DOI: 10.1007/s11099-014-0066-1 PHOTOSYNTHETICA 52 (4): 636-640, 2014

BRIEF COMMUNICATION

Proton concentration in the thylakoid membranes can regulate energy distribution between the two photosystems

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Abstract

The aim of our study was to investigate the role of protons in regulating energy distribution between the two photosystems in the thylakoid membranes. Low pH-induced changes were monitored in the presence of a proton blocker, N,N'-dicyclohexylcarbodiimide (DCCD). When thylakoid membranes were suspended in a low-pH reaction mixture and incubated with DCCD, then a decrease in the fluorescence intensity of photosystem II (PSII) was observed, while no change in the intensity of photosystem I (PSI) fluorescence occurred according to the measured fluorescence emission spectra at 77 K. Since low pH induced distribution of energy from PSII to PSI was inhibited in the presence of DCCD, we concluded that pH/proton concentration of the thylakoid membranes plays an important role in regulating the distribution of the absorbed excitation energy between both photosystems.

Additional key words: light-harvesting complex; luminal pH; nonphotochemical quenching; oxygen consumption; oxygen evolution; spinach.

Photosynthetic reactions in plants and green algae fix CO_2 by converting solar energy into electrochemical energy with the contribution of sequentially functioning PSII and PSI. In the nature, unexpected changes in light intensity could lead to overexcitation of the photosystems. Photosynthetic efficiency is one of the key factors which regulate plant growth and development. To balance the rate of photosynthesis under changing environmental conditions, several mechanisms have been evolved, such as protective, nonphotochemical quenching (NPQ), state transitions, and *etc*. Among these mechanisms, quenching of chlorophyll (Chl) fluorescence (q_E), a feedback regulatory process of PSII dissipates excessive light energy safely as heat on luminal acidification of the thylakoid membranes (Tokutsu and Minagawa 2013).

In higher plants, induction of q_E depends on activation of the xanthophyll cycle (Horton *et al.*1996, Niyogi *et al.* 1997) as well as on the sensing of luminal acidification or proton concentration by PsbS, a protein homologous to light harvesting complex (LHC) proteins (Li *et al.* 2004). Electron microscopic studies have revealed that PsbS protonation regulates the macro-organisation of PSII complexes in the grana membranes of higher plant chloroplasts (Kereiche *et al.* 2010). A role of PsbS in regulating cyclic electron flow by controlling the tight coupling between cytochrome b_6f and PSI was proposed (Roach and Krieger-Liszkay 2012). Protonation of PsbS drives concerted conformational changes in light harvesting complex of PSII (LHCII) leading to q_E (Kiss *et al.* 2008). Direct interactions of LHCs of PSII with PsbS monomers have been recently shown to regulate NPQ in *Arabidopsis* (Wilk *et al.* 2013). This sensing of pH by PsbS is a natural device to protect PSII from damage. Protonation of PsbS induces a change in the macro-organization of the thylakoid membranes (Horton *et al.* 2008, Kereiche *et al.* 2010) that results in the aggregation of LHCII proteins and/or induces conformational change(s) in LHCII, generating energy-quenching sites in the LHCII aggregates (Johnson *et al.* 2011) and establishing a low-fluorescence state that is measurable as NPQ. Thus both PsbS protein and zeaxanthin are thought to have crucial roles in q_E quenching in higher plants. It is also known that the LHC plays a vital role in the energy distribution between the two photosystems (Allen and Forsberg 2001, Cardol *et al.* 2003). LHC is not a static structure but it is able to interact with both the photosystems to maximize photosynthetic efficiency (Iwai *et al.* 2010).

Received 22 January 2014, *accepted* 4 April 2014.
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Abbreviations: DCCD − N,N'-dicyclohexylcarbodiimide; NPQ – nonphotochemical quenching; q_E – energy-dependent quenching.
Acknowledgement: Supports from University Grant Commission, India (No. F.5-26/2007(BSR) to T. Tongra and from Department of Science and Technology, India (INT/RFBR/P-173) to A. Jajoo are thankfully acknowledged.

It is well known that illumination of dark-adapted chloroplasts leads to the alkalization of stroma and acidification of thylakoid lumen and generates proton gradient and electrical potential difference across the thylakoid membrane (Kramer *et al.* 1999). Low pH in thylakoid lumen is a result of proton release from water molecules in the oxygen-evolving complex and proton influx from stroma which is mediated by plastoquinone. It is evident that the pH of the medium significantly affects the proton flux across the thylakoid membranes and also suggests that under physiological condition, the luminal pH in chloroplasts declines to around pH 5.5. At this pH, a fine tuning of the other electron transport components occurs to ensure proper functioning of PSII (Kramer *et al.*1999, Waloszek and Wieckowski 2004).

Energy distribution between the two photosystems is regulated by a number of factors including light intensity (Wollman 2001, Tikkanen *et al.* 2012) and ionic environment of the thylakoid membrane (Jajoo *et al.* 1998*,* Singh-Rawal *et al.* 2010). To maintain maximal rates of photosynthesis at limiting light intensities, plants have evolved a mechanism that enables them to optimize the balance of incoming light energy between PSI and PSII (Bergantino *et al.* 2003*,*Tikkanen *et al.* 2012) by the mechanism called "state transitions" (Minagawa 2011).

In higher plants, effects of low pH on photosynthetic machinery are well documented. At low luminal pH, protonation of carboxyl groups of PSII Chl *a*/*b* antenna proteins induces conformational change in the PSII antenna which in turn generates energy-quenching centers (Horton *et al.* 1996). It was hypothesized that low luminal pH might have a role to drive state transitions (Singh-Rawal *et al.* 2010) and it induces structural reorganization in the thylakoid membranes (Jajoo *et al.* 2012, 2014). The PsbS monomer to dimer ratio in isolated thylakoid membranes varies with luminal pH, where at acidic pH, the monomeric and the active form for NPQ prevails (Bergantino *et al.* 2003).

DCCD has been shown to (*1*) bind to the lumen-exposed carboxyl groups of antenna proteins (Horton *et al.*1996*,* Walters *et al.* 1996*,* Jahns and Heyde 1999) and (*2*) inhibit the low pH-induced fluorescence quenching (Ruban *et al.* 1998, Liu *et al.* 2008). DCCD binds to the acidic sites close to the lumen surface (as found for CP26), and putative Chl-binding sites in the hydrophobic interior (as for CP29) (Ruban *et al.* 1998). At the site of water oxidation, DCCD stops the extrusion of protons into the lumen and at the site of plastoquinone reduction*,* it stops the uptake of protons from the medium (Jahns *et al.* 1988). DCCD also alters the pH dependence of violaxanthin de-epoxidase activity (Jahns and Heyde 1999). Thus it is widely used as a proton blocker.

Singh-Rawal *et al.* (2010) suggested that acidification of lumen leads to the protonation of specific glutamate residues that induces a conformational change in LHCII resulting in the detachment of LHCII from PSII and its migration and attachment towards PSI. In other words, all these observations suggest that protons in the lumen bind to certain residues (may be PsbS or LHCII or some others) and thus control various elements of nonphotochemical quenching. In this study, we blocked the proton binding sites by treating the thylakoids with a proton blocker DCCD. In such case, free binding sites are not available for protonation of any of the residues and thus energy distribution caused by low pH-induced changes should not be observed. The work presented in this paper provides a new insight regarding the mode of action of proton concentration in thylakoid membranes.

Thylakoid membranes were prepared from fresh market spinach (*Spinacia oleracea*) in the following manner: The spinach leaves were washed with distilled water, dried, deveined, and then kept overnight in a refrigerator for dark incubation. Approximately 100 ml of isolation medium (0.33 M sucrose, 50 mM HEPES-NaOH, pH 7.5, 1 mM $MgCl_2$, and 10 mM NaCl) was used per 20 g of spinach leaves. Homogenate was then filtered through four layers of nylon gauze and four layers of cheese cloth. Filtrate was then centrifuged at 7,500 × *g* for 8 min at 4ºC. The pellet was suspended in shock medium (50 mM HEPES-NaOH, pH 7.5, 1 mM $MgCl_2$, 10mM NaCl) and kept in the dark for 3 min. The homogenate was again centrifuged at 7,500 × *g* for 8 min at 4ºC. The pellet was resuspended in isolation medium (pH 7.5). The Chl concentration of fresh membranes was measured following Porra *et al.* (1989) and the suspension was kept in liquid nitrogen till further use.

Thylakoids were treated at different pH (7.5, 6.5, and 5.5) by suspending the membranes in isolation medium of different pH for 30 min in the dark. DCCD was added (from an 20 mM ethanolic stock solution to give a final concentration of 50 μM) in 2 ml of the reaction mixture of respective pH containing 20 μg Chl of thylakoid membranes. Incubation of thylakoids with DCCD was carried out at room temperature for 15–20 min in the dark. All measurements were performed five times and averaged spectra were presented.

Chl *a* fluorescence emission spectra at low temperature (77 K) were recorded in a spectrofluorimeter (*FP-6300 model*, *Jasco*, Japan) equipped with a liquid nitrogen dewar container with a continuous flow of nitrogen gas at room temperature from beneath to avoid formation of ice crystals from the water vapour. The reaction mixture and measurement conditions are described in Singh-Rawal *et al.* (2010).

PSII activity was measured in terms of [μmol(O_2 evolved) mg(Chl)$^{-1}$ h^{-1}]. The PSI activity was measured in terms of [μmol(O_2 consumed) mg(Chl)$^{-1}$ h^{-1}] by monitoring N, N, N, N-tetramethyl-p-phenylendiamine (TMPD) to methyl viologen (MV) reaction. Both measurements were carried out using a Clarke-type oxygen electrode (*Hansatech*, King Lynn, UK). Reaction mixture and measurement conditions are as described in Singh-Rawal *et al.* (2010).

The redox state of P700 was monitored by measuring the absorbance changes at 820 nm using *PAM-100*

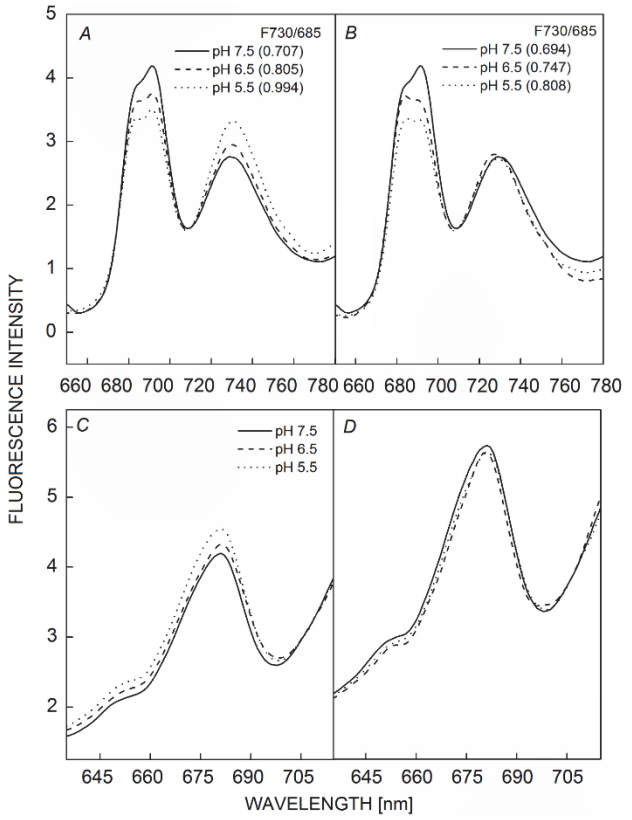

Fig. 1. Low-temperature (77 K) fluorescence emission spectra of the thylakoid membranes treated with different pH (7.5, 6.5, and 5.5) without addition of N,N'-dicyclohexylcarbodiimide (DCCD) (*A*) and in the presence of DCCD (*B*). Low-temperature fluorescence excitation spectra of the thylakoid membranes treated with different pH (7.5, 6.5, and 5.5) without addition of DCCD (*C*) and in the presence of DCCD (*D*). The emission spectra have been normalized at 710 nm, while the excitation spectra have been normalized at 705 nm.

fluorimeter (*Walz*, Germany). The samples were illuminated for 30 s by far red light in order to oxidize P700. Re-reduction of $P700^+$ in the dark was then recorded.

Measurement of a spectrum at low temperature (typically at liquid nitrogen temperature, 77 K) sharpens the peaks and reveals the details of small shoulders. At 77 K, three maxima were resolved in the spectrum at about 685, 695, and 730 nm (Fig. 1*A*) believed to originate predominantly from Chl *a* in CP47 of PSII, a specific Chl in CP43 of PSII and PSI, respectively (Lambrev *et al.* 2010). Low-temperature fluorescence emission spectra were measured in spinach thylakoid membranes treated with different pH (7.5, 6.5, and 5.5). As evident from Fig. 1*A*, lowering of the bulk pH of the thylakoid membranes resulted in a consistent decrease in the fluorescence intensity at 685 and 695 nm and a simultaneous increase in the intensity of the peak at 730 nm. An increase in the F_{730}/F_{685} ratio suggested that PSI received more energy at the expense of PSII, or in other words, the state transition took place (Singh-Rawal *et al.* 2010). Decrease in fluorescence of PSII with the high proton concentration/acidification of thylakoid membranes suggests a low pH inducible mechanism in PSII that dissipates excess light energy, measured as the q_E. Another probability is that binding of both protons and xanthophylls to specific sites in the PSII antenna causes a conformational change within LHCIIs that switches PSII units into a quenched state, generating energy-quenching site(s) in the LHCII and establishing a low fluorescence state that is measurable as NPQ (Horton *et al.* 2005*,* Johnson *et al.* 2011*,* Tokutsu and Minagawa 2013). Low pH can also lead to accumulation of $P680^+$. The result is that whatever amount of energy is trapped by LHCs it is not utilized properly in the photochemical reaction of PSII. Hence, the overall fluorescence was reduced with increasing acidification or proton concentration of thylakoid membrane by decreasing pH from 7.5 to 5.5. It is indeed known that under state transitions, the fluorescence emission is inversely proportional to the yield of PSII photochemistry and it is proportional to the size of its light-harvesting antenna (Singh-Rawal *et al.* 2010).

However, when thylakoid membranes treated with different pH were incubated with 50 μM of DCCD, a decrease in PSII fluorescence at low pH, but no increase in PSI fluorescence was observed in the fluorescence emission spectra (Fig. 1*B*). Decrease in PSII fluorescence might occur because of activation of energy-dependent fluorescence quenching (q_E) which is a type of NPQ. However, in the presence of DCCD, no change in PSI fluorescence indicated that PSI had received extra energy. Probably DCCD treatment led to covalent modification in carboxyl groups of LHCII subunits which are involved in protonation. Further rearrangement of the molecule finally forms the stable dicyclohexyl-N-acyl urea (Azzi *et al.* 1984). Hence the ion-binding sites accommodating the structure have no bound H^+ available for transport because of neutralization of the conserved carboxylic groups. DCCD has been proposed to influence the ring structure in a way that mimics the arginine residue in the subunit (Pogoryelov *et al.* 2009*,*Mizutani *et al.* 2012).

Fig. 1*C* represents fluorescence excitation spectra (77 K) at 730 nm at each pH value, normalized at 710 nm. The results showed that the thylakoid membranes treated at pH 6.5 and 5.5 received more excitation energy from Chls absorbing at around 683–684 and 654–655 nm, indicating that the absorption cross-section of the PSI light-harvesting complex (LHCI) had increased because of the association of LHCII. Furthermore, no change in PSI fluorescence excitation spectra was observed with DCCD treatment (Fig. 1*D*).

To confirm the results obtained with 77K fluorescence emission measurements, rates of electron transport through PSII and PSI were monitored at different pH (Table 1). It is clear that at low pH, rates of electron transfer through PSII decreased, while that of PSI increased. Increased rate of re-reduction of $P700^+$ also suggested that PSI is getting more electrons at low pH. However, in the presence of DCCD, pH-induced effects

Table 1. Change in the rates of electron transport of PSII and PSI under different pH conditions in the presence and absence of N,N'-dicyclohexylcarbodiimide (DCCD).

Treatment	Rate of oxygen evolution [μmol($O_{2\ evolved}$) mg(Chl)$^{-1}$ h^{-1}]	Rate of oxygen consumption [μmol($O_{2\ consumed}$) mg(Chl)$^{-1}$ h^{-1}]	$P700^+$reduction kinetics [s]
pH 7.5	257 ± 12	345 ± 15	16.2 ± 0.5
pH 7.5 + DCCD	249 ± 10	337 ± 15	15.7 ± 0.4
pH 5.5	188 ± 12	490 ± 14	11.7 ± 0.2
pH 5.5+ DCCD	176 ± 12	463 ± 15	12.2 ± 0.4

were observed almost to the same extent. Although it is known that DCCD inhibits PSII and Cyt b_6f, rates of PSI were not found to be inhibited in the presence of DCCD. This is because of the use of artificial electron donors and acceptors which have their site of action beyond Cyt b_6f.

Our finding suggests that low pH (higher proton concentration) in the thylakoid membranes controls energy distribution in favour of PSI. If the binding of protons is inhibited by the use of a proton binder such as DCCD, pH induced effects were not observed. However, whether the change in energy redistribution is a result of spillover or state transitions is yet to be explored. Further work on mutants which cannot perform state transitions (*e.g.*, *stn7*) may resolve this issue.

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ERRATUM DOI: 10.1007/s11099-014-0054-5

Erratum to: Accumulation of photosynthetic pigments in *Larix decidua* Mill. and *Picea abies* (L.) Karst. cotyledons treated with 5-aminolevulinic acid under different irradiation

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Erratum to: Photosynthetica
DOI: 10.1007/s11099-014-0019-8

The acknowledgements were completed in the corrected version:

Acknowledgements: This work was supported by Structural Funds of EU Project of the Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic, Bratislava, ITMS No. 26110230078 and the Slovak Research and Development Agency (APPV-20-020805). We thank Y. Fujita, J.-D. Rochaix, and B. Grimm for providing the primary antibodies used in this work. We thank MSc Robin Rigg for English correction.

The publisher and authors apologize for these errors and for inconveniences they may have caused.

The online version of the original article can be found under doi: 10.1007/s11099-014-0019-8
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