Three-dimensional architecture of phycobilisomes from *Nostoc flagelliforme* revealed by single particle electron microscopy

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Abstract Phycobilisomes are protein complexes that harvest light and transfer energy to the photo system. Here, the three dimensional structure of intact phycobilisomes from *Nostoc flagelliforme* is studied by a combination of negative stain electron microscopy and cryo-electron microscopy. Results show that the intact phycobilisomes are composed of a tricylindrical core and six rods. Each allophycocyanin cylinder presents a double-layered structure when viewed from the side and a triangular shape when viewed from the top. These characteristics indicate that allophycocyanin trimers in the intact phycobilisomes are arranged into hexameric oligomers in a parallel manner.

Keywords: Phycobilisomes; Allophycocyanins; Electron microscopy

1. Introduction

Phycobilisomes, bound to the outer surface of the thylakoid membrane, are the major light harvesting complexes of photo system II [1–4]. Phycobiliproteins and linker peptides are basic building blocks of phycobilisomes. Phycobiliproteins consist of four types: phycoerythrins, phycoerythrocyanins, phycocyanins and allophycocyanins. Linker peptides in the 27–40 kD range are divided into two categories: L\textsubscript{R}, which takes part in the assembly of rods, and L\textsubscript{RC}, which helps the attachment of rods to the adjacent core [5]. Morphologically, cyanobacterial phycobilisomes have a hemidiscoidal shape formed by different numbers of rods and a core. Although the compositions of phycobilisomes of cyanobacteria vary remarkably according to their organismal sources, the allophycocyanins in the core and the phycocyanins in rods are invariably present.

The structures of various phycobiliproteins have been determined by X-ray crystallography [6,7] and the packing mode of phycobiliproteins has been proposed [8,9]. However, the spatial arrangements of the phycobiliproteins in intact phycobilisomes have not been completely clarified. For example, it is generally believed that the functional unit of allophycocyanins is a trimer [10] and some crystal structures of allophycocyanins are trimeric [11]. However, in the crystal structure of allophycocyanins from red algae-*Porphyra Yezoensis*, two allophycocyanin trimers are associated face to face into a loose hexamer [12]. A key question now is: do the allophycocyanin hexamers exist in intact phycobilisomes? If they do, then in what way are they packed?

To address these questions, electron microscopy and single particle techniques were used for their considerable advantages in the study of protein complex structures with large molecular mass. Biochemical studies of phycobilisomes from *Nostoc flagelliforme*, a terrestrial cyanobacterium [13–15], demonstrated their simple compositions, which make them suitable for investigating the structural characteristics of phycobilisomes.

In this work, the intact phycobilisomes *Nostoc flagelliforme* were isolated and their three dimensional structures were determined from negative stain electron micrographs by random conical reconstruction. However, the missing cone resulting from this reconstruction method limits the resolution of the three dimensional structure in the z-axis. To examine the reliability of reconstruction, cryo-electron microscopy was used to collect images with azimuthal angles that cannot be achieved by negative stain microscopy. The refined structures of intact phycobilisomes were used to carefully study the spatial arrangements of the allophycocyanins.

2. Materials and methods

2.1. Phycobilisome isolation

*Nostoc flagelliforme* was collected at Sunitezuoqi, Inner Mongolia, China. The sample was rehydrated in BG-11 medium without any nitrogen source. Isolation procedures were carried out at about 20 °C as previously reported [16]. French Pressure treated cells were substituted for sonication, for better disruption.

Absorption spectra were measured with a UV-2550 spectrophotometer (Shimadzu). Low temperature (77 K) fluorescence emission spectra were measured at the excitation wavelength of 580 nm and fluorescence excitation spectra at the emission wavelength of 685 nm with a Hitachi F-4500 spectrofluorometer.

SDS–polyacrylamide gel electrophoresis was performed with 13.5% total acrylamide and 3.3% cross-linked acrylamide.

2.2. Negative stain electron microscopy

Samples of 1 mg/ml protein were loaded onto the surface of glow discharged carbon filmed grids. After removing most of the applied drop, the residual material on the film was fixed for 5 min in glutaraldehyde diluted to 1% in the same buffer and then negatively stained with 1% (w/v) potassium phosphotungstate (PTA). Samples were examined with a Philips CM 120 transmission electron microscope at

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an accelerating voltage of 100 kV under low dose mode. Electron micrographs were recorded at a magnification of 37000× on Kodak Electron Image film SO-163.

The random conical reconstruction method using SPIDER and WEB image processing packages was employed for the calculation of the three dimensional structure of phycobilisomes [17,18]. Thirty-five micrograph pairs (taken at tilt angles of 0° and 50°) were selected and digitized using an AGFA Duoscan camera system. Particles were selected from the untilted and tilted micrographs (2005 pairs). The untilted data set was rotationally and translationally aligned using a reference-free alignment algorithm [19]. The first alignment gave an average map, which was then used as the reference for a second round of alignment. A mask was constructed from the reference map, and the aligned particles were masked and analyzed by multivariance statistical analysis. The particles were classified into three clusters by hierarchical classification; the main cluster included 1510 particles. For the reconstruction, we followed the iterative reconstruction algorithm from the tilted-specimen images. Finally, the whole set of tilted specimen images were aligned to the projections of this initial model and used for six rounds of iterative back projection matching to yield the refined structures. At this step, an additional set of untilted specimen images were included in the refinement process.

3. Results and discussions

3.1. Isolation of intact phycobilisomes from Nostoc flagelliforme

The phycobilisomes from Nostoc flagelliforme were isolated by sucrose density gradient centrifugation. To examine the intactness of phycobilisomes and investigate their compositions, the absorption spectra and fluorescence spectra were measured.

The peak in the phycobilisome absorption spectrum appeared at 618 nm (Fig. 1A, left). Three further peaks were revealed by the second derivative of the absorption spectrum: the 612/625 nm maximum, which is characteristic for rod phycocyanins; the 633/644 nm maximum, which corresponds to rod-core phycocyanins; and the 656/669 nm signal, which belongs to allophycocyanins [21]. The fluorescence emission spectrum at 77 K (in Fig. 1A, right) exhibits one peak at 686 nm, indicating that all subunits are energetically linked to the terminal fluorescence transmitters—allophycocyanins and the core membrane linker peptide (LR and LRC bands) [22]. Furthermore, in the excitation spectrum at 77 K, three peaks were identified at 617, 630 and 653 nm, which agree well with the second derivative of the absorption spectrum.

SDS/PAGE analysis of phycobilisomes provides additional evidence that the isolated phycobilisomes are intact and contain only phycocyanins and allophycocyanins. In the 27–40 kD range (Fig. 1B), two linker peptides are observed in equal amounts at 29 and 34 kD, and correspond to LR and LRC, respectively, indicating that only phycocyanins and allophycocyanins exist in the isolated phycobilisomes. The presence of a 103 kD core-membrane linker polypeptide confirmed the intactness of the isolated phycobilisomes. The total mass of the intact phycobilisomes was estimated as 5–6 MDa.

3.2. Images of phycobilisomes by negative stain electron microscopy and cryo-electron microscopy

The phycobilisomes applied onto carbon film present a strongly preferred orientation because of their hemidiscoidal shape (Fig. 2A). A typical particle is characterized by a tricylindrical core with six rods radiating symmetrically from the core. Each rod consists of two domains, which correspond to two phycocyanin hexamers according to previous results. The grids were tilted by 50° to apply the random conical reconstruction (data not shown). However, direct side information of phycobilisomes could not be obtained. It is known that phycobilisomes stand upright on the thylakoid membrane in the native cell [23] and at the air–liquid interface [24] due to the hydrophobic side of the complex. Therefore, with a simulation of phycobilisomes at the air–liquid interface, images of upright phycobilisomes were directly acquired by cryo-electron microscopy. The upright phycobilisomes have a rectangular shape with dimensions of 41×12 nm (Fig. 2B). The two-dimensional average map of phycobil-
somes in the side view (Fig. 3B, left) was calculated by single particle analysis and eight high-density domains were identified in the core area. The intensities of the domains are not equal, and the inner four domains have higher intensity than the outer four domains.

3.3. Phycobilisome reconstruction

After random conical reconstruction and six cycles of refinement, the final three-dimensional map was calculated. In Fig. 3, representative class averages are shown with the corresponding reprojection of the three-dimensional map (Fig. 3A and B). Good agreement between these images is observed despite the lack of detail in the reprojection from the side view (Fig. 3B, right). The phycobilisome reconstruction at 2.8 nm resolution (Fig. 3C) reveals the phycobilisomes of length ~41 nm, width ~31 nm and height ~10 nm (Fig. 4A and B), including three triangular allophycocyanin cylinders, and six rectangular phycocyanin rods (11 × 10 nm). Sections cut through the three-dimensional structure show that the allophycocyanin cylinder has a double-layered structure from the side view (Fig. 4C) and a triangular shape with a side length of 10–11 nm from the top view (Fig. 4D).

3.4. The arrangements of allophycocyanins

To further study the arrangements of the allophycocyanins in the intact phycobilisomes, a model of the allophycocyanin cylinder was generated using the crystal structure of allophycocyanin trimers (Fig. 4E). In this model, two trimers were packed tightly face to face into a hexameric oligomer, then two hexameric oligomers were stacked loosely into a cylinder (Fig. 4F, upper). The allophycocyanin trimers were arranged in a parallel manner, resulting in 3-fold symmetry of the cylinder when viewed from the top (Fig. 4F, lower). This model exhibits striking similarity to the density map of the allophycocyanin cylinder shown in Fig. 4C and D. The height of the al-
Cylinders were aligned to form a 3-fold symmetrical core via interactions at the triangle vertex (Fig. 4 G, lower). The scale bar represents 10 nm. (B) Viewed from the side. (C) Section through the lowest rods and allophycocyanin cylinders represented as density contours. The position of this section is indicated by the short arrow and the allophycocyanin cylinder is marked by a triangle. The bar in (A); the two-layer structure in the allophycocyanin cylinder is visible. (D) A density contour representation of the central section of the reconstruction. (E) Representation of the crystal structure of allophycocyanin trimer from Porphyra Yezoensis viewed from the side (upper) and down the 3-fold axis (lower). (F) Molecular model of the allophycocyanin cylinder in which the crystal structure of allophycocyanin trimers has been stacked into a two-layered cylinder. Upper: side view of the model, where two trimers are packed face to face into a hexameric oligomer, then two hexameric oligomers are packed loosely into a cylinder. Lower: top view of the model. In this model, four allophycocyanin trimers are aligned in a parallel manner leading to the 3-fold symmetry. (G) Model of phycobilisome core. Upper: viewed from side, four high-density domains and four low-density domains were observed. Lower: viewed from the top, three allophycocyanin cylinders were aligned to form a 3-fold symmetrical core via interactions at the triangle vertex.

The structural characteristics of the phycobilisome core can be deduced from this allophycocyanin cylinder model. Three allophycocyanin cylinders are aligned into a 3-fold symmetrical core via interactions at the triangle vertex (Fig. 4G, lower). The existence of four high-density domains and four low-density domains in the model when viewed from the side (Fig. 4G, upper) correlate well with the eight unequal density domains in the average map of phycobilisomes in vitreous ice (Fig. 3B, left). The linker peptides should be involved in the allophycocyanin cylinder organization; however, their positions are still unclear at this level of resolution. Further studies are needed to determine the precise orientation and position of the linker peptides in the intact phycobilisomes.

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