CHAPTER 4

The Light-Harvesting Complexes of Photosystem I in *Chlamydomonas reinhardtii:* a time-resolved fluorescence study

Clotilde Le Quiniou, Roberta Croce

ABSTRACT

Photosystems (PS) I and II form the center of the photosynthetic process since they harvest sunlight and use its energy to drive charge separation. The light harvesting capacities of both photosystems are assured by two types of antenna: the core and the peripheral antennae. The peripheral antenna is composed of Light-Harvesting complexes (LHC) that differ between organisms. In the green alga *Chlamydomonas reinhardtii*, nine LHCI compose the peripheral antenna of PSI. Despite a very similar chromophore organization, the nine monomers have different spectroscopic properties, in particular regarding the content and energy of the red forms, Chls absorbing at longer wavelengths than the bulk Chls. In this study, we have characterized the decay kinetics of each LHCI monomer by measuring time-resolved fluorescence. We observe a similar decay time of 1.9 ns for all the monomers. This lifetime is shorter than that of the antenna complexes of PSII. This difference is not due to the presence of the red forms in LHCI, as the data show that these Chls do not act as quenchers.

INTRODUCTION

During photosynthesis, organisms transform light energy into useful chemicals. The first steps of this process rely on a set of multi-protein complexes, among which are photosystems (PS) I and II (1). Both PSs are crimped with pigments that harvest light and transfer excitation energy to the reaction centers (RC) of PSI and PSII, where it is used to promote charge separation (3). Pigments are specifically organized either in the core antenna, which is directly connected to the RC, or in the peripheral antenna, which in plants and green algae can be further divided into subunits called Light-Harvesting Complexes (LHC). The LHCs differ in type and number in different organisms (177, 187). Our focus here is on the LHCs of PSI, called LHCIs (or Lhcas), of the green alga *Chlamydomonas reinhardtii*. *C. reinhardtii* LHCIs properties are discussed in comparison with those of the better characterized LHCIs of the model plant *A. thaliana* and of the Lhc complexes of PSII (LHCII).

In *A. thaliana*, six *Lhca* genes were present (41) among which *Lhca1-4* are the ones that form the PSI peripheral antenna (28, 42, 43). These pigment-protein complexes are associated with the core in all conditions and are present in a 1 to 1 ratio with it (188). Two more gene products, Lhca5 and Lhca6, are present in sub-stoichiometric amount and their exact location has not been determined yet (41, 44, 114). Nine *Lhca* genes (1-9) have been found in *C. reinhardtii* (45, 46, 151), whose products are present in the PSI-LHCI complex (48). 127 chlorophylls (Chls) and 27 carotenoids (Cars) are estimated to be associated with the PSI peripheral antenna of *C. reinhardtii* (189), 70 more Chls and 14 more Cars than in the peripheral antenna of higher plants (5).

The structures of the LHCIs of higher plants have been recently obtained at high resolution (5, 29) revealing a highly conserved organization of both aproproteins and pigments. The remaining minor structural differences seem to have a large impact on the biochemical (e.g. pigment binding affinity) and spectroscopic properties of the complexes, which differ considerably between the four Lhcas (69, 190). In particular, each Lhca shows different absorption in the far-red part of the spectrum. This is due to a different content of the so-called red forms, which are Chls *a* that have their S_1 energy level lower than the one of the RC (74-77), or more generally of the bulk Chls *a* (63) and are typical of PSI (69). In higher plants, the excitonic pair formed by Chls *a* 603 and 609 (nomenclature Liu 2004) is responsible for the lowest energy absorption band in all LHCs (79, 81, 157, 191). The strength of the interaction is modulated by the ligand of Chl 603 which is an asparagine (Asn) in the "red" Lhcas Lhca3 and Lhca4 (108). Changing this Asn into a histidine leads to the loss of the red absorption and fluorescence in Lhca3 and Lhca4 (108). It was shown that in Lhca4, the red forms are due to a mixing of this lowest exciton state with a charge transfer (CT) state (82, 84). A similar origin was suggested for the red forms associated with the other Lhcas (63, 80).

Monomeric LHCIs cannot be isolated to homogeneity, and reconstitution *in vitro* was then used to determine the biochemical and spectral properties of the individual complexes of *C. reinhardtii* (63). All *C. reinhardtii* LHCIs showed similar major pigment binding and spectroscopic properties despite a low sequence identity. As compared to the LHCIs of higher plants, the LHCIs of *C. reinhardtii* have higher affinity for Chl *b* binding (62, 63), with the exception of Lhca3 (nomenclature from (45)), which is however very unstable upon *in vitro* reconstitution. On average, the Chl a/b ratio of the reconstituted *C. reinhardtii* Lhca monomers was 2.2 (±0.9 when Lhca3 is excluded from the calculation) in agreement with the Chl a/b ratio of the PSI-LHCI supercomplex (48), considering 98 Chls *a* in the PSI core (5) in addition to the 85 Chls contained in the peripheral antenna. Low Chl a/b ratios were found in Lhca4, 7 and 8 complexes where a glutamine (Gln) residue is present as ligand for Chl 606. Gln residue at this site has previously been shown to increase the selectivity for Chl *b* of both 606 and 609 sites in plant LHCs (192, 193). The nine LHCIs of *C. reinhardtii* have been clustered in three groups according to their red form content: the "blue" Lhcas (Lhca1, Lhca3 and Lhca7), the "intermediate" Lhcas (Lhca5, Lhca6 and Lhca8) and the "red" Lhcas (Lhca2, Lhca4 and Lhca9) (63). However, in *C. reinhardtii*, the red forms of the "red" LHCIs are at higher energy than those of the complexes of plants but they show common properties (63): (i) an Asn residue acts as ligand for Chl *a* 603 and (ii) they have a very broad bandwidth suggesting a CT origin. And finally, Lhca9 of *C. reinhardtii* has a Stokes shift of 15

nm, smaller than that of the "red" Lhcas of higher plants (80, 153) but larger than the "blue" Lhcas (191).

The presence and properties of the LHCI subunits influence the overall kinetics of PSI, slowing down the trapping time (90, 114, 163, 184). It was also shown that LHCIs have a multi-exponential decay (83, 112, 194-197), which is attributed to multiple conformations assumed by a monomer, each characterized by a specific lifetime and spectrum (112, 197). The analyses of the Lhcas of *A. thaliana* have shown that the conformation that harbors the reddest forms, also has the longest lifetime. However, in general the Lhcas lifetime is shorter than that of Lhcbs (197, 198).

To complete previous work on the properties of LHCIs in *C. reinhardtii* (63), time-resolved fluorescence of the nine Lhcas was measured, their decays were compared to those of *C. reinhardtii* Lhcbs and discussed on the basis of the red form content of each Lhcas.

MATERIALS AND METHODS

Sample preparation - Lhcas were reconstituted *in vitro* as in (63). For a detailed description of the reconstitution procedure see (199). Lhcb monomers were prepared as in (65).

Spectroscopic measurements -

Steady state measurements - Absorption and fluorescence spectra were measured at room temperature (RT) on a Varian Cary 4000 UV-Vis spectrophotometer and a Fluorolog spectrofluorimeter (Jobin Yvon Horiba) respectively. For fluorescence measurement, samples were diluted below OD 0.07 cm^{-1} at the maximum in the Q_y in order to avoid self-absorption.

Time-resolved fluorescence measurement with a TCSPC (Time-Correlated-Single-Photon-Counting) set-up - The fluorescence decay kinetics of reconstituted Lhca antenna from *C. reinhardtii* were measured with the Lifetime Spectrometer FluoTime 200 (MCP-PMT detector) after excitation of 468 nm. The FWHM of its IRF is around 82 ps (due mainly to the pulse duration of 78 ps) and the time resolution is around 50 ps (channel spacing of 8 ps). The spectral bandwidth was 8 nm (monochromator ST-9030, 1200 lines/mm grating with 1.0 mm slits). The repetition rate was 10 MHz and the energy per pulse was 10 pJ (average power of 100 µW) which corresponds to far less than one photon per pulse per complex. Practically, the signal count rate has been set at less than 1% of the excitation repetition rate frequency. The samples were diluted at OD 0.07 cm^{-1} at the Q_Y maximum to avoid self-

absorption (buffer 10 mM Tricine (pH 7.8), 0.03% α-DM, 0.5 M sucrose) and the fluorescence measured at 10°C, every 4 nm between 650 nm and 750 nm.

Data analysis of time-resolved measurements - The sequential analysis (148, 167, 168) was done as described in (184). The deconvolution and the fitting were made in TRFA Data Processor v 1.4 (chi² ranging between 1.033 and 1.353). The IRF was measured with pinacyanol chloride whose lifetime is 6 ps in methanol (117). The DAS were calculated manually, to correct for the sensitivity of the TCSPC detector, by the following formula: $\mathrm{DAS_n}(\lambda)=\frac{\mathrm{I}(\lambda)*\mathrm{p_n}(\lambda)}{\sum_n(\mathrm{p_n}(\lambda)*\tau_n)}$, with $\mathrm{p_n}$ the amplitude of the exponential decay (not convoluted with the IRF) associated to the *n*-th component and I is the total emission (equivalent to the steady state emission) at a specific detection wavelength.

The average decay time τ_{av} is calculated with the following formula: $\tau_{av}=\sum_n(\tau_n.\mathrm{A_n})\,/\sum_n \mathrm{A_n}$ with $\mathrm{A_n}$ the area under the DAS of the *n*-th component (i.e. its total amplitude) and $\mathrm{A_n}/\sum_n \mathrm{A_n}$ the relative amplitude (see (184) for details). Care is taken to extract from the kinetics any influence of the disconnected species (Table 1). The relative initial population of the free Chls (%) was extracted from the amplitude of the 3rd DAS. For that, steady state fluorescence emission upon 468 nm excitation was compared to the one upon 500 nm. The emission difference upon 468 nm excitation compared to 500 nm was assumed to be related mainly to free Chls *b*. In case of a very high percentage of disconnection, the 3rd lifetime was discarded from the calculation of the average lifetime (Lhca3). Also the range considered to calculate the area under the DAS permits to exclude the region where free Chls dominate the emission (Lhca3, Lhca5).

	Relative initial population of the free Chls (%)	DAS range considered to calculate the relative DAS area (nm)
Lhca1	3.3%	650-740
Lhca2	5.7%	650-750
Lhca3	26.0% 3rd lifetime discarded	674(c)-750
Lhca4	3.6%	650-760
Lhca5	1.9%	674(c)-740
Lhca6	3.9%	650-750
Lhca7	4.7%	650-750
Lhca8	3.4%	650-750
Lhca9	2.1%	650-750

Table 1: Percentage of free Chls and DAS range considered for the calculation in order to exclude the free Chls contribution.

RESULTS

Absorption and emission spectra at RT of all nine Lhcas are shown in Figure 1. Differences between the complexes can be observed in the ratio between the main absorption peak and the shoulder around 650 nm, which mainly reflects the differences in the binding affinity for Chl *a* or *b*. All complexes have their absorption maximum above 677 nm with the exception of Lhca3, 5 and 7. In the case of Lhca3 and Lhca5 the presence of disconnected Chls contributes to the blue shift as observed before (63). Lhca2, Lhca4 and Lhca9 have the largest absorption above 700 nm (Figure 1A) as well as the reddest emission (Figure 1B). However, the fluorescence spectra of all Lhcas have larger contributions above 700 nm than the spectrum of Lhcb monomers, indicating the presence of red-absorbing species in all nine Lhcas.

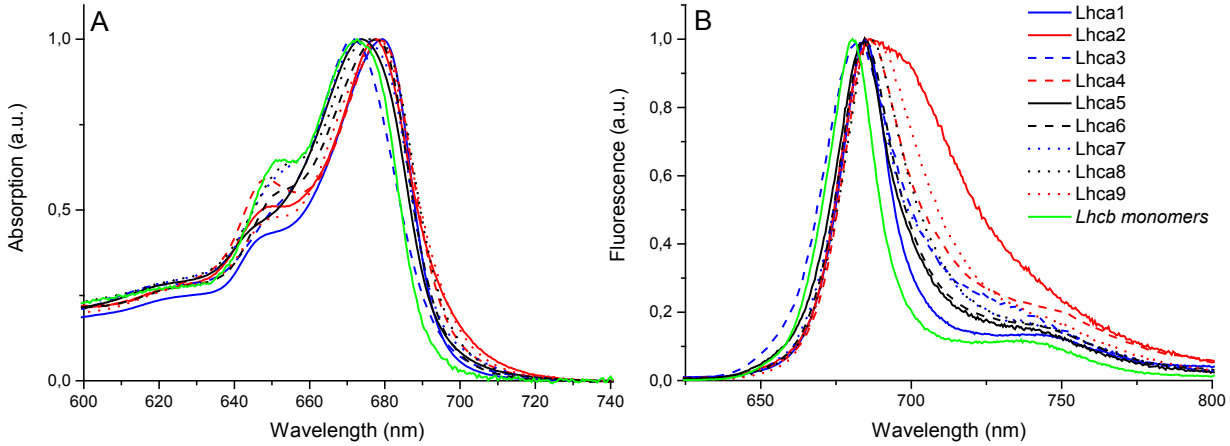

Figure 1: A) Absorption spectra at room temperature (RT) of the "blue" (blue), "intermediate" (black) and "red" (red) Lhcas normalized to 1 at the maximum in the Q_y region and B) Emission spectra upon 500 nm excitation at RT, normalized to the maximum. Both absorption and emission are compared to the ones of Lhcb monomers (green).

To estimate the relative amount of the red forms in the different complexes, the percentage of Q_y absorption (630-750 nm) and of fluorescence emission above 700 nm were calculated. The results are reported in Table 2 and they indicate that the contribution of red forms increases in this order: Lhca1<Lhca6<Lhca3<Lhca5<Lhca8<Lhca7<Lhca4<Lhca9<Lhca2 in absorption and Lhca1<Lhca5<Lhca3<Lhca6 < Lhca7<Lhca8< Lhca4 <Lhca9<Lhca2 in emission.

	Relative absorption above 700 nm	Relative emission above 700 nm
Lhca1	1.1%	34.3%
Lhca2	3.5%	54.0%
Lhca3	1.7%	36.6%
Lhca4	2.2%	47.0%
Lhca5	1.9%	35.0%
Lhca6	1.2%	37.8%
Lhca7	2.1%	40.1%
Lhca8	2.0%	40.5%
Lhca9	3.0%	45.2%
Lhcb monomers	*0.7%*	*25.6%*

Table 2: Percentage of absorption and emission above 700 nm

To determine the excited state lifetimes of the Lhcas, time-resolved fluorescence of each complex was measured upon 468 nm excitation by using a TCSPC set-up (see Materials and Methods). The data were globally analyzed by a sequential model (see Materials and Methods). The lifetimes and decay associated spectra (DAS) of all nine Lhcas are presented in Figures 2A and B. The decay of all complexes was well fitted by three or four components. Since four components did not significantly improve the fit, the three components description was used. The lifetimes range between 0.26-0.69 ns for the 1st component, between 1.6-2.6 ns for the 2nd component and between 3.6-4.8 ns for the 3rd component. The ~2 ns component dominates the decay of all the Lhcas (Figure 2A).

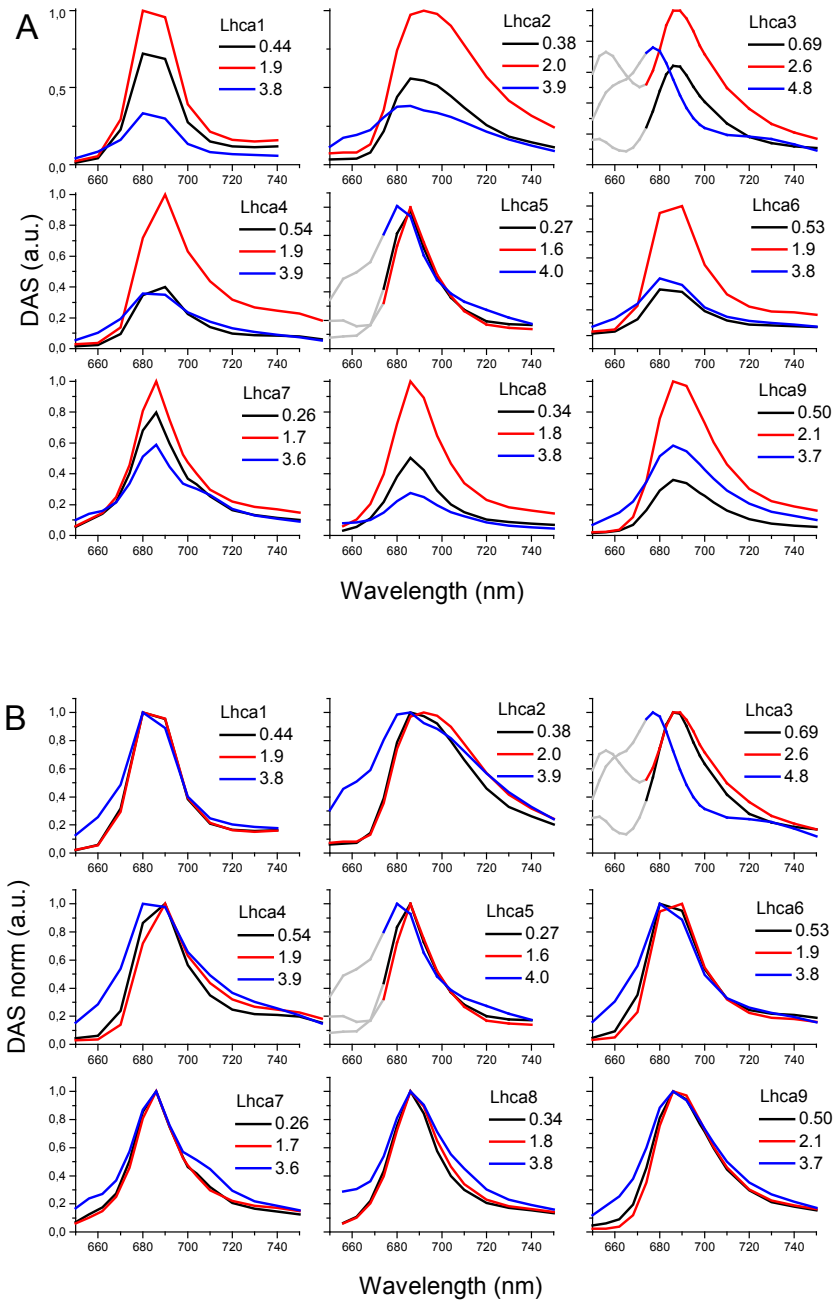

Figure 2: DAS obtained from the sequential analysis of Lhcas time-resolved fluorescence measured at 10°C: A) scaled DAS with the 2nd component's maximum at 1; B) normalized DAS of all the components. The samples were excited at 468 nm.

When normalized to the maximum (Figure 2B), the shape of the first and second DAS are similar for most of the complexes, with the exception of Lhca2, Lhca4 and Lhca8 in which the second component is more red-shifted. In all the complexes the third DAS shows some increased emission in the blue, indicating the presence of disconnected Chls. This is particularly visible for Lhca3 and Lhca5, which also show a contribution of Chl *b* emission in

the second DAS. However, for most of the complexes the third component has an increased intensity in the far-red, indicating that part of the emission originates from an Lhc conformation enriched in red forms. An increased red-shift in the component characterized by a long lifetime was observed before for plant LHCI (112).

When comparing the DAS of the individual Lhcas (Figure 3), a large difference in the red form content of the complexes becomes visible. Lhca2 shows the largest amplitude in the far red for all three components, followed by Lhca9, Lhca3, Lhca4, Lhca6, Lhca7, Lhca8, Lhca5 and finally Lhca1 for the first component, which is not influenced by the presence of "free" pigments.

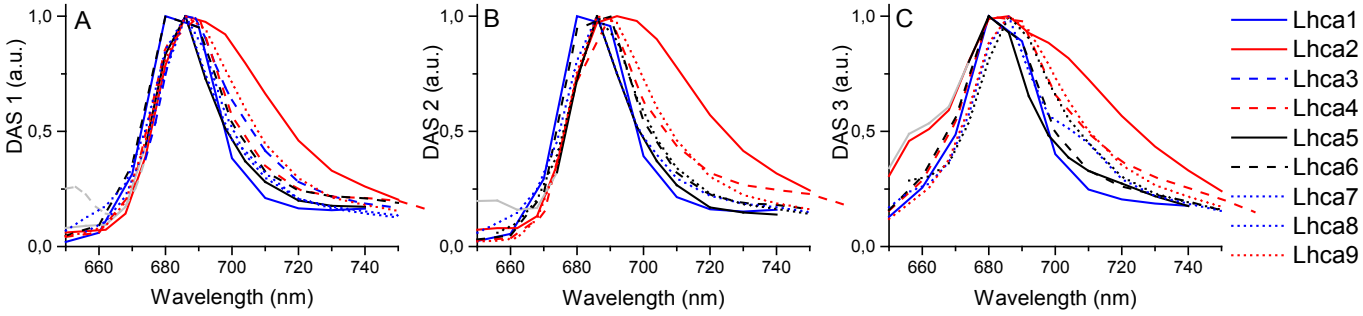

Figure 3: DAS of the "blue" (blue), "intermediate" (black) and "red" (red) Lhcas grouped by component: A) 1st component (0.26-0.69 ns), B) 2nd component (1.6-2.6 ns) and C) 3rd component (3.6-4.0 ns). The spectra are normalized to their maximum. DAS from Lhca3 are not presented since dominated by the free pigment emission.

The percentage of disconnected Chls (i.e. Chls that do not participate in the excitation energy transfer within the complex) in each sample was evaluated (see Materials and Methods, Table 1) in order to calculate the average decay time of the individual Lhca. For most of the complexes the average decay times are in the 1.7-2.1 ns range (Table 3). Lhca9 shows a slightly longer lifetime (2.3 ns), but far shorter than that of the Lhcb complexes of *C. reinhardtii* (64).

	tau1	tau2	tau3	Average lifetime (ns)
Lhca1	0.44 (35.5)	1.9 (49.0)	3.8 (15.5)	1.7
Lhca2	0.38 (27.6)	2.0 (53.7)	3.9 (18.7)	1.9
Lhca3	0.69 (36.7)	2.6 (63.3)	4.8 (-)	1.9
Lhca4	0.54 (22.1)	1.9 (56.2)	3.9 (21.7)	2.0
Lhca5	0.27 (33.3)	1.6 (32.8)	4.0 (33.9)	2.0
Lhca6	0.53 (20.9)	1.9 (54.9)	3.8 (24.2)	2.1
Lhca7	0.26 (33.8)	1.7 (41.7)	3.6 (24.5)	1.7
Lhca8	0.34 (27.6)	1.8 (56.9)	3.8 (15.5)	1.7
Lhca9	0.50 (18.3)	2.1 (49.4)	3.7 (32.3)	2.3
Lhcb monomer	0.35 (10)	2.9 (49)	4.4 (41)	3.3

Table 3: Table of lifetimes and relative amplitude (in brackets) estimated from the sequential analysis of Lhcas fluorescence decays (detection between 650 and 750 nm) compared to the Lhcb monomers (64). The relative contributions of disconnections in the amplitudes of the DAS have been estimated and excluded for the calculation on Lhcas (see Materials and Methods). However, the presence of disconnected species leads to a relatively high uncertainty (7%) of the average decay time.

DISCUSSION

The red forms of LHCI are known to have a CT character (80, 84), which is normally associated with a relative dark state and can behave as a quencher (82, 200). Studies of the fluorescence properties of LHCI of plants by time-resolved (112, 197) and single molecule spectroscopy (113), have revealed that these complexes switch between different conformations characterized by blue and red-spectra. However, differently than expected the conformation with the most red-shifted emission was shown to have the longest lifetime (112), suggesting an inverse relationship between red form content and quenched states. The nine Lhcas of C*. reinhardtii* have different contents of red forms and thus allows a systematic investigation of the effect of these forms on the excited state lifetimes of the complexes.

The fluorescence decays of the nine reconstituted Lhcas in *C. reinhardtii* are well described with three components of 0.4 (± 0.1) ns, 1.9 (± 0.3) ns and 3.8 (± 0.1) ns. The average decay times were all around 1.9 ns (± 0.2), significantly shorter than the average decay time of Lhcbs (64), but very close to that of the Lhcas of plants (112, 197).

It is interesting to observe that not only the average lifetime, but also all the three lifetimes that describe the decay of Lhcas are shorter than the three lifetimes that characterize the decay of LhcbMs of *C. reinhardtii* (64). This indicates that although all Lhcs exist in different conformations, the Lhcas are always present in a more quenched state than the Lhcbs.

In the case of Lhca2, which is the most red-shifted complex, the longest DASs also showed a higher amount of red forms, indicating that as it is the case in plants, the low energy forms are not acting as quenchers. However, a direct correlation between the energy/amount of red forms and the lifetime of the complexes could not be observed as all Lhcas have a very similar lifetime.