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**Determination of the topography and biometry of chlorosomes by atomic force microscopy**

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*Key words:* atomic force microscopy, biometry, chlorosomes, freeze-drying, green sulfur bacteria, topography, transmission electron microscopy

**Abstract**

Isolated chlorosomes of several species of filamentous anoxygenic phototrophic bacteria (FAPB) and green sulfur bacteria (GSB) were examined by atomic force microscopy (AFM) to characterize their topography and biometry. Chlorosomes of *Chloroflexus aurantiacus*, *Chloronema* sp., and *Chlorobium* (*Chl.*) *tepidum* exhibited a smooth surface, whereas those of *Chl. phaeobacteroides* and *Chl. vibrioforme* showed a rough one. The potential artifactual nature of the two types of surfaces, which may have arisen because of sample manipulation or AFM processing, was ruled out when AFM images and transmission electron micrographs were compared. The difference in surface texture might be associated with the specific lipid and polypeptide composition of the chlorosomal envelope. The study of three-dimensional AFM images also provides information about the size and shape of individual chlorosomes. Chlorosomal volumes ranged from ca. 35 000 nm3 to 247 000 nm3 for *Chl. vibrioforme* and *Chl. phaeobacteroides*, respectively. The mean height was about 25 nm for all the species studied, except *Chl. vibrioforme,* which showed a height of only 14 nm, suggesting that GSB have 1–2 layers of bacteriochlorophyll (BChl) rods and GFB have ~4. Moreover, the average number of BChl molecules per chlorosome was estimated according to models of BChl rod organisation. These calculations yielded upper limits ranging from 34000 BChl molecules in *Chl. vibrioforme* to 240000 in *Chl. phaeobacteroides,* values that greatly surpass those conventionally accepted.

*Abbreviations:* AFM – atomic force microscopy; BChl – bacteriochlorophyll; *Chl*. – *Chlorobium*; *Cfl.* – *Chloroflexus*; FAPB– filamentous anoxygenic phototrophic bacteria; GSB–

green sulfur bacteria; TEM– transmission electron microscopy, Csm– chlorosome; F and S chlorosomes– chlorosomes from FAPB and GSB, respectively.

**Introduction**

Chlorosomes are unique among all the lightharvesting complexes described in photosynthetic organisms (Blankenship et al. 1995; Olson 1998). Unlike the antenna complexes in higher plants (Kühlbrandt et al. 1994), purple bacteria (McDermott et al. 1995) or dinoflagellates (Hoffmann et al. 1996), chlorosomes of green photosynthetic bacteria do not need a protein scaffold to organize the pigment molecules (Holzwarth et al. 1990). In turn, their chlorosomal antennae are composed of densely packed bacteriochlorophyll (BChl) molecules, which form rod elements by specific pigment–pigment interations. These BChl rods are surrounded by a glycolipid monolayer of monogalactosyl diglyceride, which also contains a few small polypeptides – the so-called Csm proteins – (Holo et al. 1985; Chung and Bryant 1996). In cells, chlorosomes are observed by transmission electron microscopy (TEM) as bodies attached to the inner face of the cytoplasmic membrane (Oelze and Golecki 1995). Atomic force microscopy (AFM) is a scanning probe technique that can resolve the surface of biological and non-biological samples with a resolution of 0.5–1.0 nm. Since the invention of AFM (Binnig et al., 1986), this technique has proven to be very powerful and has been used to study three-dimensional (3D) images of biomolecules (Möller et al. 1999; Engel and Müller 2000; Seelert et al. 2000), protein dynamics (Viani et al. 2000) and metabolite biosynthesis (Schneider et al. 1999). With respect to 3D images, AFM has a great advantage over other

techniques such as NMR or X-ray crystallography. The latter produce high-resolution atomic data, but require relatively large amounts of pure analyte in a particular solution or crystalline state. NMR has already provided a few models to describe the rod-like organization of BChl molecules inside chlorosomes (Mizoguchi et al. 1996); however, this technique fails when an overall view of chlorosomal topography is required. In addition, unlike other pigment–protein antenna complexes, the high lipophilic nature of chlorosomes hampers any attempt to crystallise these structures. AFM is, therefore, the most suitable technique to study 3D images of chlorosomes. This technique has already been used to report changes in the shape of chlorosomes of *Chloroflexus* (*Cfl.*) *aurantiacus* when exposed to hexanol (Zhu et al. 1995). Here we examined the topography and biometry of chlorosomes of five species of green anoxygenic photosynthetic bacteria that cover all phylogenetic and physiological groups. Two distinct types of chlorosomal surface, smooth and rough, were observed by AFM and freeze drying for TEM. The linear dimensions and volume of individual chlorosomes were also measured by AFM. The data allowed the estimation of the number of BChl molecules and layers of BChl rods inside the chlorosomes.

## Materials and methods

### *Growth conditions of selected organisms and isolation of chlorosome*

We selected five representative species of all the phylogenetic and physiological groups of green photosynthetic bacteria (Table 1). All green sulfur bacteria (GSB) strains were grown using Pfennig mineral media (Trüper and Pfennig 1992), in the presence of either sulfide or thiosulfate under saturating light conditions (>50 $\mu$mols of photons m−2 s−1). The growth medium for *Chloronema* sp. consisted of mineral medium (Trüper and Pfennig 1992) supplemented with ammonium acetate (2 mM), propionate (1 mM), $\alpha$- ketoglutaric acid (1 mM), yeast extract (0.5 g l−1) and casaminoacids (0.1 g l−1). *Cfl. aurantiacus* was grown in PE medium (Hanada et al. 1995). Cells were harvested by centrifugation at the beginning of the stationary phase. Chlorosomes were isolated on a sucrose gradient prepared in 50 mMTris- HCl pH 8.0 and 2 M NaSCN (Arellano et al. 2000), followed by flotation sucrose gradients prepared with the same buffer to further purification (Steensgaard et al. 1997). Fresh fractions were immediately processed for freeze-drying and stored at –80 ◦C until use. *Atomic force microscopy* All sampleswere diluted and immediately poured onto freshly cleaved muscovite mica discs in 5 $\mu$l aliquots. The discs were then dried under argon in a vacuum chamber. The samples were imaged under room air conditions with a Nanoscope III MultiMode (Digital Instruments). To minimize sample damage by the tip, we used the TappingModeTM technique (Zhong et al. 1993; Zhu et al. 1995). A stiff 125 $\mu$m silicon cantilever (spring constant of 20–40 N m−1) with an integral tip oscillated near its resonance frequency of about 300 kHz. The damping of the amplitude was monitored with an optical laser beam and detector system. Images were obtained and analysed using the Nanoscope III DI software. AFM scanners are calibrated in the factory. However, a second calibration is usually performed to avoid problems with

accurate measurements. First of all the device is calibrated using a gold grid with a repetitive pattern of 1 × 1 $\mu m^2$. Later, for higher resolutions, calibration is done using monocristals of known net parameters. In AFM, mica is used as calibration sample with a net parameter of 5.2 Å. 'Z' direction is also calibrated by measuring the height of the monoatomic step of the 10 Å mica. For measurements above 10 nm, the error due to the lack of this second fine recalibration is negligible.

*Table 1.* Main characteristics of the species of green photosynthetic bacteria under study

| Characteristic | *Cfl. aurantiacus* | *Chloronema* sp. | *Chl. tepidum* | *Chl. phaeobacteroides* | *Chl. vibrioforme* |
|---|---|---|---|---|---|
| Strain | J-10-fl | UdG 9001 | ATCC49652 | CL1401 | NCIB8327 |
| Phylogenetic affiliation [a] | FAPB | FAPB | GSB | GSB | GSB |
| Physiological traits | thermophile mixotroph | thermophile mixotroph | mesophile photoautotroph | mesophile photoautotroph | mesophile photoautotroph |
| BChl | *c* | *c* + *d* | *c* | *e* | *d* |
| Carotenoids | *β*-carotene | chlorobactene | chlorobactene | isorenieratene | chlorobactene |
| Cell shape | filamentous | filamentous | rod | rod | vibrio |
| Colour | orange-brown | green | green | brown | green |
| Optimal temperature (°C) | 55 | 20 | 45 | 25 | 25 |
| NaCl requirements | – | – | – | – | 2% |
| Electron donor | organic matter | 0.5 mM $H_2S$ | 5 mM $Na_2S_2O_3$ | 2 mM $H_2S$ | 5 mM $Na_2S_2O_3$ |

[a]FAPB – filamentous anoxygenic phototrophic bacteria; GSB – green sulfur bacteria.

*Freeze-drying for transmission electron microscopy*

Freshly isolated chlorosomes were attached to coverslips and cryofixed by immersion in liquid propane at −190 °C. Each frozen coverslip was transferred to a freeze-etch unit (BAF-060, Baltec, Liechtenstein) and freeze-dried at −90 °C at $10^{-7}$ mbar for 90 min. The chlorosomal surface was shadowed in a rotary motion with 1 nm of platinum at an angle of 23°. Replicas were then reinforced with 10 nm of carbon at an angle of 75°, removed

from the coverslips in 30% hydrofluoric acid and digested in bleach for 12 h. Finally, replicas were repeatedly washed in distilled water before being mounted on formvar-coated grids and observed in a Hitachi 800 transmission electron microscope.

## Results and discussion

### *Topography*

Chlorosomes of green photosynthetic bacteria are oblong or ellipsoid bodies (Oelze and Golecki 1995 and references therein). Although changes in the shape and size of these antenna complexes greatly depend on the species and growth conditions, no previous study has reported that chlorosomes could also have distinct surface topographies. Zhu et al. (1995) reported that native chlorosomes of *Cfl. aurantiacus* appeared as smooth bodies under AFM. Similar results have been obtained in this study for chlorosomes isolated from *Cfl. aurantiacus*, *Chloronema* sp. (Figures 1A and 1C) and *Chl. tepidum* (Figure 2A). However, those from *Chl. phaeobacteroides* and *Chl. vibrioforme* exhibited a rough surface with protrusions along the whole surface (Figures 2C and 2E).

There are two possible explanations for the two types of chlorosomal surface: (i) they are artefacts generated during chlorosome isolation or during AFM analysis, or (ii) the chlorosomal surface differs among species because of intrinsic variations in lipid and Csm protein composition. The isolation of chlorosomes was identical for all the species studied; moreover, the chaotropic agent NaSCN was present in the isolation buffers, thereby

increasing chlorosomal stability (Gerola and Olson 1986). It is, therefore, highly unlikely that the same isolation method have different effects on the chlorosomal surface of the five species of green photosynthetic bacteria studied. Several authors have reported that AFM can deform soft biological samples (Czajkowsky et al. 2000; Boulbitch 2000). Since the use of different tapping forces provides information about the compressibility and the extent of damage that can be caused to a sample (Hansma et al. 1997), we tested whether the tapping tip could alter the chlorosomal surface. After a series of tests using different AFM force settings, no changes in chlorosomal topography or height were observed. We, therefore, conclude that deformations caused by the tip in Tapping Mode (TM) AFM were negligible.

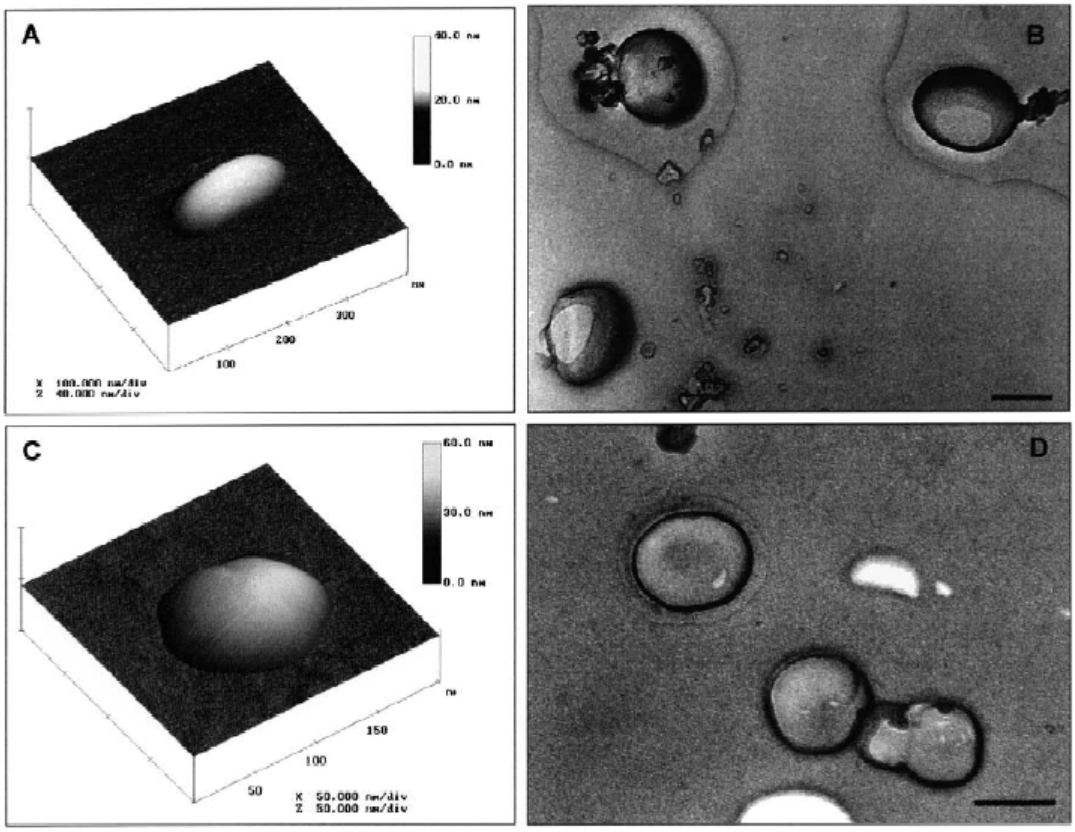


*Figure 1.* Chlorosomes of FAPB representatives imaged by TM-AFM (A, C) and freeze drying (B, D). (A and B) chlorosomes of *Cfl. aurantiacus*; (C and D) chlorosomes of *Chloronema* sp. Bars: 100 nm.

The rough surface of chlorosomes of *Chl. phaeobacteroides* and *Chl. vibrioforme* could also be explained by the shrinking of dehydrated chlorosomes. In AFM contact modesoft biological samples have been reported to collapse when dehydrated (Czajkowsky et al. 2000). To test this, freeze-dried replicas of chlorosomes observed by TEM were compared with those observed under AFM. Comparison of these images has already been used in discussions about the effect of these techniques on the surface of some crystallised biological complexes (Walz et al. 1996; Fotiadis et al. 1998; Scheuring et al. 2000).Comparison of these images has already been used in discussions about the effect of these techniques on the surface of some crystallised biological complexes (Walz et al. 1996; Fotiadis et al. 1998; Scheuring et al. 2000). Our results show that dehydration did not change the surface in either technique, since both TEM and AFM images showed chlorosomeswith one or the other type of surface, independently of the technique (Figures 1 and 2).

It is plausible that the difference in chlorosomal surface is due to the intrinsic nature of the chlorosomes. Although little information is available concerning the precise biochemical composition of the chlorosomal monolayer in green photosynthetic bacteria, lipid composition clearly differs between species (Schmidt 1985; Schmidt et al. 1980; Holo et al. 1985). Likewise, chlorosomes of several species of *Chlorobium* and *Chloroflexus* have a different Csm polypeptide composition (Stolz et al. 1990; Cahill and Stolz 1995; Chung and Bryant 1996; Foidl et al. 1997). Furthermore, distinct arrangements of BChl rods inside the chlorosome matrix could also explain changes in the chlorosomal surface. Examination of

chlorosomes of *Chl. limicola* and *Cfl. aurantiacus* by freeze-fracture and freeze-etching TEM (Staehelin et al. 1978; Staehelin et al. 1980) suggests that rod elements fill the chlorosome, with an orientation nearly parallel to its long axis. From such an organization, a smooth surface is conceivable and our results in *Cfl. aurantiacus* and *Chl. tepidum* support this hypothesis.

However, the protrusions observed in the chlorosomes of mesophile GSB could suggest a different arrangement of BChl rods inside the chlorosome; however, further studies are needed to confirm this hypothesis.

*Table 2.* Biometry of chlorosomes by AFM and estimated number of BChls per chlorosome, assuming single and double ring of BChls per rod in FAPB and GSB, respectively (Holzwarth and Schaffner 1994; Prokhorenko et al. 2000; Steensgaard et al. 2000). See text for details.

| Organism | $n$[a] | Width (nm) | Length (nm) | Height (nm) | L/W[b] | Volume ($\times 10^3$ nm$^3$) | BChls/Csm |
|---|---|---|---|---|---|---|---|
| *Cfl. aurantiacus* | 7 | 97 ± 25 | 166 ± 37 | 24 ± 5 | 1.7 | 152 ± 86 | 198 000 |
| *Chloronema* sp. | 10 | 82 ± 12 | 98 ± 11 | 24 ± 4 | 1.2 | 79 ± 44 | 103 000 |
| *Chl. tepidum* | 8 | 104 ± 14 | 194 ± 20 | 26 ± 4 | 1.9 | 165 ± 47 | 157 000 |
| *Chl. phaeobacteroides* | 18 | 101 ± 23 | 209 ± 36 | 29 ± 6 | 2.1 | 247 ± 82 | 235 000 |
| *Chl. vibrioforme* | 5 | 51 ± 12 | 150 ± 3 | 14 ± 2 | 2.9 | 35 ± 16 | 33 000 |

[a] Number of chlorosomes measured for averaging.
[b] Length to width ratio.
Csm – chlorosome.

*Biometry*

Antenna size is an important issue in the study of green photosynthetic bacteria. The determination of the pigment loading capacity of chlorosomes is controversial and the number of rods per chlorosome is yet to be elucidated. Some authors have estimated chlorosomal size using pigment-based calculations, such as the BChl *c* to BChl *a* ratio (Feick et al. 1982) or dynamic light scattering (Wang et al. 1995).

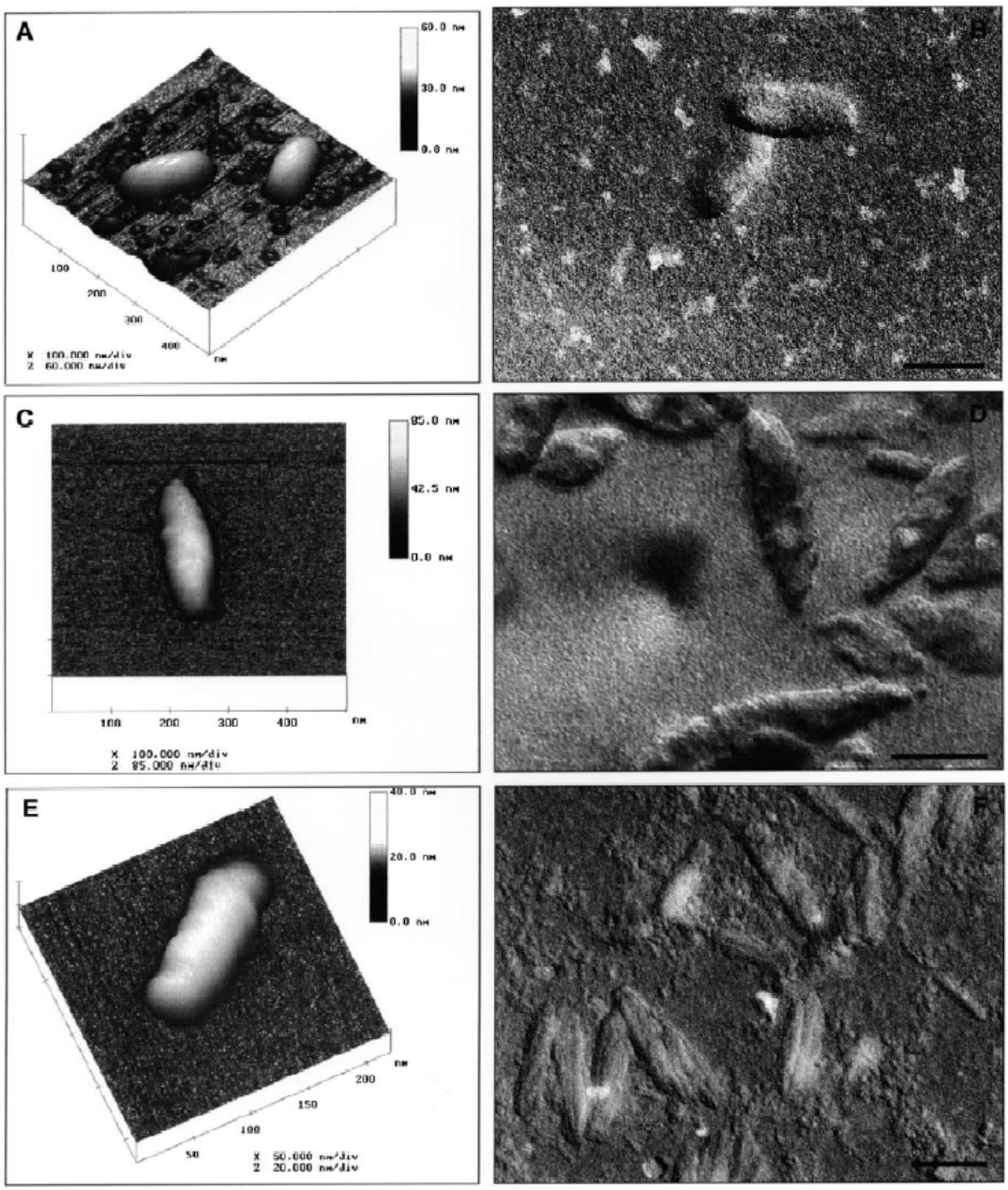


*Figure 2.* Chlorosomes of GSB representatives imaged by TM-AFM (A, C and E) and freeze drying (B, D and F). (A, B) chlorosomes of *Chl. tepidum*; (C, D) chlorosomes of *Chl. phaeobacteroides*; (E, F) chlorosomes of *Chl. vibrioforme*. Bars: 100 nm.

The biometry of chlorosomes has traditionally relied on direct measurements by TEM. Chlorosomal width and length have usually been determined from negative staining and/or freeze-fracture and freeze-etching micrographs, whereas height has been measured in ultrathin sections of chemically fixed cells (Golecki and Oelze 1987; Foidl et al. 1997; Arellano et al. 2001). Because chlorosomes are ellipsoids or oblongs, their volume has been estimated using the corresponding formula according to the linear dimensions measured by TEM (Golecki and Oelze 1987) Our results (Table 2) show that the AFM-determined linear dimensions were larger than those obtained by TEM (Blankenship et al. 1995; Olson 1998). The interaction of chlorosomes with the mica substrate might cause some deformation of chlorosomes although the comparison of AFM values with TEM measurements did not confirm this possibility (data not shown). Chlorosomes from GFB (F-chlorosomes) have been reported to be approximately 100 nm, 20–40 nm, 10–20 nm (length, width and height, respectively) using TEM. Similar dimensions were reported by Zhu et al. (1995) using AFM in tapping mode. However, we found that chlorosomes of *Cfl. aurantiacus* were ellipsoid and were larger in all three dimensions (166 nm, 97 nm, 24 nm), also using TEM (data not shown), and that chlorosomes of *Chloronema* sp. were oblong and had a similar length and width (L/W 1.2). AFM also yielded higher width and length values for chlorosomes of GSB (S-chlorosomes) than those obtained by TEM (70–180 nm, 30–60 nm) (see Olson 1998). The height of most F- and S-chlorosomeswas very similar (24–29 nm), except for *Chl. vibrioforme*, which never exceeded 16 nm and showed a constant L/W ratio of approximately 3. The heights observed suggest that the number of

stacking layers of BChl rods varies from 4 in FAPB to 1–2 in GSB, accepting that the diameter of BChl rods are 5.2 nm and 10 nm, respectively (Olson 1998).

New models for BChl organization in F- and Schlorosomes have been recently proposed (Prokorenko et al. 2000; Steensgaard et al. 2000). In these models, the rods in F-chlorosomes consist of a tubular micelle of 18 stacks of BChl whereas rods in S-chlorosomes form a double tube consisting of 20 and 30 stacks for inner and outer BChl rings, respectively (V. Prokhorenko, personal communication). The distance between chlorins (Mg-Mg atoms) was assumed to be 6.5 Å and 6.7 Å for FAPB and GSB, respectively. The number of BChl molecules per F- or S-chlorosomes was calculated according to these data and AFM-measured volumes (Table 2). The resulting numbers for chlorosome packaging greatly exceed the values reported previously (Blankenship et al. 1995; Olson 1998).

In conclusion, AFM has proven to be a useful technique to examine the topography of chlorosomes and to accurately determine their linear dimensions and volume. It has revealed two distinct chlorosomal surfaces, smooth and rough, which are most probably associated with the intrinsic properties of the chlorosomes than with changes caused by the scanning technique itself.

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