Direct Observation of Protein Secondary Structure in Gas Vesicles by Atomic Force Microscopy

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ABSTRACT The protein that forms the gas vesicle in the cyanobacterium *Anabaena flos-aquae* has been imaged by atomic force microscopy (AFM) under liquid at room temperature. The protein constitutes "ribs" which, stacked together, form the hollow cylindrical tube and conical end caps of the gas vesicle. By operating the microscope in deflection mode, it has been possible to achieve sub-nanometer resolution of the rib structure. The lateral spacing of the ribs was found to be 4.6 ± 0.1 nm. At higher resolution the ribs are observed to consist of pairs of lines at an angle of ~55° to the rib axis, with a repeat distance between each line of 0.57 ± 0.05 nm along the rib axis. These observed dimensions and periodicities are consistent with those determined from previous x-ray diffraction studies, indicating that the protein is arranged in β-chains crossing the rib at an angle of 55° to the rib axis. The AFM results confirm the x-ray data and represent the first direct images of a β-sheet protein secondary structure using this technique. The orientation of the GvpA protein component of the structure and the extent of this protein across the ribs have been established for the first time.

INTRODUCTION

Gas vesicle protein and structure

Gas vesicles are hollow structures that provide buoyancy in various aquatic microorganisms. The gas vesicles in cyanobacteria have the form of hollow cylindrical tubes closed at each end by a hollow conical end cap; both the central cylinder and the end caps are formed from ribs, which are either turns of a shallow spiral or stacks of hoops (Walsby, 1972). Chemical analysis of gas vesicles has revealed that only protein is present. Two main types of gas vesicle protein (Gvp) have been identified in the cyanobacterium *Anabaena flos-aquae*. The major protein, GvpA, is known to form the ribs; it accounts for about 90% of the mass in gas vesicles. The complete amino acid sequence of *Anabaena* GvpA indicates a highly hydrophobic protein of 70 amino acids and M_r 7397 Da (Hayes et al., 1986). The minor constituent, GvpC, is a larger, more hydrophilic protein of M_r 21,985 Da (Hayes et al., 1988). It can be removed by rinsing the gas vesicles with the detergent sodium dodecyl sulfate (Walsby and Hayes, 1988) or with 6 M urea (Hayes et al., 1992). The gas vesicles remain intact without collapsing when GvpC is removed, and it is therefore concluded that GvpC is located on the outside of the ribbed structure formed by GvpA (Walsby and Hayes, 1988).

X-ray crystallographic studies on partially oriented gas vesicles indicate that the ribs of the structure have a width of 4.57 nm and a thickness through the wall of 1.95 nm, and that they contain a subunit that repeats at intervals of 1.15 nm along the rib axis (Blaurock and Walsby, 1976). The volume of this unit cell, 10.25 nm^3, corresponds with that of a protein of approximately 7.5 kDa and therefore clearly corresponds with that of GvpA.

Atomic force microscopy

AFM has been applied to the study of biological materials at different levels of structural organization. Images of living cells (Häberle et al., 1992), metaphase chromosomes (Rasch et al., 1993), single DNA molecules (Murray et al., 1993), and crystals of amino acids (Manne et al., 1993) have been obtained. Previous AFM investigations of bacterial wall protein have indicated the usefulness of the technique in determining large-scale surface features (Blackford et al., 1991), the three-dimensional relationship of structural components (Southam et al., 1993), and defect structure (Devaud et al., 1992); in revealing interactions between surface proteins and antibody molecules (Mulhern et al., 1992; Ohnesorge et al., 1992); and in imaging the two-dimensional structure of the hexagonally packed intermediate layer of *Deinococcus radiourans* (Schabert et al., 1992). Whereas most attempts to observe protein secondary structure using scanning probe microscopy have not been successful, scanning tunneling microscopy of a two-dimensional crystal layer of poly(γ-benzyl-L-glutamate) has revealed details of the α-helical backbone (McMaster et al., 1990; Breen and Flynn, 1992).

AFM of individual proteins adsorbed on surfaces has been hampered by the mobility of the absorbed molecules and their inherent flexibility. A locally rigid protein system will have greater stability and provide a means for assessing the resolution limits of the AFM for biological systems. We have imaged the surface structure of the gas vesicle at high resolution and correlated the data with information from previous structural studies.

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MATERIALS AND METHODS

Gas vesicle preparation

Gas vesicles were isolated from the strain of *Anabaena flos-aquae* CCAP 1403/13f used in previous studies. The gas vesicles were released from cells lysed by osmotic shrinkage in 0.5 M sucrose, and concentrated by repeated centrifugally accelerated flotation. The gas vesicles, purified as described previously (Walsby and Buckland, 1969), were filtered through a Sartorius membrane filter of pore size 1.0 μm and then dialyzed for 2 days at 5°C against distilled water.

Atomic force microscopy imaging

For investigation by AFM, 10-μl samples of gas vesicles were then sprayed onto freshly cleaved mica from a distance of 18 cm. The suspending water rapidly evaporated, leaving the gas vesicles mounted on the surface. (The same purification and spray process was used for complementary TEM imaging. The TEM used was a Jeol 1200-EX operating at 80 kV.) The AFM used was a Nanoscope III Multimode microscope (Digital Instruments, Santa Barbara, CA). All of the AFM images were obtained using 200-μm-long cantilevers with unmodified integrated pyramidal tips and a nominal force constant of 0.06 N m^{-1} (Digital Instruments). The general distribution of vesicles was checked using the large-area J-scanner (~140 μm scan area). For all the images presented here the A-scanner (maximum scan area 1.07 μm) was used. The sample was imaged under propanol (spectrophotometric grade; Aldrich) to minimize the contact force between the probe tip and the sample (Weisenhorn et al., 1992) and to reduce possible surface disruption by the probe. This technique has been successfully employed in previous AFM studies of biomolecules (Hansma et al., 1992). Propanol was not observed to have any effect on the gas vesicle structure. Although the gas vesicle is a rigid structure (Walsby, 1982) able to withstand the application of hydrostatic pressure of several bars with little diminution in volume, at a certain critical pressure the cylindrical structure collapses to a flattened envelope (Walsby, 1991). The gas vesicle also collapses during spray deposition, probably under pressure generated by surface tension as the drops dry.

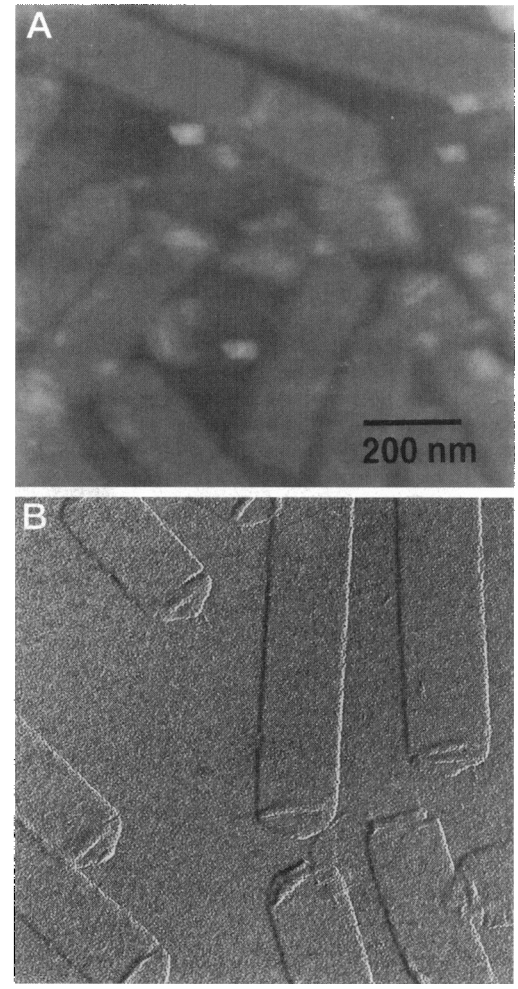

FIGURE 1 AFM and TEM images of gas vesicles at approximately the same magnification. (*a*) AFM image of a spread of gas vesicles in which the data were collected in the constant force mode. The imaging force was 1 nN. (*b*) TEM image of gas vesicles prepared in the same way, and then Pt-C shadowed.

RESULTS AND DISCUSSION

Fig. 1 *a* shows a typical distribution of gas vesicles after spray deposition as imaged by AFM. It can be seen from Fig. 1 that the structure of the flattened cylindrical tube and conical end-cap was preserved. A few of the gas vesicles are fractured and some of them are folded. A typical TEM result (Fig. 1 *b*) shows evidence of the ribbed structure perpendicular to the long axis of the gas vesicle.

The usual AFM mode for investigating biological structures is the constant force mode, in which feedback control is used to keep the cantilever deflection (and thus force) constant. An alternative mode, which has rarely been used for the study of biological samples, is the deflection mode (Putman et al., 1992; Henderson et al., 1992). In this mode the *z*-height of the sample piezo is constant, and variations in the deflection of the cantilever are recorded as a function of position. The height information, or image contrast, in the deflection or error signal mode image represents a deflection transient as the tip encounters a topographical or frictional change. The flat surface of an intact gas vesicle should be an ideal specimen for this mode of imaging, as there are no large-scale variations in sample height to obscure any fine surface detail.

By using both the trace and retrace directions of the same scan line, images of the same area in the two different modes were collected and are shown in Fig. 2. To optimize the scan parameters for the deflection signal, the scan rate was increased, and the amplifier gains were reduced substantially, compared with typical values used in the constant force mode. The relatively fast rate of 20 Hz had the additional benefit of minimizing any instrumental drift during the scan. The deflection mode as used here does not yield true height information; however, on the particular surface studied here, the loss of true height information is not important, as it is the spatial separation of protein features that is of primary interest. The effect of these changes on the conventional topographic signal is shown in Fig. 2 *a*. Although the underlying stucture is resolvable, the image appears indistinct. By comparison the deflection mode image (Fig. 2 *b*) shows much more detail. The edge of the gas vesicle appears to be rough and of irregular height,

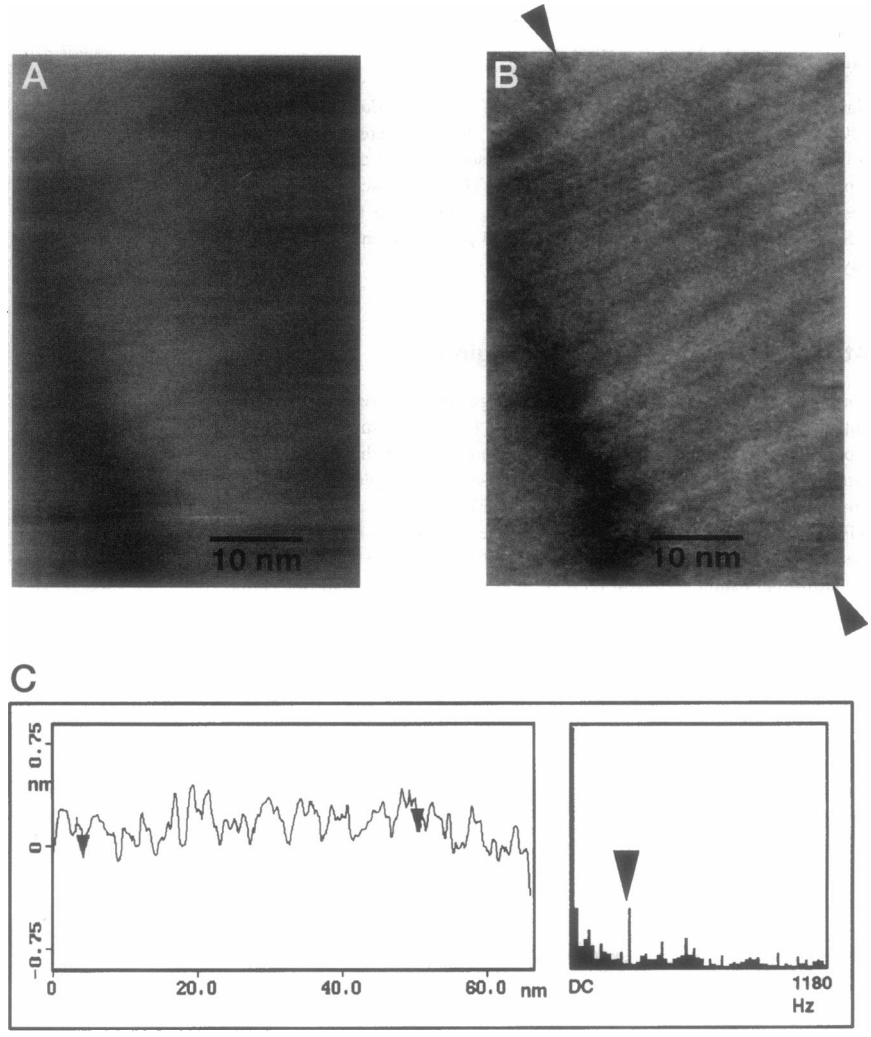

FIGURE 2 Simultaneous acquisition of (*a*) constant force and (*b*) deflection mode data from an area of a single gas vesicle. The constant force data were collected on the trace scan, and the deflection data were collected on the retrace scan. The scan area is 42.4 × 60.0 nm in each image. Except for flattening of the data set, it is unprocessed. The arrows in *b* indicate the line along which the profile was measured. (*c*) Line profile, and 1-D Fourier transform of the line data. The Fourier transform was performed using on-line Digital Instruments image-processing software. The arrows in the line profile, separated by 46.4 nm, define 10 repeats of the rib structure. The prominent peak in the 1-D transform occurs at 4.57 nm.

probably as a result of the collapse of the vesicle during sample deposition. The clearly resolved bands in the image are the ribs of the gas vesicle. From the line profile data and the frequency spectrum along the line, a rib periodicity of 4.6 ± 0.1 nm is obtained. This is in excellent agreement with the data from x-ray crystallography. The error bound was not determined from real-space measurements but estimated from the width of the corresponding peak in the frequency spectrum of the line profile data. For the purposes of measurement from the line profile, the data set was median filtered using Nanoscope III software.

Another example of gas vesicle rib morphology from another area is shown in Fig. 3 *a*. In this image the substructure of the GvpA protein along the ribs is more clearly seen. Each of the ribs appears to consist of repetitive "blocks," which define an angle of approximately 55° to the vertical rib axis, as measured in a clockwise direction. These repetitive structures were not observed on surfaces free of gas vesicles. The x-ray diffraction data (Blaurock and Walsby, 1976) indicated that the GvpA protein is in the form of paired β-chains that cross the ribs at an angle of approximately 35° to the longitudinal axis of the gas vesicle, or 55° to the rib axis. The 1.15-nm repeat along each rib as measured by x-ray diffraction is explained by this angle and the typical repeat spacing (0.930 nm) (Rees and Sternberg, 1984) between the pairs of β-chains in the antiparallel β-sheet. The sense of the 55° angle (i.e., to the right or left of the rib axis) cannot be determined by x-ray diffraction of the aligned cylindrical structures because both the front and back faces of the cylinders produce diffractions in opposite senses, resulting in x-ray patterns symmetrical in all four quadrants. Moreover, although the x-ray data suggested that much of the GvpA protein is in the β-sheet conformation, it did not indicate how far across the rib the β-chains extended. The AFM data resolve for the first time the orientation of these molecules. From these images it is apparent that the GvpA β-chain crosses much of the full width of the rib, but that there is a clear break between adjacent ribs.

The repetitive "blocks" are shown in greater detail in Fig. 3 *b*, a magnified version of Fig. 3 *a*. The line profile data (Fig. 3 *c*) reveal two periodicities along the rib: one of 1.12 nm and a second of 0.57 nm. Estimated as for Fig. 2, the error bound for these measurements is found to be ±0.05 nm. Close inspection of the image shows the "blocks" to be

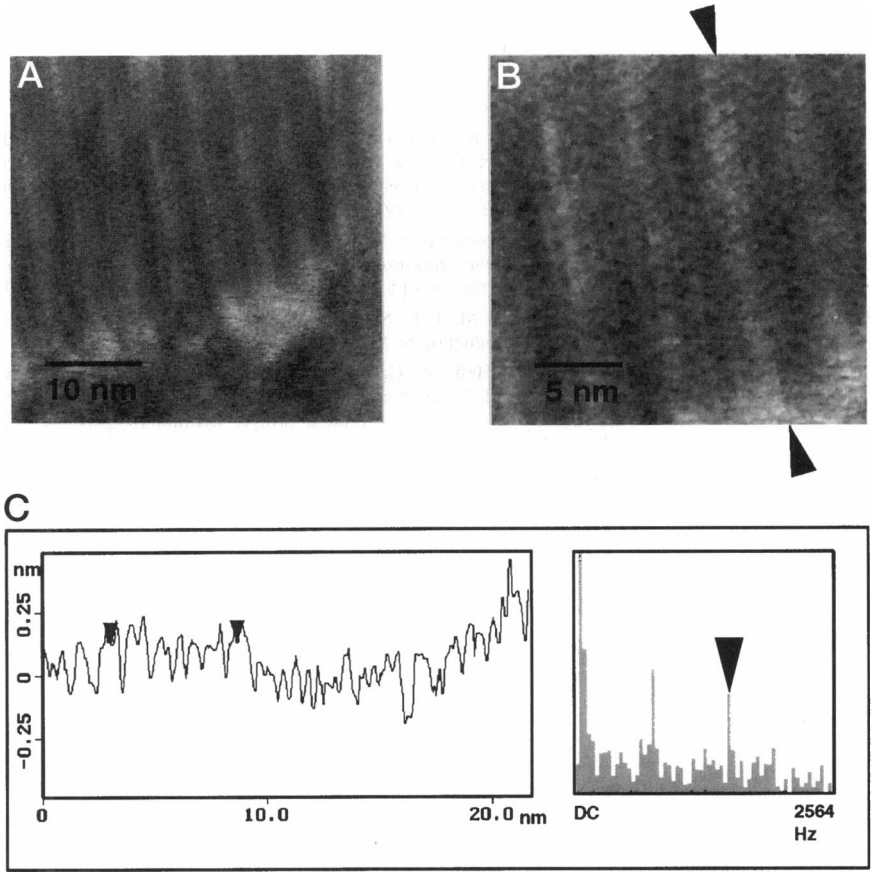

FIGURE 3 (*a*) Deflection data from another gas vesicle collected at 20 Hz scan frequency. The data were median filtered (3 × 3). The scan area is 40 × 40 nm. (*b*) Part of the same area at higher magnification. Arrows indicate the position of the line profile. (*c*) The line profile along part of the rib structure, and the associated Fourier transform. There are two distinct periodicities in the profile, which the Fourier analysis gives as 0.57 nm and 1.12 nm (marker positioned over 0.57 nm repeat). The two markers in the line profile are 5.62 nm apart, which for the five repeats gives a periodicity of 1.12 nm.

made up of pairs of lines. These lines are the origin of the smaller periodicity. The markers are positioned in the grooves of the main peaks of the line profile. These grooves separate the two side peaks of each of the main peaks. These side peaks originate from the individual β-chains in the protein structure. The measured spacing of 1.12 nm along the rib is consistent with the 1.15-nm repeat of pairs of β-chains indicated by x-ray diffraction. The additional periodicity of 0.57 nm, which is very distinct in the Fourier spectrum, is consistent with the perpendicular spacing of 0.465 nm between two adjacent β-chains in an antiparallel β-sheet, if these chains are oriented at 55° to the rib axis, and suggests that individual β-chains in each pair have been imaged.

CONCLUSIONS

The high-resolution imaging of the gas vesicle protein structure represents a new achievement in scanning probe microscopy of biological material. The β-sheet secondary protein structure indicated by previous x-ray measurements has been confirmed by the direct imaging and analysis of the structure by AFM. Furthermore, new information on the orientation and extent of the GvpA protein in the ribs has been obtained. The collapsed gas vesicle is well suited to AFM investigation because of its rigid, relatively flat morphology. Operating the AFM in deflection mode seems to offer advantages in revealing the fine structure of flat biological objects compared with the more commonly used constant force mode. It is unclear exactly why this mode of imaging should offer better resolution of the fine structure of the gas vesicle. The flatness of the sample is of major significance, although the faster scan speeds, compared with those normally used in constant force mode imaging, will minimize any effect of piezo drift and sample movement. The results described here indicate that the right combination of sample preparation and imaging mode can yield ultra-high resolution of biological structures, at a level more normally associated with flat, inorganic materials.

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