Reconstitution and AFM Observation of Photosynthetic Membrane Protein Assembly in Planar Lipid Bilayers

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In purple photosynthetic bacteria, light-harvesting complex 2 (LH2) and the light harvesting-reaction center complex (LH1-RC) play the key roles of capturing and transferring light energy and subsequent charge separation. These photosynthetic apparatuses form a molecular assembly; however, how the assembly influences the efficiency of energy conversion is not yet clear. To address this issue, direct observation of the assembly at the molecular level is necessary to analyze its function. In this study, we reconstituted photosynthetic membrane proteins into artificial lipid bilayers and directly observed their assembly by AFM. The absorption spectra of the reconstituted proteins showed characteristic Qy bands of bacteriochlorophyll a that were identical to those of intact proteins. AFM observation of the reconstituted membranes revealed that LH2 and LH1-RC were successfully assembled into the lipid bilayer, and their observed structures were in good agreement with corresponding crystallographic structures. Specifically, binary proteins, i.e., LH2/LH1-RC and LH2/LH1, which form a densely packed molecular assembly, could be clearly identified at the molecular level by this method of observation. Energy transfer from LH2 to LH1-RC in a reconstituted lipid bilayer was observed by steady-state fluorescence spectroscopy. Enhanced energy transfer was confirmed in the membrane phase compared to that in a homogeneous micellar solution. Such reconstituted molecular assemblies are useful experimental platforms to investigate the relationship between supramolecular arrays and function. [DOI: 10.1380/ejssnt.2011.15]

Keywords: Atomic force microscopy; Bioimaging and engineering; Self-assembly; Biological molecules–proteins; Biological aspects of nano-structures; Photosynthetic membrane proteins; Lipid bilayer; Energy transfer

I. INTRODUCTION

In a purple photosynthetic bacterial membrane, two types of membrane protein-pigment antenna complexes, light-harvesting complex 2 (LH2) [1] and light harvesting (LH1)-reaction center (RC) complex (LH1-RC) [2], perform the transfer and capture of light energy and subsequent charge separation. These photosynthetic apparatuses form a molecular assembly; however, how the assembly influences the efficiency of energy conversion is not yet clear. To address this issue, direct observation of the assembly at the molecular level is necessary to analyze its function. In this study, we reconstituted photosynthetic membrane proteins into artificial lipid bilayers and directly observed their assembly by AFM. The absorption spectra of the reconstituted proteins showed characteristic Qy bands of bacteriochlorophyll a that were identical to those of intact proteins. AFM observation of the reconstituted membranes revealed that LH2 and LH1-RC were successfully assembled into the lipid bilayer, and their observed structures were in good agreement with corresponding crystallographic structures. Specifically, binary proteins, i.e., LH2/LH1-RC and LH2/LH1, which form a densely packed molecular assembly, could be clearly identified at the molecular level by this method of observation. Energy transfer from LH2 to LH1-RC in a reconstituted lipid bilayer was observed by steady-state fluorescence spectroscopy. Enhanced energy transfer was confirmed in the membrane phase compared to that in a homogeneous micellar solution. Such reconstituted molecular assemblies are useful experimental platforms to investigate the relationship between supramolecular arrays and function. [DOI: 10.1380/ejssnt.2011.15]

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In a purple photosynthetic bacterial membrane, two types of membrane protein-pigment antenna complexes,
molecular-level strategies for the construction of artificial photosynthetic antenna systems.

One approach to fabricate molecular assemblies is to reconstitute antenna proteins into a lipid bilayer supported on a solid substrate [8, 9]; this enables the formation of a stable protein assembly for structural and functional analyses by AFM, spectroscopic, and electrochemical measurements. For instance, observation of fluorescence resonance energy transfer and photocurrent generation of a molecular assembly consisting of LH2 and LH1-RC on a solid support (e.g., electrode) gives useful information about the mechanism of the photochemical event [10, 11]. A method for reconstituting these antenna proteins into a planar lipid bilayer, instead of using a native membrane, would be useful because the essential components can be extracted and artificially assembled to focus on structural and functional analyses.

In this study, we reconstituted these photosynthetic antenna proteins into lipid bilayers and directly observed their supramolecular assemblies by high resolution AFM. The antenna proteins, LH2, LH1-RC, and LH1, were clearly observed in the reconstituted membrane at the molecular level. The AFM images revealed the formation of a supramolecular assembly of the proteins. Enhanced energy transfer from LH2 to LH1-RC was observed in the reconstituted membrane compared with that in a homogeneous micellar solution. Such an approach using reconstitution is useful to investigate the relationship between the structure of a molecular array and its function.

II. EXPERIMENTAL

A. Materials

Unless stated otherwise, all chemicals were obtained commercially and used without further purification. Phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-rac(1'-glycerol)] (DOPG), and asolectin were purchased from Avanti Polar Lipids, Inc. and SIGMA. The surfactants used were n-octyl-β-D-glucoside (OG, DOJINDO) and N, N-dimethyldecylamine N-oxide (LDAO, Fluka). LH2 and LH1-RC were isolated from purple photosynthetic bacteria by methods reported for Rps. palustris 2.1.6 (LH2 and LH1-RC) [2], Rb. sphaeroides 2.4.1 (LH2) [12], and Rb. sphaeroides puc705BA (LH1) [13].

B. Reconstitution of photosynthetic antenna proteins into lipid bilayers

A proteoliposome suspension consisting of phospholipids (DOPG, DOPC, or asolectin) and photosynthetic membrane proteins (LH2 and/or LH1-RC (or LH1)) was prepared via dialyzing a co-micellar solution of 0.78 wt% OG in 20 mM Tris-HCl buffer (pH 8.2) containing phospholipid and protein at an appropriate molar ratio (lipid/LH2/LH1-RC (or LH1)) against a Tris-HCl solution (20 mM, pH 8.2) for 30 h. The lipid/protein(s) ratio was fixed at 500/1. For example, the ratios (lipid/LH2/LH1-RC (or LH1)) for the reconstituted samples of LH2-alone, LH1-RC-alone, LH2+LH1-RC, and LH2+LH1 were 500/1/0, 500/0/1, 1000/1/1, and 2000/3/1, respectively. The resulting proteoliposomal solution was substantially purified by a sucrose density-gradient centrifugation method [14]. In brief, the proteoliposomal solution (1 ml) was thoroughly mixed with 1 ml of sucrose/Tris-HCl solution (60% w/w) (20 mM, pH 8.2) containing 0.01% of Triton-X 100. The mixed solution was put into a 10-ml centrifugal precipitation tube, in which sucrose layers were formed with 2 ml of 20, 10, 5, and 2.5% of sucrose solutions and 0.3 ml of buffer solution. The sucrose layers were ultracentrifuged at 150,000×g for over 1 h. The fraction containing proteoliposome, ~15% of sucrose solution, was collected to use for AFM observation.

C. Absorption and fluorescence spectra

Near-infrared absorption spectra of antenna proteins in reconstituted lipid bilayers were acquired with a HITACHI U-2800 spectrometer. Steady-state fluorescence spectra were obtained using a spectrometer composed of a CCD detector (Spec-10: 100BR/LN; Roper Scientific), monochromators (SP-150M for excitation and SP-306 for emission; Acton Research Co.), and a lamp house (tungsten halogen light source, TS-428DC; Acton Research Co.). All data were obtained at room temperature, with excitation at 800 nm and with an exposure time of 10 sec.

D. AFM observation of reconstituted antenna proteins in planar lipid bilayers

The purified proteoliposomal solution was put onto a freshly cleaved mica surface followed by addition of CaCl2 solution. After ~1 h of incubation, the solution was gently replaced with milli-Q water. Images were taken with a Picoplus5500 (Molecular Imaging) or a modified-JSPM5200 (JEOL) using the acoustically oscillated mode at room temperature. Cantilevers used were BL-AC40TS (OLYMPUS) and NCH (Nano World), respectively. All images were recorded under aqueous conditions in a liquid cell.

III. RESULTS AND DISCUSSION

A. Absorption spectra of photosynthetic antenna proteins reconstituted in lipid membranes

Bacteriochlorophyll a (Bchl a) bound to the photosynthetic antenna proteins exhibits characteristic absorption bands (Qy) depending on the type of complex; this makes peak assignment and analysis easy in binary protein systems. Figure 1 shows NIR-absorption spectra of LH2 (A), LH1-RC (B), and LH2+LH1-RC (C) in micellar (dashed line, 0.1 wt% LDAO in 20 mM Tris-HCl, pH 8.2) and reconstituted liposomal (solid line, 20 mM Tris-HCl, pH 8.2) solutions. For LH2 (A), Qy bands were observed at 800 and 858 nm in the both of micellar and liposomal solutions. Similarly, for LH1-RC (B), Qy bands at 802 and 879 nm, which correspond to accessory Bchl a in RC and B880 of LH1 complexes, respectively, were observed in both micellar and liposomal solutions. These results...
indicate that LH2 and LH1-RC complexes were successfully reconstituted into lipid bilayers without denaturation. For the LH2+LH1-RC coexisting solution (C), Qy bands were observed at 800 nm, and in the broad range of 830-920 nm in both micellar and liposomal solutions, because of the coexistence of LH2 and LH1-RC. The molar ratio of LH1-RC/LH2 in the reconstituted membrane can be estimated as 0.94 by peak analysis. The molar ratio of incorporated proteins is highly proportionate to that in the co-micellar solution in the preparation of the liposomal membrane.

B. AFM observation of supramolecular assembly of antenna proteins in reconstituted planar lipid bilayers

Direct observation of the assembly at the molecular level is necessary for investigating the relationship between a supramolecular array and its function. Planar membranes on a mica substrate were formed from each reconstituted proteoliposome, enabling us to directly observe the photosynthetic antenna protein assemblies by AFM. Figure 2(A) shows an AFM image of a planar lipid bilayer without proteins as a reference. Planar bilayer patches were observed; such planar membrane patches are formed via vesicle adhesion and subsequent rupturing on the substrate [15, 16]. Figure 2(B) shows the height profile of the lipid patch along the dashed line indicated in (A). The observed height corresponds to the thickness of a single lipid bilayer (∼4 nm) whose surface is very smooth.

Figure 3 shows AFM images of LH2 and LH1-RC reconstituted in a planar membrane. The antenna proteins, LH2-only (A), LH1-RC-only (B), and LH2+LH1-RC (C), were observed as being incorporated into lipid bilayer patches. In the LH2-only membrane (A), a cluster of ring structures (d = ∼6 nm) that corresponds to the X-ray crystallographic structure of LH2 was observed (white arrows) [1]. The objects circled with dotted rings are also LH2 molecules, which were placed in a lower position within the membrane plane than those indicated by the white arrows. Such displacement of LH2 molecules has been observed when an LH2-containing vesicular membrane transforms into a planar membrane on a substrate [17]. The LH1-RC-only membrane shows a protrusion (H-subunit of RC, height from the membrane surface, h = 3-4 nm) surrounded by an ellipsoidal structure (LH1, d = ∼11 nm, h = ∼2 nm) (white arrows). These architectures were in good agreement with the corresponding crystallographic structures [2]. Some H-subunits of RCs come off during imaging by the scanning force of the tip (black arrows). In the LH2+LH1-RC coexisting membrane (C), although the resolution of the image was low, characteristic molecular clustering was observed. The height profile along the dashed line in image C is shown in Fig. 3(D). The membrane surface is marked by “m” in C and D. The clustering parts indicated by white arrows typically consist of ∼4 nm of protrusions from the membrane surface (indicated by * in C and D). This part corresponds to the H-subunit of RC in the LH1-RC molecule. In the circled areas with dotted lines in C, protrusions of ∼2 nm from the membrane surface could be assigned to LH2 molecules (marked by ** in C and D). These images suggest that the antenna proteins in the reconstituted lipid bilayers have a tendency to form a densely packed assembly similar to that in a photosynthetic bacterial membrane. Such spontaneous assembly formation is meaningful for functional analysis.

When we tried reconstitution of LH1-RC isolated from Rb. sphaeroides, an LH1-reconstituted membrane (without RC) was unexpectedly obtained. The absorption spectrum of the membrane is shown in Fig. 4(A) (dashed line), which lacks the absorption band of RC (805 nm). The AFM image of the LH1-reconstituted membrane is shown in Fig. 4(B). Ring structures (d = 10-11 nm) were clearly observed. The ring size is in good agreement with
the reported value [18]. The absorption spectra of the LH1+LH2 coexisting membrane are shown in Fig. 4(A) (solid line). The spectral shape is characteristic of the overlapped profiles of LH2 and LH1 spectra. An AFM image of the LH2+LH1 coexisting membrane (Fig. 4(C)) shows the assembly of ring structures that have two diameters (d = 6 nm and 10-11 nm). These are reasonably assignable to LH2 and LH1, respectively. Again, LH2 and LH1 also form a densely packed assembly in the membrane. The high resolution images obtained for this antenna assembly provide useful information of the structure given that each molecule is distinguishable. The apparent distribution of these proteins seems rather heterogeneous.

In this study, we used LH2s and LH1-RCs isolated from Rps. palustris and Rb. sphaeroides. The structure and function of the LH2s from these bacteria can be assumed to be identical in a broad sense. LH1-RC molecules from Rps. palustris and Rb. sphaeroides are distinctive; the former is monomeric and the latter is dimeric [6]. The LH1 complex lacking RC as shown in Figs. 4(A) and (B) exhibited a monomeric form. It has been reported that the PufX membrane protein associated with the LH1-RC complex (Rb. sphaeroides) contributes to the dimerization of the complex and that the complex without PufX is a monomer [6]. It is likely that the formation of the monomeric LH1 (Fig. 4(B)) results from spontaneous release of PufX and RC from the LH1-RC during the reconstitution process. In order to demonstrate energy transfer from LH2 to LH1-RC, hereafter, we used LH2 and LH1-RC isolated from Rps. palustris as shown in Fig. 3(C) for the sake of simplicity.

C. Intermolecular energy transfer from LH2 to LH1-RC in the reconstituted membrane

AFM images of LH2 and LH1-RC clearly exhibited assembly at the molecular level. In this membrane phase, where the antenna proteins are densely arranged as shown in Fig. 3(C) (LH2 and LH1-RC, Rps. palustris 2.1.6), one may expect distinctive energy transfer from LH2 (energy donor) to LH1-RC (energy acceptor) when compared with that in a homogeneous micellar solution. Intermolecular fluorescence resonance energy transfer from LH2 to LH1-RC was observed in micellar (Fig. 5(A)) and liposomal (Fig. 5(B)) solutions. In the case of the LH2-only membrane (black line), fluorescence from B850 was observed upon irradiation at $\lambda_{em} = 800$ nm (B800 of LH2). The LH1-RC-only membrane (gray line) showed very weak fluorescence upon excitation of the B880 band. When the LH1-RC was added into the LH2 micellar solution (spectrum colored in blue in Fig. 5(A)), fluorescence from LH2 ($\lambda_{em} = 867$ nm) was slightly suppressed, and the full-width at half maximum was broadened from 42 nm to 47 nm. This indicates that energy transfer from LH2 to LH1-RC takes place in the micellar solution. In sharp
FIG. 4: Absorption spectra (A) and AFM images of LH1 (*Rb. sphaeroides* puc705BA) (B) and LH1+LH2 (*Rb. sphaeroides* 2.4.1) reconstituted membranes (C). In (A), dashed and solid lines represent absorption spectra of reconstituted LH1-only and LH1+LH2 coexisting membranes, respectively. For (B) and (C), typical structures of LH1 and LH2 are indicated by white and black arrows, respectively. The LH1 and LH1+LH2 coexisting proteoliposome solutions were prepared by detergent removal from co-micellar solutions composed of β-OG, lipid, and protein (lipid/LH2/LH1 ratios = 500/0/1 (LH1-only) and 2000/3/1 (LH2+LH1 coexisting) (mol/mol)), followed by purification with sucrose density-gradient centrifugation. Sample preparation for AFM observation is described in detail in the text. The image was recorded under aqueous conditions (milli-Q) with the oscillation mode of AFM. Scale bars: 50 nm.

FIG. 5: Fluorescence spectra of LH2 and/or LH1-RC in micellar (A) and liposomal (B) solutions. Spectra of LH2-only, LH1-RC-only, and the LH2+LH1-RC coexisting system are indicated by black, gray, and blue lines, respectively. LH2 and LH1-RC were isolated from *Rps. palustris* 2.1.6. The molar ratios of LH1-RC/LH2 in both coexisting micellar and liposome systems were estimated to be 0.94 by peak analysis of the absorption spectrum. Spectra were acquired in buffer solution (micelle, 20 mM Tris-HCl, pH 8.2, 0.1 wt% LDAO; liposome, 20 mM Tris-HCl, pH 8.2) at room temperature. $\lambda_{ex} = 800$ nm.

contrast, when both LH2 and LH1-RC complexes were incorporated into a liposomal membrane (spectrum colored in blue in Fig. 5(B)), fluorescence from LH2 ($\lambda_{em} = 867$ nm) was significantly suppressed, and that from LH1-RC ($\lambda_{em} = 895$ nm) was prominently enhanced. This clearly shows enhanced energy transfer from LH2 to LH1-RC in the reconstituted membranes. As shown in the AFM images of the LH2/LH1-RC assembly, the antenna proteins formed densely packed clusters. This is likely responsible for the enhanced energy transfer from LH2 to LH1-RC.

Structural analysis by AFM at the molecular level provides insights that aid in understanding the relationship between the structure of the antenna protein assembly and its function, e.g., energy transfer and subsequent charge separation events.

IV. CONCLUSIONS

To address the relationship between the assembly of photosynthetic antenna proteins and their function, we reconstituted photosynthetic protein complexes LH2 and LH1-RC into planar lipid bilayers and directly observed their assembly by AFM. AFM observations revealed supramolecular assemblies of these antenna proteins at molecular level resolution. Absorption spectra showed that the photosynthetic proteins were successfully reconstituted into lipid bilayers. Enhanced energy transfer from LH2 to LH1-RC was observed in the reconstituted membrane phase. The combination of the reconstitution method and high resolution AFM observation provides useful information on the relationship between the assembly of antenna proteins and their function through spectroscopic and electrochemical analysis.

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