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Reversible membrane-reorganizations during photosynthesis *in vivo* – revealed by small-angle neutron scattering

Gergely Nagy^{*,†}, Dörthe Posselt[‡], László Kovács[§], Jens K. Holm[‡], Milán Szabó[§], Bettina Ughy[§], László Rosta[†], Judith Peters^{*,||,¶}, Peter Timmins^{*}, Győző Garab[§]

^{*}*Institut Laue-Langevin, BP 156, F-38042, Grenoble Cedex 9, France*

[†]*Research Institute for Solid State Physics and Optics, Hungarian Academy of Sciences, BP 49, H-1215, Budapest, Hungary*

[‡]*IMFUFA, Department of Science, Systems and Models, Roskilde University, DK-4000 Roskilde, Denmark*

[§]*Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, BP 521, H-6701, Szeged, Hungary*

^{||}*Université Joseph Fourier Grenoble I, BP 53, F-38041, Grenoble Cedex 9, France*

[¶]*Institut de Biologie Structurale Jean Pierre Ebel CEA-CNRS-UJF, F-38027, Grenoble Cedex 1, France*

Corresponding author:

Győző Garab

Address: Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, P.O.Box 521, H-6701, Szeged, Hungary

Telephone: 0036-62-433131

Fax: 0036-62-433434

E-mail: gyozo@brc.hu

Synopsis:

We determined characteristic repeat distances of the photosynthetic membranes in living cyanobacterial and eukaryotic algal cells and in intact thylakoid membranes isolated from higher plants with time-resolved small-angle neutron scattering. This non-invasive technique reveals light-induced reversible reorganizations in the seconds to minutes time scale, which appear to be associated with functional changes *in vivo*.

Short title: Neutron scattering reveals membrane-reorganizations during photosynthesis *in vivo*

Keywords: chloroplast thylakoid membranes, cyanobacteria, diatom, light-induced reorganizations, membrane ultrastructure, SANS

Abbreviations used: CD, circular dichroism; DCMU, (3-(3,4-dichlorophenyl)-1,1-dimethylurea); EM, electron microscopy; FCPs, fucoxanthin-chlorophyll proteins; LHCI, main light harvesting complex of PSII; PMS, N-methyl phenazonium methosulphate; PSI, photosystem I; PSII, photosystem II; Q, scattering vector; RD, spatially averaged repeat distance; SANS, small-angle neutron scattering

Introduction:

In order to increase the efficiency of light capturing, most photosynthetic organisms have evolved highly organized multilamellar membrane systems. They also exhibit remarkable structural and functional flexibility, which enables these organisms to carry out short term adaptations and long term acclimations in response to changes in environmental conditions [1-6]. Our knowledge concerning the molecular mechanisms of photosynthesis has advanced greatly in the past two decades, owing to the availability of high resolution structural information of the protein components and their supercomplexes as well as detailed information from sophisticated spectroscopic techniques [7]. In contrast, much less is known about the self-assembly and regulation of membrane ultrastructures under different environmental conditions.

To obtain detailed and accurate information on the membrane ultrastructure of photosynthetic organisms and their structural flexibilities during photosynthesis, non-invasive techniques are required, such as neutron scattering, which offers unique structural information on complex membrane systems under physiological conditions [8;9]. The neutron scattering length is very different for hydrogen and deuterium, thus allowing for systematic contrast variation in an aqueous environment by varying the H₂O/D₂O ratio in the suspension medium [10;11]. Depending on this ratio we can highlight protein and/or lipid parts of complex biological membrane systems. While interatomic distances are determined using diffraction methods, small-angle neutron scattering (SANS) can be used to determine the repeat distances in multilamellar membrane systems with long-range order. Accordingly, recording the scattered intensity as a function of the scattering vector, Q , a Bragg peak positioned at Q^* determines a spatially averaged repeat distance (RD), characteristic of the entire statistical population: $RD = 2\pi/Q^*$.

The membrane ultrastructure of photosynthetic organisms depends largely on its composition and on the structure and arrangement of the integral and membrane-associated protein complexes. In oxygenic photosynthetic organisms, the initial steps of the conversion of light energy into chemical energy occur in flattened vesicular bilayer structures, called the thylakoid membranes. These membranes separate the inner aqueous phase, the lumen, and the outer aqueous phase. The membranes contain two photochemical systems: photosystem I (PSI), responsible for the production of NADPH, carrying the reducing power, and photosystem II (PSII), which catalyses the oxidation of water. The thylakoid membranes also embed the cytochrome b₆/f complex and the ATP synthase. The energy supply for photosynthesis is provided mainly by extended arrays of accessory light harvesting antenna complexes, which increase the effective absorbance cross sections of the photosystems. These complexes are either integral membrane proteins, as in higher plants and most algal cells, including the diatoms, or large external protein aggregates, the phycobilisomes, as in cyanobacteria and red algae. The presence of external proteins anchored to the membrane and polypeptide sections protruding from the thylakoid lamellae toward the outer aqueous phase poses restrictions for the distances between adjacent thylakoid membranes [12], thus limiting the packing density of membranes and their possible minimum RDs. Regarding the membrane ultrastructure and

regulatory functions in different classes of photosynthetic organisms nature displays astounding variations in various organisms from primitive bacteria to higher plants.

In this work we performed SANS experiments on three basically different classes of oxygenic photosynthetic organisms: i) a cyanobacterium – these prokaryotic organisms are the progenitors of chloroplasts in green plants, ii) a diatom species – representative of the major group of algae, which, due to their specific lifecycle, play an essential role in the regulation of atmospheric CO₂ concentration, and iii) granal chloroplasts isolated from a higher plant – representing the most abundant and one of the most complex membrane systems in the Biosphere. By using SANS we were able to study photosynthetic membrane ultrastructures of these species *in vivo*, determine the membrane RDs and in particular, their time-resolved light-induced reversible changes.

Experimental:

Alga cultures, growth conditions and sample preparation

The PAL mutant cells of *Synechocystis* PCC 6803 were grown photoautotrophically in BG 11 medium [13] supplemented with 5 mM HEPES NaOH (pH 7.5) at 30 °C under continuous illumination at a photon flux density of 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. *Phaeodactylum tricornutum* (1090-1a, obtained from the Culture Collection of Algae, Göttingen (SAG)) was cultivated as described in [14] at a photon flux density of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with light/dark periods of 16h/8h at 19 °C. Cells were harvested from the logarithmic (or exponential) growth phase, by centrifugation (5000 g, 5 min) and resuspended for SANS measurements in ~95% (v/v) D₂O-containing culture medium to a chlorophyll content of 200-500 $\mu\text{g/ml}$.

Thylakoid membranes were freshly isolated from market spinach. Leaves, after main ribs being removed, were homogenized in ice cold grinding medium containing 20 mM Tricine (pH 7.6), 0.4 M Sorbitol, 10 mM MgCl₂, 10 mM KCl and filtered with 6 layers of medical gauze pads. Remaining debris was removed by centrifugation (200 g, 2 min). The supernatant was centrifuged for 5 min at 4000 g; the pellet was resuspended in osmotic shock medium containing 20 mM Tricine (pH 7.6), 5 mM MgCl₂ and 5 mM KCl. This was then centrifuged for 5 min at 7000 g. The pellet obtained was washed in D₂O-containing grinding medium (pD 7.6) and centrifuged for 5 min at 7000 g. Thylakoid membranes resuspended in the same medium to a chlorophyll concentration of 1-2 mg/ml were stored at 4 °C and used within 4 h. Aliquots from the stock solutions were supplemented with 100 μM N-methyl phenazonium methosulphate (PMS) to catalyse cyclic electron transport around PSI or with 1 mM methylviologen for providing electron acceptor for the whole chain, linear electron transport; the uncouplers, nigericin and NH₄Cl were used at 2.5 μM and 4 mM concentrations, respectively. For SANS measurements, isolated thylakoid membranes were oriented with their membrane normal approximately parallel to the applied field in an electromagnet of 1.5 T field strength with the field vector perpendicular to the neutron beam. D₂O was purchased from Euriso-top, all other chemicals were obtained from Sigma-Aldrich.

SANS

Neutron scattering experiments were performed on the D22 small angle neutron scattering instrument at the Institut Laue-Langevin (ILL) in Grenoble, France, where a high flux research reactor provides a continuous neutron beam (<http://www.ill.eu/instruments-support/instruments-groups/instruments/d22/>). Neutrons thermalized in a cold source were monochromatized by a mechanical velocity selector to obtain a neutron wavelength (λ) of 6 Å ($\Delta\lambda/\lambda \approx 10\%$). Sample suspensions filled in 1 mm quartz cuvettes were mounted in a temperature controlled sample holder placed in the neutron beam, defined by a 10 mm × 6.5 mm size aperture in front of the sample. The sample temperature was maintained at 15 °C. Neutrons, scattered from the sample were detected with the aid of a position sensitive ^3He multidetector with 128×128 pixels, each pixel having a size of 8×8 mm². The sample-to-detector distance was set to 2.45 m or 8 m; the corresponding collimation distances, which determine the beam divergence, were 2.8 m and 8 m, respectively. With these instrument settings we could cover a Q-range of 0.008 – 0.2 Å⁻¹. Integration time of the data acquisition was varied between 1 s and 5 min; the data storage time was ~1 s. Pilot experiments on the Yellow Submarine SANS instrument at the Budapest Research Reactor were carried out as described in [15;16]. Additional experiments were also performed at SANS II, Paul Scherrer Institute, Switzerland.

Data Treatment

Data reduction of two dimensional scattering data (example shown in Supplementary Figure 1) recorded during the experiments was performed by the Graphical Reduction and Analysis SANS Program for MatlabTM (GRASP), developed by Dr. Charles Dewhurst at the ILL. The data counts for each pixel, n , and errors, \sqrt{n} , were normalised to the number of beam monitor counts and multiplied by a constant (standard monitor – set to be 10⁷). Correction for detector efficiency and absolute calibration was performed by normalisation to the scattering from a 1 mm thick H₂O sample. Buffer and sample holder scattering together with instrument background were measured and subtracted from the sample scattering taking the transmissions of sample and buffer/sample holder into account. The 2D scattering signal thus obtained was radially averaged along circles around the beam centre position for unoriented samples. In case of oriented samples, the data were radially averaged in 2 sectors centred around each opposite Bragg peak and with 45° opening angles.

Structural information was deduced from the radially averaged scattering curves as follows. For plant thylakoid membranes and for *Phaeodactylum tricornutum* at low [0.023 Å⁻¹, 0.053 Å⁻¹] Q, the scattering profile was fitted with the sum of a Gaussian and a power function. For the PAL mutant of *Synechocystis* PCC 6803 the scattering curve was fitted with the sum of two independent Gaussians and a power function. Peak positions of the Gaussians, Q^* , were used for determining repeat distances of the different thylakoid membrane assemblies ($RD = 2\pi/Q^*$).

Results and Discussion:

The thylakoid membranes in most cyanobacteria are arranged into concentric or radial arrays [17]. The packing density of membranes in these organisms is determined by the phycobilisomes: the distance between two adjacent membranes (the interthylakoidal

space) was found to be 460 and 40 Å, in the presence and absence of phycobilisomes, respectively - determined by electron microscopy on the wild type and a mutant unicellular cyanobacterium *Synechocystis* PCC 6803 [18]. In the wild type cells no Bragg peak could be discerned in the Q-range corresponding to a RD between 550 and 600 Å, which would be expected with the above cited interthylakoidal space and a thylakoidal contribution of about 125 Å [cf. [19], discussed below]. This is most probably because of strong forward scattering of the whole cells and the smearing effect of the hemispherically shaped membrane-anchored phycobilisomes. In contrast, the PAL mutant [20] of *Synechocystis* PCC 6803, which is devoid of phycobilisomes, exhibited a reasonably sharp Bragg peak at 0.032 \AA^{-1} (Figure 1A), corresponding to a RD of $\sim 190 \text{ \AA}$ (Table 1). The measured RD values, representing statistically averaged values, are somewhat higher than expected based on electron microscopy data: a thylakoid vesicle thickness of 125 Å plus the 40 Å interthylakoidal space [18].

In diatoms the chloroplast thylakoid membranes are loosely appressed and organised into groups of three [21;22]. Each of these membranes contains both PSII and PSI, along with their common light harvesting antenna complexes, the fucoxanthin-chlorophyll proteins (FCPs). In the diatom *Phaeodactylum tricornutum*, we obtain a RD of $\sim 170 \text{ \AA}$ (Table 1). This RD evidently requires a tight packing of membranes – given the even distribution of PSII, PSI and FCPs [21]. In plants, such an arrangement of PSII and PSI complexes would require a somewhat larger RD of $\sim 185 \text{ \AA}$: PSI protrudes about 40 Å while both PSII and its main light harvesting complexes (LHCII) are extended by about 20 Å into the interthylakoidal space; the membrane thicknesses are $\sim 40 \text{ \AA}$ each, and the luminal spacing is $\sim 45 \text{ \AA}$ [12;19]. In diatoms, however, FCPs possess considerably smaller loop segments [21;23] than LHCII - thus allowing a smaller interthylakoidal space and smaller RD. Concerning the location of the ATP synthase in diatoms, with its estimated large, $\sim 140 \text{ \AA}$ protrusion [24], it is clear that this enzyme can only be accommodated in the ‘end’ membrane, i.e. on the outer surfaces of the groups of three thylakoid membranes. This implies that the thylakoid in the middle must be energetically coupled, evidently via interconnected luminal spaces and contiguous bilayer membranes, to the two end membranes.

In the chloroplasts of higher plants, cylindrical stacks of granum thylakoids, with typical diameters of 300–600 nm and containing 5–20 thylakoids are interconnected by non-appressed, so-called stroma thylakoid membranes [25]. With this lateral heterogeneity, there are two RDs in the system, corresponding to the grana stacks and stroma lamellae, respectively. In grana, which in the stacked region contain PSII and LHCII but no PSI, RD can be small indeed, a RD_{grana} of 157 Å (with 45 Å luminal and 32 Å interthylakoidal space) has recently been determined by cryo-EM for spinach chloroplasts [19]. RD_{grana} was found to be 167 Å in dark-adapted Arabidopsis leaves after cryo-immobilization and freeze-substitution (H. Kirchhoff and Z. Reich, personal communication). In contrast, $\text{RD}_{\text{stroma}}$ must be considerably larger, due to the presence of PSI and the ATP synthase in these membrane sections. Indeed, in freshly isolated intact spinach thylakoids we find well defined Bragg peaks corresponding to RDs between 285–300 Å (Figure 1C, Table 1). These RDs can accommodate a $\sim 125 \text{ \AA}$ thick thylakoid vesicle and allow $\geq 160 \text{ \AA}$ protrusions between the lamellae. For reasons probably related to symmetry and/or lack

of contrast, the first order Bragg peak of the multilamellar grana stacks is missing – under all experimental conditions tested. (For theoretical treatment of the small angle scattering of granal thylakoid membranes, see [26].) Instead, as will be shown elsewhere, grana, as well as PSII membrane-pairs devoid of the interconnecting stroma thylakoids, i.e. pairs of adjacent stacked membranes isolated from granal chloroplasts, display characteristic scattering in the Q-range between 0.065 and 0.07 Å⁻¹.

In order to investigate if these membrane ultrastructures are stable under different conditions and in particular during the photosynthetic functions, we carried out time resolved SANS experiments. These measurements on the PAL mutant of *Synechocystis* PCC 6803 revealed, to our knowledge for the first time, rapid light-induced RD changes of the thylakoid membranes in living cyanobacterial cells (Figure 1A). The magnitude of these changes, which probably originate from variations in the surface charges during photosynthesis, is rather small (~ 10 Å), rendering them almost certainly undetectable by EM. It is also noteworthy that the changes were fully reversible in the dark. This phase was rather slow, which suggests the existence of two states, a light adapted state and a dark adapted state of the thylakoid membrane RDs. Similar membrane reorganizations are observed in *Phaeodactylum tricornutum* cells (Figure 1B), where the full reversibility is also slow (30-60 min), similar to the relaxation after the photoprotective non-photochemical quenching of the fluorescence in the antenna in the dark [27;28], and to the light-induced reorganizations in the chiral macroorganization of the pigment system detected by CD spectroscopy [29]. The structural flexibility of diatoms might be related to their well known ability to survive large fluctuations in light intensity in mixing waters [30;31]. With regard to higher plants, our studies reveal unexpectedly high structural flexibility of stroma thylakoids. Fully reversible light-induced reorganizations, observable already in 2-dimensional scattering profiles (Supplementary Figure 1), could be recorded with a time-resolution of several seconds, which affected both RD and the degree of the lamellar order - manifested in shifted peak positions and broadened and diminished Bragg peaks, respectively (Figure 1C). It is interesting to note that the light-induced changes in the RD were smaller when whole chain, instead of PSI cyclic electron transport, was operating, i.e. when PMS was replaced with methylviologen (Supplementary Figure 2). These reorganizations in plant thylakoid membranes closely resemble the light-induced shrinking of thylakoid membranes, detected earlier by electron microscopy as well as by light scattering, and which has been shown to be driven by transmembrane ΔpH generated during photosynthesis [32]. In perfect agreement with these data, we show that uncouplers eliminate the light-induced RD changes, a shrinkage, in isolated plant thylakoid membranes (Supplementary Figure 3A and B). In contrast, NH₄Cl had no significant effect on the light-induced RD change, a swelling, in the diatom *P. tricornutum* (Supplementary Figure 3C and D), showing that the light-induced transmembrane ΔpH in the two systems do not play the same role in the membrane reorganizations. While the mechanism of the light-induced membrane reorganizations in diatoms remains to be explored, it is to be noted that the macro-organization of membranes, the roles of transmembrane proton gradients generated upon illumination and via chlororespiration in the dark as well as some details of the regulatory mechanisms of non-photochemical quenching display significant differences in the two systems [33;34].

In summary, in the past decades a wealth of information has accumulated on different reorganizations which are part of the multilevel regulatory mechanisms of photosynthetic organisms and which affect the overall membrane organization [1-6;32-38]. As demonstrated in this paper, SANS allows the determination of RDs of the membrane ultrastructure in different photosynthetic organisms and the monitoring of their time - resolved reorganizations during photosynthesis, information hitherto not available. This technique is thus suitable for the fast and accurate determination of repeat distances on the entire population of multilamellar, inherently heterogeneous biological membrane systems *in vivo*. Our data provide clear evidence for the occurrence of small but well discernible membrane reorganizations during photosynthesis, which appear to be linked with basic regulatory mechanisms.

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Tables

Organism	Calculated RD
PAL mutant of <i>Synechocystis</i> PCC 6803	
batch 1	$195 \pm 1 \text{ \AA}$
batch 2	$180 \pm 1 \text{ \AA}$
<i>Phaeodactylum tricornutum</i>	
batch 1	$169 \pm 1 \text{ \AA}$
batch 2	$171 \pm 1 \text{ \AA}$
Spinach - stroma lamellae of isolated intact thylakoids	$301 \pm 1 \text{ \AA}$ (largest) $286 \pm 1 \text{ \AA}$ (smallest)

Table 1. Repeat distance (RD) values of dark adapted thylakoid membranes in living cyanobacterial (*Synechocystis*) and eukaryotic algal cells (the diatom *P. tricornutum*), and of the stroma lamellae of isolated intact spinach thylakoid membranes.

Cells and isolated thylakoid membranes were suspended in D₂O-containing reaction media. The standard errors characterize the uncertainty of the peak positions calculated from the scattering curves fitted with the sum of a Gaussian and a power function for the corresponding section of SANS (cf. Figure 1). The data on the PAL mutant and *P. tricornutum* were obtained from two different batches; variations of RD between independent measurements on the same batch were smaller than 1.5%. Independent measurements on the same batch of isolated spinach thylakoids under the same conditions yielded essentially identical values but during storage RD tended to increase by several percent.

Figure 1. Effect of illumination on the SANS profiles of thylakoid membranes in living cyanobacterial cells (A), and diatoms (B) and in suspensions of isolated plant thylakoid membranes (C).

(A) the PAL mutant of *Synechocystis* PCC 6803, (B) the diatom *Phaeodactylum tricornutum*, and (C) isolated spinach thylakoid membranes – the SANS signal of stroma lamellae. Insets, time courses of the light-induced variations in the repeat distances, calculated from the first order Bragg peaks, and their dark recovery phases. The additional peaks seen at Q values at around 0.048 \AA^{-1} (A) and 0.065 \AA^{-1} (B) are proposed to originate from paired membranes of adjacent thylakoids. The samples were illuminated for different time periods with white light of 2000 (A), 1200 (B) and 1700 (C) $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photon flux densities, as indicated by the light (light on) and dark (light off) horizontal bars.

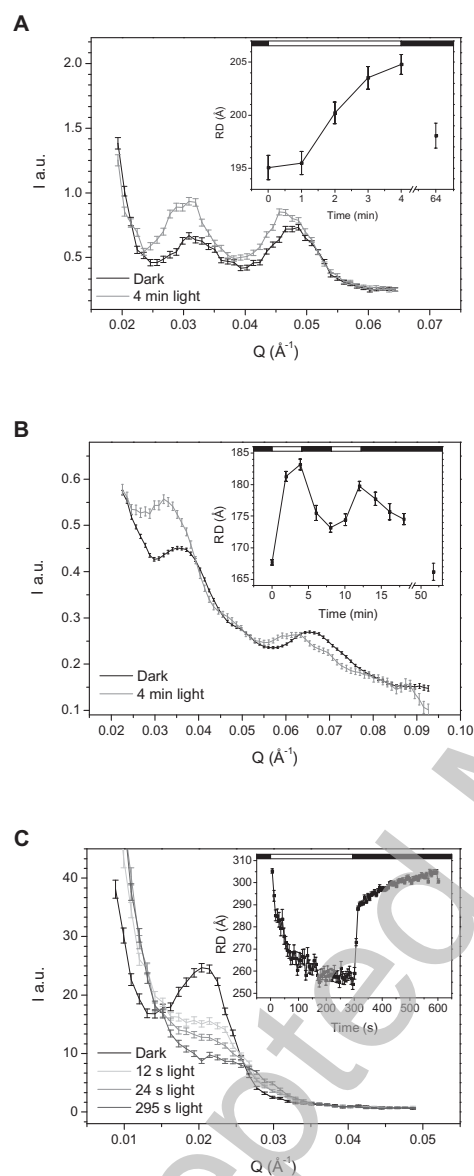


Figure 1