PhaM Is the Physiological Activator of Poly(3-Hydroxybutyrate) (PHB) Synthase (PhaC1) in Ralstonia eutropha

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Poly[(R)-3-hydroxybutyric acid] (PHB) and related polyhydroxalkanoates (PHAs) are important storage compounds widespread in prokaryotes that enhance survival of the cells in times of starvation (1, 2). PHAs are accumulated under conditions of unbalanced growth (high C-to-N ratio of nutrients) in the form of insoluble inclusions (granules) and can amount up to 90% of the cellular dry matter (storage-PHB). PHB and related PHAs are considered environmentally friendly materials because of their synthesis from renewable resources and their biodegradability via specific PHB/PHA depolymerases (3, 4). PHAs are meanwhile commercially available products, e.g., as packaging materials, but, remarkably, the use of PHAs as high-technology materials available products, e.g., as packaging materials, but, remarkably, the use of PHAs as high-technology materials in the medical field has also made considerable progress (5). PHB in vivo plays also a role in form of PHB-calcium-polyphosphate complexes (6, 7), and recently it was shown that PHBylation of channel protein TRPM8 modulates channel function in mammalian cells (8). This work focuses on the function of PHB as a storage compound in the model organism of PHB research, Ralstonia eutropha H16 (9). The biochemical pathway leading to PHB is rather simple and requires only two enzymatic steps starting from acetyl coenzyme A (acytetyl-CoA) to the monomeric precursor of PHB, 3-hydroxybutyryl-CoA (3HB-CoA) that is polymerized to PHB by PHB synthase PhaC1 (for reviews, see references 1 and 2). Despite the simplicity of the biochemical pathway to the polymer molecule, PHB granules in vivo are highly organized functional units for which the designation “carbonsome” has been suggested (10). The basis for the complexity is the finding of a large and still increasing number of polypeptides that constitute the surface layer (11) of PHB granules in vivo. Nineteen proteins apparently are associated with the proteinaceous surface layer of PHB granules in R. eutropha H16. PHB granule-associated proteins (PGAPs) of R. eutropha H16 include (i) PHB synthase PhaC1 (12–14), (ii) seven PHB depolymerases (PhaZs, PhaZa1 to PhaZa5, PhaZd1, and PhaZd2) (15–18), (iii) two PHB oligomer-hydrolases (PhaZb and PhaZc [alternative designations PhaY1 and PhaY2]) (19, 20), (iv) seven phasins (PhaP1 to PhaP7) (21–24), and (v) two DNA- and PHB-binding proteins (PhaR and PhaM) (25–28). At present, it is not known which and how many of these individually identified PGAPs are expressed and localized on a PHB granule at a given time in vivo. Recent studies of our lab on the function of PhaM suggested that this multifunctional protein specifically binds to DNA and to PHB synthase (PhaC1). PhaM apparently is responsible for attachment of PHB granules to the bacterial nucleoid and ensures that both daughter cells get an almost equal number of PHB granules from the mother cell during cell division (27, 29) as has been suggested for PhaF in medium-chain-length-PHA-accumulating Pseudomonas putida (30, 31).

PHB synthase PhaC1 is the key enzyme in PHB synthesis and its biochemical characteristics were investigated in several contributions in the past (for reviews on PHA synthases, see references 32, 33, 34, and 35; for archaeal PHB synthases and PGAPs in Archaea, see references 36 and 37). It is well known that Cys319 of R. eutropha PhaC1 is the active site to which the precursor, 3-hydroxybutyryl-CoA, is bound via a thioester bond (38, 39). Purification of active PHB synthase from native organism or in recombinant form turned out to be rather difficult. Recombiantly (Escherichia coli) expressed His6-tagged PhaC1 could be purified but the enzyme was isolated in a low activity form. A lag phase of several minutes and a low specific activity were generally observed in in vitro PHB synthase assays (38, 40, 41). Interestingly, the lag phase could be reduced if a detergent was present in the assay mixture. Nonionic detergents such as hecameg [6-O-(N-heptyl-carbamoyl)-methyl-α-D-glucopyranoside], which apparently accelerates the formation of PhaC1 dimers. We identified the PHB granule-associated protein (PGAP) PhaM as the natural primer (activator) of PHB synthase activity. PhaM was recently discovered as a novel type of PGAP with multiple functions in PHB metabolism. Addition of PhaM to PHB synthase assays resulted in immediate polymerization of 3HB coenzyme A with high specific activity and without a significant lag phase. The effect of PhaM on (i) PhaC1 activity, (ii) oligomerization of PhaC1, (iii) complex formation with PhaC1, and (iv) PHB granule formation in vitro and in vivo was shown by cross-linking experiments of purified proteins (PhaM, PhaC1) with glutardialdehyde, by size exclusion chromatography, and by fluorescence microscopic detection of de novo-synthesized PHB granules.

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Overexpression and purification of His<sub>6</sub>-tagged *R. eutropha* PHA synthase. High expression of His<sub>6</sub>-PhaC1 from recombinant *E. coli* BL21(DE3)/pLys harboring pET28a-phaC1 was obtained by induction with 0.1 mM IPTG (isopropyl-β-D-thiogalactoside) at an optical density at 600 nm of ~0.6 and 4 h of subsequent incubation at 30°C. His<sub>6</sub>-PhaC1 was purified in the presence of 0.05% (wt/vol) hecagam via Ni-agarose chromatography according to a protocol described in detail previously (40). Imidazole and other low-molecular-weight molecules were removed by size exclusion chromatography (HiPrep 26/10, desalting column, 53-ml bed volume) and exchanged against 20 mM potassium phosphate buffer (pH 7.5) containing 5% (vol/vol) glycerol and 0.05% (wt/vol) hecagam. Purified His<sub>6</sub>-PhaC1 was concentrated to 4 mg/ml via ultrafiltration and was then shock-frozen in liquid nitrogen and stored at ~−80°C until use. Purification of PhaM-His<sub>6</sub> and PhaP5-His<sub>6</sub> was performed as described previously (43).

**PHB synthase assay.** PHB synthase activity was assayed at 30°C both continuously and discontinuously as follows. For the continuous assay, the assay mixture (a total volume of 1 ml) contained 0.02 to 0.10 mM 3-hydroxybutyryl coenzyme A (3HB-CoA; Sigma), 0 to 300 mM purified His<sub>6</sub>-PhaC1, 0 to 600 nM purified His<sub>6</sub>-PhaM, 1 mM Ellman’s reagent (dithionitrobenzoate [DTNB]) in 150 mM potassium phosphate buffer (pH 7.2), and 0.2% (vol/vol) glycerol. The reaction was initiated by the addition of PHB synthase. Liberated coenzyme A reacted with DTNB to give yellow-colored nitro-thiobenzoate (NTB). The formation of NTB was monitored spectroscopically at 412 nm by using the molar absorption coefficient for NTB of 13,600 M<sup>−1</sup> cm<sup>−1</sup>. PHB synthase activity could be assayed online for a few minutes. However, PhaC1 was sensitive to Ellman’s reagent, presumably because a reaction of Ellman’s reagent with the active-site cysteine and PhaC1 became inactive within several minutes. Therefore, PhaC1 activity was also discontinuously assayed. The reaction principle was the same as for the continuous assay except that Ellman’s reagent was absent and that the reaction was performed in a 0.2-ml volume with 3HB-CoA concentrations up to 0.5 mM in an Eppendorf tube incubator; the reaction was started by the addition of PHB synthase. Aliquots of the reaction mixture (20 μl) were taken at various time intervals and immediately terminated by mixing with 40 μl of 10% (wt/vol) trichloroacetic acid. Precipitated protein was removed by centrifugation, and the concentration of released free coenzyme A was determined by the addition of 145 μl of 1 mM DTNB in 0.5 M potassium phosphate (pH 7.8) to 55 μl of terminated assay mixture. The concentration of formed NTB (i.e., the absorbance at 412 nm) was determined after a 10-min incubation step at room temperature by using a microplate reader. Using the discontinuous assay method, the PHB synthase was stable for the complete assay time. The reaction was limited by the amount of substrate (3HB-CoA) and could be restored by addition of fresh 3HB-CoA. One unit of PHB synthase activity corresponds to the formation of 1 μmol of NTB/minute. The specific activity values mentioned in Results are based on at least two biological and three technical replicates.

**Trypsin treatment of PhaM.** Purified PhaM-His<sub>6</sub> (1.75 μg) was incubated with trypsin (1.25 μg) in PHB synthase assay buffer at 37°C overnight. The reaction was stopped by the addition of trypsin inhibitor (25 μg). A trypsin-treated PhaM preparation was then investigated for PhaC1-activating effect in the discontinuous PHB synthase assay in comparison to a control without trypsin.

**In vitro synthesis of PHB granules.** PHB synthesis was performed in 80 μl of PHB synthesis buffer consisting of 150 mM potassium phosphate buffer (pH 7.2), containing 0.2% (vol/vol) glycerol, 1 mM 3-hydroxybutyryl-CoA (Sigma) and ~160 mM purified His<sub>6</sub>-PhaC1. In some experiments, purified PhaM-His<sub>6</sub> (0 to 120 mM) was also present. Aliquots (10 μl) were removed at various time intervals, and the reactions were terminated by the addition of 5 μl of ethanol containing 1 μg of Nile red/ml. Next, 7-μl portions of each sample were examined for the formation of PHB by fluorescence microscopy. Controls in which PHB synthase or the substrate (3HB-CoA) was omitted showed no formation of Nile red-stainable PHB granules.

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**TABLE 1** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics or features&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source or reference</th>
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<sup>a</sup> Amp<sup>+</sup>, ampicillin resistance; Km<sup>+</sup>, kanamycin resistance.
Fluorescence microscopy. Fluorescence microscopy was carried out as described previously (27) on a Zeiss Axioplan fluorescence microscope using Metaview/Metamorph software (Visitron Systems). All microscopic investigations were performed in triplicate. Typical images are shown.

Chromatography methods. All chromatography steps were performed at room temperature. Eluted fractions were collected immediately on ice. C-terminal His6-tagged PhaM and N-terminal His6-tagged PhaC1 proteins were expressed in E. coli JM109 or BL21(DE3) using the pJOE4036.1 expression vector (PhaM) or pET28a (PhaC1) and purified via standard nickel-agarose (Amersham) affinity chromatography flow columns. For analytical size exclusion chromatography, the buffer was 150 mM potassium phosphate (pH 7.2) containing 0.2% (vol/vol) glycerol (SEC buffer). Chromatography was carried out on a Äkta Purifier System equipped with a Superdex 200 column (prep-grade HR 10/30, geometric column volume \( V_c \) of 23.41 ml, equilibrated with SEC buffer operated at a flow rate of 0.5 ml/min). The column void volume \( V_v \) was determined with blue dextran 2000 to 8.41 ml. One hundred microliters of a 7.5 to 15.1 \( \mu \)M purified His6-PhaC1 solution or 100 \( \mu \)l of a 4.5 to 10.6 \( \mu \)M purified PhaM-His6 solution (or mixtures of PhaC1 and PhaM after a 30-min incubation step on ice) were applied to the column, respectively, and eluted with SEC buffer (detection at 280 nm). The apparent molecular weights of His6-PhaC1 and PhaM-His6 were determined after calibration of the column with standard proteins (Bio-Rad gel filtration standard, catalog-no. 151-1901; GE Healthcare LMW gel filtration calibration kit, catalog no. 28-4038-41). To this end, the partition coefficient \( K_p \) for each protein was calculated using the elution volume \( V_e \) and the formula: 

\[
K_p = \frac{V_v - V_e}{V_e - V_0}
\]

Cross-linking. For in vitro cross-linking studies different mixtures (total volume, 10 \( \mu \)l) of PhaM (300 \( \mu \)g/ml) or PhaC1 (4 mg/ml) in SEC buffer (pH 7.5) were mixed and stored on ice for 30 min before 0.5 \( \mu \)l of a freshly prepared glutardialdehyde (1,5-pentanedial) solution (2.5%) was added. The mixture was subsequently incubated at 37°C for 5 min. The reaction was stopped by addition of 1 M Tris-HCl (pH 8). Samples were mixed with 5.5 \( \mu \)l of 3X loading buffer and analyzed by SDS-PAGE and subsequent staining with colloidal Coomassie brilliant blue G-250.

Other techniques. Protein determination was performed according to the bicinchoninic acid method. Conjugation experiments with E. coli S17-1 harboring the pBBR1MCS2::PphaC1::phaM as the donor and R. eutropha ΔphaP1 as the recipient were performed by cross-streaking both strains directly on selective medium (mineral salts medium with 0.5% [wt/vol] fructose and appropriate antibiotics). Transconjugants appeared after 2 to 3 days at 30°C and were purified and checked for the presence of plasmids.

RESULTS

His6-tagged PHB synthase (His6-PhaC1) and PhaM-His6 were purified as described in Materials and Methods. Both proteins were \( \approx 95\% \) pure as judged by SDS-PAGE analysis. When purified His6-PhaC1—as isolated—was used in the discontinuous PHB synthesis assay, a lag phase of 2 to 4 min was reproducibly observed until the polymerization reaction started and proceeded with maximal velocity. This lag phase is well known from literature (33, 38, 40–42). We first speculated that the active center of PhaC1, Cys319, possibly could be present in an inactive, oxidized form. The background for this assumption was the sensitivity of PhaC1 to Ellman’s reagent, DTNB, which is a thiol oxidizing agent. If oxidation of Cys319 was the reason for the partially inactive state, reducing agents such as glutathione should be able to reactivate PhaC1. However, when we preincubated concentrated PhaC1 with an excess amount of glutathione and started the PHB synthesis assay by the addition of the PhaC1-glutathione mixture to the assay solution, the lag phase of the PHB polymerase reaction was still present and essentially not affected at all (data not shown; the effect of glutathione on the cleavage of Ellman’s reagent to give NBT resulted in a modest rise of the baseline of the reaction and was taken into account). In conclusion, we found no experimental support for the assumption that Cys319 of PhaC1—as isolated—could be partially present in an inactive (oxidized) form.

When we tested the influence of hecameg on His6-PhaC1 activity, we found that 0.05% hecameg partially and 0.25% hecameg strongly reduced the lag phase to 1 min compared to a control with no addition (Fig. 1A). This finding is in agreement with previous contributions of others (41). In our earlier studies on PHB granule formation, we showed by fluorescence microscopy that newly synthesized PHB granules occurred very rapidly within 5 min after application of PHB permissive conditions in starved (PHB-free) cells of R. eutropha (i.e., after transfer of starved cells to fresh NB-glucanate medium) (24). If a PHB granule of \( \approx 250 \) nm in diameter is formed in the cells within only 5 min, it is unlikely that PHB synthase has a significant lag phase of 2 to 4 min in vivo. Rather, it seems that the constitutively expressed PHB synthase is “waiting” for its substrate (3HB-CoA) in an active form. As soon as the cells are fed with glucanate, the central Entner-Doudoroff pathway and PhaA/PhaB proteins provide 3HB-CoA units that are polymerized immediately to PHB. Therefore, we assume that PHB synthase is present in vivo in an active state and that no lag phase is required. However, which component could be responsible for the conversion of inactive (monomeric) PhaC1 to active dimeric or oligomeric PhaC1?

We have previously identified PhaM as a novel PGAP protein with phasin-like and DNA-binding properties (27). We showed by bimolecular fluorescence complementation that PhaM and PhaC1 were localized close to each other in vivo in R. eutropha (43). We therefore hypothesized that PhaM could also be important in vivo for PHB synthase activity. Figure 1B shows the time course of PhaC1-catalyzed PHB synthesis in the presence of different amounts of purified PhaM. The concentration of His6-PhaC1 was \( \approx 165 \) nM in all experiments. The typical lag phase of 2 to 3 min was observed until maximal PHB synthase activity was reached, when no PhaM was added (control, black circles). The presence of only 3.2 or 6 nM PhaM reduced the lag phase to \( \approx 1 \) min or 30 s (gray circles, data only for 3.2 nM are shown). Preincubation of PhaC1 (165 nM) with PhaM (6 nM) and start of reaction by the addition of 3HB-CoA had no beneficial effect compared to the same reaction in which the reaction was started by the addition of PHB synthase (graph not shown). When the reaction was performed in the presence of 16 or 32 nM PhaM, the lag phase was reduced to 15 to 30 s, and the specific activities increased from 4.8 U/mg \( \sim 3\)-fold compared to the control with no PhaM to 16.4 U/mg. The time necessary to start the reaction was 15 s (i.e., the addition of PhaC1, mixing, taking the first sample, and stopping the reaction). Remarkably, reduction of the lag phase and an increase in the specific activity were not affected by heating of PhaM to 95°C for 20 min (open circles in Fig. 1C), and this indicated a high thermostability of PhaM. When the same experiment was repeated with an independently purified PhaM preparation (0, 10, 25, 50, and 100 nM PhaM tested at a constant PhaC1 concentration of 165 nM), an almost complete reduction of the lag phase and 3-fold increase in specific activity (\( \sim 15 \) to \( \sim 17 \) U/mg) was obtained with \( \approx 25 \) nM PhaM. Higher concentrations of PhaM (50 and 100 nM) had no significant additional effect on the reaction (data not shown). However, trypsin treatment of PhaM destroyed the activating effect of PhaM (Fig. 1C). When PhaM was
placed by bovine serum albumin (BSA; up to 600 nM tested) or phasin PhaP5 (up to 1.5 μM tested), no reduction of the lag phase was determined, and the typical lag phase and low specific activity were observed again (graphs not shown). The activating effect of PhaM on PHB synthase activity was also observable in the continuous assay (Fig. 1D). His8-PhaC1 became inactivated before a significant activity could be observed (three graphs in light, medium, and dark gray at the bottom of Fig. 1D). However, when the reaction was started in the presence of PhaM significant PHB synthase activity was determined within the first 5 min (three different 3HB-CoA concentrations tested, the upper three black graphs in Fig. 1D) before all synthase molecules became inactivated. When fresh PHB synthase (PhaC1) was added to an already inactivated reaction (in the presence of PhaM) PHB synthesis was resumed for a few minutes (Fig. 1D, bottom right). This finding confirmed that the presence of DTNB in the assay mixture is inhibitory and results in inactivation of PhaC1 within minutes. Despite the negative effect of the presence of DTNB in the continuous assay, the activating effect of PhaM on PHB synthase activity was clearly seen in the first minutes of the assay. In conclusion, both activity assays clearly showed that small amounts of PhaM are sufficient to specifically reduce the lag phase of the PHB synthase reaction and to increase the specific activity. This activating (“priming”) effect of PhaM was stronger than that of hecameg, of any other detergent (42) or other proteins (e.g., PhaP5 and BSA). Our findings suggest that PhaM is the physiological “primer” or “activator” of the PHB synthase reaction.
PhaM-PhaC1 complex formation in the presence of glutardialdehyde. The PHB synthase experiments shown above clearly demonstrated the priming effect of PhaM on PHB synthesis. To find independent evidence for the interaction of PhaM with PhaC1 and to estimate the size of the protein complexes, we performed cross-linking experiments. To this end, variable amounts of PhaC1 and PhaM were mixed in the presence of glutardialdehyde (for details, see Materials and Methods). The aldehyde groups form covalent bonds with free amino groups of proteins and due to the five carbon atoms spacer between the two carbonyl oxygen atoms glutardialdehyde can cross-link different proteins that are localized close to each other. The complexes formed were then separated by SDS-PAGE. Lanes 2 to 4 of Fig. 2 show the individual proteins (PhaM-His6, lane 2), His6-PhaC1 (lane 3), and a mixture of both without glutardialdehyde (lane 4). The arrows point to the subunits of PhaM and PhaC1. When PhaM and PhaC1 were cross-linked individually the monomer band of PhaM disappeared completely and high-molecular-mass products of PhaM and glutardialdehyde (lanes 7 to 10). 0.6, 1.2, 2.4, and 6 μM PhaM in lanes 7 to 10, respectively).

PhaM-PhaC1 complex formation in the absence of glutardialdehyde. To characterize and to test the stability of the formed PhaC1-PhaM complexes PhaC1 was allowed to react with different amounts of PhaM in the absence of glutardialdehyde and the complexes were separated after a 30-min incubation step on ice by size exclusion chromatography on a Superdex 200 column (Fig. 3). When the amount of PhaM was increased from 4.5 to 10.6 μM, the PhaC1 monomer peak (at ~14.82 ml) strongly decreased in favor for a HMW peak (10.69 to 11.28 ml) corresponding to an apparent molecular mass in the range of ca. 300 to 400 kDa. The area of the high-molecular-mass peak increased with increasing amounts of PhaM, and the area of the PhaC1 monomer peak decreased. When this HMW peak was separated by SDS-PAGE, both PhaC1 and PhaM bands were present, and no lag phase was observed in the PHB synthase assay using the HMW fraction. In contrast, only PhaC1 (without detectable amounts of PhaM) was detected in the 14.8-ml monomer peak (data not shown). As expected, this monomeric PhaC1 preparation showed a lag phase in PHB synthase assay. Notably, the peak of PhaM alone eluted slightly later from the Superdex 200 column (11.06 ml corresponding to ~326 kDa) than the peak of the PhaM-PhaC1 complex (10.69 ml corresponding to ~380 kDa). However, a precise resolution of this difference is difficult because of limited resolution of the column in the HMW region. These data clearly show that PhaC1 and PhaM interact with each other and form stable complexes at least under conditions of size exclusion chromatography.
raphy. We conclude that this HMW PhaM–PhaC1 complex represents the active, “primed” form of PHB synthase.

In vitro and in vivo synthesis of PHB granules in the presence of PhaM. Purified His$_6$–PhaC1 was used to synthesize PHB in vitro in an 80-μl assay in the absence or presence of PhaM–His$_6$ as described in Materials and Methods. The reaction was allowed to proceed for one up to 8 min before the reaction was terminated by the addition of ethanolic Nile red solution. The Nile red-stained PHB granules were visualized by fluorescence microscopy. As shown in Fig. 4, the number of granules increased, and the average size of the granules decreased in the presence of PhaM. No granules were detected in the absence of PhaC1. A similar effect of PhaM was observed in vivo: when PhaM was constitutively overexpressed in a R. eutrophaphaP1 background, the cells produced PHB granules.

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**Figures**

**Fig. 3.** Size exclusion chromatography of PhaM, PhaC1, and of PhaM–PhaC1 complexes. Analytical gel filtration was carried out on a Superdex 200 column (for details, see Materials and Methods). Calibration of the column was performed using the following standard proteins: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), myoglobin (17 kDa), RNase (13.7 kDa), aprotinin (6.5 kDa), and vitamin B$_{12}$ (1.35 kDa). The elution profiles of 670-, 158-, 44-, 17-, and 1.35-kDa standard proteins are indicated (dashed line). The elution profiles of purified PhaM–His$_6$ ($V_e = 11.06$ ml) and of purified His$_6$–PhaC1 ($V_e = 14.82$ ml) and of mixtures containing different amounts of PhaM and PhaC1 are shown. Void volume of the column (A, unspecific protein aggregates) was determined as 8.41 ml. M and D, monomer and dimer of PhaC1. HMW, high-molecular-weight fraction. Note that the increasing amounts of PhaM added to PhaC1 led to a decrease of the PhaC1 monomer peak and to the formation of HMW products.

**Fig. 4.** In vitro synthesis of PHB granules in the absence (A) or presence (B) of PhaM. Purified PhaC1 (160 nM) was incubated with 1 mM 3-HB-CoA and with or without PhaM (120 nM). Samples were removed at different time intervals, terminated by the addition of an ethanolic Nile red solution, and examined by fluorescence microscopy for the formation of PHB as described in Materials and Methods. The formation of PHB granules was visible in phase-contrast images and fluorescence images by staining with Nile red. The addition of PhaM led to a rapid increase of more and smaller PHB granules. Scale bar, 5 μm.
a large number of Nile red-stainable granules in the middle (nucleoid) region of the cells during growth on PHB permissive conditions (NB-glucconate) (Fig. 5). Apparently, the elevated number of PhaM molecules led to an increased number of PHB granule initiation sites. Control cells (R. eutropha ΔphaP1 with empty plasmid) produced only one or two subterminal or polar-localized PHB granules.

**DISCUSSION**

The molecular events happening at the onset of PHB granule formation in PHB-accumulating bacteria have kept PHA researchers engaged for many years. Several models of PHB granule formation were proposed in the past. The budding model (32, 33) assumes initiation of PHB synthesis at or in the cytoplasmic membrane via membrane-attached or membrane-integrated PHB synthase. The budding model was favored by our group previously (10, 44, 45), and this assumption was experimentally based on microscopic observation of periphery- or cell pole-localized PHB granules at the early stages of PHB granule formation in growing R. eutropha cells. The budding model could be recently excluded at least for R. eutropha by electron cryo-tomography (46). The authors of that study never observed PHB granules at or near the cytoplasmic membrane. The second model, the micelle model, assumes the presence of soluble PHB synthase molecules that start to synthesize PHB molecules once the cellular metabolism has provided enough substrate molecules (3HB-CoAs). A consequence of the micelle model is that the initiation locus of PHB granules should be anywhere in the cytoplasm. The micelle model was the favorite model for many PHA researchers in the past. However, this model cannot explain the nonrandom localization of “early” PHB granules, i.e., the frequent observation of PHB granules near the cell center in nongrowing cells (46–48) or the finding of periphery-located PHB granules in growing cells (24, 45, 49). Nucleoid occlusion of PHB granules (50) in growing cells might be one explanation for the frequent observation of periphery-localized PHB granules, and this assumption would allow to keep the micelle model. However, the identification of PhaM as a novel PHB- and DNA-bound protein (27, 29) and the in vivo interaction of PhaC1 and PhaM in R. eutropha (43) is in disagreement with the assumption of a random localization of PhaC1 in the cytoplasm. It is more compatible with a third model, the scaffold model, in which the PHB synthase PhaC1 is attached to an internal cellular structure, most likely to the bacterial nucleoid. Depending on the physiological state of the cells (growing versus nongrowing) PHB granules appear more often in the central region (bound to the resting nonreplicating nucleoid in stationary cells) or in the cell periphery (bound to the dividing nucleoid in growing cells). At present, we do not know whether the attachment site of PHB granules to the nucleoid is random or is specifically controlled by the cells.

To answer the question which of the different models of PHB granule formation is correct, it is necessary to understand the events that happen at the onset of the polymerization reaction on the molecular level. Fortunately, the PHB synthase reaction can be performed and assayed in vitro using purified PHB synthase molecules and commercially available or self-synthesized monomers (3HB-CoA). In principle, the PHB synthase reaction does not need any cofactors given that PHB synthase and substrate (3HB-CoA) are present under appropriate physical conditions (aqueous environment, appropriate pH and temperature). Recently, Cho and coworkers of the Sinskey-Stubbe group elegantly purified PHB synthase from recombinant R. eutropha cells in which the chromosomal phaC1 gene had been replaced by a strep2-tagged phaC1 gene. Active PHB synthase without lag phase was purified in a HMW complex form with soluble PHB (350 kDa) and phasin protein PhaP1. Low-molecular-weight PhaC1 was also present (in monomeric and dimeric form), but this fraction required a lag phase for maximal PHB synthase activity. The authors concluded that the PHB synthase of the HMW complex form represents the “primed” active synthase. However, it remained unsolved which of the complex constituents (PHA, PhaP1, other components) was responsible for the activating effect of PHB synthase. The authors also identified minor amounts of a few (at least four additional) proteins that copurified with strep2-tagged PhaC1 one of which
was a 32-kDa protein. This 32-kDa protein had been identified in our group as PhaM shortly before the Cho paper was finished. However, the effect of PhaM on PhaC1 activity was not described either by Cho et al. or by us. Later, we found by bimolecular fluorescence complementation that PhaM interacts with PhaC1 in vivo in R. eutropha and is responsible for attachment of PHB granules to the bacterial nucleoid (27, 29). In the present study, we were able to discover a new (additional) function of PhaM: as little as ~16 nM PhaM was sufficient to switch ~165 nM PhaC1 from the inactive (lag phase) to the active (no lag phase) form. Moreover, the specific activity of the PhaM-activated form of PhaC1 was considerably higher (~17 versus ~5 U/mg) compared to the maximal activity determined for PhaC1 in the absence of PhaM (Fig. 1). Our findings suggest that one PhaM molecule is sufficient to activate approximately 10 to 11 molecules of PhaC1. Even if we take into consideration some error in protein determination, it is evident that PhaM is required only in substoichiometric amounts. Because of the tendency of purified PhaM to disintegrate the PhaM to PhaC1 ratio in the complex may be even lower (~1:12). This finding is in agreement with the low abundance of the 32-kDa characteristic of PhaM is its insensitivity to heat treatment. Incubation of PhaM at 95°C for 20 min did not prevent the activating effect of other proteins, such as BSA or PhaP5 (the present study) or PhaP1 (28), even when they were applied in large excess, were not able to convert PhaC1 into the active form. Another interesting characteristic of PhaM is its insensitivity to heat treatment. Incubation of PhaM at 95°C for 20 min did not prevent the activating effect of PhaM (Fig. 1). A comparable heat stability of a PGAP is known for PhaM (Fig. 1). A comparable heat stability of a PGAP is known for phasin ApdA of Rhodospillum rubrum (51, 52).

Taken together, the results of Cho et al., the results of the present study, and our previous results on PhaM suggest that PhaM, PhaC1, and presumably DNA itself or a component that is bound to DNA are the constituents for the initiation complex of PHB granules. Our in vitro studies show that DNA per se is not necessary for the formation of the active initiation complex, but in vivo the DNA-binding properties of PhaM apparently direct the initiation complex to the nucleoid region that could possibly constitute the scaffold to which the emerging PHB granules become attached. Phasins such as PhaP5 or PhaP1 are not required for active PHB synthase, and we conclude that both phasins are not involved in the initiation complex formation and probably attach to PHB after the onset of PHB granule formation. Future research will show whether other, not-yet-discovered molecules play a role in the formation and subcellular localization of PHB granules in R. eutropha in particular and of PHA granules in PHA accumulating bacteria in general.

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