Two-Photon Excited Fluorescence from Higher Electronic States of Chlorophylls in Photosynthetic Antenna Complexes: A New Approach to Detect Strong Excitonic Chlorophyll *a*/*b* Coupling

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ABSTRACT Stepwise two-photon excitation of chlorophyll *a* and *b* in the higher plant main light-harvesting complex (LHC II) and the minor complex CP29 (as well as in organic solution) with 100-fs pulses in the Q_y region results in a weak blue fluorescence. The dependence of the spectral shape of the blue fluorescence on excitation wavelength offers a new approach to elucidate the long-standing problem of the origin of spectral "chlorophyll forms" in pigment-protein complexes, in particular the characterization of chlorophyll *a*/*b*-heterodimers. As a first result we present evidence for the existence of strong chlorophyll *a*/*b*-interactions (excitonically coupled transitions at 650 and 680 nm) in LHC II at ambient temperature. In comparison with LHC II, the experiments with CP29 provide further evidence that the lowest energy chlorophyll *a* transition (at ~680 nm) is not excitonically coupled to chlorophyll *b*.

INTRODUCTION

Usually, organic molecules with π-electron systems show fluorescence from higher excited states (if at all) only with very small quantum yield. There are few known exceptions, e.g., azulene. Conventional one-photon excitation of this emission from higher excited states (usually with wavelengths in the blue or near-ultraviolet spectral region) often suffers from overlap with fluorescence from the environment of the molecule under investigation (solvents, impurities, etc.). A principal improvement in selectivity of detection of emission from higher excited states can be achieved by stepwise two-photon excitation of fluorescence with pulsed lasers (Terenin et al., 1966; König et al., 1974). In this case mostly the first singlet excited electronic state (S_1) serves as the intermediate level. Usually, the environment absorbs much to the blue as compared with the organic molecule of interest. Excitation of the environment is circumvented this way. Moreover, considerably higher intensities would be required to excite the environment by simultaneous two-photon absorption via virtual intermediate states (compare Teuchner et al., 2000, Eqs.1 and 2, for a quantitative relationship), thus the latter can be avoided easily.

To the best of our knowledge no systematic study of (stepwise excited) fluorescence from higher states of chlorophylls (Chls) in the protein matrix of photosynthetic light-harvesting complexes has been reported so far. Such fluorescence does not only characterize a loss channel in the excitation energy transfer chain in these antenna complexes, but, as we will show in the following, may contain valuable information on spectral heterogeneity ("Chl-forms"). The latter refers to the broadening of the red-most absorption band of Chls in pigment-protein complexes as compared with the band shape of the same pigments dissolved in organic solvents. The broadened spectral profiles can be resolved (more or less formally) into a number of subbands. The origin of these subbands, their number in different pigment-protein complexes, as well as their role(s) in photosynthesis have been a matter of controversial debate since decades. A recent summary of the subband problem for the plant main light-harvesting complex (LHC II) is given by Leupold et al. (1999). A multitude of spectroscopic techniques and in particular each progress in laser-spectroscopic methodology has been evaluated for its possible contribution to solve this basic problem in photosynthesis research.

In the following we will illustrate that stepwise two-photon excited fluorescence from higher excited states (corresponding to the B_x/Soret-region of the absorption spectra) provides a new approach to elucidate the origin of "Chl-forms," especially of those subbands, which are the result of strong electronic coupling between Chls *a* and *b*. This is exemplified with trimeric LHC II, and comparative investigations were made with the related monomeric minor photosystem II antenna complex CP29 (the *lhcb4* gene product). To support interpretation of these spectra, stepwise two-photon excited fluorescence spectra of Chls *a* and *b* in diluted organic solution are also presented.

MATERIALS AND METHODS

Commercial Chls *a* and *b* (Sigma, St. Louis, MO) were used without further purification. LHC II was isolated from freshly harvested pea leaves after the procedure of Krupa et al. (1987). LHC II in the trimeric state was obtained in a buffer containing 10 mM Tricine (pH 7.8) and

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1.2% *n*-octyl β-D-glucopyranoside at 110 μg/ml Chl *a*+*b*. CP29 was isolated from photosystem II(PS II) membrane fragments according to Berthold et al. (1981) with some modification as described in Völker et al. (1985). PS II membranes were resuspended to 0.2 mg Chl *a*+*b* ml^{-1} in 0.8 M Tris/HCl (pH 8.35) under ambient light for 30 min. CP29 was extracted and purified following the protocol of Henrysson et al. (1989) with the following modifications: 2 mM benzamidine and 1 mM pefabloc (Merck, Rahway, NJ) were used as protease inhibitors, sulfobetaine 14 was replaced by sulfobetaine 12. Chromatographic purification was performed on a CM-Sepharose Fast Flow column under dim green light at 4°C. Equilibration and gradient buffers contained 0.1% (w/v) sulfobetaine 12 and 0.05% *n*-dodecyl β-D-maltoside. One to two rechromatographies were necessary to obtain the desired purity. Chls were dissolved in diethyl ether. Absorption and fluorescence spectra of all samples were measured before and after the two-photon excitation experiments to exclude sample degradation. All samples were adjusted to an optical density ≈ 0.1 cm^{-1} at the excitation wavelength. All stepwise two-photon excited fluorescence measurements were made at room temperature.

The equipment for stepwise two-photon excited fluorescence spectroscopy is described in detail elsewhere (Teuchner et al., 2000). In short, a Clark regeneratively amplified Ti:sapphire laser in combination with an optical parametric oscillator was used for excitation (1-kHz repetition rate; 100-fs pulse duration; 5 μJ of maximum used pulse energy; flux density of excitation, 1×10^{28} cm^{-2} s^{-1} for spectral shape measurements, and from 3×10^{26} to 2×10^{28} cm^{-2} s^{-1}, respectively, for intensity dependence measurements; the wavelengths of excitation given below are the pulses center wavelengths, their spectral bandwidths being 10 nm). Stepwise two-photon excited fluorescence was detected with a high speed photomultiplier PMH-100 and a gated photon-counting module PHC 322 (Becker and Hickl, Berlin, Germany).

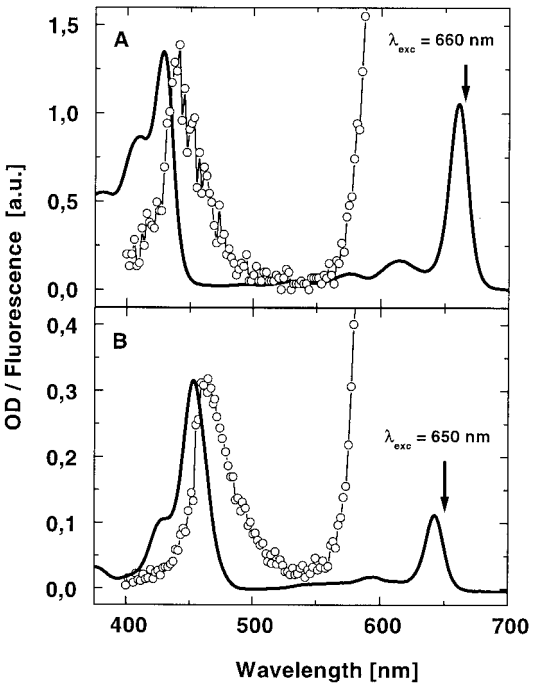

FIGURE 1 Absorption (*solid lines*) and two-photon excited fluorescence spectra (*open symbols*) of chlorophyll *a* (*A*) and chlorophyll *b* (*B*) in diethyl ether. Excitation wavelengths (λ_{exc}) are indicated by arrows, the stepwise two-photon excited fluorescence spectra are normalized to the corresponding Soret absorption peak.

RESULTS

Stepwise two-photon excited fluorescence of chlorophylls in solution

To aid interpretation of stepwise two-photon excited fluorescence of Chls in antenna complexes the corresponding spectra of Chls *a* and *b* (in diethyl ether) were measured (Fig. 1). Chl *a* was excited with 100-fs pulses at 660 nm and Chl *b* at 650 nm, i.e., close to their respective absorption maxima in the red spectral region.

Under these excitation conditions Chl *a* shows a fluorescence in the blue spectral region with maximum at 440 nm, 10-nm red-shifted to the maximum of the B_x absorption band (Fig. 1 *A*). Starting at ~560 nm the ascent of the dominant red fluorescence band is observed, containing (besides the well studied Q_y fluorescence) also emission from the Q_x state. The amplitude of the blue fluorescence band as related to this ascent gives an impression of the very low quantum yield of the blue fluorescence (being less than 10^{-4}).

Chl *b* displays an analogous behavior, its blue fluorescence peaks at 462 nm (Fig. 1 *B*). (Note, that in the following we call all emissions in the Soret-region "blue" fluorescence irrespective of the exact spectral position of the band). With respect to the interpretation of the stepwise two-photon excited fluorescence spectra of antenna complexes it is worth to note that the blue Chl *b* fluorescence is located at longer wavelengths than the corresponding Chl *a* fluorescence band. This is just opposite to the relative locations of their respective red Q_y fluorescence bands. However, also these fluorescence bands in the blue spectral region are in normal (roughly mirror image) relation to the corresponding B_x absorption bands (Fig. 1, *A* and *B*).

The excitation intensity dependence of the blue fluorescence signal of both Chls was measured over two orders of magnitude. In each case, a quadratic dependence as exemplified for Chl *b* (Fig. 2 *A*) is obtained. Also shown in Fig. 2 *A* is the result of a simulation on the basis of a model assuming stepwise two-photon absorption (Fig. 2 *B*). Notably, the theoretically conceivable simultaneous two-photon absorption (indicated in Fig. 2 *B* by the dashed arrows) does not give a measurable contribution to the blue fluorescence in the intensity range under investigation. Even if assuming a large two-photon absorption cross-section of 10^{-48} (as e.g., for rhodamin B; compare Diaspro and Robello, 2000) the simulation results in less than 1% contribution of simultaneous two-photon excitation to the blue fluorescence. For further (standard) parameters in the simulation see legend to Fig. 2 *B*. (Following a suggestion of the Associate Editor, in a qualitative experiment the intensity of the blue Chl *b* fluorescence was measured while changing the excitation wavelength from 650 nm toward the red

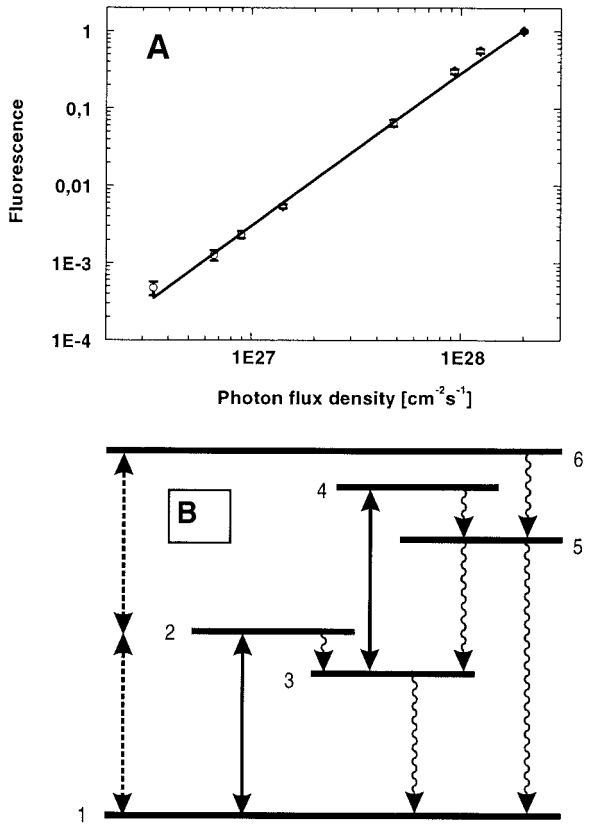

FIGURE 2 (*A*) Log-log plot of the blue fluorescence intensity of Chl *b* in diethyl ether (λ_{em} = 460 nm) versus incident photon flux density (λ_{exc} = 650 nm). Experimental data (*open symbols*), simulation (*solid line*). The simulation is based on the model depicted in *B* (compare text). The simulated curve is a straight line with a linear regression coefficient r = 0.99997 and a slope $m = 1.98 \pm 0.05$. For the simulation the following parameters were used: absorption and emission cross-sections (one-photon): $\sigma_{12} = \sigma_{21} = \sigma_{34} = \sigma_{43} = 1.6 \times 10^{-16}$ cm^2, (two-photon): $\sigma_{16}^{(2)} = 10^{-48}$ cm^4 s; relaxation constants: $k_{31} = 2.9 \times 10^8$ s^{-1}, $k_{51} = 1.75 \times 10^9$ s^{-1}, $k_{51}/k_{53} = 10^{-4}$, $k_{23} = k_{45} = k_{65} = 10^{13}$ s^{-1}. Data were taken in part from Pfarrherr et al., 1991; parameters of higher excited states were obtained from simulation of nonlinear absorption (K. Teuchner, H. Stiel, and A. Pfarrherr, unpublished results) as described for Chl *a* by Leupold et al. (1990). For the unknown simultaneous two-photon absorption cross-section a maximal value has been assumed (compare Diaspro and Robello, 2000). (*B*) Energy level scheme including absorption and relaxation channels relevant for interpretation of the blue fluorescence. Simultaneous two-photon absorption (*dashed arrows*) is indicated for completeness only (compare text). (*1*) Ground state; (*2*) first excited singlet (Frank-Condon) state; (*3*) first excited singlet state (vibronic ground state), origin of red fluorescence; (*4*) higher excited singlet (Frank-Condon) state; (*5*) higher excited singlet state, origin of blue fluorescence; (*6*) terminal state of a (hypothetical) simultaneous two-photon absorption process.

wing of the Q_y absorption band. The resultant strong decrease of fluorescence intensity (corresponding to the descending absorption) is in accordance with the mechanism of stepwise absorption of two photons. At 800-nm excitation (far away from the longest-wavelength absorption band) no blue emission was observed.)

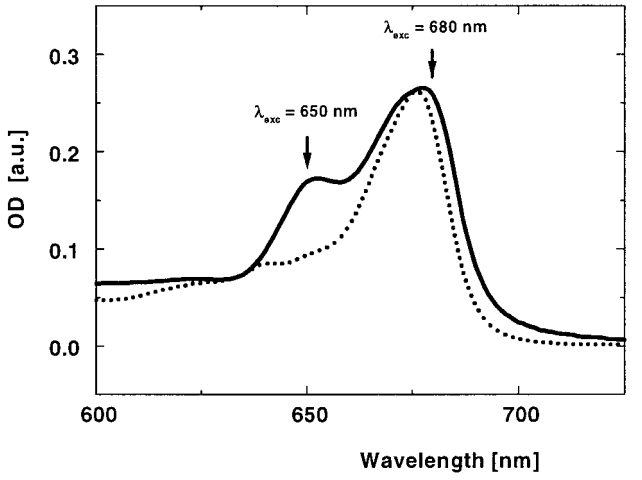

FIGURE 3 Q_y-absorption spectra of trimeric LHC II (*solid line*) and CP29 (*dotted line*), respectively. Arrows indicate excitation wavelengths of the stepwise two-photon excited fluorescence.

Stepwise two-photon excited fluorescence of chlorophylls in photosynthetic antenna complexes

Two-photon excited fluorescence spectra from higher excited states of Chls in the protein matrix of the photosynthetic antenna complexes LHC II and CP29, respectively, as

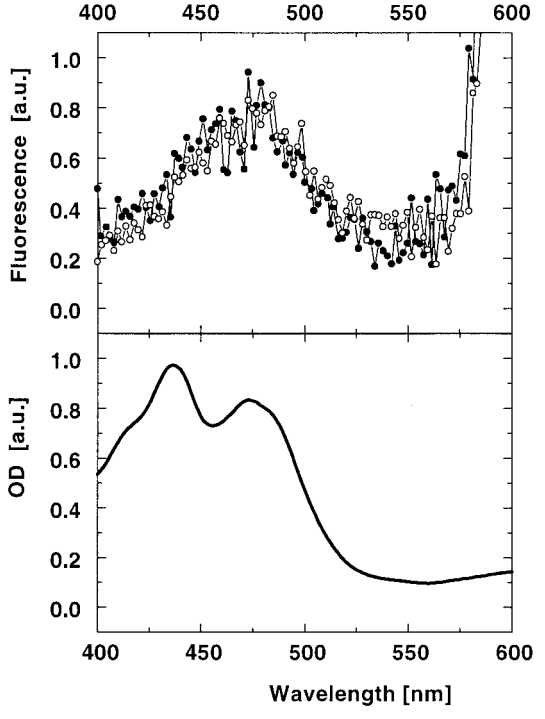

FIGURE 4 (*Upper panel*) Stepwise two-photon excited fluorescence spectra of LHC II excited at 680 nm (*open symbols*) and 650 nm (*full symbols*). (*Lower panel*) Absorption spectrum of trimeric LHC II in the corresponding spectral region.

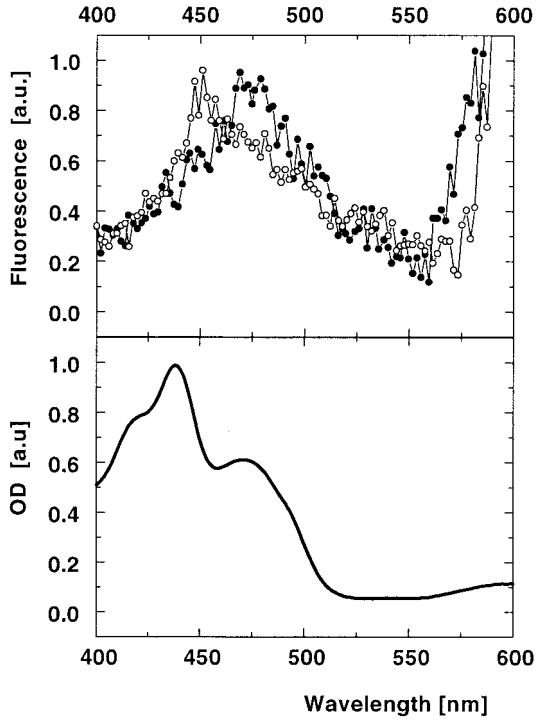

FIGURE 5 (*Upper panel*) Stepwise two-photon excited fluorescence spectra of CP29 excited at 680 nm (*open symbols*) and 650 nm (*full symbols*). (*Lower panel*) Absorption spectrum of CP29 in the corresponding spectral region.

excited in their Q_y absorption bands (Fig. 3) are shown in Figs. 4 and 5.

In the visible absorption spectrum of trimeric LHC II (compare Fig. 3 and lower panel of Fig. 4), the bands around 675 nm and 437 nm can be attributed formally (i.e., without taking strongly coupled dimers or oligomers into account) to the resolved 21 Chls *a* in the structure of the trimer (Kühlbrandt et al., 1994; Rogl and Kühlbrandt, 2000). Similarly, the bands centered around 650 nm and 473 nm can be assigned to the 15 Chls *b*. The short-wavelength fluorescence of LHC II, excited with 100-fs pulses at 680 nm (Chl *a* region), has its maximum at 477 nm (see Fig. 4, upper panel). Note, that there is also very intense "normal" red fluorescence, indicated in Fig. 4 by the ascent at ~570 nm. The weak blue fluorescence band overlaps with the absorption band peaking at 473 nm, which is assumed to be dominated by Chl *b* (with a contribution from xanthophylls). If LHC II is excited in the second maximum of the Q_y absorption band at 650 nm (Chl *b* region, Fig. 3), the spectral shape of the resulting blue fluorescence is the same as with 680-nm excitation (Fig. 4, upper panel). This is in remarkable contrast to the behavior of dissolved Chls *a* and *b*, as outlined above (Fig. 1, *A* and *B*), which show distinct, unique blue fluorescence bands. The result can be interpreted as evidence for excitonic coupling between subbands at ~650 nm and ~680 nm in the Q_y band of trimeric LHC II (i.e., the existence of strongly coupled Chl *a*/*b* dimers or even higher aggregates). For further discussion, see below.

The minor photosystem II antenna complex CP29 (the absorption spectrum of which is displayed in Fig. 3 and Fig. 5, lower panel) was subjected to a similar experimental regime (two-photon excitation at 650 and 680 nm). The absorption in the Q_y region around 650 nm (formally attributable to Chl *b*) of CP29 is rather low, corresponding to the lower Chl *b*/*a* ratio of 2:6 (compare Gradinaru et al., 1998; Pieper et al., 2000). It shows a remarkably different behavior as compared with LHC II in the two-photon excited fluorescence experiment: excitation with 100-fs pulses at 680 nm results in a weak blue fluorescence peaking at 450 nm, whereas excitation at 650 nm yields a blue fluorescence band at 475 nm (compare Fig. 5, upper panel). This behavior is expected, if the absorption subbands centered at ~650 nm and ~680 nm are not excitonically coupled, but rather belong to separate Chl *b* and Chl *a* transitions.

DISCUSSION

A conditio sine qua non for stepwise absorption of two photons (as assumed to underlie the here observed fluorescence from higher excited states of Chls) is a suitable S_1-S_x absorption in the spectral region of the S_0-S_1 transition. Such a feature has been described for a multitude of organic molecules with π-electron systems, in particular for Chls (e.g., Shepanski and Anderson, 1981; Leupold et al., 1990). Fluorescence from a higher excited state of a Chl *a* derivative after excitation at 694 nm with 1-ms pulses of a ruby laser has been observed already in the 1960s by Terenin et al. (1966). The spectral shape of the observed blue emission is, however, different from what is shown here (Fig. 1 *A*). Moreover, also the underlying mechanism (namely triplet-triplet annihilation) as assumed by these authors, is quite different.

The experimental results obtained with CP29 indicate that 100-fs pulse excitation at 680 nm elicits blue fluorescence, which can be attributed (according to the results with the dissolved pigments, vide infra, and the spectral location of the respective B_x absorption band, compare Fig. 5) to Chl *a*, whereas 650-nm excitation yields blue fluorescence typical for Chl *b*. This demonstration of the transitions at ~650 nm and ~680 nm being uncoupled is in line with the conclusions from a recent Q_y spectral substructure analysis of CP29 using nonlinear polarization spectroscopy in the frequency domain (B. Voigt, K.-D. Irrgang, J. Ehlert, W. Beenken, G. Renger, H. Lokstein, and D. Leupold, unpublished results). Hole-burning data obtained at 4.2 K also indicate that the lowest energy state of CP29 is localized on a single Chl *a* molecule (Pieper et al., 2000). However,

results obtained at cryogenic temperature must not necessarily correspond to those at ambient temperatures (vide infra).

The results obtained with CP29 also demonstrate that neither excitation energy transfer from S_1 of Chl *b* to Chl *a* nor back transfer (Chl *a* → Chl *b*) from higher excited states can effectively compete with internal excitation and deactivation mechanisms. This is particularly noteworthy with respect to the interpretation of the above-described results for LHC II. In this case, the experimental data underline that the identical blue fluorescence band shapes obtained with excitation at 650 nm and at 680 nm corroborate the above proposed coupling of the respective Chl *b* and Chl *a* transitions. For both wavelengths, excitation results after internal conversion in the population of the lowest common heterodimer state in the B_x range, which is close to the B_x level of an individual Chl *b* and which is origin of the blue fluorescence.

Regarding LHC II at ambient temperature, there are few (more or less indirect) hints for the existence of Chl *a*/*b* heterodimers in the literature (Renger and May, 1997; Valkunas et al., 1999 and references therein), but to our knowledge there has been no unequivocal experimental proof. However, a recent determination of the dipole moment of the 680-nm transition of LHC II indicated coupling of at least two Chls at ambient temperature (Schubert et al., 2002; compare also Leupold et al., 1999). Although Pieper et al. (1999, 2001) concluded from hole burning and fluorescence line narrowing studies at 4.2 K that the lowest energy Q_y state in LHC II carries the absorption intensity of approximately one Chl *a* molecule, this is not necessarily in contradiction to the presented results indicative of strong coupling and enhanced transition dipole at room temperature. Pieper et al. (1999) state explicitly that there is indication of a conformational change of the protein environment of ~120 K, which may alter excitonic coupling between the pigments.

Without going into details of subband-assignment to definite Chl binding sites in the structural models of LHC II and CP29, the results would fit nicely with an assignment of the above proposed heterodimer to the Chl a2/b2 sites in the in LHC II model (Rogl and Kühlbrandt, 2000; Remelli et al., 1999) and a missing Chl *b* at the b2-binding site in CP29 (compare Gradinaru et al., 1998).

CONCLUSIONS

Summarizing, we have shown that fluorescence from a higher excited Chl state elicited via two-photon excitation in the Q_y spectral region is a novel source of information on nature and spectral shape of "Chl-forms" in pigment-protein complexes. Stepwise two-photon excited fluorescence is especially well suited to unravel strongly coupled Chl *a*/*b* heterodimers. In general, to fully exploit the information available from stepwise two-photon excited fluorescence, the emission profiles in the blue spectral region should be registered by tuning the excitation wavelength continuously over the Q_y region of interest. The excitation pulse duration should be chosen as a compromise—between obtaining the necessary higher excited state population (in competition to fast depopulation processes) via use of short pulses and a sufficient spectral resolution (for subband analysis). A suitable compromise may be attained with pulse durations between 100 and 200 fs. In a first application of the new methodology at physiologically relevant ambient temperature, we have demonstrated the existence of a strongly coupled Chl *a*/*b*-heterodimer in LHC II. Furthermore, evidence is provided that the lowest-energy Chl *a* "form" in CP29 (at ~680 nm) is not excitonically coupled to Chl *b*.

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