PHA Synthase from *Chromatium vinosum*: Cysteine 149 Is Involved in Covalent Catalysis†

Ute Müh,‡ Anthony J. Sinskey,§ Daniel P. Kirby,|| William S. Lane,|| and JoAnne Stubbe*,‡,§

Department of Chemistry and Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, and Harvard Microchemistry Facility, 16 Divinity Avenue, Cambridge, Massachusetts 02138

Received July 30, 1998; Revised Manuscript Received October 23, 1998

ABSTRACT: Polyhydroxyalkanoate synthase (PHA) from *Chromatium vinosum* catalyzes the conversion of 3-hydroxybutyryl-CoA (HB-CoA) to polyhydroxybutyrate (PHB) and CoA. The synthase is composed of a ∼1:1 mixture of two subunits, PhaC and PhaE. Size-exclusion chromatography indicates that in solution PhaC and PhaE exist as large molecular weight aggregates. The holo-enzyme, PhaEC, has a specific activity of 150 units/mg. Each subunit was cloned, expressed, and purified as a $(His)_6$-tagged construct. The PhaC-$(His)_6$ protein catalyzed polymerization with a specific activity of 0.9 unit/mg; the PhaE-$(His)_6$ protein was inactive (specific activity <0.001 unit/mg). Addition of PhaE-$(His)_6$ to PhaC-$(His)_6$ increased the activity several 100-fold. To investigate the priming step of the polymerization process, the PhaEC was incubated with a trimer of HB-CoA in which the terminal hydroxyl was replaced with tritium ([^{3}H]-sT-CoA). After Sephadex G50 chromatography, the synthase contained ∼0.25 equiv of the labile label per PhaC. Incubation of [^{3}H]-sT-synthase with HB-CoA resulted in production of [^{3}H]-polymer. Digestion of [^{3}H]-sT-synthase with trypsin and HPLC analysis resulted in isolation of three labeled peptides. Sequencing by ion trap mass spectrometry showed that they were identical and that they each contained an altered cysteine (C149). One peptide contained the [^{3}H]-sT while the other two contained, in addition to the [^{3}H]-sT, one and two additional monomeric HBs, respectively. Mutation of C149 to alanine gave inactive synthase. The remaining two cysteines of PhaC, 292 and 130, were also mutated to alanine. The former had wild-type (wt) activity, while the latter had 0.004 wt % activity and was capable of making polymer. A mechanism is proposed in which PhaC contains all the elements essential for catalysis and the polymerization proceeds by covalent catalysis using C149 and potentially C130.

Polyhydroxyalkanoates (PHAs[1]) are polyoxoesters with properties that range from elastomers to thermoplastics (*1*−*4*). They are produced by a wide range of bacteria when they are placed in an environment of nutrient limitation (*5*). Copolymers of polyhydroxybutyrate (PHB) and polyhydroxyvalerate in the correct ratio have properties similar to the petrochemically based polypropylenes, the major component of bulk plastics (*6*). In 1997, the US market for thermoplastics was on the order of 40 million tons per year (*7*). PHAs have recently received much attention because they are biodegradable and can be generated from biorenewable sources: bacteria and plants (*8*). The major focus of many investigators is to make their production economically competitive with the polypropylenes. To achieve this goal, the requirements for the polymerization process need to be established. This paper focuses on the PHA synthase from *Chromatium vinosum* which catalyzes the formation of PHB from 3-hydroxybutyryl-CoA (HB-CoA). Evidence is presented that two cysteines and covalent catalysis play an important role in the initiation and elongation of the polymerization process.

PHA synthases from 20 organisms have now been identified. They have been divided into three classes based on their substrate specificity and subunit composition (*9*). The class I synthases, with the *Ralstonia eutropha* synthase as a prototype, are composed of a single polypeptide (∼65 kDa) proposed to be functionally active as a dimer (*10*). HB-CoA and 3-hydroxyvaleryl-CoA are substrates for these synthases. The class III synthases, typified by the *C. vinosum* enzyme (*11*), are composed of two different types of polypeptides of ∼40 kDa each. The substrate specificity of the class III synthases is similar to that observed with the class I enzymes. Finally, the class II enzymes (*12*) are composed of a single polypetide chain (∼63 kDa) and use medium-chain-length 3-hydroxyalkanoyl-CoA substrates. Polymers derived from short-chain substrates have properties of thermoplastics, while polymers of medium-chain substrates behave as elastomers (*6*).

† This work was supported by NIH grant GM 49171 to J.S. and A.J.S. and by a Feodor Lynen fellowship from the Alexander von Humboldt Foundation to U.M.

* To whom correspondence should be addressed.
‡ Department of Chemistry, MIT.
§ Department of Biology, MIT.
|| Harvard Microchemistry Facility.

[1] Abbreviations: ACP, acyl carrier protein; DTNB, dithionitrobenzoic acid; GPC, gel permeation chromatography; HB, 3-hydroxybutyrate; HB-CoA, 3-hydroxybutyryl-CoA; IPTG, isopropyl-β-D-thiogalactopyranoside; PHA, polyhydroxyalkanoic acid; PhaEC, PhaE and PhaC coexpressed and copurified; PHB, polyhydroxybutyrate; sT, a trimer of 3-hydroxybutyrate in which the terminal hydroxyl is replaced with a hydrogen; sT-CoA, saturated trimer-CoA; TFA, trifluoroacetic acid; TFE, trifluoroethanol; wt, wild-type; CID, collisionally induced dissociation; MS/MS, tandem mass spectrometry.

10.1021/bi9818319 CCC: $18.00
Published on Web 12/22/1998

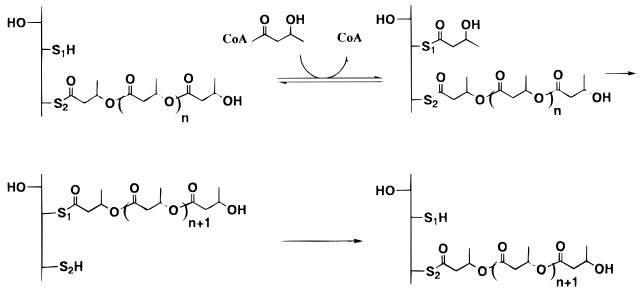


FIGURE 1: Working model for the reaction mechanism of *C. vinosum* PHA synthase. Two cysteine residues are postulated to participate in covalent catalysis: a loading site and an elongation site. A conserved serine (S90) could function in the loading of HB-CoA onto a cysteine of the enzyme or as a covalent intermediate in the chain termination process (a thioesterase). Active site bases are required to generate the thiolate anions and to deprotonate the substrate hydroxyl group, activating it for nucleophilic attack. These roles could be accomplished by conserved histidines (H303 and H331).

Alignment of all class I enzymes using the Clustal W algorithm with the default settings (*13*) reveals only 10% identity (25% similarity). When the three class III enzymes are included in the alignment, only 6% sequence identity (20% similarity) is apparent. Alignment of all PHA synthases sequenced to date reveals that only 19 residues are conserved and suggests that at least 1 cysteine, 1 serine, and 2 histidines will play an important role in catalysis.

Understanding of the mechanisms of initiation, elongation, and termination for homopolymerization processes, such as glycogenesis (*14, 15*), formation of polyphosphate (*16, 17*), and rubber synthesis (*18*−*20*), has lagged far behind our understanding of protein and nucleic acid polymerizations. To study the PHA synthase catalyzed formation of polyoxoesters, a working hypothesis is required. Fatty acid biosynthesis (*21*) and more recently polyketide biosynthesis (*22*−*24*) have provided us with a framework, shown in Figure 1, on which to design experiments. This model is further based on the conservation of residues across all three classes of synthases noted above and on the importance of thiolates noted many years ago by Merrick and co-workers (*25*). In this model, two cysteines and a serine play a key role in catalysis. Cysteine 1 (SH1) binds the monomeric substrate, much as malonyl-CoA is loaded onto a pantetheinylated acyl carrier protein (ACP) in fatty acid synthase. In both *E. coli* and mammalian fatty acid synthase, loading of malonyl-CoA proceeds through an acyl-*O*-serine intermediate on the translocating enzyme, malonyl-CoA:ACP transacylase (*26*−*28*). The conserved serine (S90 in *C. vinosum*) in all synthases could serve a similar function in PHA synthases. The second cysteine (SH2) is the site of initiation (priming) and elongation, and is analogous to the essential cysteine of β-ketoacyl-ACP synthase in fatty acid synthase (*21, 26*).

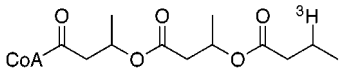
Once "primed" and loaded, the growing polymer is proposed to chain-extend by attack on the growing chain by the deprotonated hydroxyl group of the monomer. In our model, the relative positions of the attacking alkoxide group and the carbonyl group of the growing chain always remain constant. This reaction results in the elongated chain remaining temporarily on the "loading" site. From the symmetry point of view, the elongated chain is then transferred back to SH2 and the elongation process repeats itself. The chain termination process or whether the PHB always remains covalently attached to the protein is largely unknown. Recent evidence from the Steinbüchel laboratory has been interpreted to indicate that in vitro there are 25 polymer chains synthesized per molecule of *C. vinosum* PHA synthase, which implies that there is a termination step (*29*). In animal fatty acid synthase and in some members of the polyketide synthase family, a thioesterase domain containing an essential serine is required for the hydrolysis step or lactone formation (*30*−*33*). Thus, an alternative role for the conserved serine in PHA synthases might be controlling the chain length in a fashion that remains to be established. In the case of fatty acid synthase and polyketide synthase, conserved histidines have been proposed to generate thiolates or alkoxides (*34, 27, 35*), the active form of the residues required for catalysis. A similar role can be postulated for the conserved histidines in PHA synthases.

The present paper focuses on understanding the mechanism of a class III synthase from *C. vinosum* isolated in 1994 by Steinbüchel and co-workers (*11*). They demonstrated that it was composed of two subunits, designated PhaE and PhaC (*36*). PhaC from *C. vinosum*, while possessing low sequence identity with class I synthases (6%), possesses all of the conserved residues. However, all residues that are conserved among PHA synthases are present in PhaC, suggesting that it is the synthase component of the enzyme. PhaE shows no similarity to any protein sequence in the databases. The properties of the *C. vinosum* synthase were reported to be significantly different from the *R. eutropha* enzyme despite the fact that both enzymes use HB-CoA as a substrate (*36*). In an effort to understand the mechanism of the polymerization process and differences in polymerization strategies between the class I and class III synthases, we decided to investigate this enzyme. The present paper provides evidence that PhaC contains all of the elements required for the polymerization process. Our studies demonstrate that C149 plays an essential role in covalent catalysis similar to C319 from *R. eutropha* (*10*). Our studies further demonstrate that C130 also plays a key role in catalysis and may serve a role equivalent to that postulated for C319 in the second monomer in the *R. eutropha* enzyme. In addition, studies with a derivative of trimeric 3-hydroxybutyryl-CoA as a primer have given the first glimpse into the mechanism of the elongation process.

MATERIALS AND METHODS

Materials. The plasmid pds37 containing *phaEC* was a gift of Alexander Steinbüchel at the University of Münster (*11*). Synthesis of HB-CoA and a trimer of 3-hydroxybutyryl-CoA in which the terminal hydroxyl group is replaced with ^{3}H, designated saturated trimer-CoA (**1**, [^{3}H]-sT-CoA), will be described elsewhere (Wodzinska and Stubbe). [^{3}H]-3-Hydroxybutyryl-CoA ([^{3}H]-HB-CoA) was prepared as described (*37*). DNA and Edman N-terminal sequencing were carried out by the MIT biopolymers lab. Peptide molecular weight determinations were made on a Sciex Model 365 mass spectrometer, also by the MIT biopolymers lab. Sequence analysis of tryptic peptides was performed using nanospray (*38, 39*) by the Harvard Microchemistry Facility. Collisionally induced dissociation (CID) spectra were recorded with a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA) operated in the Tune Plus

mode at 30−35% relative energy. Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs. Oligonucleotides were obtained from Genosys Inc. Sequence alignments were carried out with Clustal W (*13*).



I: [^{3}H]-saturated trimer-CoA

Construction of a phaEC Expression Plasmid and Site-Directed Mutagenesis of phaC. DNA manipulation followed standard protocols (*40*). Plasmid pET-UM4, which places expression of *phaEC* under the control of a tightly regulated T7*lac* promoter in pET26b (Novagen), was constructed by the following steps. (i) A ∼300 bp PCR product encoding the 5′ end of *phaE* was obtained from pds37 (*11*) using TGC TCT AGA CGC CCA ACA CCA CGC GCC and TCT TGG TCC AGA GCT CCA AGC CGT TGC CGG as primers. The first primer hybridizes 17 bps upstream of the start codon of *phaE* and encodes an *Xba*I site (underlined). The second primer hybridizes at the *Sac*I site (underlined) in *phaE*. The PCR product was digested with *Xba*I and *Sac*I and ligated into pET26b that had been cut with the same enzymes. The resulting plasmid was called pET-UM2. (ii) To create pET-UM4, the rest of *phaE* and all of *phaC* were obtained from pds37 on a ∼2300 bp restriction fragment (*Sac*I−*Sph*I/blunt-ended with mung bean nuclease) and ligated into pET-UM2 (*Sac*I−*Hin*dIII/blunt-ended with Klenow fragment).

All three cysteines in *phaC* were mutated individually to alanine using the QuikChange method (Stratagene) according to the instructions from the manufacturer. Plasmid DNA, purified from a *dam* methylating strain of *Escherichia coli* (DH5α) and containing the DNA insert to be mutated, was the template for a primer extension reaction with two oligonucleotides, each containing the desired mutation and complementary to opposite strands of the vector. Temperature cycling reaction with *Pfu* DNA polymerase generates a mutated plasmid with staggered nicks. The product is treated with *Dpn*I, an endonuclease with a 4 base recognition sequence that specifically hydrolyzes methylated and hemimethylated DNA. Thus, the parental DNA template is digested, leaving behind the mutation-containing synthesized DNA which is then transformed into *E. coli*.

For the purpose of the mutagenesis reaction, casettes surrounding the area to be mutated were subcloned. A ∼780 bp fragment of *phaC* was PCR-amplified from pET-UM4 using CCG GCC AGG ACG TCT ACC TG and CGC GGA TCC **CTC GAG** CGT TAT CGC TCG TTG AGC CAC T as primers. The first primer hybridizes to the *Aat*II site (underlined) in *phaC*. The second primer hybridizes to the 3′-end of *phaC* and introduces an *Xho*I site (boldface) followed by a *Bam*HI site. The PCR fragment was digested with *Aat*II and *Bam*HI, and cloned into the same sites of pNEB193 (New England Biolabs). Sequencing revealed that during this procedure a silent mutation was inadvertently introduced—glutamate 324 is now coded for by GAA instead of GAG. Mutations were then introduced using the following primers (and their respective complements): GGC TAC ATC GAC CGC GCC GTG GAC TAT CTG CGC (Cys130→Ala); CAA TCT GCT CGG TAT CGC TCA GGG CGG CGC CTT C (Cys149→Ala); CCT GAA GGA CAT CAC CGC CCC GGT GCT CAA CAT C (Cys292→Ala). The cassettes were excised with *Aat*I and *Xho*I and ligated into the same sites of pET-UM4, yielding pET-UM23 (C130A), pET-UM21 (C149A), and pET-UM24 (C292A).

Construction of Plasmids for Production of PhaE-His$_6$ and PhaC-His$_6$. PhaE and PhaC with (histidine)$_6$-tags at their carboxy termini were cloned separately under control of a *trc* promoter. The expression vectors were derived from pTrc-UM7 as follows. (i) Construction of pTrc-UM7: a ∼2600 bp fragment carrying *phaEC* was cloned into the *Eco*RI−*Sma*I-digested pTrcN (*37*) in a two-step procedure essentially as described above for pET-UM4 except that the 5′ primer encoded an *Eco*RI site rather than an *Xba*I site. (ii) PhaE-His$_6$: the 3′ end of *phaE* was PCR-amplified from pds37 using primers GAA GCG CAT GTC GAT CCT GG and CGG CTG CAG TCA GTG ATG GTG ATG GTG ATG CTC GAG GGA TCC ATC GGC CGG ATT GGT CTT GG. The first primer hybridizes 101 bp 5′ to the *Eco0109* I site in *phaE*. The second primer hybridizes to the 3′ end of *phaE*, codes for the histidine tag, and includes a *Pst*I site (underlined). The resulting ∼300 bp PCR product was cut with *Eco0109* I and *Pst*I, and ligated into the same sites of pTrc-UM7 to make pTrc-UM9, the plasmid used for expression of PhaE-His$_6$. (iii) PhaC-His$_6$: *phaC* was amplified by PCR from pds37 with CCG GAA TTC GGA TCC GGC TCA CCA GAC AGA ATC AG and CGG GGT ACC TCA GTG ATG GTG ATG GTG ATG CTC GAG TCG CTC GTT GAG CCA CTT ACC as primers. The first primer hybridizes 32 bp upstream of the start codon for *phaC* and includes an *Eco*RI site (underlined). The second primer hybridizes to the 3′ end of *phaC* and encodes a histidine-tag and a *Kpn*I site (underlined). The ∼1100 bp PCR product was digested with *Eco*RI and *Kpn*I, and ligated into the same sites of pTrc-UM7 to make pTrc-UM11, the plasmid used for expression of PhaC-His$_6$. Each construct was confirmed by sequencing.

Purification of PHA Synthase. PHA synthase was purified from strain BL21(λDE3) carrying plasmid pET-UM4. An overnight culture (50 mL) that had been grown at 37 °C in LB medium plus 15 μg/mL kanamycin was used at a dilution of 1:100 to inoculate 5 × 1 L of the same medium. This culture was grown at 37 °C with vigorous shaking to an OD_{600} of 0.6, at which time expression of *phaEC* was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; Gibco BRL, Life Technologies) to a final concentration of 0.1 mM. After growth for an additional 3 h, 5 g/L cells were harvested by centrifugation and used for purification immediately or stored at −80 °C.

The following steps were performed at 4 °C. The cells (∼25 g wet weight) were resuspended and homogenized in 40 mL of buffer A (20 mM potassium phosphate, pH 7.8, 50 mM NaCl, 0.5 mM EDTA). Cells were disrupted by two passes through a French pressure cell at 12 000 psi, and cell debris was removed by centrifugation at 8000*g* for 30 min. The supernatant was recovered and centrifuged at 30000*g* for 45 min. This supernatant was designated the crude extract. The crude extract was made 30% in ammonium sulfate (176 g/L) and centrifuged at 18000*g*, 4 °C, for 30 min. The supernatant was made 60% in ammonium sulfate (198 g/L) and centrifuged at 18000*g*, 4 °C, for 30 min. The pellet was dissolved in buffer A and dialyzed against 2 L of buffer A for a minimum of 5 h. The dialysate was centrifuged for 20

min at 18000*g* to remove any insoluble material, and loaded onto a DEAE-Sephacel column (Sigma, 40 mL) that had been equilibrated with buffer A. The column was washed with 5 volumes of buffer A containing 100 mM NaCl, and then PHA synthase was eluted with a linear gradient of NaCl (100−500 mM; 250 mL × 250 mL; 3 mL/min). The major fractions containing PHA synthase, which eluted at ∼200 mM NaCl, were pooled, concentrated by ultrafiltration (Amicon cell, YM30 membrane), and dialyzed against 2 L of 10 mM potassium phosphate, pH 7.8, for a minimum of 5 h. The dialysate was loaded onto a BioGel HTP hydroxyapatite column (BioRad, 100 mL) that had been equilibrated with the same buffer. The column was washed with 1 volume of this buffer, and then PHA synthase was eluted with a linear gradient of phosphate (10−500 mM, 750 mL × 750 mL, 1 mL/min). Fractions containing PHA synthase, which eluted at ∼150 mM potassium phosphate, were pooled and concentrated by ultrafiltration to 10 mL. The protein was loaded onto a Sephacryl S-300 molecular sieving column (Pharmacia, 360 mL) equilibrated with 50 mM potassium phosphate, pH 7.8, 150 mM NaCl. The column was run at a flow rate of 2.5 mL/min, and PHA synthase eluted at ∼150 mL. Fractions containing pure protein were pooled, concentrated by ultrafiltration, and dialyzed against 20 mM potassium phosphate, pH 7.8, 50 mM NaCl. Aliquots of the protein were quick-frozen in liquid N_2 and stored at −80 °C. No loss of activity was detected even after 6 months storage. PMSF (phenylmethylsulfonyl fluoride) was not added during the purification since we observed that it irreversibly inactivated the enzyme. An identical procedure was followed for purification of PHA synthases with cysteine to alanine mutations. Appropriate fractions were pooled based on analysis by SDS−PAGE (*41*).

Expression and Purification of PhaC-His$_6$ and PhaE-His$_6$. PhaC-His$_6$ and PhaE-His$_6$ were purified from *Escherichia coli* strain BL21 harboring pET-UM9 or pET-UM11, respectively. Growth and induction were as described above for PHA synthase, except that the antibiotic was 100 μg/mL ampicillin and induction was with 1 mM IPTG. After 3 h of additional growth, PhaE-His$_6$ was about 10% of total cell protein as judged by SDS/PAGE and staining with Coomassie blue, and PhaC-His$_6$ was <1% of total cell protein.

The following steps were performed at 4 °C. Cells (4 g) were resuspensed in 6 mL of buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and broken by two passages through a French pressure cell at 12 000 psi. After centrifugation at 18000*g* for 45 min, most of the PhaE-His$_6$ was found in the pellet and was discarded. The soluble protein was purified further. PhaC-His$_6$, on the other hand, remained soluble. Hexa-histidine-tagged proteins were purified over His-Bind resin (Novagen, 1.5 mL) according to the manufacturer's protocol, except that the elution buffer contained 0.2 M imidazole. About 6 mg of PhaC-His$_6$ and 1 mg of PhaE-His$_6$ were obtained from 1 L of culture.

Enzyme Assays. PHA Synthase Activity. During purification, PHA synthase activity was monitored with a continuous assay (*42*) in which free CoA reacts with dithionitrobenzoic acid (DTNB). A 50 mM stock solution of DTNB in 0.1 M $KHCO_3$ is prepared fresh before each use. The assay mixture contains the following in a final volume of 1 mL: 25 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM DTNB; and 60 μM HB-CoA. Reactions were perfomed at 30 °C and started by addition of 10−50 nM enzyme. The ΔA_{412} nm was monitored using $\epsilon_{412} = 13\,600\ cm^{-1}\ M^{-1}$ for the released thiolate (*43*).

In all other experiments, synthase activity (CoA release) was measured with a discontinuous assay. Reactions were conducted at 30 °C and contained the following in a final volume of 250 μL: 20 mM Tris-HCl, pH 7.5; 50 mM NaCl; enzyme (20 nM wt synthase, 5 μM PhaC-His$_6$, or C130A); and 1 mM HB-CoA. At various times, 20 μL aliquots were removed from the reaction mixture and quenched with 50 μL of 10% trichloroacetic acid. After brief centrifugation to remove the precipitated protein, 68 μL was added to 382 μL of 1 mM DTNB in 0.5 M potassium phosphate, pH 7.8, and A_{412} was measured. One unit was defined as 1 μmol of substrate min^{-1}.

Assay for 3-Hydroxybutyrate. 3-Hydroxybutyrate dehydrogenase (Sigma Type II from *Rhodopseudomonas sphaeroides)* was used to assay for 3-hydroxybutyrate (HB) according to a published procedure (*44*). The dehydrogenase oxidizes HB to acetoacetate with concomitant reduction of NAD, resulting in an absorbance change at 340 nm.

Western Blotting. New Zealand white rabbits were immunized against PhaEC by HRP, Inc. Antibodies specific for PhaE and for PhaC were purified (*45*) by absorbing 50 μg of PhaE-His$_6$ or PhaC-His$_6$ onto nitrocellulose membrane (BioBlot-NC from CoStar) and incubating overnight with 400 μL of antibody serum diluted 1:1 in phosphate-buffered saline (*40*). After removal of the depleted serum and extensive washing of the membrane with phosphate-buffered saline, antibodies were eluted with 200 μL of 0.1 M glycine hydrochloride, pH 2.5. The pH was then adjusted to neutrality with 0.1 volume of 1 M Tris-HCl, pH 8.0, and bovine serum albumin and glycerol were added to 0.1 mg/mL and 50% final concentration, respectively. Purified antibodies were stored at −20 °C. Western blotting was performed according to standard protocols (*40*). Protein was separated by SDS−PAGE and transferred in a semi-dry-blot apparatus (Hoefer) to a PVDF membrane (2 microns pore size, BioRad) at 100 mA for 90 min. The membrane was treated with blocking buffer (5% dry milk powder in phosphate-buffered saline) for 1 h and incubated with the purified antibody at 1:400 dilution in blocking buffer for 1 h. After extensive washing with phosphate-buffered saline, the membrane was incubated for 1 h with anti-rabbit IgG, coupled to horseradish peroxidase (Amersham), diluted 10^4-fold in blocking buffer. The membrane was washed extensively with phosphate-buffered saline, and detection of immobilized antigens was carried out with ECL Western blotting detection reagents from Amersham.

Quantitating the Ratio of PhaE to PhaC. The ratio of PhaE to PhaC was estimated by two methods. First, Coomassie-stained SDS−PAGE gels were analyzed by density scanning with a Fluor-S Multimager from BioRad. Second, PHA synthase was injected onto a Vydac C18-RP HPLC column (4.6 × 250 mm), equilibrated in 40% acetonitrile, 0.08% TFA in water. The protein was eluted using a gradient from 40 to 90% acetonitrile in 50 min at a flow rate of 0.8 mL/min. Elution was monitored at 280 nm. PhaE and PhaC were characterized by N-terminal sequencing and by Western blotting with antibodies specific for PhaE and PhaC. From the elution profile at 280 nm, the ratio of PhaE to PhaC was calculated based on the area underneath the respective peaks, corrected for the different theoretical extinction coefficients

(*46*) for PhaE (ϵ_{280} = 1.72 M^{-1} cm^{-1}) and PhaC (ϵ_{280} = 1.23 M^{-1} cm^{-1}).

Covalent Labeling of PHA Synthase with [^{3}H]-sT: Stability of Label. PHA synthase (95 μM) was reacted with 1 mM [^{3}H]-sT-CoA (3.1×10^7 cpm/μmol) at room temperature for 4 min, at which time the protein was denatured by addition of guanidinium chloride to a final concentration of 6 M. After 10 min at room temperature, enzyme was separated from unincorporated [^{3}H]-sT-CoA by passing the sample over a G-50 Sephadex column (Sigma, 22 mL) that had been equilibrated in 2 M urea and one of the following buffers: 0.1 M ammonium bicarbonate, pH 8.5; 0.1 M potassium phosphate, pH 7.0 and 6.0; 0.1 M ammonium acetate, pH 4.0; or 0.1 M glycine hydrochloride, pH 2.0. Fractions containing PHA synthase were pooled and analyzed by scintillation counting. The pooled, denatured, and ^{3}H-labeled protein in each of the above buffers was incubated at 37 °C. Aliquots were removed at times and loaded onto the Sephadex G-50 column in the same buffer. Radioactivity eluting with synthase was quantitated by scintillation counting.

Isolation of [^{3}H]-sT-Labeled Peptides. In a final volume of 73 μL, 86 μM PHA synthase (500 μg) and 1.0 mM [^{3}H]-sT-CoA (3.1×10^7 cpm/μmol) were reacted in 20 mM potassium phosphate, pH 7.8, 50 mM NaCl at room temperature for 4 min. The reaction was stopped by the addition of 219 μL of 8 M guanidinium chloride (6 M final concentration) and incubated for 10 min at room temperature. The sample was then loaded onto and eluted from a Sephadex G-50 column (22 mL, 4 °C) equilibrated in 2 M urea, 0.1 M potassium phosphate, pH 6.0. [The urea had been recrystallized from methanol and deionized immediately before use by treatment with resin AG 501-X8(D) from BioRad.] Fractions that contained protein were pooled and digested for 30 min at 37 °C with trypsin (TPCK-treated, Sigma) at a trypsin-to-synthase ratio of 1:4. The reaction was stopped by making the sample 5% in acetonitrile and by adjusting the pH to ~2 with trifluoroacetic acid (TFA). The entire sample was injected onto a Vydac RP-C18 HPLC column (4.6 × 250 mm) equilibrated in 5% acetonitrile and 0.08% TFA in water. The peptides were eluted using an acetonitrile gradient as follows: 5−30% in 0−50 min; 30−50% in 50−70 min; and 50−90% in 70−90 min. The concentration of TFA was 0.08%, and the flow rate 0.8 mL/min.

Gel Permeation Chromatography (GPC) Analysis of PHB. PHA synthase (50 μg of wt enzyme, PhaC-His_6, or C130A mutant enzyme) was reacted with 1 mM [^{3}H]-HB-CoA (3.8×10^4 cpm/μmol) at 30 °C for 30 min, 30 min, and 9 h, respectively. Protein was digested with 50 μg of proteinase K (Boehringer Mannheim Biochemicals) at 37 °C for 30 min, and the reaction mixture was lyophilized. The residue was extracted 3 times with 1 mL of trifluoroethanol (TFE). The combined organic phases were concentrated to 100 μL under a stream of nitrogen, and the sample was injected onto a Shodex KF-807L gel permeation column (Showa Denko K.K.). The column was eluted with 100% TFE at 35 °C and a flow rate of 1 mL/min. Fractions were collected every 30 s and analyzed by scintillation counting.

Chemical Competence of [^{3}H]-sT-Synthase. [^{3}H]-sT-CoA was incorporated into synthase in a 73 μL reaction mixture that contained 86 μM PHA synthase (500 μg) and 1.0 mM [^{3}H]-sT-CoA in 20 mM potassium phosphate, pH 7.8, 50 mM NaCl. The reaction was allowed to proceed for 4 min at room temperature, at which time the enzyme was separated from unincorporated radiolabel on a Sephadex G-50 column (22 mL, equilibrated in 20 mM potassium phosphate, pH 7.0) at 4 °C. Fractions of 1 mL were collected, and an aliquot was removed for scintillation counting. The remainder of each fraction was frozen in liquid nitrogen to prevent loss of [^{3}H]-sT from the enzyme while determining which fractions contained the synthase. The synthase fractions were then thawed and pooled, and aliquots were removed for further analysis. One aliquot (200 μL) was used to determine whether enzyme-bound [^{3}H]-sT could be chased into polymer. PHA synthase (50 μg, final concentration 4 μM) was made 50 mM NaCl and 1 mM HB-CoA and incubated at 30 °C for 30 min. The sample was then treated with proteinase K (50 μg) for 30 min at 37 °C and lyophilized. A second aliquot (200 μL), to provide a standard for [^{3}H]-sT, was prepared by digesting 50 μg of [^{3}H]-sT-labeled PHA synthase with 50 μg of proteinase K for 30 min at 37 °C, followed by lyophilization. A third aliquot was used to determine the amount of [^{3}H]-sT still bound covalently to the synthase just prior to the chase with HB-CoA. Recall that the labeled synthase is chemically labile. PHA synthase (25 μg) was denatured with 6 M guanidinium chloride for 10 min at room temperature and eluted from a Sephadex G-50 column that had been equilibrated in 2 M urea, 0.1 M glycine hydrochloride, pH 2.0. The eluted protein was analyzed by scintillation counting. Aliquots from the first and the second experiments were lyophilized, extracted into 3 mL of TFE, and analyzed by GPC. [^{3}H]-PHB, that was prepared with synthase and [^{3}H]-HB-CoA as described above, served as a standard for the retention time of polymer produced under identical conditions.

RESULTS

PHA Synthase Expression and Purification. Studies by Steinbüchel reported cloning, sequencing, and expression of the *C. vinosum* synthase (*11*, *36*, *29*). They purified PHA synthase to homogeneity and reported a specific activity of 60 units/mg. In an effort to determine whether the synthase activity resides on PhaC as suggested by sequence alignments, they independently expressed each subunit and used crude extracts of these individually expressed subunits to assay for activity. Their failure to detect activity under these conditions led to their conclusion that both subunits are essential to catalyze polymerization.

Our original efforts to purify the synthase used pds37 constructed and provided by the Steinbüchel lab. In our hands, expression from this plasmid was constitutive, which presumably led to the ~50% proteolysis of PhaE that was observed. To avoid this problem, PhaEC was recloned into a vector under the control of a T7*lac* promoter. After growth and induction with IPTG, the synthase constituted ~25% of the soluble protein with specific activity in the crude extract of 10 units/mg (Table 1). An alternative purification procedure to that originally published (*36*) was developed due to our inability to reproduce the results from their dye-based affinity resin. From 25 g of bacteria, 60 mg of protein (Figure 2) was isolated by standard procedures (Table 1). Purification was followed using a continuous assay in which DTNB is included in the assay mixture, the concentration of HB-CoA

Table 1: Purification of PHA Synthase[a]

step	volume (mL)	total units	sp act. (units/mg)[b]	protein (mg)	protein concn (mg/mL)
crude extract	82	26500	12	2200	27
30−60% AS	21	9690	23	910	44
DEAE-Sephacel	71	16700	59	280	4.0
hydroxyapatite	260	8670	64	120	0.45
S-300	38	4460	73 (150)[c]	64	1.7

[a] From 25 g of cells. [b] All specific activities were determined with a continuous assay at 60 μM HB-CoA. One unit corresponds to 1 μmol/min. [c] Specific activity measured in a discontinuous assay using 1 mM HB-CoA.

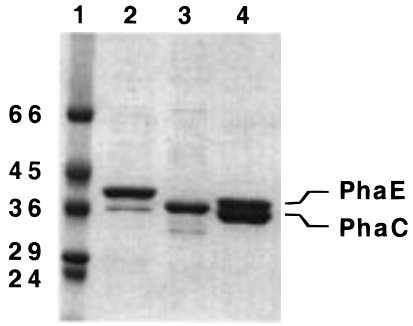


FIGURE 2: SDS−PAGE of purified PHA synthase. Lane 1, molecular mass markers; lane 2, PhaE-His_6 (41.3 kDa); lane 3, PhaC-His_6 (40.6 kDa); lane 4, PhaC (39.8 kDa) and PhaE (40.5 kDa) copurified. Approximately 3 μg of protein was loaded in each lane.

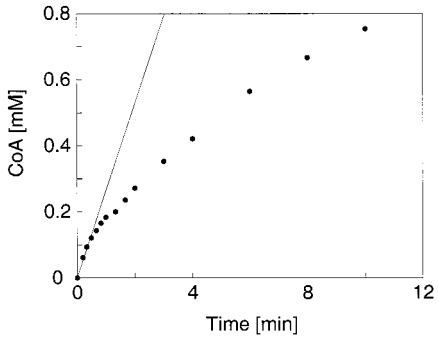


FIGURE 3: Time course of CoA release by PHA synthase (18 nM) from 1 mM HB-CoA at 30 °C. CoA release was monitored in a discontinuous assay using DTNB (*43*).

is subsaturating at 60 μM, and CoA release is monitored. In this assay, the specific activity of our protein (73 units/mg) is comparable to that previously reported (*36*). Since DTNB reacts readily with thiolates and these residues have been proposed to play an important role in the polymerization process, we also monitored CoA release using a discontinuous assay. In this assay, the kinetics are biphasic (Figure 3). In the first phase of this reaction, we measure a significantly higher specific activity of 150 ± 50 units/mg than that determined in the continuous assay. The ease of the continuous assay makes it the method of choice during protein purification. However, the discrepancies between the two methods suggest that the discontinuous assay should be used in all other analyses.

Previous studies, using rocket immunoelectrophoresis, reported a ratio of PhaE to PhaC of 4:1 to 1.5:1 (*36*). We have reinvestigated the subunit stoichiometry by several methods. SDS−PAGE (Figure 2, lane 4) and densitometry

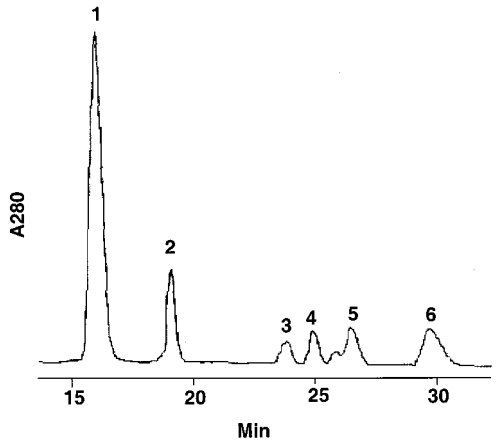


FIGURE 4: RP chromatography of PHA synthase. PhaE and PhaC were eluted from a Vydac C18-RP HPLC column monitored at 280 nm, using 0.08% TFA and a linear gradient from 40% to 90% acetonitrile. Eluted peaks were identified by Western blotting and by N-terminal sequencing to be PhaE (peak 1) and PhaC (peaks 2−6).

scanning of Coomassie-stained gels reveal that PhaC and PhaE are present in approximately stoichiometric amounts when copurified. However, proteins of the same size can stain differently, and therefore a second method of assessment of the stoichiometry was undertaken. The synthase (440 μg) was placed in 0.08% TFA, and PhaE and PhaC were separated on a Vydac C18 RP-HPLC column (Figure 4). Fractions that contained protein were collected and characterized by Western blotting and N-terminal sequencing by the Edman method (*47*). While PhaE eluted as a single sharp peak (1, Figure 4), PhaC eluted as five separate peaks (2 through 6, Figure 4). The area underneath the elution trace monitored at 280 nm was quantitated. The ratio of the area under peak 1 was compared to the sum of the areas under peaks 2 through 6, while correcting for the difference in extinction coefficients at 280 nm for PhaE and PhaC. Using this method, the relative ratio of PhaE to PhaC was estimated to be about 1. Thus, in our hands the ratio of PhaC to PhaE appears to be different than that previously reported (*36*). The reason for the discrepancy is unknown. To examine the native molecular mass, preliminary studies using size-exclusion chromatography on Superose 6 in 0.1 M potassium phosphate, pH 7.8 at 0.2 mL/min, were carried out and revealed two large molecular aggregates, one of 360 ± 50 kDa and one of 520 ± 50 kDa (data not shown). Further studies are required to determine what factors influence the aggregation state.

Is PhaC Sufficient for Synthesis of PHB? Despite the differences in size between PhaC (40 kDa, class III) from *C. vinosum* and PhaC (65 kDa, class I) from *R. eutropha*, sequence alignments suggest that the two proteins are 24% identical in the regions in which they overlap. Thus, given the His-tagged methods available to rapidly isolate proteins from crude extracts, we decided to investigate the question of whether PhaC alone could function as a synthase. PhaC and separately PhaE were recloned with a His_6-tag at their C-termini and placed in an expression system under the control of a *trc* promoter. Each protein was purified to >90% homogeneity using Ni-affinity chromatography (Figure 1, lanes 2 and 3). Purified PhaC-His_6 had a specific activity of 0.9 unit/mg, whereas PhaE-His_6 had no detectable activity (lower limit of detection: 0.001 unit/mg). Interestingly,

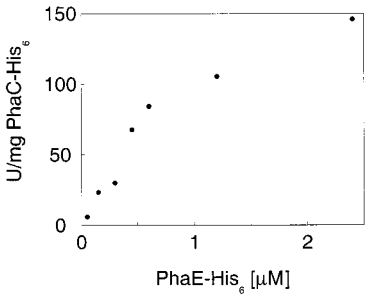


FIGURE 5: Reconstitution of holoenzyme from PhaC-His_6 and PhaE-His_6. All reaction mixtures contained 0.3 μM PhaC-His_6 and the indicated concentration of PhaE-His_6. A discontinuous assay was used, and specific activities were calculated based only on PhaC-His_6.

addition of a 10-fold excess of PhaE-His_6 to PhaC-His_6 stimulated activity by several 100-fold (Figure 5) to levels only 2-fold below that obtained with PhaEC. (It should be noted that the specific activity for the reconstitution experiments is based on milligrams of PhaC only, while that for the native holoenzyme is based on a 1:1 ratio of PhaE:PhaC. Thus, the specific activity of native PhaEC is about 150 units/mg, equivalent to 300 units/mg of PhaC.) The inability to restore full activity at approximately stoichiometric amounts of PhaE and PhaC might mean that the best conditions for reconstition have not yet been found and/or that a substantial fraction of PhaE-His_6 is inactive. It is important to point out that the activity measurements were obtained using the discontinuous assay. The activity of PhaC-His_6 is 20-fold lower using the continuous assay performed in the presence of 1 mM HB-CoA (data not shown). These results suggest that PhaC-His_6 alone is particularly susceptible to inhibition by DTNB.

Because the DTNB-based methods only measure CoA release, two additional assays were carried out on PhaC-His_6 to ensure that polymer formation and not hydrolysis of the HB-CoA ester was the source of the CoA release. First, reaction mixtures were analyzed for HB using HB dehydrogenase. No butyrate was detected, indicating that PhaC-His_6 does not simply hydrolyze HB-CoA. Analysis of the reaction products by gel permeation chromatography (GPC) demonstrated directly that PhaC-His_6 makes polymer, albeit with a lower molecular mass than that synthesized by wt synthase (Figure 6, top). Upon reconstitution of PhaC-His_6 with PhaE-His_6 and incubation with HB-CoA, the product elutes with a retention time comparable to product synthesized by PhaEC (data not shown).

Biochemical Identification of a Reactive Cysteine. Our model for the polymerization process (Figure 1) suggests PHA synthase from *C. vinosum*, like the enzyme from *R. eutropha*, employs covalent catalysis using an *S*-acyl-enzyme intermediate to synthesize PHB from HB-CoA. To identify the amino acid(s) involved in covalent catalysis, [^{3}H]-sT-CoA, **1**, previously used to identify a catalytically important cysteine in PHA synthase from *R. eutropha* (*10*), was investigated. This analogue, which lacks the terminal hydroxyl group, was previously shown to function as a primer of the *R. eutropha* polymerization reaction by eliminating the lag phase in the release of CoA. Furthermore, incubation of the [^{3}H]-sT-synthase with HB-CoA revealed that the resulting polymer was radiolabeled. A similar experiment has now been carried out to determine if a cysteine is also

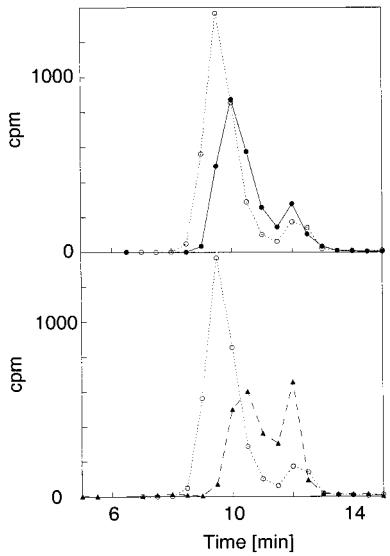


FIGURE 6: Analysis of polymer synthesized by PhaC-His_6 and by the C130A mutant enzyme. Reaction mixtures contained 5 μM protein and 1 mM [^{3}H]-HB-CoA. After removal of protein by digestion with proteinase K, the reaction products were analyzed by GPC. Product from PhaC-His_6 reaction (closed circles, top panel); product from C130A-PhaEC reaction (closed triangles, bottom panel); product from PhaEC reaction (open circles, top and bottom panels). Small molecules eluted at 12.5 min.

involved in covalent catalysis of polymer formation with the *C. vinosum* synthase.

In the case of the *R. eutropha* synthase, ~1 equiv of [^{3}H]-sT was incorporated per dimer of enzyme. The modification was remarkably stable to subsequent steps, including incubation for 17 h at pH 8.0 during trypsin digestion of synthase into peptides (*10*). In contrast, a similar experiment with the *C. vinosum* synthase resulted in ~0.25 equiv of [^{3}H]-sT per mole of PhaC, assuming PhaC and PhaE are present in a 1:1 ratio. In addition, the stability of the label is markedly different from that of the *R. eutropha* synthase. Understanding the stability is essential to designing a protocol to isolate labeled peptides. The stability was investigated under conditions compatible with protein digestion and HPLC purification of peptides. At neutral pH, in 2 M urea, the label is lost from the *C. vinosum* synthase within minutes. In 2 M urea at pH $\leq$4.5, the covalent linkage is stable for 20 h, while in 2 M urea at pH 6.0 the label has a half-life of ~30 min. These studies imply that digestion needs to be carried out rapidly under slightly acidic conditions (pH 6.0), but that the label should be stable to normal peptide purification in TFA and acetonitrile.

Once the stability of the label was established, two experiments were carried out to identify the polypeptide and the amino acid labeled. First, to determine if the label is attached to PhaE or PhaC, [^{3}H]-sT-PhaEC was solubilized in TFA and acetonitrile and chromatographed on a Vydac C18-RP HPLC column as described above. All of the radiolabel (96% recovery) resided on PhaC. Interestingly, the label was found exclusively in peak 2 (Figure 4), and the ratio of the area under peak 2 in comparison to that under peaks 3−6 had shifted from 0.4 to 2.9 (data not shown). The basis for the unusual chromatographic behavior of PhaC in TFA−acetonitrile is not understood, but it is interesting that labeling with sT changes the conformation or aggregation state.

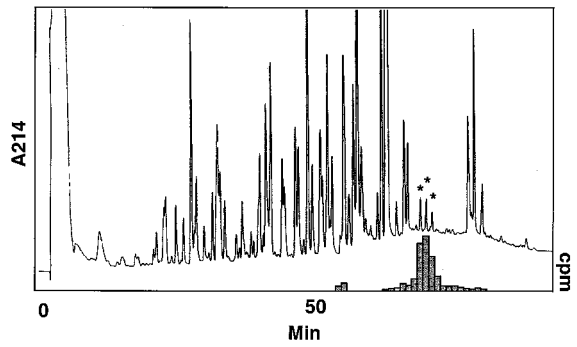


FIGURE 7: Isolation of [^{3}H]-sT-labeled peptides. PHA synthase modified by incubation with [^{3}H]-sT-CoA was isolated and digested with trypsin, and the resulting peptides were separated by HPLC on a Vydac RP-C18 column. The A_{214} was monitored, and aliquots of the eluted fractions were analyzed by scintillation counting, shown as shaded rectangles.

Table 2: Edman Degradation and Electrospray Mass Spectroscopic Analysis of Peptides from Trypsin Digestion of [^{3}H]-sT-PHA Synthase

peptide	N-terminus	calcd peptide mass (Da)	determined mass (Da)	unaccounted mass (Da)
1	EAHGV	3426.98	3669.6	242.6[a]
2	EAHGV	3426.98	3755.1	328.3[b]
3	EAHGV	3426.98	3841.3	414.3[c]

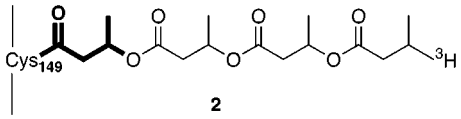
[a] The mass of sT is 242.27 Da. [b] sT + HB = 242.27 + 86.08 Da. [c] sT + $(HB)_2$ = 242.27+172.18 Da.

In an effort to isolate a labeled peptide, [^{3}H]-sT-PHA synthase was digested with high concentrations of trypsin at pH 6.0 for 30 min. The reaction was then stopped by addition of TFA to pH 2.0, inactivating trypsin and stabilizing the remaining covalently attached label. Analysis of the digestion products by Vydac C18-RP HPLC and scintillation counting revealed three peptides that coeluted with 62% of the injected radioactivity (Figure 7). This implies close to quantitative recovery of label given the half-life of the linkage. These three peptides eluted at ~43% acetonitrile and were base-line-separated. Thus, after a single chromatography step, the peptides were essentially homogeneous.

Each peptide was characterized by N-terminal sequencing using Edman degradation (*47*) and by molecular mass using mass spectrometry (Table 2). All three peptides contained an N-terminal E136 of PhaC. The mass of peptide 1 (3669.6 Da) agrees well with that predicted for the peptide EAHGVDKVNLLGI**C**QGGAFSLMYSALHPDKVR (3426.98 Da) plus a saturated trimer moiety (Table 3). Peptide 1 contains C149, which is the only universally conserved cysteine among PHA synthases and the most likely site of acylation (*10*). C149 is in a sequence context very similar to C319 previously identified in the *R. eutropha* enzyme as the most likely site of acylation.[2] Peptide 1 also contains two lysines, indicating that the trypsin digestion did not go to completion.

The remaining two peptides, 2 and 3, are 86 and 172 atomic mass units larger than peptide 1. CID in an ion trap was used to identify the site of the label and the molecular mass of the attached fragment. The full-scan spectrum of peptide 2 contains ions for $[M + 3H]^{3+}$ through $[M + 6H]^{6+}$ charge states which allows verification of the peptide's molecular mass as 3755.1 Da (Table 2). Each of these ions was subjected to CID, but the $[M + 3H]^{3+}$ and $[M + 4H]^{4+}$ species provided the most informative fragments. Fragmentation of the $[M + 3H]^{3+}$ ion produced a series of Y^{1+} ions which verified the putative sequence from G[16] through K[30] and a series of Y^{2+} ions which verified the sequence from G[4] through I[13] (Table 3). Close examination of the Y^{1+} series clearly indicated that neither serine residue in the peptide had been modified. E[1] through V[5] were identified through N-terminal Edman sequencing. The entire peptide sequence has therefore been verified except for the C[14] and Q[15] residues and the last two C-terminal residues (Table 3). Fragmentation of the $[M + 4H]^{4+}$ ion produced the spectrum shown in Figure 8. Here a series of Y^{2+} ions defines the sequence from L[10] through G[16], clearly demonstrating that any modification is contained on the C[14] residue and no other. Additional ions, mainly Y^{3+} and B^{2+} species, are labeled in Figure 8. The observed fragment ion of *m*/*z* 215 (1204.76 − 989.22, Figure 8) is consistent with the mass of 242 (sT) + 86 (HB monomer) + 103 (the Y fragment associated with C) multiplied by 2, the ionic charge. A similar analysis has also been carried out on peptides 1 and 3.

In the case of peptide 2, Cys with the addition of 328.3 atomic mass units is equivalent to that of a saturated tetramer, which is a saturated trimer that has been extended by one HB unit, **2**:



2

A similar analysis on peptide 3 indicates that the C149 is modified with saturated trimer in addition to two HB units. The source of two monomers of HB was initially confusing, as the synthase was exposed only to **1**. However, **1** had been stored for several weeks at pH 4.7 and 4 °C. HPLC analysis revealed that during this time period **1** had been hydrolyzed to give ~7% HB-CoA (*48*). Thus, during the incubation with [^{3}H]-sT-CoA, the synthase was also reacted with approximately stoichiometric amounts of HB-CoA, consistent with an average of one molecule of substrate-derived HB unit per peptide. Quite inadvertently, we have caught the synthase in the process of elongation! These observations indicate that the generation of oligomeric products of controlled length on the enzyme should be feasible and suggest that this synthase process can catalyze a "living" polymerization (*49, 50*).

Chemical Competence of [^{3}H]-sT-Synthase. The labeling of synthase on Cys149 suggests that this residue plays a role in covalent catalysis during polymerization. If the hypothesis is correct, then incubation of [^{3}H]-sT-synthase with HB-CoA should result in formation of [^{3}H]-PHB. This experiment is complicated by the fact that the acylated synthase is labile at the pH required to make labeled polymer. However, despite this problem, GPC chromatography of the resulting product reveals that ~50% of the radioactivity is associated with a large molecular mass polymer (Figure 9). The size of the polymer is similar to that of a control in which [^{3}H]-

[2] Previous studies with *R. eutropha* identified a peptide containing C319, but methods of analysis precluded us from establishing that the cysteine was actually modified.

Table 3: Peptide 2 Sequenced by Tandem Mass Spectrometry[a]

residue:	1	2	3	4	5	6	7	8	9	10	11	12	13	**14**	15	16
assignment:	[E]	[A]	[H]	G	V	D	K	V	N	L	L	G	I	**C***	Q	G
residue:	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
assignment:	G	A	F	S	L	M	Y	S	A	L	H	P	D	K	V	R

[a] Brackets, the three amino-terminal residues could not be defined unambiguously (see text); **C***, mass of cysteine plus 328.3 atomic mass units.

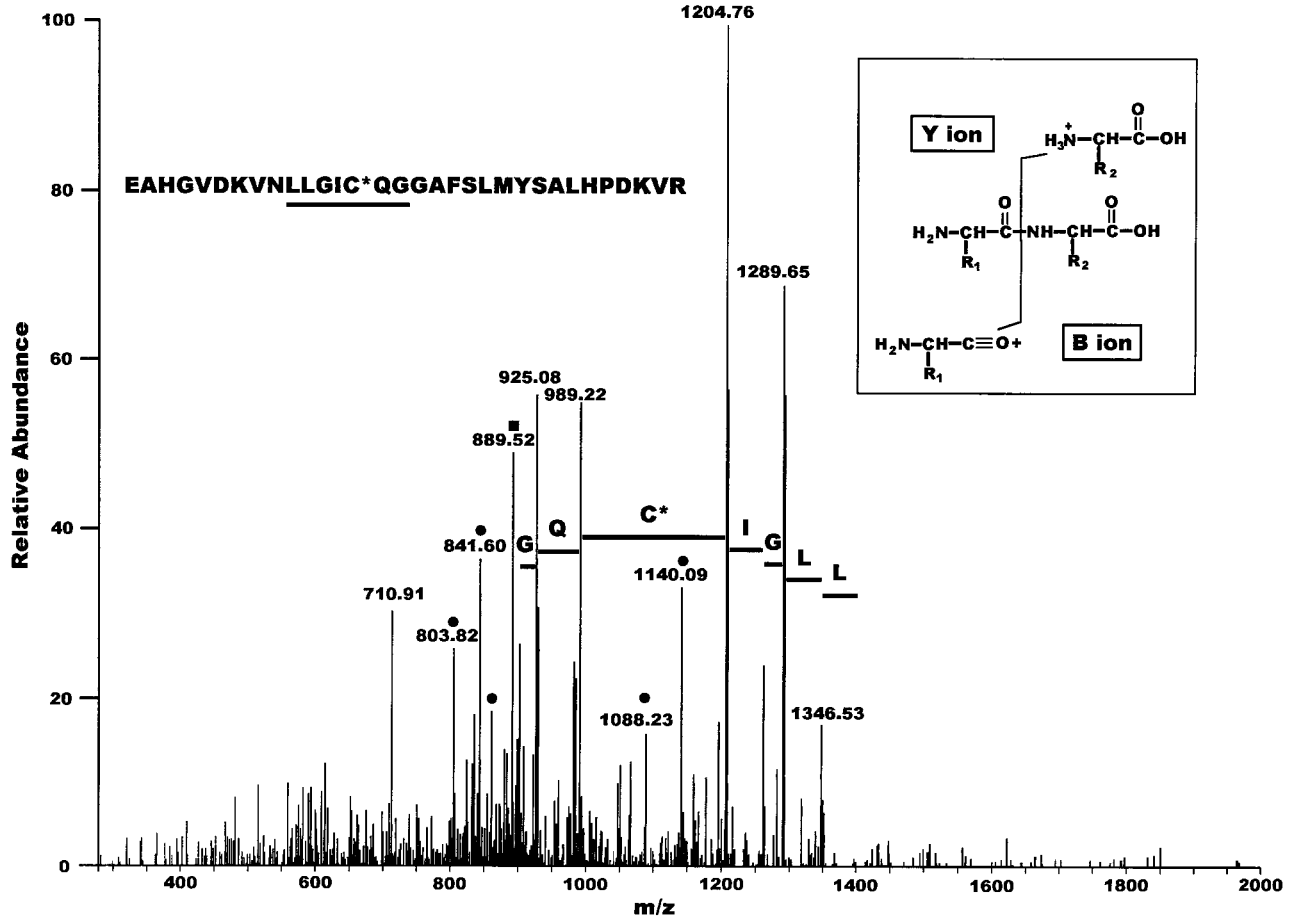


FIGURE 8: CID mass spectrum produced from the fragmentation of the $[M+4H]^{4+}$ ion. A series of Y^{2+} ions (see inset) defines the peptide sequence from L[10] through G[16]. Y^{3+} ions (●) and B^{2+} ion (■) are labeled for clarity. The mass of residue C* is consistent with a cysteine modified with the addition of 328 Da.

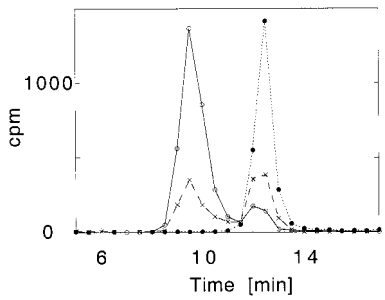


FIGURE 9: Label from [^{3}H]-sT synthase can be chased into polymer by incubation with HB-CoA. [^{3}H]-sT-synthase was incubated with cold HB-CoA, treated with proteinase K to remove protein, and analyzed by GPC and scintillation counting. Reaction product (dashed line, crosses); tritiated polymer standard (solid line, open circles); [^{3}H]-sT (dotted line, closed circles).

HB-CoA is incubated with synthase and analyzed under identical conditions. The remaining radioactivity is isolated with small molecules, consistent with the experimental observation that 30% of the counts had already been lost from the enzyme prior to addition of HB-CoA to start the polymerization.

Genetic Identification of Important Cysteine Residues. Our model in Figure 1 proposes that two thiolates are involved in covalent catalysis. To provide further evidence for the importance of C149 and assess whether an additional cysteine might play a role in catalysis, site-directed mutagenesis of all three cysteines in PhaC was carried out. Each was mutated to an alanine, and the mutated enzymes were expressed and isolated in a fashion very similar to wt synthase. The C292A synthase and wt synthase had similar activities in the crude extract (14.5 and 15.6 units/mg, respectively), while C130A and C149A mutant enzymes lacked detectable activity under these conditions.

To set a lower limit on detectable activity, the C130A and C149A synthases were purified to homogeneity. Protein yields and elution behavior were comparable to those of the wt enzyme at each purification step, suggesting that these proteins were properly folded. The specific activity of the purified C130A protein was 0.004 unit/mg (Figure 10) and

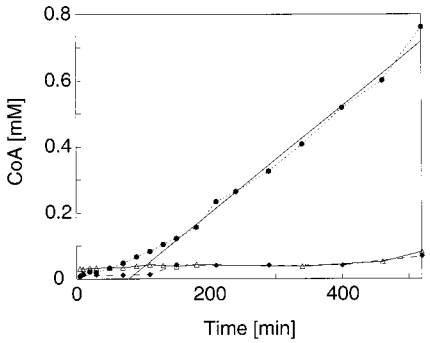


FIGURE 10: Time course of CoA release by C149A and C130A PhaEC. Reaction mixtures contained 5 μM enzyme and 1 mM HB-CoA. At the indicated times, an aliquot was removed and assayed for CoA. C149A-PhaEC (open triangles), C130A-PhaEC (closed circles), no protein control (closed diamonds).

was preceded by a long lag phase. In contrast, the activity of the C149A synthase was below the lower limit of detection (0.001 unit/mg). When high concentrations of C149A protein were incubated with substrate, no significant CoA release was detected (data not shown), demonstrating that the C149A protein did not undergo single turnover indicative of protein acylation.

To rule out the possibility that C130A mutant enzyme simply hydrolyzes the HB-CoA instead of generating polymer, the reaction mixture was assayed for HB enzymatically. No HB was detected. Furthermore, incubation of the reaction mixture for 9 h and analysis of the product mixture by GPC demonstrate directly that the mutant enzyme makes polymer, albeit with a lower molecular mass than that synthesized by wt synthase (Figure 6, bottom). About 60% of the eluted radioactivity was in the polymer fraction, which is comparable to the percentage of consumed substrate determined by assaying an aliquot of the reaction mixture for CoA release (not shown).

Incubation of Mutant Synthases with [^{3}H]-sT-CoA. As a further probe of the structural intactness of the mutant synthases and to compare their properties with wt synthase, each was incubated with [^{3}H]-sT-CoA. As anticipated, no radiolabel was recovered with C149A protein under conditions in which 0.25 equiv of label was observed with the wt protein. C130A synthase, on the other hand, incorporated approximately 0.25 mol of [^{3}H]-sT per mole of enzyme. As with the wt enzyme, the label was stable at pH 4.5 but not at pH 6.0. These results support the importance of C149 in catalysis.

DISCUSSION

Studies of the Steinbüchel group laid the groundwork for a detailed mechanistic study on the class III PHA synthases (*11*, *36*, *29*). The synthase from *C. vinosum* differs markedly in structure from the *R. eutropha* class I synthase (*37*, *10*), while maintaining a similar substrate specificity. The *R. eutropha* enzyme exists as a monomer of 64 kDa in equilibrium with a dimer, the latter thought to be the active form. In contrast, the *C. vinosum* enzyme appears to be composed of a 1:1 mixture of PhaC and PhaE, each of molecular mass ~40 kDa, which exist in large molecular mass aggregates. The question can then be posed as to whether these two classes of synthases, which catalyze the same reaction, do so by the same reaction mechanism.

To address this question, we have developed an alternative method to isolate large amounts of homogeneous PHA synthase. Several important insights were gained about this system during synthase purification. First, in contrast to previous results, *C. vinosum* synthase is not cold-sensitive. The reason for this discrepancy is not understood, but may be related to use of PMSF as a protease inhibitor by Liebergesell et al. (*36*). PMSF also functions as a synthase inhibitor (data not shown). Second, the continuous and the discontinuous assays using Ellman's reagent to monitor CoA release do not yield equivalent results. Not surprisingly, given the results presented here, DTNB inhibits the reaction. While the continuous assay facilitates monitoring of protein from column profiles, it should not be used for quantitative assay of the polymerization process. Finally, and in contrast to previous reports (*36*), we have shown that PhaC is active as a synthase in the absence of PhaE. The failure of previous workers to detect activity in crude extracts most probably resulted from use of the continuous DTNB assay. Our studies have shown that PhaC alone is much more susceptible to such inhibition in comparison with PhaEC.

With large amounts of homogeneous protein available, mechanistic studies were undertaken in an effort to define the similarities and/or differences between the class I and class III enzymes. The kinetics of polymerization between these two classes of synthases are very distinct. With the *R. eutropha* enzyme, a long and variable lag phase precedes CoA release. Interestingly, the lag phase is greatly diminished by preincubation of [^{3}H]-sT-CoA, a putative primer. This decrease of the lag phase is accompanied by conversion of the monomeric to the dimeric form of the enzyme, and only the latter appears to be covalently labeled with 1 equiv of [^{3}H]-sT per protein dimer (*10*).

In contrast, CoA release catalyzed by *C. vinosum* synthase shows no apparent lag phase, but also exhibits biphasic kinetics. In the case of class I enzyme, we proposed that chain elongation is much faster than the priming process, a proposal that accounted for our inability to isolate covalently labeled cysteine-containing peptides when enzyme was incubated with stoichiometric amounts of HB-CoA (*37*). Once the protein was acylated in an appropriate fashion, elongation proceeded rapidly, and thus it was either the case that enzyme was not modified, or that enzyme was modified with a long polymer. The altered kinetics with *C. vinosum* synthase suggest that incubation of enzyme with stoichiometric amounts of HB-CoA could result in isolation of acylated protein. Experiments of this type have thus far been unsuccessful, but in retrospect the lack of success is most probably associated with the lability of the thioester linkage of the acylated protein.

Does the difference in kinetics of CoA release preclude similarities in mechanism between these two classes of enzymes? Our studies with [^{3}H]-sT-CoA suggest a mechanistic congruence, namely, the importance of a specific cysteine (C319 from *R. eutropha* and C149 from *C vinosum*) in covalent catalysis. Studies presented here provide the first direct evidence that the cysteine is acylated. Furthermore, quite serendipitously, these studies gave us a glimpse of the elongation process itself. Isolation of a tetrameric (**2**) and pentameric HB-labeled synthase demonstrated directly that elongation occurs at a rate comparable to the initiation process.

In the present studies, the [^{3}H]-sT was separated from the ^{3}H-labeled synthase prior to isolation of labeled peptide, and only 0.25 equiv of label per PhaEC was detected. Given that our studies reveal that at least the C149 linkage is stable in acid and that the elongation step is slow, experiments done under turnover conditions and quenching with acid should be informative regarding the role of a second cysteine.

The question of the importance of a second thiolate in covalent catalysis is thus still an open one. In the case of the *R. eutropha* synthase, two possibilities have been suggested for the source of the second thiolate. One was, in analogy with fatty acid synthases, that posttranslational modification of a serine by CoA and a phosphopantetheinyl transferase provides the second thiolate (*37*, *51*). A second possibility, the one favored at this point (Stubbe, unpublished), is that the active form of the class I enzyme is the dimer and that the active site is at the interface of the two subunits. In this model, C319 alternates between the role of the loading thiol and the role of the elongation thiol as described in Figure 1.

With the *C. vinosum* enzyme, mutagenesis studies indicate that the source of the second thiolate could be C130. This cysteine is conserved among class III synthases, suggesting its importance. The C130A mutant still catalyzes PHB formation, but at a rate 10^4-fold lower than that of the wt enzyme. The fact that this mutant enzyme is acylated to the same extent as the wt enzyme with [^{3}H]-sT-CoA suggests that it is appropriately folded. The slow rate of polymerization may result from its inability to form the normal acylated intermediate in the pathway of polymer formation. Possibly HB-CoA bound noncovalently could replace the first acylation step. It must bind in order to form acylated enzyme as well. Precedence for a mechanism in which substrate-acylated CoA can substitute for a substrate-acylated enzyme has been described for citrate lyase from *Klebsiella aerogenes* (*52*). Similarily, it has been shown for two biotin-dependent multisubunit enzymes, glutaconyl-CoA decarboxylase and 3-methylcrotonyl-CoA carboxylase, that the carboxytransferase subunit will react either with biotin carrier subunit or with free biotin (*53*−*55*). In C130A PHA synthase, the greatly reduced rate could result from inappropriate positioning of the 3-hydroxyl group of the bound HB-CoA for deprotonation and nucleophilic attack on the growing strand. Further studies are required to determine if C130 plays such a role.

Our studies (*10*) thus far suggest that both classes of synthases have a cysteine, in a homologous sequence context, that plays an essential role in polymerization. The presence of a second thiol group remains to be determined. If such a group is present, its source and role in covalent catalysis and the kinetics of the polymerization process would have to be different in the two systems. Comparison of parallel studies in the class I and class III enzymes should reveal the details of the initiation process, the elongation process, and the termination process, and, importantly, whether the elongation process is processive. The tools are now available to shed light on these questions.

ACKNOWLEDGMENT

We thank Jola Wodzinska for synthesizing [^{3}H]-sT-CoA. We also thank Alexander Steinbüchel for sending us plasmid pds37.

REFERENCES

1. Barham, P. J., Keller, A., Otun, E. L., and Holmes, P. A. (1984) *J. Mater. Sci. 19*, 2781−2794.
2. Bluhm, T. L., Hamer, G. K., Marchessault, R. H., Fyfe, C. A., and Veregin, R. P. (1986) *Macromolecules 19*, 2871−2876.
3. Bloembergen, S., Holden, D. A., Bluhm, T. L., Hamer, G. K., and Marchessault, R. H. (1989) *Macromolecules 22*, 1663−1669.
4. Doi, Y. (1990) *Microbial Polyesters*, VCH Publishers, Inc., Weinheim.
5. Steinbüchel, A. (1991) *Biomaterials. Novel Materials and Biological Sources* (Byrom, D., Eds.) Macmillan Publishers Ltd., Basingstoke.
6. Müller, H.-M., and Seebach, D. (1993) *Angew. Chem., Int. Ed. Engl. 32*, 477−502.
7. The Society of the Plastics Industry (1997) *Facts & Figures of the US Plastics Industry*, New York.
8. Steinbüchel, A. (1992) *Curr. Opin. Biotechnol. 3*, 291−297.
9. Steinbüchel, A., Hustede, M., Liebergesell, M., Pieper, U., Timm, A., and Valentin, H. (1992) *FEMS Microbiol. Rev. 103*, 217−230.
10. Wodzinska, J., Snell, K. D., Rhomberg, A., Sinskey, A. J., Biemann, K., and Stubbe, J. (1996) *J. Am. Chem. Soc. 118*, 6319−6320.
11. Liebergesell, M., and Steinbüchel, A. (1992) *Eur. J. Biochem. 209*, 135−150.
12. Huisman, G. W., Wonink, E., Meima, R., Kazemier, B., Terpstra, P., and Witholt, B. (1991) *J. Biol. Chem. 266*, 2191−2198.
13. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res. 22*, 4673−4680.
14. Smythe, C., and Cohen, P. (1991) *Eur. J. Biochem. 200*, 625−631.
15. Alonso, M. D., Lomako, J., Lomako, W. A., and Whelan, W. J. (1995) *FASEB J. 9*, 1126−1137.
16. Robinson, N. A., Clark, J. E., and Wood, G. W. (1987) *J. Biol. Chem. 262*, 5216−5222.
17. Ahn, K., and Kornberg, A. (1990) *J. Biol. Chem. 265*, 11734−11739.
18. Light, D. R., and Dennis, M. S. (1989) *J. Biol. Chem. 264*, 18689−18697.
19. Light, D. R., Lazarus, R. A., and Dennis, M. S. (1989) *J. Biol. Chem. 264*, 18598−18607.
20. Tarshis, L. C., Proteau, P. C., Kellogg, B. A., Sacchettini, J. C., and Poulter, C. D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15018−15023.
21. Smith, S. (1994) *FASEB J. 8*, 1248−1259.
22. Hutchinson, C. R., and Fujii, I. (1995) *Annu. Rev. Microbiol. 49*, 201−238.
23. Simpson, T. J. (1995) *Chem. Ind.*, 407−411.
24. Tsoi, C. J., and Khosla, C. (1995) *Chem. Biol. 2*, 355−362.
25. Griebel, R., Smith, Z., and Merrick, J. M. (1968) *Biochemistry 7*, 3676−3681.
26. Cronan, J. E. J., and Rock, C. O. (1996) *Escherichia coli and Salmonella typhimuricum* (Neidhardt, F. C., et al., Eds.) ASM Press, Washington, D.C.
27. Rangan, V. S., and Smith, S. (1996) *J. Biol. Chem. 271*, 31749−31755.
28. Rangan, S. V., and Smith, S. (1997) *J. Biol. Chem. 272*, 11975−11978.
29. Jossek, R., Reichelt, R., and Steinbüchel, A. (*1998*) *Appl. Microbiol. Biotechnol. 49*, 258−266.
30. Bibb, M. J., Biro, S., Motamedi, H., Collins, J. F., and Hutchinson, C. R. (1989) *EMBO J. 8*, 2727−2736.
31. Donadio, S., and Katz, L. (1992) *Gene 111*, 51−60.
32. Yu, J. H., Leonard, T. J. (1995) *J. Bacteriol. 177*, 4792−4800.
33. Pieper, R., Ebert-Khosla, S., Cane, D., and Khosla, C. (1996) *Biochemistry 35*, 2054−2060.
34. Pazirandeh, M., Chirala, S. S., and Wakil, S. J. (1991) *J. Biol. Chem. 266*, 20946−20952.
35. Yuan, L., Nelson, B. A., and Cary, G. (1996) *J. Biol. Chem. 271*, 3417−3419.

36. Liebergesell, M., Sonomoto, K., Madkour, M. H., Mayer, F., and Steinbüchel, A. (1994) *Eur. J. Biochem. 226*, 71−80.
37. Gerngross, T. U., Snell, K. D., Peoples, O. P., Sinskey, A. J., Cushai, E., Masamune, S., and Stubbe, J. (1994) *Biochemistry 33*, 9311−9320.
38. Wilm, M. S., and Mann, M. (1994) *Int. J. Mass Spectrom. Ion Processes 136*, 167−180.
39. Wilm, M. S., and Mann, M. (1996) *Anal. Chem. 68*, 1−8.
40. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY.
41. Laemmli, U. K. (1970) *Nature 227*, 680−685.
42. Valentin, E. H., and Steinbüchel, A. (1994) *Appl. Microbiol. Biotechnol. 40*, 699−709.
43. Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.
44. Williamson, D. H., and Mellanby, J. (1974) *Methods Enzymol. 4*, 1836−1840.
45. Pringle, J. R., Adams, E. E. M., Drubin, D. G., and Haarer, B. K. (1991) *Methods Enzymol. 194*, 565−602.
46. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
47. Edman, P., and Begg, G. (1967) *Eur. J. Biochem. 1*, 80−91.
48. Jencks, W. P., (1969) *Catalysis in Chemistry and Enzymology*, Dover Publications, Inc., New York.
49. Grubbs, R. H., and Coates, G. W. (1996) *Acc. Chem. Res. 29*, 85−93.
50. Lynn, D. M., Mohr, B., and Grubbs, R. H. (1998) *J. Am. Chem. Soc. 120*, 1627−1628.
51. Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., and Walsh, C. T. (1996) *Chem. Biol. 3*, 923−936.
52. Buckel, W., Ziegert, K., and Eggerer, H. (1973) *Eur. J. Biochem. 37*, 295−304.
53. Lynen, F., Knappe, J., Lorch, E., Jütting, G., Ringelmann, E., and Lachance, J.-P. (1961) *Biochem. Z. 335*, 123−167.
54. Schiele, U., Niedermeier, R., Sturzer, M., and Lynen, F. (1975) *Eur. J. Biochem. 60*, 259−266.
55. Bendrat, K., and Buckel, W. (1993) *Eur. J. Biochem. 211*, 697−702.

BI9818319