Low-pH induced reversible reorganizations of chloroplast thylakoid membranes - as revealed by small-angle neutron scattering

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Abstract

Energization of thylakoid membranes brings about the acidification of the lumenal aqueous phase, which activates important regulatory mechanisms. Earlier Jajoo and coworkers (2014 FEBS Lett. 588:970) have shown that low pH in isolated plant thylakoid membranes induces changes in the excitation energy distribution between the two photosystems. In order to elucidate the structural background of these changes, we used small-angle neutron scattering on thylakoid membranes exposed to low p^2H (pD) and show that gradually lowering the p^2H from 8.0 to 5.0 causes small but well discernible reversible diminishment of the periodic order and the lamellar repeat distance and an increased mosaicity – similar to the effects elicited by light-induced acidification of the lumen. Our data strongly suggest that thylakoids dynamically respond to the membrane energization and actively participate in different regulatory mechanisms.

Keywords: chloroplast thylakoid membranes, lamellar repeat distance, low pH and p^2H, small-angle neutron scattering (SANS)

Highlights:

1. Thylakoid membranes exposed to low p^2H studied by small-angle neutron scattering
2. Acidification causes reversible shrinkage and diminished lamellar order
3. SANS changes induced by low pH resemble those due to light-induced lumenal acidification

Abbreviations: p^2H (pD), deuterium analogue of pH; NPQ, non-photochemical quenching; qE, the energy-dependent component of NPQ; $\Delta\mu_H^+$, transmembrane electrochemical potential gradient; PSI, photosystem I; PSII, photosystem II; LET, linear electron transport; CD, circular dichroism; SANS, small-angle neutron scattering; q, scattering vector; I, intensity; q*, center position of the Bragg peak; RD, repeat distance; φ, azimuthal angle; I(φ), angular dependency of the scattering intensity; FWHM, full width at half maximum

1. Introduction

In photosynthesis charge separation followed by vectorial electron transport is coupled to proton translocation processes. This creates a transmembrane electrochemical potential gradient ($\Delta\mu_H^+$) between the inner and outer aqueous phases of the photosynthetic membranes - in chloroplasts, the lumenal and stromal sides, respectively, of the thylakoid membranes. $\Delta\mu_H^+$, which is utilized for the synthesis of ATP, consists of electrical field and ΔpH components of ~10^5 V cm^{-1} and ~2-3 pH units, respectively. They modulate the electron transport rate via different feedback regulatory mechanisms. The transmembrane electric potential gradient is required for metabolite and protein transport across the membranes [1]. The ΔpH component (i.e., the acidification of the lumen) is involved, perhaps most prominently, in the photoprotective mechanisms of non-photochemical quenching (NPQ) of the first singlet excited state of chlorophyll-a [2]. In particular, qE, the energy-dependent component of NPQ depends on the acidification of the lumen [3]. It is generally agreed that NPQ requires the structural flexibility of thylakoid membranes. In fact, there are several reports demonstrating the involvement of structural changes at different levels of structural complexity [4-14]. Some of these changes might be directly linked to the generation of ΔpH, e.g., via the redistribution of ions in the 'electrolyte' following the generation of $\Delta\mu_H^+$ [15, 16] and, in particular, upon the acidification of lumen and the binding of protons to different polypeptide residues [2, 17, 18].

In general, the effects of pH on many physiological processes in plants are well established and significant work has been done to explore its effects on different photosynthetic processes. In addition to the involvement of lumenal acidification in NPQ, acidic lumen leads to inhibition of Photosystem II (PSII) activity due to a reversible dissociation of Ca^{2+} from the water splitting enzyme [19]. In vitro, the oxygen evolving complex loses Ca^{2+} at pH<6.0, inhibiting water splitting and rendering the PSII reaction center inactive [20]. The linear electron transport (LET) can also be down-regulated via back-pressure due to the build up of ΔpH [21]. The photosynthetic machinery in plants is endowed with a strong ΔpH-dependent control mechanism of LET from cytochrome b6f to PSI. By using the pgr5 mutant of Arabidopsis, which is deficient in strong light-induced ΔpH, it has been shown that PSI also plays role in excess energy dissipation and the control of LET [22].

In earlier works, structural and functional changes have been induced by exposing isolated thylakoid membranes to low pH [23-25]. By using 77K fluorescence excitation and emission spectroscopy on isolated spinach thylakoid membranes, it has been shown that low pH induces a redistribution of the excitation energy between the two photosystems. By analysing data obtained on state-transition and NPQ mutants of Arabidopsis, it has been shown that the increase in the 77K emission of PSI and the concomitant quenching of PSII fluorescence in thylakoid membranes exposed to low pH cannot be accounted for by state transitions. They originate from a PsbS-protonation dependent spillover of the excitation energy from PSII to PSI. It has also been shown, by using circular dichroism (CD) spectroscopy of isolated thylakoid membranes, that low pH induces substantial but essentially fully reversible changes in the chiral macroorganization of the protein complexes without noticeable changes in the excitonic interactions, i.e., at the level of bulk pigment-protein complexes [23]. These reorganizations in the CD were similar to those induced by light [26-28]. Here, in order to obtain more information on the nature of these membrane reorganizations we used small-angle neutron scattering (SANS) and investigated the effect of low pH on the multilamellar organization of isolated pea thylakoid membranes. Our data reveal a small but well discernible low-pH induced shrinkage ($\leq$ 2 nm) in the repeat distance of the grana thylakoid membranes and a diminishment in their periodic order, which is accompanied by an increased mosaicity of the membranes.

2. Materials and Methods

2.1. Isolation of thylakoid membranes. Thylakoid membranes were isolated as described earlier [29] from freshly harvested three-weeks-old pea leaves (*Pisum sativum*, Rajnai törpe) grown in a greenhouse at 20–22 °C in soil under natural light conditions. Briefly, leaves were homogenized in ice-cold grinding medium A, containing 20 mM Tricine (pH 7.6), 0.4 M sorbitol (or 0.3 M NaCl [30]), 5 mM $MgCl_2$ and 5 mM KCl, and filtered through six layers of medical gauze pads. After discarding the remaining debris by centrifugation at 200×*g* for 2 min, the supernatant was centrifuged for 5 min at 4000×*g* and the pellet was resuspended in 10 ml osmotic shock medium containing 20 mM Tricine (pH 7.6), 5 mM $MgCl_2$ and 5 mM KCl. After a short, 5–10 s, osmotic shock, breaking the envelope membrane and allowing the replacement of the stroma liquid with the reaction medium, the osmolarity was returned to isotonic conditions

by adding equal volume of double strength medium. This suspension was then centrifuged for 5 min at 4000×*g*. The thylakoid samples were stored at 4 °C until further treatments and/or use in the experiments.

pH treatments. The pH/p^{2}H was adjusted on the suspension medium without thylakoids. The p^{2}H was measured with a pH meter, while using the correction factor described in [31]. In order to maintain the same pH/p^{2}H values in the suspension medium containing the thylakoids, the thylakoid samples were washed twice with reaction medium A adjusted to different pH values (pH 7.5, 6.5, 5.5 or 4.5), for thermoluminescence (TL) measurements, or in the same, D_2O-based medium, to p^{2}H (pD) 8.0, 7.0, 6.0 and 5.0, for SANS experiments. The chlorophyll concentration was adjusted to 1–2 mg/ml for SANS measurements, and 1.3 mg/ml for TL measurements. The pH/p^{2}H-treated thylakoid membranes were kept in dark at room temperature for 30 min before the measurements. After 30 min, half of the samples were used in the measurements, and for the recovery experiments, the remaining samples were washed twice with reaction medium A adjusted to pH 7.5 or p^{2}H 8.0 for TL (Fig. S1) and SANS measurements, respectively; the measurements were performed after 30 min incubation at these pH/p^{2}H values.

2.3. SANS experiments. SANS measurements were performed on the SANS-II small-angle neutron scattering instrument at the Paul Scherrer Institute, Villigen, Switzerland, as previously described [29]. The wavelength, sample-to-detector distance and collimation were 6 Å, 6 m and 6 m, respectively. The collimation slit was a 24 mm diameter pinhole at the opening of the collimation section. At the end of the collimation section, i.e. directly before the sample, we used an overlapped 7 mm * 10 mm rectangular and a 10 mm diameter pinhole. The wavelength distribution ($\Delta\lambda/\lambda$) was 10 %. The isolated thylakoid membranes were measured at room temperature in a quartz cuvette of 2 mm optical path length in the presence of ~0.4 T horizontal magnetic field with the field vector perpendicular to the neutron beam. The samples were measured for 2*5 min (with sorbitol as osmotic medium) and 5*2 min (with NaCl as osmotic medium).

2.4. SANS data treatment and fitting procedures. All experimental data are normalized to the number of beam monitor counts; instrumental backgrounds and scattering from the suspending media were subtracted from the scattering profiles. The detector efficiency was calculated from background-subtracted water measurement. The primary data were treated with the Graphical Reduction and Analysis SANS Program for Matlab - GRASP (developed by Charles Dewhurst, ILL). The obtained two-dimensional data were reduced from 2D to 1D profile via radial or azimuthal averaging. The radial averaging was performed in two 75° sectors around each opposite Bragg diffraction peaks [32] in order to obtain intensity (I) versus scattering vector (q) curves.

The scattering curves were fitted with the phenomenological model expressed by the linear combination of a constant, power and Gauss functions in the q region of 0.01-0.033 $Å^{-1}$ (sorbitol) and 0.015-0.042 $Å^{-1}$ (NaCl) around the Bragg peak in order to determine the center position of the Bragg peak (q*) [32]; this value was used to calculate the thylakoid membrane repeat distance (RD), according to RD=2π/q*. In order to better visualize the shift in the position of the Bragg peak we also used the Kratky-plot ($I(q)\cdot q^2$ vs q) [33], where *I(q)* was obtained as follows: the radially averaged intensity in vertical orientation (with an opening angle of 75°) was subtracted from the radially averaged intensity in horizontal orientation (with an opening angle of 75°). Due to the magnetic orientation the Bragg peaks are significantly more pronounced in the field direction (horizontal); therefore, with this subtraction the contribution of the isotropic signal is minimized and the difference spectra exhibit better defined Bragg peaks.

In order to provide information on variations in the mosaicity of membranes, we determined $I(\varphi)$, the angular dependency of the scattering intensity. To this end, 2D SANS profiles were azimuthally integrated across the q region of 0.017-0.44 (sorbitol) and 0.025-0.040 (NaCl) $Å^{-1}$ for 360° interval with 5 pixel binning (φ is the azimuthal angle).

The I(φ) curves were fitted with the sum of a constant and two (due to the symmetric scattering profile) modified Lorentzian functions $I(\varphi) = I_{\varphi_0} + \frac{A}{(\cos^{-1}(\cos(\varphi-\varphi_0)))^2+\frac{FWHM^2}{4}} + \frac{A}{(\cos^{-1}(\cos(\varphi-\varphi_0-\pi)))^2+\frac{FWHM^2}{4}}$. The full width at half maximum (FWHM) of the Lorentzian function provides information about the anisotropy of scattering profile, thus the magnetic orientability of sample; I_{φ_0} and A are constants, φ_0 is the position of the first peak, $\cos^{-1}(\cos(\varphi))$ is applied in order to fulfil the periodic boundary conditions.

A quantitative characterization of the degree of orientation can be obtained by Hermans orientation function [34], which is defined as $f = \frac{3\langle cos^2\varphi\rangle - 1}{2}$, where $\langle cos^2\varphi\rangle = \frac{\int_0^{\pi/2} I(\varphi)\cdot cos^2\varphi \cdot sin\varphi \cdot d\varphi}{\int_0^{\pi/2} I(\varphi)\cdot sin\varphi \cdot d\varphi}$. The function takes value of 1 or -0.5 when the membranes are completely oriented parallel or perpendicular to the direction of reference, respectively, and 0 for the case of random orientation. In our case, the direction of reference is the direction of magnetic field, and for perfectly aligned sample the value would be 1.0.

2.5. Thermoluminescence measurements The measurements were carried out using a home-built thermoluminescence apparatus [35]. A single-turnover saturating flash excitation was applied at -30 ^{o}C; the heating rate was 20 ^{o}C/min [36]. These measurements were used to control the efficiency of our low pH treatments. The observed low-pH induced reversible shifts of the B-band (data not shown) were in perfect agreement with literature data [37, 38].

3. Results and discussion

As reported earlier [39], the SANS signal of magnetically oriented thylakoid membranes is dominated by well discernible scattering peaks with maxima on the 2D image in the direction parallel with the direction of the applied magnetic field (Figure 1A). Upon acidification of the suspension medium the observed diffraction peak became more flat and the Bragg peaks largely diminished (Figure 1B). Resuspending the low-pH treated thylakoid membranes in p^2H 8.0 medium largely restored the original 2D profile (Figure 1C) with well-defined Bragg peaks. It is interesting to note that these low-pH induced variations in the 2D SANS profiles closely resemble the ΔpH-dependent light-induced changes in the 2D scattering profiles of isolated thylakoid membranes [29, 32, 39].

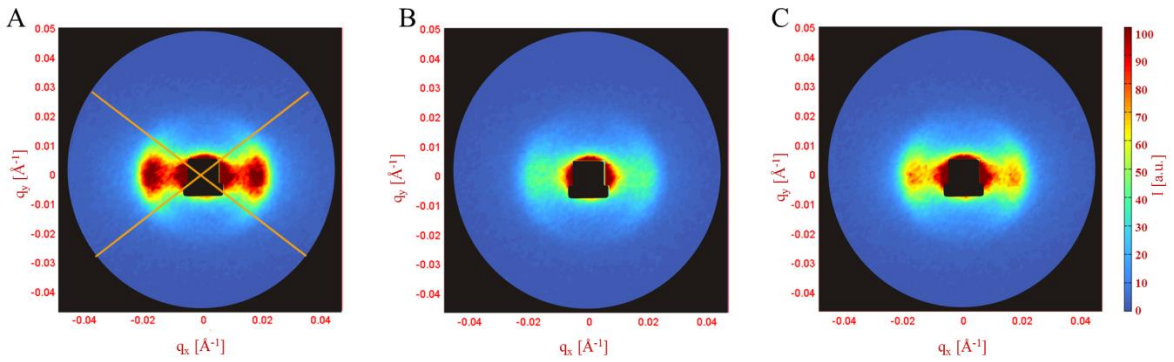

Figure 1: 2D small-angle neutron scattering profiles of magnetically oriented thylakoid membranes isolated from pea leaves and suspended in p^2H 8.0 reaction medium (A), resuspended in the same medium adjusted to p^2H 5.0 (B), and returned to the p^2H 8.0 medium (C). The magnetic field is applied perpendicular to the neutron beam, in the horizontal direction. The orange lines mark the boundaries of the sectorial averaging.

In order to obtain quantitative information about the low-pH induced reorganization of the thylakoid membranes we performed sectorial averaging of the 2D scattering curves – allowing determination of the diffraction peak (and hence the RD of the thylakoid membranes), and also investigated the angular dependency of the 2D scattering signal around the diffraction peak. In earlier studies, we have shown that the osmoticum used in the reaction medium significantly influences the structure of the isolated thylakoid membranes, and that NaCl retains much better the in-vivo structure of the thylakoid membranes than sorbitol [29]. For this reason, we performed the experiments both in sorbitol- and NaCl-based media.

The radially averaged scattering curves revealed similar and strong influence of the acidity of the suspension medium on the multilamellar arrangement of the thylakoid membranes for both types of reaction medium (Figure 2 A and B). In both cases, the diffraction peak around 0.019 Å^{-1} (sorbitol) and 0.027 Å^{-1} (NaCl) was shifted towards higher scattering vector values while its intensity was diminished. These variations are best seen using Kratky plots of the data (insets in Figure 2). The observed difference in the scattering curves of the thylakoid membranes suspended in sorbitol- and NaCl-based media is in good accordance with our earlier results [29].

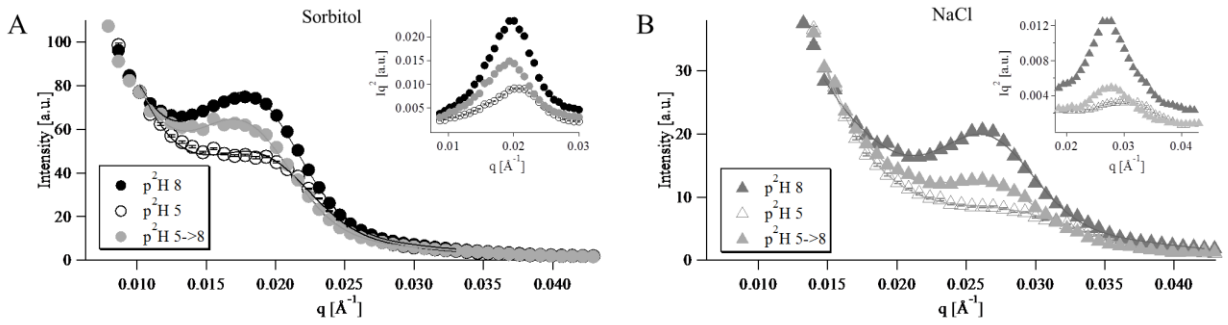

Figure 2 Sectorially averaged scattering curves of the thylakoid membranes suspended in p^2H 8.0, p^2H 5.0 and returned to the p^2H 8.0 medium. (The low p^2H treated and recovery curves are normalized to untreated curves at q values of 0.01 Å^{-1}.) The suspension medium contained, as osmoticum, sorbitol (A) or NaCl (B). The lines represent the fitted curves (for the fitting parameters see Table S1). Insets are the Kratky plot of the same data [33].

We determined the center position of the diffraction peaks and calculated the average RD of the thylakoid membranes at various p^2H conditions (Figure 3). Upon acidification RD decreased (sorbitol: from 342±1 Å (p^2H=8) to 329±1 Å (p^2H=5); NaCl: from 235±1 Å (p^2H=8) to 219±1 Å (p^2H=5)), while upon resuspension in the original medium, the original RD values were largely recovered (sorbitol: 352±1 Å (p^2H=8); NaCl: 233±1 Å (p^2H=8)); for further data see Table S2. This acidification-induced reversible shrinkage of the thylakoid membranes strongly resembles the effect of illumination, observed earlier on isolated thylakoid membranes [29, 32, 39, 40]. Similar to the light-induced SANS variations in thylakoid membranes the intensity of the Bragg peak (i.e., of the fitted Gaussian) diminished upon acidification (see Figure 3) – indicating a disorder in the periodic membrane ultrastructure. These changes were, however, not fully reversible upon resuspension in media with p^2H 8.0, especially after exposures to p^2H 5.0; these long low-pH treatments induced some irreversible changes.

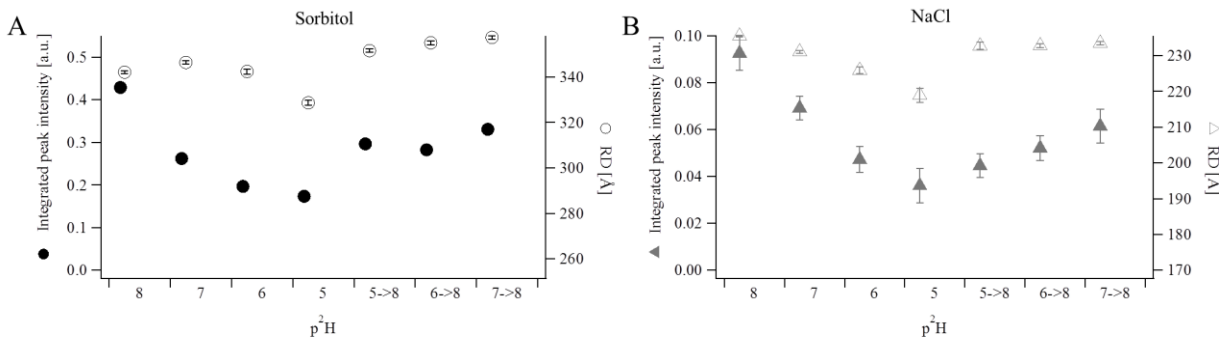

Figure 3. Physical parameters of Bragg peaks at various p^2H conditions: the calculated average RD and the integrated Bragg peak intensity (B) in sorbitol-containing medium (A) and in NaCl-containing medium (B). The errors signify the uncertainity of the fitting. For the fitting parameters see Table S1 and for the RDs values, Table S2.

As concerns the low-pH induced disorder, the analysis of the angular dependence of the SANS signal also reveals significant changes. Upon acidification the orientability of the multilamellar membrane system was significantly reduced, as shown both by the decrease in the values of the Hermans function and by the increase in the azimuthal width of the Bragg peaks (see Figure 4). For the interpretation of these changes we discuss below the origin of the broadening of the Bragg peak, including the case of thylakoid membranes suspended in p^2H 8.0 media.

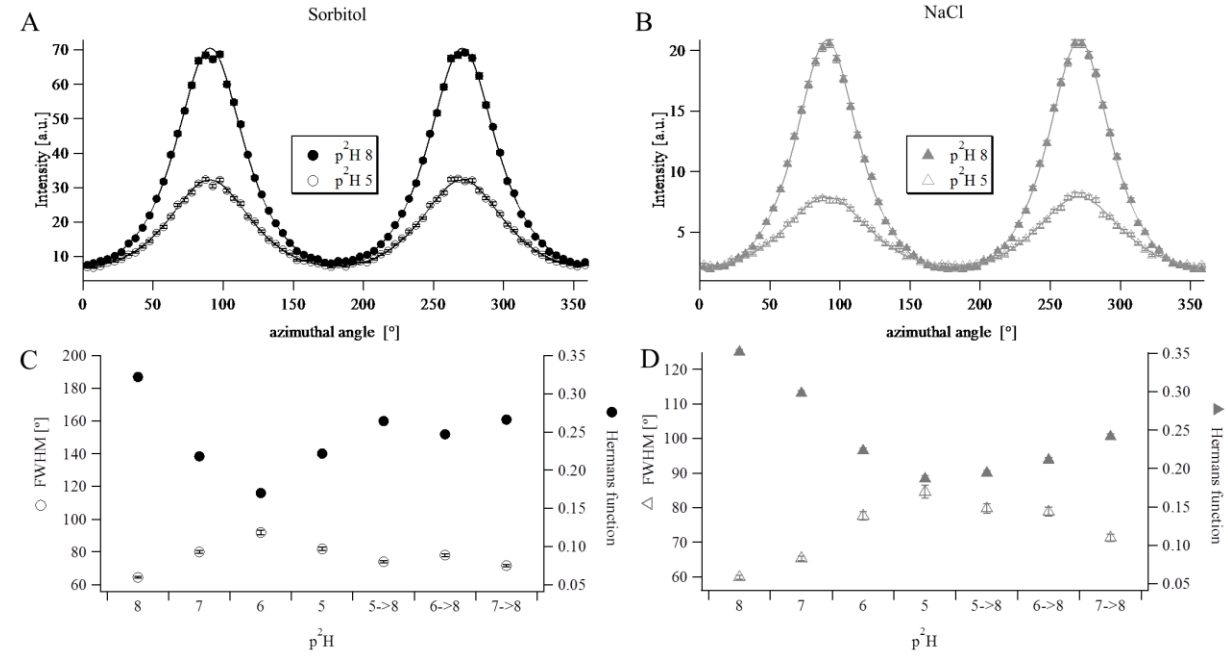

Figure 4. The angular dependences of the scattering intensity (A,B) of the thylakoid membranes suspended in p^2H 8.0 and p^2H 5.0 with the suspension media containing, as osmoticum, sorbitol (A) or NaCl (B). The lines represent the fitted curves (for the fitting parameters see Table S3); and the dependences of the full width at half maxima (FWHMs) of the Lorentz

function and the Hermans' orientation function (see Table S4) on the p^2H treatments of the thylakoid membranes with the suspension media containing, as osmoticum, sorbitol (C) or NaCl (D). The error bars in C and D signify the uncertainty of the fitting. The error bars of Hermans function are deduced from the statistics of the measurements.

In an external magnetic field intact thylakoid membranes tend to align perpendicular to the magnetic field. However, there are irregularities in the membrane system, and the chloroplasts and membranes often assume banana-shape. Therefore, not all grana sections, which give rise to the scattering peak, can be oriented exactly perpendicular to the magnetic field and the majority of membrane normals will have non-zero angle relative to the magnetic field. This leads to a relatively broad distribution of grana in Bragg condition [32], i.e. a mosaicity of the membrane sheets, which inherently also contain structural irregularities - contributing to the relatively broad width of the Bragg peak. The scattering signal from these imperfectly oriented grana will exhibit, for symmetry reasons, two Bragg peaks with a broad spread in azimuthal angles around the horizontal direction. Additional factors, which contribute to the broadening of the scattering peak, such as the detector resolution and the finite size of the incident beam at the detector (FWHM of 5.3 mm and 3.6 mm in the vertical and horizontal direction, respectively), can be neglected since their contributions are (i) small and (ii) do not differ sizeably under the conditions for samples with different p^2H treatments. The finite spectral bandwidth of the monochromatized neutron beam does not contribute to the azimuthal broadening of the Bragg peaks.

The increased azimuthal width of the Bragg peak component of the scattering signal (Fig. 4), reflecting the reduced orientability of the multilamellar membrane system upon acidification, is attributed to an increased mosaicity, i.e., an increase in the spread of membrane plane orientations.

4. Conclusion

Based on the above data, it can be concluded that the observed low-p^2H induced smearing and broadening of the Bragg peak and the increased mosaicity of the membranes, reflects a loosening in the periodic order of the thylakoid membranes that may arise from some undulations, membrane bending or other increased disorder affecting the diamagnetic anisotropy of the sample. These membrane reorganizations, along with the low-p^2H induced shrinkage, might be related to the lateral rearrangements of the protein complexes that are thought to be responsible

for the observed changes in the chiral macrodomains (i.e., in the psi-type CD) and in the distribution of absorbed excitation energy between the two photosystems – regulated by PsbS [24].

It is important to note that the low-pH induced variations in the SANS profiles and the underlying structural reorganizations of the thylakoid membranes reported here are very similar to those observed earlier upon illumination of isolated thylakoid membranes [29, 39]. Since illumination induces the acidification of the lumen in the thylakoid membranes, the present results further support our earlier conclusion that variations in the periodic arrangement of plant thylakoid membranes – evidently in concert with other membrane reorganizations (see Introduction) - participate in NPQ, a key photoprotective mechanism of green plants.

In general, these results, in line with similar observations [29, 39, 41-43], underline the remarkable flexibility of the thylakoid membrane ultrastructure, which should thus not be portrayed as simply providing a scaffold for the photosynthetic functions but also actively participating in the energy conversion steps and in different regulatory functions.

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