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The ultrastructure and flexibility of thylakoid membranes in leaves and isolated chloroplasts as revealed by small-angle neutron scattering ☆,☆☆

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ABSTRACT

We studied the periodicity of the multilamellar membrane system of granal chloroplasts in different isolated plant thylakoid membranes, using different suspension media, as well as on different detached leaves and isolated protoplasts—using small-angle neutron scattering. Freshly isolated thylakoid membranes suspended in isotonic or hypertonic media, containing sorbitol supplemented with cations, displayed Bragg peaks typically between 0.019 and 0.023 $Å^{-1}$, corresponding to spatially and statistically averaged repeat distance values of about 275–330 Å. Similar data obtained earlier led us in previous work to propose an origin from the periodicity of stroma thylakoid membranes. However, detached leaves, of eleven different species, infiltrated with or soaked in D_2O in dim laboratory light or transpired with D_2O prior to measurements, exhibited considerably smaller repeat distances, typically between 210 and 230 Å, ruling out a stromal membrane origin. Similar values were obtained on isolated tobacco and spinach protoplasts. When NaCl was used as osmoticum, the Bragg peaks of isolated thylakoid membranes almost coincided with those in the same batch of leaves and the repeat distances were very close to the electron microscopically determined values in the grana. Although neutron scattering and electron microscopy yield somewhat different values, which is not fully understood, we can conclude that small-angle neutron scattering is a suitable technique to study the periodic organization of granal thylakoid membranes in intact leaves under physiological conditions and with a time resolution of minutes or shorter. We also show here, for the first time on leaves, that the periodicity of thylakoid membranes in situ responds dynamically to moderately strong illumination. This article is part of a Special Issue entitled: Photosynthesis research for sustainability: Keys to produce clean energy.

1. Introduction

In order to improve the efficiency of capturing the incident light, oxygenic photosynthetic organisms have evolved multilamellar membrane systems, the thylakoid membrane system. These closed flattened membrane vesicles in plant and algal chloroplasts accommodate virtually all light-harvesting and energy-transducing functions. They separate the inner aqueous phase, the lumen, and the outer aqueous phase, the stroma, an important feature with regard to the energization of the membranes and the synthesis of ATP that uses the transmembrane electric field and the pH gradient. In plants, the thylakoid membranes are differentiated into granum and stroma regions (also called stacked or appressed and unstacked or non-appressed regions)—ensuring the optimal packing density of membranes [1–6].

During photosynthesis and upon changes in the environmental conditions there are significant structural and functional changes in the photosynthetic machinery, which affect the composition and macro-organization of the thylakoid membranes and the membrane ultrastructure. There are substantial ultrastructural variations between different organisms and habitats, showing that long-term adaptation

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of plants to environmental conditions affect the membrane system, the organization of which must be in harmony with the available energy supply and other conditions. As pointed out by Anderson et al. [7], re-organizations during long-term acclimation of plants are also mimicked following rapid transitions in irradiance. These are proposed to depend on reversible changes in the macro-organization of LHCII and LHCII–PSII supercomplexes within the thylakoid membrane network [8]. Reorganizations in the membrane ultrastructure and/or in the macroorganization of the complexes inside the membranes, associated with qE in excess light, have been shown to occur by several independent techniques, including biochemistry, CD spectroscopy and electron microscopy [9–11] (qE, the energy dependent component of the non-photochemical quenching of chlorophyll fluorescence). Also, state transitions have been proposed to be associated with membrane reorganizations [12–14]. In general, however, our understanding of the nature and dynamics of these reorganizations are far from being complete, especially in intact organisms under physiologically relevant conditions. This requires the use of non-destructive techniques, which nevertheless provide structural information.

Small-angle neutron scattering (SANS) is a non-invasive technique, also free of any detectable radiation damage (cf. [15] and references therein), which is particularly well suited to determine the average repeat distances (RDs) of membranes separated by aqueous phases. This information is averaged over the entire sample volume in the neutron beam. Contrast variation, using different concentrations of D_2O, can be used to identify the origin of the scattering signal. It is important to note that, in contrast to electron microscopy (EM), the sample does not require chemical or low temperature fixation and staining and the measurements can be performed in aqueous suspensions in the physiological temperature range. The signal-to-noise ratio for single runs allows time resolution from 1–2 min down to 10 s, depending on the sample and the intensity of the neutron beam [15]. Recently, SANS experiments have been carried out on isolated thylakoid membranes as well as on different cyanobacterial and algal cells [16–19]. In addition to 'static' RD values of the membranes, these SANS experiments provided evidence for the occurrence of light-induced reversible changes in the membrane ultrastructure; they revealed small, but well discernible changes in the periodicity of the thylakoid membranes both in vitro and in vivo.

While thylakoid membranes isolated from higher plants have been shown to exhibit a remarkable structural flexibility associated with the operation of the photosynthetic electron transport system and the build-up of the transmembrane ΔpH [15,16,18], no SANS data are available on whole leaves. The application of this technique would be important because some of the major regulatory mechanisms, such as the non-photochemical quenching of the excess excitation energy in the light harvesting antenna, or short- and long-term light- or temperature-adaptation mechanisms and abiotic stresses are observed in whole leaves. Recent literature data have revealed that there are significant reorganizations in the membrane ultrastructure when whole leaves are exposed to different environmental conditions leading e.g. to state transitions [13,20] or upon their exposure to high light stress [9,11,21–23]. SANS, with its unique ability of providing time-resolved structural information under physiological conditions, might open up new vistas for monitoring ultrastructural reorganizations in vivo. The major aim of this work is to explore the main SANS features of whole leaves, which, as demonstrated here, carry information on the periodic organization of the thylakoid membranes and its inherent flexibility in vivo. In addition, we also compare the structural parameters of the thylakoid membranes in situ and when isolated from the cell and suspended in different media. SANS data, in accordance with EM, show that the most frequently used reaction media might significantly alter the periodicity of membranes in comparison to that in leaves.

2. Materials and methods

2.1. Sample preparation

Tobacco (*Nicotiana tabacum* SR1) was grown for 6 months in a greenhouse at 25 °C at a photon flux density of 100 μmol photons $m^{-2} s^{-1}$ with light/dark periods of 16/8 h. Pea (*Pisum sativum*, Rajnai törpe) was grown in a greenhouse at 20–22 °C in soil, under natural light conditions. In some experiments we used freshly harvested pea leaves or even whole seedlings, transferred from greenhouse conditions to field conditions (at the site of PSI). *Arabidopsis thaliana* was grown at 25 °C in soil in a growth chamber with light/dark periods of 16/8 h; we used 6–8 week-old fully expanded leaves. Spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa*) were bought at the local market. Fresh leaves with good turgor were selected, washed in de-ionized chilled water and stored in a refrigerator in order to reduce their starch content. Before the measurements leaves were exposed to dim light or were illuminated with low intensity white light. *Monstera deliciosa*, *Schefflera arbicola*, and *Euphorbia pulcherrima* were collected from potted plants. *Hedera helix* and common grass were collected from nature.

Spinach protoplasts were isolated as described in [24]. Briefly, the leaves were cut into thin strips with a razor blade and the strips were then submerged and vacuum-infiltrated with a solution containing 1% (w/v) cellulose R10 and 0.2% (w/v) macerozyme R10 (Yakult Honsha, Tokyo, Japan) dissolved in 20 mM MES buffer (pH 5.7) containing 0.4 M mannitol. Digestion of the cell wall proceeded at room temperature for 4 h in darkness. The mixture was then filtered through a 35–75 μm nylon mesh, followed by centrifugation in a round-bottom test-tube for 2 min at 100 ×g, and re-suspension in protoplast buffer containing 50 mM HEPES-KOH (pH 7.6), 0.4 M mannitol and 10 mM NaHCO₃. Tobacco protoplasts were isolated according to [25] from 5 to 6 month old, sterilized leaves. Briefly, leaf sections were incubated in an enzyme solution containing 2% Meicelase, 0.5% Macerozyme R-10, 1/5 MS strength salts, 5 mM MES and 0.4 M sucrose (pH 5.6), in the dark at 26 °C for 16 h. The suspension was filtered through a nylon mesh (pore size 100 μm) and floated on the original enzyme solution by centrifugation at 120 ×g for 5 min. The protoplasts were collected from the top of the enzyme/sucrose layer and were washed twice in W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, pH 5.6) with centrifugation at 80 ×g for 2 min. For SANS measurements the protoplasts were transferred to D₂O buffer (50 mM Tricine, 0.4 M sorbitol, 5 mM MgCl₂ and 5 mM KCl, pD 7.5).

Thylakoid membranes were isolated, as described earlier [18], from freshly harvested pea, or from spinach purchased at the local market, or from tobacco leaves harvested in Szeged and transported on ice to the site of experiment. Before the experiments leaves were stored in a refrigerator for 1–3 days in order to reduce their starch content. Briefly, leaves (after de-veining, in the case of tobacco and spinach) were homogenized in ice-cold grinding medium containing 20 mM Tricine (pH 7.6), 0.4 M sorbitol, 5 mM MgCl₂ 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 MgCl₂ 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 pellet was washed twice in D₂O-containing grinding medium (pD 7.6). The chlorophyll concentration was adjusted to 1–2 mg/ml and the samples were stored at 4 °C and used typically for 6–8 h.

In the thylakoid membrane preparations using NaCl as the osmoticum, 0.4 M sorbitol was replaced by 0.3 M NaCl [26]. In some experiments higher concentrations of sorbitol or NaCl were used or sorbitol and NaCl were used together in the reaction medium as indicated in

the text. Thylakoid membranes were also isolated using other buffers, in particular phosphate or MES.

Before SANS measurements, in most samples part of the water content was replaced with heavy water to enhance the contrast and to reduce the background scattering [15]. Thylakoid membranes were suspended in D_2O-based reaction media as described earlier [16]. Though intact leaves can be measured without increasing the contrast for neutron scattering the diffraction peak—as also in the case of isolated thylakoid membranes [27,28]—is much better defined with increased contrast. To reach this, different procedures were used. We soaked the leaves by immersing them in heavy water for a few hours in light in the presence or absence of bicarbonate. In some cases, we illuminated detached leaves or whole plants with their petioles or roots immersed in D_2O to allow transpiration; the obtained RD_{q*}-s did not differ dramatically from each other; in particular RD < ~170 Å was not obtained under either of these conditions. Most efficiently, we used the infiltration of a detached leaf or a leaf segment. To this end, we placed them into a plastic syringe which was filled up to about 1/5 of its volume with heavy water; then the air was released from the barrel by pushing the plunger with the tip pointing upward; then, with the tip closed, the plunger was pulled up to create a vacuum; upon its release heavy water entered the tissues. This process was repeated, usually 2–4 times, until the leaf became sufficiently transparent.

2.2. Small-angle neutron scattering measurements

SANS measurements were performed at the "Yellow Submarine" instrument at the Budapest Neutron Center (BNC), Budapest, Hungary; the D11 and D22 instruments at the Institut Laue-Langevin (ILL), Grenoble, France; at the SANS-I and SANS-II instruments at the Paul Scherrer Institute (PSI), Villigen, Switzerland and at the KWS-2 instrument at the Jülich Centre for Neutron Science (JCNS), Garching, Germany. Neutrons are produced by reactor-based neutron source (BNC, ILL, JCNS) and accelerator-based neutron source (PSI). Each instrument was using neutrons moderated in a cold source and afterwards monochromatized by a velocity selector. Neutrons scattered from the sample were recorded by ^{3}He (D11, D22, SANS-I, SANS-II), ^{6}Li-scintillator (KWS-2) and BF_3 (Yellow Submarine) filled position-sensitive detectors. Different sample-to-detector distances (SDs) and sample-to-collimation distances were used; see figure legends (SD and collimation).

For SANS measurements, isolated thylakoid suspension was filled in quartz cuvettes with 2 mm path length; the chlorophyll content was typically between 1 and 2 mg/ml. In most experiments, the isolated thylakoid membranes were aligned in a magnetic field, using an electromagnet (BNC, ILL, JCNS) of ~1.5 T or a permanent magnet (PSI) of ~0.4 T field strength [15]. Leaves were also placed in 2 mm quartz cuvettes, which were usually filled with D_2O. The samples were thermostated, with temperatures adjusted between 16 and 20 °C (PSI, ILL and in some measurements at the BNC). At the JCNS and in some experiments at the BNC the measurements were performed at room temperature.

All experimental data are normalized to the number of incoming neutrons and the backgrounds, recorded with the beam blocked by cadmium (BNC, PSI, ILL) or boron carbide (JCNS), were subtracted. The two-dimensional scattering profile of an empty quartz cuvette was subtracted from the water. Empty quartz cuvette or the suitable media or the heavy water was substracted from the sample profiles. The signals were corrected for the detector efficiency, determined with the aid of light water scattering (BNC, ILL, PSI) and with the aid of precalibrated "plexiglass" (JCNS).

For data analysis, we used the "Graphical Reduction and Analysis SANS Program" package (GRASP) (developed by C. Dewhurst, ILL) and 'BerSANS-PC' software (developed by U. Keiderling, Helmholtz-Zentrum Berlin [29]). SANS patterns were reduced from 2D to 1D profile by radial averaging, which was performed in a 360° sector around the

center of the direct beam or, for magnetically aligned samples, in two 75° sectors [15]. The 1D profiles are plotted either on lin(q) − log (I) or lin(q) − lin(I) scale, depending on the better representation of the Bragg peak.

The difference between the incoming and the outgoing wave vectors of the neutron beam is the scattering vector, q:

$$q = \frac{4\pi}{\lambda} \sin\left(\frac{\Theta}{2}\right) \tag{1}$$

where Θ is the scattering angle (the angle between the wave vector of the incident and the scattered neutron beams of λ wavelength).

The scattering curves in the given regions around the Bragg peak were fitted with the following equation:

$$I(q) = I_0 + A * |q|^p + B * e^{-\frac{(q-q^*)^2}{2c^2}} \tag{2}$$

to obtain the position (q*) of the Bragg peak; i.e. it was fitted with a Gaussian for the Bragg diffraction and a power function for the 'background' scattering. RD values were calculated using the peak position of the Gaussian, q*, which we interpret as a Bragg diffraction peak (i.e. the peak between 0.02 and 0.03 $Å^{-1}$) using the formula:

$$RD_{q*} = \frac{2\pi}{q*}. \tag{3}$$

where I_0 is constant and I is the scattered intensity, p is the parameter of the power function and c is the standard deviation of the Gaussian distribution. The same equation (Eq. (2)) was used in the region around the higher q value peaks.

The features of the Bragg peak depend on the order of the multilamellar system. In particular, the width of the peak depends on the number of lattice-periods [30]. In an ideal case, one can calculate the RD, the ordering of bilayers and the number of coherently scattering bilayers from the features of the Bragg peak. Higher and narrower peaks can be found with increasing number of bilayers [30]. However, thylakoid membranes have variable number of layers and the distances might also show heterogeneity. These factors, without additional and very detailed structural information, would be difficult to take into consideration in a quantitative model. Hence, our analysis is mostly confined to the determination of the peak position—a statistically and spatially averaged value. Variations in the amplitude and broadening might originate from changes in the degree of the lamellar order, e.g. due to local undulations of the membranes, an increased heterogeneity in the widths of aqueous phases or variations in the number of stacks.

2.3. Transmission electron microscopy

Pieces of approx. 1×2 mm^2 from the middle of the leaf lamina and isolated thylakoids were fixed in 2.5% glutaraldehyde for at least 3 h. Unless otherwise stated, glutaraldehyde was buffered with 70 mM Na–K phosphate buffer (pH 7.2), while in case of thylakoids in sorbitol or NaCl containing media we added glutaraldehyde directly to the thylakoids for fixation. After fixation, the thylakoid membranes were centrifuged for 5 min at 4000 ×g, and glutaraldehyde was washed out 3 times for 15 min. Then the pellet or the leaf pieces were post-fixed in 1% OsO_4 buffered with 70 mM Na–K phosphate buffer (pH 7.2) for 2 h. After post-fixation, the excess OsO_4 was removed by washing the samples 3 times for 15 min. Post-fixation was followed by dehydration in alcohol series. Finally, the samples were embedded in Durcupan ACM resin (Fluka Chemie AG, Buchs, Switzerland). Ultrathin sections (70 nm) were cut with Reichert Jung Ultracut E microtome (Reichert-Jung AG, Vienna, Austria). The sections were stained with uranyl acetate and Reynold's lead citrate [31]. The sections were visualized by a Hitachi 7100 TEM (transmission electron microscope) at 75 kV accelerating voltage.

To measure the RD of thylakoid membranes from EM data we used two different methods, which yielded similar results. ImageJ software

was applied for both methods and sharp regions were selected where the thylakoid membranes appeared to be sliced perpendicularly to their membrane planes. We either simply measured the height of a granum and divided this distance by the number of thylakoid vesicles, or applied fast Fourier transformation (FFT) on the selected region of interest, which provided values on the periodicity of the thylakoids. This RD comprises the widths of the two aqueous phases (the lumen and the interthylakoidal aqueous phase), and twice the width of the membrane.

3. Results and discussion

3.1. Detached leaves and isolated protoplasts

As shown in Fig. 1, spinach leaves exhibit three peaks in their SANS profile, around 0.0258 ($q^*_{n\,=\,1}$), 0.0508 ($q_{n\,=\,2}$) and 0.078 $Å^{-1}$. The first peak could be related to a peak found between 0.019 and 0.023 $Å^{-1}$ [18] in isolated spinach thylakoid membranes, that has been shown to originate from Bragg diffraction of the multilamellar membrane system (see also Section 3.2). The second peak, which is often not discernible in isolated thylakoid membranes, is evidently the second-order diffraction peak, as suggested by its position at ~2q*. The high-q peak appears to be identical with the ones seen in isolated plant thylakoid membranes and in different algal cells [16,17]; and it has been proposed to originate from adjacent membrane pairs [15]. The origin of this peak is under investigation and the results will be published elsewhere.

When calculating RD_{q^*}, one obtains 244 Å, which is 50–100 Å lower than the values obtained for isolated thylakoid membranes, thus giving rise to questioning the interpretation of this peak as a stroma peak, since stroma distances are expected to be around ~300 Å [16,18]. However, the distance is also larger than the values assigned to grana repeat distances from previous EM works (see discussion in [18]). The Bragg peak in different leaves is found in the same range, typically between 0.026 and 0.030 $Å^{-1}$, corresponding to about 240 and 210 Å, respectively (Table 1). In order to clarify the origin of this peak, systematic investigations were carried out on a number of different species as well as on different regions of variegated leaves, on red bracts and on protoplasts.
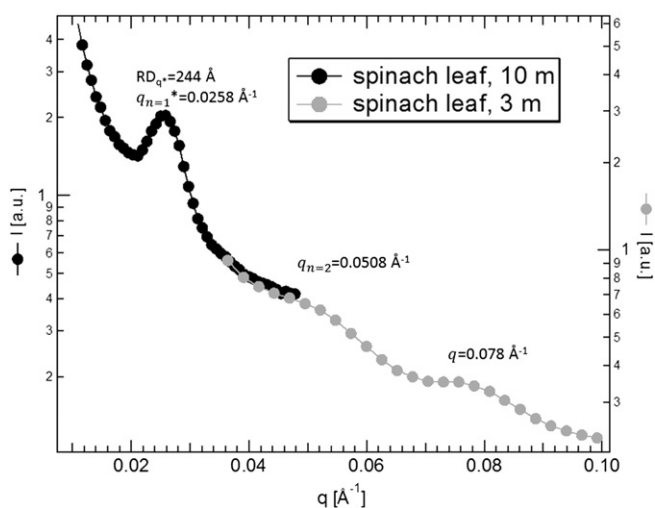

Fig. 1. SANS profile, scattering intensity (I, arbitrary units) as a function of the scattering vector q, of D$_2$O-infiltrated spinach leaf measured in two overlapping q ranges. The first peak, at 0.0258 $Å^{-1}$, which is attributed to a first order Bragg diffraction, is marked with q*, and the corresponding repeat distance (RD_{q^*}) value, calculated after fitting the curves with Eq. (2), is also given; the positions of second-order peak ($q_{n\,=\,2}$) and the high-q value peak are also indicated. The measurements were carried out at the ILL-D11 SANS instrument with a sample-to-detector distance (SD), 10 m; collimation, 10.5 m, $\lambda = 6$ Å, for the q range between about 0.01 and 0.05 $Å^{-1}$, and SD = 3 m; collimation, 4 m; $\lambda = 6$ Å for the high-q range measured between about 0.02 and 0.2 $Å^{-1}$ and plotted between 0.036 and 0.1 $Å^{-1}$.

3.1.1. The role of cell wall

Fig. 2 shows that the yellow leaf region of the variegated dwarf umbrella tree (*S. arboricola*) displays no scattering peak between 0.016 and 0.044 $Å^{-1}$, in contrast to the green leaf parts of the same plant, which exhibit a well-defined peak at 0.0277 $Å^{-1}$ ($RD_{q^*} = 227$ Å). The yellow regions have single thylakoid lamellae, whereas there is a normal thylakoid membrane system in the green parts [32]. Similar data were obtained on poinsettia (*E. pulcherrima*), where, in their flowering period, the red colored bracts display no peak in the same range, whereas the green leaves exhibit a peak at 0.0286 $Å^{-1}$, $RD_{q^*} = 220$ Å (data not shown). It has been shown that in poinsettia in the process of change from green to red color, the bracts' chloroplasts are disintegrated and the lamellar structure is destroyed [33]. These measurements show that the peaks do not arise from the cell wall, a multi-scale highly-organized structure, neither from single thylakoid membranes, present in these leaves. In general, the texture of the leaf does not seem to interfere with the SANS profiles, except for leaves with many parallel veins, such as maize or barley; nevertheless, RD_{q^*} could be determined on common grass leaves. The possibility that this peak originates from starch is also highly unlikely since the peak is observed also in leaves stored for several days in darkness. Purified (semi-)crystalline lamellae of waxy maize starch exhibit ~90 Å RD values [34], hence, even if it were present, it would not interfere with the first-order Bragg peak at around 200 Å. It is also to be noted that pilot experiments testing the SANS signal of starch isolated from leaves displayed no peak between 0.01 and 0.1 $Å^{-1}$. Hence, the peak exhibited by leaves between 0.026 and 0.03 $Å^{-1}$ must be associated with the multilamellar organization of the thylakoid membranes. This conclusion is supported by the SANS experiments on isolated spinach and tobacco protoplasts, which display smeared Bragg peaks between about 0.027 and 0.031 $Å^{-1}$ (corresponding to RD_{q^*} between ~230 and 200 Å, considerably closer to the values in leaves than in isolated chloroplasts in sorbitol-containing media (Fig. 3). Since the Bragg peak cannot arise from stroma lamellae, we assign this peak to the periodicity of thylakoid membranes in the grana. It must, however, be explained why this peak appears at much lower q, i.e. why it yields much higher RD, than expected. Most literature data, either from 'conventional' or cryo EM, give or yield RD_{EM} between about 150 and 200 Å in leaves [35–37] and somewhat higher values in isolated thylakoid membranes [38,39] (discussed also in [16] and [18]). To answer this question, we need to assess the details of both SANS and EM experiments.

3.1.2. Effect of infiltration

Although for SANS experiments the leaves do not require special treatment, infiltration with D$_2$O increases the contrast and thus this treatment is applied in most experiments. The RD of the thylakoid membranes increased in some of the leaves tested compared to non-infiltrated leaves. The magnitude of the changes depended on the plant species and on the experimental conditions. The difference might arise from different turgor of the cells or other unknown factors that control the tonicity of chloroplasts. In samples of whole pea leaves before infiltration, we observed grana with swollen end membranes, indicated with asterisk in Fig. 4; in these RD_{EM} was 211 ± 15 Å (n = 28). On the other hand, with grana without swollen end membranes RD_{EM} was 205 ± 18 Å (n = 23). In samples after infiltration, the corresponding RD_{EM} values were 230 ± 20 (n = 30) and 206 ± 10 (n = 10) Å, respectively, and the swelling of the end membranes, if it occurred, became more prominent. Overall, however, upon infiltration the average RD_{EM} increased from 208 ± 16 to 224 ± 21 Å. We noticed no significant difference between the infiltrations with heavy and light water (not shown). Typical EM images are shown in Fig. 4. Similar data were obtained on tobacco, where the RD_{EM} values increased from 189 ± 23 (n = 9) to 200 ± 16 (n = 22) Å upon infiltration. Grana of tobacco apparently did not have swollen end membranes. (Data represent the average of measurements performed on at least 9 grana from 5 different

Table 1
Calculated RD_{q^*} values, obtained from SANS measurements, of different detached leaves.

Species	Repeat distance (RD_{q^*})[Å]	Number of repetitions	condition	Instrument
Arabidopsis thaliana	225	1	Infiltrated	YS
Lactuca sativa	222	1	Infiltrated	YS
Pisum sativum	220 ± 8	2	Infiltrated	YS, SANS-II
	205	1	Control	SANS-II
Spinacia oleracea	239 ± 6	3	Infiltrated	D11, SANS-I
Nicotiana tabacum	223 ± 7	4	Infiltrated	KWS2, YS, SANS-I
	231 ± 5	2	Control	YS, SANS-II
Monstera deliciosa	222	1	Infiltrated	YS
Hedera helix	216	1	Infiltrated	YS
Schefflera arboricola	227	1	Infiltrated	YS
Euphorbia pulcherrima	220	1	Infiltrated	YS
	215	1	Control	KWS2
Epipremnum aureum	219	1	Control	D22
Common grass	254	1	Control	SANS-I

chloroplasts.) It must also be noted that in some samples we did observe RD_{EM} values which were in the range of the expected (minimum) values, based on the calculations (2x40 Å for the membranes and 2x < ~45 Å for the aqueous phases, i.e. <~170 Å (cf. [16,18]): in a series of tobacco EM images 2 out of 7 grana displayed RD of 160 Å, but 5 exhibited >190 Å; in pea, this ratio was 1 out of 17, and again all other RD_{EM} values were larger than 190 Å.

SANS experiments on pea leaves show an expansion from 207 Å, measured on D_2O-transpired pea leaf, to 215 Å after infiltration (Fig. 4). A similar increase was seen on tobacco leaves (Table 1). However, in some spinach leaves with RD_{q^*} of about 241 Å, we observed no infiltration-induced expansion. It is also to be noted here that, as shown in [18] on isolated thylakoid membranes, the RDs are independent of the D_2O/H_2O ratio.

These data strongly suggest that the RD values of the thylakoid membranes depend on the turgor of the cells. This might warrant further investigations and potentially offers applications of SANS measurements in monitoring the water potential of chloroplasts. Although the overall ultrastructure of chloroplast thylakoid membrane system is retained in a broad range of water pressure [40], our data suggest that the interlamellar distances and/or the lumenal space might be affected by the turgor of chloroplasts/leaves (even though all leaves used appeared to be 'healthy' and with normal turgor).

3.1.3. Light-induced changes

Earlier it has been shown that isolated thylakoid membranes are capable of undergoing reorganization that affect their periodic ultrastructure [15,16,18]. Upon the induction of photosystem I cycle, a substantial (20–40 Å) decrease in the RD_{q^*} and a disorganization of the membrane system was observed; the rate and magnitude of these fully reversible changes depended on the intensity of the actinic light used; these reorganizations could be inhibited by uncouplers [15,16,18]. With active full-chain linear electron transport, the changes were significantly less dramatic, <10 Å shrinkage, but still discernible [16]. Here we tested whether or not similar reorganization can also be observed in intact detached leaves.

As demonstrated with spinach in Fig. 5, the SANS profile of leaves also undergoes changes upon illumination, and these changes are largely reversible—although upon light–dark cycles the RD_{q^*} and the amplitude of the Bragg peak appears to drift to a light-adapted state from the dark-adapted state, as also seen on isolated thylakoid membranes [18]. These changes are similar to the light-induced, uncoupler-sensitive reversible SANS changes observed earlier on isolated spinach thylakoid membranes in the presence of methyl viologen—which have shown that the light-induced shrinkage of the granum thylakoid membranes is accompanied by a decrease in the long-range order of the thylakoids; in these experiments RD_{q^*} decreased from about 290 to 280 Å [16,18]. The magnitude of these changes was similar
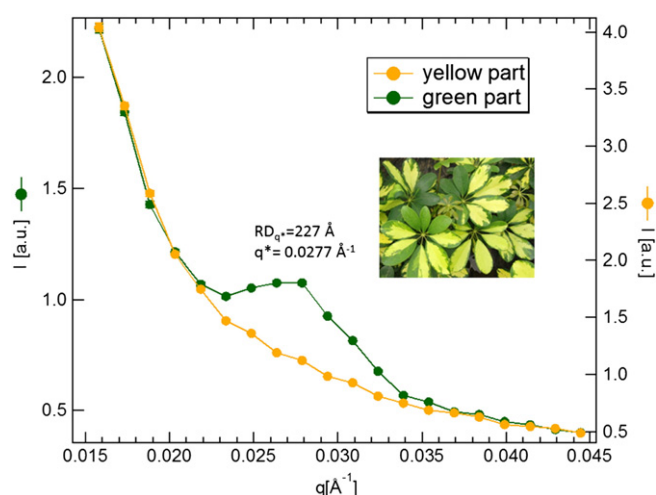

Fig. 2. SANS profiles of D_2O-infiltrated variegated *Schefflera arboricola* leaves recorded on the green and yellows parts of the leaf. The peak position and the calculated RD_{q^*} value are also indicated. The measurements were carried out at the BNC Yellow Submarine SANS instrument (SD = 5.5 m; collimation, 4 m; λ = 7.6 Å).
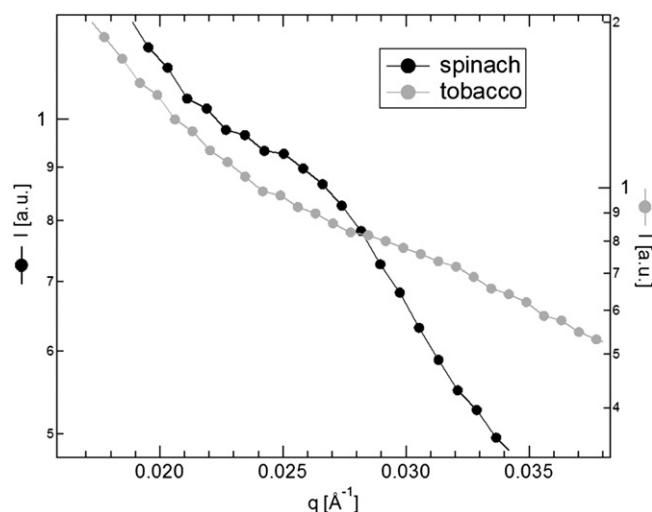

Fig. 3. SANS profiles of freshly isolated spinach and tobacco protoplasts, prepared as described in Section 2.1. The measurements were performed at the ILL-D11 (SD = 10 m; collimation, 10.5 m; λ = 6 Å) and at the PSI SANS-I instrument (SD = 11 m; collimation, 11 m; λ = 6 Å), respectively.
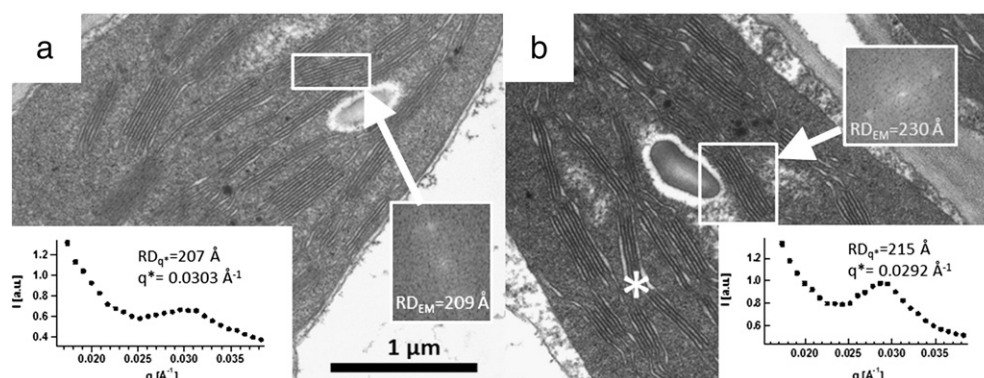
Fig. 4. EM images and SANS profiles of pea leaves before (a) and after (b) infiltration with D_2O. Prior to the SANS measurements, the leaf was enriched in D_2O via transpiration as described in Section 2.1; the same leaf, after the SANS measurement was infiltrated with D_2O and measured again. The calculated RD_{q*} and RD_{EM} values are also indicated. (The latter RD values were obtained by FFT, using ImageJ software). We observed some grana with swollen end membranes, indicated with asterisk. The SANS measurements were performed at the PSI SANS-II instrument (SD = 6 m; collimation, 6 m; λ = 5.2 Å).

in leaves but the absolute values were different: here the changes occurred from 229 to 221 Å. It is also interesting to note that these changes occur in the opposite direction than those observed in cyanobacteria [16,19] or in the diatom *Phaeodactylum tricornutum*, where the changes were insensitive to the uncoupler NH_4Cl [16,17]. It must also be elucidated how the light-induced decrease in RD_{q*} can be reconciled with the conclusion based on EM that the width of the thylakoid lumen expands in light [21]. Exposure of leaves to light under normal atmospheric conditions has been shown to lead to expansion of the grana lumenal compartment in a light-intensity-dependent manner [21]. Infiltration of the leaf and its immersion in D_2O evidently changes the availability of CO_2 to the photosynthetic machinery, which might also affect the membrane system. The exact origin and physiological significance of these SANS changes in leaves are beyond the scope of our present paper, but it can be concluded that the thylakoid membrane system in situ possesses the capability to respond dynamically to the illumination of leaves.

3.2. Isolated thylakoid membranes

In order to clarify the discrepancy between the RD data obtained on leaves and isolated thylakoid membranes, experiments were carried out
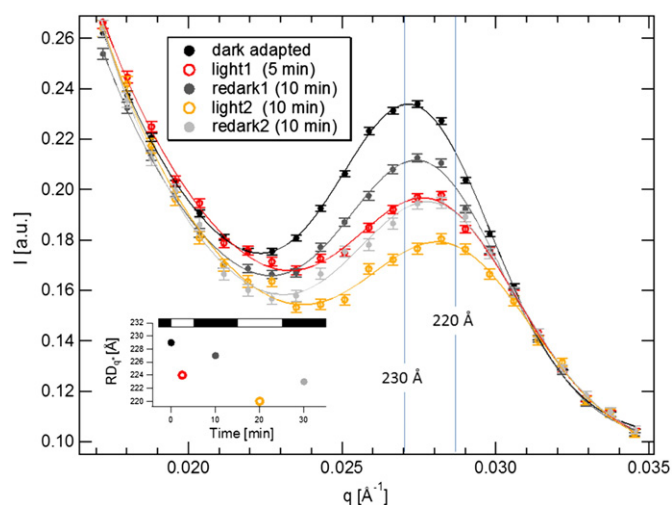

Fig. 5. SANS profiles of a D_2O-infiltrated spinach leaf segment recorded during light–dark cycles of different lengths as indicated; the leaves were illuminated with white light of 1700 µmol photons $m^{-2} s^{-1}$. The lines are fitted curves (Eq. (2)); inset, the corresponding RD_{q*} values during the light (white bars) and dark (black bars) periods, as indicated. The SANS measurements were performed at the ILL-D11 (SD = 10 m; collimation, 10.5 m; λ = 6 Å).

on the same plant material: leaves and freshly isolated thylakoid membranes suspended in different reaction media. Spinach leaves display a Bragg peak at 0.0271 $Å^{-1}$ (corresponding to 232 Å), whereas in freshly isolated thylakoid membranes the peak is found at 0.020 $Å^{-1}$ (314 Å); however when 300 mM NaCl was added to the sorbitol-containing medium, the peak shifted to 0.023 $Å^{-1}$ (273 Å) (data not shown). A similar shift has previously been induced by increasing the sorbitol concentration to 1 M plus doubling the concentration of $MgCl_2$—under these conditions q* was found at 0.0227 $Å^{-1}$ (277 Å) [18]. The shift observed in the present work depended on the concentration of NaCl. In a series of experiments, it shifted from 0.019 $Å^{-1}$ in the presence of 400 mM sorbitol to ~0.0225 and ~0.026 $Å^{-1}$ upon the addition of 100 and 500 mM NaCl, respectively; at 500 mM it appeared to saturate. This suggests that NaCl might be a more suitable osmoticum for these samples than sorbitol. In order to clarify this question we compared different isolation and reaction media and performed EM and SANS experiments under comparable conditions.

3.2.1. Effect of different osmotica and buffers

Fig. 6a shows the EM picture and SANS curve of isolated tobacco thylakoid membranes which had been suspended in an isotonic medium containing 400 mM sorbitol, as the osmoticum. It can be seen that, in good agreement with our earlier data obtained on spinach [18], the Bragg peak is found at 0.02 $Å^{-1}$, corresponding to 314 Å. The FFT of EM images yielded a somewhat lower RD value, 294 Å, on the granum analyzed in Fig. 6a, and an average RD_{EM} value of 302 ± 35 Å (mean value ± SE calculated from 16 grana measurements). Hence, these data again clearly show that the Bragg peak originates from grana rather than from stroma thylakoid membranes. To further support this conclusion, we measured the SANS profile of mesophyll thylakoid membranes isolated from lincomycin-treated [41] maize seedlings, in which the stroma thylakoid membranes are largely disorganized whereas the grana are retained [42]; RD_{q*} of ~310 Å on SANS curve of untreated plant was largely maintained in the isolated thylakoid membrane from lincomycin-treated seedlings (not shown).

It is evident that RD_{EM} and RD_{q*} values in isolated thylakoid membranes are significantly higher than those obtained on the leaves from the same plant material (Fig. 6b, see also Section 3.1.2). Although the EM and SANS data also differ somewhat from each other they both reflect a significant expansion of the membrane system upon the isolation of thylakoid membranes in sorbitol-containing isotonic medium.

When using NaCl as osmoticum during the preparation of thylakoid membranes from the same batch of leaves, the Bragg peak was found at 0.0262 $Å^{-1}$, corresponding to an RD_{q*} of 240 Å. In some protocols, NaCl, rather than sorbitol is used as osmoticum [26] EM FFT data on thylakoid membranes isolated with the same method, yielded 216 Å (Fig. 6c)—
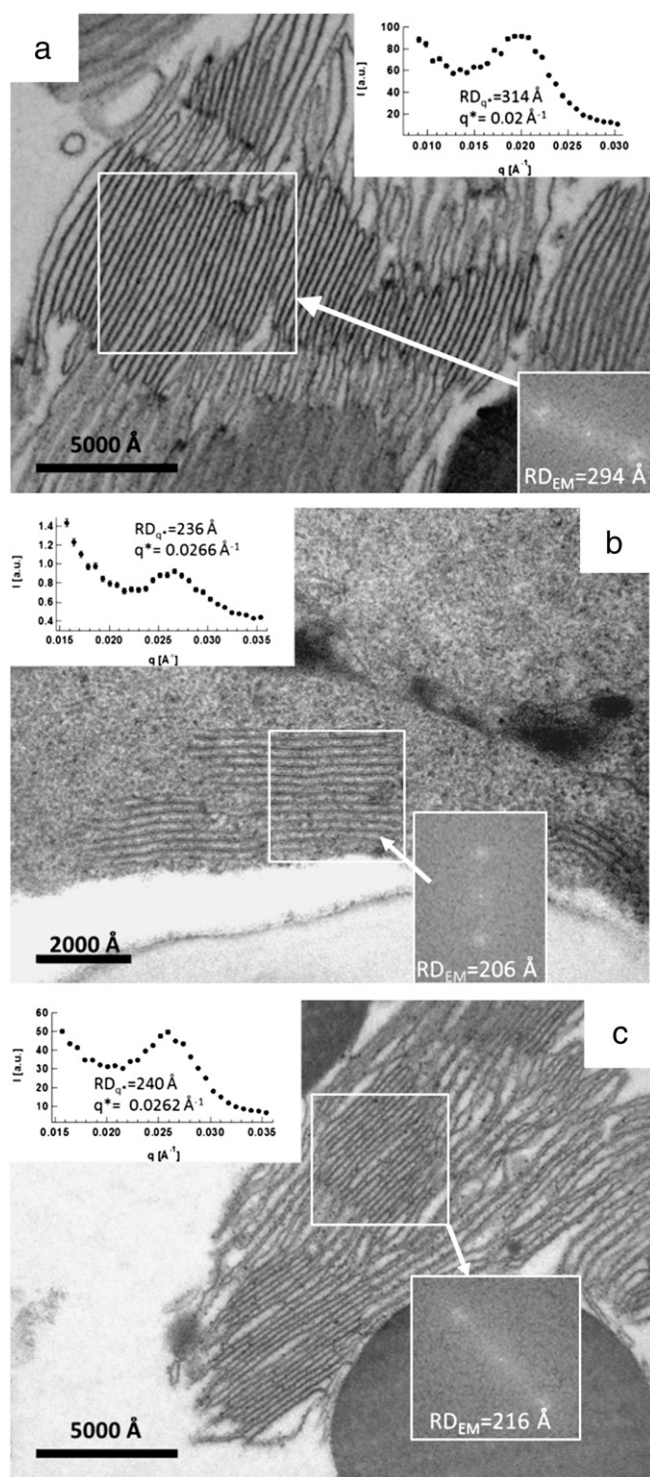
Fig. 6. SANS profiles and EM images of sorbitol isolated thylakoid membrane (a), a non-infiltrated tobacco leaf (b) and NaCl isolated thylakoid membrane (see Section 2.1). Isolated thylakoid membranes were obtained from the same batches of leaves that were used for the EM and the SANS experiments. The SANS experiments were performed at the PSI-SANS-II instrument (SD = 6 m; collimation, 6 m; λ = 6.2 Å).

and an average RD_{EM} value of 208 $\pm$ 10 Å (n = 22). It can thus be seen that apart from the differences between the EM and SANS data, the NaCl-isolated thylakoid membranes exhibit very similar RD values as the D_2O-infiltrated leaves (see Section 3.1.2). In other terms, NaCl, as osmoticum, preserves much better the membrane ultrastructure than sorbitol. We have also investigated whether RD_{q^*} depends on the buffer or the pH of the medium. However, we found that with sorbitol it was

always found in the same range, between 280 and 340 Å, when instead of Tricine we used 25 mM MES buffer at pH 6.5 or 20 mM K-phosphate buffer either at pH 7.5 or 6.5. On the other hand, also in the presence of phosphate buffer RD_{q^*} decreased from 336 Å to 243 Å when sorbitol was replaced with NaCl (not shown). In general, these data also show that SANS can be used to optimize the isolation procedure of intact chloroplasts and thylakoid membranes.

Since it appears that in the presence of NaCl the thylakoid membrane ultrastructure is considerably more compact than with sorbitol, it was of interest to test if the thylakoid membranes retain their flexibility also under these conditions. To this end, we carried out experiments in the presence of 100 μM phenazine methosulphate (PMS) on thylakoid membranes isolated either with sorbitol or with NaCl. As shown in Fig. 7, both preparations possessed the ability of undergoing light-induced reversible changes in their ultrastructures. However, as expected, the changes in the SANS profile were less marked in the presence of NaCl than with sorbitol, where RD_{q^*} shifted from 236 to 231 Å, and from 346 to 310 Å, respectively. Also, the light-induced changes in the amplitudes of the Bragg peaks were considerably smaller with NaCl than with sorbitol. The former changes resemble those observed in leaves (see Section 3.1.3), while the values with sorbitol are essentially identical with those reported earlier [16–18]. Hence it can be concluded that the structural flexibility is considerably lower in samples with the more compact organization of the membranes. Nevertheless, the fact that the changes are also observed in leaves shows that this flexibility, i.e. the capability of undergoing reversible RD changes on the timescale of minutes, is an inherent property of intact thylakoid membranes.

Another interesting feature in the samples with NaCl is the appearance of a weak scattering signal between 0.012 and 0.013 Å^{-1}, corresponding to RD_{q^*} of 523–483 Å. This signal, although migth be suspected in some SANS profiles also in the presence of sorbitol (Figs. 6a and 7a), has not been as clearly and as frequently observed in samples with sorbitol as with NaCl. This peak is assigned to originate from stroma thylakoid membranes (Fig. 7b), where we have obtained RD_{EM} of 454 Å.

3.2.2. Possible bias of SANS and the effect of glutaraldehyde fixation

As shown above, there is a systematic difference between the SANS and EM data. In particular RD_{q^*} appears to be somewhat larger than RD_{EM}, suggesting some bias in SANS and/or in EM. Similar differences can be seen in cyanobacterial cells, where most RDs derived from SANS appear to be about 10% larger than those obtained from EM [19]. In SANS, such a bias can be envisioned towards larger RDs, i.e. with wider aqueous phases; both the lumen and the interthylakoidal space are densely packed with proteins [21], which might not allow free aqueous phases to be present. Thylakoid membranes with wider aqueous phases might produce higher contrast than those with narrow aqueous phases—and thus, assuming either a heterogeneity in the width or a continuum distribution of these distances, the Bragg peak might be dominated by the scattering from thylakoid membranes with larger RDs. This possibility should be tested with carefully designed experiments. On the other hand, the discrepancy between the EM and SANS data might also be explained by distortions during the fixation procedure. The primary fixing material is glutaraldehyde. Thus, we studied the effect of this compound on isolated pea and tobacco thylakoid membranes and found that 2.5% glutaraldehyde shifted q* from 0.0219 Å^{-1} to 0.024 Å^{-1}(pea) (Fig. 8) and from q* = 0.0212 Å^{-1} to 0.0227 Å^{-1} (tobacco) (not shown). It means that RD_{q^*} of thylakoid membranes decreases by about 20 Å. To overcome this problem, a detailed statistical analysis should be carried out using cryo EM, which is free of such possible artifacts (cf. [6,21]), parallel to or under similar conditions as the SANS measurements. Nevertheless, a bias of SANS toward larger RD values, i.e. toward thylakoid membranes containing wider aqueous phases, cannot be ruled out either. This bias, however, cannot be very large because isolated spinach thylakoid membranes suspended in sorbitol-containing medium, displayed
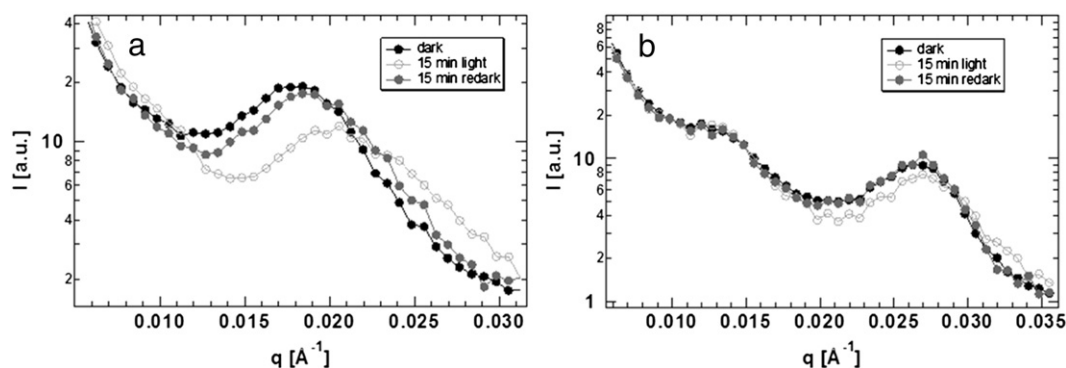
Fig. 7. SANS profiles of isolated tobacco thylakoid membranes, in the presence of 100 μM PMS, in the dark, in a steady state during a 15 min illumination (~1000 μmol photons m^{-2} s^{-1}) period and in the following re-dark period, as indicated; panels a and b, thylakoid membranes isolated and suspended in sorbitol- and NaCl-containing media, respectively (see Section 2.1). The measurements were performed at the PSI SANS-I instrument (SD = 11 m; collimation, 15 m; λ = 6 Å).

small-angle X-ray scattering (SAXS) peaks between about 0.020 and 0.023 Å^{-1}, corresponding to RD$_{q^*}$ between about 314 and 273 Å [43]. These values are very similar to those obtained with SANS. Thus, SAXS, where the contrast between the components of thylakoid membranes is determined by the differences in the electron densities, can provide complementary information about the ultrastructure of thylakoid membranes, as shown in [44]. Information on the thylakoid ultrastructure, derived from SAXS, will be reported in more details elsewhere.

4. Conclusions

In this paper we have shown that small-angle neutron scattering (SANS) provides unique structural information on the ultrastructure of grana in situ. In leaves, it yields statistically and spatially averaged repeat distance (RD) values of the thylakoid membranes. These repeat distances, which are also determined from EM image-analyses, comprise the widths of the two aqueous phases, the lumen and the interthylakoidal space, and twice the width of the membrane.

The two techniques, EM and SANS, yield basically very similar data. Minor differences in the RD values obtained with the two techniques might originate from bias of SANS due to the possible dependence of the scattering intensity on the width of the aqueous phase (which can

lead to variations in the contrast), and a shrinkage induced by glutaraldehyde fixation on the other hand, shown here by SANS measurements on isolated thylakoid membranes.

Nevertheless, both the average EM and SANS data (which ab ovo provide statistically averaged values) yield higher RD values (~200 Å) than anticipated, which suggests that the granum ultrastructure is somewhat looser than thought based on the generally accepted structural parameters, suggesting RD values typically between about 160 and 180 Å. Looser stacking of adjacent membranes may facilitate the lateral movement of the membrane components compared to a tight stacking. As already pointed out, the functional plasticity of thylakoid membranes observed upon a variety of changes in the environmental conditions requires significant changes in the macro-organization of the thylakoid membranes.

It must also be pointed out that, somewhat surprisingly, sorbitol, which is the most frequently used osmoticum in the reaction media, leads to a substantial expansion of the thylakoid membranes, which also appears to affect the structural flexibility of the thylakoid membranes. In contrast, NaCl, as an osmoticum, mimics much better the stromal liquid—the structural parameters and flexibility of the isolated thylakoid membranes match quite closely those obtained in leaves.

While EM contains more detailed information, SANS, as a non-invasive technique, provides data on samples without low temperature or chemical fixation or staining, and can, in general, be measured on samples under physiological conditions. Furthermore, SANS yields an averaged information over the entire sample volume in the neutron beam, providing much higher statistical accuracy than what is achievable with EM. Since SANS profiles on leaves can be measured in less than a minute, it opens the way for dynamic investigations. In other terms, as also shown here, it is a suitable technique to monitor the structural flexibility of the multilamellar thylakoid membrane system in situ, e.g. upon the exposure of leaves to moderately intense light.

It must also be noted that the high-q value peak of the SANS signal, too, appears to carry important information, most likely on the structure of the adjacent membrane pairs, but its elucidation requires further studies.

Our investigations have revealed that the RDs depend on the turgor of the leaf tissue and/or of the chloroplasts. In fact, infiltration of the leaves with D$_2$O, for SANS measurements leads to an increase of the RD values. This, the movement of water at the cellular or organelle level might be involved in RD changes, a question that warrants further investigations. Also, the mechanism and significance of the light-induced reversible RD changes, reported here for the first time on leaves, are to be investigated in more detail. In general, SANS might help in the better understanding of the macro-organization and structural flexibility of the multilamellar thylakoid membrane system in situ, in leaves during photosynthesis and upon the exposure of plants to different biotic and abiotic stresses.
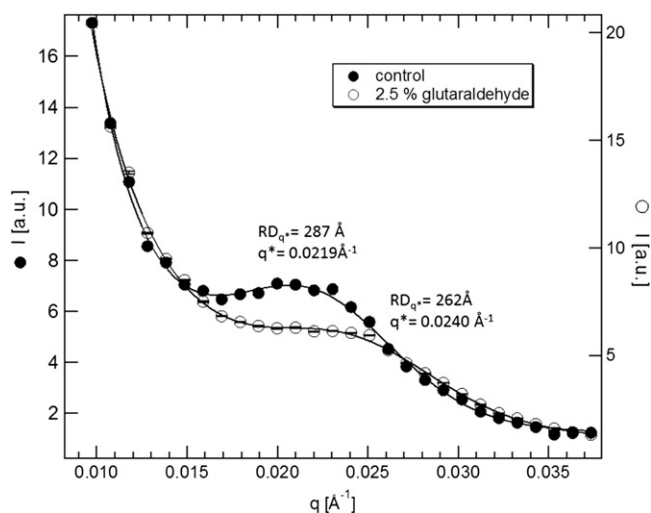

Fig. 8. Effect of glutaraldehyde on the SANS profile of pea thylakoid membranes isolated and suspended in the sorbitol-containing reaction medium (see Section 2.1). The calculated RD$_{q^*}$ values are indicated in the figure based on the fitted curves, which are also plotted. The measurements were performed at the BNC Yellow Submarine (SD = 5.42 m; collimation, 4 m; λ = 11.3 Å).

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