Kinetics of structural reorganizations in multilamellar photosynthetic membranes monitored by small-angle neutron scattering^{*}

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Abstract. We demonstrate the power of time-resolved small-angle neutron scattering experiments for the investigation of the structure and structural reorganizations of multilamellar photosynthetic membranes. In addition to briefly summarizing our results on thylakoid membranes isolated from higher plants and in unicellular organisms, we discuss the advantages and technical and methodological limitations of time-resolved SANS. We present a detailed and more systematic investigation of the kinetics of light-induced structural reorganizations in isolated spinach thylakoid membranes, which show how changes in the repeat distance and in the long-range order of the multilamellar membranes can be followed with a time resolution of seconds. We also present data from comparative measurements performed on thylakoid membranes isolated from tobacco.

1 Introduction

Small-angle neutron scattering (SANS) has proved to be an especially useful tool for the study of the structure of biological systems in solution [1–3]. A key component for the success of the technique is the large difference in the scattering amplitude of neutrons for hydrogen and for deuterium. Furthermore SANS (as neutron scattering methods in general) has practically no destructive influence on the sample, which makes the method ideal for measuring biological systems both *in vitro* and *in vivo*. Compared to electron microscopy SANS can provide structural information from the biological system without the need of staining or fixation, facilitating the investigation of structural responses to variations in different environmental parameters. A further difference between the two techniques is that SANS provides structural information averaged over

the entire sample volume in the neutron beam. Finally, due to the generally low neutron cross-section of sample environment materials it is relatively easy to provide a complex sample environment for neutron scattering experiments, which is particularly important for biological samples.

Early neutron scattering studies of oriented photosynthetic membranes of the unicellular *Euglena gracilis* and of spinach, reported in 1982 [4] showed how structural parameters of the periodic thylakoid membranes parallel to the membrane normal can be deduced. We do not know about continuation of these pioneering works from the subsequent decades. The presently available high-flux SANS instruments, however, made it possible to characterize thylakoid membranes in a significantly shorter time, which—as we reported in [5,6], and described here in more detail—allows the investigation of reversible reorganizations during photosynthesis in the ultrastructure of thylakoid membranes in different physico-chemical environments, such as osmolarity and ionic strength, and *in situ*, in different living organisms. Other, nonphotosynthetic complex biological membrane systems, such as mi-

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tochondria, can also successfully be studied with SANS and characteristic membrane distances or even complete structural reorganizations can be revealed [7,8].

One of the salient features of the structural reorganization processes in biological systems is their characteristic time dependence, which arises from the combination of the instantaneous effects induced by an external stimulus (*e.g.* photon absorption, a (bio)chemical reaction or change in the environmental conditions), and the subsequent relaxation. Due to the nondestructive nature of neutron scattering the structural changes can be followed in real time also in live or biochemically, photochemically functioning samples.

A restricting factor for observing time-dependent processes in neutron scattering is the relatively low probability of a scattering process, compared to the typical neutron beam flux of existing sources. With single-shot SANS experiments kinetic processes can be monitored with a time resolution of a fraction of a second, depending on the scattering power of the sample coupled with the necessary statistics of the accumulated data [9].

If the phenomena to be studied allow the process to be repeated in a similar manner by applying periodic perturbations — such as an oscillating external electric magnetic field — then stroboscopic measurements become possible, allowing the time resolution of the experiment to be improved practically down to the limits of the neutron detection and signal processing, provided the long-term stability and repeatability of the process can be maintained for a time sufficient for accumulating the necessary statistics [10]. Reproducibility is also the key component of the stopped-flow-based SANS experiments. In this technique, several solutions are rapidly mixed with flexible flow rates allowing observation of structural changes during the early stages of the induced reactions [11].

The complexity of the biological systems may limit the applicability of strictly stroboscopic techniques, because during the repeated stimuli the samples might not fully revert to their original state. However, when the processes under investigation are reversible repetitive techniques can be used. Also, if the investigated processes are relatively slow, allowing relatively long integration times, single-shot measurements can be performed. In favorable cases such measurements are reproducible, allowing to improve the quality of the accumulated data by averaging over several individual measurements.

The kinetics of ribozyme folding, temperature-induced fibrillar protein aggregation or protein gelation are some examples of such experiments [12–15]. The above-mentioned processes in biological systems are mostly irreversible, and by studying them we can understand how components of the system become dysfunctional. It is equally interesting to study biological systems under physiological conditions and during their functional activities. This is the focus of our studies.

Our present study deals with processes in thylakoid membrane systems *in situ*, *i.e.* in their native, cellular environments or in suspensions under physiologically relevant conditions. We use time-resolved SANS for studying

these multilamellar biological membranes in order to determine the kinetics of their light-induced reversible reorganizations, changes in their membrane ultrastructure — which was shown to be highly flexible [16], during the functioning of the photosynthetic machinery.

The light reactions of oxygenic photosynthesis take place in the thylakoid membranes, flattened vesicles, which in higher plants and various unicellular organisms are organized into multilamellar membrane systems, ensuring an efficient capturing of light energy. In higher plants, they are arranged in stacked layers, called the grana, which are interconnected by stroma thylakoid membranes winding around the granum “pillar” in a quasi-helical structure [17,18]. While during the recent decades our knowledge of the molecular basis of the photosynthetic processes has advanced significantly, the roles of large scale structures of the thylakoid membranes and their dynamic features in particular are still poorly understood; thus, non-destructive structure investigating techniques which also allow time-resolved measurements, such as SANS, are of paramount importance.

In this paper, we give a general overview of the technical details, practical issues, advantages and limitations of the time-resolved SANS experiments performed on isolated thylakoid membranes and on unicellular photosynthetic organisms, and focus our attention on detailed analyses of the kinetic experiments performed on isolated plant thylakoid membranes.

2 Materials and methods

2.1 Sample preparation

Spinach thylakoid membranes for the experiments on D22 at the Institut Laue-Langevin (ILL) Grenoble, France, and for prior pilot experiments at the Yellow Submarine of the Budapest Neutron Centre (BNC), were freshly isolated according to [5], resuspended in D₂O containing grinding medium to a chlorophyll concentration of 1–2 mg/ml and were stored at 4 °C and used within 4 h.

Tobacco thylakoid membranes for SANS I experiments, at the Paul Scherrer Institute (PSI), Villigen, Switzerland, were freshly isolated from tobacco leaves. Tobacco (*Nicotiana tabacum* SR1) was grown for 6 months in a greenhouse at 25 °C at a photon flux density of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with light/dark periods of 16/8 h. After the main ribs had been removed, leaves 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 cheese cloth. The remaining debris was removed by centrifugation at 200 g for 2 min. The supernatant was centrifuged for 5 min at 4000 g. The pellet was resuspended in 10 ml osmotic shock medium containing 20 mM Tricine (pH 7.6), 5 mM MgCl₂ and 5 mM KCl in order to remove the envelop membrane and the water soluble proteins from the stroma aqueous phase. After 5 s, the osmolarity was returned to isotonic conditions by adding 10 ml double osmotic strength (0.8 M) medium. This was then centrifuged

for 5 min at 4000 g. The pellet obtained was washed and re-suspended in D₂O-containing grinding medium (pD 7.6). The chlorophyll concentration of this thylakoid membrane suspension was adjusted to 1–2 mg/ml and the samples were stored at 4 °C until use.

2.2 Small-angle neutron scattering

2.2.1 Experimental setup

Small-angle neutron scattering experiments were performed on the D22 SANS instrument at the ILL, and on the SANS I [19] instrument at the PSI. Both instruments use a continuous neutron beam which is monochromatized by a velocity selector. The applied wavelength (λ) and wavelength spread ($\Delta\lambda/\lambda$) values were 6 Å and 10%, respectively, on both instruments. The suspensions of the isolated thylakoid membranes were filled in 2 mm quartz cuvettes and were mounted in a temperature controlled sample holder (D22) or in a conventional temperature controlled sample changer (SANS I) with a set temperature of 20 °C. Samples could be illuminated in the sample holders directly during the SANS measurements. For this, Schott lamps KL2500 (at ILL) and KL1500 (at PSI) were used with a flexible fiberoptic light guide.

The neutron beam was defined with a 10 mm × 6.5 mm (D22) or a 10 mm × 7 mm (SANS I) size aperture in front of the sample. The neutrons scattered from the samples were detected with two dimensional position-sensitive ³He detectors with 128 × 128 pixels, with a pixel size of 8 × 8 mm² (D22) and 7.5 × 7.5 mm² (SANS I). The sample to detector distance and collimation were 8 m, 8 m (D22) and 11 m, 11 m (SANS I). These instrument settings covered the Q -range of 0.008–0.05 Å^{−1} on D22 and 0.006–0.09 Å^{−1} on SANS I.

In order to increase the scattering intensity, the isolated thylakoid membranes were aligned with their membrane normal perpendicular to the neutron beam in a horizontal direction. (For the mechanism of magnetic orientation, see sect. 3.1.2). For this, a magnetic field of ~ 1.5 T, generated by a conventional electromagnet was used at D22. On SANS I special sample holders with permanent magnets were mounted on the temperature controlled sample changer, creating a magnetic field of ~ 0.4 T. The field vector was perpendicular in both cases to the neutron beam.

Both types of magnetic orientation have their advantages and drawbacks. When an electromagnet is used for the experiments, a higher degree of orientation of the thylakoid membranes can be achieved due to the higher and more homogeneous magnetic field compared to the setup with permanent magnets, however the available electromagnets are usually not equipped with thermostated sample changers, requiring continuous presence and manual sample change at the experiments. Furthermore due to spatial restrictions the temperature control is more precise and illumination with a light guide is more convenient when the samples are mounted on a thermostated sample holder with small permanent magnets.

2.2.2 Data treatment

Primary treatment for raw data collected on D22 and SANS-I was performed with the Graphical Reduction and Analysis SANS Program for Matlab - GRASP (developed by Charles Dewhurst, ILL) and “BerSANS-PC” software (developed by U. Keiderling, HZB [20]), respectively. The collected two-dimensional data counts were normalized to the number of incoming neutrons pixel by pixel and the corresponding errors were normalized accordingly. Background scattering (from the empty cell in case of H₂O or the empty cell and buffer in case of samples) was subtracted, along with the instrumental background (measured while blocking the beam with a cadmium plate) taking into account the appropriate sample transmissions. Data were corrected for variations in the detector efficiency by normalizing to a background subtracted water sample (with thickness of 1 mm). On the obtained two dimensional data radial averaging was performed in a 360° sector around the center of the direct beam in the case of unoriented samples and in two 45° sectors for oriented samples at D22 (see fig. 1) and two 75° sectors at SANS I. Larger sector size was used on SANS I because the peaks were less sharp, due to the lower magnetic field applied. The radially averaged curves were used for deriving structural information.

Scattering curves of thylakoid membranes isolated from higher plants were fitted with the following equation:

$$I(Q) = I_0 + A|Q|^p + \frac{B}{w\sqrt{\frac{\pi}{2}}} e^{-\frac{2(Q-Q^*)^2}{w^2}}, \quad (1)$$

i.e. with the sum of a power function and a Gaussian with peak position at Q^* , integrated intensity of B , and full width at half maximum (FWHM) of $\sqrt{2 \cdot \ln(2)} \cdot w$. At the moment it is not clear and it warrants further investigations if relevant structural information can be deduced from the fitting parameters of the power function; this article is dealing only with fitting parameters of the Gaussian component. Repeat distances (RDs), or lamellar periodicity, for the thylakoid membranes (fig. 2) were calculated from the peak positions of the Gaussians based on the Bragg equation $RD = 2\pi/Q^*$.

2.2.3 Time-resolved experiments

In time-resolved experiments performed on D22 we recorded the two-dimensional scattering data with a time resolution ranging from 2 s to 5 min, while on SANS I the time resolution was 1 min. In the data analysis procedure, the individual radially averaged scattering curves were re-grouped for longer time intervals if it was required to increase the statistical accuracy of the curves used for further treatment. A time parameter is associated with each scattering curve, which is defined by the time elapsed between the moment of the last change of the sample environmental parameters (*e.g.*, start or end of illumination) and the moment the scattering data is read out from the detector. The fitting parameters for eq. (1), along with the calculated RD, were plotted as a function of the time parameter values (see sect. 3.2).

Fig. 1. Effect of external magnetic field on the SANS profiles of isolated spinach thylakoid membranes. Scattering profiles showing Bragg peaks attributed to the periodic arrangement of granum thylakoid membranes; recorded with the 2D detector of the D22 SANS instrument. Corresponding instrumental and sample background are subtracted and the intensities are corrected for detector efficiency. Colors represent the differential scattering cross-section values on a logarithmic scale. The applied magnetic field is 0 T (A) and 1.5 T (B). The white sectors represent the area of radial averaging in the two cases. Comparison of the radially averaged SANS signals from the two experiments (C).

Fig. 2. Main structural parameters of the thylakoid membranes of higher plants. Lamellar periodicity of the granum thylakoids (RD_{granum}) and of the stroma thylakoids (RD_{stroma}). Thickness of the inner (Luminal space) and outer (Interthylakoidal space) aqueous phase.

3 Results and discussion

3.1 Overview

We have performed experiments on various photosynthetic organisms in order to reveal relevant structural information on the periodicity and structural flexibility of different thylakoid membrane systems. The experiments ranged from the study of unicellular organisms, such as cyanobacterial, diatom or green algal cells, through thylakoid membranes isolated from different higher plants [5,6,21], to the investigation of entire leaves. Of particular interest with regard to the dynamic features, we paid special attention to the effects of illumination by different light intensities and dark relaxations and under different experimental conditions (*e.g.*, the presence of reagents, spectrum of illumination) affecting the operation of the photosynthetic apparatus.

The most important experimental parameter in kinetic measurements is the time resolution. Several factors determine how long measuring time is required to obtain data of sufficient statistical accuracy to extract precise structural information, in our case about the periodicity of the thylakoid membranes.

The neutron flux available on the used SANS instrument is of primary importance. Most of our measurements were performed on one of the best instruments at steady state reactor sources, the D22 diffractometer at the ILL, with a single wavelength and wavelength distribution setup; therefore, we can consider these measurements as benchmark for our similar future experiments.

3.1.1 Sample composition

The required measuring time is strongly influenced by the amount of thylakoid membranes relative to the total organic material present in the system; which affects the signal/noise ratio. Evidently, shorter measuring times are required for isolated membranes with low background

from other organic materials. Furthermore, the concentration of the thylakoid membranes in the sample suspension is significantly higher for isolated membranes than in whole cells, resulting in a higher signal/noise ratio for isolated thylakoid membranes. However, when measuring light-induced changes the optical density should still allow a relatively uniform illumination of the sample.

The investigated samples are suspended in D₂O-based reaction media and the contrast between the thylakoid membranes and the surrounding aqueous phase is strongly influenced by the protein content of the aqueous phase and by the protein composition in the bilayer lipid membrane, both of which depend on the investigated species. Since the contrast between the sample components can be very different for neutrons and for X-rays, small-angle X-ray scattering can provide complementary information about the structure of the thylakoid membranes, as demonstrated in [22].

The number of stacked thylakoids also varies from sample to sample, typically ranging from 5–20 for the granal thylakoids [23,24] to 3 for the algal group of diatoms [25], which also influences the intensity of the observed Bragg-peak and consequently the required measuring time (c.f. [26]).

3.1.2 Magnetic orientation

Multilamellar thylakoid membranes can be oriented in an external magnetic field with their membrane normals tending to be parallel to the direction of the magnetic field [27] due to the diamagnetic anisotropy of the thylakoid membranes [28]. The diamagnetic anisotropy, a collective property of these samples, are thought to be given rise by the vectorial sum of the diamagnetism of pigment, protein and lipid molecules. In granal chloroplasts this is largely determined by the ordered arrays of their main light harvesting antenna complexes [29], and by the long-range order of the complexes [30], as well as by the geometrical shape of the membranes [31]. We have demonstrated earlier that this method can be very useful to increase the signal-to-noise ratio [5,6,21], since the orientation increases the number of membranes positioned in a Bragg-diffracting position. Furthermore, most of these Bragg-diffracting thylakoids are oriented into a similar direction. Thus the intensity of the diffraction peak strongly increases, while less background is needed to be subtracted due to using sectorial masks for the radial integration. As a representative example, detector images and the radially averaged scattering signals from magnetically aligned and randomly oriented thylakoid membranes are shown in fig. 1.

The magnetic orientability of the isolated chloroplasts or thylakoid membranes (“chloroplasts” without their envelope membranes and the soluble stromal proteins) is impaired above a certain concentration, probably due to adhesive interaction between independent adjacent chloroplasts, or due to physical constraints of their rotational degree of freedom. Therefore, the chlorophyll concentration

usually should not exceed a value of 1–2 mg/ml. For the investigation of light-induced structural changes, this may not be optimal because the absorbance of samples with this chlorophyll concentration is relatively high. (With an optical pathlength of 2 mm, it is somewhat higher than 2 in the red absorbance maximum.) Nevertheless, these experimental conditions are suitable for functional investigations, as tested by independent control experiments.

Among the systems investigated orientation by a magnetic field could be applied only for isolated thylakoid membranes and for the diatom *P. tricornutum*, where the thylakoid membrane assembly exhibit an inherent structural anisotropy, with nearly planar, parallel membranes, which brings about a nonzero diamagnetic anisotropy for the membrane system. This, together with the concentration of the thylakoid membranes in the sample suspension (see sect. 3.1.1) explains why measuring times of down to seconds were applicable for the case of thylakoid membranes and tens of seconds for diatoms, while scattering data were required to be collected for at least minutes, e.g., for cyanobacteria, where the membranes are arranged in concentric arrays and thus are not amenable for magnetic alignment. These are practical limits for the time resolution in kinetic SANS experiments on different photosynthetic membranes.

3.2 Rapid light-induced reorganizations in isolated thylakoid membranes

We performed SANS experiments on thylakoid membranes freshly isolated from spinach leaves, in the presence of 100 μ M PMS (N-methyl phenazonium methosulfate), which catalyses cyclic electron transport around photosystem I (PSI), one of the main protein supercomplexes involved in the conversion of light energy to chemical energy. We could observe a well-defined diffraction peak which appeared on the radially averaged scattering curves at $Q^* \approx 0.022 \text{ \AA}^{-1}$. Earlier [5,6] we have interpreted this peak to originate from the periodic arrangement of the stroma thylakoid membranes. However, our recent experiments performed on thylakoid membranes in different reaction media suggest that this peak originates from the periodic arrangement of swollen granum thylakoids (R. Ünneper, G. Nagy, K. Solymosi, O. Zsiros, L. Rosta, D. Posselt, G. Garab, unpublished data).

Upon illumination, we observed fully reversible light-induced reorganisations, which could clearly be seen in the radially averaged scattering profiles, a decrease in the total intensity and a shrinkage of RDs. Figure 3 shows the time course of the light-induced changes and of the dark readaptation for isolated spinach thylakoid membranes. The figure also shows curves calculated by performing the fitting of a Gaussian and a background as detailed above and subtracting the resulting power functions from the corresponding experimental scattering curves. These curves (B and D in fig. 3), compared to fig. 3A published earlier [5], characterize further the effect of illumination on the membrane ultrastructure, and answer the question as to what extent the changes in the scattering profiles

Fig. 3. Radially averaged scattering curves (A and C) and scattering curves after subtraction of the fitted power function (B and D) for spinach thylakoid membranes in the presence of $100 \mu\text{M}$ PMS during the illumination (A and B) with white light of $1700 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ photon flux density and during the dark adaptation (C and D). (Figure A is reprinted from [5]: with permission of Portland Press.)

can be ascribed to the decrease of the RDs per se, or are there additional changes involved. As shown in fig. 3B the possibility that the shift in the peak position can be interpreted as a selective disordering of a subpopulation of thylakoid membranes with higher repeat distance is unlikely: although there is a clear asymmetry in the measured shape of the SANS signal, induced during the dark-to-light transition, but the signals with higher Q -values, outside the band in the dark, indicate that there is a real decrease in (at least a significant part of) the membrane RDs. At the same time, the shift is accompanied with diminishing peak intensities and higher FWHM, and—as demonstrated in fig. 3C and D—all these changes are reversible in the dark. The dark reversibility of these light-induced reorganizations shows that they are physiologically relevant changes.

It is also to be noted that these changes were accompanied by a smearing in the 2D SANS profiles (see fig. 1 in [5])—indicating that the magnetic orientability of the sample decreased. This shows that additional changes occur in the sample—evidently in the shape of the membranes or in their internal order since, as pointed out above, the magnetic orientability depends on the shape

of membranes and on the presence of ordered arrays of the complexes. This observation indicates that this type of changes in the membrane ultrastructure can also be monitored by 2D SANS measurements on magnetically alignable samples. The analysis of these changes is, however, beyond the scope of the present paper. As mentioned above, it is also an interesting question what is the information content of the scattering signal outside the area of the Bragg peak, which is fitted with a power function, and which can most clearly be observed, essentially without the influence of the Bragg peak, by sectorial averaging of the 2-dimensional data at perpendicular direction relative to the Bragg peaks. The same question relates to the scattering profile of membranes oriented with their plane perpendicular to the propagation of the neutron beam. This type of experiment can be done by using a magnet with its field vector parallel to the neutron beam, or by using samples in which the magnetic alignment is trapped in a gel, experiments in progress (R. Ünneper, G. Nagy, O. Zsiris, G. Káli, L. Rosta, G. Garab).

Concerning the kinetics of the light-induced changes, it is of interest to investigate the time course of some of the fitted parameters (center position, intensity and

Fig. 4. Time course of different fitted parameters: the center position (A), FWHM (C) and integrated intensity (D) of the Bragg peak for the scattering curves obtained during illumination (with white light of $1700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ photon flux density) and during dark relaxation of the isolated spinach thylakoid membranes (in the presence of $100 \mu\text{M}$ PMS), and of the corresponding calculated RD values (B). The illumination and dark periods are shown as white and dark bars, respectively. (Figure B is reprinted from [5] with the permission of Portland Press.)

FWHM of the peak and calculated RD), which are presented in fig. 4. These figures also show that the light-induced shrinkage of the granum thylakoid membranes are accompanied by a decrease in the long-range order of the thylakoids, as indicated by the increased FWHM and the decrease in the integrated intensity of the peak. It is also of interest that, as shown in fig. 4D, the drop in the total intensity is much faster than the shift of peak position. This shows that these reorganizations cannot be ascribed to a single process and demonstrates the complexity of these reorganizations. In this context it is interesting to note that CD measurements on granal thylakoid membranes under comparable conditions revealed similar characteristics, with very similar kinetic components in the psi-type CD signals, a fast and a slower component and clear indication of the reversible disassembly of macrodomains with long-range chiral order of the complexes [32,33]. These light-induced changes occurred without affecting the molecular architecture of the constituent complexes, as shown by the invariance of the excitonic CD bands, and were fully reversible. It is also to be noted that the illumi-

nations applied in the above SANS and CD measurements do not induce any noticeable increase in the temperature of the sample. (For the origin of CD signals at different levels of the structural hierarchy see [34] and [35]).

In the observed structural reorganization, the shift of the Bragg peak was always followed by the change of its width. Since the width of the peak is affected by the finite instrumental resolution, and it is not constant at different Q -values, we calculated the possible influence of the varying resolution function to the observed peak width. The resolution function for D22 as a function of Q was provided by the GRASP data treatment program, based on the equation [36]

$$\Delta Q^2 = Q^2 \left(\frac{1}{2\sqrt{2}\ln 2} \frac{\Delta\lambda}{\lambda} \right)^2 + \left[\left(\frac{4\pi}{\lambda} \right)^2 - Q^2 \right] \Delta\theta^2, \quad (2)$$

where in our experiments $\frac{\Delta\lambda}{\lambda} = 0.1$, and $\Delta\theta$ is estimated by GRASP from the attenuated direct beam measurement, based on the equation [36]: $\Delta Q(Q=0) = \left(\frac{4\pi}{\lambda} \right) \Delta\theta$.

Fig. 5. Q dependency of the calculated instrument resolution in our experiments on D22, showing little FWHM changes in the Q range, relevant for the center position of the observed Bragg peak (A). Peak broadening upon illumination in normalized representation, together with the calculated instrument resolution at $Q = 0.0215 \text{ \AA}^{-1}$. Bragg peak curves are created from fig. 3B via dividing by the maximum value of the fitted Gaussians and shifting the curves to be centered around $0 \text{ \AA}^{-1}$ (B).

Estimated FWHM values for the instrument resolution are shown in fig. 5A.

Figure 5A reveals that in the Q range $0.02\text{--}0.025 \text{ \AA}^{-1}$, *i.e.* where the center position of the peak can be found, the calculated FWHM of the instrument resolution is in the range $0.0038\text{--}0.0042 \text{ \AA}^{-1}$. We have to note that the shape of the direct beam is asymmetric in our experiments—being slightly wider in the vertical direction than in the horizontal direction. Therefore, when radial averaging is applied over a horizontal sector, the width of the resolution function is expected to decrease. Furthermore, from the weak Q dependency of the instrument resolution in the range of the possible peak center positions we can conclude that the increased FWHM of the peak cannot be the result of the shift of the Bragg peak alone; on the contrary, it reveals a decrease in the order of the granum thylakoids. The origin of this phenomenon is yet unknown. This effect of the light-induced desorganization of the membrane system is demonstrated in fig. 5B, where normalized peaks are shown together with the instrument resolution function (similarly as described in [37]), allowing a better visualization of the widening of the Bragg peak during illumination.

3.3 Light-intensity-dependent kinetics of the structural reorganizations in isolated thylakoid membranes

The effect of the illumination on the structural rearrangements observed by psi-type CD was earlier found to be strongly light intensity dependent [33]. To investigate in more detail the nature of these reorganizations we performed SANS experiments with a sub-minute time resolution and followed the light-induced changes in isolated spinach thylakoid membranes for different light intensities. The time course of the granum RD changes during the illumination and the consecutive dark period

(fig. 6) reveal that increasing light intensities result in increased repeat distance changes and in significantly different kinetics, to a large extent similar to the CD kinetics. Although more systematic series of experiments and also temperature-dependent kinetic measurements would be needed for obtaining a precise correlation, it is clear that both CD and SANS reflect well discernible and rapid reorganizations during photosynthetic functions, which appear to affect the membrane ultrastructure.

During these SANS experiments the data acquisition time was 1 s, which—including a ~ 1 s data storage time—results in a ~ 2 s time resolution. For a better representation we summed the radially averaged scattering curves in groups of 5, to obtain the curves in fig. 6, and performed the curve fitting and calculation of the RD values on these curves. Average RD changes during the ~ 3 min light or dark-time intervals were calculated by averaging two consecutive light-dark cycles for each light intensity (see table 1). As shown, *e.g.*, in fig. 6D there are major RD changes, especially in the dark adaptation period during the first ~ 10 s. Therefore, we used the original ~ 2 s time resolution for the investigation of the kinetics of the RD changes. Illumination with light intensities of $330 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photon flux density and higher appears to lead to a state with an equilibrium RD. We fitted an exponential function to the RD data *vs.* time and the characteristic time constants from the fits are presented in table 2.

We observed that increasing light intensities result in faster kinetics of the light-induced RD changes. This result is in accordance with the one presented in [33], where the initial rate of the light-induced CD changes was found to increase with increasing light intensity. Dark adaptation after illumination with light intensities of $650 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photon flux density and higher appears to induce RD changes with two significantly different time constants as shown in table 2.

Fig. 6. Time courses of the calculated granum thylakoid RD values in isolated spinach thylakoid membranes in the presence of 100 μM PMS during illumination (with white light of 150 (A), 330 (B), 650 (C) and 1200 (D) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ photon flux density) and their dark recoveries.

Table 1. RD changes in isolated spinach thylakoid membranes during illumination with different light intensities and in the dark following the illumination period.

		Illumination intensity [$\mu\text{mol photons m}^{-2} \text{s}^{-1}$] photon flux density				
		150	330	650	1200	1700
RD change [Å]	Illumination	-5 ± 1	-11 ± 1	-22 ± 1	-33 ± 1	-37 ± 2
	Dark adaptation	6 ± 1	11 ± 1	23 ± 1	33 ± 1	37 ± 2

Table 2. Kinetic parameters determined by curve fitting from the time courses of the light-induced RD changes during illumination with different light intensities and in the dark following the illumination period.

			Illumination intensity [$\mu\text{mol photons m}^{-2} \text{s}^{-1}$] photon flux density			
			330	650	1200	1700
Illumination	Decay time constant [s]		38 ± 6	35 ± 4	27 ± 2	19 ± 1
Dark relaxation	Rise time constant [s] (Relative contribution)	fast	–	2.4 ± 0.9 (56%)	1.9 ± 0.4 (67%)	0.8 ± 0.4 (63%)
		slow	41 ± 4	98 ± 39 (44%)	64 ± 13 (33%)	155 ± 85 (37%)

Fig. 7. Effect on illumination on the radially averaged SANS signal of tobacco thylakoid in a reaction medium containing $100 \mu\text{M}$ PMS, under $\sim 1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ photon flux density white light and redark (A). Time course of the corresponding calculated granum thylakoid RD values (B).

We have to note that, unlike the static structural parameters, the light-induced structural changes of the granum thylakoids vary significantly between different batches. Therefore, in our experiments it was crucial to perform the comparative measurements on the same sample or on samples from the same batch. Samples from the same batch investigated with few hours time difference also revealed that during storage the RD tended to increase by several percent. This increase was accelerated when illumination was applied.

3.4 Functional basis of the observed light-induced structural changes

Comparative experiments were performed on isolated thylakoid membranes in the presence of different reagents during illumination with fixed ($1700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) photon flux density in order to reveal the functional origin of the observed structural changes and the results were presented in [5]. Light-induced structural changes of thylakoid membranes isolated from spinach were observed in the presence of $100 \mu\text{M}$ PMS, *i.e.* during the functioning of the PSI cycle. By changing the reagent present in the sample suspension, or by changing the color of the illuminating light, using farred light, absorbed preferentially by photosystem I, we were able to prove that the observed RD changes can be elicited by the PSI cyclic electron transport alone. We could also prove that the observed light-induced changes in the grana RD are driven by the transmembrane ΔpH , which is generated during the vectorial electron and proton transport processes. Further experiments are needed to clarify the question to what extent the RD changes contain variations in the membrane thickness and the lumenal and interthylakoidal aqueous phases.

It is to be noted that in this paper we confine our data treatment to the diffraction peaks of thylakoid membranes around $0.02\text{--}1 \text{ \AA}^{-1}$. A more complete analysis, such as the one by Nallet *et al.* [38] presented on a model system, of our highly organized membrane assemblies, and even of

a somewhat simpler, cyanobacterial membrane system [5, 39], was not attempted due to their high complexity. Here, our attention is focused on the Bragg peak and its small but well-discernible light-induced variations. In order to perform a deeper analysis in a broader Q range and to establish the influence of the form factor, more information would be required on the composition and molecular architecture of these membranes; first, this could probably be attempted on a simpler system, *e.g.*, on isolated photosystem II, so called BBY, membrane fragments (see [40]).

3.5 Time-resolved experiments on thylakoid membranes isolated from tobacco

As a comparative study we investigated isolated thylakoid membranes of *Nicotiana tabacum* which were also capable of undergoing light-induced reversible structural changes, as shown by SANS (see fig. 7). For the illumination experiments as in the case of spinach thylakoids we added PMS at $100 \mu\text{M}$ concentration, which, in the dark, had negligible influence on the scattering signal.

The averaged RD of the granal thylakoid system in tobacco appears to be larger ($349 \text{ \AA} \pm 11 \text{ \AA}$) than in spinach ($294 \text{ \AA} \pm 7 \text{ \AA}$) and within the statistical error similar to our earlier results on pea thylakoids ($345 \text{ \AA} \pm 11 \text{ \AA}$). In our experiments, tobacco thylakoids were exposed to $\sim 1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ photon flux density white light for a longer period than in our earlier SANS experiments on spinach or pea. Nevertheless, they retained full reversibility. The RD is decreasing under illumination like in the case of spinach or pea, and it occurs, along with the overall decrease in the scattering intensity. The magnitude of the light induced $\sim 50 \text{ \AA}$ shrinkage is comparable to those observed on pea and spinach. Surprisingly the calculated decay ($178 \text{ s} \pm 15 \text{ s}$) and rise ($210 \text{ s} \pm 20 \text{ s}$) time constants were relatively slow compared to observed values for spinach thylakoids. However, in order to make better comparison between thylakoids isolated from these two plants, systematic light-intensity-dependent studies are needed to be performed.

3.6 Unicellular organisms

As reported earlier, we have also investigated the diatom *Phaeodactylum tricornutum* and determined the repeat distances of its thylakoid membranes. For the first time on living algae, we observed the occurrence of light-induced reversible reorganizations in the lamellar order of the thylakoid membranes [5]. The light-induced reversible swelling of the membranes was correlated with the light-induced reversible increase in the psi-type CD signal [21], which, as pointed out above, originates from the long-range chiral order of the chromophores and depends on the 3D organization of the pigment system [35]. On this system we could perform kinetic experiments with a time resolution of tens of seconds and observed structural reorganizations on the minutes timescale during the illumination period, while full reversibility during dark adaptation was found to be significantly slower (in the range of 30–60 min). This latter timescale is comparable to the dark-relaxation time of the light-induced reorganizations in the chiral macrodomains (measured by CD) [41] and also with the relaxation time of the photoprotective nonphotochemical quenching of the chlorophyll-a fluorescence [42], implicating that these reorganizations play an important role in regulatory processes. It is interesting to note that in contrast to the isolated thylakoid membranes, where the light-induced shrinkage was driven by the transmembrane ΔpH , the light-induced reversible swelling of ~ 10 Å in magnitude in the living diatom cells was not sensitive to uncouplers abolishing the ΔpH .

During our experiments performed on the PAL mutant of cyanobacterium *Synechocystis PCC 6803* — devoid of the membrane bound, large light harvesting complexes, the phycobilisomes [43] — the periodicity of the thylakoid membranes was determined together with light-induced RD changes, also small (~ 10 Å) but well-discernible dark-reversible light-induced shrinkage was observed [5].

The underlying physical mechanism(s) of these changes in whole algal and cyanobacterial cells remain to be elucidated. Nevertheless, it is clear that the small but well-discernible reorganizations in the membrane ultrastructure — which can be monitored by SANS at a good time resolution and under physiological or physiologically relevant conditions — play important physiological roles and their study will contribute significantly to our understanding of key regulatory processes in plants and algae. Such structural changes and time resolutions can hardly be achieved reliably by electron microscopy. Similarly, our recent SANS experiments on intact leaves of different plants, in which we have been able to identify the repeat distances of thylakoid membranes and have started investigations on light-induced changes in wild type and different mutant leaves (R. Ünnepp, G. Nagy, O. Zsiros, L. Rosta and G. Garab, in preparation), especially when combined with parallel functional studies (such as chlorophyll-a fluorescence induction and absorbance transients to characterize photosynthetic functions) will be of high value in understanding the roles of ultrastructural changes of the thylakoid membranes in the physiology of plants.

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