High-resolution AFM topographs of *Rubrivivax gelatinosus* light-harvesting complex LH2

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Light-harvesting complexes 2 (LH2) are the accessory antenna proteins in the bacterial photosynthetic apparatus and are built up of $\alpha\beta$ -heterodimers containing three bacteriochlorophylls and one carotenoid each. We have used atomic force microscopy (AFM) to investigate reconstituted LH2 from *Rubrivivax gelatinosus*, which has a C-terminal hydrophobic extension of 21 amino acids on the α -subunit. High-resolution topographs revealed a nonameric organization of the regularly packed cylindrical complexes incorporated into the membrane in both orientations. Native LH2 showed one surface which protruded by ~6 Å and one that protruded by ~14 Å from the membrane. Topographs of samples reconstituted with thermolysin-digested LH2 revealed a height reduction of the strongly protruding surface to ~9 Å, and a change of its surface appearance. These results suggested that the α -subunit of *R. gelatinosus* comprises a single transmembrane helix and an extrinsic C-terminus, and allowed the periplasmic surface to be assigned. Occasionally, large rings (~120 Å diameter) surrounded by LH2 rings were observed. Their diameter and appearance suggest the large rings to be LH1 complexes.

Keywords: atomic force microscopy/light-harvesting complex/molecular interaction/sidedness/two-dimensional crystallization

Introduction

Purple photosynthetic bacteria provide an ideal experimental system for describing the assembly and organization of photosynthetic systems. Absorption of light and its conversion into chemical energy is performed by highly organized transmembrane pigment–protein complexes: the light-harvesting complexes 2 (LH2) and 1 (LH1) and the reaction center (RC). In addition to the wealth of information from biochemistry, molecular biology and spectroscopy, structural data have advanced our understanding of the single components of the bacterial photosynthetic apparatus and have provided a model for its functional mechanism. According to this

model, the light energy initially is trapped by the peripheral antenna LH2 complexes and the excitation energy is transferred to LH1 complexes that are closely associated with the RC. The subsequent photon-induced charge separation in the RC initiates a cyclic electron transport and the formation of an electrochemical proton gradient across the membrane (Papiz *et al.*, 1996; Sundström *et al.*, 1999).

Different types of LH antenna complexes have been isolated from various species of purple bacteria and their structures solved at atomic resolution. These cylindrical complexes are assembled from small polypeptides α and β , which both span the membrane once as α -transmembrane helices. Together they form a heterodimer that binds three bacteriochlorophyll *a* pigments (Bchl) and one carotenoid. McDermott *et al.* (1995) first solved the structure of LH2 from *Rhodospseudomonas acidophila* by X-ray crystallography, revealing a membrane-spanning cylinder, formed by nine $\alpha\beta$ -heterodimers. Atomic structures from three-dimensional crystals of *Rhodospirillum molischianum* LH2 revealed an octameric arrangement (Koepeke *et al.*, 1996). Electron crystallographic data were acquired on two-dimensional crystals of LH2s from *Rhodovulum sulfidophilum* and *Rhodobacter sphaeroides*, both exhibiting a nonameric organization (Montoya *et al.*, 1995; Walz *et al.*, 1998). The structure of the LH1 complex, however, is still unknown at atomic resolution. An electron crystallographic projection map of *Rhodospirillum rubrum* LH1 at 8.5 Å showed a similar ring-like structure, in this case consisting of 16 $\alpha\beta$ -heterodimers (Karrasch *et al.*, 1995). The diameter of the LH1 complex suffices to accommodate a RC within the ring, a model confirmed by analyses of two-dimensional crystals of LH1–RC core complexes from *R. sphaeroides* (Walz and Ghosh, 1997; Stahlberg *et al.*, 1998).

In spite of the wealth of information available on the individual components of the photosynthetic apparatus of photosynthetic bacteria, their supramolecular organization in the membrane is poorly understood. Models of interaction between LH2, LH1 and the RC have been established to explain the highly efficient energy transfer of a photon trapped by an LH2 antenna complex to LH1 and finally the RC (Sundström *et al.*, 1999). The structural model for understanding light capture and transfer is a close packing of ring-like structures, with LH2 complexes surrounding the LH1–RC core complex. However, the size of the rings of the different LH complexes as well as the organization of LH1 complexes around RCs allowing an efficient quinone/quinol transfer are still under debate (Francia *et al.*, 1999; Jungas *et al.*, 1999; Frese *et al.*, 2000; Loach, 2000).

Here we present a structural study of the LH2 complex from *Rubrivivax gelatinosus*. Compared with LH2s from other species, the most distinct feature is related to the

sequence of the α -polypeptide chain, which has a C-terminal extension that is 21 amino acids longer than the C-terminus of α -subunits from *R.acidophila*. As proposed on the basis of its hydrophobicity, this extension could be folded into a second transmembrane helix, leading to an α -subunit spanning the membrane twice in a hairpin structure (Brunisholz *et al.*, 1994). Recent electron microscopy studies of *R.gelatinosus* LH2 reconstituted in two-dimensional crystals provided projection maps of negatively stained samples revealing a cylindrical assembly of *R.gelatinosus* LH2 complexes with a 9-fold symmetry, with inner and outer diameters similar to those reported from X-ray models of the nonameric *R.acidophila* LH2 (McDermott *et al.*, 1995; Ranck *et al.*, 2001). Comparison of the projection maps from two-dimensional crystals of native and truncated LH2, in which the C-terminal extension has been digested by thermolysin, did not allow any extra density to be detected inside or outside the nonameric ring. Therefore, the location of the C-terminal extension could not be identified.

We have used the atomic force microscope (AFM) (Binnig *et al.*, 1986) to measure the surface topography of LH2 of *R.gelatinosus* in a native environment and to locate the C-terminal extension. As previously reported, this method allows protein surfaces to be imaged at sub-nanometer resolution (Schabert *et al.*, 1995; Müller *et al.*, 1995; Scheuring *et al.*, 1999; Engel and Müller, 2000; Fotiadis *et al.*, 2000). Topographs of LH2 complexes had a lateral resolution of ~ 8 Å and a vertical resolution of ~ 1 Å, and confirmed the nonameric organization of the $\alpha\beta$ -heterodimers determined by electron microscopy (Ranck *et al.*, 2001). The cylindrical complexes were found to protrude strongly (~ 14 Å) on one side and weakly (~ 6 Å) on the other side of the membrane. The heights of the protruding rings provide information about the position of the LH2 cylinder with respect to the lipid bilayer. Imaging two-dimensional crystals reconstituted with digested LH2 revealed a height reduction of the strongly protruding rings to ~ 9 Å, while weakly protruding rings remained unchanged. This suggests the extrinsic localization of the C-terminal domain of the α -subunit of *R.gelatinosus* and allows the periplasmic surface to be assigned. In addition, together with LH2 nonamers (diameter ~ 50 Å), occasional large rings (diameter ~ 120 Å) were imaged. Their dimensions and appearance suggest these rings to be a minor LH1 contaminant. Although only seven large rings were found in the work presented here, they all exhibited a closed structure, in contrast to the open structure of *R.sphaeroides* LH1 complexes reported by Jungas *et al.* (1999). The AFM offers a unique possibility to address this question by studying native bacterial membranes.

Results

Rubrivivax gelatinosus LH2 protein is built up of the α - and the β -polypeptides consisting of 71 and 51 amino acids. Sequence alignment with LH2 of *R.acidophila* (using Clustal_W) and hydropathy analysis led to the topology model displayed in Figure 1A. The C-terminal extension is shown in a putative extrinsic configuration. The thermolysin cleavage site has been determined by HPLC and mass spectroscopy (Ranck *et al.*, 2001) and is indicated by

the triangle. The limited proteolysis is illustrated by the silver-stained SDS gel in Figure 1B, which was obtained after solubilization of two-dimensional crystals reconstituted from native and digested LH2. Native LH2 complexes run with an apparent mol. wt of 115 kDa, while digested complexes run at 82 kDa, reflecting the removal of 20 amino acids from each α -subunit of the complex with a concomitant change of the electrophoretic mobility. The sharp band documents the specific and effective cleavage of the protein. Figure 1C displays the absorption spectra of the native (black line) and truncated LH2 (gray line) after reconstitution into lipid bilayers. Both spectra show the characteristic absorption bands of carotenoids at 460, 488 and 517 nm, the Q_x Bchl band at 595 nm and the Q_y bands at 802 and 859 nm, indicating native Bchls and carotenoid-binding sites (see also Ranck *et al.*, 2001).

Large two-dimensional arrays of LH2 complexes were produced by detergent removal from a micellar solution containing the *N,N*-dimethyldodecylamine *N*-oxide (LDAO)-purified protein supplemented with egg phosphatidylcholine and *n*-octyl- β -D-thioglucoopyranoside (OTG), a detergent that has recently been reported to increase the size of the reconstituted two-dimensional crystals significantly (Chami *et al.*, 2001; see also Materials and methods). The use of bio-beads for controlled detergent removal allowed reconstitution of two-dimensional crystals in a few hours, avoiding any denaturation of the LH2 complexes.

Using a high ionic strength buffer (10 mM Tris-HCl pH 7.2, 150 mM KCl, 25 mM $MgCl_2$), two-dimensional crystals of native and pre-digested *R.gelatinosus* LH2 could be firmly attached to the mica AFM support (Schabert and Engel, 1994). To acquire high-resolution topographs, the buffer was exchanged carefully (10 mM Tris-HCl pH 7.2, 150 mM KCl) to achieve slightly repulsive force curves (data not shown) on the reconstituted two-dimensional crystals (Müller *et al.*, 1999). Under such conditions, the two-dimensional crystals of both sample types remained attached to the mica for hours. The height measured for the lipid bilayer (native sample 41.9 Å, digested sample 41.2 Å) and for the crystals (native sample 64.5 Å, digested sample 60.7 Å) indicated that the C-terminus is an extrinsic domain that protrudes from the membrane (see Table I and Figure 2A and B).

At higher magnification, the LH2 rings became distinct, revealing the two different sides of the transmembrane cylindrical complexes (Figure 2E). They are regularly packed in alternative orientations. Unit cells that house two rings have dimensions of $a = 82$ Å, $b = 133$ Å and $\gamma = 90^\circ$. Arrows in Figure 2E indicate defects in the crystal lattice where less protruding rings were more clearly visible than in the crystalline areas between the strongly protruding surfaces. Such areas were also identified at lower magnification (see arrows in Figure 2C and D) and allowed their height to be measured, 60.4 Å (native sample) and 57.4 Å (digested sample), as summarized in Table I.

In agreement with results from electron microscopy studies (Ranck *et al.*, 2001), high-resolution topographs (Figure 3A, C, E and G) revealed that *R.gelatinosus* shares the circular nonameric organization of LH2 subunits with *R.acidophila* (McDermott *et al.*, 1995), *R.sphaeroides* (Walz *et al.*, 1998) and *R.sulfidophilum* (Montoya *et al.*,

Fig. 1. (A) Topology model of *R. gelatinosus* LH2 (strain S1; gene sequence accession No. AF312921). The α - (consisting of 71 amino acids) and the β -polypeptide (consisting of 51 amino acids) cross the membrane once each. The boxed regions in the sequence correspond to α -helical stretches in the *R. acidophila* LH2 structure, after sequence alignment [using Clustal_W (<http://www.ch.embnet.org/software/ClustalW.html>)]; amino acids surrounded by circles in dark gray are predicted to be transmembrane [using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html)], those surrounded by circles in light gray are predicted to be α -helical [using GOR4 (http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl/page=npsa_gor4.html)]. The triangle indicates the thermolysin cleavage site on the C-terminus of the α -polypeptide. The arrow points towards the center of the nonamer in the membrane plane. (B) Silver-stained SDS–polyacrylamide 10% (w/v) gel. Columns from left to right: (1) markers at 97.4, 66.2, 42.7 and 31.0 kDa; (2) thermolysin (37 kDa); (3) crystals of native LH2, band at 115 kDa; (4) crystals of thermolysin-treated LH2, band at 82 kDa. (C) Absorption spectra of native and thermolysin-cleaved LH2 reconstituted into lipid bilayers. The bacteriochlorophyll and carotenoid absorption spectra do not change upon cleavage of the C-terminus of the α -subunit (native LH2, black line; digested LH2, gray line). The absorption spectra document the native state of the protein: arrows 1–3, carotenoid peaks at 460, 488 and 517 nm, respectively; arrow 4, Q_x peak at 595 nm; arrow 5, Q_y peak at 802 nm; arrow 6, Q_y peak at 859 nm.

Table I. LH2 dimensions measured with the AFM under physiological conditions

	Native LH2 sample		Digested LH2 sample	
	Average $\pm$ SD	<i>n</i>	Average $\pm$ SD	<i>n</i>
Overall thickness				
lipid bilayer	41.9 $\pm$ 2.5 Å	37	41.2 $\pm$ 2.5 Å	23
up-and-down 2D crystal	64.5 $\pm$ 2.8 Å	46	60.7 $\pm$ 1.6 Å	33
one-sided 2D crystal	60.4 $\pm$ 1.4 Å	11	57.4 $\pm$ 2.5 Å	81
Dimensions of large protrusions				
height (lipid–top) ^a	13.9 $\pm$ 1.7 Å	72	8.9 $\pm$ 1.4 Å	110
height (ring center–top) ^b	7.6 $\pm$ 2.0 Å	73	5.3 $\pm$ 0.9 Å	33
diameter of top ^c	49.0 $\pm$ 0.5 Å	3 ^d	49.0 $\pm$ 0.5 Å	3 ^d
volume/subunit	3297 $\pm$ 517 Å ³	^e	1765 $\pm$ 353 Å ³	^e
Dimensions of small protrusions				
height (lipid–top) ^a	5.6 $\pm$ 0.8 Å	152	5.4 $\pm$ 0.5 Å	34
height (ring center–top) ^b	5.6 $\pm$ 1.0 Å	24	5.3 $\pm$ 0.8 Å	137
diameter of top ^c	54.0 $\pm$ 0.5 Å	3 ^d	54.0 $\pm$ 0.5 Å	3 ^d
volume/subunit	1001 $\pm$ 161 Å ³	^e	952 $\pm$ 182 Å ³	^e

^aHeight difference between the top of the ring and the lipid surface.

^bHeight difference between the top and the center of the ring.

^cRing diameter measured at its top after rotational averaging.

^dAverage of three independent AFM topographs at different nominal magnifications scaled with respect to the unit cell dimensions found in electron microscopy.

^eVolumes and deviations calculated taking standard errors in height measurements and the standard deviation determined by single particle averaging (Schabert and Engel, 1994) into account.

1995). The reconstituted LH2 complexes (Figure 3A–D) protruded by 13.9 ± 1.7 Å from one side of the membrane (Table I; Figure 3A and B), but only by 5.6 ± 0.8 Å on the other side (Table I; Figure 3C and D). These lower rings

could be imaged best close to the edges of sheets (see arrows in Figure 2C), where unidirectionally inserted complexes were found to assemble into a hexagonal closest packing with unit cell dimensions of $a = b = 76$ Å

Fig. 2. Height measurements of LH2 two-dimensional crystals adsorbed to mica in buffer (10 mM Tris-HCl pH 7.2, 150 mM KCl, 25 mM MgCl₂) and imaged under physiological conditions (10 mM Tris-HCl pH 7.2, 150 mM KCl). (A) AFM topograph of double and multilayered areas which can be distinguished clearly from single layer crystals by their height (full image size, 4 μ m; full gray scale, 30 nm). (B) Section analysis along the white line in image (A). The two-dimensional crystals show a uniform height of 64.5 ± 2.8 Å ($n = 46$) (vertical scale bar: 100 Å). (C) AFM topograph of an LH2 sheet containing particles in up-and-down crystalline packing in the center and crystalline areas exposing only the lower surface on the edges (full image size, 700 nm; full gray scale, 10 nm). (D) Section analysis along the white line in image (C). The two different surface types are clearly visible. While the central region shows a characteristic section analysis for up-and-down packing with strong surface corrugation, the edge areas appear smooth and show a height of only 57 Å above the mica support (vertical scale bar: 50 Å). (E) Medium magnification image of a crystalline sheet of LH2 rings. At this magnification, the ring structure of the complexes is already clearly visible. The crystals show coherence only over small regions, and lattice displacements of half a unit cell are frequent. While the ring structure of the high side is clearly visible in the crystalline areas, the lower circles can be seen better within the crystal defects (bottom right) (full image size, 400 nm; full gray scale, 4 nm). (F) Section analysis along the white line in image (E). The height of LH2 rings can be measured directly from such section analyses of raw data (vertical scale bar: 20 Å).

and $\gamma = 60^\circ$. The membranes reconstituted in the presence of digested LH2 revealed a very similar overall organization of the protein (Figure 3E–H). The weakly protruding (5.4 ± 0.5 Å) surface showed a surface appearance identical to that of the native sample (compare Figure 3G and H with 3C and D). Relatively large areas with complexes exposing their lower surface to the tip allowed the right-handed twist of the protruding structure to be resolved (Figure 3G and H). The band shift in the gel (Figure 1B) documents a complete cleavage of the C-terminus of the α -subunit. Accordingly, a change in the strongly protruding surface was observed in the digested sample. Its height was reduced to 8.9 Å over the lipid bilayer (Figure 3E and F), and the cleaved rings had a width of 28 ± 0.5 Å (Figure 3A). Thus, the volume integral over the protruding structure changed from ~ 3300 Å³ to ~ 1800 Å³ (Table I), compatible with the loss of the C-terminus.

As displayed in Figure 3, the lower rings had a larger diameter than the higher rings. Taking unit cell dimensions determined by electron microscopy (Ranck *et al.*, 2001) for scaling the AFM data, the respective diameters were 54.0 Å for the lower ring and 49.0 Å for the higher ring (see Table I). In addition, we have determined the

ellipticity of native complexes by aligning the rings (see Figure 3A and C) with respect to an elliptical reference (see Materials and methods). The ratio b/a was 0.95 for the cytoplasmic side and 0.91 for the periplasmic side.

In membranes reconstituted from an LH2 preparation in which the hydroxyapatite column purification step was omitted (see Materials and methods), a few giant rings (diameter ~ 120 Å) were imaged at submolecular resolution. The more abundant smaller LH2 rings (diameter ~ 50 Å) in their vicinity were found to be separated from the large rings by a minimum top ring distance of 35 ± 3 Å (Figure 4). The merged rotational power spectra of 68 LH2 rings showed a strong maximum corresponding to the 9-fold symmetry (data not shown). The seven large rings from the same images, however, exhibited a much stronger intensity for 8-fold symmetry than for 9-fold symmetry, together with weak signals corresponding to 12- and 16-fold symmetries (data not shown).

Discussion

The AFM is now a powerful tool in membrane protein research. This progress is the result of improved instrumentation as well as optimized recording conditions

Fig. 3. High-resolution raw data AFM topographs and corresponding averages (all in 15° tilted representation and corresponding heights). Averages were calculated after a single particle alignment and 9-fold symmetrized. (A) Raw data topography of the strongly (~14 Å) protruding surface of the native LH2 complex. The 9-fold symmetry is visible in the raw data. (B) Average of image (A) (full gray scale: 14 Å). (C) Raw data topography of the weakly (5.6 Å) protruding surface of the native LH2 complex. (D) Average of image (C) (full gray scale: 6 Å). (E) Raw data topography of the strongly (~9 Å) protruding surface of the digested LH2 complex. (F) Average of image (E). The loss of protruding structure compared with (B) localizes the C-terminal position of the α -subunit (full gray scale: 9 Å). (G) Raw data topography of the weakly (5.4 Å) protruding surface of the digested LH2 complex. (H) Average of image (G). The proteolysis had no influence on the topography of the weakly protruding surface (full gray scale: 6 Å). Raw data are displayed with a full gray scale corresponding to 20 Å. Scale bars represent 100 Å in the raw data and 20 Å in the averages.

(Engel and Müller, 2000). The AFM allows information to be acquired on the membrane-protruding structure of single proteins. Such information is difficult to obtain with cryo-electron microscopy where the membrane-embedded parts are preserved, but connecting loops and protruding termini are often distorted. Furthermore, the heights of membranes and loops can be measured accurately in buffer solution with the AFM (Müller *et al.*, 1999), poorly ordered single particles can be recognized and imaged at high resolution (<10 Å) (Scheuring *et al.*, 1999; Fotiadis *et al.*, 2000; Seelert *et al.*, 2000) and sidedness assignments can be obtained directly from raw data (Scheuring *et al.*, 1999).

LH2 complexes are rings of $\alpha\beta$ -heterodimers, each subunit crossing the plasma membrane of the photosynthetic bacteria once. For *R. acidophila* LH2, the α -polypeptide contains a perpendicular transmembrane α -helical segment, which constitutes the inner surface of the ring, while the β -polypeptide is a transmembrane helix that is tilted by ~15° and forms the periphery of the cylinder (McDermott *et al.*, 1995). The LH2 of *R. gelatinosus* carries a large C-terminus on the α -polypeptide of ~35 amino acids (half of the polypeptide), with a hydrophobic extension of 21 amino acids (Figure 1).

High-resolution AFM topographs of reconstituted arrays of LH2 complexes revealed the nonameric organization of the LH2 rings (Figure 3). This finding disagrees with a previous report that suggested a heptameric LH2 complex based on ultracentrifugation experiments of solubilized LH2 rings and single-particle negative stain electron microscopy (Jirsakova *et al.*, 1996), but it corroborates a recent electron crystallographic study of the same two-dimensional crystals (Ranck *et al.*, 2001). Projection maps of negatively stained samples showed the ring-like nonameric organization of the $\alpha\beta$ -heterodimers,

Fig. 4. (A and B) Raw data AFM topographs showing an ~120 Å ring surrounded by LH2 nonamers revealing top ring distances between the complexes of ~35 Å (see Discussion; scale bar, 100 Å; full gray scale, 20 Å).

with outer and inner diameters of ~66 and 30 Å, respectively (Ranck *et al.*, 2001). However, these projection maps of negatively stained samples did not show major differences between digested and undigested LH2 complexes. In contrast, topographs of membranes packed with native and digested LH2 recorded by AFM revealed a thermolysin-induced change of height and surface appearance of the strongly protruding surface. This allowed an unambiguous identification of the extrinsic C-terminus of the α -polypeptide (Figure 3), ruling out the model of a buried transmembrane C-terminus (Brunisholz *et al.*, 1994). Thus, the surface protruding ~14 Å from the membrane represents the periplasmic side, while the cytoplasmic surface housing the N-termini protrudes by only ~6 Å. The latter height defines the position of the LH2 complex within the bilayer. The volume change of the periplasmic protrusions from ~3300 Å³ to ~1800 Å³ (Figure 3; Table I) induced by thermolysin corresponds to a calculated change of 10 amino acids. This is far fewer than the 20 amino acids removed (Figure 1A and B), but the result can be explained by the flexibility of the uncleaved C-terminus protruding far out of the membrane surface (14 Å). In addition, the height (9 Å) and width (28 Å) of the digested periplasmic ring suggest that the extrinsic helical segment, predicted to have a length of 15 Å (Figure 1A), lies on the membrane surface.

The strongly protruding periplasmic surface that is thought to comprise the 35 C-terminal amino acids of the α -polypeptide and six C-terminal amino acids of the β -polypeptide (Figure 1) has a smaller diameter

(~49 Å) than the cytoplasmic protrusions (~54 Å) housing 11 N-terminal amino acids of the α -polypeptide and 14 N-terminal amino acids of the β -polypeptide (Table I). Since the α -subunits face the inner diameter of the LH2 rings in *R.acidophila* (McDermott *et al.*, 1995), the more protruding structure is expected to form a narrower cylinder, corroborating the sidedness assignment based on proteolytic cleavage.

Spectroscopic studies have indicated that LH2 rings are flexible, assuming elliptical configurations with a *b/a* ratio of 0.85 (Van Oijen *et al.*, 1999). Because crystallographic packing could stabilize the cylindrical complexes, we have analyzed the ellipticity of loosely packed LH2 complexes. A small deviation from cylindrical symmetry was indeed observed. However, the values found for the *b/a* ratio (0.95 on the cytoplasmic side and 0.91 on the periplasmic side) are significantly smaller than that found from spectroscopic analysis.

The high signal-to-noise ratio of AFM topographs allows single non-ordered proteins to be imaged at submolecular resolution (Scheuring *et al.*, 1999; Fotiadis *et al.*, 2000; Seelert *et al.*, 2000). Figure 4A and B shows rings with a diameter of ~120 Å, which are in close contact with several LH2 rings exhibiting diameters of ~50 Å. Top ring contact distances between large and LH2 rings are ~35 Å. Based on the agreement of their appearance and their diameter with that of LH1 (Karrasch *et al.*, 1995), we speculate that the large rings are minor LH1 contaminants.

In conclusion, we have imaged the surfaces of the nonameric complexes from *R.gelatinosus* LH2 with the AFM and identified the periplasmic surface with the C-terminus of the α -polypeptide. The high signal-to-noise ratio of images acquired with the AFM allowed recognition of different protein complexes that work together in the native membrane. As a next step in understanding the photosynthetic apparatus, native membranes will be imaged directly with the AFM under physiological conditions. This will resolve questions related to the oligomerization states of different LH complexes *in situ* and to the open/closed configuration of the LH1 rings.

Materials and methods

Materials

All phospholipids were of the highest purity and were purchased from Avanti Polar Lipids. LDAO (30% solution) was from Fluka and OTG was from Sigma. Thermolysin was purchased from Boehringer. Bio-Beads SM2 (25–50 mesh) from Bio-Rad were washed extensively with methanol and water before use as described (Levy *et al.*, 1990). All other reagents were of analytical grade.

Isolation, purification and proteolysis of LH2 complex

The light-harvesting LH2 complex was isolated from photosynthetically grown *R.gelatinosus* cells (strain S1) essentially as described previously (Jirsakova *et al.*, 1996), with slight modifications (Ranck *et al.*, 2001). Briefly, solubilization of the broken cells with LDAO was followed by two successive chromatographic purifications on DEAE–Sephacrose FF and Sepharose CL-6B (Pharmacia) columns. Further purification was achieved by chromatography on a hydroxyapatite (Biosepra) column eluted in a buffer containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA and 0.1% LDAO.

A limited digestion by thermolysin (4 h incubation time at 22°C, enzyme/LH2 molar ratio = 20) was performed on purified LH2 in LDAO solution, and its effect was analyzed by denaturing SDS–PAGE and by MALDI–TOF mass spectroscopy as described elsewhere (Ranck *et al.*, 2001).

Biochemical and biophysical techniques

Protein concentration was determined from the absorption at 854 nm using $\epsilon = 382 \text{ mM}^{-1}\text{cm}^{-1}$ (Sturgis *et al.*, 1995) and values of 12 530 and 10 933 Da for the molecular weights of the native and the thermolysin-cleaved LH2, respectively (Ranck *et al.*, 2001). Absorption spectra of the vesicles were recorded on a Cary 2300 spectrophotometer.

Vesicles containing native or cleaved LH2 were analyzed by SDS–gel electrophoresis on silver-stained 10% acrylamide gels under oxidizing conditions.

Reconstitution and two-dimensional crystallization

Two-dimensional crystallization of native and truncated LH2 complexes was performed as described previously (Chami *et al.*, 2001; Ranck *et al.*, 2001). Briefly, purified LH2 complexes were diluted to ~0.5 mg/ml in a buffer containing 10 mM Tris–HCl pH 8.0, 400 mM NaCl, 0.1% LDAO and supplemented with 20 mM OTG. Then egg phosphatidylcholine was added at lipid to protein ratios ranging from 0.3 to 0.5 (w/w) and the micellar solution allowed to equilibrate for ~1 h in the dark at room temperature. Detergent removal was performed through three successive additions of 5 mg of SM2 Bio-Beads for 1 h each, according to the batch procedure previously described by Rigaud *et al.* (1998). After 3 h of stirring in the presence of polystyrene beads at room temperature, the reconstituted material was kept at 4°C for AFM analysis.

Atomic force microscopy

Mica prepared as described by Schabert and Engel (1994) was used as support and freshly cleaved before every experiment using scotch tape. To check the cleavage quality, the mica was imaged in 30–50 μl of adsorption buffer (10 mM Tris–HCl pH 7.2, 150 mM KCl, 25 mM MgCl_2). Subsequently, 1 μl of protein crystal solution (0.1 mg/ml) was injected into the adsorption buffer drop on the mica surface. After 2 h, the sample was rinsed carefully with recording buffer (10 mM Tris–HCl pH 7.2, 150 mM KCl). The recording buffer was optimized to achieve high resolution as described (Müller *et al.*, 1999). Imaging was performed with a commercial Nanoscope III AFM (from Digital Instruments, Santa Barbara, CA) equipped with a 120 μm scanner (J-scanner) and oxide-sharpened Si_3N_4 cantilevers with a length of 100 μm ($k = 0.1 \text{ N/m}$; Olympus Ltd., Tokyo, Japan). The sharpest tips adequate for high-resolution imaging were cleaned in 1% SDS and subsequently washed three times in distilled water. The AFM was operated in contact mode applying forces below 0.2 nN at a scan frequency of 4–6 Hz. The instrument was calibrated using layered crystals of MoTe_2 as described previously (Müller and Engel, 1997).

Image processing

AFM images were calculated as 15° tilted surface representations using Image SXM (Figures 3A, C, E and G, and 4A and B). Rings were aligned translationally and angularly and averaged by a single particle averaging protocol using the SEMPER image processing system (Figure 3B, D, F and H; Saxton *et al.*, 1979). In order to determine the ellipticity of the LH2 complexes, an average topograph was calculated and artificially stretched into an ellipse, which was used as a reference for single particle averaging. Subsequently, the top distances of the long and short axes of the protein ellipsoid were measured.

Volumes in $\text{\AA}^3$ were calculated directly using *x*, *y* and *z* dimensions of AFM topograph averages; amino acid numbers were deduced using $1.35 \text{ \AA}^3/\text{Da}$ for average protein density in secondary protein structure, and 110 Da as average molecular weight of one amino acid.

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