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Purification of Polyhydroxybutyrate Synthase from Its Native Organism, *Ralstonia eutropha*: Implications for the Initiation and Elongation of Polymer Formation in Vivo

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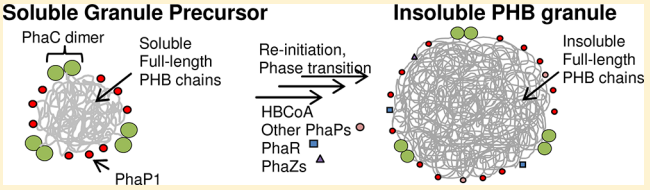
Supporting Information

ABSTRACT: Class I polyhydroxybutyrate (PHB) synthase (PhaC) from *Ralstonia eutropha* catalyzes the formation of PHB from (*R*)-3-hydroxybutyryl-CoA, ultimately resulting in the formation of insoluble granules. Previous mechanistic studies of *R. eutropha* PhaC, purified from *Escherichia coli* ($PhaC_{Ec}$), demonstrated that the polymer elongation rate is much faster than the initiation rate. In an effort to identify a factor(s) from the native organism that might prime the synthase and increase the rate of polymer initiation, an N-terminally Strep2-tagged *phaC* (Strep2-$PhaC_{Re}$) was constructed and integrated into the *R. eutropha* genome in place of wild-type *phaC*. Strep2-$PhaC_{Re}$ was expressed and purified by affinity chromatography from *R. eutropha* grown in nutrient-rich TSB medium for 4 h (peak production PHB, 15% cell dry weight) and 24 h (PHB, 2% cell dry weight). Analysis of the purified PhaC by size exclusion chromatography, sodium dodecyl sulfate−polyacrylamide gel electrophoresis, and gel permeation chromatography revealed that it unexpectedly copurified with the phasin protein, PhaP1, and with soluble PHB (M_w = 350 kDa) in a "high-molecular weight" (HMW) complex and in monomeric/dimeric (M/D) forms with no associated PhaP1 or PHB. Assays for monitoring the formation of PHB in the HMW complex showed no lag phase in CoA release, in contrast to M/D forms of $PhaC_{Re}$ (and $PhaC_{Ec}$), suggesting that PhaC in the HMW fraction has been isolated in a PHB-primed form. The presence of primed and nonprimed PhaC suggests that the elongation rate for PHB formation is also faster than the initiation rate in vivo. A modified micelle model for granule genesis is proposed to accommodate the reported observations.



Polyhydroxybutyrate (PHB) synthase (PhaC) catalyzes the polymerization of (*R*)-3-hydroxybutyryl-coenzyme A (HBCoA) to PHB. These polyoxoesters are produced and packaged into insoluble inclusions or granules by a variety of bacteria as carbon storage compounds, when the bacteria are faced with nutrient-limited growth conditions in the presence of an abundant carbon source.[1−5] When the environment is more conducive to replication, the biopolymers are degraded and the liberated energy and monomers are used for cell growth and division. PHBs and copolymers of PHB and polyhydroxyvalerate (PHV) are of general interest because they are biodegradable thermoplastics. Understanding the polymerization process resulting in high-molecular weight and low-polydispersity PHB and the genesis of PHB granules is important for optimizing the metabolic engineering of organisms to produce PHB/PHV and other polyoxoesters in an economically competitive fashion with environmentally unfriendly, petroleum-based plastics.[6,7] Thus far, synthases have primarily been isolated from recombinant expression in *Escherichia coli*, which does not have the biosynthetic machinery for PHB production.[8−13] This paper reports the first isolation of a synthase from its native organism, *Ralstonia eutropha*, and the unexpected observation that it copurifies with PHB and the phasin protein PhaP1 in a soluble form. The former observation suggests isolation of PHB-primed PhaC and the latter observation isolation of soluble granule precursors, providing insight into insoluble granule formation.

R. eutropha, which possesses a class I synthase, is the best characterized PHB-producing organism.[1,4,14,15] Biochemical studies using *R. eutropha* synthase expressed and purified from *E. coli* ($PhaC_{Ec}$) have shown that the polymerization process involves covalent catalysis requiring an active site cysteine.[16] Kinetic studies monitoring CoA release as an indicator of polymer formation reveal a lag phase that is dependent on the concentration of the enzyme[8,14,17] and that the lag phase can be reduced and the activity increased by artificially priming $PhaC_{Ec}$ with a trimer or saturated trimer of HBCoA [$(HB)_3$ CoA or sTCoA (Figure 1)].[14,16] The synthase has been shown to exist in monomeric and dimeric forms, and priming of the synthase with sTCoA shifts the equilibrium to the dimeric form, suggesting that the synthase is active as a dimer.[16] Kinetic studies further reveal that the elongation rate is much faster than the initiation rate; that is, a small amount of $PhaC_{Ec}$ catalyzes formation of a large PHB polymer, while most of the PhaC remains unreacted.[8,18] If the kinetics of polymerization are similar in vivo, then it is difficult to understand how the cell

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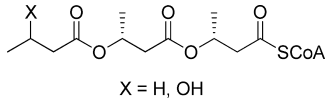

Figure 1. Structure of $(HB)_3$CoA and sTCoA. Primers for in vitro production of PHB by $PhaC_{Ec}$. X = OH for $(HB)_3$CoA. X = H for sTCoA.

controls the generation of granules in which all of the proteins involved in PHB homeostasis are located on their exterior with amorphous PHB on their interior.[19−22] Thus, we proposed that a factor(s) might be present in *R. eutropha* that primes the synthase, allowing control of the polymer initiation rate relative to its elongation rate that, in turn, would facilitate controlled granule formation.

A number of protein factors have been identified by their association with purified PHB granules or by bioinformatics, but no candidates for a protein initiation factor(s) have been reported.[3,4] The phasin protein PhaP1 is of particular interest as it is the most abundant granule-associated protein.[21] Its expression is induced by PHB,[23] and its expression levels increase concomitant with PHB production.[24,25] In contrast, PhaC is constitutively expressed[25,26] at substantially lower levels than PhaP1. At maximal PHB production in rich, tryptic soy broth (TSB) medium or in nitrogen-limited medium (PHB_{high}), the granules range in size from 0.2 to 0.5 μm in diameter and each granule has one to two molecules of PhaP1 per PHB chain and 50−100 PhaC molecules per PHB chain requiring many chain termination and reinitiation events during granule formation.[25] PhaP1 controls the amount,[23,24] size, and number of granules[21] and likely prevents PHB crystallization.[27] While other putative phasin proteins (PhaP2−PhaP5, PhaP1 homologues) have been identified, their expression levels are low compared to that of PhaP1.[28−30] Other proteins associated with granules include PhaR, a transcription factor that negatively regulates PhaP1 expression,[31,32] and the PhaZ proteins, which are intracellular depolymerases.[33−35] These protein factors are associated with the surface of the PHB granules based on immunogold labeling studies,[20,21,31] but only PhaP1 contributes substantially (14−28%) to the granule surface coverage.[25,36]

Our initial objective in the studies described here was to identify an initiation factor via isolation of the synthase from its native organism. For this purpose, a strain of *R. eutropha* was constructed in which an N-terminal Strep2-tagged *phaC* gene was integrated in place of the wild-type *phaC* gene in the *R. eutropha* genome to express an affinity-tagged synthase under its endogenous promoter. We chose rich TSB medium for growth conditions, under which PHB is transiently produced (Figure S1 of the Supporting Information). Under these conditions, the amount of PHB in the cells increases to a maximum of ∼15% of the cell dry weight (cdw) at 4 h and then declines to 2% over 24 h because of degradation of the PHB chains.[25] To facilitate PhaC purification, we initially chose to isolate the synthase after cultivation for 24 h to minimize the amount of PHB in the cells and, thus, the amount of PhaC associated with insoluble granules precluding its isolation.[8,25,37] The affinity tag allowed rapid purification of PhaC, which was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and by size exclusion chromatography (SEC). Surprisingly by SEC, PhaC eluted in a soluble high-molecular weight (HMW) fraction containing PHB and PhaP1 as well as in a dimeric/monomeric form without PHB or PhaP1. Furthermore, assays of the PhaC in the HMW fraction revealed no lag phase in CoA release. Thus, although no candidate protein for an initiation factor was identified by SDS−PAGE, our studies suggested that a PHB-primed synthase was isolated for the first time.[16] We then focused on purification of PhaC from cells cultivated in TSB medium for 4 h (Figure S1 of the Supporting Information) when PHB production was maximized. Our prior transmission electron microscopy (TEM) studies showed that small, "initiating" granules as well as 0.2 μm diameter granules are present at this time point,[25] and recent cryoelectron tomography studies suggest nascent granules are continually generated during PHB production.[38] Therefore, we predicted that this growth condition might allow isolation of soluble granule precursors, providing insight into the mechanism of granule formation. Again, a soluble HMW fraction that includes PhaC, PhaP1, and high-molecular weight PHB was isolated by SEC. These results suggest that we have isolated a primed synthase from a native organism and that PHB functions in this capacity. They also suggest that the elongation rate for PHB polymerization is much faster than the initiation rate in vivo. Finally, the isolation of a soluble PhaC−PhaP1−PHB complex precludes a membrane budding model for the mechanism of granule genesis and suggests an alternative micelle model.

EXPERIMENTAL PROCEDURES

Materials. All strains and plasmids used in this study are listed in Table 1. All chemicals were purchased at the highest available purity from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). *Taq* DNA polymerase was purchased from Qiagen (Valencia, CA), and other DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). (*R*)-3-Hydroxybutyryl-CoA (HBCoA) was synthesized as previously described.[15]

Protein Quantitation and Sequencing. The PhaC concentration was based on A_{280} (ε = 162000 M^{-1} cm^{-1}).[15] For heterogeneous mixtures of protein, PhaC and PhaP1 concentrations were determined by quantitative Western blotting, as detailed below. Total protein concentrations in heterogeneous mixtures were determined by the Bradford assay using BSA as a standard.[39] Protein N-terminal sequencing was performed by the Tufts University School of Medicine Core Facility.

Enzyme Assays. Assays were conducted as previously described[14] with the concentration of PhaC being 30−60 nM. The average specific activity from three assays performed at three different enzyme concentrations is reported where one unit of activity is defined as 1 μmol of CoA released per minute.[14]

Construction of an N-Terminally Tagged Strep2-$(G_4S)_3$-*phaC* (*strep2-phaC*) for Expression in *E. coli*. For construction of *strep2-phaC*, *phaC* from *R. eutropha* strain H16 was amplified by polymerase chain reaction (PCR) using the forward primer phaCSTREPFW and the reverse primer phaCSTREPRV (Table 2) to introduce a *Bam*HI restriction site followed by the coding sequence for a $(G_4S)_3$ linker at the 5′ end of *phaC* and a *Hin*dIII restriction site at the 3′ end. The amplified gene was cut with the appropriate restriction enzymes and ligated into pET51b (Novagen), containing the gene sequence for an N-terminal Strep2-$(G_4S)_3$ tag, to produce pCJB16. The portion of pCJB16 containing the tagged *phaC*

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Table 1. Strains and Plasmids

strain	genotype	ref
R. eutropha		
Re1034	H16, Δ*phaC*	23
Re1052	H16, Δ*phaP1*	24
Re1053	Re1034, Δ*phaP1*	this work
Re2016	Re1034 with N-terminal $(His)_6$-tagged *phaC* gene inserted into Δ*phaC* locus	this work
Re2018	Re1034 with N-terminal Strep2-tagged *phaC* gene containing a $(G_4S)_3$ linker inserted into Δ*phaC* locus	this work
Re2019	Re1053 with N-terminal Strep2-tagged *phaC* gene containing a $(G_4S)_3$ linker inserted into Δ*phaC* locus	this work
Re2017	Re1034 with N-terminal Strep2-tagged *phaC* C319A mutant gene containing a $(G_4S)_3$ linker inserted into Δ*phaC* locus	this work
E. coli		
BL21(DE3)	strain for protein expression from pET-based vectors.	Novagen
S17-1	strain for conjugative transfer of plasmids into *R. eutropha*	64
plasmid	description	ref
pET51b	N-terminal Strep2 tag cloning plasmid, confers amp resistance	Novagen
pJQ200mp18Km	gene replacement plasmid, confers kan resistance and sucrose sensitivity by *sacB* gene	41
pJV7	pJQ200mp18Km containing *phaC* deletion allele in the multiple-cloning site; *phaC* deletion allele has a *Swa*I restriction site inserted between 5′ upstream and 3′ downstream fragments of *phaC*	40
pCJB16	pET51b with N-terminal Strep2-tagged *phaC* gene containing a $(G_4S)_3$ linker between the tag and PhaC, confers amp resistance	this work
pCJB4	pJV7 with N-terminal Strep2-tagged *phaC* gene containing a $(G_4S)_3$ linker in the *Swa*I restriction site, gene replacement plasmid	this work
pCJB16-C319A	pCJB16 with C319A mutation, confers amp resistance	this work

was sequenced by the Massachusetts Institute of Technology Biopolymers Laboratory.

Integration of *strep2-phaC* into the *R. eutropha* Chromosome in Place of wt-*phaC*.[23,24] Plasmid pJV7,[40] a derivative of pJQ200mp18Km containing kan resistance (kan^R) and sucrose sensitivity (*sacB*) markers for homologous recombination,[41] was utilized for integration of *strep2-phaC* into the *R. eutropha* chromosome. To generate pJV7, we inserted the deletion allele of $phaC_{Re}$ (Δ*phaC*) into the *Bam*HI site in the multiple-cloning region of pJQ200mp18Km. The Δ*phaC* allele consists of the 0.41 kb sequence located immediately upstream of the $phaC_{Re}$ open reading frame (ORF) ligated adjacent to the 0.45 kb sequence located immediately downstream of the $phaC_{Re}$ ORF via a *Swa*I restriction site. For insertion of *strep2-phaC* into pJV7, the gene was first excised from pCJB16 by digestion with *Hin*dIII and *Xba*I. The gene was blunt ended using the Klenow fragment (New England Biolabs) and then ligated into the *Swa*I site of pJV7 to create pCJB4 (Table 1).

The resulting plasmid was introduced into *R. eutropha* Re1034 (Δ*phaC*)[23] by conjugation with *E. coli* S17-1/pCJB4. Transconjugants of *R. eutropha*, in which pCJB4 was inserted into the genome in the first recombination event, were selected by being plated on tryptic soy agar supplemented with 0.2% fructose, 10 μg/mL gentamicin (gent) to select for *R. eutropha*, and 300 μg/mL kanamycin (kan) to select for the recombination event. These transconjugants were then grown in TSB medium supplemented with 10 μg/mL gent for 24 h at 30 °C and spread on tryptic soy agar with 0.2% fructose, 10 μg/mL gent, and 5% sucrose to select for the second recombination event in which plasmid pCJB4 is excised from the genome to produce the desired mutant strain. All sucrose resistant colonies were tested for kan sensitivity to ensure the loss of the pCJB4 insert. They were also tested for PHB production, indicating the presence of a functional Strep2-$PhaC_{Re}$. PCR was used to confirm the presence of a *strep2-phaC* gene replacement. The resulting strain, Re2018, was used for purification of Strep2-$PhaC_{Re}$.

Construction of *C319A-strep2-phaC*. Primers *phaC*-C319A and *pha*CC319A-GC (Table 2) were used in conjunction with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to mutagenize the codon for the active site cysteine (TGC) to an alanine (GCG) and create pCJB16-C319A (Table 1). The resulting *C319A-strep2-phaC* was excised from pCJB16-C319A and inserted into the *R. eutropha* chromosome as described above.

Growth of *E. coli* BL21(DE3)/pCJB16 and Purification of Strep2-$PhaC_{Ec}$. All culture medium was supplemented with 100 μg/mL ampicillin (amp). A single colony of BL21(DE3)/pCJB16 cells from an LB plate was used to inoculate 5 mL of LB culture broth. Cells were grown overnight at 37 °C. The entire culture was used to inoculate 1 L of LB at 37 °C. Cells were grown to an OD_{600} of 0.6, and the expression of Strep2-$PhaC_{Ec}$ was induced by addition of 0.1 mM isopropyl β-thiogalactoside (IPTG). Cells were then grown at 30 °C for an additional 2 h. Cells (~5 g wet weight) were pelleted by centrifugation at 3000*g* and 4 °C. The cell pellet was resuspended in 25 mL of 100 mM Tris-HCl (pH 8.0) and 0.05% Hecameg and lysed using a French pressure cell (two passes at 12000 psi). The resulting cell lysate was centrifuged at 100000*g* to remove cell debris. The clarified lysate (25 mL, 3.5 mg of protein/mL) was loaded onto a Strep-tactin column (IBA, GmbH, Göttingen, Germany; 10 mL column volume) pre-equilibrated with buffer A [100 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA]. The lysate and column were incubated at 4 °C for 15−20 min. The column was washed with 5 × 20 mL of buffer A containing 0.05% Hecameg. Strep2-$PhaC_{Ec}$ was eluted from the column with 30 mL of buffer A containing 0.05% Hecameg, 1 mM desthiobiotin, and 10% glycerol, and 5 mL fractions were collected. Hecameg detergent

Table 2. Primers Used in This Study

name	sequence[a]
phaCSTREPFW	5′-GAGTAGGGATCCCATGGGCGGCGGCGGCTCCGGCGGCGGCGGCTCCGGCGGCGGCGGCTCCGCGACCGGCAAAGG CGCGGCAGCTTCCAC-3′
phaCSTREPRV	5′-CTAGTGAAGCTTTCATGCCTTGGCTTTGACGTATCGCCC-3′
phaCC319A	5′-ACGTGCTCGGCTTCGCGGTGGGCGGCACCA-3′
phaCC319A-GC	5′-TGGTGCCGCCCACCGCGAAGCCGAGCACGT-3′

[a]Restriction sites underlined.

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(Sigma) was added to the buffers to improve recoveries of Strep2-PhaC from the column as previously described.[8] Concentrations of Hecameg were 10-fold below the critical micelle concentration. Fractions containing Strep2-PhaC as determined by SDS−PAGE and A_{280}/A_{260} were pooled and concentrated using a Vivaspin 6 concentrator (Sartorius AG, Göttingen, Germany) to 5 mg/mL and stored at −80 °C. Protein (12.5 mg/g of cells) with a specific activity of 39 ± 11 units/mg was obtained.

Growth of Re2018 for Purification of Strep2-PhaC$_{Re}$. All culture medium was supplemented with 10 μg/mL gent, and all growth occurred at 30 °C. A single colony of *R. eutropha* Re2018 (Table 1) was used to inoculate 5 mL of TSB culture broth. The culture was grown overnight and was used to inoculate 250 mL of TSB in a 2.8 L baffled flask, which was then also grown overnight. The 250 mL culture was used to inoculate, to an initial OD_{600} of 0.5, either 3 L of TSB in a 4 L BioEngineering (Wald, Switzerland) L1523 fermentor or 8 L of TSB in a 10 L New Brunswick (Edison, NJ) Microferm fermentor. Cells were grown for 24 h at 400 rpm in the 4 L fermentor (Strep2-PhaC$_{Re24h}$) or 4 h at 500 rpm in the 10 L fermentor (Strep2-PhaC$_{Re4h}$). In both fermentations, aeration was maintained by sparging with air at 1 volume of air per volume of culture per minute. The pH was not controlled. Cells were harvested by centrifugation at 3000*g* for 20 min at 4 °C, giving a cell paste of 20 g/L (24 h culture) and 4 g/L (4 h culture).

Purification of Strep2-PhaC$_{Re24h}$. The cell pellet (40 g) was resuspended in 240 mL of 100 mM Tris-HCl (pH 8.0) and 0.05% Hecameg and lysed by two passes through a French pressure cell at 12000 psi. The cell debris was removed by centrifugation at 100000*g*, and 10 mL of Strep-tactin resin was added to the supernatant, which was stirred at 4 °C for 30 min. The resin was loaded into a column and was washed with 100 mL of buffer A containing 0.05% Hecameg. Strep2-PhaC$_{Re24h}$ was eluted using 30 mL of buffer A containing 0.05% Hecameg, 1 mM desthiobiotin, and 10% glycerol, and 5 mL fractions were collected. Fractions containing Strep2-PhaC as determined by SDS−PAGE and A_{280}/A_{260} were pooled and concentrated using a Vivaspin 6 concentrator to 8 mg/mL and stored at −80 °C. Protein (0.14 mg/g of cells) with a specific activity ranging from 30 to 90 units/mg was obtained, based on three different purifications.

Purification of Strep2-PhaC$_{Re4h}$. The purification was similar to that for Strep2-PhaC$_{Re24h}$ except that a cell pellet of 1 g was resuspended in 5 mL of 100 mM Tris-HCl (pH 8.0), 0.05% Hecameg, and 2 units/mL DNase (New England Biolabs). The crude extract was incubated with the DNase for 30 min during cell lysis. After lysis, cell debris was removed by centrifugation at 30000*g*, and 5 mL of the Strep-tactin resin was added to the supernatant. All wash and elution volumes described above were reduced by a factor of 2. Protein (0.72 mg/g of cells) with a specific activity ranging from 35 to 100 units/mg was obtained, based on four different purifications.

Size Exclusion Chromatography (SEC). Purified Strep2-PhaC$_{Ec}$, Strep2-PhaC$_{Re4h}$, or Strep2-PhaC$_{Re24h}$ (500 μg) was injected onto a Bio-Sil SEC 250-5 column [M_w range of 10000−300000 Da (Bio-Rad, Hercules, CA)] attached to a Waters 515/2487 high-performance liquid chromatography (HPLC) system at room temperature (RT). The eluent consisted of 50 mM KP_i (pH 7.6), 150 mM NaCl, and 0.05% Hecameg, and the flow rate was 0.5 mL/min. Fractions (200 μL) were collected in a 96-well plate on ice. Each fraction was monitored for A_{280} using a plate reader (Bio-Rad Ultramark EX). Fractions corresponding to the high-molecular weight (HMW), dimeric, and monomeric Strep2-PhaC species were pooled and concentrated to 1 mg/mL protein using a Vivaspin 6 concentrator. Samples were analyzed via SDS−PAGE and assayed for activity and PHB content via the crotonic acid assay.[42] Retention times of the different fractions containing Strep2-PhaC were compared with retention times of molecular weight standards (Bio-Rad): bovine thyroglobulin (M_w of 660 kDa, elution time of 13.8 min), bovine γ-globulin (158 kDa, 16.1 min), chicken ovalbumin (44 kDa, 17.1 min), horse myoglobin (17 kDa, 20.9 min), and vitamin B_{12} (1350 Da, 24.0 min).

Quantification of PHB in Whole Cells and PHB Associated with PhaC$_{Re}$. *R. eutropha* H16 (wt) and Re2018 (*strep2-phaC*) cells grown in TSB as described above were dried at 80 °C (10−20 mg dry weight) and used to determine the levels of PHB in whole cells as described below. To determine the amount of PHB associated with purified protein samples [Strep2-PhaC$_{Ec}$ (2 mg), Strep2-PhaC$_{Re4h}$ (120 μg), Strep2-PhaC$_{Re24h}$ (1 mg) or fractions of Strep2-PhaC$_{Re}$ (30−500 μg) isolated by SEC−HPLC; amounts determined by Bradford assays], each sample was added to a borosilicate tube and heated overnight in vacuo at 80 °C. Samples were then dissolved in 1 mL of concentrated H_2SO_4, and PHB quantitation was performed using the crotonic acid assay.[42] Samples or dilutions of samples (25 μL) were injected onto a Bio-Rad Aminex HPX-87H organic acid column attached to an Agilent 1100 series HPLC system and eluted at a flow rate of 0.6 mL/min at 50 °C with 5 mM H_2SO_4. Elution was monitored by A_{210}. The retention time for crotonic acid was 29 min. Standard curves of peak area versus PHB concentration (2−200 μg/mL) were generated using commercially available PHB (Sigma).

Quantitative Western Blotting. Samples of purified Strep2-PhaC$_{Re4h}$ before SEC and the HMW fraction of Strep2-PhaC$_{Re4h}$ (20−30 ng of protein) were resolved via 10% SDS−PAGE for Strep2-PhaC or 15% SDS−PAGE for PhaP1. Protein was blotted onto a PVDF membrane (Immun-Blot, Bio-Rad) at 100 V for 80 min on ice using a Criterion wet blotting apparatus (Bio-Rad). The blotting buffer for Strep2-PhaC sample consisted of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, and 0.01% (w/v) SDS, and for PhaP1, the blotting buffer was identical with 15% (v/v) methanol. Blots were blocked in 3% (w/v) milk in Tris-acetate-EDTA (TAE) buffer containing 0.1% (v/v) Tween 20. The primary rabbit antibodies for PhaP1 were purified by acetone powder extraction using the Re1052 (Δ*phaP1*) strain.[24,43] Blots were incubated with anti-PhaC antibody (diluted 1:5000) or anti-PhaP1 antibody (diluted 1:2500) in blocking buffer for 1 h at RT. Goat anti-rabbit antibody, conjugated with horseradish peroxidase (Thermo Scientific, Rockford, IL), was used as the secondary antibody (1:2000). All membranes were developed using SuperSignal West Femto chemiluminescent detecting reagents (Thermo Scientific). Signals were detected using a Bio-Rad ChemiDoc XRS imager and analyzed with Quantity-One 1-D (Bio-Rad). The protein standard curves were generated with Strep2-PhaC$_{Ec}$ (0.5−10 ng) and PhaP1 (2−15 ng) purified from *E. coli*.

For quantitative whole cell Western blots, samples were normalized on the basis of the OD_{600} of the cells used for sample preparation. Generally, 10 μL of the cell suspension (OD_{600} from 1 to 10, depending on whether a PhaP1 or PhaC Western blot was performed) in Laemmli buffer was loaded.[44]

For standard curve generation, Re1052 and Re1034 (Table 1) cell samples in Laemmli buffer were spiked with 2−15 ng of purified PhaP1 and 0.5−10 ng of purified Strep2-PhaC$_{Ec}$, respectively, immediately prior to gel loading for blotting consistency. All blotting conditions were the same as described above.

Extraction of PHB and Gel Permeation Chromatography (GPC) Analysis. Soluble and insoluble fractions of the crude extract of Re2018 or Strep2-PhaC$_{Re4h}$ (3−200 mg) were lyophilized in a glass vial. PHB was extracted directly from the resulting residue by refluxing in chloroform at 70 °C for 48 h with stirring. After being cooled to RT, the samples were filtered through a 4 mm syringe filter containing a 0.45 μm PTFE membrane (Whatman, Piscataway, NJ) to remove insoluble material. Sample volumes were adjusted to a final PHB concentration of 3 mg/mL as determined by the crotonic acid assay. Samples (100 μL) were injected onto 2 × (300 mm × 7.5 mm) Plgel Olexis columns (Varian, Palo Alto, CA) attached to an Agilent 1100 series HPLC system fitted with refractive index detection, and products were eluted at a flow rate of 1 mL/min at 30 ° C with chloroform. Retention times of sample peaks were adjusted on the basis of an internal isopropanol standard (retention time of 21.0 min) and compared with polystyrene narrow molecular weight standards (Varian) with sizes (retention times) of 1.1 kDa (18.6 min), 3.1 kDa (17.9 min), 10 kDa (17.0 min), 25 kDa (16.4 min), 72 kDa (15.6 min), 210 kDa (14.9 min), 490 kDa (14.2 min), 1800 kDa (13.2 min), and 5000 kDa (12.9 min). To determine the M_p, M_n, and M_w of each sample, Agilent GPC add-on (revision B.01.01) was used.

RESULTS

Construction of the *strep2-phaC* Gene and the Corresponding Replacement Strain Re2018. To purify PhaC from the native organism, several gene constructs encoding different N-terminal affinity-tagged PhaCs with various linkers for expression in both *E. coli* and *R. eutropha* were generated. Initially, a (His)$_6$-PhaC with no linker was investigated. Expression and isolation of this construct from *E. coli* yielded homogeneous protein and excellent recoveries. However, attempts to purify the same protein after integration of the gene into the *R. eutropha* genome were unsuccessful because of weak binding of the tag to the Ni^{2+} affinity resin.[45] Thus, a Strep2 tag, with the amino acid sequence WSHPQFEK, was investigated.[46] Ultimately, the *strep2-phaC* construct encoding a linker with the triplicate repeat of four glycines and one serine, $(G_4S)_3$, between the tag and PhaC was chosen for further study. Isolation of the protein expressed in *E. coli* BL21(DE3) yielded a homogeneous enzyme with a lag phase in CoA release (Figure 2) and a specific activity of 39 ± 11 units/mg, similar to the activity of the untagged PhaC previously reported, 40−50 units/mg.[16] The variability in the specific activity is due to difficulty in identifying the linear part of the activity curve as described in Figure S2 of the Supporting Information.

Given the comparable activity of Strep2-PhaC$_{Ec}$ and untagged-PhaC, the gene construct was incorporated into the *R. eutropha* genome in place of wt-*phaC* to generate strain Re2018.[23,24] Rich TSB medium[25] was chosen for growth of Re2018 instead of nitrogen-limited medium[24] for maximal PHB production, to minimize the amount of PhaC bound to insoluble granules that has previously defied purification by many groups.[8,37] Growth of Re2018 in TSB medium resulted in maximal production of PHB by 4 h (15%), which then dropped substantially by 24 h (Figure S1 of the Supporting Information) as PHB is degraded to replace the depleted carbon source. A similar trend has been observed for wt *R. eutropha* H16 in TSB medium.[25] Analysis of the amounts of PhaC in the Re2018 and wt strains using quantitative Western blots with whole cells showed 35-fold upregulation in the mutant strain during parallel growths in shake flasks (Figure S3A of the Supporting Information). The level of expression of phasin protein PhaP1 was increased 1.5-fold (Figure S3A of the Supporting Information). The upregulation of PhaC production was not expected, is not understood, and is likely the result of insertion of the *strep2* tag into the *phaCAB* operon. Recent studies have shown that insertions into the *phaCAB* operon can affect expression from this operon.[47] The upregulation of PhaC may fortuitously have played a role in our ability to isolate soluble granules described subsequently.

Purification of Strep2-PhaC$_{Re24h}$. Initial purification efforts focused on Strep2-PhaC from cells grown in TSB medium for 24 h (Figure S1 of the Supporting Information), conditions that minimize the amount of PhaC associated with insoluble granules. Chromatography on a Strep-tactin column yielded a highly purified enzyme with a specific activity ranging from 30 to 90 units/mg [Strep2-PhaC$_{Re24h}$ (Table S2 of the Supporting Information)], based on three different purifications. In contrast with PhaC$_{Ec}$ assayed at the same concentration, however, there was no lag phase in the release of CoA (Figure 2), unexpectedly suggesting the presence of a PHB-primed synthase.[16] The broad range of specific activities measured is likely associated with variability in the extent of primed synthase within and between different purifications (Figure 2 and Figure S2 of the Supporting Information).[16]

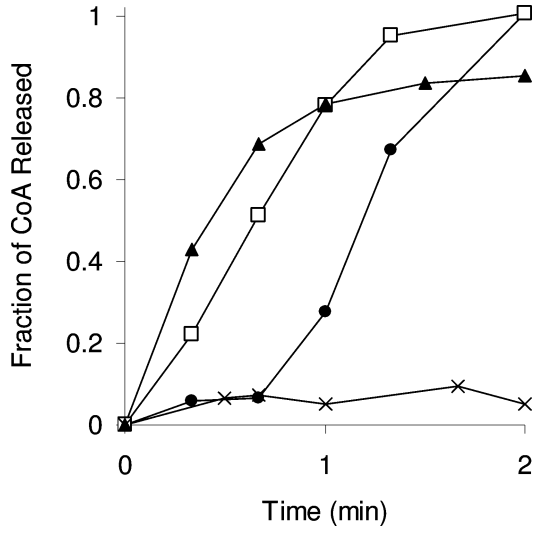

Figure 2. Time course of release of CoA from HBCoA at 30 °C catalyzed by Strep2-tagged synthases purified from *R. eutropha* after growth in TSB for 24 and 4 h and from *E. coli*. The reaction mixtures for Strep2-PhaC$_{Re24h}$ (□), Strep2-PhaC$_{Ec}$ (●), and C319A Strep2-PhaC$_{Re}$ (×) contained 60 nM enzyme and 750 μM HBCoA. The reaction mixture for Strep2-PhaC$_{Re4h}$ (▲) contained 60 nM enzyme and 600 μM HBCoA. HBCoA concentrations used in the assays are well above the K_m (100 μM).[17]

Identification of Proteins That Copurify with Strep2-PhaC$_{Re24h}$. By affinity chromatography isolation of Strep2-PhaC$_{Re24h}$ from the host organism, we hoped to identify a

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factor(s) that could increase the initiation rate of the polymerization process relative to the elongation rate and consequently provide insight into the priming process in vivo. Thus, we examined affinity-purified Strep2-PhaC$_{Re24h}$ by SDS–PAGE (Figure 3A, lane 2). In addition to PhaC (64 kDa), five proteins with molecular weights of 20, 32, 45, 60, and 75 kDa were observed and subjected to N-terminal sequencing analysis. Each of the copurifying proteins was identified (Table S1 of the Supporting Information), but none appeared to be a reasonable candidate for a primer of the synthase.[35]

The 60 and 75 kDa proteins are annotated as the β and α subunits, respectively, of an acetyl/propionyl-CoA carboxylase (GenBank entries Q0KA97 and Q0KA96), the first committed step of fatty acid biosynthesis.[35] The α subunit of this enzyme is biotinylated, is known to copurify with the β subunit, and therefore likely copurified with PhaC because of their affinity for the Strep-tactin resin. These same proteins were previously reported to copurify with Strep2-tagged [NiFe]-hydrogenases from *R. eutropha* H16[48] using this resin. The 45 kDa protein is annotated as a hypothetical protein (GenBank entry Q0K3J8). A BLAST search showed that the protein is 84% identical to a sulfotransferase in *R. eutropha* JMP134 and is an unlikely candidate for PhaC priming. The 32 kDa protein is identified as a hypothetical, membrane-associated protein (GenBank entry Q0KFC8). The gene is located downstream of a crotonase-like enoyl-CoA hydratase and upstream of an α–β hydrolase family esterase annotated to be in the patatin phospholipase superfamily, suggesting that this protein is involved in fatty acid metabolism.[35] However, while our paper was under review, Jendrossek and co-workers identified this protein in a bacterial two-hybrid screen using PhaC as the bait protein.[49] From their studies, they hypothesized that it may be responsible for binding the granules to the bacterial nucleoid through its interactions with PhaC during cell segregation and granule formation. They renamed this protein PhaM. We will return to this protein in the Discussion.

The most striking observation is the identification of the 20 kDa protein as PhaP1, the most abundant protein associated with PHB granules and calculated to cover 14–28% of the granule surface.[25,36] PhaP1 was eliminated as a priming factor because the presence of recombinantly purified PhaP1 was shown to increase the lag phase and decrease the rate of the linear phase in activity assays of PhaC with HBCoA (Figure S4 of the Supporting Information), contrary to expectations for a priming factor. No other phasin proteins (PhaP2–PhaP5) were detected.[28,30] The identification of PhaP1 is supported by its absence in Strep2-PhaC$_{Re24h}$ purified from a Δ*phaP1* strain, Re2019 (Figure 3B, lane 2). The absence of PhaP1 in the Δ*phaP1* strain was previously demonstrated using Western blots.[24] The 20 kDa protein observed is biotin carboxyl carrier protein, likely copurifying because of its affinity for the Strep-tactin resin. It is unclear why this protein is observed only in Strep2-PhaC$_{Re24h}$ isolated from the Δ*phaP1* strain. Finally, in a similar experiment, a Strep2-PhaC$_{Re24h}$ mutant with its active site cysteine replaced with alanine (C319A) was purified from a C319A Strep2-PhaC$_{Re}$ strain, Re2017. It also failed to reveal PhaP1 (Figure 3B, lane 3). Thus, the detection of PhaP1 requires active PhaC, suggesting the importance of PHB for their copurification.

Strep2-PhaC$_{Re24h}$ Contains an Active High-Molecular Weight Fraction by SEC That Contains PHB and PhaP1. An assay of Strep2-PhaC$_{Re24h}$ revealed the absence of a lag phase in CoA release (Figure 2), supporting the presence of a primed PhaC. Our previous in vitro studies revealed that primed PhaC is predominantly in the dimeric form.[16] SEC,

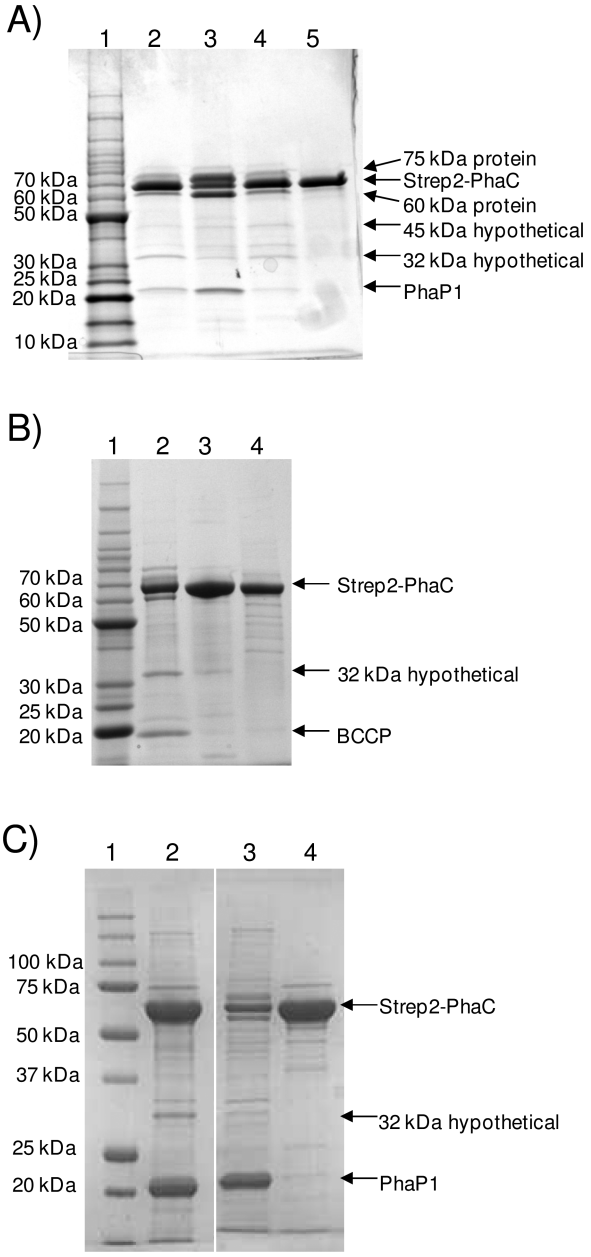

Figure 3. SDS–PAGE gels of unfractionated, SEC-purified HMW, dimer, and monomer fractions of the Strep2-tagged synthases purified from *R. eutropha* strains after growth in TSB for 24 and 4 h and from *E. coli*. (A) Gradient SDS–PAGE (4 to 15%) gel of Strep2-PhaC$_{Re24h}$ SEC fractions: lane 1, molecular weight standards; lane 2, unfractionated Strep2-PhaC$_{Re24h}$; lane 3, HMW fraction; lane 4, dimer fraction; lane 5, monomer fraction. (B) Gradient SDS–PAGE (4 to 15%) gel of Strep2-PhaC purified from a Δ*phaP1 R. eutropha* strain, C319A Strep2-PhaC purified from a *C319A-strep2-phaC R. eutropha* strain, and Strep2-PhaC purified from *E. coli* as controls: lane 1, molecular weight standards; Lane 2, Strep2-PhaC purified from a Δ*phaP1 R. eutropha* strain; lane 3, C319A Strep2-PhaC$_{Re}$; lane 4, Strep2-PhaC$_{Ec}$. (C) SDS–PAGE (10%) gel of Strep2-PhaC$_{Re4h}$ SEC fractions: lane 1, molecular weight standards; lane 2, unfractionated Strep2-PhaC$_{Re4h}$; lane 3, HMW fraction; lane 4, dimer/monomer fraction. Strep2-PhaC, PhaP1, biotin carboxyl carrier protein (BCCP), and copurifying 32, 45, 60, and 70 kDa proteins identified by Edman sequencing are labeled accordingly.

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however (Figure 4A), revealed in contrast to expectations the presence of a HMW protein fraction based on its short retention time, as well as later eluting fractions containing dimeric and monomeric synthase, similar to Strep2-PhaC$_{Ec}$ (compare panels A and C of Figure 4). Assays of the pooled HMW fractions exhibited no lag phase in CoA release and a specific activity of 35 ± 12 units/mg (Table S2 of the Supporting Information). In contrast, the monomeric and dimeric forms of Strep2-PhaC$_{Re24h}$ exhibited lag phases, although they had similar specific activities (30 ± 7 units/mg). SDS−PAGE analysis of the SEC fractions showed that PhaP1 is associated with only the HMW fraction (Figure 3A, lane 3). The small amount of PhaP1 in the dimer fraction (Figure 3A, lane 4) is due to the poor resolution of HMW and dimeric fractions. We note that an aggregate fraction is observed with Strep2-PhaC$_{Ec}$ (Figure 4C), also previously found in other purifications of PhaC from *E. coli*.[15] In contrast with the HMW fraction observed with Strep2-PhaC$_{Re24h}$, however, the aggregate fraction of Strep2-PhaC$_{Ec}$ exhibits a low specific activity (~10 units/mg) compared with those of the dimer/monomer fractions and is comprised of only PhaC.

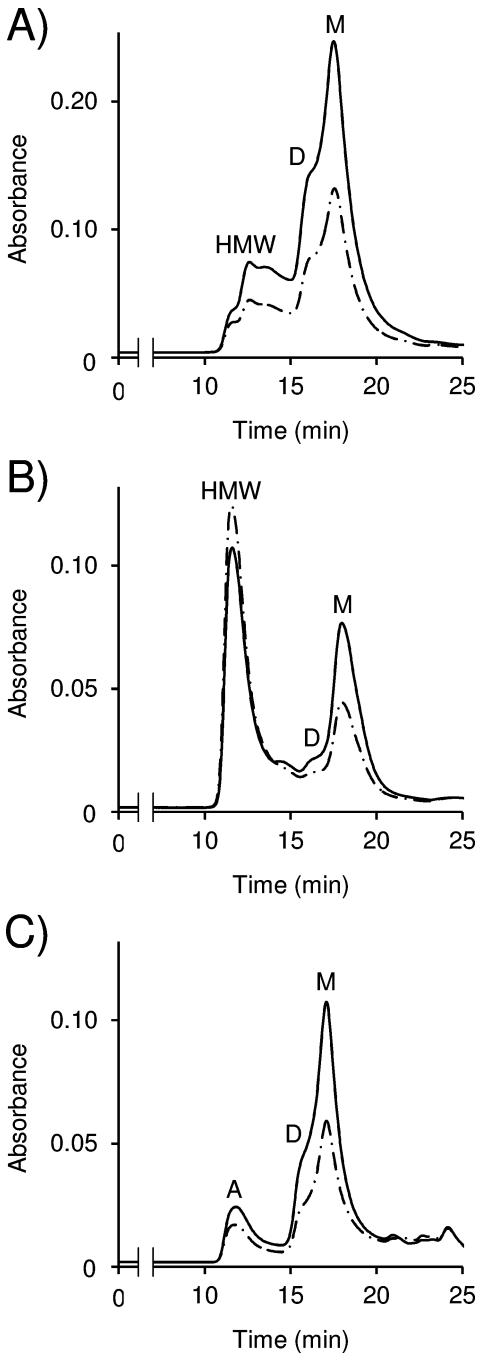

Figure 4. Bio-Sil SEC250-5 size exclusion chromatography of Strep2-tagged synthases purified from *R. eutropha* after growth in TSB for 24 and 4 h and from *E. coli*. (A) SEC of Strep2-PhaC$_{Re24h}$. (B) SEC of Strep2-PhaC$_{Re4h}$. (C) SEC of Strep2-PhaC$_{Ec}$. Peaks D and M are the dimer and monomer fractions, respectively. Peaks labeled HMW are the high-molecular weight fractions containing PHB. Peak A is a protein aggregate, containing no PHB. The solid line shows the absorbance at 280 nm; the dotted line shows the absorbance at 260 nm.

C319A Strep2-PhaC$_{Re24h}$ had no HMW fraction (Figure S5 of the Supporting Information), suggesting that the HMW fraction of Strep2-PhaC$_{Re24h}$ contained PHB. Analysis of all the SEC fractions from the experiment and mutant control for PHB via the crotonic acid assay[42] revealed its presence only in the HMW fraction. The observation of a soluble HMW fraction containing PHB, PhaC, and PhaP1 was unexpected and suggested that this fraction may result from precursors to granules or resolubilized breakdown products of insoluble granules given its isolation at 24 h (Figure S1 of the Supporting Information). This soluble, primed Strep2-PhaC$_{Re}$ could be providing insight about polymer initiation and granule genesis, and thus, we next attempted isolation of Strep2-PhaC$_{Re}$ during maximal PHB production at 4 h (Figure S1 of the Supporting Information), a time when granule initiation is more frequently occurring.[25,38]

Purification of Strep2-PhaC$_{Re4h}$. After growth in TSB medium for 4 h, 83% of the total PHB in crude extracts is in the insoluble fraction, while 17% is soluble. Affinity chromatography of the soluble fraction resulted in a reduced flow rate for the column, suggesting some sample precipitation, and significant loss of activity in the column flow through and washes, suggesting inaccessibility of the Strep2 tag. A summary of the Strep2-PhaC$_{Re4h}$ purification is given in Table 3 and Figure S6 of the Supporting Information. Despite the low overall recovery of units, highly purified, active Strep2-PhaC$_{Re4h}$ (35−100 units/mg) was isolated.

Table 3. Activity for the Purification of Strep2-PhaC$_{Re4h}$

purification step	volume (mL)	protein (mg)	activity (units/mg)[a]	total units	% yield
crude extract[b]	5.0	140	3.4	470	100
soluble fraction	4.3	120	2.8	340	72
insoluble fraction	2.1	15	8.7	130	28
flow through	7.2	89	1.2	110	22
washes	60	28	1.1	31	6
pure sample	0.52	0.94	36	34	7

[a]One unit of enzyme activity is defined by 1 μmol of CoASH released per minute. [b]The crude extract for Strep2-PhaC$_{Re4h}$ purification originated from 1 g of cells.

SEC Reveals a HMW Fraction with Strep2-PhaC$_{Re4h}$, PhaP1, and PHB. SEC of Strep2-PhaC$_{Re4h}$ revealed a HMW fraction (Figure 4B) in addition to dimeric and monomeric PhaC. Some DNA copurified with the HMW fraction despite

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incubation with DNase for 30 min during cell lysis (dashed line in Figure 4B and Figure S6 of the Supporting Information). In light of recent data of Jendrossek and co-workers for PhaM, it is possible that DNA is present due to association of granules with the nucleoid.[49] The HMW fraction isolated via SEC was assayed for activity; it exhibited no lag phase in CoA release and had a specific activity of 47 ± 13 units/mg. The pooled monomeric and dimeric fractions of Strep2-PhaC$_{Re4h}$ showed a lag phase and a specific activity of 38 ± 8 units/mg (Figure 5

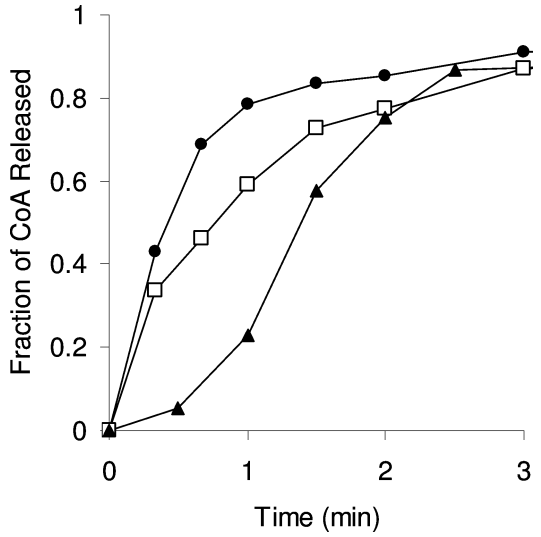

Figure 5. Time course of release of CoA from HBCoA at 30 °C catalyzed by unfractionated Strep2-PhaC$_{Re4h}$ and HMW and dimer/monomer SEC fractions of Strep2-PhaC$_{Re4h}$. The reaction mixtures contained 600 μM HBCoA and either 60 nM unfractionated Strep2-PhaC$_{Re4h}$ (●), 60 nM HMW fraction (□), or 80 nM dimer/monomer fraction (▲). Assays for the unfractionated, HMW, and dimer/monomer fractions of Strep2-PhaC$_{Re24h}$ are similar to the assays for the respective Strep2-PhaC$_{Re4h}$ fractions.

and Table S2 of the Supporting Information), similar to Strep2-PhaC$_{Re24h}$ and Strep2-PhaC$_{Ec}$. SDS−PAGE analysis revealed PhaP1 in both the unfractionated material and the HMW fraction (Figure 3C, lanes 2 and 3, respectively). Quantitative Western blots of the HMW fraction from four different preparations gave PhaP1:PhaC molar ratios from 2:1 to 4:1 (Figure S7 of the Supporting Information). The 32 kDa (PhaM),[49] 60 kDa, and 75 kDa proteins were also observed (Figure 3C, lanes 2 and 3), while the 45 kDa protein was absent. Crotonic acid assays of the SEC fractions show PHB associated with only the HMW fraction. The recovery of PHB from the SEC column is 15−30%, likely associated with "granule" precipitation (Figure S6 of the Supporting Information). The HMW and dimeric/monomeric PhaC fractions suggest that in vivo, the elongation rate is faster than the initiation rate.

***M*$_w$ of PHB in Strep2-PhaC$_{Re4h}$.** While the HMW fraction migrates as a large aggregate on the SEC matrix, we did not know how a PHB polymer or oligomers would affect the retention time of PhaC in this matrix. The size of the PHB has important implications for understanding the priming of PhaC and initiation of granule formation. To determine the size distribution of the PHB associated with purified Strep2-PhaC$_{Re4h}$, we extracted the PHB by refluxing it in chloroform for 2 days. Crotonic acid assays revealed 74−100% of the total PHB was recovered. This material was analyzed by gel permeation chromatography (GPC) and gave a weight average molecular weight (M_w) of 350 kDa with a polydispersity index (PDI) of 1.7 relative to polystyrene standards. No attempt was made to isolate PHB from the HMW fraction of SEC because of the poor recovery from the column and the requirement for large amounts of material for GPC analysis. Control experiments using GPC to measure the M_w of the PHB isolated from chloroform extraction of the soluble and insoluble crude cell extract fractions showed M_w values of 320 and 400 kDa and PDIs of 1.7 and 2.1, respectively. Our results indicate that high-M_w PHB chains can be soluble, potentially providing important insight into how PHB is packaged before it becomes an insoluble inclusion.[3,4]

Our GPC results indicate that this mutant strain produces PHB chains that are shorter than the isogenic wt strain grown under identical conditions (M_w of 990 kDa with a PDI of 1.9), similar to our previous measurements made after growth in TSB medium for 4 h in a shake flask.[25] These results can be rationalized, as PhaC levels in the mutant strain are substantially higher (35-fold) than in the wt strain and previous studies in *E. coli* have shown that the molecular weight of PHB is decreased with increased synthase levels.[50]

Finally, we also looked for $(HB)_n$ oligomeric chains in the extracted PHB from Strep2-PhaC$_{Re4h}$, which may be initially involved in priming the synthase. GPC, in which the refractive index signal is proportional to the mass of PHB and independent of its chain length,[51] failed to reveal any $(HB)_n$ oligomers. If short oligomers were present, they are <5% of the extracted material by mass given the limit of detection of this method.

Strep2-PhaC$_{Re4h}$:PhaP1:PHB Ratios. Our previous studies of the wt strain showed that the PhaC:PhaP1:PHB molar ratio in whole cells, assuming PhaC and PhaP1 are monomers, was ~0.01:1:1 after growth in TSB medium for 4 h.[25] The observation of a 1:1 PHB:PhaP1 ratio suggested an important mechanistic role for PhaP1. The low PhaC:PHB ratio of 0.01:1 suggested that PhaC can reinitiate polymer formation after chain termination when PHB chains of defined length are generated.[18,25] Using the M_w of 350 kDa and the amounts of PHB and protein determined by crotonic acid assays and quantitative Western blots, respectively, the Strep2-PhaC:-PhaP1:PHB molar ratio was calculated to range from 1:4:1 to 3:9:1 in the HMW fraction (based on three different preparations) compared to 0.1:0.6:1 in the Re2018 mutant whole cells grown for 4 h in TSB (Table 4). In whole cells from

Table 4. Strep2-PhaC:PhaP1:PHB Molar Ratios in the HMW Fraction of Strep2-PhaC$_{Re4h}$ and in Whole Cells

purification step	Strep2-PhaC$_{Re}$	PhaP1	PHB
HMW fraction	1−3	4−9	1
Re2018 whole cells	0.1	0.6	1
wt whole cells[a]	0.01	1	1

[a]From ref 25.

the Re2018 strain, as with the wt strain, the PhaP1:PHB ratio is ~1:1. However, in the HMW fraction, the ratio is increased to 4−9:1. Although there are several explanations for this observation, we suggest that in the HMW fraction, several PhaP1 molecules associate with a high-molecular weight PHB chain to form a structure to facilitate polymer solubility and that this PhaC−PhaP1−PHB complex may be the initial,

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nucleating species for granule formation. With time, as more and more HB units are added to this species, the PhaP1:PHB ratio decreases from 4−9:1 to 1:1 and the Strep2-PhaC:PHB ratio decreases from 1−3:1 to 0.1:1 (Table 4). The Strep2-PhaC:PHB ratio in the HMW fraction of 1−3:1 is within error of the stoichiometry of one PHB chain per PhaC dimer observed when the synthase is artificially primed in vitro.[16] This ratio thus suggests that the HMW fraction contains primed synthase in which the primer is $(HB)_n$, where n is large but less than or equal to ~4000 (350 kDa). A large PHB chain serving as the primer is consistent with the elongation rate of PHB formation being much greater than the initiation rate demonstrated in vitro.[8,18] The copurification of PhaC, PhaP1, and PHB and changes in their ratios provide the basis for our modified micelle model for granule genesis described subsequently (Figure 6A2).

DISCUSSION

In vitro assays of all synthases examined to date have elongation rates for PHB formation that are much faster than the initiation rates.[8,10−13,18,52] This observation has made it challenging to study the initiation or priming process and to formulate a model for granule genesis that prevents cellular toxicity associated with nonspecific hydrophobic binding of cellular components to the PHB. We therefore undertook a search for a protein(s) in *R. eutropha* that might be involved in increasing the rate of initiation by a priming process similar to our previous observations with artificial primers [Figure 1, $(HB)_3$CoA and sTCoA].[16] Because PhaC has never been isolated from its host organism because of its prevalence on insoluble inclusions,[8] our approach was to engineer a strain of *R. eutropha* to express an affinity-tagged PhaC. The strategy was successful, and a highly purified Strep2-tagged $PhaC_{Re}$ was isolated from native *R. eutropha* for the first time (Figure 3).

Studies with this PhaC have allowed a number of important observations to be made about the initiation and elongation steps of PHB polymerization. First, the activity assays with Strep2-$PhaC_{Re}$ showed no lag phase in CoA production, distinct from the case in recombinantly expressed $PhaC_{Ec}$, suggesting that $PhaC_{Re}$ is primed. GPC analysis of the PHB isolated with $PhaC_{Re}$ suggests that it is primed by $(HB)_n$, where n is large. Thus, while our initial efforts were focused on the identification of a "protein" primer, our studies reveal that the primer is likely $(HB)_n$ itself.[53] Second, SEC analysis revealed that the purified material contained not only the soluble HMW complex but also unmodified dimeric/monomeric PhaC. The observation of two populations of PhaC suggests that the polymer elongation rate is much faster than the initiation rate as previously observed in vitro.[8,18] Thus, we are still left with the issue of how granule genesis is controlled.

We believe that insight into this mechanism has been provided by the unexpected isolation of soluble PhaC in a complex with PhaP1 and high-molecular weight PHB chains. On the basis of the mole ratios of these species within the complex, a modified micelle model for granule genesis (Figure 6A2) is presented. While the HMW complex is kinetically competent at PHB production, the demonstration that it is on pathway to insoluble granules will require additional experimental approaches.

Models for Granule Genesis. In vitro studies have shown that granules can be generated with HBCoA and PhaC only, although they are much larger and the PHB has a higher

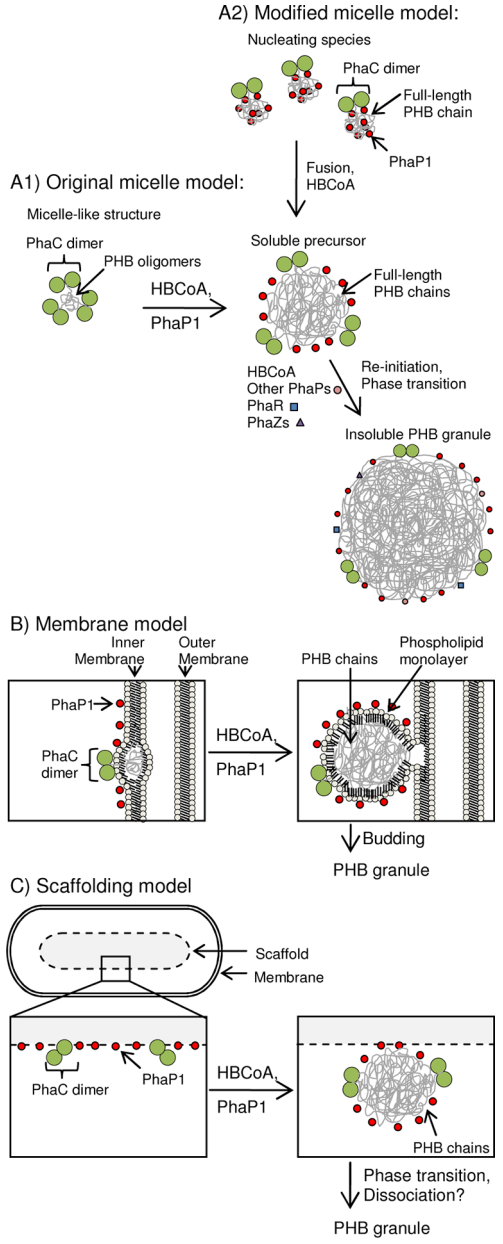

Figure 6. Models for granule formation. PhaC and PhaP1 are represented as green and red spheres, respectively. PhaP1 is represented as a monomer in the figures but may have higher oligomeric states (i.e., trimer). (A) Micelle models for granule formation. In the original micelle model (A1), several short PHB chains covalently attached to PhaC associate to form an initial micelle-like structure, to which PhaP1 later binds in a soluble granule precursor. The original micelle model assumes that the polymer elongation rate is controlled relative to the initiation rate to allow short PHB chains to aggregate. In the modified micelle model (A2), single, full-length PHB chains covalently attached to PhaC associate with PhaP1 early on in a nucleating species to allow the polymer elongation rate to be faster than the initiation rate. Several nucleating species fuse together to form a soluble granule precursor. The soluble granule precursor later undergoes a phase transition to form the mature granule. (B) Membrane model. In this model, PhaC and PhaP1 bind to the inner leaflet of the inner membrane with PhaC synthesizing PHB chains into the membrane. Eventually, the nascent granule buds from the membrane with PhaC, PhaP1, and a phospholipid monolayer found at the surface. (C) Scaffolding model. In this model, PhaC and PhaP1 interact with a protein and/or DNA scaffold in the cytoplasm of the cell, which serves as the starting point for granule formation.

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molecular weight than granules found in vivo.[54−56] Thus, granules can form by self-assembly. Therefore, it is not surprising that a self-assembly micelle model was the first proposed mechanism for granule genesis.[57] However, recently, a membrane budding model[4,58,59] and a scaffolding model[25,60] have also been considered as alternatives.

Our studies have led us now to favor a modified micelle model. It differs from the original micelle model (Figure 6A1) in which $(HB)_n$ oligomers (n is small) covalently attached to PhaC were proposed to aggregate to form a micelle-like structure in the absence of PhaP1.[19,20] This model requires a controlled elongation rate so that short PHB chains have sufficient time to form the micelle structure.[19,20] Our observations that the elongation rate is faster than the initiation rate in vivo and that PhaP1 levels are relatively high at an early stage of granule genesis have led to our modified micelle model (Figure 6A2). In our model, the initial nucleating species consists of a PhaC dimer covalently attached to one PHB chain [$(HB)_n$ where n is large] with which several PhaP1s (four to nine monomer units) are associated to maintain solubility (Figure 6A2). Exposed hydrophobic patches of $(HB)_n$ in this species allow fusion with similar nucleating species and give rise to a soluble granule precursor. A mixture of the nucleating species and soluble granule precursors is proposed to comprise the HMW fraction of Strep2-$PhaC_{Re4h}$. The PhaC:PHB chain molar ratio of 1−3:1 in the HMW fraction (Table 4) and the lack of a lag phase in CoA release are consistent with our in vitro studies of primed synthase, with one PHB chain per dimeric synthase.[16] The phase transition from a soluble to an insoluble granule requires addition of HB units and reinitiation of polymer formation. Recall that mature granules found in Re2018 and wt whole cells have many more PHB chains than PhaC molecules (Table 4, ∼100:1 in wt and ∼10:1 in Re2018 grown in TSB).[25] During this phase transition, the PhaP1:PHB molar ratio decreases, eventually reaching 0.6:1 in Re2018, similar to the ratio of ∼1:1 in wt cells under TSB growth conditions.[25] Finally, other proteins associated with PHB homeostasis (PhaR and PhaZs) were not observed with the purified Strep2-$PhaC_{Re4h}$. Because these proteins have previously been shown to associate with mature PHB granules by either insoluble granule isolation or immunogold labeling,[28,30,31,33] they are proposed to bind to the granule exterior as the PhaP1:PHB ratio decreases to 1:1 and more exposed PHB becomes accessible to the granule surface.

A second model for granule genesis, the membrane budding model, is based on the analogies with lipid bodies and their biogenesis in eukaryotic systems.[4,61] In this model (Figure 6B), PhaC and PhaP1 bind to the inner leaflet of the plasma membrane with PHB chains from PhaC extended into the membrane. The PHB granules subsequently bud from the membrane, with a monolayer of phospholipid and PhaC and PhaP1 residing on the granule surface. Previous fluorescence studies have been interpreted to support this model.[58,59] In the past year, however, a number of observations that suggest that this model is unlikely have been published. First, our examination of recent microarray data reporting changes in gene expression associated with PHB production revealed downregulation of the biosynthetic pathways associated with phospholipids found in *R. eutropha* membranes: phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.[27,29] This is the opposite of expectations for the budding model in which each granule is covered with a lipid monolayer. Second, cryoelectron tomography has recently been used to examine granule surfaces and localization in *R. eutropha* during PHB production in nitrogen-limited growth medium.[38] These studies revealed discontinuous surface coverage of the granules inconsistent with a lipid monolayer. No granules (of 450 examined) were budding from the membrane. Finally, our observation in this paper of the soluble PhaC−PhaP1−PHB complex is at odds with a membrane budding model as a lipid-coated PHB would likely be insoluble. Thus, in the case of *R. eutropha*, the membrane budding model for granule genesis is unlikely.

A scaffolding model (Figure 6C) (where the scaffold was an unspecified macromolecule) was previously proposed on the basis of negative staining TEM of *R. eutropha* at early stages of granule genesis during growth in TSB medium and in nitrogen-limited medium.[25,60] The granules appeared to be associated with "dark-stained features" in the center of the cells, which we now believe to be the bacterial nucleoid. A nucleoid scaffolding model (Figure 6C) recently became more interesting as Jendrossek and co-workers, using *R. eutropha* PhaC as bait in a screen for protein−protein interactions via a two-hybrid method,[49] identified an interaction with a previously uncharacterized *R. eutropha* protein, which they designated PhaM (26.6 kDa). This protein has PAKKA-like motifs at its C-terminus, proposed in other systems to be involved in "nonspecific" DNA binding.[62,63] From a variety of additional studies, they proposed that PHB granules bind to the nucleoid via this protein during granule formation and cell segregation. In the latter case, this binding would provide a mechanism for equal segregation of granules among daughter cells.[49,62] It is interesting to note that the 32 kDa protein that copurified with Strep2-$PhaC_{Re}$ at both 4 and 24 h is PhaM. From our biochemical and biological studies described above, the modified micelle model for granule formation (Figure 6A2) could be integrated with a scaffolding model in which the scaffold would be bacterial nucleoid. Thus, a nucleoid scaffolding model cannot at present be distinguished from a random cytoplasmic micelle model.

■ SUMMARY

The results presented here provide the first glimpse into the initiation process of granule formation in *R. eutropha*. The studies suggest that PhaC is primed by rapid acylation and elongation of its active site cysteine by HBCoA, in accord with in vitro studies in which the elongation rate is much faster than the initiation rate.[8,18] The soluble HMW complex of PhaC, PhaP1 and high-molecular weight PHB is capable of adding HB units to the primed PhaC in a kinetically competent fashion. The molar ratios of these species relative to those observed on mature granules have led to our modified micelle model for granule genesis that provides the basis for future studies.

■ ASSOCIATED CONTENT

Supporting Information

Identification of proteins copurified with Strep2-$PhaC_{Re}$, activities of Strep2-$PhaC_{Re4h}$ and Strep2-$PhaC_{Re24h}$ SEC fractions, PHB production in wt and Re2018 cells during cultivation in TSB medium, variability in specific activities of Strep2-$PhaC_{Ec}$ and Strep2-$PhaC_{Re}$, whole cell Western blots for quantification of PhaP1 and Strep2-PhaC in wt and Re2018 strains, Bio-Sil SEC250-5 size exclusion chromatography of C319A Strep2-$PhaC_{Re}$, flowcharts accounting for activity, protein, and PHB content during purification of Strep2-$PhaC_{Re4h}$, and Western blots for quantification of PhaP1 and

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Strep2-PhaC in the HMW fractions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

PHB, polyhydroxybutyrate; PHV, polyhydroxyvalerate; Strep2-$PhaC_{Ec}$, N-terminally Strep2-$(G_4S)_3$-tagged PhaC purified from *E. coli*; Strep2-$PhaC_{Re}$, N-terminally Strep2-$(G_4S)_3$-tagged PhaC purified from *R. eutropha*; Strep2-$PhaC_{Re24h}$, Strep2-PhaC purified from *R. eutropha* culture after growth in TSB medium for 24 h; Strep2-$PhaC_{Re4h}$, Strep2-PhaC purified from *R. eutropha* culture after growth in TSB medium for 4 h; SEC, size exclusion chromatography; HMW, high-molecular weight; HBCoA, 3-hydroxybutyryl-CoA; $(HB)_n$, oligomers of 3-hydroxybutyrate of length n; gent, gentamicin; kan, kanamycin; amp, ampicillin; sTCoA, analogue of $(HB)_3$-CoA in which the terminal HO group is replaced with a H; TSB, tryptic soy broth; TAE, Tris-acetate-EDTA buffer; IPTG, isopropyl β-thiogalactoside; OD_{600}, optical density at 600 nm; RT, room temperature; cdw, cell dry weight; A_{280}, absorbance at 280 nm; TEM, transmission electron microscopy.

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