Immunocytochemical Analysis of Poly-β-Hydroxybutyrate (PHB) Synthase in *Alcaligenes eutrophus* H16: Localization of the Synthase Enzyme at the Surface of PHB Granules

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Antibodies raised against the *Alcaligenes eutrophus* poly-β-hydroxybutyrate (PHB) synthase polypeptide were used for immunocytochemical localization of the synthase enzyme in whole cells and purified PHB granules. The data presented demonstrate for the first time that the synthase enzyme is located on the surface of the PHB granule rather than being incorporated inside the granule during its formation. From these basic observations and data from the recent literature, a model of granule assembly is proposed.

Poly-β-hydroxybutyrate (PHB) accumulates as granular inclusions in the cells of a large number of bacterial species grown under conditions of nutrient limitation (2). In both *Zoogloea ramigera* (8, 9, 15, 16, 25, 27, 28) and *Alcaligenes eutrophus* (19, 20), the condensation of two acetyl coenzyme A (CoA) units to form acetoacetyl-CoA by the enzyme β-ketothiolase is followed by a stereospecific reduction step catalyzed by an $NADP^+$-specific acetoacetyl-CoA reductase to form D-β-hydroxybutyryl-CoA, the substrate for PHB synthase. Both the β-ketothiolase and acetoacetyl-CoA reductase are soluble cytosolic enzymes, whereas the PHB synthase is found attached to the PHB granules.

Purified granules contain around 98% PHB by weight, with the remainder being made up of proteins and lipids (17, 22). It was previously concluded that the physical state of the polymer inside the granules in vivo was that of a crystalline solid, similar to that of the extracted polymer (12, 14, 21). More recently, nuclear magnetic resonance studies of whole cells have provided the first evidence that the "granule" is in fact in a mobile, amorphous state in vivo (3, 4). Both the presence of plasticizers including water (4, 18) or lipid (22) and the kinetics of the crystallization process (6, 10) have been proposed to describe how the granules are maintained in the amorphous state inside the cell. Additional studies have concluded that the PHB granules inside the cell are surrounded by a membrane containing the synthase enzyme and that the D-3-hydroxybutyryl-CoA monomers are transported across the membrane in conjunction with polymerization (5, 13, 24, 32).

Our current research activities are focused on understanding the role of the PHB synthase in the biogenesis of the PHB granules in *A. eutrophus*. In this regard there are two main issues: (i) the mechanism by which the PHB synthase initiates and carries out the condensation of literally thousands of D-3-hydroxybutyryl-CoA units to form the polymer chains and (ii) the role of this mechanism and the PHB synthase protein in the assembly of the PHB granules. We report here the immunocytochemical localization of the PHB synthase on the surface of the granules and describe a working hypothesis for granule formation.

Preparation and analysis of anti-PHB synthase antibodies.
In order to obtain purified *A. eutrophus* PHB synthase protein for the purpose of preparing antibodies, the C-terminal region of the *phbC* gene from plasmid pAeT42 encoding residues 86 to 589 of the PHB synthase (26) was inserted in frame into the glutathione S-transferase (GST) fusion protein expression plasmid pGEX2T (30) to obtain plasmid pGAS1. Following growth and induction of *Escherichia coli* JM101
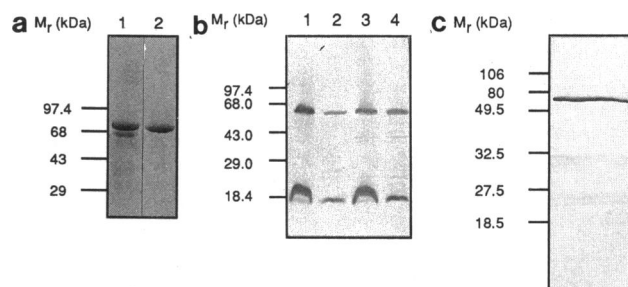

FIG. 1. Western blot analysis of protein extracts from *A. eutrophus*. (a) Proteins from the purified inclusion bodies were separated on SDS–4 to 15% polyacrylamide gradient gels (Bio-Rad, Richmond, Calif.) and stained with Coomassie blue. Lane 1, 10 μg of inclusion body protein; lane 2, 5 μg of electroeluted full-length GST-PHB synthase protein. (b) *A. eutrophus* cells were grown under different conditions, and extracts were prepared as described in the text. Protein samples were separated on SDS–4 to 15% polyacrylamide gradient gels electroblotted onto nitrocellulose filters (pore size, 0.4 μm; BA85, Schleicher & Schuell) and screened with anti-PHB synthase antibodies diluted 1:2,000. Material cross-reacting with anti-PHB synthase antibodies was visualized with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate and horseradish peroxidase color development reagent (Bio-Rad). The locations and molecular masses of the prestained molecular mass markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; and β-lacto-globulin, 18.4 kDa) are indicated by the bars. Lanes 1 to 4, 20 μg of protein from cells grown in carbon-rich medium (lane 1, pellet fraction; lane 2, supernatant fraction) and minimal medium with no carbon (lane 3, pellet fraction; lane 4, supernatant fraction). (c) The sample lane contained 10 μg of protein from PHB granules which had been purified by sucrose density gradient centrifugation. The locations and molecular masses of the prestained molecular weight markers are indicated by the bars.

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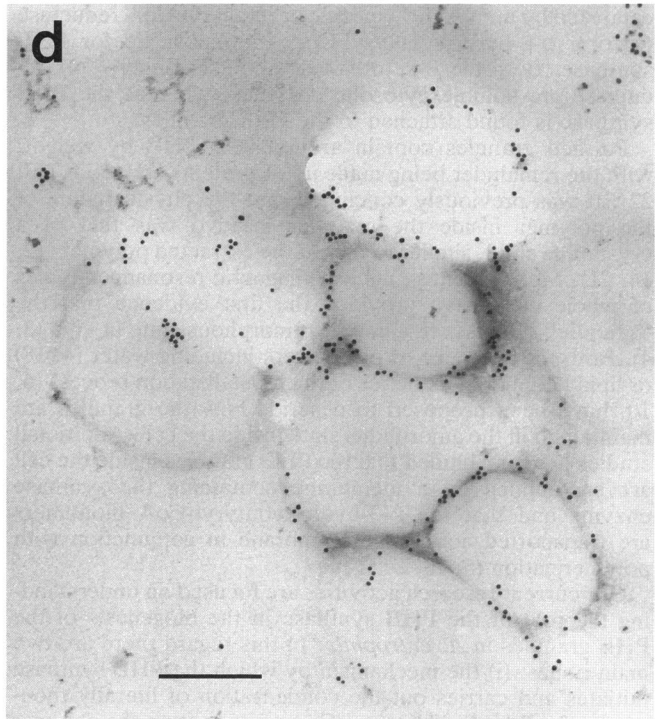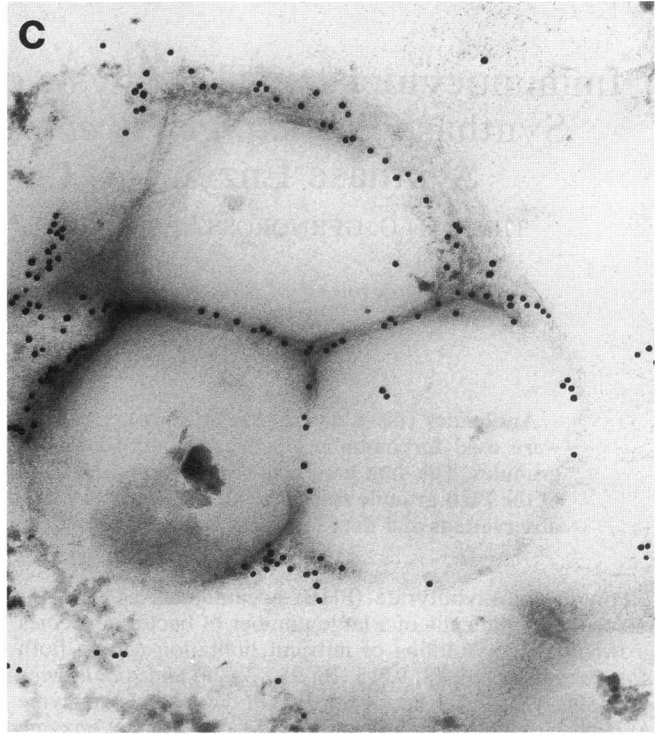
containing plasmid pGAS1 (30), the fusion protein was recovered as densely packed inclusion bodies, washed successively with phosphate-buffered saline (PBS) containing 6 M urea and then 8 M urea and finally resuspended in PBS containing 0.16% sodium dodecyl sulfate (SDS). SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the purified protein revealed two bands (Fig. 1a) identified as the full-length and a truncated GST-PHB synthase fusion protein by N-terminal amino acid sequence analysis. This material was used for the production of antibodies in New Zealand White rabbits. Anti-GST-PHB synthase immunoglobulin G was purified on a protein G affinity column (Pharmacia, Piscataway, N.J.).

A. eutrophus H16 (ATCC 17699) was grown in Trypticase soy broth (TSB) or minimal medium (MM) as previously described (26). To prepare cells containing high levels of PHB, 500-ml cultures of MM containing fructose (1.0% [wt/vol]) and 0.02% (wt/vol) NH_4Cl were inoculated with washed cells from 50 ml of TSB culture and incubated for 48 h at 30°C. To prepare samples in which PHB should not accumulate, 200 ml of TSB grown cells was harvested, washed with MM, and transferred to 500 ml of fresh, carbon-free MM supplemented with 0.1% NH_4Cl and incubated for a further 48 h at 30°C. Cell extracts were prepared as described previously (26). Crude cell lysates prepared as described above were used without further treatment or fractionated by centrifugation at 10,000 rpm for 10 min at 4°C. Protein concentrations were determined by the method of Bradford (7) with bovine albumin as a standard.

Western blot (immunoblot) experiments were performed on these protein extracts, and results are shown in Fig. 1b. Control experiments, performed with rabbit anti-GST antibodies (AMRAD, Borunia, Victoria, Australia), identified no cross-reacting proteins in the *A. eutrophus* extracts (data not shown). The anti-PHB synthase antibodies clearly bind to a polypeptide with an apparent M_r of 64,000 (Fig. 1b, lanes 1 to 4). The size of this protein is consistent with the molecular weight of PHB synthase predicted from the nucleotide sequence of the *phbC* gene (26). Cell extracts of *A. eutrophus* mutant strains (26) in which the *phbC* gene had been disrupted by Tn5 insertion were analyzed and found not to have the M_r 64,000 band (data not shown). From these results, we conclude that the antibodies raised by using the GST-PHB synthase fusion protein clearly cross-react with the *A. eutrophus* PHB synthase. Additional small molecular polypeptides (M_r around 16,000) were also detected in all of the samples (Fig. 1b, lanes 1 to 4).

Proteins extracted from sucrose-density-gradient-purified (16) PHB granules were also analyzed, and the results are shown in Fig. 1c. In this case, we again detect a strong signal from the 64-kDa band, but the density gradient separation has removed the small-molecular-weight proteins which cross-react with the anti-PHB synthase-antibodies in Fig. 1b. The nature and role of these smaller-molecular-weight proteins are unknown.

Immunogold localization of PHB synthase in *A. eutrophus* cells. Proteins cross-reacting with purified anti-PHB synthase serum were localized by immunogold labelling of thin sections prepared from *A. eutrophus* cells and purified PHB granules. Samples were fixed overnight at 4°C in a solution of 1% paraformaldehyde–0.1% glutaraldehyde in 0.1 M sodium phosphate buffer and rinsed three times for 5 min (each) in sodium phosphate buffer prior to embedding the pellet in 1% agar. Dehydration was performed by successive washes in ethanol solutions: 30 and 50% ethanol (30 min, 4°C), 70 and 90% ethanol (60 min, −5°C), and two times with 100% ethanol (30 min, −5°C). Low-temperature embedding was performed with a Lowicryl K4M kit (Chemische Werke Lowi, Waldkraiburg, Germany) essentially as described by Simon et al. (29). Sections (70 nm) were prepared on Pioloform-coated nickel grids (200 mesh; Pella, Redding, Calif.) by using a Sorval MT 5000 microtome.

Immunostaining of thin sections was done as follows: grids were blocked for 25 min in blocking solution (TBS-Tween 20 [TBST] containing 3% [wt/vol] nonfat dry milk [filtered through a 0.2-μm-pore-size filter] and rinsed for 5 min in TBST. Specimens were incubated with anti-PHB synthase antibody in TBST at various dilutions overnight at 4°C. Specimens were then washed for 5 min in three washes of TBS. Gold-labelled goat anti-rabbit antibodies (Auroprobe, Amersham) were briefly centrifuged (2,000 rpm, 30 s) in a Eppendorf microcentrifuge to remove aggregated material, diluted 1:20 in TBS, and used immediately. Binding of goat anti-rabbit antibodies was carried out for 1 h, and the grids were subsequently washed twice in TBS (5 min) and finally in distilled water. Sections were stained with uranyl acetate (5 min) and then with lead citrate (2 min) and viewed on a JEOL 1200 CX electron microscope at 80 kV.

In whole cells of *A. eutrophus* grown in the absence of a carbon source, the synthase is essentially randomly distributed throughout the cytoplasm, as indicated by the immunogold labelling (Fig. 2a, black dots). In this case, we also observed smaller granule-like structures in many of these cells even though they were grown for 48 h with no carbon source other than the endogenous PHB accumulated during growth on TSB. Cells cultured under conditions in which PHB accumulates (Fig. 2b) contain large PHB granules, and the immunogold label is located around the edge of the granule. Immunostaining of the purified PHB granules which do not contain the cross-reacting small-molecular-weight proteins (Fig. 1b) clearly demonstrates the association of the synthase enzyme on the surface of the granule (Fig. 2c and d). Although the synthase enzyme is bound to the granules, we have not yet determined if this is because of a covalent linkage of the growing polymer chain to an active site residue in the synthase or, simply, a hydrophobic interaction. Control experiments performed using preimmune serum or the rabbit anti-GSH antibodies gave no signal. In all cases (Fig. 2), virtually no background signals were obtained on the Lowicryl matrix.

Conclusion and model for PHB granule assembly. These results clearly demonstrate for the first time the location of the synthase enzyme on the surface of the PHB granules. Recent evidence indicates that the polymer in the granules is in an amorphous, mobile state. On the basis of these data, we have begun to further develop a working model of PHB granule formation (Fig. 3). Assuming that the process starts in the total absence of PHB and that the growing polymer

FIG. 2. Immunogold localization of proteins cross-reacting with anti-PHB synthase antibodies on thin sections of *A. eutrophus* cells. Immunogold labelling (black dots) was performed as described in Materials and Methods on whole cells grown in carbon-rich medium (a) or carbon-free medium (b). (c and d) PHB granules were also purified from cells grown in carbon-rich medium and similarly labelled. The samples were incubated overnight with anti-PHB synthase antibodies at dilutions of 1:50 (a and b) or 1:100 (c and d). Bars, 200 nm.
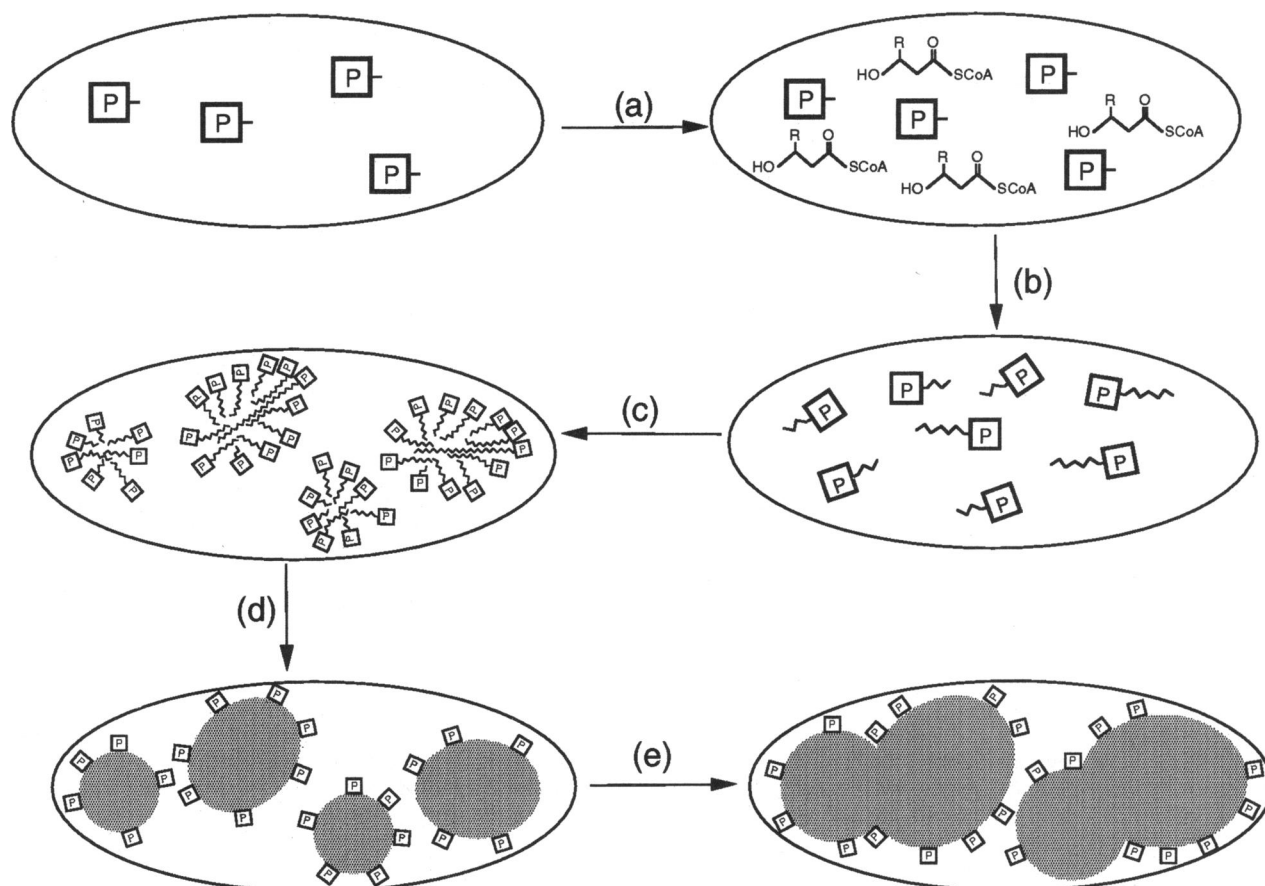
FIG. 3. Model of PHB granule assembly in *A. eutrophus*. A model of PHB granule accumulation in *A. eutrophus* cells is illustrated. The model is described in detail in the text. PHB synthase molecules are indicated by the [P] free in the cell cytoplasm (a) and in the presence of accumulating concentrations of D-3-hydroxybutyryl-CoA monomers (b). (c) The growing polymer chains are then presented as the zig-zag lines eventually becoming dense granules, indicated by the shaded regions (d). (e) As the granules increase in size, they may be forced to fuse together.

chain remains bound to the synthase enzyme, granule formation would proceed as follows. (i) The metabolism of the cell would reach a stage at which free CoA becomes depleted, acetyl-CoA accumulates and D-3-hydroxybutyryl-CoA is produced. (ii) The synthase enzyme molecules would be primed (mechanism unknown), polymerization of the substrate would take place, and the polymer chains would begin to grow by extrusion from the synthase. (iii) Increased chain length would make the growing polymer chains more hydrophobic (31). This would generate a number of growing hydrophobic polymer chains free in the aqueous cell cytoplasm, each attached at one end to a hydrophilic synthase (the synthase is a soluble cytoplasmic enzyme in the absence of PHB [Fig. 3a]), resulting in the assembly of a number of amphipathic molecules in the aqueous cytoplasm.

The hydrophobic polymer chains form a micelle-like structure in which the synthase enzyme forms a skin around the granule, effectively separating the hydrophobic polymer from the aqueous cytoplasm. This concept is not entirely new, as Griebel et al. (17) previously discussed the possibility that the polymer-synthesizing enzymes aggregate into a micellar form. (iv) Polymerization continues by effectively taking the substrate from the aqueous cytoplasm and extruding the growing polymer chain into the increasingly densely packed amorphous hydrophobic center of the granule, in

effect pushing the synthase away from the center of the granule. (v) Inside an individual cell, several granule foci could be formed initially, but as they grow the intracellular space becomes limited. Granules are forced to fuse as the polymer continues to accumulate, squeezing the synthase out onto the periphery. Coalescence of PHB granules during cell growth has been reported in the literature (4). In this model, other hydrophobic or amphipathic proteins or metabolites, e.g., phospholipids, may partition at the surface of the granules. It is not clear whether these molecules are essential for granule formation or are simply trapped there by virtue of their amphipathic nature.

The driving force for this process is the availability of substrate and the free energy generated by the release of CoA by the condensation reaction catalyzed by the synthase. As long as there is sufficient substrate available, the synthase can incorporate it into polymer, and the limitation on production would then become the internal volume of the cell. This process may be occurring concurrently with PHB degradation, as has been reported for *A. eutrophus* (11).

Neither a typical membrane nor membrane-bound polymerization machinery (14) is required to explain the observations, and this model incorporates the recent findings that the polymer remains in an amorphous state in the native granule (1, 3, 4, 6, 10, 22, 23). There are insufficient data to

make any conclusions with respect to the ratio of the number of synthase molecules to the number of polymer chains. We are currently working with purified PHB synthase to examine the requirements for granule formation in vitro. For the first time, we will be able to test if the purified synthase and its substrate alone in an aqueous solution are sufficient for polymer synthesis and granule formation and, if not, to determine what additional factors are required.

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