LOW pH INDUCED ALTERATIONS IN THE THYLAKOID MEMBRANES MORPHOLOGY AND FLUIDITY

Svetozar Stoichev, Tonya Andreeva, Iren Tsonova, Mira Busheva, Stefka Taneva, Sashka Krumova

Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences
Acad. Georgi Bonchev Str., Block 21, Sofia 1113, Bulgaria
e-mail: svetlio85@abv.bg

ABSTRACT

The first event that triggers the photoprotective mechanism non-photochemical quenching (NPQ) is the protonation of the thylakold lumen upon saturating illumination. Here we study the effect of protonation on the overall morphology of thylakoid membranes by means of atomic force microscopy and on the fluidity of the lipid matrix following the spectroscopic properties of the lipophilic fluorescent dye merocyanine 540 (MC540).

The protonation of the thylakoid membranes induced significant increase in their area and height, and hindered the incorporation of MC540 in the lipid phase – an indicator for rigidification of the membrane or at least of the stroma exposed membrane layer accessible for the fluorescent probe. These changes in the thylakoids architecture and lipid phase behavior are most probably a result from the protonation-induced protein reorganizations in the granal membrane regions.

Keywords: *non-photochemical quenching, thylakoid membranes, merocyanine 540, atomic force microscopy, fluorescence spectroscopy*

INTRODUCTION

During photosynthesis the electron flow from photosystem II (PSII) to photosystem I (PSI) is conjugated with directed movement of protons (H^+) from the stroma to the thylakoid lumen that generates a pH gradient across the thylakoid membrane (TM) that reaches its maximum in conditions of excess light (1). Over-excitation is harmful for the photosynthetic organisms and can cause damage in its pigment-protein structures. Higher plants, therefore, possess several mechanisms against such an over-illumination conditions, dissipating the potentially harmful excess energy as heat – non-photochemical quenching of chlorophyll *a* fluorescence (NPQ) (2,3). *In vivo* the protonation is an initiating signal for series of consecutive conformational changes leading to NPQ (4). It is believed that the changes in the macroorganization of the photosynthetic complexes caused by protonation are an important part of the photoprotection mechanisms.

The *in vitro* protonation of thylakoids mimics (concerning the thylakoids morphology) the effect of strong light and was demonstrated that it leads to decrease in the membrane thickness and in the spacing of grana membranes (5). By means of AFM we have recently observed the same effect in isolated grana membranes and further showed that these effects are accompanied by lateral rearrangements of the PSII supercomplexes and formation of extended domains of the major light harvesting complex of PSII (LHCII) (6) in line with earlier reports (7,8). At present, it is known that the decrease of the lumenal pH of TM either *in vivo* in high light conditions or *in vitro* when triggering NPQ, is sensed mainly by the lumen-exposed glutamate residues of the PsbS subunit of PSII and of LHCII. The following structural and special rearrangements of pigment-protein complexes are believed to be of considerable physiological importance for light harvesting optimization and photoprotection (8,9).

The main purpose of the present study is to study the changes in the thylakoid architecture and lipid phase induced by the protonation of the thylakoid lumen and thus to get an insight in the initial steps of NPQ. Thylakoids in two functional states – partly deprotonated (as model for dark-adapted condition) and protonated (as model for high light–adapted condition) states were studied.

The obtained results provide a more detailed picture of the structural changes occurring in thylakoid membranes upon NPQ initiation.

MATERIALS AND METHODS

Plant material

The experiments were performed on thylakoid membranes isolated from 10-14 days-old pea (*Pisum sativum RAN-1*) plants, grown as hydroponic culture at controlled light intensity of 60 μmol $m^{-2}s^{-1}$ photon flux density, 23°C, 57% humidity and 12h photoperiod.

Thylakoid membrane preparation

Thylakoid membranes were isolated according to Harrison and Melis (1992). Before the microscopic and spectroscopic measurements the thylakoid membranes were washed and suspended in a buffer containing 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose and either 50 mM Tricine-NaOH (pH 7.8) or 40 mM MES-NaOH (pH 5.3).

Merocyanine 540 fluorescence

For analysis of the physical state of the thylakoid lipid matrix we studied the spectral characteristics of the lipophilic fluorescent probe merocyanine 540 (MC540). Room temperature (20 ^{o}C) excitation spectra of MC540 were recorded on a Jobin Yvon JY3 spectrofluorimeter at 590 nm detection wavelength (1 nm step upon 450–575 nm excitation), excitation and emission slits of 10 nm. The samples (with concentration of 20 μg Chl/mL^{-1}) were treated with MC540 (Sigma-Aldrich Co.) at a final concentration of 0.2 μM. The spectra of thylakoid membranes incubated with MC540 were corrected for the Chl emission (in the 450-575 excitation range) of thylakoid membranes devoid of MC540. Data shown are representative of 6 independent experiments.

Atomic force microscopy

Atomic force microscopy (AFM) imaging (resolution 512 x 512 pixels) was performed in tapping mode in air using Nanoscope V AFM (Bruker Inc.) with silicon scanning tips (Tap300Al-G, Budget Sensors, Innovative solutions Ltd, Bulgaria), tip radius <10 nm, at a resonance frequency of 150±75 kHz and scan-rate of 0.2 Hz. 100 μl of freshly isolated thylakoids in buffer containing 2% (v/v) glutaraldehyde was deposited on a poly-L-lysine coated mica. After 30 min the mica surface was rinsed with the suspension buffer and gently dried with a nitrogen flow. The AFM images were analysed using NanoScope 6.13R1 software. Data shown are representative of 4 independent experiments.

RESULTS AND DISCUSSION

Thylakoid membrane morphology in partly deprotonated and protonated state

It has long been noticed that light changes the thylakoid membranes morphology; electron microscopy and light scattering studies have shown that illumination with red light (as well as *in vitro* acidification of the medium) leads to tighter and more extensive grana stacking (5). Recently AFM PSII specific illumination was found to trigger increase in the thylakoids height, the number of stacks in granas and the membrane stiffness (10). The observed effects were assigned to lumen expansion driven by the pH gradient and the consequently induced state transitions (10). Wheeler and Fagerberg (2000) have also shown that the light intensity moderates the thylakoids morphology - fast changes in the thylakoids architecture were related to broadening and shortening of the grana stacks upon transition from high to low light. Pfeiffer and Kupinska (2005) have also revealed that granas are better defined upon light adaptation and largely disintegrated upon extensive dark adaptation.

In this work the thylakoid membranes morphology of partly deprotonated (pH 7.8) and protonated (pH 5.3) states was compared (Fig. 1 C-H). Two parameters, thylakoid area and height

(Fig. 1 A, B) were determined from the AFM images. The data showed significant differences between partly deprotonated and protonated thylakoids – the area of the protonated is about 2.5 times larger than that of partly deprotonated samples (Fig. 1 A); the same trend holds true for the thylakoids height, it increased with 40 % at pH 5.3 compared to pH 7.8 (Fig. 1 B).

Our data are fully in line with the PSII light induced swelling of thylakoid membranes shown by Clausen et al. (10). However, it should be noted that in our case the observed effect of pH on the thylakoids volume is stronger than that reported in (10) probably due to species specificity regarding the extent of grana stacking between pea and *Arabidopsis*. The larger size of thylakoids at pH 5.3 is probably due to the spatial reorganization of their granal and stromal regions and consequently higher grana stacking (as observed by Murakami and Packer (5) and Semenova (11)). These authors however did not report large low pH induced increase in thylakoids volume as seen by us, probably due to the different approach applied (lack of sucrose and $MgCl_2$ in the medium, etc.) and thus the different physical state of the thylakoid membranes.

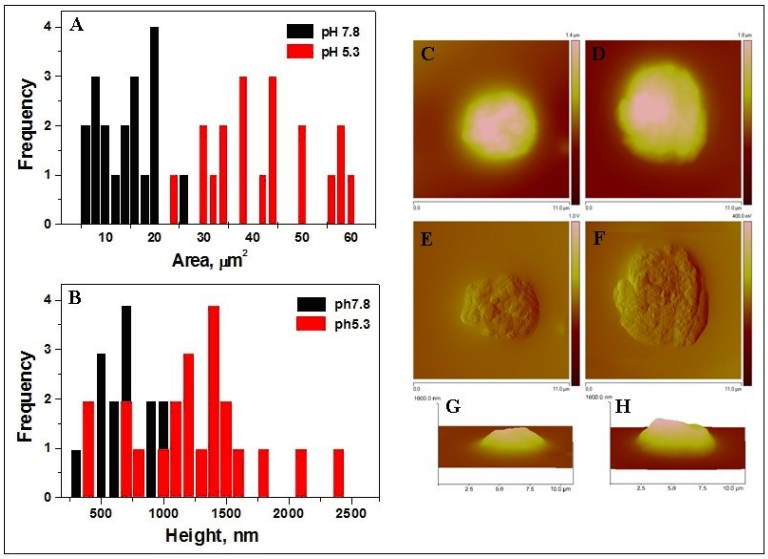

Figure 1. *Histogram plots of the area (A) and height (B), 2D AFM images of intact thylakoid membranes deposited on mica at pH 7.8 (C, E) and pH 5.3 (D, F), and their 3D representation (G, H). Scanning size area – 11x11 μm.*

Thylakoid membrane fluidity in partly deprotonated and protonated state

Applying steady-state fluorescence we studied the packing of the lipid molecules in thylakoid membranes (in partly deprotonated and protonated state) by the lipophilic fluorescent marker MC540 (Fig. 2); its fluorescence spectra reflect changes in the fluidity of their immediate surrounding (18); when embedded into model and native membranes MC540 is sensitive to the existence of domains with different phase and different degree of packing of the lipid molecules (13,14). In lipid bilayers MC540 molecules are incorporated in the membrane as monomers (15,16), but may also exist as dimers (located on the surface of the membrane), or aggregates – in solution (17). The excitation spectra of MC540 when embedded in thylakoids are characterized by a 566 nm emission peak originating from deeply incorporated in the lipid phase MC540 monomers, and a 536 nm shoulder corresponding to surface (stroma) exposed MC540 dimers; their ratio, E566/536, being a marker for the extent of lipid packing (18).

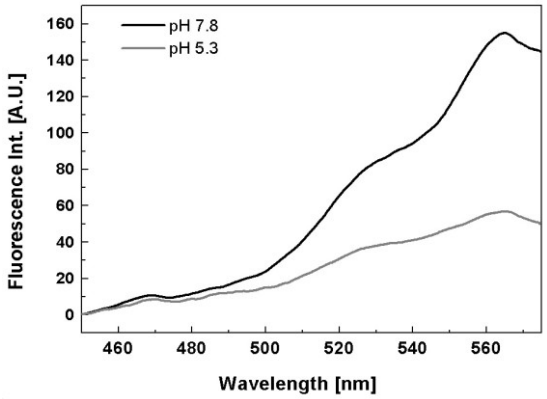

Figure 2. *Excitation spectra of MC540 incorporated in partly deprotonated (black line) and protonated (gray line) thylakoid membranes. The emission was recorded at 590 nm.*

The E566/E536 ratio at pH 5.3 was lower by about 21 %, as compared to pH 7.8 (Table 1), indicative for hindered incorporation of MC540 in the thylakoid membranes at the lower pH, at least in the stroma exposed membrane layer which is accessible for MC540.

Table 1. *Fluorescence intensity ratio E566/E536 (mean ± SD) determined for MC540 incorporated in TM at pH 7.8 and pH 5.3.*

pH	E_{556}/E_{536}
7.8	1.70 ± 0.1
5.3	1.34 ± 0.1

The observed effects strongly indicates that the *in vitro* protein protonation is accompanied with tighter packing of the lipid molecules. This might be induced either by: (i) protonation-induced protein rearrangements in granas, which might also affect the lipid-protein and lipid-lipid interactions; (ii) increased grana size which hinders the MC540 incorporation (our unpubished data show that the incorporation of MC540 is largely hindered in isolated grana fragments but readily incorporated in isolated stromal lamellae) or (iii) by the direct protonation of the lipid head groups and consecutive change in their physical state.

CONCLUSIONS

In this work we clearly demonstrate that *in vitro* reduction of pH to values achieved upon excess light induces expansion of thylakoid membrane system accompanied by tightening of the lipid matrix packing. It can be assumed that these morphological changes are a prerequisite for the accomplishment of the the multicomponent photoprotective mechanism NPQ.

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