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Polyamines induce aggregation of LHC II and quenching of fluorescence *in vitro*

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ABSTRACT

Dissipation of excess excitation energy within the light-harvesting complex of Photosystem II (LHC II) is a main process in plants, which is measured as the non-photochemical quenching of chlorophyll fluorescence or qE. We showed in previous works that polyamines stimulate qE in higher plants *in vivo* and in eukaryotic algae *in vitro*. In the present contribution we have tested whether polyamines can stimulate quenching in trimeric LHC II and monomeric light-harvesting complex b proteins from higher plants. The tetramine spermine was the most potent quencher and induced aggregation of LHC II trimers, due to its highly cationic character. Two transients are evident at 100 μM and 350 μM for the fluorescence and absorbance signals of LHC II respectively. On the basis of observations within this work, some links between polyamines and the activation of qE *in vivo* is discussed.

1. Introduction

The plant light-harvesting complex of photosystem II (LHC II) functions not only to absorb and deliver solar energy to the PS II reaction centers, but also to protect them from overexcitation. LHC II dissipates excess solar energy as thermal energy under high light conditions, in a process that is referred to as non-photochemical quenching (NPQ) [1]. In the past three decades, the interest has been focused on high energy quenching (qE), the rapidly inducible and reversible component of NPQ, which is triggered when ΔpH across the thylakoid membrane is increased. Although the molecular mechanism of LHC II quenching remains unresolved, the predominant hypothesis claims that LHC II acts as a molecular switch by changing its internal pigment organization [2–5].

The main players in qE are low lumenal pH [6] and xanthophyll cycle carotenoids [7]. In plants, PsbS is also necessary for qE with plants lacking PsbS showing significantly delayed activation of qE [8]. Apart from low pH and zeaxanthin, other agents, such as the presence of dibucaine, have been shown to stimulate quenching *in vivo* and *in vitro* [9–13]. Moreover, it has been shown that for minor LHCb proteins (CP29, CP26 and CP24), addition of dibucaine results in a greater quenching of fluorescence than that for the major LHC II (Lhcb1, Lhcb2 and Lhcb3) [14]. The mode of action of dibucaine is not clear. Dibucaine, a local anesthetic, is a tertiary amine that induces qE in the dark and acts as a "decoupler". This amine stimulates qE via LHC II aggregation [13]. Nature may exploit the endogenous capacity of LHC II to aggregate and quench efficiently the energy of light using natural amines in a similar manner to dibucaine. Thus, biogenic amines that occur normally in chloroplasts and are implicated in the photosynthetic process are putative candidates for this role.

The main polyamines (PAs) are produced and oxidized in chloroplasts of higher plants and over the years, a trickle of results has suggested that PAs play a crucial role in photosynthesis [15]. PAs are low in molecular weight aliphatic amines found in all cells of plants, animals and microbes. Three forms of them occur in plants: free, bound and conjugated to phenolics. Bound PAs are normally found in the PS II and LHC II of higher plants [16]. PAs cause significant alterations in the secondary structure of PS II [17] and at higher concentrations detach oxygen evolving complex [18,19]. *In vitro*, the free pool of the diamine putrescine (Put), has been shown to play a positive role in photophosphorylation [20] and recent *in vivo* data show that Put increases $\Delta\psi$ component of proton motive force[21]. The higher PAs, spermidine (Spd) and spermine (Spm) quench maximal fluorescence of dark adapted tobacco leaf disks and stimulate NPQ at low light and decrease it at high light [22]. Recently, it was shown that tobacco plants over-expressing a maize chloroplast transglutaminase have about double thylakoid-associated PAs and increased qE in comparison to the wild type [23]. Moreover in isolated LHC II from green algae, Spd and Spm increase fluorescence quenching at 680 nm [24]. Hence, a link between antenna properties and PAs is recently established. However, their mode of action is still not clear. Furthermore, the three types of PAs interactions with antenna (free amines,

Abbreviations: LHC II, light-harvesting complex II; Chl, chlorophyll; Neo, neoxanthin; Vio, violaxanthin; PAs, polyamines; Spm, spermine; Spd, spermidine; Put, putrescine; ΔpH, thylakoid proton gradient; qE, ΔpH-dependent quenching of chlorophyll fluorescence; DM, dodecyl-β-D-maltoside; q_F, fluorescence quenching.

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covalently bound and non-covalently bound PAs) have still ill-defined roles. It is known that all three PAs are found in LHC II [25]. Normally, Spd is the most abundant but Spm is a better substrate for transglutaminases *in vitro* [26]. It is accepted that they occur in the micromolar to a few millimolar range in plant cells. Recent studies from our group indicate that upon illumination they accumulate in lumen [21]. Based in previous works with amines it is anticipated that amines could reach 90 mM in lumen for a concentration of about 2 mM in stroma [27]. Upon shuttering of actinic light it is anticipated that ΔpH between stroma and lumen will be minimized and PAs will "return" to stroma. qE is the result of this membrane energization. Here one should keep in mind that all stroma factors are depleted upon plastid isolation thus PAs are lost during *in vitro* experiments. One has to add exogenously the PAs in order to investigate their effects. Thus this work mimics to some extent the natural polyamine environment of LHC II.

In this study, we tested whether PAs stimulate quenching of LHC II from higher plants *in vitro*. As a model we used spinach LHC II and checked for indications of aggregation. We have used conventional fluorescence and absorbance spectroscopy to study the formation of the dissipative conformation of LHC II at two pH values that simulate *in vivo* conditions. Time courses and acid jumps quantify the rapidness of the phenomenon and allow direct comparisons with previous works with dibucaine [13,14]. Moreover, we present the Raman shift at selected regions that shed light on a novel aspect of LHC II aggregation.

2. Materials and methods

2.1. Preparation of the LHC II complex

Thylakoids from spinach leaves were isolated according to Bassi et al. [28]. The LHC II was isolated as in Ref. [29] with minor modifications. Membranes corresponding to 500 μg of Chl were solubilized in 1 ml of 14 mM HEPES (pH 7.5 NaOH) and 0.6% dodecyl-β-D-maltoside (β-DM). The solubilized material was centrifuged at 12,000 *g* for 10 min to eliminate unsolubilized material and then fractionated by ultracentrifugation (210,000 *g*, 19 h, 4 °C) on a sucrose gradient. Gradients were formed directly in the tube by freezing at −80 °C and thawing at 4 °C on a 0.65 M sugar solution containing 14 mM HEPES (pH 7.5 NaOH) and 0.01% β-DM. The green bands corresponding to monomeric LHCb proteins and trimeric LHC II were harvested with a syringe and stored at −25 °C.

2.2. Gel electrophoresis

The protein composition of the LHC II preparation was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [30]. Before loading on SDS-PAGE proteins were precipitated by 10% TCA (final concentration). A protein amount containing 15 μg Chl was loaded in each lane and proteins were separated on a 16% acrylamide gel. Protein bands were colored with Coomassie R250 brilliant blue.

2.3. HPLC analysis

Pigments were extracted by addition of 500 μl ethylacetate, 500 μl distilled water, and a spatula of NaCl to 250 μl of the different fractions collected from the sucrose gradients. The samples were vortexed and centrifuged for 5 min at 13,000 *g*. The organic phases were collected and the pigments dried in a gentle N_2 stream. The dried pigments were dissolved in 80% acetone prior to injection into a Shimadzu Liquid Chromatography apparatus (LC-10AD) equipped with a column (C18, 2.1×200 mm, 5 μm particle size Hypersyl, Hewlett-Packard, USA) and a SPD-M10A diode array detector (Shimadzu SPD-M10A). Pigments were separated by applying a gradient of acetonitrile:methanol (75:25) and H_2O according to Ref. [31]. The chromatograph peaks were quantified by integration of the 445 nm trace and comparison to a calibration curve of purified standards of neoxanthin (Neo), violaxanthin (Vio), lutein (Lut), chlorophyll (Chl) a and Chl b.

2.4. Spectroscopic measurements

Absorption spectra were obtained using an Ocean Optics USB4000 spectrophotometer at room temperature and a DH-2000-BAL light source. Samples were diluted in 14 mM HEPES (pH 7.5) and 20 μM DM at 3.5 μM Chl concentration.

Steady state room temperature fluorescence spectra were recorded using a LS-50B spectrophotometer luminometer (Perkin Elmer). LHC II was diluted in 14 mM HEPES (pH 7.5) with different concentrations of DM at a Chl concentration of about 0.1 μM. For the polyamine titration, salts were added into the cuvette, before the LHC II addition. Acidification was achieved by addition of a small aliquot of HCl to give a final pH 5.7. Excitation wavelength was set at 440 nm, with an excitation slit at 15 nm, whereas the emission slit was 5 nm and scan speed 500 nm/min. The preparations were quite stable on ice for at least 8 h. Spontaneous quenching due to DM decrease from 200 μM to 6 μM was less than 10% for the time needed for spectra collection.

Raman measurements were performed at room temperature using a Nicolet Almega XR Raman spectrometer equipped with a 473 nm blue laser (15 mW) as an excitation source and 672 lines/mm grating by a thermoelectrically cooled (−49 °C) CCD camera. The beam was focused on the sample through a confocal microscope equipped with a 10× objective. Spectra were recorded by accumulation of 2 scans while the exposure time for each sample was 5 s. The setting of the power of the excitation laser was 100%. All spectra were taken with the use of Nicolet OMNIC software in the range between 400 and 4000 cm^{-1}. Samples were in 14 mM HEPES (pH 7.5) and 40 μM DM at 3.5 μM Chl concentration.

Chl concentration was determined from the absorbance of 80% acetonic extracts [32].

2.5. In silico analysis

The models of Fig. 8 were plotted with the PyMOL Molecular Graphics System, version 1.3, Schrödinger LLC (www.pymol.org). Pdb entries were for spinach CP 29 (PDB ID: 3PL9) [33], for spinach LHC II (PDB ID: 1RWT) and for pea LHC II (PDB ID: 2BHW) [34].

3. Results

3.1. Characterization of the isolated LHC II

The upper bands of the sucrose gradient corresponding to monomeric LHCb proteins and trimeric LHC II were used for the polyamine treatments (Fig. 1A). Absorption spectra and SDS-PAGE of the preparations are illustrated in Fig. 1. The fully denaturating SDS-PAGE showed that according to previous works [35–38], band 1 contained the minor LHCb proteins CP 29, CP 26, and CP 24 as well as monomers of LHC II and band 2 contained the trimeric LHC II (Fig. 1B). The room temperature absorption spectrum of LHC II showed maxima at 435 nm and 675 nm (Chl a) and at 473 nm and 652 nm (Chl b) as well as some shoulders in the blue region of the spectrum resulting from xanthophylls (Fig. 1C). The Chl a/b ratio of the trimeric LHC II preparation was close to 1.55. This type of rough measurement of the purity of LHC II indicates that each monomer retains about 8 Chl a and 5 Chl b molecules. By further HPLC analysis (Fig. 1D), a pigment complement of 13 Chl, 1.92 Lut, 0.76 Neo and 0.65 Vio per polypeptide is obtained for trimeric LHC II (Table 1). In agreement with previous works we assumed that each LHC II monomer of the trimer has 13 Chls [29]. This number is a good approximation of the pigment

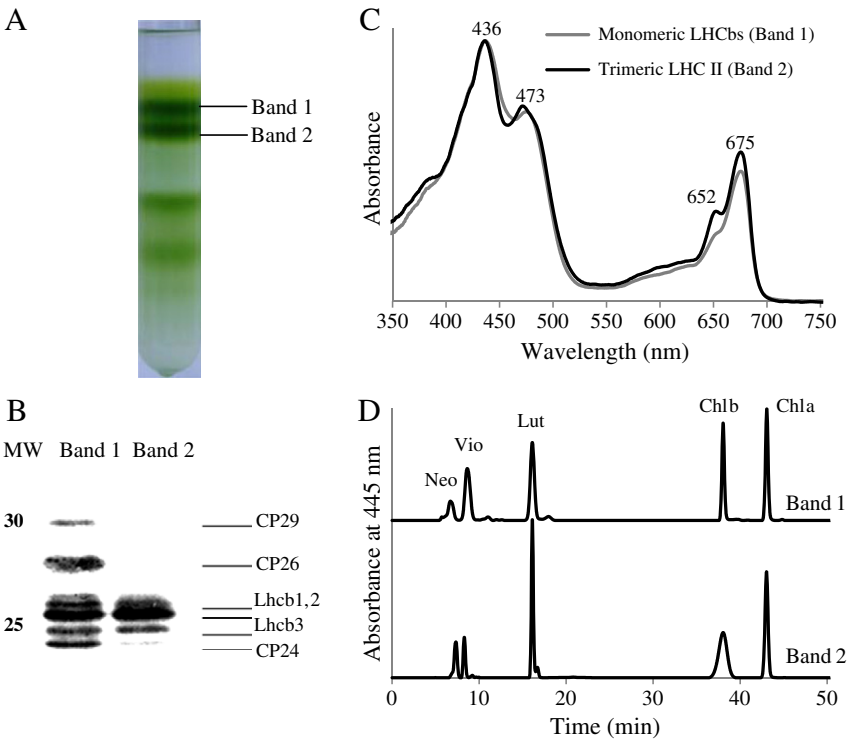

Fig. 1. A. Sucrose density gradient profile of solubilized thylakoids. For each tube, the two fractions indicated were harvested. B. SDS-PAGE analysis of the two upper bands. The identity of each Coomassie band is indicated according to previous works [33,35–37]. C. Absorption spectra of these bands, normalized to the maximum in the blue region. D. HPLC pigment profiles of the two bands.

content of the LHC II monomer. In the same table we provide the pigment profile of the band 1 (LHC II, CP29, CP26, CP24) and we assume that on average 13 Chls are bound to the apoproteins. This is only a rough approximation of the real pigment content but allows a more convenient comparison between the trimeric LHC II and monomeric LHCbs. As an alternative way of presenting the same results we provide in parenthesis in the same column the pigment profile per 100 Chls. The relatively high content of carotenoids of band 1 indicates that our monomeric population (band 1) seems to have lost at least 30% of the Chl and thus the differences from the native state are expected to be big. For this reason the obtained information with these subcomplexes should be interpreted very cautiously and further studies are needed before unequivocal conclusions are drawn.

3.2. The impact of polyamine treatment on isolated LHC II monomers and trimers

Given that *in vitro* aggregation of LHC II is inhibited in DM concentrations larger than its critical micelle concentration (cmc), we diluted our initial solutions to final detergent concentrations below 160 μM [13]. At sub-cmc concentrations LHC II is already found in small aggregates [1,39], while addition of protons or inorganic cations has been found to induce the formation of larger aggregates [14,40]. Spm induces changes in the spectroscopic properties of LHC II (Fig. 2). Addition of Spm causes an increase in absorption around 502 and 685 nm and the appearance of two characteristic minima at around 430 and 470 nm, similar to the low pH effect (Fig. 2A). It was reported that these changes reflect aggregation of LHC II trimers [13,40,41]. Fig. 2B shows the change in the LHC II emission spectrum induced by the addition of 50 μM Spm.

Fig. 3 shows the *in vitro* quenching effect of PAs in LHC II trimer fluorescence at ambient temperature before and after acid jump. Fully fluorescent LHC II (suspended in 200 μM DM) was injected into low DM buffer (6 μM DM) in the presence of selected PAs or cations. Dilution at 6 μM DM at pH 7.5 resulted in a spontaneous quenching that was larger in the presence of PAs (Fig. 3, up down dashed arrows), while acidification resulted in a ~30% additional quenching (Fig. 3a). The presence of 25 μM Spm caused a significant large quenching of LHC II fluorescence (Fig. 3e). Almost the same decrease in fluorescence intensity at pH 7.5 was observed using an order of magnitude greater concentration of Spd (Fig. 3d) and two orders of magnitude greater concentration of Put and $MgCl_2$ (Fig. 3b, c) than that of Spm. Using 250 μM of Spm, it was possible to mimic fully the pH-induced quenching by amine-induced quenching. Addition of Spm, before (0.025 mM Fig. 3e) or after (0.25 mM Fig. 3f) the LHC II sample injection, caused a very strong decrease in fluorescence intensity at pH 7.5, and only a small amount of additional quenching (~10%) was observed by acidification (Fig. 3e, f).

The dependence of the two spectroscopic signals on the Spm concentration is shown in Fig. 4. The relationship between the amplitudes of Chl fluorescence quenching (q_F) at 681 nm and $\Delta A_{502\ nm}$ at pH 7.5 is initially linear, but saturates at high concentrations of Spm (Fig. 4A). At least two effects are expected to happen at this range of Spm concentration: a scattering effect due to formation

Table 1
Pigment to protein stoichiometry of LHCb complexes. The data are presented as the pigment content per monomer (mon) normalized at 13 Chls and normalized to 100 Chl a + b (numbers in parentheses). The values are the average of 2 measurements on two different samples.

	Neo/mon	Vio/mon	Lut/mon	Chl/mon
Monomeric LHCbs	0.90 ± 0.04 (6.93)	2.11 ± 0.18 (16.23)	2.53 ± 0.04 (19.50)	13 (100)
Trimeric LHC II	0.76 ± 0.04 (5.84)	0.65 ± 0.14 (5.00)	1.92 ± 0.05 (14.74)	13 (100)

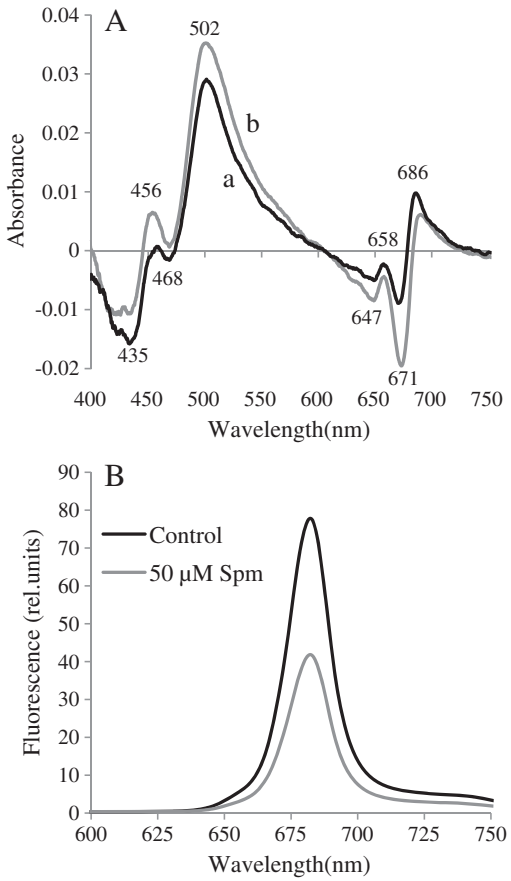

Fig. 2. A. Absorption difference spectra for quenched minus unquenched LHC II trimers. The unquenched samples were dissolved in 200 μM DM, whereas the quenched were diluted in 20 μM DM, (a) at pH 5.7 or (b) treated with 0.35 mM Spm at pH 7.5. B. Fluorescence spectra at ambient temperature of the trimeric LHC II, before (black line) and after the treatment with 50 μM Spm (gray line).

of large particles via LHC II aggregation and absorbance differences due to changes in conformation/oligomerization of LHC II. Scattering is more intense as the wavelength decreases for a given particle size. On the other hand conformational/oligomerization changes produce more clear peaks for example around 502 nm. In order to better understand this latter relationship, both Chl fluorescence quenching and $\Delta A_{502\ nm}$ data were plotted against the Spm concentration. It is shown that Chl fluorescence quenching has a markedly lower transition midpoint than the absorption change. The inflection point for q_F is found around 100 μM Spm, while the midpoint of the absorption change is shifted to 350 μM (Fig. 4B, dashed line).

The mode of action of PAs on monomeric LHCb proteins and trimeric LHC II fluorescence at 6 μM DM was similar at pH 7.5, even though fluorescence quenching of monomeric LHCbs reaches saturation at lower concentrations of Spm (Fig. 5). All PAs stimulated quenching of LHC II complexes, Spm being the most potent quencher. The relative rank of effectiveness was Spm > Spd > Put. Interestingly, PAs caused high quenching in monomeric LHCb proteins that was close to the quenching effect of acidification (Fig. 5A, dotted line). In the case of LHC II trimers Put caused total quenching at a similar extent to acidification and the higher polyamines were even more potent quenchers (Fig. 5B). Please note that maximal quenching (dotted lines in Fig. 5) corresponds to the quenching induced by low pH (pH 5.7). At concentrations of 100 μM and 200 μM DM, near below and above the cmc respectively, addition of acid to lower the pH to 5.7 had negligible effect.

3.3. Raman spectra of the LHC II treated with PAs

As shown in Fig. 6A, all Raman spectra are dominated by an intense band at 1523 cm^{-1} (called ν_1) which arises from C=C stretching vibrations. At the high frequency region of Raman spectra, two additional formyl stretching mode contributions are observed at around 1630 and 1647 cm^{-1} for Spm treated LHC II and LHC II diluted in low pH, which are not exhibited by the untreated LHC II. Moreover, the presence of Spm results in a downshift of 5 cm^{-1} of the bands at 1625, 1615 and 1604 cm^{-1} arising from the stretching modes of the methine bridges, or contributions of those modes (Fig. 6A). ν_2 band, which arises from C–C stretches coupled either to C–H in-plane bending or C–CH_3 stretching, is also affected by Spm in both monomeric LHCbs and LHC II trimers. As clearly shown in Fig. 6B for LHC II trimers, new bands appear at 1113, 1125 and 1206 cm^{-1}. The

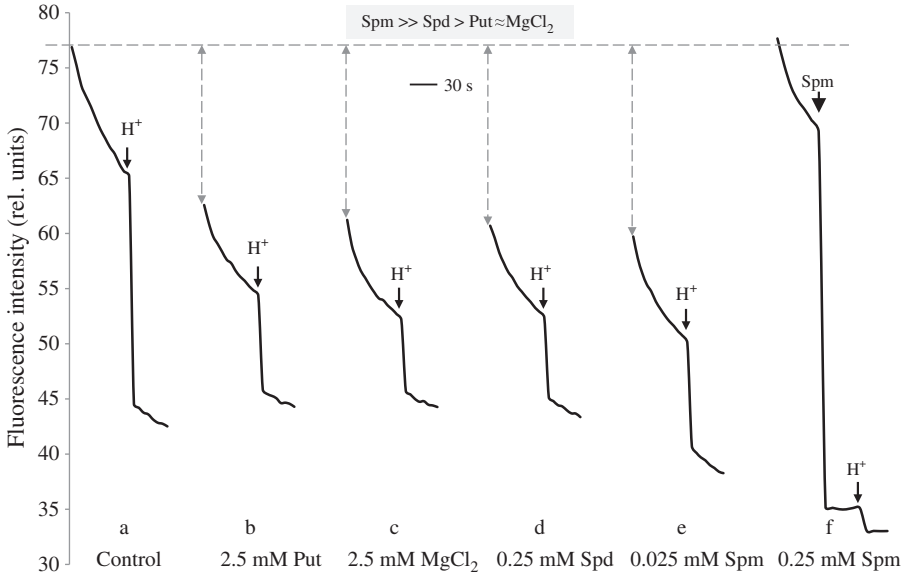

Fig. 3. Chl fluorescence quenching in LHC II trimers upon dilution into 14 mM HEPES (pH 7.5), 6 μM DM. (a) Control; (b) 2.5 mM Put; (c) 2.5 mM $MgCl_2$; (d) 250 μM Spd; (e) 25 μM Spm; and (f) 250 μM Spm added prior to acidification (fat arrow). Black arrows indicate addition of HCl to change the pH of the medium from 7.5 to 5.7. Up down dashed arrows represent the quenching caused by the presence of the cations The samples were excited at 440 nm, and the fluorescence emission was detected at 681 nm. The chlorophyll concentration was about 0.1 μM.

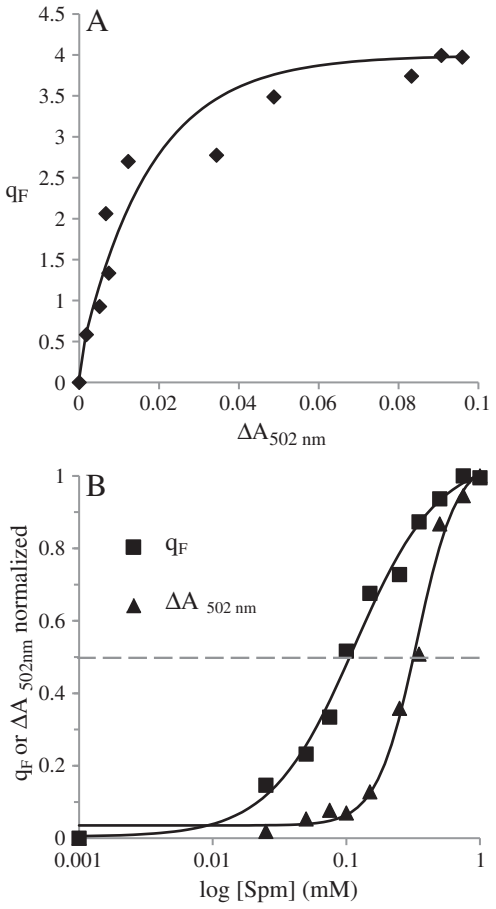

Fig. 4. A. Relationship between $\Delta A_{502\ nm}$ and Chl fluorescence quenching in LHC II trimers upon Spm titration. B. Dependence on the Spm concentration of normalized Chl fluorescence quenching (■) and absorption changes around 502 nm (▲). Quenching was calculated as (Fm − F)/F, where Fm is the fluorescence intensity recorded for a sample diluted into 20 μM DM at pH 7.5 and F is the quenched fluorescence level.

frequency of ν_3 band, which arises from CH_3 in-plane rocking vibrations, is split in two components at 997 and 1006 cm^{-1} (Fig. 7a, b). The satellite of the main ν_3 band at around 1025 cm^{-1} is only present in the Spm treated spectrum of LHC II but has also appeared in the Spm treated spectrum of isolated Chl a in ethanol (Fig. 7, dashed line). The LHC II spectrum has a band at 1041 cm^{-1} (Fig. 7a), consistent with the position of isolated Chl a in ethanol (Fig. 7c), whereas for both Spm treated LHC II and isolated Chl a this band is upshifted about 10 cm^{-1} (Fig. 7b, d).

4. Discussion

As shown by absorption and fluorescence spectroscopy PAs alter LHC II properties (Figs. 2, 3) in a similar way to Mg^{2+} and dibucaine and induce aggregation [13,40–44]. A drop in Chl fluorescence yield at ambient temperatures and an increase in 700 nm fluorescence at cryogenic temperatures have also been observed in isolated thylakoids [45,46] and plant leaves [47] under qE conditions, indicating an aggregation quenching model of qE *in vivo* [48,49]. Recently, direct structural evidence has been provided that qE involves the clustering of LHC II within the thylakoid membrane, explaining the consistently observed spectroscopic link between qE and aggregation of LHC II *in vitro* [8]. Even though it has been suggested that the aggregation quenching mechanism observed *in vitro* and NPQ *in vivo* could be the same [44,50], the exact quenching site and the quenching units participating remain a question [51]. On the other hand due to differences between the LHC II 3D network *in vitro* and the structural changes that are happening during qE *in vivo*, all data from isolated LHC II, including those of the present study, should be viewed with caution. In agreement with previous works [13,14,52] it has been shown here that the extent of quenching for the monomeric LHCbs is greater than that for the trimeric LHC II complex (Fig. 5). At this point we should repeat that our preparations of minor antenna contain to a small extent some LHCb 1–3. A rather overlooked process that could be of importance in aggregation/quenching phenomena is the post translational polyaminylation of LHCbs [23]. Both trimeric LHC II and CP29, CP26 and CP24 are normal substrates of the plastidal

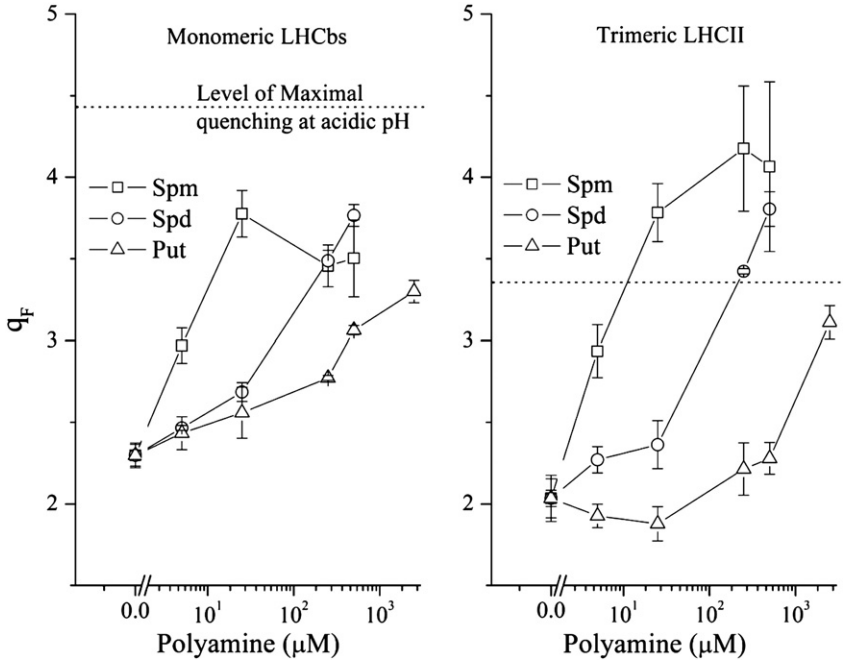

Fig. 5. Log-concentration response of LHCb monomers (A) and LHC II trimers (B) fluorescence produced by Spm (■), Spd (●) and Put (▲) at pH 7.5. The dotted lines correspond to the total quenching of the complexes at pH 5.7. Data were obtained upon dilution into 14 mM Hepes, pH 7.5, 6 μM DM at a Chl concentration of 0.1 μM. The values which are ± standard error are obtained from 3 to 6 replicates. Polyamines were present in the dilution buffer. Quenching was calculated as (Fm − F)/F, where Fm is the fluorescence intensity recorded for a sample diluted into 200 μM DM and F is the quenched fluorescence level. Note that q_F at 0 mM of amine added is not zero. This is due to the quenching of fluorescence upon dilution of 200 μM DM to 6 μM.

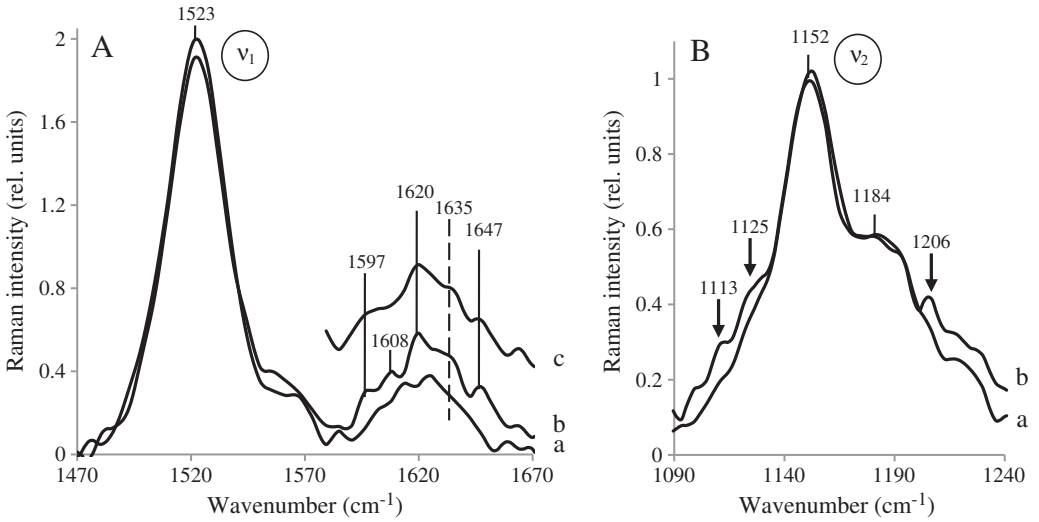

Fig. 6. ν_1 and high frequency (A) and ν_2 (B) regions of carotenoid Raman spectra induced by 473 nm excitation for LHC II (a) and LHC II treated with 1 mM Spm (b). For the high frequency region, the spectrum of LHC II in low pH is also shown for comparison (c).

TGase [26,53] and putative sites of qE [3,54]. It has also been demonstrated that a plastidial transglutaminase activity in maize polyaminylates LHC II in a light dependent way [26]. Thus, it has been speculated that they change conformation upon polyaminylation, which in turn promotes dissipation [23]. On the other hand differences in the composition of the band could be responsible for this effect.

Polyamines at 6 μM DM were shown to induce fluorescence quenching of LHC II even in the absence of ΔpH, similar to the effect of dibucaine under similar experimental conditions (Figs. 3, 5) [13,14]. The observed quenching becomes larger as the cationic charge of the amine increases (Put<Spd<Spm). Therefore, it seems likely that aggregation might be induced by nonspecific electrostatic interactions. Similar effects were reported in LHC II treated with monovalent, bivalent and trivalent inorganic cations [40]. An attempt to illustrate the number of negative charges and their distribution in the surface of spinach LHC II and CP 29 from available 3D structural data is shown in Fig. 8. Please note that some residues from the N terminal are missing in these structures (13 from spinach LHC II, 87 from CP 29, 9 from pea LHC II). It is evident that positively charged molecules (such as amines or inorganic cations) can interact via coulombic forces mainly with the stroma and lumen side of LHC II and CP 29. Moreover molecules with spaced positive groups such as Spm (4 positively charged groups) can more easily fit the spotted profile of negative charges of LHC II (red regions in Fig. 8) and neutralize it so that repulsion between different complexes is minimized. This is more clear in LHC II from pea mainly because the resolution is better. In this case, the screening of the negative charged parts of the macromolecule by the cation reduces the repulsion between the proteins [55]. In agreement with Kirchhoff et al. [40], this *in vitro* study supplies evidence that the fluorescence quenching is not exactly correlated with LHC II aggregation. The transition observed by fluorescence quenching takes place at a significant lower Spm concentration than the transition observed by absorption changes, which correspond to the degree of aggregation (Fig. 4). It has been suggested that the fluorescence changes recorded are related to changes in the lateral microorganization within already formed small aggregates due to the low detergent concentration [40]. Thus, a possible model for polyamine-induced aggregation of LHC II could comprise a surface charge screening until larger aggregates can be formed. A more recent mechanism that involves an internal conformation change causing quenching will be discussed below regarding Chl b3.

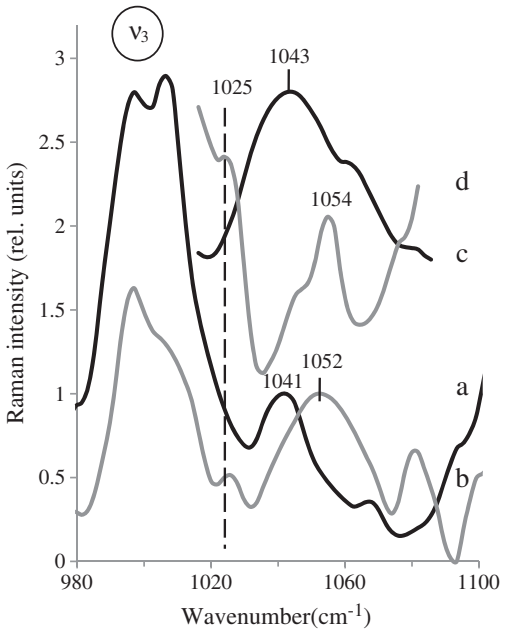

Fig. 7. ν_3 region of the LHC II (a) and LHC II treated with 1 mM Spm (b) Raman spectra, induced by 473 nm excitation. The Raman spectra induced by 473 nm excitation of isolated Chl a (c) and isolated Chl a treated with Spm in a molar ratio 1:30 (d) dissolved in ethanol, are shown for comparison.

It has been shown, in previous works, that polyamines also increase fluorescence quenching of isolated LHC II from green algae [24], while *in vivo*, Spm and Spd induce NPQ in higher plants under low light conditions [22]. Hence, Spm has a larger capacity for quenching both *in vitro* and *in vivo* while Put is the less potent. This higher sensitivity of LHC II to the higher polyamines suggests a more specific binding of Spm and Spd to LHC II. Although Spd is less potent than Spm the physiological role of Spd could be more important on the grounds that Spd is the most abundant polyamine bound to LHC II [26]. Further information about the nature of the molecular interactions responsible for the aggregation can be obtained from Raman spectra.

The high frequency region of Raman spectra evidence at least two Chl b populations, free from interactions in the trimer, having formyl carbonyl groups vibrating at different frequencies in the presence of Spm or at low pH (Fig. 6A). One, at 1647 cm^{-1}, corresponding to a formyl group either involved in a medium molecular interaction or

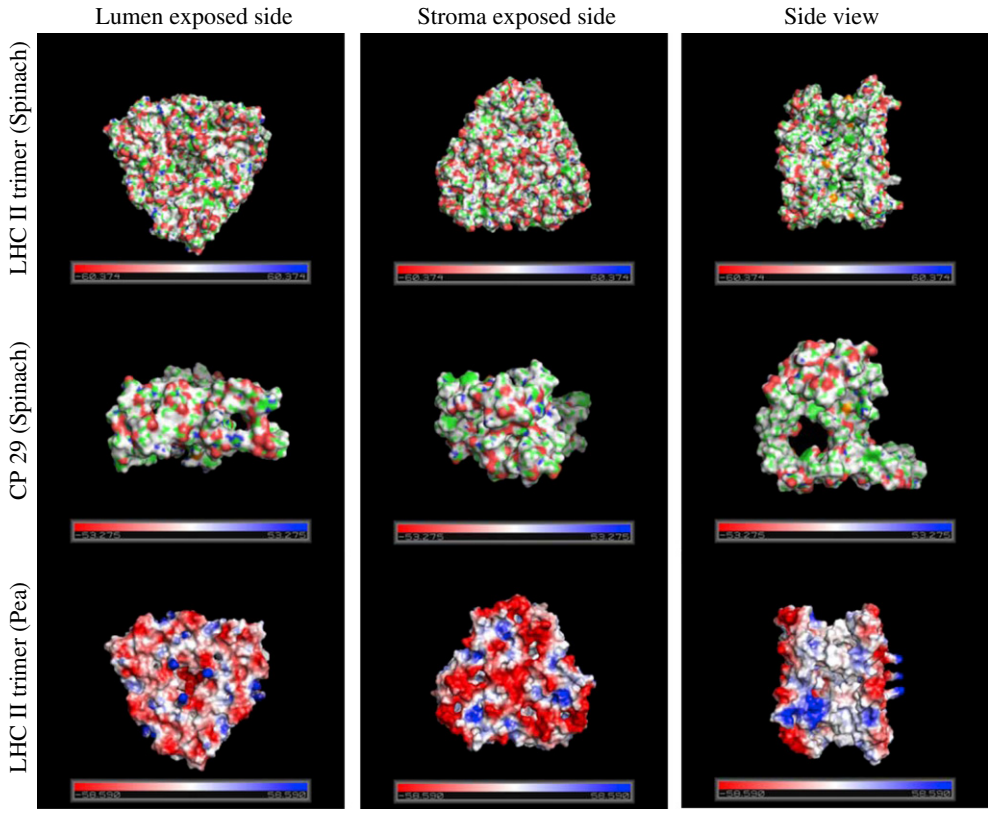

Fig. 8. Surface charges of LHC II (from spinach and pea) and CP 29 (from spinach). Rough models of electrostatic surfaces demonstrate that negative charged spots (red) exist at stroma and lumen exposed sides of the complexes. This pattern of negative spots makes easy the interaction with molecules with spaced charged positive groups. Polyamines and in particular the biogenic Spm molecules have 4 positively charged moieties that could bind via columbic forces to LHC II and CP 29 and explain some of the results of the present as well as previous works. Images were created in PyMOL for spinach CP 29 (PDB ID: 3PL9), for spinach LHC II (PDB ID: 1RWT) and for pea LHC II (PDB ID: 2BHW). Models were plotted with the PyMOL Molecular Graphics System, version 1.3, Schrödinger LLC (www.pymol.org).

in a polar environment, and another at 1630 cm^{-1}, indicating a formyl group involved in a weak strength hydrogen bond with its peptide environment [56]. The formation of H-bonds to formyl groups of Chl b molecules upon aggregation of LHC II complexes is well documented [3,56,57]. The presence of the intense ν_1 carotenoid band impairs observation of the Chl modes at the same region, which would give information about the coordination state of their central Mg ion. In a recent work, we have shown that polyamines can bind axially to isolated chlorophylls via the Mg [58]. In the present study, the downshift of the band at 1615 cm^{-1} to 1608 cm^{-1} indicates that the equilibrium of a Chl molecule has moved towards the 6th coordination [59]. This peak at 1608 cm^{-1} is not present at the spectrum taken at low pH, indicating a specific interaction caused by Spm. Spm could have a direct or an indirect effect. More particularly, the new ligand of Mg could be either a Spm molecule or a neighboring side chain that has approached the Chl due to conformational changes induced by Spm treatment. A putative site of this new 6th coordination could be Chl 8 of LHC II (b3 Chl that is available in LHC II, CP 29 and CP 26) (nomenclature of [34]). We showed recently that the domain that ligates Chl 8 has similar structure to the domain of heme in myoglobin that modulates the positioning of the metal in heme. This domain could provide to Chl 8 the 6th ligand which in turn will affect the positioning of the Mg. A possible interpretation of our results (as well as former results in the field of amine and cation stimulation of qE *in vitro*) is that amines or/and amine-induced aggregation lead to deeper insertion of the Mg in the macrocycle and this quenches fluorescence of one Chl (*e.g.* Chl 8) creating a sink that quenches the fluorescence of the LHC II. In line with this concept the positive peak in the region of 470–600 nm could originate from a reinsertion of Mg from an out-of-plane position in an in-plane position as reported for Chl b [58]. Thus a b3 Chl (available in LHC II, CP 29, CP 26) with the metal out of plane will show high fluorescence and low absorbance at the 535 nm region and the same Chl with the metal in plane will show quenched fluorescence and higher absorbance at the 535 nm region. Future experiments could answer whether this domain of LHC II could play such a role during qE response. Also Chl b3 is a Chl b and thus not the best candidate for fluorescence quenching.

According to Ruban et al. [60], one can assign the satellite bands in ν_2 region at 1113, 1125 and 1206 cm^{-1} to neoxanthin (Neo). The association of trimeric LHC II into small oligomers has been shown to induce a distortion of the Neo molecule [61]. Although Neo is not required for quenching, changes in its conformation have been correlated to qE *in vivo* [4]. Another feature of the Raman spectra giving clues as to the identity of the molecular interactions responsible for the Spm induced aggregation is shown in Fig. 7. Bands at 1041 and 1052 cm^{-1}, for LHC II and Spm treated LHC II respectively, can be assigned to C–O stretching modes coupled to skeletal vibrations [62]. Here, these modes are probably attributed to Chl a, which has a similar spectrum to LHC II. Interestingly, FTIR measurements have revealed that the interaction between isolated Chl a and 9-cis violaxanthin (Vio) leads to the same upshift of this band [63]. Taking into account that 9-cis Vio can mimic the molecular properties and functions of Neo in LHC II [64], the interpretation of Neo distortion upon aggregation is supported. However, on the other hand, in the absence of carotenoids, the interaction of isolated Chl a with Spm results in a maximum at 1054 cm^{-1} consistent with that of Spm treated LHC II. This is an indication that the band at 1052 cm^{-1} is determined by direct Chl a–Spm interaction in the complex. However, in order to support more strongly its role in qE further experiments are required. The next stage of this work will involve incorporation of LHCb

proteins into liposomes, examining the effect of ΔpH and PAs on Chl fluorescence.

This experimental approach has been (over) exploited by other groups, but a major issue, still not solved, is whether random 3D aggregates of LHCs in solution might be a good model for qE *in vivo*, where LHCs move in 2 dimensional membranes and only particular contacts are possible. Moreover, aggregation and quenching in LHCs can be obtained in many different ways: by adding mono-bi-trivalent cations, by lowering the pH, by removing the detergent, by adding zeaxanthin, by increasing pressure, by adding PAs (this paper) or other ions, by crystallization [8]. A reasonable assumption is that in isolated LHC II there are more quenching possibilities than *in vivo*. However, one of these paths might be the one used by nature. Future experiments will elucidate the quencher. Noncovalently bound PAs at present are not the best candidate for the quenching mechanism and we see our work as a must do series of experiments in order to shed light on possible *in vivo* interactions. Furthermore, recent works tested the hypothesis regarding concentration of PAs (namely putrescine) in lumen during the illumination of thylakoids *in vivo* [21]. At present it seems more probable than before that a gradient of amines is formed in the chloroplast *in vivo* and that the concentration of PAs that experience the antenna proteins (as well as all other thylakoid proteins) is many times higher than previously described. Assuming that PAs occur in the micromolar to few millimolar range in plants and in chloroplasts and that ΔpH between stroma and lumen is about 2 then one expects up to 100 times more PAs in lumen during illumination (*e.g.* 0.5 mM PAs in lumen of dark adapted samples could lead to 50 mM upon illumination).

4.1. Concluding remarks

Polyamines stimulate quenching both in spinach LHC II and monomeric LHCbs. The transient of quenching has a midpoint of 100 μM for the tetramine Spm which is more potent quencher than Spd and Put. In addition, Spm treatment induces aggregation of LHC II. Raman spectra indicate that polyamine induced changes at LHC II mimic to a great extent the effect of protons (*i.e.* acidification of the suspension). On the grounds that *in vivo* protons trigger qE the reported results open new ways to address the long standing question of the mechanism of qE. Although under *in vivo* conditions protons accumulate in a vectorial way in relation to LHC II, our preparations shed some light to interactions of polyamines with this subcomplex.

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