ELSEVIER

Biochimica et Biophysica Acta 1556 (2002) 1–5

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Rapid report

Direct evidence for excitonically coupled chlorophylls *a* and *b* in LHC II of higher plants by nonlinear polarization spectroscopy in the frequency domain

Maria Krikunova[a,b], Bernd Voigt[a], Heiko Lokstein[c,*]

[a] *Max-Born-Institut für Nichtlineare Optik und Kurzzeitspektroskopie, Max-Born-Str. 2a, D-12489 Berlin, Germany*
[b] *A.N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, Moscow 119899, Russia*
[c] *Institut für Biologie/Pflanzenphysiologie, Humboldt-Universität zu Berlin, Unter den Linden 6 (Sitz: Philippstr. 13), D-10099 Berlin, Germany*

Received 30 July 2002; accepted 6 August 2002

Abstract

Occurrence of excitonic interactions in light-harvesting complex II (LHC II) was investigated by nonlinear polarization spectroscopy in the frequency domain (NLPF) at room temperature. NLPF spectra were obtained upon probing in the chlorophyll (Chl) *a*/*b* Soret region and pumping in the Q_y region. The lowest energy Chl *a* absorbing at 678 nm is strongly excitonically coupled to Chl *b*.

Keywords: Chlorophyll; Excitation energy transfer; Excitonic interaction; LHC II; Laser spectroscopy; Nonlinear polarization spectroscopy in the frequency domain

Photosynthetic antenna systems capture photons and transfer excitation energy to the reaction centers where photochemistry occurs. Most abundant in higher plants and extensively studied—in particular since its crystal structure was obtained with 3.4 Å resolution [1]—is the main light-harvesting complex (LHC II); for a recent review of its spectroscopic properties, see Ref. [2]. LHC II comprises homo- and mixed trimers of the closely related gene products Lhcb1, Lhcb2 and Lhcb3, according to the nomenclature of Jansson [3]. The absorption spectrum of LHC II in the Q_y region is broadened and somewhat red-shifted as compared to monomeric chlorophyll (Chl) in organic solvents. This is attributed to spectral heterogeneity, that is, the existence of unresolved subbands (so-called Chl forms). The phenomenon of Chl forms may be explained by energetically inequivalent binding sites (pigment–protein interactions) which lead to shifts of the respective pigments absorption bands. Pigment–pigment interactions (excitonic coupling) as an additional source of spectral heterogeneity are usually assumed to be of minor importance for LHC II and related complexes; see, for example, Refs. [2,4–6]. Notably, vanishingly small as well as strong pigment–pigment interactions appear to occur together in the purple bacterial peripheral light-harvesting complex (LH2) of which a high-resolution crystal structure has been obtained [7]. LH2 was shown to consist of a circular aggregate formed by strongly excitonically coupled BChls *a* (B850) and an essentially non-interacting BChl *a* ring (B800) [8]. Electron crystallography of trimeric LHC II revealed that 12 Chls (7 Chls *a* and 5 Chls *b*) per monomeric subunit are densely packed [1]. Center-to-center distances between certain Chls are comparable to the diameters of the molecules, rendering the existence of excitonic interactions among them highly probable. However, at the current resolution, Chls *a* and *b* were not directly distinguishable, so their assignment to the identified binding sites [1] remains tentative. Moreover, orientations of transition-dipole moments of the pigments have not been determined. Thus, theoretical calculations of the extent of excitonic interactions yielded widely varying results [2,5,9,10].

Abbreviations: CD, circular dichroism; Chl, chlorophyll; CP29, minor chlorophyll *a*/*b*-binding protein of M_r 29 kDa; EET, excitation energy transfer; LHC II, light-harvesting complex II; NLPF, nonlinear polarization spectroscopy in the frequency domain

* Corresponding author. Tel.: +49-30-2093-6477; fax: +49-30-2093-6337.
E-mail address: lokstein@mbi-berlin.de (H. Lokstein).

0005-2728/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0005-2728(02)00326-2

Nevertheless, there are some hints for strong excitonic coupling among Chls in LHC II. Circular dichroism (CD) spectra of LHC II show a considerable rotational strength that has been taken as indicative of excitonic interactions [11]. However, an estimate of the coupling strength from CD spectra has not been achieved so far. Ultrafast spectroscopic studies have revealed multiphasic excited state kinetics with lifetimes ranging from about 100 fs to a few nanoseconds [2]. To explain the ultrafast excitation energy transfer (EET) steps observed in one- and two-color pump-and-probe experiments, a model based on excitonically coupled Chl *a*/*b* heterodimers has been developed [10]. However, fluorescence and transient absorption spectroscopic data have also been evaluated in the framework of Förster-type EET [2,4–6]. The results of a recent study [12] employing complementary nonlinear laser spectroscopic techniques also favor the existence of strong excitonic interactions in LHC II. Unfortunately, the experiments did not suffice to distinguish the possibilities whether the lowest energy state in LHC II is formed by a homo- (Chl *a*/Chl *a*) or a heterodimer (Chl *b*/Chl *a*). In LHC II and the minor chlorophyll *a*/*b*-binding protein of M_r 29 kDa (CP29), stepwise two-photon excitation with 100-fs pulses in the Chl *a*/*b* Q_y region elicits a weak blue emission [13]. The dependence of the spectral profile of this fluorescence on excitation wavelength together with a comparison to the properties of Chls in solution are consistent with the existence of strongly coupled Chls *a*/*b* heterodimers in LHC II. Nonlinear polarization spectroscopy in the frequency domain (NLPF) with the minor Photosystem II antenna complex CP29 (being structurally related to LHC II) revealed strong excitonic coupling between certain Chls *a* and *b* [14]. In the current study, we employ a similar approach to investigate occurrence of excitonic coupling between Chls *a* and *b* in LHC II.

LHC II was isolated from freshly harvested pea leaves according to the procedure of Krupa et al. [15]. LHC II in the trimeric state was obtained in a buffer containing 10 mM Tricin (pH 7.8) and 0.06 % *n*-dodecyl β-D-maltoside (Glycon, Luckenwalde, Germany). Absorption, CD and fluorescence spectra were measured before and after the NLPF experiments to monitor sample integrity. NLPF spectra were recorded applying a 90° set-up [14] (see also Fig. 1). Pump (λ_p) and probe (λ_t) beams (characterized by a spectral linewidth of about 0.05 cm^{-1}) are obtained from dye lasers (DCM in DMSO or Coumarin 47/102 for the Q_y and Soret regions, respectively) synchronously pumped by an excimer laser (pulse duration 15 ns). The sample (absorbance ≅ 1.1 at 675 nm) is located in a home-made flow-through cell based on a 5-mm quartz cell (Hellma, Müllheim, Germany) which does not show detectable birefringence. At a 5-Hz repetition rate of the lasers, the sample volume is exchanged after each shot. NLPF spectra were recorded by tuning the pump laser beam from 640 to 690 nm (Chl *a*/*b* Q_y absorption region) and probed in the Chl *a*/*b* Soret region (between 435 and 480 nm at 5-nm steps). Pump beam intensities of about 5×10^{15} photons cm^{-2} $pulse^{-1}$ warrant validity of the χ^3 approach for description of NLPF spectra [16]. NLPF pump and probe spectral ranges are indicated in Fig. 2. NLPF theory, the rationale of this particular NLPF approach, and relevant NLPF model spectra have been described previously [14,16]. The advantages of the current NLPF set-up (in particular, homogeneous illumination of the probed sample volume) are described in detail by Voigt et al. [17].

To detect strong excitonic interactions between Chls *a* and *b* in LHC II, a special variant of the NLPF technique was employed [14]: The approach relies on recording NLPF spectra pumped in a lower energetic absorption band and probed in a (spectrally well-separated) higher energetic absorption band (with negligible overlap and, hence, no trivial EET occurring among them). The NLPF response does not disappear in this case only if the pigments were strongly excitonically coupled. This effect originates from depletion of the *common* ground state of the interacting pigments and enables unequivocal detection of excitonic interactions [14,16]. The visible range absorption spectrum of trimeric LHC II is shown in Fig. 2. Two major absorption regions can be distinguished. The region between 630 and

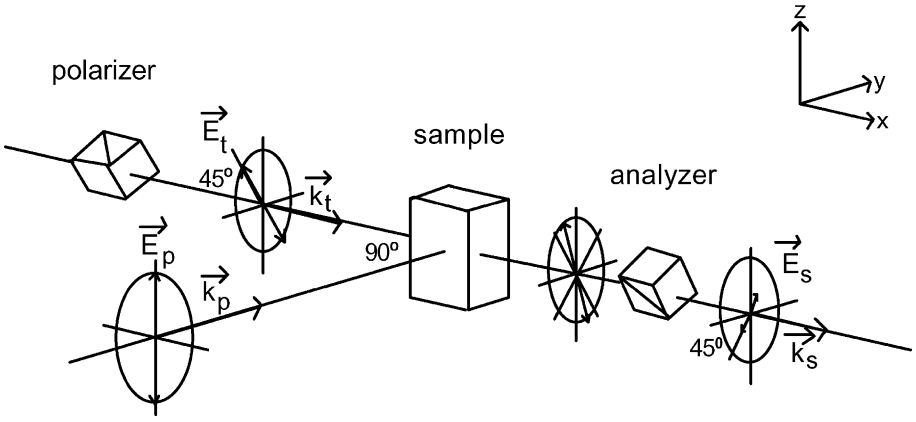

Fig. 1. Principle of NLPF. Pump, probe and signal fields ($\vec{E}$) are distinguished by the indices p, t and s, respectively, as well as the corresponding wave number vectors ($\vec{k}$). The NLPF signal is the component of the signal field which is perpendicular to the incident probe field.

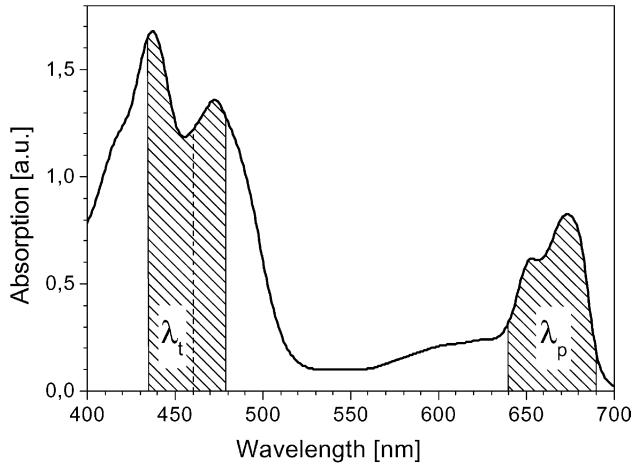

Fig. 2. Absorption spectrum of trimeric LHC II. NLPF probe (λ_t) and pump (λ_p) regions are indicated by shading. The vertical dashed line marks the wavelength (460 nm) above which Chl *a* absorption is negligible; cp. Ref. [17].

690 nm corresponds to the $S_0 \rightarrow S_1$ transition (Q_y band) of Chls *a* and *b*. The prominent bands centered at about 650 and 675 nm can be assigned to Chl *b* and *a*, respectively. The form(s) absorbing at intermediate wavelengths (around 660 nm) cannot be assigned with certainty, yet. The second prominent absorption region in LHC II (between 350 and 520 nm) is the so-called Soret band of Chls *a* and *b*. Note, that also the xanthophylls absorb in the same region (for a discussion of possible xanthophyll contributions to NLPF spectra, see below). More specifically, Chl *a* absorbs between 350 and 460 nm (peaking at about 435 nm). Chl *b* absorbs roughly between 420 and 500 nm, peaking at 480 nm. Hence, the energetic order of Chl *a* and *b* energy levels is reversed in the Soret region (as compared to the Q_y region). NLPF spectra probed at λ_t=435, 445 and 455 (A) and 465, 470, 480 nm (B) and pumped from 640 to 690 nm in the Chl *a*/*b* Q_y region are displayed in Fig. 3. Probe wavelengths of the NLPF spectra in Fig. 3A are all located in the Chl *a* Soret band (with minor absorption of Chl *b* increasing toward longer wavelengths). No contribution of Chl *b* to the NLPF signal can be detected for λ_t=435 nm. Probing at λ_t=445 and 455 nm reveals a minor Chl *b*-associated feature (centered at about 652 nm) in parallel to the accruing Chl *b* absorption. The NLPF spectra display a marked red-shift of their Chl *a*-associated maxima with increasing λ_t. The latter fact indicates that the spectral heterogeneity of the Q_y band is reflected in the Soret band, too. However, this phenomenon may be exploited in a high-resolution subband analysis of the Q_y band, a goal that has not been accomplished so far despite considerable effort. A detailed analysis of the spectral substructure is complicated by the different orientation of the dipole moments of the respective transitions and will be dealt with in a forthcoming paper. However, from the data in Fig. 3A, no unequivocal inference toward excitonic interactions between certain Chls *a* and *b* can be drawn since trivial EET would obscure effects of them.

A deconvolution of the LHC II Soret-absorption spectrum has revealed that Chl *a* absorption in LHC II is negligible above 460 nm [18]. Hence, probe wavelengths of the NLPF spectra in Fig. 3B are exclusively located in the Chl *b* Soret region. Consequently, most of the NLPF signal derives from the Chl *b* partition of the Q_y band (635–655 nm)—in case of λ_t=465 nm even the entire signal. A shift of the NLPF signal of the Chl *b*-associated band with shifting

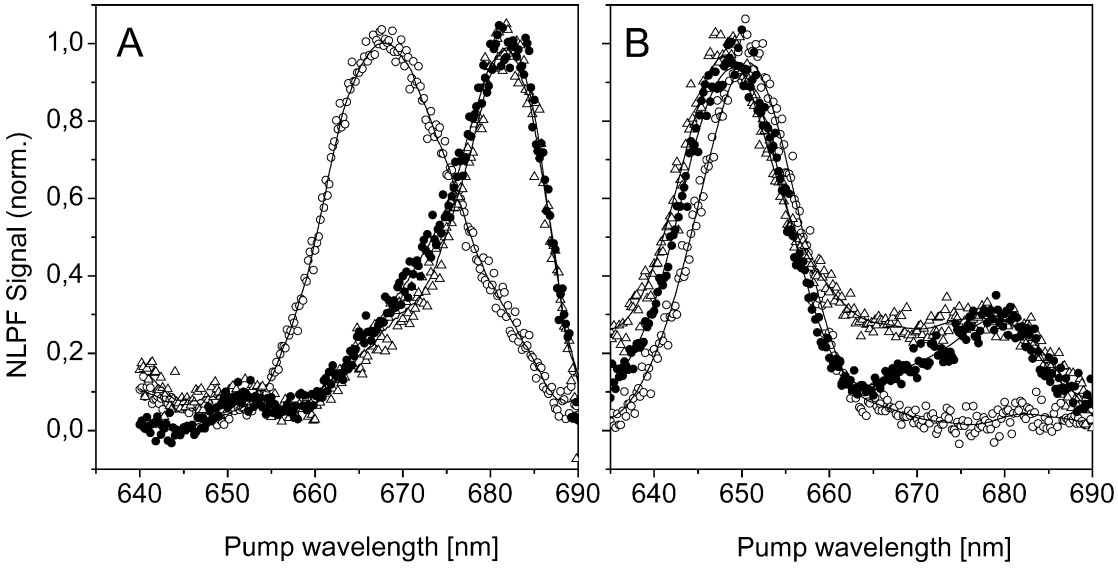

Fig. 3. NLPF spectra of LHC II obtained at different probe wavelengths; (A) 435 nm (open circles), 445 nm (open triangles) and 455 nm (full circles) in the Chl *a* Soret (and xanthophyll absorption) region; (B) 465 nm (open circles), 470 nm (full circles) and 480 nm (open triangles) corresponding to the Chl *b* Soret (and xanthophyll absorption) regions.

λ_t (indicative of spectral heterogeneity) can also be observed—it is, however, much less pronounced than in the Chl *a* range.

Remarkably, NLPF spectra obtained at λ_t=470 and 480 nm exhibit an additional feature in the spectral range of Chl *a* (at about 678 nm). The latter feature is observed even though λ_t is beyond the Chl *a* Soret region ($\lambda > 465$ nm). In other words, exciting a Chl *a* low-energy Q_y state (670–690 nm), a NLPF response is obtained in the far apart higher energy Chl *b* Soret region. This observation can only be explained assuming strong excitonic coupling between certain Chls *a* and *b*; cp. also Ref. [14]. One could object that the NLPF signal obtained upon probing in the 465- to 480-nm region may derive (at least in part) from ground state depletion of the xanthophylls (absorbing in the same region) via interaction with Chl *a* (EET, either singlet–singlet or triplet–triplet). However, the observation that the Chl *a*-associated NLPF signal (around 678 nm) is absent at λ_t=465 nm (strong xanthophyll absorption as compared to Chl *a*; cp. Ref. [18]) argues against this possibility. Further arguments dispelling possible contributions of xanthophyll or low-dipole strength Chl *a*-excited states can be found in Ref. [14]. The observation of strong excitonic Chl *a*/*b* interactions has also been made in NLPF experiments with the structurally related antenna complex CP29 [14]. Nevertheless, marked differences are also revealed between these antenna complexes: whereas in LHC II the lowest energy Chl *a* (absorbing at about 678 nm) appears to be part of a strongly excitonically coupled pigment cluster (involving also Chl *b*), the Chl form with the lowest energy in CP29 can be attributed to a not excitonically coupled Chl *a* [14]. The interacting pigments in CP29 were assigned to a Chl *a* absorbing at 670 nm and a Chl *b* absorbing at 640 nm. These observations are consistent with the assignment of the longest wavelength band to Chl *a2* in the LHC II structure [19–21] the binding site of which is lacking in CP29. This is corroborated by a variety of studies both at room and low temperatures, for example, Refs. [13,19–22]. In LHC II and CP29, stepwise two-photon excitation with 100-fs pulses in the Q_y region of Chls *a* and *b* elicits a blue fluorescence with a quantum yield less than 10^{-4} [13]. The blue emission profiles of LHC II (peaking at 475 nm, and hence, can be assigned as originating from the Chl *b* Soret band) are virtually identical upon either Chl *a* (680 nm) or Chl *b* (650 nm) excitation. This observation is consistent with the existence of strongly coupled Chls *a* and *b* in LHC II. Under similar conditions, two peaks—one at 450 nm (upon Chl *a* excitation) and one at 475 (upon Chl *b* excitation) are observed in CP29 [13]. Nonlinear absorption of 120-fs pulses indicates the existence of a spectral form with about two-fold increased absorption cross section in the red wing of the Q_y band of trimeric LHC II (as compared to monomeric Chl *a*) [12]. The results were corroborated by intensity-dependent NLPF experiments [12]. A spectrum of the NLPF saturation parameter, ξ, as generated from these experiments indicated that a spectral form emitting at 682 nm is characterized by a 2.2 ($\pm$0.8) increased absorption and emission dipole strength. The most obvious explanation for the observed enhancement is excitonic interaction between Chl molecules forming the terminal emitter in LHC II. From a very recent steady-state study of superradiance, it was concluded that the lowest energy state in LHC II carries at most 1.18 the dipole strength of free Chl *a* (in solution), that is, is essentially monomeric [23]. However exciton delocalization is strongly time dependent and steady-state superradiance measurements provide only the final (thermalized) value [24]. It has to be mentioned that NLPF measurements are carried out under quasi-stationary conditions (the 15-ns pump pulse duration is considerably longer than all relaxation time constants of the system under investigation). The measured NLPF signal, however, contains only the nonlinear sample response on the pump field and, hence, is fundamentally different from the steady-state conditions in the fluorescence yield measurements [16]. In conclusion, NLPF provides a direct access to assay excitonic interactions in LHC II under physiological conditions.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (SFB 429, TP A2) is gratefully acknowledged. M.K. was supported by postgraduate scholarships by the DAAD and the Stifterverband für die Deutsche Wissenschaft (Dr. E. und H. Diener-Stiftung). The authors acknowledge helpful discussions with Dr. D. Leupold.

References

[1] W. Kühlbrandt, D.N. Wang, Y. Fujiyoshi, Nature 367 (1994) 614–621.
[2] H. van Amerongen, R. van Grondelle, J. Phys. Chem., B 105 (2001) 604–617.
[3] S. Jansson, Biochim. Biophys. Acta 1184 (1994) 1–19.
[4] E. Giuffra, G. Zuccelli, D. Sandona, R. Croce, D. Cugini, F.M. Garlaschi, R. Bassi, R. Jennings, Biochemistry 36 (1997) 12984–12993.
[5] G. Trinkunas, J.P. Connelly, M.G. Müller, L. Valkunas, A.R. Holzwarth, J. Phys. Chem., B 101 (1997) 7313–7320.
[6] G. Cinque, R. Croce, A. Holzwarth, R. Bassi, Biophys. J. 79 (2000) 1706–1717.
[7] G. McDermott, S. Prince, A. Freer, A. Hawthornthwaite-Lawless, M. Pariz, R. Cogdell, N. Isaacs, Nature 374 (1995) 517–521.
[8] D. Leupold, H. Stiel, K. Teuchner, F. Nowak, W. Sandner, B. Ücker, H. Scheer, Phys. Rev. Lett 77 (1996) 4675–4677.
[9] E.I. Iseri, D. Gülen, Eur. Biophys. J. 30 (2001) 344–353.
[10] T. Renger, V. May, Phys. Rev. Lett. 84 (2000) 5228–5231.
[11] P.W. Hemelrijk, S.L.S. Kwa, R. van Grondelle, J.P. Dekker, Biochim. Biophys. Acta 1098 (1992) 159–166.
[12] A. Schubert, W. Beenken, H. Stiel, B. Voigt, D. Leupold, H. Lokstein, Biophys. J. 82 (2002) 1030–1039.
[13] D. Leupold, K. Teuchner, J. Ehlert, K.-D. Irrgang, H. Lokstein, Biophys. J. 82 (2002) 1580–1585.
[14] B. Voigt, K.-D. Irrgang, J. Ehlert, W. Beenken, G. Renger, D. Leupold, H. Lokstein, Biochemistry 41 (2002) 3049–3056.
[15] Z. Krupa, N.P.A. Huner, J.P. Williams, E. Maissan, D.R. James, Plant Physiol. 84 (1987) 19–24.

[16] W. Beenken, J. Ehlert, J. Chem. Phys. 109 (1998) 10126–10137.
[17] B. Voigt, F.R. Nowak, W. Beenken, Meas. Sci. Technol. 10 (1999) N7–N11.
[18] C.C. Gradinaru, I.H.M. van Stokkum, A.A. Pascal, R. van Grondelle, H. van Amerongen, J. Phys. Chem., B 104 (2000) 9330–9342.
[19] H. Rogl, W. Kühlbrandt, Biochemistry 38 (1999) 16214–16222.
[20] R. Remelli, C. Varotto, D. Sandonà, R. Croce, R. Bassi, J. Biol. Chem. 274 (1999) 33510–33521.
[21] H. Rogl, R. Schödel, H. Lokstein, W. Kühlbrandt, A. Schubert, Biochemistry 41 (2002) 2281–2287.
[22] J. Pieper, K.-D. Irrgang, M. Rätsep, J. Voigt, G. Renger, G.J. Small, Photochem. Photobiol. 71 (2000) 574–581.
[23] M.A. Palacios, F.L. de Weerd, J.A. Ihalainen, R. van Grondelle, H. van Amerongen, J. Phys. Chem., B 106 (2002) 5782–5787.
[24] M. Dahlbohm, T. Pullerits, S. Mukamel, V. Sundström, J. Phys. Chem., B 105 (2001) 5515–5524.