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Ultrafast chlorophyll *b*–chlorophyll *a* excitation energy transfer in the isolated light harvesting complex, LHC II, of green plants

Implications for the organisation of chlorophylls

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Abstract

The excitation energy transfer between chlorophyll *b* (Chl *b*) and chlorophyll *a* (Chl *a*) in the isolated trimeric chlorophyll-*a*/*b*-binding protein complex of spinach photosystem 2 (LHC II) has been studied by femtosecond spectroscopy. In the main absorption band of Chl *b* the ground state recovery consists of two components of 0.5 ps and 2.0 ps, respectively. Also in the Chl *a* absorption band, at 665 nm, the ground state recovery is essentially bi-exponential. In this case is, however, the fastest relaxation lifetime is a 2.0 ps component followed by a slower component with a lifetime in the order of 10–20 ps. In the Chl *b* absorption band a more or less constant anisotropy of $r = 0.2$ was observed during the 3 ps the system was monitored. In the Chl *a* absorption band there was, however, a relaxation of the anisiotropy from $r = 0.3$ to a quasi steady state level of $r = 0.18$ in about 1 ps. Since the 0.5 ps component is only seen upon selective excitation of Chl *b* we assign this component to the energy transfer between Chl *b* and Chl *a*. The other components most likely represents redistribution processes of energy among spectrally different forms of Chl *a*. The energy transfer process between Chl *b* and Chl *a* can well be explained by the Förster mechanism which also gives a calculated distance of 13 Å between interacting chromophores. The organisation of chlorophylls in LHC II is discussed in view of the recent crystal structure data (1991) Nature 350, 130].

Key words: Chlorophyll *a*; Chlorophyll *b*; Energy transfer; Light-harvesting complex; Femtosecond spectroscopy; Anisotropy; Photosystem 2; LHC II

1. Introduction

The very first steps in photosynthesis of green plants is the excitation of accessory pigments in the antenna system of a photosynthetic unit. This is followed by a chain of energy transfer steps that funnels the excitation energy from the antenna to the reaction centre where its trapping leads to charge separation [1]. The light-harvesting antenna of photosystem 2 consists of different Chl *a*/*b* proteins [2] of which the most abundant is LHC II [3,4]. LHC II consists of several distinct but closely homologous polypeptides in the 25–28 kDa range [5]. Each of these polypeptides non-covalently binds 8 chl *a* and 7 chl *b* molecules [3]. It is believed that LHC II is organized as trimeric units in the photosynthetic membrane [5–7]. Different sub-populations of LHC II can be isolated, and although they show different polypeptide composition, the pigment content and pigment organization of the LHC II sub-populations is very similar [5]. The structure of LHC II has recently been determined to 6 Å resolution by electron crystallography [3]. The structure shows that the chlorophylls are arranged in two levels close to the stromal and lumenal side of the photosynthetic membrane. The upper level harbours eight chlorophylls organized in a ring-like fashion while the lower level, presumably located at the lumenal side, contains seven chlorophylls. The resolution of the crystal structure is, however, insufficient for identifying individual molecules as Chl *a* or Chl *b*. Based on spectroscopic data it has been suggested that Chl *b* is organized in trimeric configuration, the so-called van Metter–Shepanski–Knox model [8–10]. The crystal structure, however, rules out this possibility. The kinetics of excitation energy transfer between Chl *b* and Chl *a* in LHC II has been studied using picosecond absorption spectroscopy [11,12]. It was, however, not possible to resolve the energy transfer between Chl *b* and Chl *a* probably due to

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Abbreviations: Chl, Chlorophyll; LHC II, light-harvesting chlorophyll *a*/*b*-protein complex of photosystem 2; PS II, photosystem 2; OGP, *n*-octyl-β-D-glucopyranoside; DM, dodecylmaltoside.

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limited time resolution in these studies [11,12]. In addition, the measurements were performed in the presence of high concentrations of Triton X-100 in which LHC II is unstable and its oligomeric configuration undefined. With a better time resolution Eads et al. [13] were able to study this particular process in the photosynthetic algea, *Chlamydomonas reinhardtii* mutant C2, and found that the energy transfer occurred within 1 ps for this system. The study [13] thus demonstrated the need for better time resolution and in this work we have reexamined the excitation energy transfer process between chlorophylls in isolated trimeric LHC II and its sub-populations using a time resolution of about 100 fs. This has enabled us, for the first time, to fully resolve the energy transfer between Chl *b* and Chl *a* in isolated native trimers of LHC II. The obtained transfer rates were used to estimate the distances between interacting chlorophylls. It was found that the Förster model gives dinstances in agreement with the crystallographic data [3].

2. Materials and methods

Isolation of the bulk LHC II was performed according to [14]. Briefly, isolated thylakoid membranes were solubilized with Triton X-100 and LHC II was isolated by sucrose gradient centrifugation, followed by 300 mM KCl induced aggregation [15] of the purified LHC II. The final LHC II preparation was suspended in 0.05% DM (Biomol), 25 mM HEPES pH 7.5, or 1% (w/v) OGP (Sigma) 25 mM HEPES pH 7.5, rapidly frozen in liquid nitrogen and stored at −70°C. Subpopulations of LCH II, [5] were isolated from solubilized photosystem 2 membranes [14] by non-denaturing isolelectric focusing using the flat-bed Ultradox method described in [16]. The resolved LHC II subpopulations were collected in 1% *n*-octyl-*β*-D-glucopyranoside, 25 mM HEPES, pH 7.5. The purity and polypeptide composition of the LHC II-preparations were analysed by SDS-PAGE [17] at 4°C and at room temperature [18]. In the spectroscopical experiments LHC II were diluted in 1% OGP 40 mM sucrose, 8 mM NaCl, 8 mM Tricine, pH 7.5. LHC II trimers were diluted the medium 20 mM HEPES pH 7.5 with 1% (w/w) OGP. Under these conditions LHC II is mainly in a trimeric configuration [3,5]. Typical absorbance in the femtosecond experiments was an OD (optical density) of 5–6 per cm and around 0.2 in the fluorescence experiments. The femtosecond light source consisted of a modelocked CW Nd:YAG laser (Spectra Physics) synchronously pumping a kiton red cavity dumped dye laser (Spectra Physics). Femtosecond pulses were obtained by compressing the optical pulses before and after the dye laser with fiber prism compressors (Spectra Physics). The duration of the optical pulses was typically around 100 fs (FWHM) and the bandwidth of the pulse was approximately 5 nm (FWHM). The experiments were performed with the well known absorption-recovery technique at room temperature and the polarisation of the probe pulses was set at parallel, perpendicular or magical angle polarisation relative to the polarisation of the excitation pulse with a Fresnel romb. The time resolved anisotropy was then calculated according to the standard expression:

$$r(t) = \{I_{para}(t) - I_{perp}(t)\}/\{I_{para}(t) + 2\,I_{perp}(t)\}.$$

The time-resolved isotropical data were analyzed with the program Spectra processor (S. Savikhin Software).

Steady-state fluorescence spectra and excitation spectra at room temperature were carried out using a Spex fluorolog 112.

The Förster radius of Chl *b* was determined according to Kawski et al. [19] by calculating the overlap integral between Chl *b* emission spectra and Chl *a* absorption spectra, Chls were diluted in diethyl ether, and was found to be 42 Å. For Chl *b* we used an extinction coefficient of 56,000 $M^{-1} \cdot cm^{-1}$ and for Chl *a* 90,000 $M^{-1} \cdot cm^{-1}$ [20]. The fluorescence lifetime of Chl *b* was calculated from the radiative lifetime and the quantum yield of Chl *b* and was found to be 3.0 ns.

The radiative lifetime was determined from the integrated absorption spectra. The quantum yield was determined using Cresyl violet in ethanol whose fluorescence quantum yield is 0.54 [21] as a reference substance. Chl *b* and Chl *a* were purchased from Sigma and were used without further purification.

3. Results and discussion

Both isolated bulk LHC II and the LHC II sub-populations had chlorophyll *a*/*b* ratios of 1.0–1.2 and showed major absorption peaks at 675 and 652 nm due to Chl *a* and Chl *b*, respectively. Two different LHC II subpopulations were studied in this work, one containing only a single 27 kDa polypeptide, the other containing both the 27 kDa and a 25 kDa polypeptide in a 1:1 ratio [4]. The bulk LHC II had a 27 kDa to 25 kDa ratio of 4:1. In spite of the difference in polypeptide composition, the energy transfer dynamics were identical in all isolated LHC II fractions showing that the different polypeptides coordinate chlorophyll in the same manner. After selectively exciting Chl *b* at 650 nm the system recovered to the ground state with two exponential phases (Fig. 1). The first phase had a lifetime of ~ 0.5 ps and the amplitude associated with this component was 80% of the total signal intensity. The second step had a lifetime of ~ 2 ps with an amplitude of 20%. At 665 nm the 0.5 ps component was not observed, instead the fastest relaxation phase now had a lifetime of ~ 2.0 ps followed by a second relaxation phase with a lifetime of 15–20 ps (Fig. 2). In this case the 15–20 ps component was the most intensive with a relative amplitude of 70–80%. Since the 0.5 ps component only appeared when Chl *b* was excited we assign this component to the energy transfer process between Chl *b* and Chl *a*. This transfer

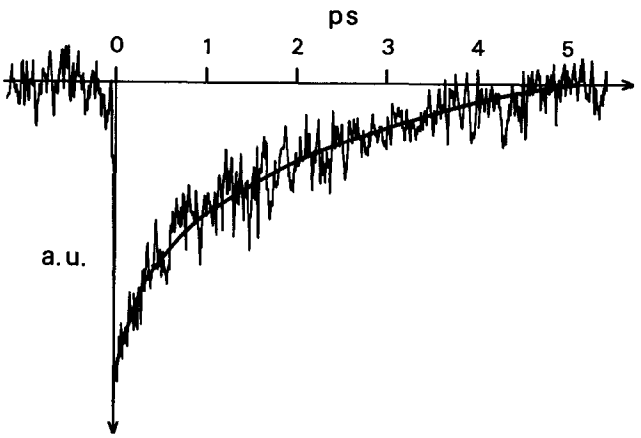

Fig. 1. Absorption recovery experiment at 650 nm on LHC II trimers of 25 and 27 kDa chlorophyll proteins with magic angle polarization between excitation and analyzing pulses. The best fit of the data gave 0.5 ps component, of 80% of the total signal amplitude, and a 2.0 ps component with the remaining 20% of the signal amplitude.

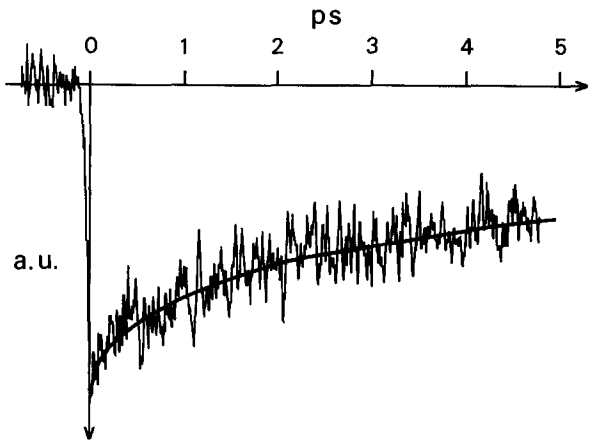

Fig. 2. Absorption recovery experiment at 665 nm on LHC II trimers 25 and 27 kDa chlorophyll proteins with magic angle polarization between excitation and analyzing pulses. The best fit of the data gave 2.0 ps component, of 20% of the total signal amplitude, and a 10–20 ps component with the remaining 80% of the signal amplitude.

time is in good agreement with what Eads et al. [13] observed for a photosynthetic algea in a fluorescence up-conversion experiment. It should also be mentioned that there does not seem to excist an initial faster component that is limited by the intrumental response profile. The more long-lived components, i.e. the 2 ps and 15–20 ps components probably represents transfer and redistribution of the excitation energy between different spectral forms of Chl *a*. The appearence of the 2 ps component at 650 nm could also be explained by the fact that even at 650 nm there is substantial absorption by Chl *a*. The red edge of the femtosecond pulses, which have a bandwidth of about 5 nm (FWHM), also promotes direct excitation of Chl *a*. An estimate shows however, that no more 1–2 Chl *a* per Chl *a*/*b* monomer is excited at 650 nm. The Förster mechanism or the so-called weak dipole–dipole interaction deals with the situation when chemically distinghuishable molecules such as Chl *a* and Chl *b* are interacting and provides an explicit relation between transfer time and the distance and relative orientation between energy exchanging chromphores [22]. Although there excists several spectral indications of excitonic interactions between chlorophylls of the same type and also between Chl *b* and Chl *a* a more recent study by Schmidt et al. [23] shows that there are only indications of interactions in circular dichroism spectroscopy between identical Chl's, but not between Chl *b* and Chl *a*. Subsequently, we have found that our data (in contrast to Eads et al. [13]) can be reasonably well interpreted within the Förster mechanism. Furthermore, we have recently shown that the Förster mechanism is valid for closely interacting chromophores in allophycocyanin (APC) [24], phycocyanin (CPC) [25] and phycoerythrocyanin trimers (PEC) [26] of the phycobilisome in red algea and cyanobacteria. Using a Förster radius of 42 Å, a fluorescence lifetime of 3.0 ns for Chl *b* and a orientation factor of 1.5, which corresponds to a planar configuration of interacting chromophores (Eisinger et al. [27]), and a rate constant of $(0.5\ \text{ps})^{-1}$ we calculate a distance of 13 ± 1 Å between interacting Chl *b* and Chl *a* molecules.

This should be compaired to the 13–14 Å distance that Kuhlbrandt et al. [3] estimated between the two layers of chlorophylls close to the stromal and lumenal sides of the membrane resolved in the crystal structure. This means that the 0.5 ps component could represent the energy transfer process between these two layers of chlorophylls in a monomeric unit assuming a Förster-type of energy transfer process.

The anisotropy associated with Chl *b* has a rather constant value of $r = 0.19$ during the coarse of the measurement. It should, however, be mentioned that the two data points closest to time zero with an anisotropy around 0.27 indicates an anisotropy decay not fully resolved in this experiment. The initial decay of the anisotropy thus seems to occur within 100 fs and probably reflects energy transfer closely situated Chl *b* chromophores (9–11 Å). This is in principle the same behaviour of the anisotropy that was observed by Gillbro et al. [11] although the anisotropy in that case was as high as 0.4 for more than 5 ps. Recent studies reveals, however, that the anisotropy observed in this study should be considered as a quasi steady state anisotropy. Kwa et al. [12] observed, for instance, no anisotropy during the coarse of their measurement and according to Hemelrijk et al. [28] there is only very little polarisation around 650 nm even at 77 K.

Our results indicate that during the excited state life time of Chl *b* there is only limited movement of excitation energy between different Chl *b* molecules. Instead it appears as if the energy is very rapidly localized on a few Chl *b* molecules, with their transition dipoles distinct from parallel orientation, that donates their excitation

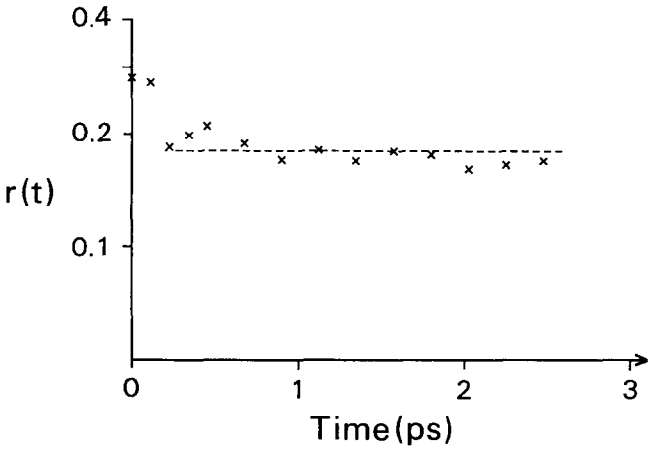

Fig. 3. Time time resolved anisotropy $r(t)$ at 650 nm for LHC II trimers 25 and 27 kDa chlorophyll proteins.

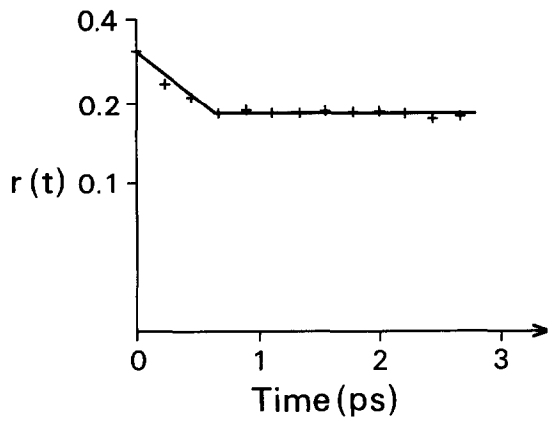

Fig. 4. The time resolved anisotropy $r(t)$ at 665 nm for LHC II trimers 25 and 27 kDa chlorophyll proteins, The anisotropy decays from 0.3 to 0.2 in 0.8 ps. The anisotropy was constant during the remaining 4 ps of the measurement.

energy to Chl *a* molecules. Chl *a* has at early times an anisotropy of 0.30 which drops to 0.18 in approximately 1 ps. These initial fast changes must be the result of energy transfer between different Chl *a*'s with different orientation in the Chl *a/b* complex. This is in principle the same behaviour as observed by Gillbro et al. [11] although the steady state level in that case was around 0.28 and the relaxation of the initial phase was much slower, 20 ps. It was, however, not possible to trace any anisotropy in the steady state fluorescence experiments around 665 nm in this case (data not shown).

Since the anisotropy at 650 nm at early times was distinct from $r = 0.4$ only indicating a fast relaxation, some initial depolarization has occurred on the hundred femtosecond timescale. A plausible explanation for this could be the excited state absorption (ESA) [12]. ESA has been found to dominate the relaxation process at 640 nm but at 645–665 nm Kwa et al. [12] found that the signal consists of both ESA and photobleaching. The fact that the anisotropy is not equal to 0.4 during the course of the measurements indicates that the initial excitation is not staying on only one Chl *b* and from there transfer to Chl *a*, but there seems to be ultra fast transfer between two Chl *b* chromophores. This is in contrast to the situation in for instance the APC trimer where an anisotropy of $r = 0.4$ is observed during the course of the measurement [24]. Turning to the anisotropy of Chl *a* the development with time indicates transfer to more redshifted forms of Chl *a*. Since the anisotropy of Chl *a* reaches some quasi steady state level during the first 5 ps when we monitored the system, this probably reflects a slower transfer of energy to other spectral forms of Chl *a*. Steady state fluorescence anisotropy indicates, that the system has a low order and it is only at very low temperatures that it is possible to detect some polarization at wavelengths longer than 690 nm [28]. Based on our observations we propose the following organisation of chlorophylls in the monomeric unit of LHC II. The fastest component we could resolve was the 0.5 ps lifetime of excited Chl *b* which is the time for transfer of excitation energy to Chl *a*. This was also observed in the fluorescence study by Eads et al. [13]. With this transfer time and assuming a Förster-type interaction mechanism, we calculated a distance between interacting chromophores of ± 13 Å which is similar to the separation between the two Chl layers (13–14 Å) in the crystal structure of Kuhlbrandt et al. [3]. It thus seems possible that each layer harbours only one type of Chl, namely *a* or *b*. Presumably then the eight Chl's in the upper level of the crystal structure is of *a* type and the remaining seven in the lower level is of *b* type. If there was a mixture of Chl's *a* or *b* in each level then one should expect a faster excited state relaxation of Chl *b* representing an energy transfer process to very closely situated Chl *a*. In our LHC II measurements, we can, however, not observe any isotropic component faster than 500 fs. Thus, our data is not consistent with a model where a Chl *a* and Chl *b* are evenly distributed between the two chlorophyll layers in LHC II but rather with a model where the Chl *a*'s are present in the upper layer and the Chl *b*'s are located in the lower lumenalfacing side of the lipid bilayer. We believe that this model can best be tested by a combination of future high-resolution structural data and femtosecond spectroscopy.

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