

## S31-003

### Molecular origin of red pigments in a peripheral light-harvesting antenna of Photosystem I: Ultrafast absorption spectroscopy of recombinant Lhca4

AN Melkozernov<sup>1,2</sup>, S Lin<sup>1,2</sup>, VHR Schmid<sup>3</sup>, E Lago-Places<sup>3</sup>, H Paulsen<sup>3</sup>, and RE Blankenship<sup>1,2</sup>

<sup>1</sup>Department of Chemistry and Biochemistry and <sup>2</sup>Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ, 85287-1604, USA.

[alexander.melkozernov@asu.edu](mailto:alexander.melkozernov@asu.edu)

<sup>3</sup>Institut für Allgemeine Botanik, Johannes Gutenberg-Universität, 55099 Mainz, Germany. [vschmid@mail.uni-mainz.de](mailto:vschmid@mail.uni-mainz.de)

**Keywords:** LHCI, antenna, femtosecond spectroscopy, energy transfer, low energy chlorophylls

#### Introduction

A remarkable feature of Photosystem I in higher plants and green algae are chlorophylls absorbing and emitting at energies lower than P700. A dominant portion of the red fluorescence comes from a peripheral light-harvesting antenna (LHCI), specifically from its LHCI-730 subpopulation. One of the constituents of the LHCI-730 heterodimer, the Lhca4 subunit, was found to harbor the low energy (red) pigments emitting at 730 nm (Tjus et al. 1995; Schmid et al. 1997; Knoetzel et al. 1998). Excitation energy transfer processes in the LHCI-730 and molecular organization of the red pigments that lead to the excitation energy localization are poorly understood. 77 K transient absorption difference spectra of Lhca4 upon excitation of Chl *b* revealed the presence of several ultrafast energy transfer processes with yet-unresolved lifetimes (Melkozernov et al. 2000a). Two major energy transfer processes include a 400-600 fs energy transfer between spectral forms of Chl *b* and Chl *a* followed by 3-5 ps energy equilibration between Chl *a* molecules - including those involved in the red pigment's transition at 705 nm. A significant red shift of the C-705 spectral form as compared to the major Chl *a* spectral form at 679 nm might be a result of a strong excitonic interaction of the Chl *a* molecules localizing excitations in the Lhca4. Ihalaïnen et al. (2000) suggested the presence of one Chl *a* dimer per LHCI-730 based on steady state spectroscopy of native LHCI complexes. Recently, Schmid et al. (2001) obtained evidence of Chl *b* involvement in the long-wavelength transition in Lhca4 based on steady state spectroscopy analysis of reconstituted Lhca4. In this work we use ultrafast transient absorption spectroscopy at 8 K to probe coupling of Chl *a* and Chl *b* in the recombinant Lhca4 polypeptides with changed pigment occupancy as compared to the wild type Lhca4.

#### Materials and methods

**Preparation of reconstituted Lhca4.** Lhca4 protein overexpression and reconstitution of polypeptides with mixtures of Chl *a*, Chl *b* and xanthophylls were described earlier (Schmid et al. 2001). The E102S mutation of the Lhca4 polypeptide was obtained by the PCR based method described by Chen and Przybyla (1994). Isolation of reconstituted Lhca4 (r-Lhca4) by density gradient ultracentrifugation and pigment analysis was as in Schmid et al. (2001).

**Femtosecond transient absorption spectroscopy.** For time-resolved absorption spectroscopy the samples with an OD of  $\sim 1$  per 0.12 cm were resuspended in 20 mM Tricine-NaOH, pH 7.8 containing 0.04 %  $\beta$ -dodecylmaltoside, 67% glycerol and frozen to 8 K using a closed-cycle helium cryostat (APD Cryogenics). Transient absorption difference spectra of r-Lhca4 polypeptides were measured on 3, 5 or 50 ps time scales in the 600-750 nm spectral region using the femtosecond spectrometer described earlier (Melkozernov et al. 2000b). 200 fs laser pulses (fwhm = 4-6 nm) were used to excite Chl *b* molecules in the  $Q_y$  absorption band at 635, 640, 645 and 650 nm and carotenoids in the  $S_2$  absorption band at 502 or 510 nm. Global analysis of the kinetics was performed as described earlier (Melkozernov et al. 2000b).

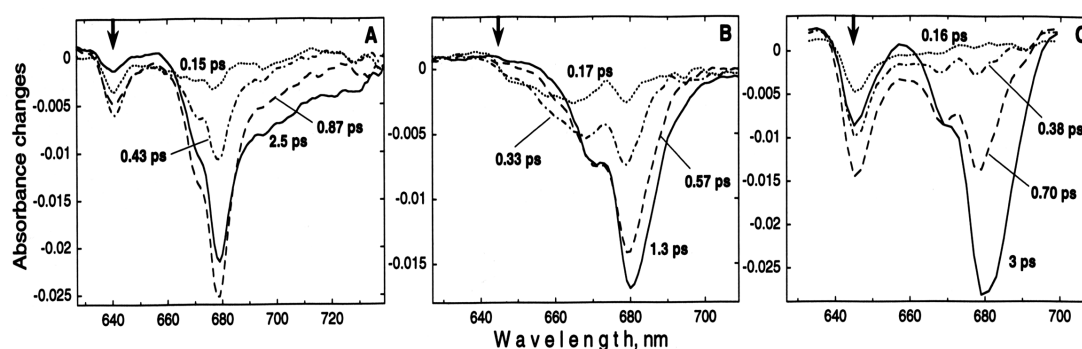
## Results and Discussion

**Pigment content of r-Lhca4.** Table 1 illustrates the pigment content of the samples used in the study. WT Lhca4<sub>Chl *a+b*</sub> binds  $\sim 2$  Chl *b* and 4-5 Chl *a*, while all pigment-binding sites ( $\sim 7$ ) in WT Lhca4<sub>Chl *a*</sub> are occupied by Chl *a*. E102S mutation results in a loss of Chl *b* and Chl *a* upon refolding.

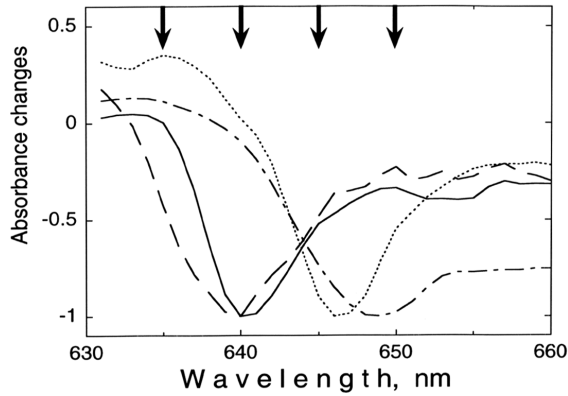
**Table 1.** Chlorophyll content of r-Lhca4 polypeptides (mol relative to 1 mol Lutein) determined by reversed phase HPLC.

Sample (abbreviation)	Chl <i>b</i>	Chl <i>a</i>	Chl <i>a+b</i>
Wildtype r-Lhca4 (WT Lhca4 <sub>Chl <i>a+b</i></sub> )	1.89	4.79	6.68
Wildtype r-Lhca4 with all binding sites occupied by Chl <i>a</i> (WT Lhca4 <sub>Chl <i>a</i></sub> )	0	6.78	6.78
r-Lhca4 with E102S mutation (E102S Lhca4 <sub>Chl <i>a+b</i></sub> )	1.34	4.23	5.57

**Excitation dynamics in wild type Lhca4 at 8K.** The binding of 6-7 pigments by the WT Lhca4<sub>Chl *a+b*</sub> protein results in the appearance of six Chl spectral forms in the  $Q_y$  transition region of Chl *a* and *b* (Schmid et al. 2001). Based on the 77 K derivative spectroscopy of r-Lhca4 these spectral forms were assigned to Chl *b* (C-644), Chl *a* (C-661, C-673, C-681 and C-687). The C-705 red pigment's transition was tentatively assigned to the Chl *a*-Chl *b* dimer (C-705). Fig. 1A illustrates the spectral evolution of



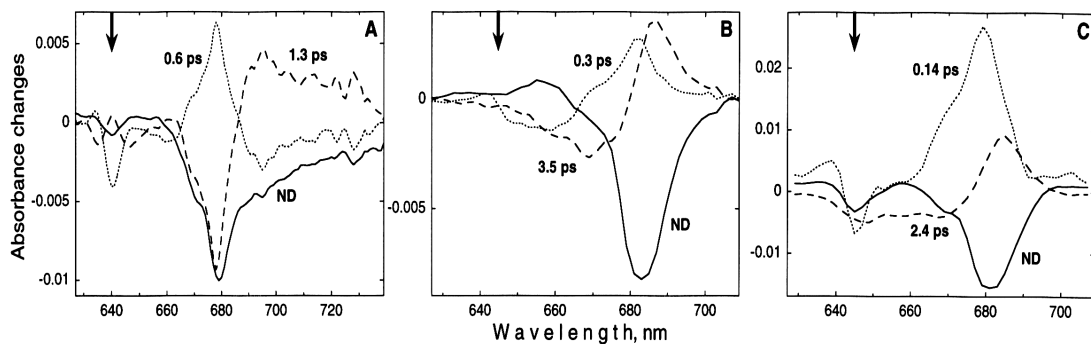
**Figure 1.** Transient absorption difference spectra of WT Lhca4<sub>Chl *a+b*</sub> (A), WT Lhca4<sub>Chl *a*</sub> (B) and E102S Lhca4<sub>Chl *a+b*</sub> (C) detected at 8 K on the 3 ps time scale with excitation in the region of Chl *b*  $Q_y$  transition. Spectra are shown at representative pump-probe delays. Arrows indicate the excitation wavelengths: 640 nm (A) and 645 nm (B, C). Spectral width of the excitation pulses was  $\sim 5$ -6 nm.



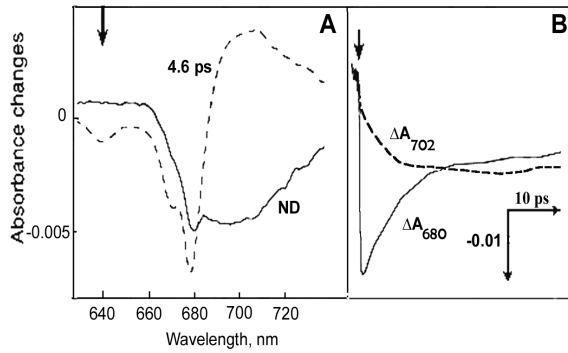
**Figure 2.** Excitation wavelength dependence of the early transient spectra of WT Lhca4<sub>Chl a+b</sub> in the region of Chl *b* Q<sub>y</sub> absorption. Excitations are at 635 nm (225 fs, dashed line), 640 nm (205 fs spectrum, solid line), 645 nm (267 fs spectrum, dotted line) and 650 nm (225 fs spectrum, dot-dashed line). Spectra are normalized to the  $\Delta A$

the Chl *b* excitation in WT Lhca4<sub>Chl a+b</sub> within 3 ps detected at 8 K. At 150-300 fs after the excitation, the transient absorption changes show a photobleaching of the Chl *b* absorption band at 640 nm accompanied by photobleachings in the region of Chl *a* absorption with three peaks at 670, 679 and 696 nm. These absorption changes might indicate excitonic coupling of closely located Chl *b* and Chl *a* molecules or unresolved ultrafast energy equilibration (faster than the time resolution).

The  $\Delta A_{640}$  band recovers very quickly due to ongoing subpicosecond energy transfer to neighboring Chl *a* molecules (Fig. 1A). An excitation wavelength dependence study of the Chl *b* Q<sub>y</sub> absorption band (Fig. 2) indicates two spectral pools of Chl *b* at 639-641 nm and at 647-649 nm that obviously reflect the absorption of two Chl *b* molecules in WT Lhca4<sub>Chl a+b</sub> (Table 1) contributing to the Chl *b* Q<sub>y</sub> transition at 644 nm in 77 K steady state absorption spectra (Schmid et al. 2001). Global analysis of the kinetics on a 3 ps time scale reveals two energy transfer processes with lifetimes of 0.6 ps and 1.4 ps and a nondecaying (ND) on this time scale component (Fig. 3A). The major donors and acceptors of the excitation in the 0.6 ps energy transfer process are Chl *b*-640 and Chl *a*-679, respectively. Small negative bands at 660 nm and 696 nm in the 0.6 ps-DAS indicate the decay of photobleaching of Chl *a*-661 (a spectral form detected by 2nd derivative spectroscopy) and the possible excitonic coupling of Chl *b*-640 with C-696 transition, respectively. A 1.3 ps-DAS illustrates the spectral profile of excitation energy equilibration that involves two spectral forms of Chl *b* (two negative amplitudes at 635 and 647 nm, see also Fig. 2) and C-679 (a major



**Figure 3.** Decay Associated Spectra obtained after global analysis of transient absorption spectra on the 3-5 ps time scale in WT Lhca4<sub>Chl a+b</sub> (A), WT Lhca4<sub>Chl a</sub> (B) and E102S Lhca4<sub>Chl a+b</sub> (C). Data are fitted to a sum of 3 exponential components with a fixed lifetime of nondecaying component.



**Figure 4.** Excitation energy transfer towards red pigments in WT Lhca4<sub>Chl a+b</sub> at 8 K. (A) Decay Associated Spectra obtained after global analysis of transient absorption spectra on the 50 ps time scale. Data are fitted to two exponential components; (B) Transient kinetics of absorption changes at 680 and 702 nm.

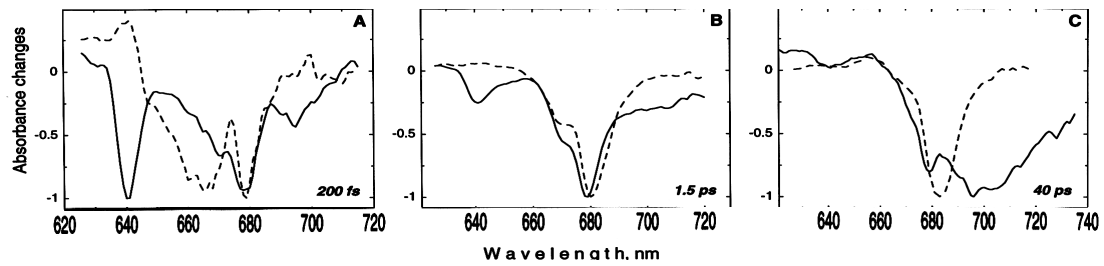
negative band) as energy donor sites and C-696 (a positive band) as an energy acceptor site. On a longer time scale, the excitation dynamics in the 695-720 nm region include a further red shift due to a process of energy redistribution with a lifetime of 4-6 ps (Fig. 4A,B). Similar processes were observed earlier at 77 K in WT r-Lhca4 (Melkozernov et al. 2000a). We suggest kinetic heterogeneity on the picosecond time scale with the faster phase (1.3 ps-DAS in Fig. 3A) reflecting energy transfer to C-696 and the slower 4-6 ps phase reflecting the localization of energy on inhomogeneously broadened red pigment's transition indicated in Fig. 4A by a broad ND spectrum with a  $\Delta A$  peak around 705 nm.

***Energy transfer pathways in recombinant Lhca4 with changed pigment occupancy.***

In contrast to the WT Lhca4<sub>Chl a+b</sub> (Fig. 1A), early transient absorption spectra of Lhca4 with all pigment-binding sites occupied by Chl *a* detected with excitation at 645 nm lack two spectral pools of Chl *b* and the C-696 spectral form (Fig. 1B). Data suggest that the replacing of Chl *b* involved in the red pigment's transition by Chl *a* in WT Lhca4<sub>Chl a</sub> disrupts pigment-pigment or pigment-protein interactions causing the red shift. Since the WT Lhca4<sub>Chl a</sub> polypeptide contains 6-7 Chl *a* molecules and no Chl *b* (Table 1), the spectral broadening around 640-660 nm in the early transient spectra might be attributed to the vibrational relaxation of the excited Chl *a* spectral form C-661 observed by global analysis in WT Lhca4<sub>Chl a+b</sub> (Fig. 3A, 0.6 ps-DAS) and by 2nd derivative spectroscopy. Similar relaxation processes were observed in a Chl *a*-containing PSI core from *Synechocystis* (Melkozernov et al. 2001). However, energy transfer processes faster than time resolution in this region cannot be ruled out.

Global analysis (Figure 3B) of the kinetics detected on a 5 ps time scale reveals two energy transfer processes illustrated by the 0.3 ps-DAS and 3.5 ps-DAS. The 0.3 ps process is an overlap of the vibrational relaxation of Chl *a* C-661 and subpicosecond energy transfer to the neighboring Chl *a* C-681. The picosecond energy transfer process with a rate of 3.5 ps is a spectral equilibration between Chl *a* spectral forms C-661, C-673, C-681 and C-687. The energy acceptors, C-681 and C-687, probably localize the excitation in WT Lhca4<sub>Chl a</sub>. This process is followed by the excitation decay as indicated by a ND spectrum centered at 683 nm (nanosecond decay).

Fig. 5 illustrates the spectral differences of WT Lhca4<sub>Chl a+b</sub> that binds 4-5 Chl *a* and ~2 Chl *b* and WT Lhca4<sub>Chl a</sub> with binding sites for Chl *b* occupied by 2 Chl *a* molecules. For both samples excited at 640 nm, the spectral difference in the transient spectra at 200 fs pump-probe delay accounts for the changes in the pigment-binding sites of Chl *b* replaced by Chl *a* (Fig. 5A). All Chl *a* molecules in the WT Lhca4<sub>Chl a</sub> contribute to the spectral forms C-661, C-673, C-681 and C-687. In transient spectra detected at 1.5 ps after the excitation (Fig. 5B) the major differences between the two



**Figure 5.** Transient absorption spectra of WT Lhca4<sub>Chl a+b</sub> (solid line spectra) and WT Lhca4<sub>Chl a</sub> (dashed line) detected at 8 K and excitation at 640 nm. The spectra are compared at ~200 fs (A); 1.5 ps (B) and 40 ps (C) pump-probe delay. Spectra are normalized to the highest  $\Delta A$ .

samples remain in the region of  $Q_y$  absorption of Chl *b* and the red pigment's absorption. The second pool of Chl *b* (C-648 in Fig. 2) can be seen in the 1.5 ps spectrum of the WT Lhca4<sub>Chl a+b</sub> as a broad shoulder around 650 nm. At this time delay the red pigment's transition is already broad and extended to 720-730 nm. The possible spectral evolution of the band from 696 nm (seen as a transient band in the 200 fs spectrum) to 705 nm (seen as a maximum of photobleaching in the long-lived spectra) might be due to energy transfer between two different transitions (C-696 and C-705). Sample heterogeneity and stimulated emission contribute to the width of this band (see Fig. 5C). In the WT Lhca4<sub>Chl a</sub>, the C-687 spectral form identified by the positive amplitude in the 3.5 ps-DAS in Fig. 3B might originate from a blue shift of the C-696 excitonic band in the WT Lhca4<sub>Chl a+b</sub> as a result of replacement of Chl *b* by Chl *a*. This suggests that steric hindrance of formyl group in C-7 position of Chl *b* might contribute to the red spectral shift.

The glutamic acid residue at position 102 of the Lhca4 polypeptide, probably located towards the stromal side of the membrane, might be a possible candidate for the binding of red pigment(s) specific for LHCI (Rupprecht et al. 2000). The spectroscopic consequence of the site-directed mutation of the glutamic acid against a serine residue at position 102 of the Lhca4 polypeptide is the lack of the red pigment's transient absorption band at early times with excitation of Chl *b* (Fig. 1C) and carotenoids (data not shown). Comparison of the 0.15 ps transient spectrum in WT Lhca4<sub>Chl a+b</sub> (Fig. 1A) and the 0.16 ps spectrum in the E102S Lhca4 (Fig. 1C) indicates a loss of possible excitonic coupling of Chl *b* and nearby Chl *a* molecules. Later the C-673 and C-681 spectral forms of Chl *a* are populated via a Chl *b*-to-Chl *a* energy transfer (see 0.2 ps-DAS in Fig. 3C) which is faster in the mutant than in the wild type Lhca4<sub>Chl a+b</sub> (0.5-0.6 ps). This may be a direct consequence of the loss of chlorophyll upon refolding of the mutated protein. We suggest that the E102S Lhca4<sub>Chl a+b</sub> lacks the Chl *b*-640 (Fig. 2) that is involved in the C-696 transition. The decay of the photobleaching of the remaining Chl *b*-647 is slower than that of Chl *b*-640 in the WT Lhca4<sub>Chl a+b</sub> as can be seen in the transient absorption spectra (Fig. 1C) and DAS (Fig. 3C). The increased contribution of slower kinetic processes might result from kinetic heterogeneity in the sample or the presence of uncoupled Chl *b*.

**Excitation of carotenoids in recombinant Lhca4.** To probe the location of the two Chl *b* molecules relative to carotenoids in the r-Lhca4 we studied the transient

absorption difference spectra in the 600-750 nm region upon excitation of carotenoids in the region of S<sub>2</sub> absorption (500-510 nm) (data not shown). The excitation of carotenoids induces delayed photobleaching of the Chl *a* spectral forms in the 660-680 nm spectral region due to an ultrafast Car-Chl *a* energy transfer. No transient changes were observed in the region of Chl *b* Q<sub>y</sub> absorption within the time resolution of the spectrometer. The photobleaching of the Chl *a* spectral forms at 670 and 680 nm suggests a close contact of the Chl *a* molecules with the carotenoid molecules. Most possibly the 4-5 Chl *a* molecules in the WT Lhca4<sub>Chl a+b</sub> are bound to Chl *a* binding sites in the center of the complex that are conserved in light-harvesting polypeptides (Bassi et al. 1999). Two Chl *b* molecules in the WT Lhca4<sub>Chl a+b</sub> are less sensitive to the excitation of carotenoids, suggesting their peripheral location relative to the central Chl *a* molecules.

In conclusion, the transient absorption difference spectra of Lhca4 at ~200 fs after the excitation into Q<sub>y</sub> transition of Chl *b* provide evidence of spectral coupling of Chl *b* and the pigments responsible for the C-696 spectral form. The C-696 transition might represent a dimer of Chl *a* affected by nearby Chl *b*. On a picosecond time scale the band evolves into a broad low energy absorption band centered at 705 nm and attributed to the red pigments that localize the excitation in LHCI. The observed two spectral pools of Chl *b* at 640 and 647 nm correspond to two Chl *b* molecules bound to the Lhca4. The data suggest that the E102S point mutation in the Lhca4 polypeptide impairs the coupling of Chl *b*-640 and the C-696 transition. One possible result of the replacement of the Chl *b* that affects the C-696 transition by Chl *a* is a 10 nm blue shift of the C-696 transition indicating involvement of the formyl group in the C-7 position of Chl *b* into the observed spectral changes.

**Acknowledgments.** This work was supported by NSF grant MCB-0091250 to REB, DFG grant Schm 1203/2-1 and 2-3 to VHRS and HP. This is publication No. 497 of the Center for the Study of Early Events in Photosynthesis at Arizona State University.

## References

- Bassi R, Croce R, Cugini D, Sandona D (1999) *Proceedings of National Academy of Sciences of USA* **96**, 10056-10061.
- Chen B and Przybyla AE (1994) *BioTechniques* **17**, 657-659.
- Ihalainen JA, Gobets B, Sznee K, Brazzoli M, Croce R, Bassi R, van Grondelle R, Korppi-Tommola JEI, Dekker JP (2000) *Biochemistry* **39**, 8625-8631.
- Knoetzel J, Bossmann B, Grimme LH (1998) *FEBS Letters* **436**, 339-342.
- Melkozernov AN, Lin S, Schmid VHR, Paulsen H, Schmidt GW, Blankenship RE (2000a) *FEBS Letters* **471**, 89-92.
- Melkozernov AN, Lin S, Blankenship RE (2000b) *Biochemistry* **39**, 1489-1498.
- Melkozernov AN, Lin S, Blankenship RE, Valkunas L (2001) *Biophysical Journal* **81**, 1144-1154.
- Rupprecht J, Paulsen H, Schmid VHR (2000) *Photosynthesis Research* **63**, 217-224.
- Tjus SE, Roobol-Boza M, Pålsson LO, Andersson B (1995) *Photosynthesis Research* **45**, 41-49.
- Schmid VHR, Cammarata KV, Bruns BU, Schmidt GW (1997) *Proceedings of National Academy of Sciences of USA* **94**, 7667-7672.
- Schmid VHR, Thome P, Rühle W, Paulsen H, Kühlbrandt W, Rogl H (2001) *FEBS Letters* **56**, 453-457.