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Light-harvesting complex II in monocomponent and mixed lipid–protein monolayers

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Abstract

Monomolecular layers at the air–water interface were formed directly with isolated largest light-harvesting pigment–protein complex of Photosystem II (LHC II) or out of egg yolk lecithin (EYL) liposomes containing incorporated LHC II. Pure protein monolayers showed a mean area of 1400 Å^2 per molecule at the air–water interface. Monolayers were deposited onto glass slides by means of Langmuir–Blodgett (LB) technique. Chlorophyll fluorescence of LHC II–LB and EYL–LHC II–LB films proved energetic coupling of chlorophyll *a* and *b*, thus indicating native conformation of LHC II within the monolayers. Scanning force microscopy (SFM) revealed ring-like structures formed in monocomponent protein layers as well as in mixed protein–lipid films. These results suggest that a structural arrangement of LHC II is favoured in a lipid environment but that the protein has itself a strong tendency for structural complex rearrangement in our system.

Keywords: Light-harvesting complex II; Monolayer; Langmuir–Blodgett film; Scanning force microscopy; Photosynthesis

1. Introduction

The largest light-harvesting pigment–protein complex of Photosystem II (LHC II) is an antenna protein in higher plants comprising almost half of the total pool of the main photosynthetic accessory pigment chlorophylls [1]. Embedded within thylakoid membranes, LHC II and accessory pigment–protein complexes capture light energy followed by energy transfer towards the reaction centre of Photosystem II, and seem to play an important role in photoprotection [2,3]. Excess levels of excitation energy in the thylakoid membrane is dissipated by various processes including the xanthophyll cycle. A three-dimensional structure of LHC II was determined on an atomic scale on the basis of electron crystallography [4]. The monomer of LHC II is a 60 Å thick protein, of a roughly elliptical cross-section (30×50 Å) [4]. LHC II easily forms trimers [4] and possesses a high tendency to form more complex structures such as macroaggregates [5,6]. The possible involve-

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ment of pigments of the xanthophyll cycle in such structural alterations are in discussion [7,8].

Preparation of monomolecular films by the Langmuir–Blodgett (LB) technique is a well-known technique to prepare ordered arrays of amphiphilic molecules which are currently investigated in detail by a variety of analytical techniques (for a recent review see Ref. [9]). LB films with integrated membrane proteins could serve as ideal models to investigate protein structures, protein–pigment and protein–lipid interactions in detail. Several protein complexes of the photosynthetic machinery have been integrated into LB monolayers and investigated, e.g., LB films of bacterial reaction centres on various substrates [10–14]. Protein–lipid interactions of a bacterial antenna protein [15] and isolated reaction centres of Photosystem II [16] have been studied in LB films, but plant LHC II was not reported to be prepared as a monolayer.

Scanning probe microscopic techniques (scanning force microscopy (SFM) and scanning tunnelling microscopy (STM)) allow the probing of structural details of molecular layers supported on solid supports. These techniques have been extensively used to evaluate molecular order in mono- and multilayered LB films [17–19] and proved to be an effective high-resolution method for the characterisation of thin films on the nanometer scale. Deposition processes, packing phenomena, defect structures, pinholes, phase transition behaviour, but also local frictional and elastic properties of a variety of LB films, have been measured with SFM [9,19]. Biological high-resolution SFM studies [20] of membrane proteins have been done successfully with proteins incorporated in stabilised synthetic phospholipid bilayers [21], with proteins which form two-dimensional arrays after reconstitution [22] or are highly ordered in the natural membrane [23–26].

In recent years several studies reported on structural investigations of supported photosynthetic pigment–protein complexes. Individual Photosystem II particles in plant Photosystem II membranes on mica could be detected by SFM [27], and two-dimensional crystals of Photosystem II derived from spinach thylakoids were analysed by SFM on highly-ordered pyrolytic graphite after titanium coating [28]. Yamada et al. imaged hexagonally packed photoreaction units of bacterial chromatophore membranes on glass by SFM [29]. Platinised Photosystem I reaction centres physisorbed on flat gold substrates were studied with STM [30], and Seibert showed an STM image of platinum replicas of the Photosystem II enriched granal membrane fragments [31]. Also, uncoated chloroplasts were imaged in solution by STM [32,33].

In the present work we describe a way to prepare Langmuir monolayers at the air–water interface composed of LHC II alone or from egg yolk lecithin (EYL) liposomes containing incorporated LHC II, using isopropanol as spreading reagent. LB technique was used successfully to transfer such monolayers on hydrophilic glass. Spectroscopic analysis of these defined thin films proved the structural and the pigment integrity of LHC II. Finally, an analysis of the surface structure of supported LHC II in monocomponent and mixed lipid–protein monolayers deposited onto glass was carried out with SFM for the first time.

2. Materials and methods

LHC II was isolated from 10-day-old rye leaves (*Secale cereale* L., cv. Pastar) according to the original procedure by Krupa et al. [34]. Liposomes containing 5 mg egg yolk lecithin/ml buffer (25 mM Tricine–KOH (pH 8.0)) were formed and LHC II was incorporated following the procedure described in detail previously [35]. Lipid was purchased from Sigma (St. Louis, MO, USA).

A method of LHC II and EYL deposition at the air–water interface was developed in order to avoid denaturation of protein while forming monolayers. Pure LHC II or EYL–LHC II liposomes could be successfully deposited at the air–water interface when protein or liposome suspensions were supplemented with 25% (by volume) isopropanol. Monomolecular layers were formed in a 10×40 cm Teflon trough and compressed with a speed of 2 cm/min along the longer axis. Redistilled water (distilled a third time from $KMnO_4$) was used as a subphase. Surface pressure was measured and monitored by NIMA Technology tensiometer, model PS3 (Coventry, UK). Monolayer compression, surface pressure measurements and data acquisition were controlled by our own software. The initial surface area of deposition was

4000 Å^2 per single LHC II monomer. Langmuir–Blodgett films were deposited onto glass with a speed of 0.5 cm/min at a constant surface pressure of 15 mN/m using a Lauda Filmlift, model FL-1E (Lauda, Königshofen, Germany). The round glass slides (diameter 1 cm) used for film deposition were cleaned with detergent and finally extensively washed with redistilled water, then kept overnight under vacuum ($< 10^{-2}$ mbar) in order to remove remaining impurities and to prevent further contaminants. The deposition ratio was determined to be close to 1, similarly to monolayers formed with chlorophyll and cytochrome *c* [36]. Monolayer compression and deposition was carried out at 24 ± 1°C.

Fluorescence measurements were carried out with a Shimadzu RF 5001-PC spectrometer, and electronic absorption spectra were recorded with Shimadzu 160A-PC UV-Vis spectrometer.

Scanning force microscopy (SFM) was carried out with dry samples either in contact mode on a NanoScope III (scan speeds higher than 2 Hz) or in tapping mode on a BioScope (Digital Instruments, Santa Barbara, CA, USA) with scan speeds around 1 Hz. Microfabricated monocrystalline silicon tips with force constants ranging from 0.06 to 0.17 N/m were used for contact mode imaging (from Lot, Darmstadt, Germany). Silicon tips with resonance frequencies between 315 and 375 kHz (Digital Instruments TESP, L=12.5 μm) were used for imaging in tapping mode. All SFM-images are raw data excepting correction for image levelling.

3. Results and discussion

Reconstitution of integral membrane proteins in model membranes is a common way to investigate structure–function relationships. Liposomes have been often the first choice to reintegrate isolated LHC II in lipid environment for use in spectroscopic studies, e.g. fluorescence behaviour, Raman spectroscopy and many others [8,34,35,37]. The preparation of LB films with integrated LHC II pigment–protein complexes is a powerful methodology to study photochemical processes within given geometries, protein–lipid interactions or structural organisations of the protein complex on flat, solid substrates using surface-sensitive methods like SFM.

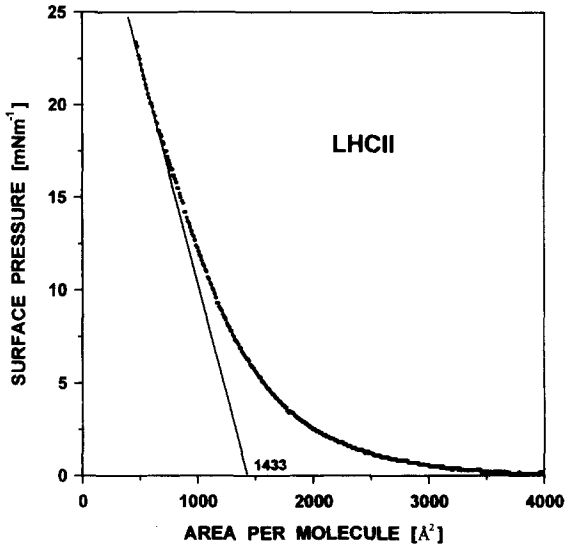

Fig. 1. Surface pressure–molecular area isotherm of a pure LHC II monolayer. Isolated LHC II suspended in 25 mM Tricine–KOH buffer (pH 8.0) supplemented with 25% (by volume) isopropanol was spread at the air–water interface and the resulting protein monolayer was compressed.

LHC II pigment–protein complexes could be spread successfully at the air–water interface using non-destructive concentration of isopropanol. This solvent was selected among several water miscible organic solvents as one which did not lead to pigment extraction and protein denaturation at relatively high concentrations required to disintegrate large LHC II aggregates. Surface pressure–molecular area isotherms as shown in Fig. 1 were obtained by compressing the monolayer at the air–water interface. The Chl *a*/Chl *b* molar ratio of 1.14 was calculated from spectrophotometric measurements of chlorophyll concentrations in our LHC II preparations and binding of 8 chlorophyll *a* (Chl *a*) molecules and 7 chlorophyll *b* (Chl *b*) molecules per monomer of LHC II was deduced, which is in accordance with Ref. [4]. The molecular area close to 1430 Å^2, as estimated from the linear increase in surface pressure (see legend to Fig. 1), corresponds well with the molecular dimensions of LHC II cross-section (roughly elliptical, 30×50 Å) obtained from crystallographic studies [4]. From this cross-section a molecular area between 1180 Å^2 (elliptical cross-section) and 1500 Å^2 (rectangular cross-section) may be expected taking into consideration molecular packing phenomena at the air–water interface. Such close

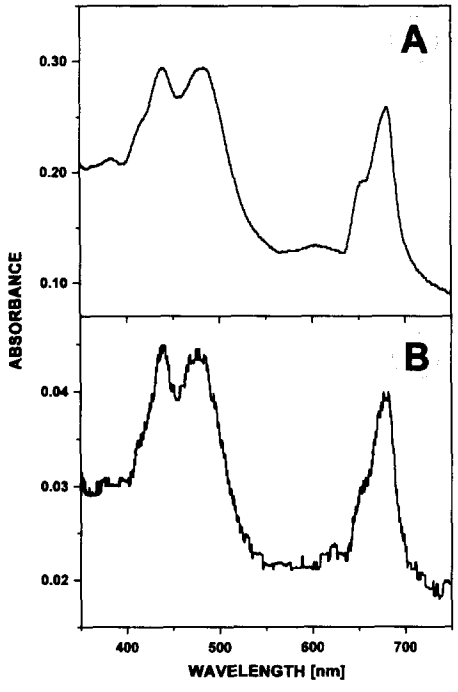

Fig. 2. Absorption spectrum of LHC II in a suspension in 25 mM Tricine–KOH buffer, pH 8.0 (A) and in a monocomponent LHC II Langmuir–Blodgett film (B). A pure protein monolayer was formed at the air–water interface (as in Fig. 1) and deposited at a surface pressure of 15 mN/m on both sides of a glass slide by the Langmuir–Blodgett technique.

correspondence of measured and expected molecular area indicates that the spreading procedure produces homogeneous protein monolayers where we can exclude the appearance of large aggregated bulk structures which could probably move into the subphase, and protein arranged in multilayers.

An absorption spectrum of LHC II in a single monolayer deposited at two sides of glass lamella is shown in Fig. 2. Except for an effect of light scattering observable in the short-wavelength region due to the monolayer (clean glass lamella recorded as a control), the spectrum of the LHC II film resembles closely that of isolated LHC II [8], i.e., no spectral changes indicative of pigment separation (protein denaturation) could be observed (see Fig. 2). Chlorophyll pigments clearly remain protein-integrated. The integrity of LHC II supported as a monolayer on glass could be proven by the observation of active excitation energy exchange between photosynthetic pigments bound to these antenna proteins. Fig. 3 presents fluorescence emission spectra of LHC II–LB excited at 440 nm and at 470 nm, the wavelengths characteristic of absorption of Chl *a* and Chl *b*, respectively. A single Chl *a* emission band and a lack of a separate short-wavelength-shifted maximum of Chl *b* indicates an efficient Chl *b*–Chl *a* excitation energy transfer. Deposition out of medium with a higher fraction of isopropanol (> 30%) than those applied in this study (25%) resulted in energetic uncoupling between chlorophyll pigments as monitored by chlorophyll fluorescence emission measurements (not shown). Both spectroscopic characterisations of protein LB films, absorption and fluorescence, prove efficient transfer of LHC II to glass lamella, preserving its photosynthetic functions active.

The same holds true for LHC II incorporated first to EYL liposomes and deposited to the air–water interface as a mixed lipid–protein monolayer. With a lipid to protein ratio of 430 in the lipid–protein system and the molecular area of EYL molecule obtained from mono-component monolayer (40 $Å^2$, not shown) one expects a mean area per one LHC II

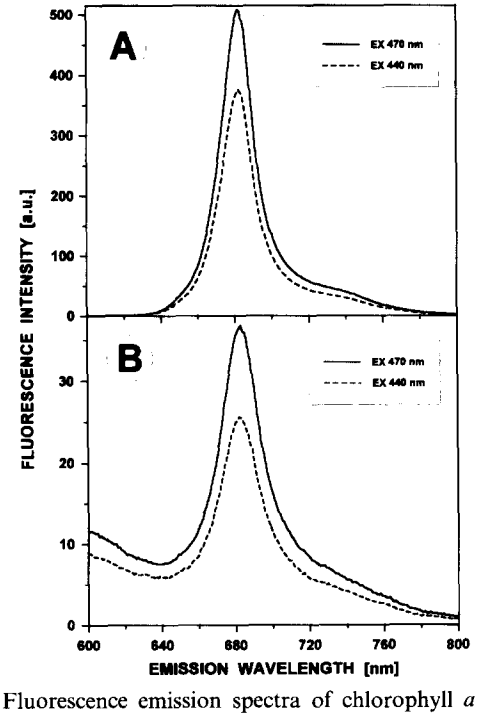

Fig. 3. Fluorescence emission spectra of chlorophyll *a* in LHC II in a suspension in 25 mM Tricine–KOH buffer, pH 8.0 (A) and in a monocomponent LHC II Langmuir–Blodgett film (B, as in Fig. 2). The pigments were excited at 440 nm (excitation of chlorophyll *a*) or at 470 nm (excitation of chlorophyll *b*). Slit widths for excitation and emission were set to 5 nm and 3 nm, respectively.

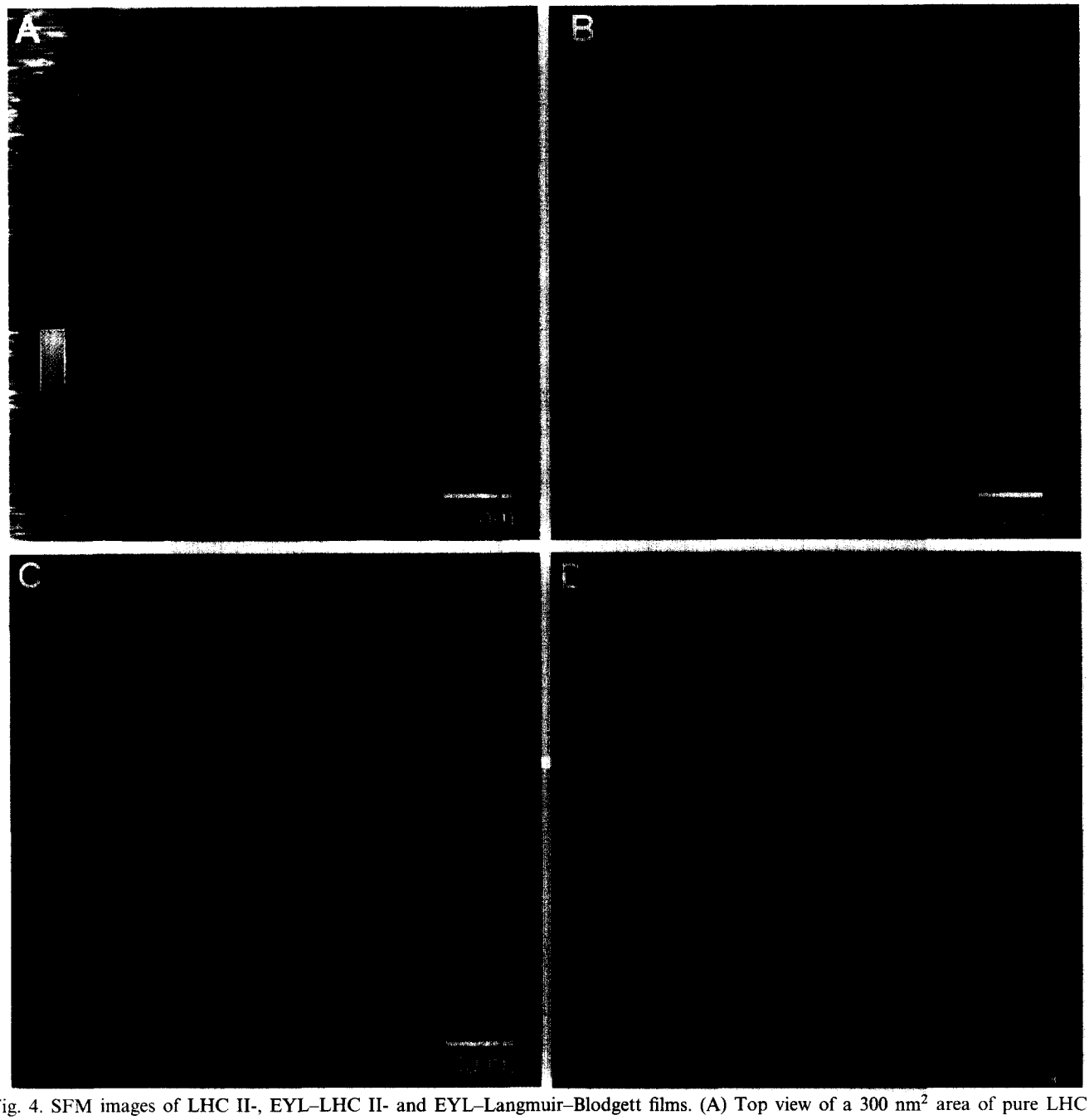

Fig. 4. SFM images of LHC II-, EYL–LHC II- and EYL–Langmuir–Blodgett films. (A) Top view of a 300 nm^2 area of pure LHC II deposited from a monolayer onto glass as obtained by tapping mode SFM. Surface topography of (B) egg yolk lecithin lipid monolayer film formed from lipid liposomes containing LHC II and (C) monolayer formed from pure lipid liposomes as obtained by SFM in contact mode. The colour bar in A indicates a z-range of 2.5 nm and is valid for B and C also. (D) Three-dimensional view of a zoomed area of EYL lipid monolayer containing LHC II as obtained by SFM in contact mode.

molecule as large as 18 630 $Å^2$ in a mixed film. Instead we have observed a reproducible value close to 15 000 $Å^2$ (not shown). This is an indication that about 27% of the molecules from the lipid matrix are not present at the air–water interface, most probably covering both hydrophobic and a second hydrophilic surfaces of a large protein (see also Fig. 1 in Ref. [35] for a discussion). Taken into account that some 40 lipid molecules per LHC II monomer remain firmly attached to the protein during the iso-

Fig. 5. (A) Top view of a small area imaged by SFM in contact mode on EYL monolayers containing LHC II. (B) A corresponding cross-section along the white line in A.

lation procedure [38], even about one-third of all lipids present in a mixed lipid–protein system do not contribute to the layer formed at the air–water interface. The structural integrity of the LHC II complex within a monolayer film, as demonstrated in Figs. 2 and 3, may also be sensitive to lipid rearrangements at the air–water interface and probably also during the deposition process (see discussion of Fig. 5 below).

Typical topographic images obtained by scanning

a given area of the supported monolayer containing LHC II alone or as lipid–protein mixture are shown in Fig. 4. SFM imaging of monocomponent LHC II LB films revealed a very flat surface with height differences of about 2.5 nm, and with randomly localised pores surrounded by ring-like structures (Fig. 4A). Best image quality was achieved with tapping mode in the case of the monocomponent film. It could be that the LB film with pure protein bearing only its own lipid layer(s) is not very suitable for the scanning process due to film instability and small lateral movements of the LHC II complex. In contrast, mixed protein—lipid monolayers were easier to image in contact mode. The very same distinctive structures as in pure LHC II LB films are found also in mixed protein–lipid monolayers (Fig. 4B) but not in pure lipid monolayers (Fig. 4C). About 50 of such ring-like structures per μm^2 are present in the protein containing lipid monolayer. Fig. 4D shows a higher magnification image of one single ring-like structure. These images are in clear contrast to the protein-free lipid monolayer where no similar structures could be detected.

The observed behaviour of the LHC II protein complex in our model system may illustrate a relevant arrangement of the membrane protein, but further studies have to clarify if our artificial model system represents directly an organisation of LHC II in the thylakoid membranes. As mentioned above, pores are clearly detectable only in monolayers containing LHC II. At present we do not fully understand the mechanisms responsible for the formation of the observed structural organisation. It is possible that LHC II by itself assembles already at the air–water interface within such kinds of superstructure. Alternatively, if the protein uniformly distributes at the air–water interface, the LHC II forms the ring-like structures only during deposition onto glass or afterwards during relaxation of the film. Support-dependent (re)organisation of LHC II in monocomponent as well as in mixed lipid–protein monolayers can be potentially involved but is specific to this protein complex. An energy minimisation process at sites, for example, of little film defects, lipid phase variations, small differences in local electric charge densities, and during the drying process may be responsible for the formation of the observed structures.

Fig. 5A presents a zoom to three individual ring-like structures observed in a mixed lipid–protein LB film. The cross-section through two ring-like structures (see Fig. 5B) reveals clearly three different height levels. One intermediate layer is indicated by the horizontal line in Fig. 5B. In a next layer, the depressions in the ring-like structures are about 2 nm deep and finally, 10 nm wide circumpherical rings, which are about 0.6 nm higher than the intermediate layer, are formed around the holes. One would have expected a flat lipid layer with half of the integral membrane protein embedded in the supported lipid monolayer and half of the protein protruding by at least one monolayer thickness. From the cross-section it emerges that the protein is submerged in lipids which may arrange in an additional layer, probably formed with lipids from the isolated LHC II complex and lipid molecules rearranging at the air–water interface (see discussion above).

Assuming that LHC II organises in our model system as round-shaped circles with internal diameters of 150–250 Å and external diameters of 300–500 Å (from SFM images, see Figs. 4 and 5), one can suggest that 50–140 LHC II monomers may be potentially involved in a formation of the structural arrangement. Despite the uncertainty of the exact cause of formation of ring-like structures (protein self-organisation/response to external conditions) within the LB films, the present study demonstrates the potential ability of LHC II for such aggregation. Organisation of LHC II into 'rings' corroborates with recent findings on its chiral nature as demonstrated by means of circular dichroism technique [39]. A ring-like molecular structure with internal pore formed by the antenna protein would provide an interesting model of organisation for Photosystem (PS) II, considering that a dimer of reaction centre with a diameter of about 200 Å [40] fits exceptionally well into the observed ring interior. Such organisation of photosynthetic units would favour excitation–energy transfer as in the case of ring-like pigment–antenna structure known from bacterial photosynthetic apparatus [41].

According to the current model of the PS II, based on electron crystallography and high resolution, single-particle electron microscopic images [42–45], the so-called PS II super complex, comprises a dimer of the main reaction centre proteins, two sets of mono-

meric minor antenna proteins and only two LHC II trimers, each associated with one reaction centre. Very recently Boekema et al. [46] reported binding of two additional LHC II trimers to the PS II dimeric super-complex. A number of 8 LHC II trimers per PS II dimer may also be deduced on the basis of statistical estimations [1], but is still below the number of calculated LHC II monomers involved in the formation of a typical ring-like structure observed in our preparations, as discussed above. This implies that for the case that such structures are formed in the thylakoid membranes, they may be rather associated with the State I–State II transition and migration of the major PS II antenna proteins within the thylakoid membranes. In fact the dimension of the LHC II lamellar aggregates reported by Garab and co-workers to be present in the photosynthetic apparatus [39,47–51] corresponds well to the structures observed in our model system. The assembly of LHC II in aggregated structures is recognised to be the important mechanism of protection against decomposition of the photosynthetic apparatus under strong illumination [6–8]. In this respect it is worth mentioning that a ring-like structure of LHC II as observed in the present work would provide conditions for both delocalisation and quenching of electronic excitation by conversion into heat and efficient thermal equilibration with the surrounding environment. In the case of the ring-like structure, the external surface is higher than in the case of a compact structure, so the thermal equilibration with the lipid matrix may be expected to be more efficient in the ring-like structure as compared with a compact aggregate. A mechanism of the thermal equilibration of the antenna proteins seems to be particularly important considering the fact that relaxation of a single excited porphyrin ring in LHC II would increase the local temperature by more than 100 K [52].

In this respect, further investigations with spectroscopic and microscopic techniques are needed to understand details of the molecular interactions responsible for the formation of specific aggregated structures of LHC II in supported lipid layers.

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