Monolayer film of phycobilisome–thylakoid membrane complexes from *Spirulina platensis*

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Abstract

Monolayer films of phycobilisome-thylakoid membrane complexes isolated from *Spirulina platensis* were prepared at air/aqueous solution interface by using the Langmuir-Blodgett technique. The film preparation was optimized with 0.5 M phosphate buffer (pH 7.0) as sub-phase at 20 °C. The monolayer was transferred into grids and into mica surface for observing the surface image of the complexes by transmission electron microscopy and atomic force microscope, respectively. The shape of complexes was disk-like with the diameter of about 50 nm and the thickness of about 35 nm. The absorption and fluorescence spectra of the complexes in the monolayer were consistent with those in buffer solution, which suggests that the complexes in the monolayer preserve the basic functional groups of photosynthetic apparatus and can be used as a model to investigate the structural connection and functional association of the light-harvesting antenna with the reaction centres.

Additional key words: absorption and fluorescence emission spectra; atomic force microscopy; cyanobacteria; Langmuir-Blodgett technique; transmission electron microscopy.

Introduction

In cyanobacteria and red algae, phycobilisomes (PBSs) attached perpendicularly to the stroma surface of the thylakoid membrane are the most important in harvesting photons for photosynthesis, while the photosystem 1 (PS1) and photosystem 2 (PS2) are the photosynthetic reaction centres where a series of photochemical processes occur (Holzwarth *et al*. 1991, Govindjee *et al*. 1995). Up to now, the crystal structures for PS1, PS2, and most of the phycobiliproteins in PBSs were determined (Schirmer *et al*. 1987, Duerring *et al*. 1990, Brejc *et al*. 1995, Stec *et al*. 1999, Jordan *et al*. 2001, Zouni *et al*. 2001). In contrast to large amount of information on PBSs and the photosystems individually, it is still not clear what is the structural connection and functional association of PBSs with the photosystems. The energy transfer efficiency from PBSs to the photosystems can be affected by temperature, state transition, *etc*. (Mullineaux *et al*. 1990, Mullineaux 1993, Sarcina *et al*. 2001). Preparation of monolayer films by the Langmuir-Blodgett (LB) technique has been applied for obtaining ordered arrays of amphiphilic molecules (Schwarts 1997). The monolayers of integrated membrane proteins have been taken as ideal models to investigate the structure and function of protein complexes (Yasuda *et al*. 1994, Fang *et al*. 1995, Kalabina *et al*. 1996, Pepe and Nicolini 1996). Several protein complexes from the photosynthetic apparatus have been integrated into monolayers, *e.g*. the light-harvesting complex 2 (Kernen *et al*. 1998, Gruszecki *et al*. 1999) and PS2 (Shao *et al*. 1999) from green plants, allophycocyanin (He *et al*. 1996) from a cyanobacterium, *etc*. The PBS-thylakoid membrane complex is an integrated and simple photosynthetic apparatus, which is composed of the PBS binding on the surface of the thylakoid membrane, and PS2 and PS1 in the thylakoid membrane. The LB film of PBS-thylakoid membrane complexes has not been reported yet.

In the present work, a method for preparing the monolayer of PBS-thylakoid membrane complexes from *Spirulina platensis* at the air-water interface is reported by using ethanol as spreading reagent. The monolayer of the complexes was prepared successfully by using the LB technique. Spectroscopic analysis of this monolayer proved the structural and functional integrity of the complexes. Finally, the surface image of monolayer of the complexes was observed with transmission electron microscope (TEM) and atomic force microscope (AFM), from which the size, the shape, and the thickness of the complexes in monolayer were determined.

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Materials and methods

Preparation of the PBS-thylakoid membrane complexes was done by the method of Brimble and Bruce (1989) with some modifications. The harvested cells were washed by the growth media and then re-suspended in the growth media to an absorbance of 1.0 at 680 nm. The cells in the suspension were ultrasonically broken in an ice-bath during 15 min and then centrifuged for 10 min at 2 000×*g* to remove the unbroken cells. The supernatant loaded onto the 11.5 cm^3 step sucrose gradient was centrifuged for 7 h in an angle head rotor (*Beckman Ti-41*) at 50 000×*g*. All sucrose solutions contained 20 mM *Tricine* (pH 8.0). A blue-green band at approx. 1.0 M sucrose containing the PBS-thylakoid membrane complexes was collected.

Preparation of monolayer of PBS-thylakoid membrane complexes: A new method for preparation of the LB films of PBS-thylakoid membrane complexes was developed. The film was prepared with a *KSV1100 LB* apparatus (*LB*, Helsinki, Finland). In order to avoid destroying the structure of the complexes, the LB films were prepared with 0.5 M phosphate buffer (pH 7.0) as the sub-phase and 3 % (v/v) ethanol–water solution as the spreading solvent. The ethanol–water solution of the complexes was spread with a micro-syringe onto the phosphate buffer sub-phase surface. The ethanol solvent was allowed to evaporate for 15 min prior to compressing, and the monolayer was stabilized at 20 mN m^{-1} for 30 min before horizontal lifting. All experiments were carried out at about 20 °C.

Absorption spectra were measured with a *UV2001* Ultra-Vis spectrophotometer (*Hitachi*, Japan) with an uncoated quartz plate as a reference. Fluorescence emission spectra were measured on a *F4500* spectrofluorometer (*Hitachi*, Japan).

Electron microscopy: TEM grids were covered with a continuous carbon film. The thickness of the carbon film was 5–10 nm. The complex monolayer at the air-water interface was transferred at surface pressure 10 mN m^{-1} by a horizontal attachment mode. The monolayer on the grid was immediately negatively stained with 1 % (m/m) UO_r-acetate. The electron micrographs were taken under low accelerating voltage (100 kV) using the *Hitachi H-800* electron microscope.

Atomic force microscopy: The monolayer was compressed to a constant surface pressure (10 mN m^{-1}), and the film was transferred onto the freshly cleaved mica plate using horizontal attachment mode. AFM images of the monolayers were recorded on a *Nanoscope III Multimode* system (*Digital Instruments*, Santa Barbara, CA, USA) with a silicon cantilever (resonance frequency 300 kHz; spring constant 35 N m^{-1}) using the tapping mode. AFM images are shown in the height mode without any image processing.

Results and discussion

The monolayers for PBS-thylakoid membrane complexes: LB is a powerful method to integrate PBS-thylakoid membrane complexes into a monolayer so that the spatial and functional association of the antenna system with the photosynthetic reaction centres can be investigated by using high-resolution technologies. The PBS-thylakoid membrane complex is an amphiphilic supermolecule system with hydrophilic PBS and lipophilic membrane which can be readily spread into monolayer. But it is crucial to look for a suitable spreading solvent in which the proteins will not be denatured and the complex will not be dissociated. In this work, ethanol was added to the solution to promote spreading by decreasing the surface tension of sub-phase. Using the ratio of A_{624}/A_{280} to characterize the degree of denaturation of PBS (Szalontai *et al*. 1987), the degree of denaturation of the proteins is shown in Fig. 1 with a series of contents of ethanol in ethanol–water solution. PBS was not seriously denatured when the volume fraction of ethanol was less than 5 %. At larger relative ethanol content A_{624}/A_{280} was less than 4, indicating PBS denaturing. Therefore, the % ethanol aqueous solution was chosen as spreading solvent.

Surface pressure-molecular area isotherms (Fig. 2) were obtained by compressing the monolayer at the air-water interface. The surface pressure can reach as much

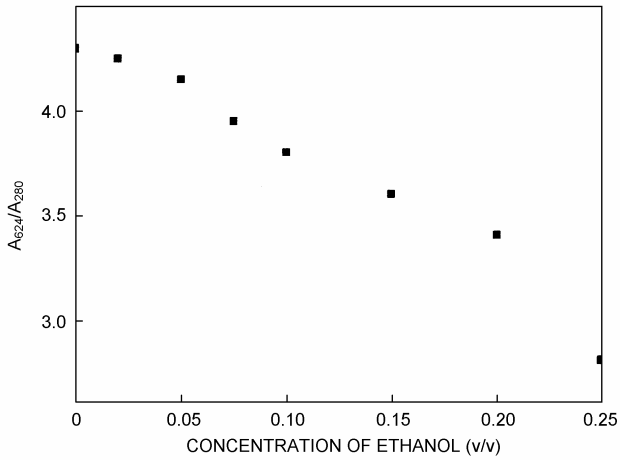

Fig. 1. Dependence of ratios of absorbance at 624 and 280 nm (A_{624}/A_{280}) on volume fractions of ethanol in ethanol–water solutions of PBS-thylakoid membrane complex.

as 40 mN m^{-1}, which indicates that the complexes possess very good film-forming property at the air-water interface. Because the molecular mass of the complexes was not determined, the molecular area of the complexes could not be obtained from the surface pressure-molecular area isotherm. The monolayer for TEM and AFM observations was prepared at 10 mN m^{-1}, which guaranteed that the complexes were organized in single layer and could be easily recognized in the film.

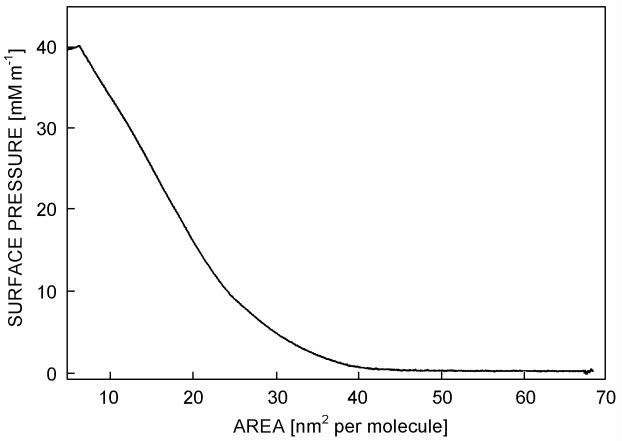

Fig. 2. Surface pressure–molecular area isotherm of monolayer of the PBS-thylakoid membrane complex. The suspension of isolated complexes in 0.5 M phosphate buffer (pH 7.0) supplemented with 3 % (by volume) ethanol was spread at the air-water interface and the protein monolayer was compressed.

Absorption and fluorescence spectra of PBS-thylakoid membrane complexes in monolayers (Fig. 3): Absorption spectra of the complexes in the monolayer deposited on both sides of quartz plate (*solid line*) and in buffer solution (*dashed line*) were measured at room temperature (Fig. 3*A*). The spectrum of the monolayer resembled closely that of the complexes in buffer solution (Li *et al*. 2001; cf. also spectra of intact cells in Venkataramanaiah *et al*. 2003). The partially resolved peaks could be assigned to chlorophyll (Chl) *a* (436 and 678 nm), carotenoids (490 nm), and phycobilisomes (624 nm). The spectral similarity confirmed that the complex was basically not dissociated and protein was not denatured (Fig. 3*A*), which was also confirmed by the excitation energy transfer between PBSs and the photosystems. Fig. 3*B* shows the fluorescence emission spectra of the complexes in monolayer (*solid line*) and in buffer solution (*dashed line*) excited at 580 nm. At this selective excitation, the partially resolved peaks could be reasonably ascribed to PBS (662 nm), PS2 (685 nm), and PS1 (720 nm). Hence the energy transfer from PBSs to the photosystems in monolayer was still as efficient as that in buffer (Fig. 3*B*). Further, when Chl *a* molecules were selectively excited at 436 nm (Fig. 3*C*), the fluorescence emission peak at 662 nm (from PBSs) implied that the uphill energy transfer from the photosystems to PBSs could also occur (Fig. 3*C*, *solid line*). The efficiencies for both downhill and uphill transfer were lower than those in buffer solution (Figs. 3*B,C*, *dashed lines*), because the PBSs are immovable in the solid film so that they lose the function to regulate the excitation energy distribution between the two photosystems (Biggins and Bruce 1989). In addition, PBSs are mobile even in physiological conditions (Mullineaux *et al*. 1997, Sarcina *et al*. 2001), while in the solid film, a PBS fixed at somewhere might be not optimally coupled with the photosystems. Anyway, the basic spectroscopic characterization of the

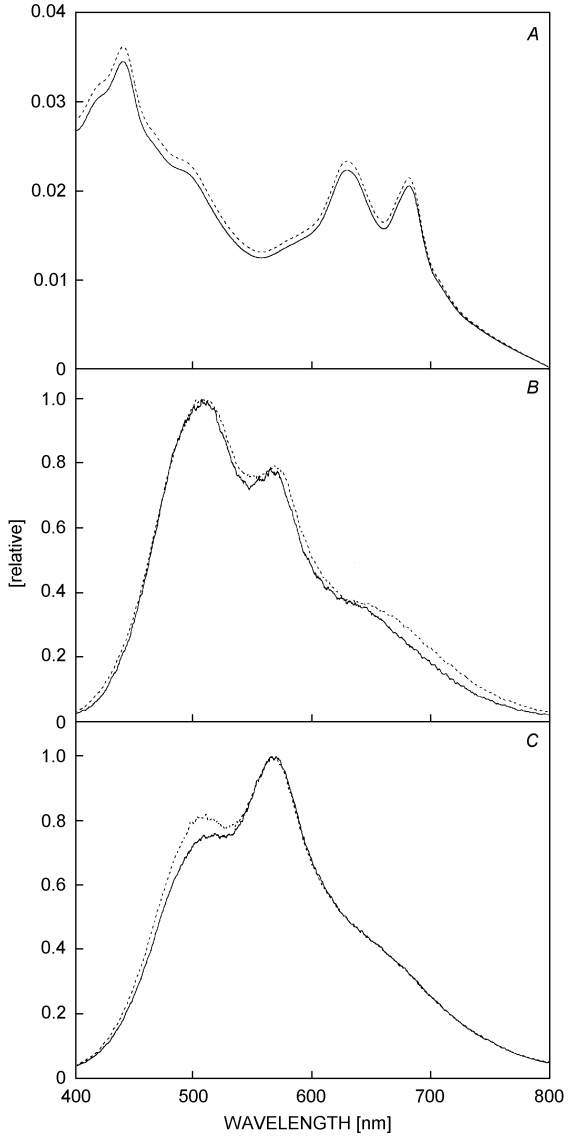

Fig. 3. Absorption (*A*) and fluorescence emission (*B, C*) spectra [relative] of PBS-thylakoid membrane complexes in monolayer (*solid line*) and in 0.5 M phosphate buffer, pH 7.0 (*dashed line*). (*A*) The monolayer was formed at the air-water interface and deposited on both sides of a quartz plate at a surface pressure of 20 mN m^{-1}. (*B, C*) The pigments were excited at 580 (*B*) (exciting PBSs) or 436 (*C*) nm and the spectra were normalized at 655 (*B*) or 680 (*C*) nm.

monolayer is consistent with those of the complexes in buffer solution, which indicates that the complexes in the monolayer preserve the basic characteristics of the photosynthetic apparatus.

The size of the dimeric PS2 is about 12 nm and that of the trimeric PS1 is about 20 nm (Boekema *et al*. 1994). According to the environment, the length of PBS can be changed from 40 to 70 nm and its height from 30 to 90 nm (MacColl *et al*. 1998). These data indicate that the length of PBS on the surface of thylakoid membrane is larger than that of PS2 and PS1, and PS2 and PS1 are imbedded in the thylakoid membrane. Therefore, only the size of the PBS can be observed under the TEM and AFM.

The TEM and AFM photographs of the monolayer of the complexes (Figs. 4 and 5) show the disk-like shape of the complexes in the monolayer with the diameter of about 50 nm and the height of 35 nm. These results indicate that the size and height of the complexes are consistent with the estimated values of the complexes (Bryant *et al*. 1979, Rosinski *et al*. 1981, Glazer 1984, Zouni *et al*. 2001). Therefore, the prepared LB film is a monolayer. However, some images of the complexes are cylindrical (see Fig. 4*A*) with the length of about 100 nm and the diameter of about 50 nm. Two or more complexes are probably aggregated into a cylinder. When the complexes were denatured, the shape of the complexes changed from the disk-like into irregular (Fig. 4*B*). In the buffer solution, the complexes were also disk-like (Fig. 4*C*) but changed into the irregular shape when they were denatured (Fig. 4*D*). Thus the shape of the biologically active complexes is disk-like either in monolayer

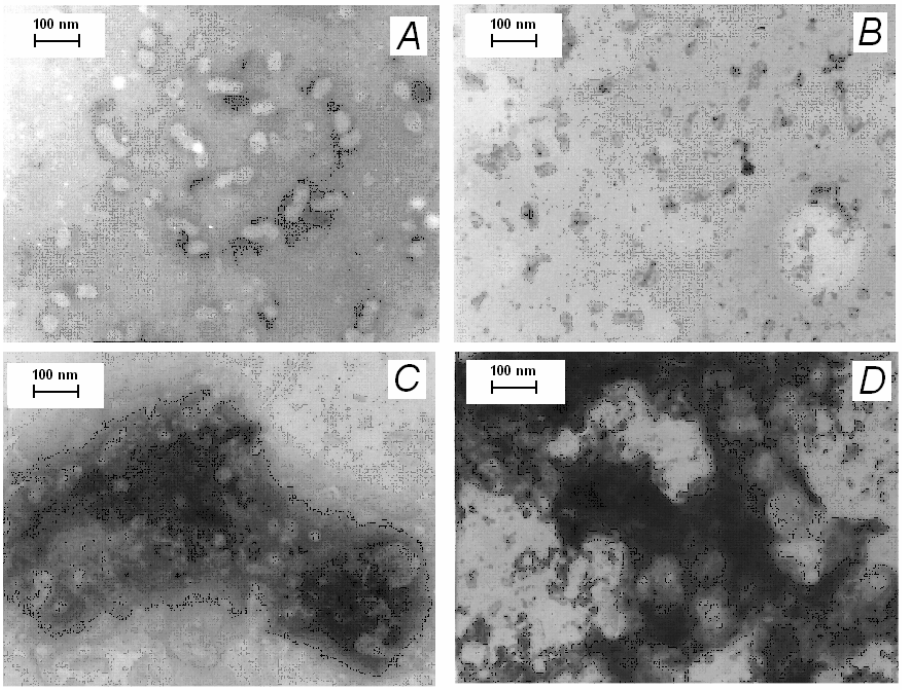

Fig. 4. Transmission electron micrographs of monolayer of the complexes transferred onto hydrophobic grid by horizontal transfer at surface pressure 10 mN m^{-1} (*scale bars* of 100 nm). (*A*) The non-denatured complexes in monolayer. (*B*) The denatured complexes in monolayer. (*C*) The non-denatured complexes in 0.5 M phosphate buffer. (*D*) The denatured complexes in 0.5 M phosphate buffer.

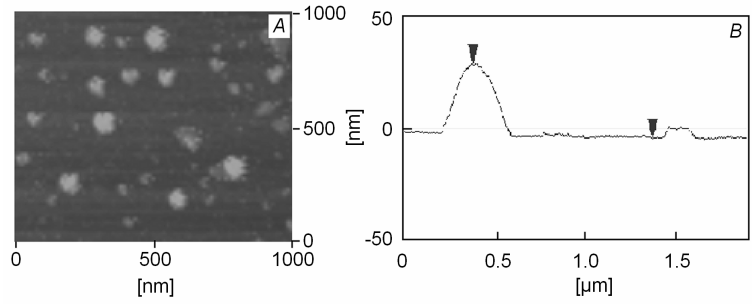

Fig. 5. AFM images of monolayer of the PBS-thylakoid membrane complexes on the freshly cleaved mica sheets (*A*) and section analysis (*B*).

or in solution, while the shape of the denatured complexes is irregular either in monolayer or in solution.

Summarizing, a monolayer of the PBS-thylakoid membrane complexes was organized at the air-water interface without using any surfactant. The TEM and AFM photographs of the monolayer demonstrated that the shape of the complexes was disk-like while that of the denatured complexes was irregular, not only in the monolayer but also in the buffer solution. The diameter and thickness of a complex in the monolayer were about 50 and 35 nm, respectively. The absorption and fluorescence spectra and the excitation energy transfer behaviour proved structural similarity of the complexes in the monolayer film to those in buffer solution, suggesting that the complexes in the monolayer preserve the basic characteristics of the photosynthetic apparatus. Overall, the monolayer of the complexes can be prepared by the reported method, and the complexes in the monolayer films can be used as a model for further study on structural connection and functional association of PBSs with the photosystems.

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