In vitro investigation of the photoprotection mechanism of Light Harvesting Complex II

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CHAPTER 3

Fluorescence lifetimes of LHCII in a lipid environment: aggregation, low pH and the presence of PsbS

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ABSTRACT

Plant Light-Harvesting Complexes have a built-in capacity to switch function between light harvesting and quenching of excitation energy, protecting the photosynthetic apparatus against over-excitation via a regulatory feedback response. In their chloroplast thylakoid membranes, non-photochemical quenching (NPQ) is activated as a response to a low luminal pH and controlled by the pH-sensing protein Photosystem II subunit S, PsbS. Several studies proposed that PsbS could directly interact with the peripheral light-harvesting complexes (LHCII) at low pH. In this study, we systematically tested the influence of low pH and PsbS on the fluorescence lifetimes of membrane-embedded spinach LHCII. The proteoliposome preparations contained LHCII in mild quenched states, aimed to mimic the fluorescence conditions of dark-adapted leaves. We found that under those conditions, neither acidification nor the presence of PsbS or the combination of both did have a significant effect on the LHCII Chl fluorescence lifetimes. The results support a view in which the functional role of PsbS consists of re-organizing the thylakoid membrane under stress, rather than creating direct quencher states.

INTRODUCTION

In naturally fluctuating light conditions, plants need to constantly adjust their photosynthetic antenna to the incoming light intensities to prevent themselves from over-excitation and photodamage. They have developed sophisticated feedback mechanisms that regulate light harvesting. In high-light conditions, NPQ processes are activated to dissipate a large part of the incoming excitations as heat [1].

Light-Harvesting Complexes (LHCs) that are associated with PSII have the ability to reversible switch their conformation into a quenched state, which is assumed to be the cause of excitation quenching during NPQ [2]. Single-molecule experiments have shown that individual LHCs can fluctuate between fluorescent and quenched states [3,4]. In bulk experiments, the quenched state is produced upon aggregation of LHCs [5,6]. Aggregation-dependent quenching has also been observed in native thylakoid membranes, where aggregates of the peripheral Light-Harvesting Complex, LHCII, are formed under high-light conditions [7,8].

The fast energy component of NPQ, called qE, in plants is reversibly controlled via the pH-sensing protein Photosystem II subunit S (PsbS), of which the action is triggered by acidification of the inner compartments of chloroplasts, the thylakoid lumen [9,10]. Switching back from high to moderate light conditions, PsbS also accelerates fast de-activation of the quenching process [11]. The fast qE process is connected to a slower quenching process, termed qZ [12]. In qZ, lumen acidification activates the enzyme violaxanthin de-epoxidase (VDE) to catalyse the conversion of the carotenoid violaxanthin (Vio) to zeaxanthin (Zea). qZ quenching likely occurs due to the binding of Zea to specific LHCs [13]. The presence of Zea has been proposed to catalyse qE quenching and for Zea-accumulating membranes faster fluorescence induction has been observed [14] [15].

The membrane protein PsbS is the only member of the LHC multi-gene family that does not bind pigments in specific locations. Instead of acting as a light harvester, this protein solely functions as a pH sensor [16]. PsbS is activated under high-light conditions, and its activation is proposed to involve protonation of specific glutamic-acid residues at low lumen pH and monomerization of PsbS dimers [16]. Recent studies showed that PsbS interacts with the LHCII polypeptides (Lhcb1, Lhcb2, and Lhcb3) or with the minor antenna proteins (Lhcb4, Lhcb5, and Lhcb6) under influence of the xanthophyll cycle [17]. The molecular mechanism for qE excitation quenching has not been resolved and the mechanistic trigger that activates the process via PsbS has not been clarified. Molecular interactions of PsbS with LHCs might induce conformational changes of the antenna proteins into quenched states. PsbS has also been suggested to induce supramolecular rearrangements of LHCs and PSII reaction center complexes [18]. According to current models, the activation of PsbS at low pH concerns the protonation of two key residues and a monomerization step [16,19]. Monomeric PsbS may disconnect LHCII from PSII after which the released LHCII complexes could form clustered aggregates that produce dissipative states via an aggregation-dependent quenching mechanism [20,21].

Liposomes form suitable model systems to investigate protein-lipid interactions in membranes of reduced complexity compared to natural biological membranes and dissect the functions of selective membrane components. Various studies have investigated the

properties of LHCII proteoliposomes [4,5,22]. Liu *et al.* demonstrated that PsbS could be refolded directly in LHCII-reconstituted membranes [23]. Wilk *et al.* co-reconstituted PsbS in liposomes with very low amounts of LHCII to prevent self-aggregation quenching, and showed that under those conditions the LHCII fluorescence was quenched in the presence of both PsbS and Zea [24]. Their work does not report on the effects of acidification though, which is known to activate PsbS *in vivo*.

The rapid qE response occurs on the time scale of seconds before the enzymatic process of xanthophyll conversion has taken place, is activated by low pH, and requires PsbS. To investigate the origin of this response mechanism, we performed a systematic fluorescence lifetime analysis on PsbS-LHCII and LHCII-only proteoliposomes at neutral and low pH. For our membrane model, we carefully choose the protein to lipids ratio, PLR expressed in mole of protein per mole lipids, so that the fluorescence of LHCII-only proteoliposomes at neutral pH would mimic the dark-adapted state of leaves *in vivo*. In dark-adapted leaves, the fluorescence of the PSII antenna is moderately quenched and LHCII has an average lifetime of ~2 ns, which is considerably shorter than the lifetime of ~4 ns that are observed for LHCII in detergent solutions [25] and suggests that LHCII exists as small aggregates in thylakoid membranes.

MATERIALS AND METHODS

LHCII EXTRACTION

Light-Harvesting Complexes were purified from spinach leaves (*Spinacia oleracea*) as described previously [5]. Briefly, LHCII trimer complexes were isolated via sucrose gradient. The trimeric green band of LHCII was extracted using a needle. Purified LHCII pigment-protein complexes were characterized by absorption spectroscopy. The buffer was exchanged into HEPES 50 mM, NaCl 100 mM, pH 7.5, n-Dodecyl *β*-D-maltoside (*β*-DM, Sigma) 0.03% and concentrated using Amicon Ultra 2 ml centrifugal filters with cut off of 30 K (Millipore) before to store the protein at -80 °C until use.

PsbS REFOLDING

Psbs genes from *Physcomitrella patens* and *Spinacia oleracea* were overexpressed in *E. coli* and purified using the protocol from Krishnan *et al.* [26]. The unfolded PsbS was stored at 4ºC until further use. PsbS was refolded in presence of the detergent *β*-DM. According to the refolding protocol, PsbS was mixed with heating buffer (NaOAc 20 mM, NaCl 100 mM, LDS 4%, sucrose 25%, pH 7.5) and heated to 98ºC for 1 minute. The appropriate amount of detergent (*β*-DM) was added to the mixture. The addition of 200 mM KCl and 30 minutes incubation at 4ºC precipitates LDS, thus allowing ß-DM to refold PsbS. Aggregated LDS was pelleted by spinning at 20000 x g for 10 minutes at 4ºC. The concentration of PsbS protein in mg/ml was determined from SDS page analysis.

PREPARATION OF ASOLECTIN LIPOSOMES

Liposomes were prepared according to Crisafi & Pandit with the following adjustments [5]. Asolectin lipids from soybean (Sigma) were dissolved in chloroform to a concentration of 5 mg/ml. With a stream on N_2, the organic solvent was evaporated from the chloroform/asolectin solution collected in a round-bottom flask. To remove all traces of solvent an extra step of evaporation in a rotary evaporator (R3000, Buchi) was performed. The phospholipid bilayer was then hydrated with the desired buffer (HEPES 50 mM, NaCl 100 mM, pH 7.5) and the round bottom flask was rotated at low rpm using the rotary evaporator for 1-2 hours. After that, if the bilayer was not completely solubilized, the solution was sonicated for 1 min (2210, Branson). The liposome suspension was exposed to 10 freeze/thaw cycles followed by extrusion through polycarbonate membranes of 400 and 200 nm pore size, using a mini-extruder (Avanti polar lipids). Sizes of liposome preparations were determined by dynamic light scattering (DLS, Malvern Zetasizer Nano ZS) equipped with a Peltier controlled thermostatic cell holder.

PREPARATION OF PROTEOLIPOSOMES

For protein insertion, preformed liposomes were destabilized by the addition of 0.03% of *β*-DM to facilitate insertion of LHCII and PsbS into the liposome membranes. LHCII

complexes were added to the liposome suspension and incubated for 1 hour. Bio-beads were added to the suspensions in 2 steps. The first incubation step was performed at 4ºC overnight while gently rotating the sample using a roller mixer (SRT9D, Stuart). Subsequently, the Bio-beads were refreshed and the solution was again incubated for 2-3 hours. The proteoliposome suspensions were centrifuged at 20000 x g for 10 min at 4 °C using a table-top centrifuge (3K18, Sigma) to remove non-incorporated LHCII and PsbS that forms aggregate pellets [27]. LHCII insertion and removal of LHCII aggregate pellet were confirmed by running LHCII and LHCII-PsbS proteoliposomes over a sucrose gradient that showed a single green band containing the proteoliposomes [28]. The reported protein to lipid ratios (PLRs) are defined as moles of LHCII or PsbS trimers per moles of total lipids. The concentration of LHCII trimers was determined from the molar extinction coefficient for LHCII (trimers) at 670 nm, ε= 1,638,000 M^{-1} cm^{-1} [29].

CIRCULAR DICHROISM EXPERIMENTS

CD spectra were recorded on a J-815 spectropolarimeter (Jasco) equipped with a Peltier element. The wavelength range was from 350 to 750 nm, data pitch 1 nm, response 2 s, bandwidth 2 nm and scanning speed of 50 nm/min at 20 ^{0}C using a 1 cm quartz cuvette (Hellma).

UV-VIS ABSORBANCE EXPERIMENTS

Absorption spectra were recorded on a UV-1700 PharmaSpec spectrophotometer (Shimadzu) with the wavelength range set from 350 to 750 nm using 1x1cm cuvette.

STEADY-STATE FLUORESCENCE EXPERIMENTS

Fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian), collecting emission spectra from 660 to 720 nm using 3x3 mm quartz cuvettes. The optical density of the sample preparations varied from 0.03 to 0.07 cm^{-1} at 650 nm. The excitation wavelength was set at 650 nm or 475 nm.

TIME-RESOLVED FLUORESCENCE EXPERIMENTS

TRF measurements were performed using a FluoTime 300 (PicoQuant) time-correlated photon counter spectrometer. Samples were held in a 1x1 cm quartz cuvette that was thermostated at 20 ^{o}C and excited at 440 nm using a diode laser (PicoQuant). Fluorescence decay traces were fitted with multi-exponential using a χ^2 least-square fitting procedure.

SDS-PAGE GEL ANALYSIS

For SDS-page analysis, proteoliposomes were pelleted and re-suspended in HEPES buffer and analysed on 12,5% polyacrylamide running gel with 4% stacking gel. Gels were stained using Silver Stain Plus kit (Bio-Rad) according to the standard Bio-Rad protocol. Molecular masses were estimated via a molecular standard, precision plus protein dual colour (Bio-Rad).

RESULTS

THE RELATIONSHIP BETWEEN LHCII CONCENTRATION AND FLUORESCENCE QUENCHING

To separate the effects of PsbS and low pH from the effects of self-aggregation on the fluorescence quenching of LHCII, we aimed to have a clear overview of how concentration and quenching of LHCII in liposomes depend on the protein to lipid ratio (PLR). Several studies, including the work from the previous chapter, report on the fluorescence yields of LHCII proteoliposomes in different PLR regimes. To have an overview, we combined data results from different reports in one graph, Figure 3.1, where the quenching of LHCII proteoliposomes is plotted against the PLRs. To generate this plot, the relative fluorescence yields of the LHCII proteoliposomes were calculated, taking the reported average fluorescence lifetimes for LHCII proteoliposomes and using the fluorescence of LHCII in detergent, reported in the same studies, as a reference for 100% fluorescence.

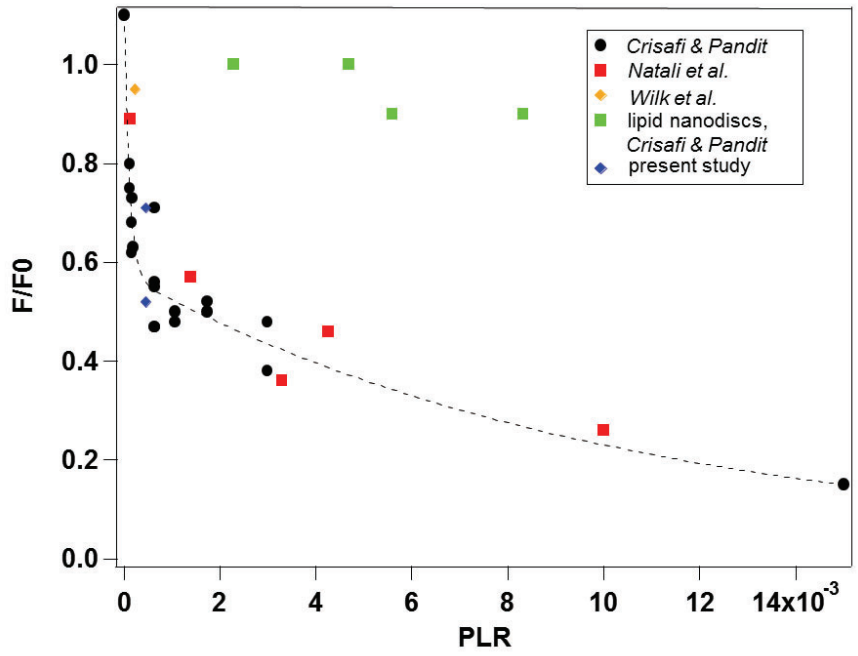

Figure 3.1 Fluorescence yield of LHCII proteoliposomes and LHCII lipid nanodiscs versus the PLRs. Data results from three different studies are plotted. Crisafi and Pandit [5] used liposomes prepared from asolectin, while Natali et al. [4] and Wilk et al. [24] used mixtures of thylakoid lipids. Data points, excluding those from lipid nanodiscs, are fitted with a double exponential fit.

As shown in Figure 3.1, the data from different sources follows a remarkably similar trend, considering that different preparation techniques were used and that our study used asolectin proteoliposomes, while other studies used liposomes prepared from thylakoid lipid mixtures of which the major content is galactolipids. At very low PLRs, a

steep dependence of the fluorescence is observed with increased LHCII concentrations that levels at ~50% quenching as described in Chapter 2. In this PLR regime, the liposomes contain only a few proteins per vesicle according to single-molecule and electron freeze-fracture microscopy [4,24].
At higher PLRs, there is a more gradual increment of quenching with the increase of LHCII concentrations. The dataset could not be fitted properly with a single exponential or sigmoidal curve and was fitted with two exponentials. The fluorescence of LHCII lipid nanodiscs is also plotted. LHCII complexes that are captured in nanodiscs are prevented from self-aggregation. Under those conditions, no significant quenching occurs, the small decrease at high PLRs is due to minor fractions of small aggregates trapped within the nanodiscs, showing that quenching in LHCII proteoliposomes is not induced by protein-lipid interactions *per se* [4,5].
The fluorescence yield of 50% around the breakpoint in the curve in Figure 3.1 corresponds to an average fluorescence lifetime of ~2 ns, which resembles the fluorescence lifetimes of dark-adapted leaves with closed reaction centres [15]. For our proteoliposome preparations, we choose to use a PLR of 6.5×10^{-4} for LHCII in lipids, which is close to the breakpoint in the slope of the curve. Note that the protein density in native thylakoids is much higher than in our proteoliposomes and we did not intend to reproduce the *in vivo* protein density, which would lead to the formation of large LHCII aggregates that strongly quench the fluorescence. In native membranes, the involvement of LHCII in super complexes may prevent the formation of such large LHCII aggregates.

ANALYSIS OF PsbS PROTEOLIPOSOMES

Proteoliposomes were prepared from asolectin lipids because those would give better reproducibility for quantitative LHCII protein insertion than liposomes prepared from galactolipid mixtures that mimic the natural thylakoid lipid composition. As shown in Figure 3.1, the trend for aggregation-dependent quenching of LHCII in asolectin liposomes (*Crisafi & Pandit*) is similar to liposomes prepared from a mixture of thylakoid lipids [5]. A PsbS: LHCII ratio of 1:1 was used. This ratio was much higher than the natural occurrence of PsbS in thylakoid membranes and was used to increase the probability that PsbS and LHCII would interact.
LHCII insertion in the liposomes was confirmed by the absence of LHCII aggregate pellet in reconstitution solutions and by sucrose gradient analysis that showed a green band containing the LHCII proteoliposomes. To test the insertion of PsbS, we performed an SDS-page gel analysis on PsbS-only proteoliposomes. Western blotting was not applied as we noticed that PsbS can give a false-positive reaction in anti-Lhcb1 Western blots (Figure A3.1).
Is recently demonstrated that PsbS forms an equilibrium of monomers and dimers in detergent solutions [26]. At low pH, the equilibrium is shifted towards the monomeric form. However, aging of the protein solutions shifts the equilibrium towards the dimeric form, both for neutral and acidic solutions. To check if the initial oligomeric state of PsbS would influence the dimerization state inside membranes, we tested PsbS insertion in

preformed liposomes under four different conditions. Proteoliposomes were prepared at pH 5.0 or pH 7.5, and for both conditions, two PsbS preparations were used containing PsbS in different monomer-dimer equilibria. One sample was three days old and contained more dimers than monomers according to SDS-page analysis. The other PsbS sample was freshly prepared and contained more monomers than dimers. The results of our insertion tests confirmed that PsbS was inserted into the liposomes under all tested conditions. Remarkably, the test shows that PsbS fractions run as a monomer band in the SDS-page gel, irrespective of the pH conditions or oligomeric state before insertion (Figure 3.2).

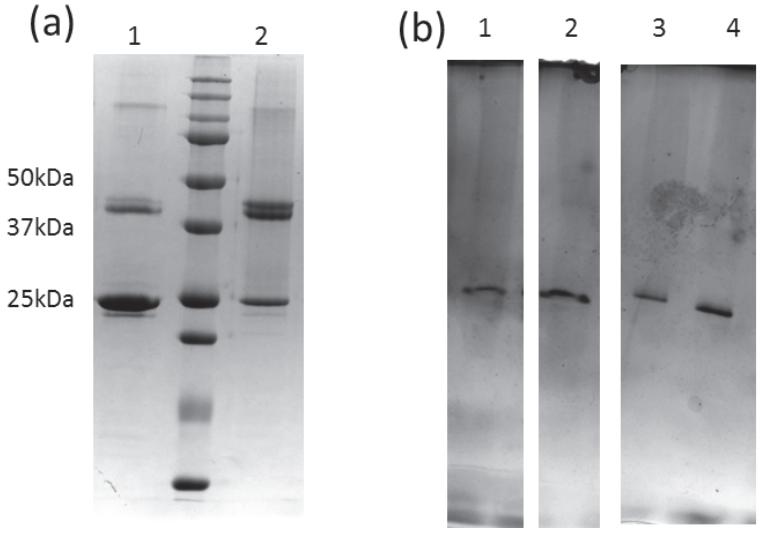

Figure 3.2 (a) SDS-page gel of PsbS in detergent solution before liposome insertion. Lane 1 contains PsbS that was predominantly in the monomeric form and lane 2 contains PsbS that is predominantly in the dimeric form. The middle lane contains the protein marker. (b) PsbS proteoliposomes confirming PsbS insertion (silver stain gel). Lane 1 and 2: proteoliposomes prepared at pH 5.0 using sample 1 (1) or sample 2 (2). Lane 3 and 4: proteoliposomes prepared at pH 7.5 using sample 1 (3) or sample 2 (4).

When the insertion was performed in pH 5.0 buffers, larger pellets of PsbS were observed containing proteins that were not inserted in the liposomes. Therefore, we proceeded with PsbS reconstitution in pH 7.5 buffer solutions. From a comparison of the gel band intensities of PsbS proteoliposomes to those of the removed pellet, we estimated that ~80% of the PsbS was inserted in the liposomes under pH 7.5 conditions (Figure A3.2).

FLUORESCENCE LIFETIME ANALYSIS OF LHCII-PsbS AT NEUTRAL AND LOW pH

TRF experiments were performed on LHCII-only and LHCII-PsbS proteoliposomes that were equilibrated at pH 7.5 or at pH 5.0. The compared LHCII-only and LHCII-PsbS proteoliposomes were always prepared using the same liposome batches since some variation in the fluorescence were found from batch to batch. To equilibrate the proteoliposome preparations at pH 5.0, we used a protocol in which suspensions prepared at pH 7.5 were acidified by injection of HCl followed by the addition of nigericin, a

liposoluble compound, in order to equilibrate pH conditions inside and outside the proteoliposomes [30]. Dynamic Light Scattering (DLS) measurements on liposomes before and after acidification and nigericin treatment confirmed that there were no significant changes in the average diameter sizes of the proteoliposomes (Table A3.1). Upon acidification, the contribution of very large particles (>500 nm), disappeared in the DLS size distribution diagrams and therefore the average diameter is slightly shifted to lower values.

Table 3.1 presents the results of TRF experiments that were performed on LHCII-only and LHCII-*patens* PsbS proteoliposomes.

Sample	**A_1%**	**τ_1**	**A_2%**	**τ_2**	**A_3%**	**τ_3**	**τ_{av}**
LHCII proteoliposomes pH 7.5	28.4	4.4	52.7	2.6	18.9	0.8	2.7
LHCII proteoliposomes pH 5.0	21.9	4.5	56.0	2.5	22.1	0.8	2.6
LHCII-PsbS proteoliposomes pH 7.5	21.5	4.6	58.0	2.6	20.4	1.0	2.7
LHCII-PsbS proteoliposomes pH 5.0	19.4	4.6	57.8	2.5	22.8	1.0	2.6
LHCII in β-DM pH 7.5	72.1	4.2	27.9	2.9	-	-	3.8
LHCII in β-DM pH 5.0	61.4	4.3	35.3	2.8	3.3	1.1	3.7

Table 3.1 Fluorescence lifetimes expressed in ns of LHCII-only and PsbS-LHCII proteoliposomes.

For comparison, the fluorescence lifetimes of LHCII in 0.03% β-DM detergent solutions equilibrated at pH 7.5 or at pH 5.0 were also collected. The presented results are the average of two reconstitution experiments using the same batch of preformed liposomes. The results show that acidification causes at most ~4% reduction of the average fluorescence lifetimes, while no differences are observed comparing LHCII and LHCII-PsbS proteoliposomes. Steady-state fluorescence spectra, as shown in Figure 3.3, collected upon 440 nm excitation show a broadening and small red shoulder for the proteoliposomes compared to LHCII in β-DM solution indicative of spectral ensembles of LHCII that are in different conformational states. In these experiments, we used PsbS from *Physcomitrella patens* since we already performed an extensive analysis of this protein, and reconstituted *patens* PsbS together with LHCII extracted from spinach leaves [26].

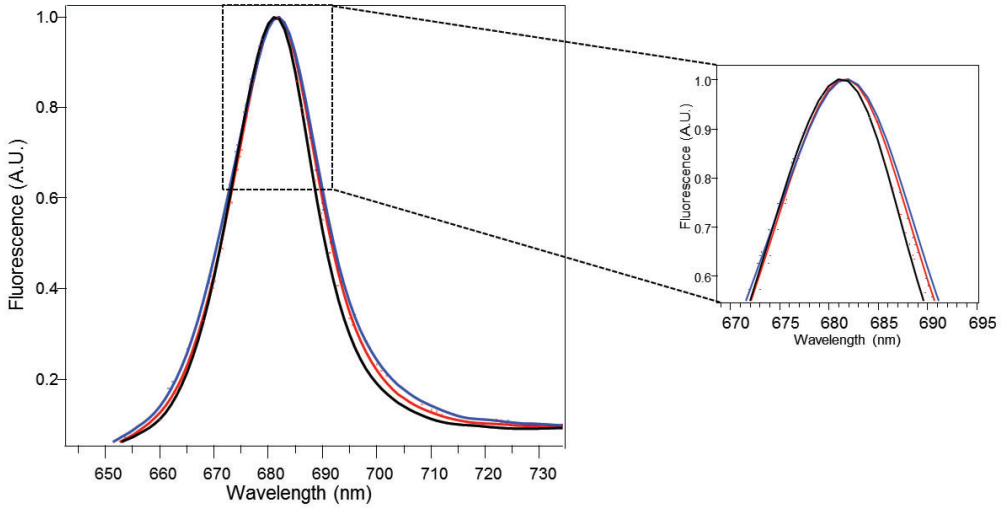

Figure 3.3 Fluorescence emission spectra of LHCII in β-DM in black, LHCII-only proteoliposomes in red and LHCII-PsbS proteoliposomes in blue at pH 7.5 normalized at the fluorescence maximum. Dashed lines: LHCII only proteoliposomes in red and LHCII-PsbS proteoliposomes blue at pH 5.0.

Because interactions between PsbS and LHCII may rely on molecular recognition sites that require protein interaction partners from the same organism, we also analysed PsbS-LHCII proteoliposomes that contained PsbS from *Spinacia oleracea*. Figures 3.4 and 3.5 show the results of a fluorescence analysis on the second set of experiments that were performed on PsbS-LHCII liposomes containing *patens* PsbS (a) or *spinach* PsbS (b). Figure 3.4 shows that the lifetime distributions of proteoliposomes containing *Patens* or *spinach* PsbS are very similar and are not significantly affected by acidification.

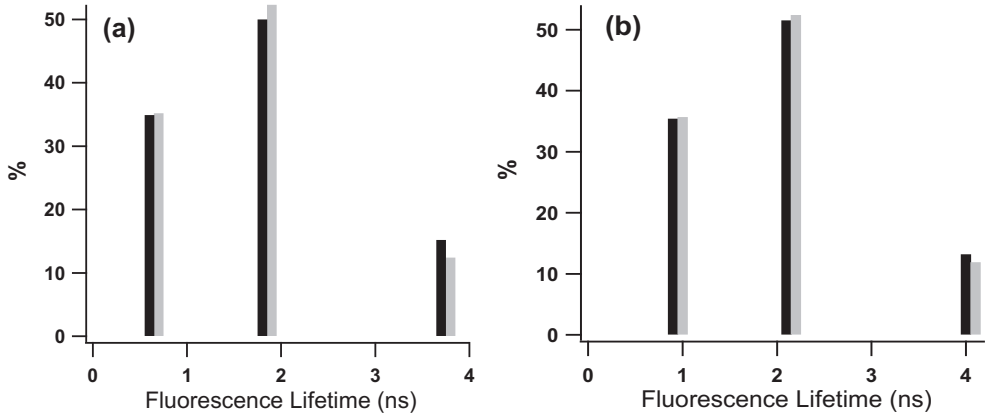

Figure 3.4 TRF analysis of PsbS-LHCII proteoliposomes containing Patens PsbS (a) or spinach PsbS (b). Black sticks: pH 7.5 conditions, grey sticks: pH 5.0 conditions.

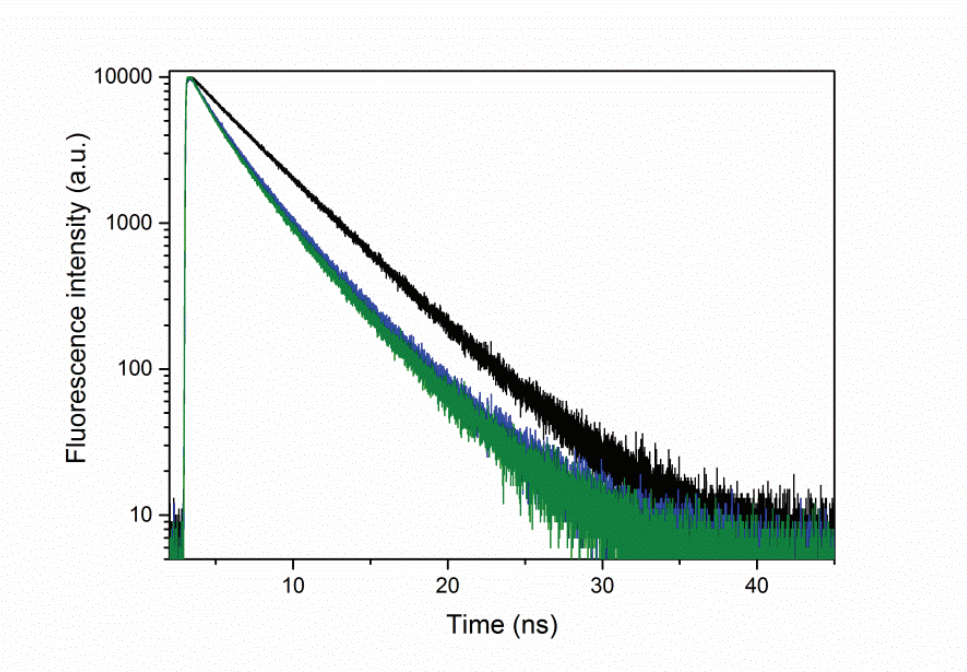

Figure 3.5 TRF experimental and fit traces of the data presented in Figure 3.3 (b). Black: LHCII in 0.03% β-DM, blue: LHCII-PsbS proteoliposomes at pH 7.5, green: LHCII-PsbS proteoliposomes at pH 5.0.

CD SPECTRAL ANALYSIS OF LHCII-PsbS PROTEOLIPOSOMES

The pigment excitonic CD spectrum of LHCII is very sensitive to the changes in the protein conformation or in the protein micro-environment. Figure 3.6 shows the excitonic CD spectra in the visible region of LHCII-only and of LHCII-PsbS proteoliposomes. PsbS does not bind any pigments and the CD bands originate from the excitonic interactions among the LHCII Chl and carotenoid pigments. The low pH CD spectra of PsbS-only and PsbS-LHCII proteoliposomes are very similar. At low pH, an increase of the negative bands at 450 nm and 461 nm is observed. The CD spectrum of LHCII in β-DM detergent is also plotted. The difference between the CD spectral shape of LHCII in β-DM and LHCII proteoliposomes is characteristic for the exchange from a detergent to lipid environment.

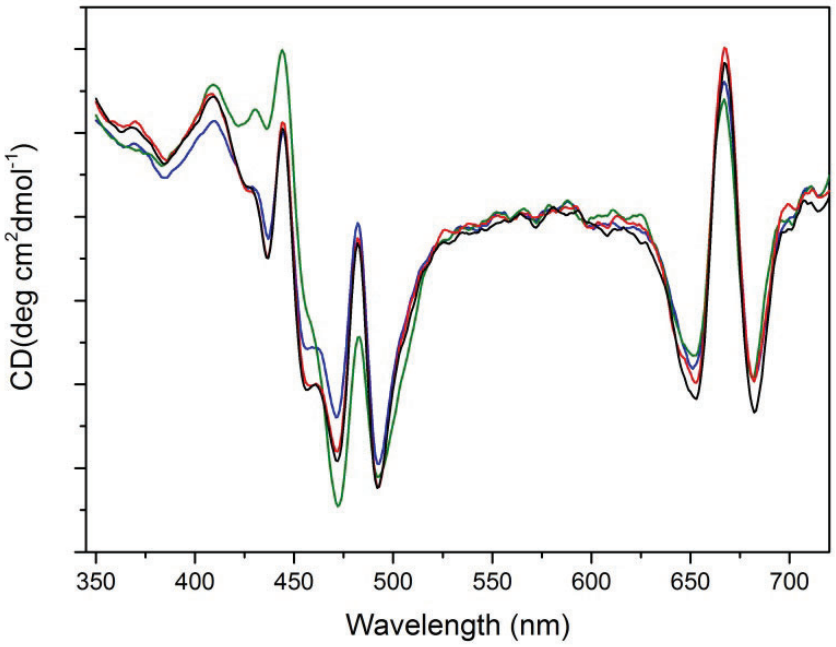

Figure 3.6 CD spectra of LHCII in 0.03% βDM at pH 7.5 (green), LHCII+ Patens PsbS proteoliposomes at pH 7.5 (blue, fluorescence τ_{av}= 2 ns), LHCII proteoliposomes at pH 5.0 (red, fluorescence τ_{av}= 1.6 ns) and LHCII+ Patens PsbS proteoliposomes at pH 5.0 (black, fluorescence τ_{av}= 1.7 ns).

Acidification of LHCII in β-DM detergent did not influence the CD spectral shape (Figure A3.3), indicating that the difference between the proteoliposome spectrum at pH 7.5 and at pH 5.0 is not due to a protonation changes at low pH that affect the LHCII conformation. The proteoliposome CD samples were obtained from different preparations that had small variations in their fluorescence lifetimes. The CD pH 5.0 samples had shorter average lifetimes, 1.6 and 1.7 ns for LHCII-only and for LHCII-PsbS proteoliposomes respectively, than the pH 7.5 sample with 2.0 ns. The small increase in quenching might have an effect on the CD spectral shape, which interestingly would

indicate that CD is a very sensitive tool for detecting quenching-related structural changes.

DISCUSSION

The analysis does not show an effect of PsbS on the LHCII Chl excited-state lifetimes, neither at neutral, nor at low pH, although the slight broadening of the fluorescence emission spectrum of PsbS-LHCII compared to LHCII-only proteoliposomes suggest that PsbS affects the LHCII fluorescence, which implies that PsbS and LHCII proteins are in physical contact with each other.

Our model aimed to mimic the fast activation of PsbS, which is presumed to rely on protonation-state changes, and we did not incorporate Zea that is responsible for the slower qZ contribution of NPQ and depends upon the activation of VDE enzymes. Wilk *et al.* performed experiments on PsbS-LHCII proteoliposomes in Zea-containing membranes [24]. On basis of their results from Western-blotting experiments, it was concluded that PsbS-LHCII heterodimers were formed, and it was shown that Zea-containing proteoliposomes were quenched via Chl-Cars interactions. We found that PsbS reacts to Lhcb1 antibodies and warn that their conclusions from Western blotting could be based on a false-positive result (Figure A3.1). Nevertheless, their data suggest that in the absence of LHCII aggregation, the combination of PsbS and Zea reduces the LHCII fluorescence yields. A much higher concentration of PsbS per lipid was used in their experiments and might be necessary to force PsbS-LHCII one-to-one interactions in an *in vitro* system.

We were not able to reproduce the results of Liu *et al.*, who detected a drop in the fluorescence intensity of PsbS-LHCII proteoliposomes upon acidification [23]. Lowering the pH of proteoliposome solutions in our case would only have a very modest effect, reducing the average fluorescence lifetimes at most ~4%, and directly monitoring the fluorescence intensity before and after acidification did not show a decrease of fluorescence intensities (*data not shown*). Liu *et al.* do not report time-resolved fluorescence experiments. Their approach consisted of direct refolding of PsbS in preformed liposome membranes [23]. We tested this method and also achieved successful refolding of PsbS directly into liposomes. However, because inspection by eye showed the presence of foam remaining in the liposome suspensions, we suspected that not all of the LDS detergent was removed with this procedure and did not continue with this approach.

The negligible effect of pH on the fluorescence lifetimes of LHCII proteoliposomes predicts that lumen acidification *per se* will not influence the quenching states of LHCII. The insensitivity to pH of LHCII proteoliposomes differs from the reported pH sensitivity of pre-aggregated LHCs in low-detergent solutions, where the lowering of the pH significantly enhanced quenching [31]. It is clear that isolated LHCII is not sensitive to pH [32] and the excitonic CD spectrum of LHCII in β-DM does not change at low pH (Figure A3.3), which means that the pH does not affect the conformational energy landscapes of LHCII. The observed increased quenching of LHCII in low-detergent solutions at low pH is associated with stronger aggregation. In liposome membranes, only

lateral aggregates are formed and aggregate cluster sizes are limited to the number of LHCII complexes per vesicle [4,5]. These differences could explain why the pH sensitivity of LHCII proteoliposomes is very low.
The PsbS proteoliposomes give new information on the self-association of PsbS in lipid membranes. PsbS in detergent solutions forms monomer and dimer bands on SDS-page gels, while in PsbS proteoliposomes only forms monomer bands. It is possible that PsbS dimers in the liposome membranes did not resist the denaturing gel conditions, which would infer that PsbS dimers are destabilized in membranes compared to dimers in detergent solutions. Further experiments using cross-linkers have to be performed to determine the oligomerization states of PsbS in liposome membranes under different pH conditions and go beyond this study.
The plot in Figure 3.1 gives an overview of aggregation-dependent quenching in different fluorescence regimes. Fluorescence induction under excess light conditions reduces the average fluorescence lifetimes from ~2 ns in dark to ~0.5 ns under qE conditions. Figure 3.1 shows that starting from conditions that mimic dark-adapted states, *i.e.* average fluorescence lifetime of ~2 ns, qE-mimicking conditions can be achieved by increasing the LHCII density by a factor of 5 to 10; an effect that was already reported by Moya *et al.* [22]. The single-molecule study of Natali *et al.* [4] suggests that at this low PLR, liposome vesicles contain several LHCII trimers. Thus, a membrane response that would create antenna re-arrangements going from a few connected LHCIIs to 10-30 interconnecting LHCIIs would be sufficient to drive the transition from dark-adapted towards qE states. The steep dependence of the LHCII fluorescence on the PLR at very low concentrations complicates the control of LHCII fluorescence states in highly diluted LHCII proteoliposomes, such as have been employed to prevent self-aggregation [24]. We warn that a fluorescence comparison of different sample preparations under those conditions, therefore, should be taken with caution.

CONCLUSIONS

The fluorescence study of our minimal membrane model does not point toward functional quenching interactions between LHCII and PsbS, neither do they show a significant effect of pH. The results strongly suggest that the pH-dependent role of PsbS during the fast qE response lies in creating membrane rearrangements and supercomplex remodelling [28,33] that could facilitate LHCII aggregation quenching, rather than in creating direct quencher states.

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APPENDIX

Western blot analysis of *spinach* LHCII and *patens* PsbS

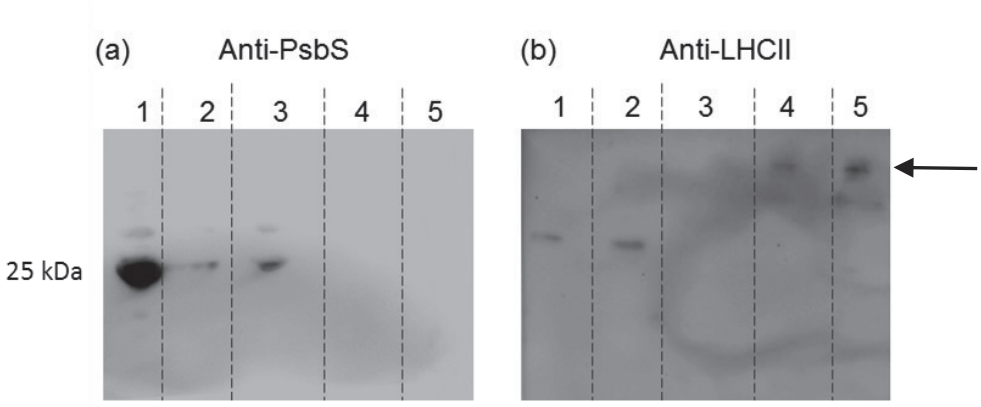

Figure A3.1. Western blotting against PsbS in (a) and against Lhcb1 in (b). In (a), lane 1 contains unfolded PsbS, lane 2 contains refolded PsbS, lane 3 contains both LHCII and PsbS and lane 4 contains LHCII proteoliposomes. Lane 5 is the marker. In (b), lane 1 contains LHCII, lane 2 contains both LHCII and PsbS, lane 3 is the marker, lane 4 contains folded PsbS and lane 5 contains unfolded PsbS. Lane 4 and 5 in (b) show that blotting against Lhcb1 detects high molecular-weight bands of PsbS aggregates (at the arrow) which is due to the unspecific binding of anti-Lhcb1 to PsbS [34].

Estimation of the efficiency of PsbS insertion into asolectin liposomes

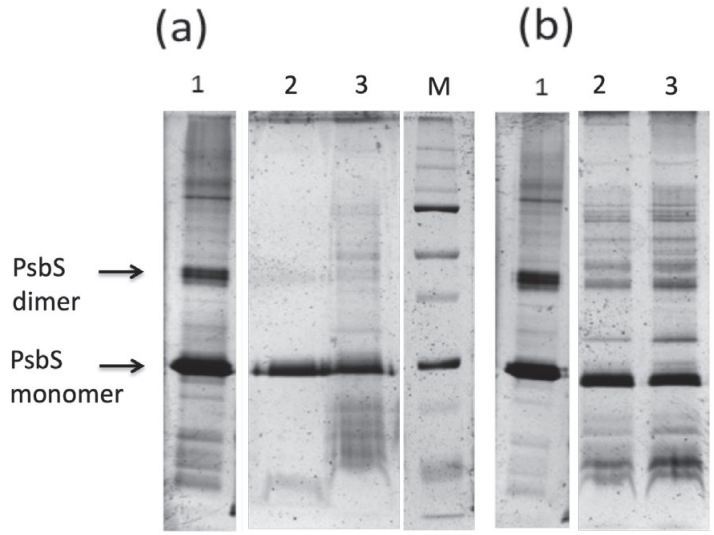

Figure A3.2 SDS-page analysis of two PsbS proteoliposome preparations. (a) 1, PsbS in β-DM, 2 and 3: PsbS proteoliposomes, M, marker. (b): 1, PsbS in β-DM, 2 and 3: PsbS aggregate pellet.

PsbS proteoliposomes prepared at pH 7.5 were separated from non-inserted protein pellet and loaded on a denaturing SDS page silver staining gel. The intensities of the bands of (a) lane 2 and 3 (PsbS proteoliposomes) and (b) 2 and 3 (PsbS pellet) were compared and corrected for the difference in original volume. The pellet was solubilized in a total volume of 41 µl of 4x crack solution from which 20 µl was loaded on the SDS-page denaturing gel. The PsbS proteoliposomes were solubilized in a total volume of 130 µl from which 15 µl was loaded. Comparing the band intensities of (a) and (b) and correcting for the difference in loading concentration, the insertion efficiency was estimated to be ~80%. The pellets in (b) contain some low-molecular-weight bands that could be of degraded protein.

Dynamic Light Scattering analysis of liposomes before and after acidification and nigericin treatment

	Z-Average (Diameter nm)	PdI Width (Diameter nm)
liposomes pH 7.5	175.7	60.1
liposomes pH 7.5 + nigericin	171.1	78.1
liposomes pH 5.0	159.8	59.1
liposomes pH 5.0 + nigericin	169.4	87.1

Table A3.1 DLS-determined diameter sizes of liposomes, confirming that acidification and nigericin treatment did not lead to destabilization of the liposomes.

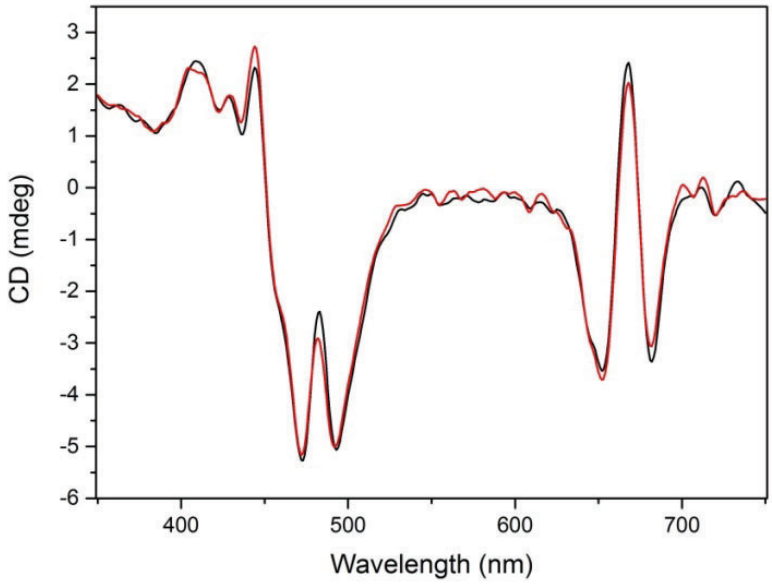

Figure A3.3 CD spectra of LHCII in 0.03% β-DM at pH7.5 (red) and at pH 5.0 (black).