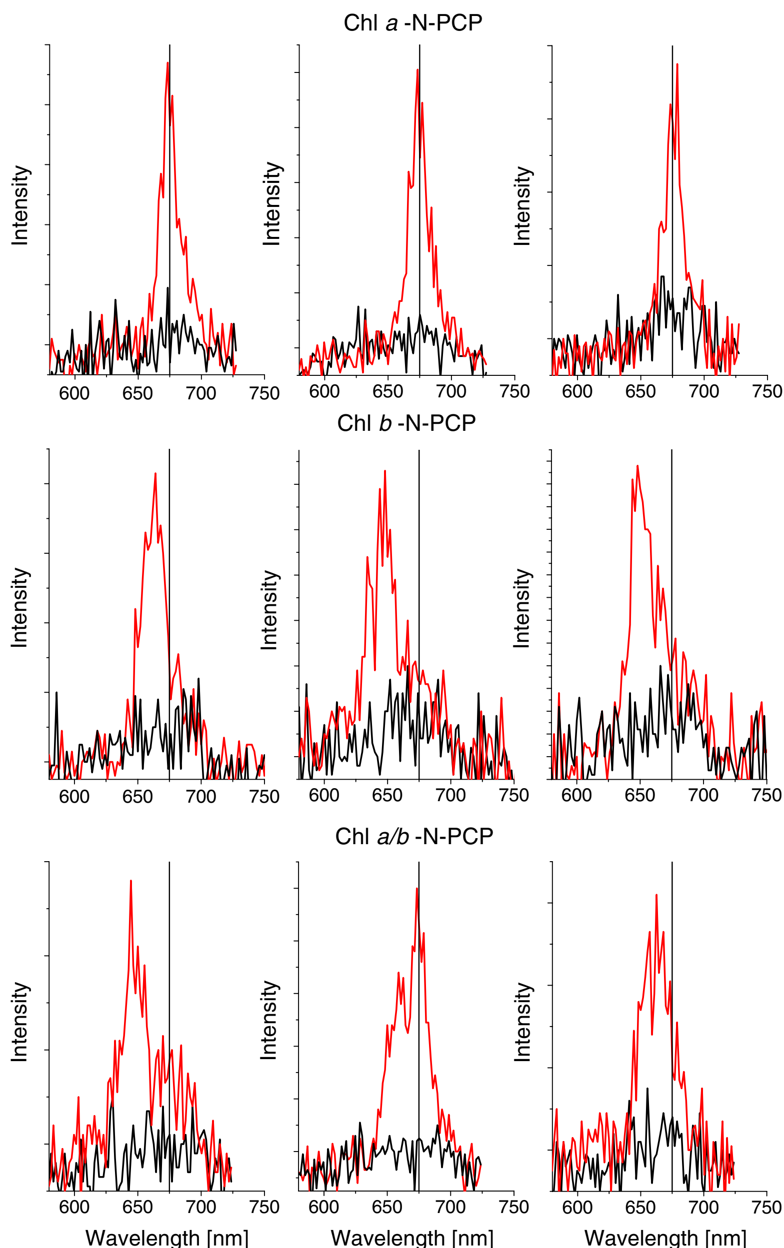

Fig. 5. Ambient temperature single molecule fluorescence spectra. Three different complexes each ($\lambda_{exc} = 532$ nm) of N-PCP reconstituted with Chl *a* (top); Chl *b* (center) and a 1:1 mixture of Chl *a* and Chl *b* (bottom). The lines at 675 nm are drawn to guide the eye. The baselines were recorded at the same position, after bleaching of the sample.

characterized by somewhat broader lines centered around 650 nm. The most interesting spectra are the ones measured for the hetero-chlorophyllous complexes, shown in the bottom row. These systems show a broadened and often split emission composed of two lines attributed to Chl *a* (∼670 nm) and Chl *b* (∼650 nm). The contribution from the short-wavelength component, associated with Chl *b* emission, is in many cases considerably larger than expected for an equilibrated emission (see above), it is even dominant in one of the samples shown. While details of the energy transfer in individual complexes are currently analyzed, the observed emission of both Chl *a* and Chl *b* from single complexes unambiguously demonstrates the successful production of hetero-chlorophyllous complexes.

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