PhaP Is Involved in the Formation of a Network on the Surface of Polyhydroxyalkanoate Inclusions in Cupriavidus necator H16

Douglas Dennis, Vicki Sein, Edgar Martinez, and Brian Augustine

Department of Integrated Natural Sciences, Arizona State University, Glendale, Arizona 85306, and Department of Chemistry and Biochemistry, James Madison University, Harrisonburg, Virginia 22807

Received 15 October 2007/Accepted 25 October 2007

Polyhydroxyalkanoate (PHA) inclusions are polymeric storage inclusions formed in some bacterial species when carbon levels are high but levels of another essential nutrient, such as nitrogen, are low. Though much is known about PHA synthesis, little is known about inclusion structure. In this study, atomic force microscopy (AFM) was employed to elucidate the structure of PHA inclusions at the nanoscale level, including the characterization of different layers of structure. AFM data suggest that underneath the inclusion envelope, there is a 2- to 4-nm-thick network layer that resides on top of a harder layer that is likely to be a crystalline lamellar polymer. The network is comprised of ~20-nm-wide linear segments and junctions that are typically formed by the joining of three to four of the linear segments. In some cases, ~50-nm globular structures that are raised ~1 to 2 nm above the network are present at the junctions. These globular structures always have a central pore that is ~15 nm in diameter. To determine if the major surface protein of PHA inclusions, PhaP, is involved in the structure of this network, inclusions from Cupriavidus necator H16 ΔphaP were examined. No network structure was detected. Instead, apparently random globular structures were found on the surfaces of the inclusions. When PhaP levels were reconstituted in this strain by the addition of phaP on a plasmid, the network was also reconstituted, albeit in a slightly different arrangement from that of the wild-type network. We conclude that PhaP participates in the formation of the inclusion network.

Polyhydroxyalkanoates (PHAs) are bacterial storage polymers that accumulate when carbon levels are high but levels of another necessary nutrient, such as nitrogen, are low. In Cupriavidus necator (formerly Wautersia eutropha), PHA synthesis occurs in multiple inclusions that are generally 200 to 1,000 nm in size (2). The inclusions contain approximately 97.5% PHA, 2% protein, and 0.5% lipid (10), although some estimates of the lipid content are higher (3). PhaP is the dominant protein species on the inclusion and, under accumulation conditions, can constitute as much as 5% of the total cell protein. Because PhaP is found at the surface of the inclusion, it is thought to be the major structural protein of the inclusion (23, 34).

The precise nature of the inclusion surface has yet to be conclusively determined. Electron microscopy (EM) studies reported more than 40 years ago supported the existence of a membrane layer at the surface of inclusions isolated from Bacillus megaterium (18). Nine years later, freeze fracture experiments expanded these results by suggesting an arrangement in which there is a membranous outer layer, a solid layer underneath it, and an inner core of soft, deformable material (7). The finding of a solid second layer has been supported by studies that demonstrated a solid, crystalline lamellar shell at or near the surface of the inclusion with a soft, noncrystalline, hydrophobic core (17). In contrast to the membrane-solid layer-amorphous core inclusion model, later studies of inclusions from Pseudomonas species imaged a regular lattice-like surface architecture at the inclusion surface that is reminiscent of a bacterial S-layer (28). Because of this, some researchers suggested that instead of an outer phospholipid layer, a protein layer, most logically composed of PhaP with embedded synthesis and depolymerizing enzymes, covers the inclusion (14, 27). For a period of time, the difference between these two models seemed to be somewhat clarified by the determination by researchers that the outermost layer is 4 nm thick (21). This implies that it cannot be a lipid bilayer, which has been measured at 7 to 13 nm for C. necator H16 (21). Similarly, it does not seem possible that it is a monolayer of PhaP, because quantitative Western blot analyses have determined that there is only enough PhaP present to cover 27 to 54% of the total inclusion surface (31, 32). With these two possibilities seemingly ruled out, a newer model of the PHA inclusion structure was proposed in which the outer layer of the inclusion consists of a phospholipid monolayer, thus conforming to the 4-nm thickness previously determined for the outermost layer (29). This model, however, has been recently challenged by EM data showing that the outer boundary layers of PHA inclusions in Caryophanon latum were found to be 14 nm thick (13). Therefore, the nature of the surface of PHA inclusions and the mode of biogenesis of these inclusions remain undefined. In an attempt to resolve the basic structure of the PHA inclusion surface, we have employed high-resolution scanning probe microscopy. Our results suggest a model of inclusion structure that is consistent with the findings of the initial EM studies in that we have imaged an envelope that covers a solid crystalline shell. In addition, we describe a heretofore uncharacterized network layer residing between the envelope and the solid, crystalline lamellar layer. Preliminary studies suggest that this network is at least partially composed of PhaP.
MATERIALS AND METHODS

Bacterial growth. Cupriavidus necator H16 was grown overnight in a nutrient broth culture, and this culture was used to inoculate a 50-ml flask at 600 nm was between 0.05 and 0.1. A supplemented minimal medium was used (24). The constituents were (per liter) 9.0 g Na₂HPO₄·12 H₂O, 1.5 g KH₂PO₄, 0.1 g NH₄Cl, 0.2 g MgSO₄·7 H₂O, 0.02 g CaCl₂·H₂O, 1.2 mg Fe(III)NH₄-citrate, 0.10 mL S6 trace elements (22), 5 ml 20% (wt/vol) Casamino Acids, 0.1 ml 0.5% (wt/vol) thiamine, and 0.4% (wt/vol) fructose. When cultures were incubated longer than 18 h, another aliquot of 0.4% fructose was added at approximately 20 h postinoculation. The culture was incubated at 30°C at a speed of 300 rpm. Cultures were normally grown to a final optical density at 600 nm of between 10 and 20.

Preparation of PHA inclusions. To minimize contamination during the isolation process, very small amounts of bacterial cells in relatively large volumes of buffer were subjected to sonication. Fifty micro-liter of cell culture was added to 1.5 ml of TN buffer (10 mM Tris [pH 7.5]-150 mM NaCl) in a microcentrifuge tube, vortexed, and pelleted (1 min at full speed). The pellet from this wash was resuspended in the 50 μl of TN buffer. In general, we added 5 μl of this cell suspension to 5 ml of buffer, but this was sometimes altered to obtain higher or lower inclusion densities. An aliquot of cell suspension was added to 5 ml of TN buffer and subjected to sonication using a Tekmar model TMX400 sonic disruptor with a tapered probe (5 mm at the tip) located about 4 mm from the bottom of a 13- by 100-mm sterile plastic culture tube (containing the cell suspension) in an ice bath. Sonication times and intensities varied depending on the level of inclusion desired, but was desired. For harsh sonication, a sonication setting of 30% power for 60 s was employed. For a harsher sonication, in which the majority of the cells were lysed, a setting of 50% power for 5 min was used. To obtain the most disruptive sonication, the cells were subjected to sonication at 60% power for 5 to 10 min. Pulse times were 3.3 s with a resting phase of 9.9 s. The degree of cell lysis and inclusion disruption varied from culture to culture (probably due to the culture age, etc.), but if over- or undersonication occurred, the procedure was redone to obtain the type of lysis desired. Immediately after sonication, the entire contents of the tube were added to a fritted filter holder (25 mm) containing a 25-mm polycarbonate filter with 200-nm pores (Osmonics; KO2CP02500). The sample was filtered quickly and washed with 5 to 15 ml of deionized water. The filter was immediately removed, fixed to a metal puck, and imaged.

Construction of C. necator H16 ΔphaP. Forward primer delphaPF (CGAGCTCGAGGAGCTAGGTAGGTCCT), containing a SacI site at the 5′ end (indicated by underlining), and reverse primer delphaPR (CTGTGCGCATCGTCAGCGAGCATGGAAGTGGCCT), containing a SacI site at the 3′ end, were employed to amplify a segment of phaP from nucleotides 395 to 1641 of the published sequence (GenBank accession number AF110426). The amplified segment was digested with SacI and Acc65I (an Acc65I site is indicated), and downstream sequences. Of particular interest to us is the network layer because it has settled into energetically more stable conical shapes (Fig. 1D). This interpretation is supported by cross-sectional analysis of the three inclusion types which shows that the enveloped and networked inclusions maintain round shapes but that the naked inclusions have settled into energetically more stable conical shapes (Fig. 1D). Of particular interest to us is the network layer because it has not yet been described and because of its physical resemblance to eukaryotic cytoskeletons. Therefore, further high-resolution AFM analyses were conducted.

In preliminary studies, it was determined that AFM cantilevers with large tip radii (10 to 25 nm) tended to increase the perceived diameters of the linear segments of the network (tip convolution) (1, 33). In some cases, the spreading was so pronounced that the network looked less like a network and more like the surface that is observed on a human brain, and the linear segments were similar to the nanostructures recently reported by Kuchta et al. (16). For this reason, AFM probes that are very uniform and very sharp were utilized, with some of them having a radius of curvature as low as 2 nm. Although they are difficult to work with on convex soft objects, when properly used, these cantilevers generate images of outstanding resolution and clarity.

In scans ranging from about 1 μm² to 8 μm² in size, networked inclusions can typically be identified by the variegation of their surfaces in contrast to the lack of variegation of enveloped and naked inclusion surfaces (Fig. 1A to C). However, little detail about surface structure can be determined at this scan size (Fig. 2A). When scan sizes below 1 μm² are used, specific surface structures begin to be observed (Fig. 2B), but their interpretation is made possible only by having imaged
and interpreted higher-resolution scans. With the highest-resolution scans, typically in the range of 150 nm² to 350 nm², the components of the network become interpretable. The network comprises a series of interconnected linear segments (Fig. 2C). In the highest-resolution images acquired, it appears that the linear segments may be composed of subunits, because faint lines perpendicular to the axes of the segments are observed, making it look like the subunits are “stacked” on top of each other to make the segments. Normally, the junctions of the linear segments are composed of three to four segments. The widths of the linear segments vary considerably, with an average width being 20 nm. This lateral measurement is subject to the error inherent in the radius of curvature of the cantilever tip (tip convolution) (1, 33), which should be minimal in this study because of the very fine tips used (~2-nm radius of curvature). A structure that is often seen at the junction of the linear segments is a globular structure that is in the range of 40 to 70 nm in diameter, with the average being ~50 nm (Fig. 2C).

These globular structures always have a central pore with a very uniform diameter of ~15 nm (Fig. 2D). The globular structures are usually 1 to 2 nm higher than the network.

Cross-sectional analysis of individual network linear segments reveals that they range in height from ~2 to 4 nm (Fig. 3A and B). It is not known at this time whether the network strands reside on the surface of the crystalline layer beneath it or whether they are partially embedded in this layer. In high-resolution AFM height scans, pores were observed in the inclusion surface between the network strands. These pores were approximately 15 nm in diameter (Fig. 3A; cross-sectional data not shown). The pores could also be detected using AFM phase imaging. In addition to the pores, faint parallel striations could often be seen in the gaps of the network in phase images (Fig. 3C). The spacing of the striations was measured at ~5 to 6 nm (Fig. 3D).

To determine if the loss of PhaP, the dominant surface protein on PHA inclusions, causes a structural change in the
surface network, a ΔphaP strain was made in which the altered PhaP contained only its first three and last two amino acids with a GGATCC linker in between. This strain, *C. necator* H16 ΔphaP, grew more slowly than the wild-type strain and synthesized larger inclusions, generally one per cell. Inclusions from *C. necator* H16 ΔphaP were much less uniform in shape than those of the wild type (Fig. 4A and B). They often had irregularly shaped perimeters. In addition, they exhibited no linear network structures. Instead, a large accumulation of globular structures was normally present on the surface (Fig. 4A and B). The number of globular structures was linked to the degree of sonication, with inclusions from more rigorously sonicated cells having little in the way of globular structure. Occasionally, globular structures with central pores were tentatively identified on an inclusion (Fig. 4C and D), but given the randomness of the surface structure, it was difficult to positively identify these as the same globular structures seen in the networked inclusions. The 5- to 6-nm parallel striations were also observed (data not shown) but less frequently, because the inclusions tended to be almost completely covered with globular material. These large inclusions were more difficult to image than inclusions from wild-type cells. Often the cantilevers gouged the inclusions at their edges and/or punctured the inclusions as they traversed their surfaces. We are unsure at this time whether this is due to the increased height signature and the inherent difficulties in traversing it or to an increase in fragility of the inclusion surface.

When plasmid-borne *phaP* under the control of its own...
promoter was introduced into \emph{C. necator} H16 \emph{ΔphaP}, the strain regained a normal growth rate and PHA accumulation levels (data not shown). The inclusions, however, were much smaller. The network was restored to the surface of the inclusion (Fig. 5A and B), but it appeared to differ slightly from the inclusion network observed in \emph{C. necator} H16 in that the linear segments were generally 5 to 10 nm wider, giving the appearance of a much more robust structure. It did not appear that there were as many junctions in the network, though this finding is somewhat subjective. The heights of the individual strands were the same as those found in inclusions from wild-type cells (cross-sectional data not shown). Pores that were \~{}15 nm in diameter were observed, but they were not as often located in distinct globular structures of \~{}50 nm in diameter (Fig. 5B). Instead, they were at the junctions of the widened network strands.

**DISCUSSION**

AFM offers an extremely powerful tool for the characterization of biological surfaces (6). Not only can the technique be used to image structures at the nanoscale level, the sample preparation required is much less severe, and thus, delicate structures can be imaged that might otherwise be compromised using traditional sample preparation techniques re-
quired for EM studies. In fact, from the time of harvesting bacterial cells from the culture, high-resolution images could be obtained within 15 min.

The AFM data support the early EM studies in that they confirm the existence of an envelope with a crystalline lamellar shell beneath it (7, 17, 18). The AFM data differ significantly from those of prior studies, however, in that AFM analysis has identified a network layer residing between the envelope and the crystalline lamellar shell. The structure of this network is somewhat reminiscent of the structure of the red blood cell cytoskeleton (Fig. 6) (30). PhaP is likely to be a component of the network because when \( \text{phaP} \) is deleted, the network is not formed, and when \( \text{phaP} \) is inserted on a low-copy-number plasmid into \( C. \text{necator} \ H16 \Delta \text{phaP} \), the network is again formed.

It could be argued that PhaP is a regulatory protein that stimulates the production of another protein that composes the network, but this ignores the fact that PhaP is the dominant protein located on the inclusion surface. Furthermore, the coverage of the network is consistent with prior studies that showed that PhaP covers 27 to 54% of the inclusion surface (31, 32) and with the fact that alteration of the PhaP levels alters the size of the inclusion (34), suggesting a structural function. A structural function is also suggested for the imaged network by the fact that removal of the network results in a conformation change in the inclusion from rounded to conical. Finally, the calculated radius of PhaP, 2.1 nm, is consistent with the height of the network (32).

The inclusion network that is formed in strains where \( \text{phaP} \) is located on a low-copy-number plasmid appears to be slightly different than the network formed on inclusions in wild-type cells. The linear portions are 5 to 10 nm wider, and the network appears to have longer linear runs between junctions (though we have not mathematically confirmed this last observation). This may be due to an increase in intracellular PhaP levels mediated by a gene dosage effect of the plasmid-borne \( \text{phaP} \) gene. It may also be that other proteins participate in the network, especially the junctions/globular structures, and that when the ratio of PhaP to these other proteins is changed, it changes the structure of the network. We are currently focusing on PhaR, an inclusion surface protein that is known to interact with PhaP, to determine if changing the PhaP/PhaR ratios alters the arrangement of the network. Experimental support for this notion comes from research on recombinant \( E. \)}
coli harboring the *C. necator* H16 biosynthesis genes (pha-CAB), in which the addition of *phaP, phaR*, or both resulted in changes in size and shape of the formed inclusions (25).

It is probable that "naked" inclusions are inclusions that have been stripped to their crystalline lamellar shell, because parallel striations that conform to the previously measured width of PHA fibrils (9) are present. These striations can also be imaged in the gaps of the network, suggesting that the network resides on top of the crystalline lamellar shell. Interestingly, ~15-nm-diameter pores were observed in the crystalline lamellar shell. Though their function is unknown, it is logical to assume that these pores provide a portal to the amorphous polymer core residing beneath the crystalline shell, both for synthesis and for depolymerization.

The pores identified in the globular structures are also intriguing. The globular structures themselves possess a fairly broad range of size, yet the pores always occur centrally, singly, and with diameters very close to 15 nm. This uniformity has structural implications. Pores of the same size have also been identified in the envelope layer (unpublished data). One might suppose that in the intact inclusion, the ~15-nm pores found in all three layers are aligned, thus providing a portal of ingress and egress through the three layers. In fact, if the pores in all three layers align, it seems likely that the pores are the sites of PHA metabolism, because there is no other plausible way that molecules could enter the inclusion fast enough to sustain the high rate of polymerization that occurs during PHA accumulation.

It is not a certainty that the networks that are imaged in this study are representative of the network in an intact inclusion. It may be that when the envelope is removed, portions of the network are lost either through physical trauma or because they are attached to the envelope. It does appear that the network is fairly easily removed from the inclusion surface, because the sum total of the AFM images acquired reveal the network in many stages of deterioration (data not shown). In some cases, only a few linear remnants are present on top of the crystalline lamellar shell. In fact, inclusions with partially degraded networks have assisted in the interpretation of the three-layer inclusion hypothesis. In this paper, only images of the most complete network are displayed.

With respect to network deterioration, it is possible that the globular structures are easily lost from the network, because in an earlier study in which inclusions resting on filters were treated with a dilute sodium dodecyl sulfate solution, many ~50-nm globular structures were imaged lying on the filter beside the inclusion but not in other areas of the filter (4). Also, if there is a 1:1:1 relationship between the pores in the crystalline lamellar layer, the pores in the globular structures, and the pores in the envelope, then there is a deficit in the number of globular structure pores. Additionally, there are many pores observed in the crystalline lamellar layer that do not have a corresponding globular structure over them. The strongest evidence for this supposition comes from the prior AFM study in which globular structures with central ~15-nm pores were observed at a high density under the surfaces of enveloped inclusions (4).

An issue to be considered is the effect of drying on the network. It has been reported that drying of the erythrocyte membrane for tapping-mode AFM in air causes a slight deformation of the attached cytoskeleton (30). This is a possibility for PHA inclusions, but because the network is apparently linked to the crystalline layer (otherwise it would be totally lost during sonication), it is unlikely that it deforms much beyond what occurs as the polymer crystallizes. Once crystallized, the surfaces of inclusions are quite stable, and they have been

---

**FIG. 5.** AFM phase images of inclusions from *C. necator* H16 Δ*phaP*(pBBR/phaP). (A) Original scan = 1.8 µm². Scale bar = 200 nm. (B) Enlargement of panel A showing the network on a single inclusion and an ~15-nm pore in a widened area of the network (white arrow). Scale bar = 200 nm.
imaged over long periods of time with no apparent alteration in their structures. To determine what effect, if any, crystallization of the polymer might have on the arrangement of the network, AFM liquid-cell studies are now being conducted.

An obvious question raised by this study is why the network has not been observed prior to this report. There are at least two reasons why this may have occurred. First, the network layer is only 2 to 4 nm thick, is not a solid layer, and is easily detached from the inclusion. It may be that during the preparation of the inclusions for electron microscopy, the network was lost, or perhaps it is simply not discernible in a cross section because of its incomplete nature. Or it may be that the layer is simply below the limits of detection for those particular EM protocols. This is not unique in biology. It is only recently that cryoelectron microscopy identified a 2- to 4-nm granular layer on the outer face of the cytoplasmic membrane in gram-positive bacteria (35) and a periplasmic space also in gram-positive organisms (20). The second explanation is that in prior studies, the envelope was removed by the EM preparation procedure, leaving only the network and the crystalline lamellar layer, which would constitute an electron-dense layer that is approximately 4 nm thick. Therefore, when previous studies reported the boundary layer as being 4 nm thick (21), it may be that the network/lamellar shell was being measured. It is well established that EM preparatory procedures are deleterious to membranes and related attached structures (5). It is noteworthy that recent EM studies have detected “regularly oriented” proteins on the surface of PHA inclusions from Caryophanon latum (13). However, the level of resolution was not high enough for a significant interpretation.

In future experimentation, we will continue to study the network layer and examine how alterations in growth conditions, bacterial species, and gene deletions alter its structure. We are also interested in the possibility that the network may

FIG. 6. AFM images of an inclusion network (A, C, and D) and an erythrocyte cytoskeleton (B). Scale bars = 100 nm. (A) Phase image of a network. In this instance, the network was found detached from an inclusion, lying on the filter substrate. This allowed imaging at a very high resolution. A putative, ~50-nm-diameter globular structure with a central pore is shown (white arrow). Original scan = 434 nm². (B) Erythrocyte skeleton imaged in tapping mode in air. Note the difference in scale between this image and the inclusion images. Taken from reference 30 with permission from the publisher. (C) Surface plot of panel A. This AFM tool renders the image more three-dimensional by tilting the image and varying the light angle. Two putative, ~50-nm-diameter globular structures are pointed out (white arrows). Others are present, however. (D) Height image of panel C. z range = 50 nm.
provide a focal point where proteins can transiently associate to mediate cellular/PHA reactions. This is suggested by recent studies in which it has been shown that phasins (PhaP) perform this function for triacylglycerol inclusions (11). It is also of interest that the 25-amino-acid, alanine-rich carboxy terminus of PhaP exhibits minor homology to the mouse clathrin coat assembly protein (GenBank accession number Q61548), a membrane-embedded protein that can recruit and release clathrin and other proteins (12). In short, we hope to gather additional evidence that will support the existence of the network layer and elucidate its function from an inclusion and cellular perspective.

ACKNOWLEDGMENTS

This work was supported by grants 0110232, 0071717, and 0520882 from the National Science Foundation.

REFERENCES


