Biochimica et Biophysica Acta 1837 (2014) 1572–1580

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

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

R. Ünnep [a], O. Zsiros [b], K. Solymosi [c], L. Kovács [b], P.H. Lambrev [b], T. Tóth [b], R. Schweins [d], D. Posselt [e], N.K. Székely [f], L. Rosta [a], G. Nagy [a,g], G. Garab [b,*]

[a] Institute for Solid State Physics and Optics, Wigner Research Centre for Physics, Hungarian Academy of Sciences, POB 49, H-1525 Budapest, Hungary
[b] Institute of Plant Biology, Biological Research Centre, Hungarian Academy of Sciences, POB 521, H-6701 Szeged, Hungary
[c] Department of Plant Anatomy, Eötvös University, Pázmány Péter sétány 1/C, H-1117 Budapest, Hungary
[d] Institut Laue-Langevin, BP 156, F-38042, Grenoble Cedex 9, France
[e] IMFUFA, Department of Science, Systems and Models, Roskilde University, DK-4000 Roskilde, Denmark
[f] Jülich Centre for Neutron Science, Forschungszentrum Jülich GmbH, 85747 Garching, Germany
[g] Laboratory for Neutron Scattering, Paul Scherrer Institute, 5232 Villigen PSI, Switzerland

ARTICLE INFO

Article history:
Received 4 November 2013
Received in revised form 4 January 2014
Accepted 28 January 2014
Available online 4 February 2014

Keywords:
Electron microscopy
Granum
Lamellar repeat distance
Small-angle neutron scattering
Thylakoid membrane

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

Abbreviations: EM, electron microscopy; FFT, fast Fourier transform; LHCII, light-harvesting complex II; PMS, phenazine methosulphate; PSII, photosystem II; q, scattering vector; RD, repeat distance; RD_{EM}, repeat distance calculated from electron microscopy; RD_{q^*}, repeat distance calculated from SANS measurement; SANS, small-angle neutron scattering; SAXS, small-angle X-ray scattering

☆☆ This article is part of a Special Issue entitled: Photosynthesis research for sustainability: Keys to produce clean energy.

* Corresponding author. Tel.: +36 62 433131; fax: +36 62 433434.
E-mail address: garab.gyozo@brc.mta.hu (G. Garab).

http://dx.doi.org/10.1016/j.bbabio.2014.01.017
0005-2728/

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], reorganizations 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 $NaHCO_3$. 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 $CaCl_2$, 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 D_2O buffer (50 mM Tricine, 0.4 M sorbitol, 5 mM $MgCl_2$ 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 $MgCl_2$ and 5 mM KCl, and filtered through six layers of medical gauze pads. After discarding the remaining debris by centrifugation at 200 ×*g* for 2 min, the supernatant was centrifuged for 5 min at 4000 ×*g* and the pellet was resuspended in 10 ml osmotic shock medium containing 20 mM Tricine (pH 7.6), 5 mM $MgCl_2$ and 5 mM KCl. After a short, 5–10 s, osmotic shock, breaking the envelope membrane and allowing the replacement of the stroma liquid with the reaction medium, the osmolarity was returned to isotonic conditions by adding equal volume of double strength medium. This suspension was then centrifuged for 5 min at 4000 ×*g*. The pellet was washed twice in D_2O-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