# Overexpression and Purification of the Soluble Polyhydroxyalkanoate Synthase from *Alcaligenes eutrophus*: Evidence for a Required Posttranslational Modification for Catalytic Activity†

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ABSTRACT: Polyhydroxyalkanoate (PHA) synthase has been expressed in *Escherichia coli* by reengineering the 5′-end of the wild-type (wt) gene and subsequent transformation of this gene into protease-deficient *E. coli* UT5600 (*ompT*⁻). Induction with IPTG results in soluble PHA synthase, which is ~5% of the total protein. The soluble synthase has been purified to >90% homogeneity using FPLC chromatography on hydroxylapatite and Q-Sepharose and has a specific activity of 5 μmol min⁻¹ mg⁻¹. The molecular weight of the PHA product is $\sim 10^6$ Da based on PlGel chromatography and calibration using polystyrene molecular weight markers. The synthase in the absence of substrate appears to exist in both monomeric and dimeric forms. Incubation of the synthase with an excess of substrate converts it into a form that is now extractable into $CHCl_3$ and sediments on sucrose density ultracetrifugation with PHA. Studies in which the ratio of substrate, 3-D-hydroxybutyrylCoA, to synthase is varied suggest that during polymerization the elongation process occurs at a rate much faster than during the initiation process. A mechanistic model has been proposed for the polymerization process [Griebel, R., Smith, Z., & Merrick, J. (1968) *Biochemistry 7,* 3676–3681] in which two cysteines are required for catalysis. This model is based on the well-characterized enzymes involved in fatty acid biosynthesis. To test this model, several site-directed mutants of synthase, selected based on sequence conservation among synthases, have been prepared. The C459S mutant has activity ~90% that of the wt protein, while the C319S and C319A synthases possess <0.01% the activity of the wt protein. CD and antibody studies suggest that the mutant proteins are properly folded. The detection of only a single essential cysteine by mutagenesis and the requirement for posttranslational modification by phosphopantetheine to provide a second thiol in many enzymes utilizing coenzyme A thiol ester substrates made us consider the possibility that posttranslational modification was required for synthase activity as well. This hypothesis was confirmed when the plasmid containing PHA synthase (pKAS4) was transformed into *E. coli* SJ16, requiring β-alanine for growth. Growth of SJ16/pKAS4 on [³H]-β-alanine followed by Coomassie staining of the protein and autoradiography revealed that PHA synthase is overexpressed and that β-alanine is incorporated into the protein. These results suggest PHA synthase is posttranslationally modified by phosphopantetheine. Mutagenesis studies and detection of phosphopantetheine suggest that the mechanistic model of Griebel et al. (1968) in which two thiols are required for catalysis is a reasonable starting point for the examination of the mechanism of the polymerization process.

The potential for utilizing biological systems as a source of biodegradable materials is becoming increasingly attractive in view of the environmental problems associated with the disposal of traditional oil-based polymers (Chu, 1985; Agrawal et al., 1992; Tamada & Langer, 1993). Poly-D-3-hydroxyalkanoates (PHAs)[1] are polyoxoesters synthesized from optically active thiol esters that are produced in many types of bacteria (Anderson & Dawes, 1990). Polyoxoesters have recently been found in mammalian systems as well (Reusch et al., 1992). Thus, a fifth class of biological polymers has been identified that is of interest from both biological and applied perspectives (Seebach, 1992; Müller & Seebach, 1993).

The family of biopolymers of interest in the present paper involves short-chain PHAs, homo or copolymers of four to five carbon units in which the monomeric building blocks are provided by (D)-3-hydroxybutyryl- and (D)-3-hydroxyvaleryl-CoA. The biosynthetic pathways for these polyoxoesters have been most extensively investigated in *Zoogloea ramigera* (Tomita et al., 1983) and *Alcaligenes eutrophus* H16 (Slater et al., 1988; Schubert et al., 1988; Peoples & Sinskey, 1989a–c). As indicated in Figure 1, their biosynthesis involves three proteins: a thiolase, a reductase, and a synthase. The thiolases and reductases from *A. eutrophus* and *Z. ramigera* have been

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[1] Abbreviations: PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; wt, wild type; IPTG, isopropyl thio-β-D-galactoside; Hecameg, 6-*O*-(*N*-heptylcarbamoyl)methyl α-D-glucopyranoside; 3-D-HBCoA, hydroxybutyryl-CoA; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, 10 mM Tris, 150 mM NaCl, 0.05% Tween 20; HRP, horseradish peroxidase; PVDF, poly(vinylidene difluoride); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); KPi, potassium phosphate; CD, circular dichroism.

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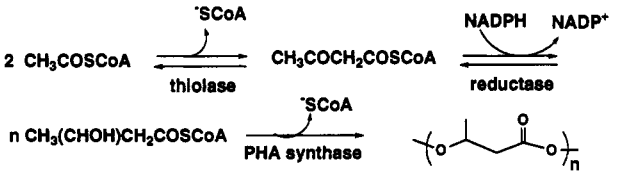



FIGURE 1: Biosynthetic pathway for PHA formation in *A. eutrophus*.

extensively studied from both biochemical and mechanistic perspectives (Masamune et al., 1989; Ploux et al., 1988; Peoples et al., 1987). The PHA synthase, both the soluble and granule-bound forms, have thus far resisted purification to a stable form that can be investigated (Haywood et al., 1989; Tomita et al., 1983).

The genes for all three enzymes from *A. eutrophus* have been cloned and expressed in *Escherichia coli*. Sequence analysis reveals that the synthase is part of a biosynthetic operon containing the *phbC–phbA–phbB* genes coding for synthase–thiolase–reductase, respectively. The synthase is a protein of 63.9 kDa, and its DNA sequence exhibits no homology with the DNA sequence of any previously reported protein. The presence of all three genes in *E. coli* results in the accumulation of large quantities of PHA (50% dry weight of the cell) (Slater et al., 1988; Schubert et al., 1988; Peoples & Sinskey, 1989c). Until recently, efforts to overexpress the synthase in the absence of the thiolase and reductase have proven unsuccessful. The present paper reports construction of a new vector that has allowed the overexpression of the soluble form of the synthase. The protein has been purified to >90% homogeneity for the first time, and in contrast to previous reports on partially purified protein, the synthase appears to be stable. Preliminary characterization of the protein is reported. Site-directed mutagenesis of several conserved cysteine residues in the protein and characterization of the resulting mutant proteins suggest that only C319 is essential for catalysis. Biochemical studies using an *E. coli* strain auxotrophic for β-alanine suggest that a second thiol is available for catalysis by posttranslational modification via a phosphopantetheine moiety. The presence of two required thiols per monomer of synthase indicates that the mechanistic model for polymerization based on the well-characterized fatty acid synthase may provide an excellent model for the PHA synthase catalyzed reaction (Rusnak et al., 1991; Griebel et al., 1968; Kawaguchi & Doi, 1992).

## MATERIALS AND METHODS

Coenzyme A, glucose dehydrogenase (specific activity 50–150 μmol $min^{-1}$ $mg^{-1}$) (*Bacillus megaterium*, Sigma), pronase E, and NADPH, have been obtained from Sigma. *Nco*I, *Eco*RI, *Not*I, *Sma*I, molecular weight standards, and T4 DNA ligase were obtained from Gibco BRL. [1-$^3$H]Glucose (15.5 C/mmol) and [$^{35}$S]ATPγS (1000 mC/mmol) were obtained from New England Nuclear. IPTG was obtained from Boehringer Mannheim. 6-*O*-(*N*-Heptylcarbamoyl)methyl α-D-glucopyranoside (Hecameg) was obtained from Vegatec (Villejuif, France). Q-Sepharose was purchased from Pharmacia, and Macro-prep hydroxylapatite 18/30 (binding capacity of 12 mg of lysozyme/g) was a kind gift of Dr. Larry Cummings, Bio-Rad Laboratories Inc. PlGel Mixed C (5 μm) column was purchased from Polymer Laboratories (Amherst, MA). C18-Sep-Pak cartridges were from Waters Inc. The Klenow fragment of DNA polymerase was obtained from Amersham. The oligonucleotides were synthesized by the MIT Biopolymers Laboratory and used without further purification. Hydroxybutyryl-CoA (HBCoA) was synthesized using *A. eutrophus* acetoacetyl-CoA reductase (20 units $mg^{-1}$) as previously described (Ploux et al., 1988).

*E. coli* strains JM105 (Yanisch-Perron et al., 1985) and UT5600 (Earhart et al., 1979) were used. Plasmid pAeT42 containing the *A. eutrophus* H16 *phb*C gene coding for the PHA synthase has been previously described (Peoples & Sinskey, 1989c). pTrc99A was obtained from Pharmacia. Bacteria were grown in 2× TY media (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) at 30 °C. Ampicillin was used in a final concentration of 100 μg/mL. Antibodies to the PHA synthase–glutathione transferase fusion protein have been prepared as previously described (Gerngross et al., 1993). The polymerase chain reaction (PCR) was performed using an Ericomp thermal cycler and an Amplitaq reagent kit from Perkin Elmer. The sequencing of protein was carried out by the MIT Biopolymer Laboratory. FPLC chromatography was carried out on a system purchased from Pharmacia.

*Preparation of [3′-$^3$H]-D-HBCoA.*[2] [1-$^3$H]-D-Glucose (100 μC), 0.2 μmol of $NADP^+$, 3 μmol of acetoacetyl-CoA and 3.5 μmol of D-glucose were dissolved in 1 mL of 20 mM Tris·HCl (pH 8.0) at 25 °C. Glucose dehydrogenase (5 units) was added followed by 20 units of acetoacetyl-CoA reductase. The mixture was incubated for 2 h at room temperature. The pH of the solution was then adjusted to 4.5 with 1 M HCl, and the reaction mixture was purified in 200-μL aliquots by loading onto 1-mL C18 Sep-Pak cartridges and washing with 1 mL of 50 mM KPi (pH 4.7). The product was eluted with 1 mL of the same buffer containing 25% methanol. The fractions containing [3-$^3$H]HBCoA were pooled, lyophilized, and stored as a 12 mM stock solution in 50% ethanol at −20 °C. The concentration was determined by $A_{260nm}$ ($\epsilon$ = 16.4 $mM^{-1}$ $cm^{-1}$) (Ploux et al., 1988) and by the coupled assay procedure described by Saito et al. (1977).

*Redesigning the PHA Synthase Gene for Overexpression.* Routine DNA manipulations were carried out as described by Sambrook et al. (1989). pTrc99A (1 μg) was digested with *Nco*I (10 units) for 1 h as described by the vendor. *Nco*I is a unique restriction site in the polylinker region of this plasmid. The resulting lineraized DNA with recessed ends was made blunt ended using the Klenow fragment (2 units) after incubation with 0.2 mM dNTPs for 15 min. The DNA was then recircularized using T4 DNA ligase (2 units), and the resulting plasmid pTrcN was transformed into *E. coli* JM105. This process removes the ATG start codon immediately downstream of the ribosomal binding site in the vector. pTrcN was characterized by restriction digest analysis subsequent to its isolation and used in the development of the new expression vector described subsequently. The 5′-region of the *phb*C gene was modified using the PCR.

Primer 1, CCGAATTCAGGAGGTTTTTATTATGGC-TACCGGCAAAGGCGCGGCAGCTTCCACGC, and primer 2, CGTGCAGCGGACCGGTGGCCTCGGCCT-TGCCC, were incubated with pAeT42 DNA linearized by digestion with *Sma*I. Primer 1 was designed to reengineer the 5′ end of the *phb*C gene and contains an *Eco*RI restriction site immediately upstream of an efficient *E. coli* ribosome binding region. Primer 2 is downstream of the *Not*I site (bp 265–297). The 316-bp amplified fragment was prepared using 35 cycles of the following program: denaturation at 95 °C, 30 s; annealing at 55 °C, 60 s; elongation at 72 °C, 60 s. Following amplification, the DNA was purified by phenol extraction and ethanol precipitation in the presence of 0.3 M

[2] This method was in part developed by Dr. Dale Drueckhammer in the laboratory of Christopher Walsh at Massachusetts Institute of Technology.

NaOAc. The DNA (1 μg) was digested to completion with *Eco*RI (20 units) and *Not*I (20 units) for 2 h. Following electrophoresis in a 1.8% agarose gel, the DNA band was visualized by ethidium bromide staining and UV light, and the gel slice was excised. The 161-bp *Eco*RI–*Not*I DNA fragment was recovered from the gel slice and purified using a Qiagen kit (Qiagen Inc., Chatsworth, CA). The 161-bp piece (200 ng) was used in a three piece ligation with the 1560-bp fragment (200 ng) isolated from the restriction digest of pAeT42 with *Not*I and *Stu*I and the 4.2-kb fragment (100 ng) of DNA resulting from restriction digestion of pTrcN with *Eco*RI and *Sma*I followed by alkaline phosphatase treatment. The fragments were ligated with 2 units of T4 DNA ligase for 16 h at 22 °C. This reaction mixture was used directly to transform competent *E. coli* JM105 cells and the resulting transformants selected on 2×TY agar plates containing 100 μg/mL of ampicillin. The resulting plasmid, pKAS4, was isolated and characterized by both restriction digest maps and DNA sequencing using the dideoxy chain termination method and Sequenase II kits (United States Biochemical Corp.).

*Optimization of the Expression of PHA Synthase Analyzed by Immunoblotting.* *E. coli* JM105 harboring pKAS4 was grown at 30 °C. At various times during the growth, 100-μL aliquots were removed, and the cells were pelleted by centrifugation. The cells were suspended in 25 μL of SDS–PAGE buffer (Laemmli, 1970) and incubated for 5 min in a boiling $H_2O$ bath. The entire sample was then loaded onto a 4–15% gradient polyacrylamide gel (Bio-Rad, Richmond, CA), and the proteins were separated by SDS–PAGE. The proteins were transferred onto nitrocellulose filters (BA85, 0.45-μm pore size, Schleicher and Schuell) using a Mini Trans-Blot Cell (Bio-Rad). Electroelution proceeded for 1 h at 300 mA in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Following transfer, the filters were blocked with TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) containing 3% nonfat dry milk for 1 h. The anti-PHA synthase antibody (diluted 1:2000) in TBST was then incubated with the filters for 1 h and washed three times with TBS (TBST minus Tween 20). The protein cross-reacting with the anti-PHA synthase antibodies was visualized using goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate and the standard HRP color development reagent (Bio-Rad). Alternatively, proteins were electroblotted onto poly(vinylidene difluoride) (PVDF) membranes in 10 mM CHAPS (pH 11) and 10% $CH_3OH$ and were subjected to sequence analysis by the automated gas-phase Edman degradation sequencing method (Matsudaira, 1987).

*Purification of PHA Synthase from E. coli UT5600.* The electroblotting procedure accompanied by analysis with anti-PHA synthase antibodies or protein sequencing revealed that, in *E. coli* JM105, extensive proteolysis accompanied the overexpression of the synthase. pKAS4 was therefore transformed into *E. coli* UT5600 and isolated from this strain. The cells were grown at 30 °C to an $A_{600}$ of 0.5–0.6 and subsequently induced by the addition of IPTG to a final concentration of 0.4 mM. The cells were then grown for an additional 4 h at 30 °C. The doubling time is typically 50 min, and yields of 8.2 g of cells/L of culture were obtained. The cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C and stored at −80 °C. Cells can be stored under these conditions for months without detectable loss of activity. All purification steps were carried out at 4 °C unless otherwise stated. Cells (8.2 g) were suspended in 55 mL of 50 mM KPi (pH 7.0) containing 5% glycerol and lysed by either sonication or by use of the French press. In the former case, lysis occurred using a sonicator (Ultrasonic Processor xL; Farmingdale, NY) turned on for 0.8 s on a power setting of 9–10 and then turned off for 0.2 s; this process was repeated over a total period of 90 s. The sample was continually cooled in an ice–salt bath, and the temperature was monitored and not allowed to exceed 4 °C. Alternatively, the cells can be lysed by two passages through a French pressure cell at 16 000 psi and 4 °C. The cell debris is then removed by centrifugation at 12000*g* for 15 min. The supernatant is isolated, filtered through a 0.45-mm membrane, made 0.05% (w/v) in Hecameg, and loaded directly onto a hydroxylapatite column (1.6 × 37 cm) which has been previously equilibrated for 30 min with 10 mM KPi (pH 7), 5% glycerol, and 0.05% Hecameg (buffer A) (flow rate 3 mL/min). The column is washed with 140 mL of buffer A subsequent to loading and eluted with a 200 × 200 mL linear gradient from 10 to 300 mM KPi–buffer A. Fractions, 10 mL, were collected, and the synthase was eluted between 110 and 150 mM KPi in 40–50 mL. The fractions containing activity can be pooled and rapidly frozen (liquid nitrogen) without significant loss in activity. This material (10 mL) in 130 mM KPi can be directly loaded onto a Q-Sepharose high-performance FPLC column (6.25 mL) which has been previously equilibrated with 40 mL of 50 mM Tris·HCl (pH 8.5 at 4 °C), 5% glycerol, and 0.05% Hecameg (buffer B). Subsequent to loading, the column is washed with 25 mL of buffer B at a flow rate of 1.2 mL/min, and 5-mL fractions are collected. A 32.5 × 32.5 mL linear gradient from 0 to 1.5 M NaCl in buffer B is then applied. Greater than 90% of PHA synthase elutes in fractions 2 and 3. Approximately 10% of the protein elutes in fractions 11 and 12 at 500 mM NaCl. The fractions are pooled and concentrated to 2.2 mg/mL using YM 30 membrane and an Amicon filtration apparatus. The protein is then rapidly frozen in liquid nitrogen and stored at −80 °C. The protein is stable for at least 1 month under these conditions. An identical protocol was used to purify the mutant synthases described below.

*Molecular Mass Determinations.* Subunit molecular mass was determined using SDS–PAGE by the procedure of Laemmli (1970). The molecular mass standards included phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactalbumin (18.4 kDa). The native molecular mass was examined using FPLC on a Superdex 200 (Pharmacia) column. Synthase (2.2 mg/mL or 0.1 mg/mL) was loaded onto the column equilibrated in 150 mM NaCl, 25 mM Tris (pH 7.5), and 5% glycerol. The following molecular mass standards were used: ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), bovine serum albumin (68 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

*PHA Synthase Assays. (A) Extraction with $CHCl_3$.* This method is a modification of the one previously described by Fukui et al. (1976). A typical assay mixture contained the following in a final volume of 40 μL: 143 mM KPi (pH 7.0), 0.62 mM [3-$^3$H]-3-D-HBCoA (specific activity $1.1 \times 10^6$ cpm/μmol), and variable amounts of PHA synthase (0.07–10 μg) depending on the purity. The reactions were started by the addition of enzyme after equilibration of the substrate in the buffer for 10 min at 25 °C. The reaction mixtures were then incubated at 25 °C for 1–30 min. The reactions are stopped by the addition of 100 μL of ice-cold 5% trichloroacetic acid with vortexing. The solution is then extracted with 0.5 mL of $CHCl_3$. The layers are allowed to separate, the protein is at the interface, 0.4 mL of the $CHCl_3$ layer is transferred to

a scintillation vial, and $CHCl_3$ is evaporated under a stream of argon and redissolved in 20 μL of $CHCl_3$. The PHA extracted is then quantified by scintillation counting after the addition of 8 mL of Scint A-XF scintillation fluid.

*(B) Loss of HBCoA Monitored Spectrophotometrically.* HBCoA has previously been reported (Fukui et al., 1976) to have a $\lambda_{max}$ at 232 nm and an $\epsilon$ of $4.5 \times 10^3$ $M^{-1}$ $cm^{-1}$. With purified synthase, the decrease in absorbance at 232 nm can be monitored spectrophotometrically and continually.

*(C) Formation of -SCoA Monitored Using Ellman's Reagent.* Coenzyme A released during the PHA synthase catalyzed reaction can be measured using Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). A typical reaction mixture (360 μL) contained 1.0 mM HBCoA and 143 mM KPi (pH 7.0). The assay mixture was preincubated for 10 min at 25 °C, after which time the reaction was started by the addition of enzyme. Aliquots (40 μL) were removed at timed intervals and stopped by the addition of 100 μL of 5% trichloroacetic acid. The precipitated protein was pelleted by centrifugation for 10 min, and an aliquot (125 μL) of the supernatant was added to 675 μL of 500 mM KPi (pH 7.5). DTNB [10 μL of a 10 mM stock solution in 500 mM KPi (pH 7.5)] was added to this mixture and incubated for 2 min at room temperature. The absorbance at 410 nm ($\epsilon$ = 13.7 $mM^{-1}$ $cm^{-1}$) was measured.

*Characterization of PHA Product by Size Exclusion Chromatography.* Assay mixtures (50 μL) containing PHA synthase and [$^3$H]HBCoA, subsequent to reaction for 30 min, were incubated with pronase (0.1 unit) in 140 mM KPi (pH 7.0) for 10 min at 25 °C. The reaction was stopped by the addition of 0.5 mL of $CHCl_3$, and the polymer was extracted by vortexing for 1 min. The $CHCl_3$ phase was dried over $MgSO_4$, filtered through glass wool, and evaporated to less than 100 μL. This solution was injected directly onto a PIGel Mixed C column and chromatographed using $CHCl_3$ as eluate at a flow rate of 1 mL/min. For calibration, polystyrene standards of low polydispersity ($M_r$ 600 Da–1860 kDa) were used (Polysciences, Inc., Warrington, PA). Fractions eluted from the column were analyzed by scintillation counting.

*Preparation of Site-Directed Mutants.* Site-directed mutagenesis was carried out as described by Thompson et al. (1988). The C319S, S319A, and C459S mutants were prepared using the following oligonucleotides:

C319S 5'-CTCGGCTTCTCCGTGGGCGGC-3'

S319A 5'-CTCGGCTTCGCCGTGGGCGGC-3'

C459S 5'-AGCTGACCGTGTCCGGCGTGCCG-3'

To create the C319S and S319A mutations, the *Sac*I fragment (570 bp) of pKAS4 was inserted into the polylinker region of M13mp18, and to create the C459S mutation, the *Sac*I–*Hind*III fragment (480 bp) was inserted into the polylinker region of M13mp19. Following mutagenesis, these fragments were sequenced and returned to pKAS4. The mutants were isolated as described for the wt synthase.

*Circular Dichroism (CD) Spectroscopy of Wild-Type and Mutant Synthases.* CD spectra were recorded at ambient temperature as the average of five scans on an AVIV spectrophotometer Model 62DS using a 0.2-mm path length cell. Samples contained PHA synthase (0.2 mg/mL) or the synthase mutants in 100 mM KPi buffer (pH 7.0), 0.005% Hecameg, and 0.5% glycerol.

*Characterization of Mutant and Wild-Type Synthases in E. coli Crude Extracts Using Antibody to Wild-Type Synthase.* *E. coli* UT5600 containing pKAS4 with either the wild-type or C319S mutant gene for synthase was grown and isolated as described above, and the crude extracts were analyzed by Western blotting analysis using the antibody for PHA synthase.

*Characterization of Synthase in the Presence of Varying Amounts of* D-*3-HBCoA by Ultracentrifugation.* Each reaction mixture contained the following in a final volume of 500 μL: 100 mM KPi (pH 8.0), 2 μg (0.017 nmol) of PHA synthase, and variable amounts of [3-$^3$H]-D-HBCoA (0–190 nmol, specific activity $1.1 \times 10^6$ cpm/μmol). The reaction was started by the addition of enzyme, and each mixture was incubated for 30 min at 25 °C and analyzed by sucrose density (1–2 M, 14 mL total volume per tube) ultracentrifugation (Fukui et al., 1976). After 3 h at 100000*g*, the tubes were punctured at the bottom and 1-mL fractions were collected. Each fraction was then analyzed by a dot blot (1 μL, applied directly to the membrane) using the anti-PHA synthase antibody as described above. Each fraction (100 μL) was also analyzed by scintillation counting.

*Evaluation of Phosphopantetheine Requirement of PHA Synthase.* The procedure of Rusnak et al. (1991) was followed with the modifications indicated below. The strains utilized in the experiments were *E. coli* SJ16 (Jackowski & Rock, 1981), SJ16/pKAS4, and SJ16/pTrcN (pTrcN is a modified pTrc99A that does not contain the PHA synthase gene). While SJ16 grew as previously described, the other two strains grew at considerably slower rates.

Single colonies of SJ16, SJ16/pKAS4, and SJ16/pTrcN were inoculated into test tubes containing 5 mL of Dex-E-B1-met medium, pH 7.0 (Rusnak et al., 1991). (Medium contains the following per L: 0.2 g of $MgSO_4$, 2 g of citric acid, 10 g of $K_2HPO_4$, 3.5 g of $NaNH_4HPO_4$, 0.4% glucose, $10^{-4}$% thiamine, and 0.002% methionine). Tetracycline (12.5 mg $L^{-1}$) was added to all samples, and ampicillin (50 mg $L^{-1}$) was added to samples with plasmid present. Cells were cultured at 37 °C for 20 h, after which time the sample containing SJ16 was faintly cloudy, while the samples containing SJ16/pTrcN and SJ16/pKAS4 showed no apparent growth. Samples were diluted 1:100 into 1 mL of fresh medium containing 0.55 μM β-[$^3$H]alanine (91.5 Ci/mmol, NEN) and were returned to incubate at 37 °C. After 25 h, IPTG was added to the samples containg SJ16/pTrcN and SJ16/pKAS4 at a final concentration of 1 mM. Additional β-[$^3$H]alanine was added to the indicated samples (Figure 7) such that the final concentration was 1.1 μM. Samples were incubated for an additional 18 h. An aliquot of cells (200 μL) was then removed from each sample and harvested by centrifugation. The samples were resuspended in 25 μL of gel sample buffer (0.0625 M Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.025% bromophenol blue) and boiled for 5 min. Proteins were resolved on a 4–15% gradient SDS–PAGE gel and visualized by staining with Coomassie blue. A prominent band at 64 kDa verified that PHA synthase was overexpressed. The destained gel was soaked in Amplify (Amersham), dried on a piece of Whatman filter paper, and autoradiographed at −70 °C on Kodak X-OMAT AR film for 18 h. SJ16/pKAS4 cells (100 mL) were cultured as described above for the assay of PHA synthase activity.

## RESULTS

*Overproduction of A. eutrophus PHA Synthase in E. coli.* The functional expression in *E. coli* of the *phb*ABC gene products encoding thiolase, reductase, and synthase, respectively, required for the biosynthesis of PHA in *A. eutrophus*

(a) Primer 1: *Eco* RI RBS
CC GAATTC AGGAGGTTTTTATT ATG-GCT-ACC-
GGC-AAA-GGC-GCG-GCA-GCT-TCC-ACG-C

Primer 2 : C-GTG-CAG-CGG-ACC-GGT-GGC-CTC-GGC-CTT-GCC-C

(b)

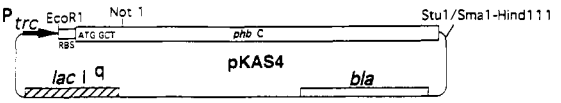



FIGURE 2: Strategy for cloning the PHA synthase gene. (a) The primers used to reconstruct the upstream region of *phb*C. (b) Construction of pKAS4 containing *phb*C.

has recently been described by several laboratories (Slater et al., 1988; Schubert et al., 1988; Peoples & Sinskey, 1989c). The transformation of *E. coli* with the *phb*C gene alone did not result, however, in the production of polymer in a nitrogen-starved medium. Peoples and Sinskey (1989c) reported using $^{35}S$-labeling studies in maxicells that low levels of expression of a protein of approximately 64 kDa were observable by SDS–PAGE and that expression of this protein appeared to be correlated with the production of very low levels of $CHCl_3$-extractable material upon incubation of [$^3$H]HBCoA with these crude cell extracts. These early studies suggested that it might be possible to overexpress the synthase in soluble form, in the absence of the thiolase and acetoacetyl-CoA reductase. The absence of these latter two gene products is desirable as they can use cellular acetyl-CoA to initiate the polymerization process leading to granule-bound, insoluble synthase. Expression in the soluble form should facilitate purification and offers the advantage that the priming and initiation processes required for polymerization can be investigated.

Therefore, in an effort to increase the level of expression of the *phb*C gene product, its ribosome binding region was redesigned using the general rules for increasing the translational efficiency in *E. coli* (MacFerrin et al., 1990). As indicated in Figure 2a, the *A. eutrophus* ribosome binding site was replaced with a strong consensus *E. coli* ribosome binding site and an 8-bp spacer, rich in Ts and As, upstream of the ATG initiation codon. In addition, the third nucleotide of the second codon was changed from GCG(Ala) to GCT(Ala) (Bagdasarian et al., 1983). PCR primers (Figure 2a) were used to generate a 161-bp fragment, which was then used to modify the N terminus of the PHA synthase gene. The modified PHA synthase gene was then placed downstream of the IPTG-inducible *trc* promoter in the vector pTrcN to produce a new vector pKAS4 (Figure 2b). This plasmid was transformed into *E. coli* JM105, and upon induction with IPTG, both analysis by Western blotting subsequent to SDS–PAGE and assay for catalytic activity revealed the production of significant levels of soluble PHA synthase. However, Western blotting analysis of the crude cell lysates revealed that subsequent to induction, in addition to the expected 64-kDa protein, there was the unexpected production of a second major protein of $M_r$ 50 kDa and several other smaller proteins. When this experiment was repeated using cells directly boiled in SDS and not subjected to cell lysis, only the 64-kDa protein was apparent. These results suggested that subsequent to cell lysis, proteolysis was occurring to degrade the PHA synthase. To test this hypothesis and to define the position of proteolysis, the 50-kDa protein was electroblotted onto a PVDF membrane (Matsudaira, 1987), and the first 10 amino acids of its N-terminal sequence were determined. As predicted by the antibody analysis, the 50-kDa protein results from a proteolytic clip of the 64-kDa protein between residues 100 and 101 ($L_{95}$-H-D-$R_{100}$-$R_{101}$-F-A-G). A search of the sequence specificity of the known *E. coli* proteases suggested that this cleavage could have been perpetrated by the *ompT* gene product, a protease found in the *E. coli* outer membrane (Sugimura & Nishihara, 1988; Earhart et al., 1979). Degradation of PHA synthase by this protease is consistent with the observation that proteolysis appears to occur only after cell lysis. In order to test this hypothesis and more importantly to prevent proteolysis of the synthase, plasmid pKAS4 was transformed into the *E. coli ompT*$^-$ strain, UT5600. The cells were grown and induced with IPTG and analyzed for proteolysis by Western blotting. The results indicate that the proteolysis has been eliminated. Furthermore, these results have been substantiated by the increase in specific activity of the synthase in crude cell extracts relative to *E. coli* JM105 as described subsequently.

*Purification of PHA Synthase.* Numerous investigators over the past decades have attempted to purify PHA synthases, both the soluble and granule-bound forms, from a variety of sources. The most successful attempt at purification of the soluble form from *A. eutrophus* was reported by Haywood et al. (1989). Subsequent to two-column chromatographic procedures, they reported a specific activity of 0.05 $\mu$mol min$^{-1}$ mg$^{-1}$ with a 2% recovery of total units. Tomita et al. (1983) have partially purified the soluble form of synthase from *Z. ramigera*, also with a 2% recovery of total units and a specific activity of 1.1 $\mu$mol min$^{-1}$ mg$^{-1}$. In both cases, the enzyme preparation was reported to be unstable, precluding its characterization or mechanistic studies.

The results of our efforts to purify the *A. eutrophus* synthase expressed in *E. coli* are summarized in Table 1 and in Figure 3. In the crude extracts, our activity is 10-fold higher than the "purified" soluble synthase from *Z. ramigera* (Tomita et al., 1983). With the reengineered gene in the crude extract, the activity is remarkably stable losing only 50% of the initial activity after standing at 4 °C for 24 h. Initial efforts to purify this protein resulted in substantial losses in activity and very broad peaks of activity from almost all chromatographic materials investigated. After extensive experimentation with a variety of detergents, it was discovered that the addition of Hecameg enhanced recovery of material from the columns and decreased the elution volumes. Therefore, all buffers were made 0.05% in Hecameg.

Previous studies suggested that anion-exchange chromatography might provide an effective purification procedure. The availability of FPLC, providing rapidity of purification, and a variety of anion-exchange materials including hydroxylapatite and Q-Sepharose have allowed successful purification of the synthase to >90% homogeneity with a 40% recovery of overall units. Based on Coomassie staining of the protein in the crude cell extracts subsequent to SDS–PAGE, we anticipated a purification of ~20-fold would be required to obtain a homogeneous protein. The protein isolated from the Q-Sepharose column has a specific activity of 5 $\mu$mol min$^{-1}$ mg$^{-1}$, and upon freezing and thawing, its activity increases to 8 $\mu$mol min$^{-1}$ mg$^{-1}$, a 16-fold overall purification. When rapidly frozen in liquid nitrogen the enzyme is stable for a period of at least 1 month.

*Molecular Mass Determination.* The subunit molecular mass of the synthase based on SDS–PAGE is 64 kDa, similar to the $M_r$ of 63.9 kDa defined by the gene sequence. Previous studies by Haywood et al. (1989) to assess the native molecular

Table 1: Purification of PHA Synthase[a]

| step | vol (mL) | total units | specific activity | protein (mg) | protein (mg/mL) | recovery (%) | purification |
|---|---|---|---|---|---|---|---|
| crude | 50 | 166 | 0.51 | 325 | 6.5 | | |
| HA[b] | 40 | 70 | 2.5 | 28 | 0.7 | 42 | 4.9 |
| Q-Sep[c] | 40 | 40 | 5.0 | 8 | 0.14 | 24 | 9.8 |
| frozen and reassayed | 40 | 64 | 8.0 | 8 | 0.14 | 38.5 | 15.7 |

[a] From 8.2 g of wet bacteria. [b] Hydroxylapatite. [c] Normalized, as only one-fourth of the active HA fractions were loaded on the Q-Sepharose column at a time.

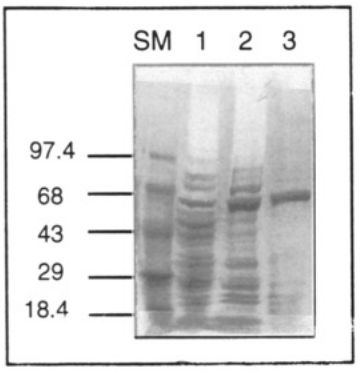



FIGURE 3: SDS, gradient gel (4–15%) describing the purification of PHA synthase; SM, standard molecular mass markers; lane 1, crude cell extracts; lane 2, pooled hydroxylapatite fractions; lane 3, pooled Q-Sepharose fractions.

mass of the synthase used crude enzyme isolated from *A. eutrophus*. Values of 150–170 kDa using gel filtration analysis and sucrose gradient ultracentrifugation, respectively, were reported. The molecular mass of the synthase isolated from its natural host, however, could be problematic as tightly bound proteins or tightly or covalently bound oligomeric or polymeric β-hydroxybutyrate (PHB) could copurify. Isolation of the enzyme from *E. coli* eliminates this complication. Our studies of the native molecular mass based on Superdex 200 size exclusion chromatography revealed two forms of the protein: one form of ~60 kDa and a second form of ~130 kDa (Figure 4a,b). The fractions containing the putative monomer and dimer were concentrated and further analyzed by Western blotting (data not shown). The proteins in both fractions cross-react with antibodies raised to the PHA synthase and indicated a protein of 64 kDa. In addition, each fraction contained polymerase activity. Concentration of each fraction followed by rechromatography revealed the same monomer-dimer mixture. Thus, the protein as isolated from *E. coli* exists as an equilibrium between monomeric and dimeric forms.

*Assay for Synthase Activity*. Previous workers have used a discontinuous assay to monitor PHA production involving the incubation of [$^3$H]HBCoA with synthase for various time periods (Fukui et al., 1976). The reactions are stopped by the addition of trichloroacetic acid, and then the product is extracted into $CHCl_3$. The $CHCl_3$ is then removed, and the residue is quantitated by scintillation counting. Results of a typical assay are shown in Figure 5a. At each concentration of protein examined, there is a lag phase which precedes the linear phase of product formation. The lag phase is longer, the lower the concentration of the protein. The rates of the reaction used to calculate the specific activities reported in Table 1 have been determined by analyzing the linear part of the curve subsequent to the lag phase. The dependence of the specific activity of the protein on the protein concentration is shown in Figure 5b. These results establish that during the purification of the synthase care must be taken to appropriately adjust the concentrations of synthase in the assay mixture

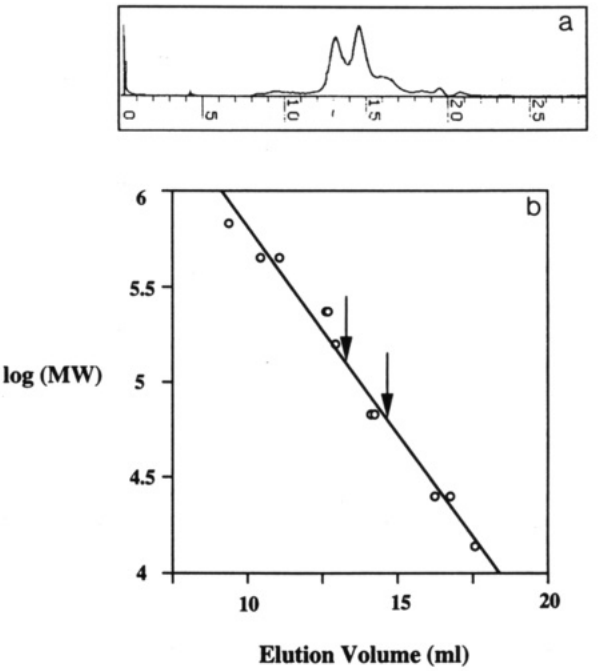



FIGURE 4: (a) Native molecular mass determination of PHA synthase using Superdex G-200 size exclusion chromatography. Two different protein-containing fractions containing synthase activity and cross-reacting with PHA synthase antibodies have been detected. (b) A plot of log (molecular mass) vs volume of elution from the Superdex column in comparison with molecular mass standards (methods) reveals molecular masses for the synthase of 60 and 139 kDa.

and a full-time course for PHA production is required to ensure that one avoids measuring the turnover in the lag phase of the reaction. Finally, it should be noted that the assays using purified synthase monitoring $CHCl_3$-extractable polymer agree within 10% with the assays monitoring hydrolysis of 3-D-HBCoA as a change in the absorbance at 232 nm or the formation of thiolate of coenzyme A using Ellman's reagent (1959).[3]

*Role of Cysteines in Catalysis*: *Posttranslational Modification of Synthase Required for Activity*. Fatty acid synthase has provided a model for thinking about the mechanism of the PHA synthase-catalyzed polymerization (Wakil, 1989; Chong & Hammes, 1990). In this model (Scheme 1), the growing chain cycles between a phosphopantetheine and a cysteine thiol to permit the alternate loading and elongation processes required for polymerization. Several experiments have been undertaken, therefore, in an attempt to define the role of thiols in the PHA synthase-catalyzed reaction.

Cloning and expression of the synthase has allowed us to use site-directed mutagenesis as a first approach. The choice

[3] Using 0.15–2 μg of synthase, the DTNB assay always gives higher specific activities than the $CHCl_3$ assay for reasons that are not presently understood.

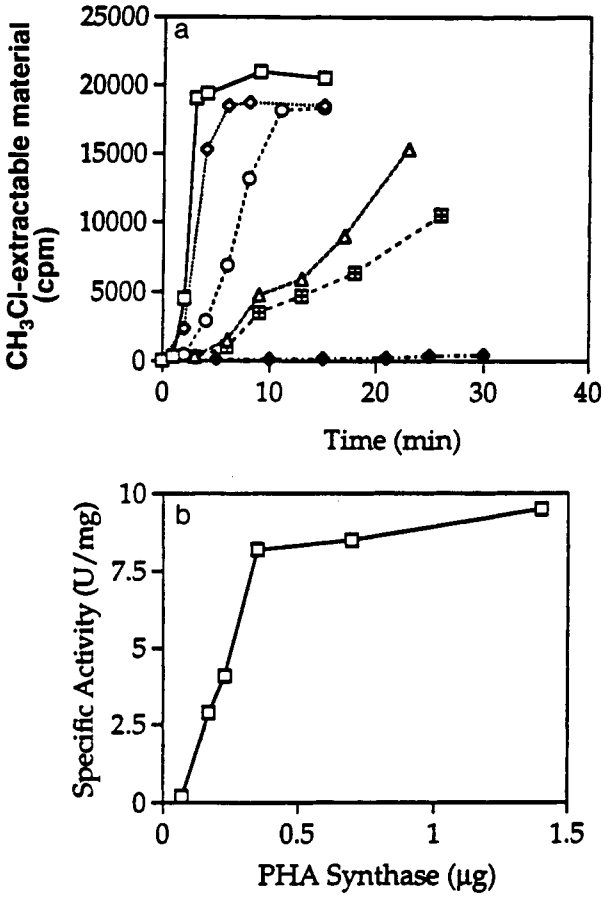
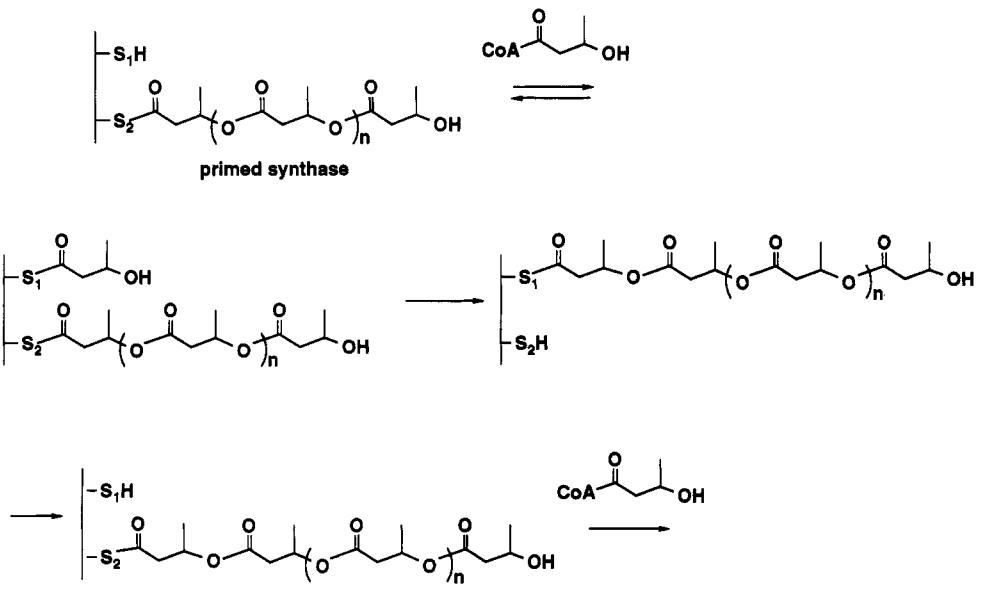



FIGURE 5: (a) Measurement of the rate of PHA formation as a function of time and enzyme concentration. (♦) 0.07, (⊞) 0.17, (○) 0.35, (△) 0.23, (◇) 0.7, (□) 1.4 μg. (b) Determination of the specific activity of PHA synthase as a function of protein concentration.

of which cysteines to mutate was governed by a comparison of the gene sequences of the 10 available synthases. This analysis revealed that only 23 of ~550 residues are conserved (Steinbuchel et al., 1992) and that only one of these residues is a cysteine [C319, numbering corresponds to the *A. eutrophus* enzyme (Peoples & Sinskey, 1989c)]. C459 is conserved among the synthases from *Alcaligenes* and *Pseudomonas*. These cysteines were therefore the initial targets of our mutagenesis studies. Cysteine 319 has been changed to a serine and to an alanine, and each mutant has been purified by a procedure identical to that described for the wt protein. The proteins have been purified to greater than 90% homogeneity as judged by SDS–PAGE (data not shown). Both the C319S and C319A synthases possess activity <0.01% of the activity of the wt protein. This activity is the lower limit of detection of our assay method. Several experiments have been carried out to ensure that the mutant proteins have been folded properly. A comparison of their CD spectra with that of the wt synthase indicates that they are superimposable and suggests that there are no gross structural differences between these proteins (data not shown). In addition, antibodies to PHA synthase were used to examine the amounts of mutant and wt synthases in crude extracts of *E. coli* UT5600. Quantitative Western blotting analysis revealed that the amount of wt and mutant proteins was identical. If the mutant proteins were improperly folded, one might have expected that they would be rapidly degraded. These two methods suggest that our inability to measure activity with C319S and C319A synthases is not due to their altered structure. The simplest interpretation of these results is that C319 is essential for catalysis. The C459S mutant was also purified to >90% homogeneity, and the activity was found to be 90% that of the wt protein. C459, therefore, plays no role in catalysis. Thus, if a model involving two thiols is to be invoked for the mechanism of the synthase reaction (Scheme 1), these studies suggest that the second thiol must be supplied by posttranslational modification with a phosphopantetheine moiety.

The second approach to identify thiols important in catalysis involved incubation of the synthase with a 1–10 fold excess of [$^3$H]HBCoA relative to the number of active sites of synthase. The expectation was that radiolabeled peptide(s) modified by $^3$H-labeled oligomeric oxoesters could be identified subsequent to proteolytic digestion of the synthase. The results of experiments in which synthase was incubated with a 0–10 000-fold excess of [$^3$H]HBCoA and the products were analyzed by sucrose density ultracentrifugation are indicated, in part, in Figure 6. In the case of the 10 and 100 equivalent excess of substrate over enzyme (data not shown), most of the synthase is located in the same position as synthase that has never been exposed to HBCoA. All of the radioactivity,

Scheme 1



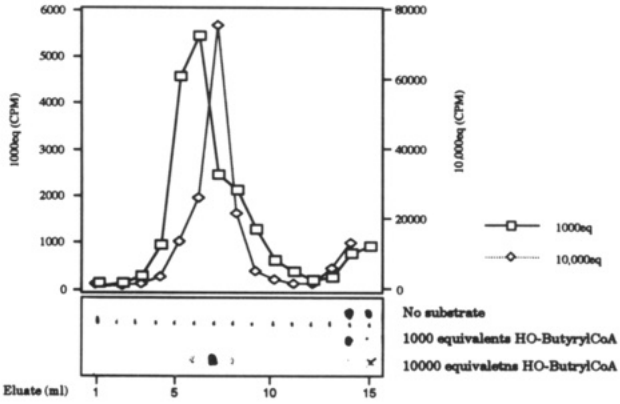



FIGURE 6: Sucrose density centrifugation of PHA synthase incubated with 0-, 1000-, and 10 000-fold excess of [$^3$H]HBCoA relative to protein. For the 1000-fold excess substrate, the scale is on the left (□). For the 10 000-fold excess, the scale is on the right (◇). The bottom panel uses dot blots and PHA synthase antibody to determine the location of the synthase.

however, sediments as a larger molecular weight aggregate that is associated with a small amount of synthase detectable using antibodies. One interpretation of these results is that, once the polymerization process is initiated, elongation occurs very rapidly and that the vast majority of synthase in solution has not yet undergone initiation. The studies with the $10^3$- and $10^4$-fold excess of substrate over synthase support this proposal. With a $10^3$-fold excess of substrate over synthase, approximately 30–50% of the synthase migrates as a large molecular weight complex, while in the case of the $10^4$-fold excess, >90% of the synthase is detectable in the aggregate (Figure 6). While these results suggest that a slow initiation process is followed by a rapid elongation process, they also indicate that this approach will not be successful in identifying covalently modified cysteine(s) of the synthase.

The ability of all of the PHA synthase to be converted into a macromolecular complex or aggregate suggests that either posttranslational modification is not required for the polymerization process or that *E. coli* have been very accommodating and have engineered extensive modification. The following experiments, therefore, were subsequently undertaken to determine whether posttranslational modification is or is not required for synthase activity. Synthases for gramicidin S, tyrocidine, enterobactin, and fatty acids have been shown to contain a 4′-phosphopantetheine as a covalent cofactor (Schlumbohm et al., 1991; Gevers et al., 1968; Rusnak et al., 1991; Chong & Hammes, 1990). The protocols described by Rusnak et al. (1991) to establish the presence of this cofactor in EntF, involved in enterobactin biosynthesis, were used as a prototype to investigate its presence in PHA synthase. *E. coli* strain SJ16, a *pan*D mutant defective in the biosynthesis of β-alanine, can be grown in minimal medium supplemented with β-[$^3$H]alanine. Subsequent to growth for 20 h, the cells are isolated and the proteins are examined on SDS–PAGE by Coomassie staining for protein and autoradiography for incorporation of β-[$^3$H]alanine (Figure 7, lane 1). In *E. coli* SJ16, four radiolabeled bands are detected: acylated acyl carrier protein (15 kDa), acyl carrier protein (20 kDa), an unknown protein of ~35 kDa, and EntF (140 kDa). These proteins are identical to those previously observed by Rusnak et al. (1991). *E. coli* SJ16 was then transformed with pKAS4 (pTrcN containing the PHA synthase gene) and pTrcN (a modified pTrc99A without the synthase gene). Growth of cells containing each of these plasmids revealed, subsequent to induction with IPTG, substantial amounts of PHA synthase

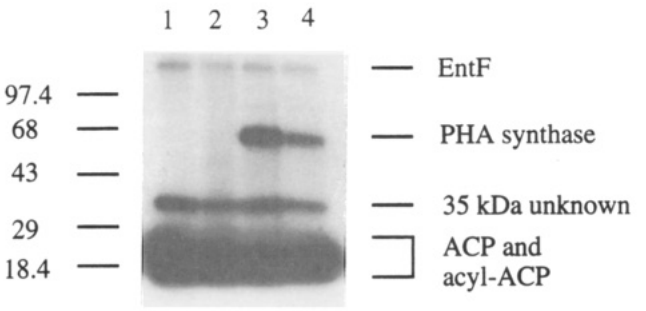



FIGURE 7: Autoradiography of an SDS gel in which SJ16/pKAS4 was grown in minimal media on β-[$^3$H]alanine. Evidence for incorporation of phosphopantetheine into PHA synthase. Lane 1, SJ16 with β-[$^3$H]alanine addition at 20 and 45 h; lane 2, SJ16/pTrcN with addition of β-[$^3$H]alanine at 20 and 45 h and IPTG induction at 45 h; lane 3, SJ16/pKAS4 with addition of β-[$^3$H]-alanine at 20 and 45 h and IPTG induction at 45 h; lane 4, SJ16/pKAS4 with addition of β-[$^3$H]alanine at 20 h and IPTG induction at 45 h. Bands observed: 15 kDa (acylated acyl carrier protein); 20 kDa (acyl carrier protein); 35 kDa (unknown); 140 kDa (Ent F).

only in the cells containing pKAS4. An experiment using β-[$^3$H]alanine was carried out using SJ16/pKAS4 and SJ16/pTrcN, and the results are shown in Figure 7 (lanes 3 and 2, respectively). With SJ16/pTrcN, the same protein bands are visible as observed with SJ16 alone (lanes 2 and 1). In the case of SJ16/pKAS4 in which PHA synthase appears to account for ~5% of the total protein, low levels of β-[$^3$H]-alanine is incorporated into the 64-kDa synthase (lane 4). As most of the PHA synthase is produced subsequent to induction with IPTG, a second addition of β-[$^3$H]alanine at the time of induction resulted in increased levels of incorporation of label into the synthase (lane 3). Assay for PHA synthase activity under the conditions shown in lanes 4 and 3 (Figure 7) revealed values of 0.05 and 0.4 unit/mg, respectively. The mechanistic implication of these efforts to identify essential cysteines will be discussed subsequently.

## DISCUSSION

Reengineering the 5′ end of the gene for PHA synthase and overexpression in an *E. coli* strain lacking the *omp*T protease has allowed successful purification to 90% homogeneity of milligram quantities of soluble *A. eutrophus* PHA synthase and several site-directed mutants. In contrast to expectations based on previous attempts to purify the soluble synthase (Haywood et al., 1989; Tomita et al., 1983), the enzyme is stable when rapidly frozen at liquid $N_2$ temperatures and stored at –80 °C.

Initial efforts to characterize the synthase focused on the determination of the size distribution of [$^3$H]PHA polymers using a variety of size exclusion chromatographic systems including Showdex, PlGel, and/or Sephacryl columns. These efforts resulted in minimal recovery of $^3$H-labeled polymer from these columns. Apparently, the protein–polymer complex either precipitates on the top of the columns and/or tightly binds to a wide variety of different column backbone matrices. However, treatment of the PHA synthase complex with pronase followed by analysis on a PlGel column resulted in the recovery of 40% of the radioactivity in a polymer of molecular mass ~$10^6$ Da. This molecular mass has been assigned based on comparison with polystyrene polymer standards. The appropriateness of this comparison remains to be established. Additional studies on the PHA synthase complex using sucrose gradient ultracentrifugation revealed the formation of very large molecular weight aggregates as well. Thus, the synthase *in vitro* is capable of producing

polymers at least as large as those isolated from *A. eutrophus*, and these polymers appear to behave as a granule-bound synthase.

Our efforts then turned to determining the kinetic parameters for this system. The kinetics of PHB formation obtained using the standard assay, however, gave very different results from those previously reported in the literature (Tomita et al., 1983; Haywood et al., 1989). The formation of polymer is preceded by a long and variable lag time (Figure 5a). Furthermore, the rate of polymer formation, measured subsequent to the lag, has a nonlinear dependence on enzyme concentration up to 0.2 $\mu$M PHA synthase (Figure 5b). This lag phase could be associated in some way with the priming and initiation process required for polymerization. Similar lag phases have been detected with glycogen synthase and rubber polymerase when the appropriate primers are lacking (Smythe & Cohen, 1991; Light & Dennis, 1989; Light et al., 1989). The priming process might well require the conversion of a monomeric to a dimeric form of the synthase, consistent with the two forms of the protein detected by size exclusion chromatography. In synthases isolated from their normal hosts, either PHB granules, in the case of the insoluble synthases, or small amounts of oligomers of PHB, in the case of the soluble synthases, could be present and serve as primers. Thus, the enzymes as isolated from their natural hosts may have been preprimed, and thus no lag phase is detectable (Tomita et al., 1983; data not shown). The observation that the lag phase is removed by preincubation of the recombinant synthase with cold 3-D-HBCoA for 10 min prior to the addition of the [$^3$H]HBCoA is consistent with this model. The details of the priming–initiation process are the subject of an ongoing investigation, and the synthase isolated from *E. coli* provides a unique system for this investigation.

The favored mechanistic model for PHA synthesis was put forth by Griebel et al. (1968) and was recently modified by Kawaguchi and Doi (1992) (Scheme 1). Its basis lies in our detailed understanding of the mechanism of fatty acid synthase and the functional role of its cysteines and thiolates in catalysis (Chong & Hammes, 1990). The model involves covalent catalysis by thiolates and is indicated in Scheme 1. In this scheme, the mechanism of the priming process has been ignored, and the primer has been covalently bound to thiolate, S2. The new monomer to be added to the growing chain is attached to a second thiolate, S1. The 3′-hydroxyl of this monomer then attacks the thiol ester of the oligomer to form an S1 covalently bound oligomer increased by 1 unit. The entire chain is then transferred back to S2 by thioltransesterification, and the entire process is repeated. This model is appealing in that the 3′-hydroxyl group to be transferred to the growing chain is juxtaposed adjacent to this chain. This is an essential requirement to make a polymer composed of 5000 monomers, if the reaction is to occur within a reasonable period of time. In the case of the fatty acid synthase system, one of the thiolates is part of a phosphopantetheine moiety attached to a serine that is part of the acyl carrier protein and the other is a cysteine.

Our initial efforts to obtain mechanistic insight into the polymerization process have focused on the identification of thiols that might be essential for PHA synthase-catalyzed polymerization. Our experimental results suggest that a single cysteine, C319, per monomer plays a key role in catalysis and that a second thiol is provided by posttranslational modification of the monomer by a phosphopantetheine.

Evidence for phosphopantetheinylation is provided by two complementary but independent experiments. Previous studies

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Field Mustard ACP | A | D | L | G | A | D | S | L | D | T | | V | E | I | V |
| Spinach ACP | S | K | L | G | A | D | S | L | D | T | | V | E | I | V |
| Barley ACP | S | E | L | G | A | D | S | L | D | T | | V | E | I | V |
| *E. coli* | E | D | L | G | A | D | S | L | D | T | | V | E | L | I |
| *Neurospora crassa* ACP | N | D | L | G | L | D | S | L | D | T | | V | E | V | V |
| Citrate Lyase | I | Q | L | E | I | D | S | I | V | K | | Q | E | F | G |
| Granaticin PK Synthase | E | E | L | G | Y | D | S | L | A | L | | M | E | S | A |
| Tetracenomycin PK Synthase | Q | D | L | G | Y | D | S | I | A | L | | L | E | I | S |
| EntF (107-122) | Q | D | L | R | V | D | S | G | K | P | L | V | F | H | Q |
| EntF (1000-1015) | F | A | L | G | G | H | S | L | L | A | M | K | L | A | A |
| PHA Synthase | L | D | L | Q | P | E | S | **S** | L | V | R | H | V | V | E |

FIGURE 8: Alignment of the sequences surrounding the putative phosphopantetheine binding sites. Serine 260 of PHA synthase is bold faced. The references are as follows: ACP from field mustard *Brassica campertris* (Rose et al., 1987); ACP from spinach *Spinacia oleracea* (Scherer & Knauf, 1987); ACP from barley *Lardeum vulgare* (Hoj & Svedsen, 1983); ACP from *E. coli* (Jackowski & Rock, 1987); ACP from *Neurospora crassa* (Brody & Mikolajczyk, 1988); citrate lyase from *Klebsiella pneumonia* (Beyreuther et al., 1978); ACP of granacticin-producing polyketide synthase from *Streptomyces violaceoruber* (Sherman et al., 1989) ACP of tetracenomycin C-producing polyketide synthase from *Streptomyces glaucescens* (Bibb et al., 1989); proposed sites for EntF involved in enterobactin biosynthesis (Rusnak et al., 1991).

of Rusnak et al. (1991) investigating enterobactin biosynthesis have established a protocol to detect proteins that are modified by phosphopantetheine. When pKAS4, a plasmid containing PHA synthase, is transformed into *E. coli* SJ16 (cells that require $\beta$-alanine for growth in minimal medium), studies using $\beta$-[$^3$H]alanine reveal that the synthase is modified (Figure 7, lanes 3 and 4). While the incorporation of this label is substantially lower than that observed with ACP and acylated ACP, this is not unexpected as most of the PHA synthase is produced subsequent to induction with IPTG and the $\beta$-[$^3$H]alanine is incorporated into ACP prior to induction during the 20-h growth phase. If additional $\beta$-[$^3$H]alanine is added concomitant with the induction of the synthase with IPTG, increased incorporation of label into the synthase is observed (Figure 7, lane 3 in comparison to lane 4). Thus, the synthase can be modified with $\beta$-[$^3$H]alanine consistent with posttranslational modification by a phosphopantetheine moiety. In addition, activity studies reveal that increasing the amount of phosphopantetheine in the protein results in a corresponding increase in polymerase activity. A second set of experiments in which the synthase is incubated with varying ratios of 3-D-HBCoA per synthase (up to 10 000:1), followed by analysis of the products by ultracentrifugation (Figure 6), also suggests that most of the synthase is active (can produce polymer) and therefore is posttranslationally modified. These results in conjunction with the $\beta$-[$^3$H]alanine labeling studies suggest that *E. coli* makes sufficient amounts of phosphopantetheine (or CoA) and the protein required to attach it to other proteins (perhaps ACP synthase) to modify the PHA synthase. When one considers that ACP is the most abundant protein in *E. coli*, that CoA pools are large in *E. coli*, and that CoA is the substrate providing the pantetheine moiety for ACP, it is perhaps not too surprising that PHA synthase can be modified in *E. coli* by the same biosynthetic pathway (Cronan & Rock, 1987). The extent of phosphopantetheinylation in PHA synthase produced in *E. coli* UT5600, however, remains to be quantitatively established.

Finally, phosphopantetheine is always found covalently attached through a phosphodiester linkage to a serine. In ACPs the sequence surrounding the modified serine has been conserved, although the putative 4′-phosphopantetheinylation site(s) of other modified proteins such as EntF and citrate lyase does (do) not show striking similarity (Figure 8) (Rusnak et al., 1991). A comparison of the sequences of 10 PHA synthases reveals a single conserved serine (S260 in the *A. eutrophus* nomenclature). Its sequence context is also shown in Figure 8. The conserved serine seems to be staggered by

one residue relative to the ACP pantetheinylation site. The residue S259 in *A. eutrophus* PHA synthase is aligned with the phosphopantetheine sites of ACPs, but this serine is not conserved in PHA synthases. The modified site in PHA synthase remains to be established, but this S260 appears to be the most likely candidate.

This paper reports the first purification of a polyoxoester synthase and provides the first insight into the mechanism of this interesting system. With the tools on hand, the details of the priming–initiation, elongation, and termination can now be elucidated. Furthermore, the substrate specificity of this enzyme can be examined in detail and could lead to new polymers with desirable thermoplastic properties not accessible in whole cells due to metabolic constraints.

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