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Class I and III Polyhydroxyalkanoate Synthases from *Ralstonia eutropha* and *Allochromatium vinosum:* Characterization and Substrate Specificity Studies[1]

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Class I and III polyhydroxyalkanoate (PHA) synthases catalyze the conversion of β-hydroxybutyryl coenzyme A (HBCoA) to polyhydroxybutyrate. The Class I PHA synthase from *Ralstonia eutropha* has been purified by numerous labs with reported specific activities that vary between 1 and 160 U/mg. An N-terminal $(His)_6$-PHA synthase was constructed and purified with specific activity of 40 U/mg. The variable activity is shown to be related to the protein's propensity to aggregate and not to incomplete post-translational modification by coenzyme A and a phosphopantetheinyl transferase. The substrate specificities of this enzyme and the Class III PHA synthase from *Allochromatium vinosum* have been determined with nine analogs of varied chain length and branching, OH group position within the chain, and thioesters. The results suggest that *in vitro,* both PHA synthases are very specific and provide further support for their active site structural similarities. *In vitro* results differ from studies *in vivo.*

Key Words: **polyhydroxybutyrate; PHA synthase; *Ralstonia eutropha; Allochromatium vinosum;* phosphopantetheinylation.**

Class I and III polyhydroxyalkanoate (PHA)[3] synthases catalyze the formation of polyhydroxybutyrate (PHB) from β-hydroxybutyryl coenzyme A (HBCoA) and polyhydroxyvalerate (PHV) from β-hydroxyvaleryl CoA (HVCoA). These polymers are synthesized by many bacteria when they find themselves in a nutrient limited environment with an available carbon source (1–3). Up to 90% of the dry weight of the bacteria can be granules composed predominantly of these polymers. Copolymers of HB and HV possess properties of thermoplastics and are biodegradable (4), which has made them potentially attractive as alternatives to the less environmentally friendly, petroleum-based plas-

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[3] Abbreviations used: ACPS, acyl carrier protein synthase; BSA, bovine serum albumin; CMC, critical micelle concentration; DAST, (diethylamino)-sulfur trifluoride; DCC, dicyclohexylcarbodiimide; DCU, *N*,*N*-dicyclohexylurea; DMF, dimethylformamide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EI, electric ionization; eq, equivalent; FAB, fast atom bombardment; HB, β-hydroxybutyrate; HBCoA, β-hydroxybutyryl coenzyme A; 4HB, 4-hydroxybutyrate; 3HDD, 3-hydroxydodecanoate; HF, hydrofluoric acid; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HP, 3-hydroxypropionate; HRMS, high resolution mass spectrum; HV, β-hydroxyvalerate; IPTG, isopropyl thio-β-D-galactoside; KMO, 1% potassium permanganate and 0.08% NaOH; KPi, potassium phosphate; LB, Luria–Bertani; MALDI, matrix assisted laser desorption mass spectrometry; NAC, *N*-acetylcysteamine; PHA, polyhydroxyalkanoate; $PhaC_{Av}$, PHA synthase of *Allochromatium vinosum*; $PhaC_{Re}$, PHA, synthase of *Ralstonia eutropha*; PHB, polyhydroxybutyrate; SA, specific activity; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sT-CoA, a terminally saturated trimer of HBCoA; TBDMS, *t*-butyldimethylsilyl; THF, tetrahydrofuran; TOF, time of flight; TMS, tetramethylsilane; wt, wild-type.

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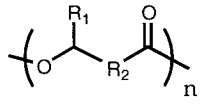


R_1 = H, CH_3, CH_2CH_3, $(CH_2)_2CH_3$, $(CH_2)_3CH_3$, $(CH_2)_4CH_3$, etc.
R_2 = CH_2, $CH(CH_3)$, $(CH_2)_2$, $(CH_2)_3$, $C(CH_3)_2$, etc.

SCHEME I

tics. Unfortunately, efforts to make them economically competitive with oil-based polymers have not yet been successful (4–6). Presently, these materials are used in a number of specialty applications (7–10) and polyoxoesters generated from 4-hydroxybutyryl CoA are being investigated for use in medical applications (11, 12). *In vivo* studies with a variety of organisms have demonstrated that 125 different hydroxyalkanoic acids can be incorporated into polyoxoesters (Scheme I) (13–18). In order to make these biopolymers more generally useful, a detailed understanding of the polymerization mechanism and enzyme substrate specificity is required.

The Class I and III PHA synthases utilize short chain alkyl groups where R1 = methyl and ethyl and R2 = CH_2 (Scheme I). The PHA synthases from *Ralstonia eutropha* (Class I) and *Allochromatium vinosum* (Class III) serve as the prototypes of their respective classes.

In 1994, we reported the first isolation of *R. eutropha* PHA synthase ($PhaC_{Re}$) with a specific activity (SA) of 5–8 U/mg (19). Since that time, purification of the same enzyme from a baculovirus expression system has appeared (20), as have other purification schemes using our expression vector (21–24). The SAs reported of "homogeneous" protein, however, vary from 1 to 160 U/mg. In an effort to obtain PHA synthase of reproducible SA and to understand the basis of this variability, we report an alternative purification procedure using an N-terminal hexahistidine tagged PHA synthase ($(His)_6$-PHA synthase) construct.

Our original purification paper (19) provided preliminary evidence, using [^{3}H]-β-alanine and a *panD*$^-$ *Escherichia coli* mutant, that pantetheinylation might be essential for PHA synthase activity. PanD generates β-alanine required for CoA biosynthesis. The discovery that specific enzymes are required for this modification (25), thus suggested an explanation for the variability of SAs reported from different labs. We also report studies to determine if incomplete posttranslational modification of the PHA synthase could provide an explanation for the variable activity. Recently, Hoppensack *et al.* created several *panD* mutants in *R. eutropha* by Tn5 insertion mutagenesis (26). Growth of these mutants on [^{14}C]-β-alanine resulted in radiolabel incorporated nonspecifically into many proteins in *R. eutropha*. In addition, they reported that they were unable to reproduce the results of our earlier studies using *panD*$^-$ *E. coli* strain SJ16 expressing $PhaC_{Re}$. They concluded from both sets of data, that pantetheinylation is not required for $PhaC_{Re}$ activity. Their studies, however, were flawed for a number of reasons, including the absence of a positive control for low levels of pantetheinylation. Thus, we would not have drawn the same conclusions from their data. Resolving the issue of posttranslational modification is therefore potentially important for defining the basis for the variable SAs of the PHA synthase. It is also important as modification would require changes in strategies for genetic engineering of PHA synthases in plants. In this report, our efforts to chemically characterize pantetheinylated PHA synthases are described.

In our original purification paper, we also reported the kinetics of the $PhaC_{Re}$ catalyzed polymerization reaction measured by CoA release using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). A variable lag phase was observed preceding a linear phase (19). A number of proposed mechanisms for this lag phase have been reported (19, 21–23), but at present the kinetics are still not understood. Recently, several laboratories have reported *in vitro* substrate specificity of the $PhaC_{Re}$ (19, 23, 24). These studies have prompted us to report the synthesis of nine substrate analogs and their interactions with the *R. eutropha* PHA synthase.

We have recently demonstrated the mechanistic similarities of the Class I $PhaC_{Re}$ and a Class III *Allochromatium vinosum* PHA synthase ($PhaEC_{Av}$) (27, 28). However, the kinetics of $PhaEC_{Av}$, while biphasic, involve a rapid release of CoA followed by a slower release of CoA. The substrate specificity of this enzyme has also been examined and provides an additional comparison of these two classes of enzymes. The differences between our *in vitro* studies and those of others, and *in vivo* results on both Class I and III PHA synthases are compared.

MATERIALS AND METHODS

Materials

Hecameg [6-O-(*N*-heptylcarbamoyl)-methyl-α-D-glucopyranoside] was obtained from Vegatec, Villejuif, France. Isopropyl thio-β-D-galactoside (IPTG) was purchased from Boehringer-Mannheim. DNA polymerase *pfu* was purchased from Stratagene, and other DNA modification enzymes and restriction enzymes were purchased from New England Biolabs. Oligonucleotides were purchased from Gibco-BRL Life Technology. Dry solvents were obtained by distillation: methylene chloride and pyridine were distilled from calcium hydride; tetrahydrofuran (THF) and diethyl ether were distilled from sodium benzophenone ketyl. Anhydrous dimethylformamide (DMF) was purchased from Sigma-Aldrich. All commercial reagents used in synthesis were purchased from Sigma-Aldrich and used as received unless otherwise indicated.

SCHEME II

Flash chromatography was carried out on a silica gel 60 (230–400 mesh, Merck). Thin layer chromatography (TLC) was performed on silica gel plates (0.25 mm, Merck). Compounds were detected by dipping them into a solution containing 1% potassium permanganate and 0.08% sodium hydroxide (KMO) and by ultraviolet light (UV). ^{1}H NMR was obtained on a Bruker-AC250 or AC300. All NMR chemical shift values were reported in δ units (ppm) using tetramethylsilane (TMS) as a reference. Optical rotation measurements were conducted on a Perkin-Elmer Model 241 Polarimeter. HPLC was performed with a Waters 510 HPLC system equipped with a Waters Automated Gradient Controller and a Waters Tunable Absorbance Detector, or a Rainin Dynamax Model SD-200 HPLC system equipped with a Dynamax Diode Array Detector (Model PDA-1). Two Alltech Econosil columns (C18, 10 μm) 250 $\times$ 4.6 mm and 250 $\times$ 22 mm were used for analytical and preparative purposes, respectively. Unless otherwise indicated, the following conditions were used for separations on the analytical column: a linear gradient of 10–50% methanol in 50 mM potassium phosphate (KPi, pH 4.7) over 50 min at a flow rate of 1.0 ml/min. Mass spectra (MS) were obtained on a Finnigan Mat (System 8200) at the MIT Chemistry Department facility. DNA sequencing and amino acid analyses were carried out by the MIT Biopolymers Laboratory.

The *A. vinosum* PHA synthase, having an SA of 140 U/mg, was isolated as previously described (29).

Construction and Purification of (His)$_6$-tagged R. eutropha PHA Synthase

Construction of (His)$_6$-tagged R. eutropha PHA synthase gene. The wild-type (wt) PHA synthase gene on plasmid pKAS4 (19) was amplified by PCR using the primers GAGTAGCATATGGCTACCG-GCAAAGGCGCG (forward) and GAGTAGGGATCCTCATGCCTTG-GCTTTGACGTA (reverse) to introduce the unique restriction sites for *Nde*I and *Bam*HI (underlined in the sequence) into the N- and C-terminus of the PHA synthase gene. The amplified PHA synthase gene was subcloned into pET14b (Novagen) cut with *Nde*I and *Bam*HI, followed by ligation to give plasmid pHAS. The portion of plasmid pHAS containing the PHA synthase gene was sequenced to confirm that no mutations occurred during the subcloning process.

Overexpression and purification of (His)$_6$-tagged R. eutropha PHA synthase. (His)$_6$-PhaC was purified from *Escherichia coli* strain BL21(DE3)pLysS harboring pHAS. The culture was grown at 30°C to an OD_{600} of 0.6, at which time expression of (His)$_6$-PhaC was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After growth for an additional 3 h at 30°C, 2.3 g/L cells were harvested by centrifugation and stored at −80°C.

All purification steps were carried out at 4°C. Cells (~13.8 g wet weight) were resuspended and homogenized in 40 ml of buffer A (50 mM KPi, pH 7.5, 300 mM NaCl). Cells were lysed by two passes through a French pressure cell at 12,000 psi, and cell debris was removed by centrifugation at 18,000 rpm for 1 h. The crude extract was mixed with Ni-NTA (~20 ml, Qiagen) that had been preequilibrated with buffer A for 30 min. The mixture was poured into a column. The column was washed with 250 ml buffer B (buffer A + 20 mM imidazole), and then with 120 ml of buffer C (60 mM imidazole, pH 7.5, 5% glycerol, 0.05% Hecameg). (His)$_6$-PhaC was eluted with buffer D (250 mM imidazole, pH 7.5, 5% glycerol, 0.05% Hecameg), and fractions of 5 ml were collected. Fractions were analyzed by 12% SDS–PAGE, and those containing the desired protein were combined, concentrated, and buffer exchanged into buffer E (20 mM KPi, pH 7.5, 5% glycerol, 0.05% Hecameg) using an Amicon with YM-30 membrane. Aliquots of the protein were quick-frozen in liquid N_2 and stored at −80°C. To remove protein aggregates, (His)$_6$-PhaC was passed through an analytical size-exclusion column (Bio-Silect SEC250, Bio-Rad) in 50 mM KPi, pH 7.8, 75 mM NaCl at a flow rate of 1 ml/min.

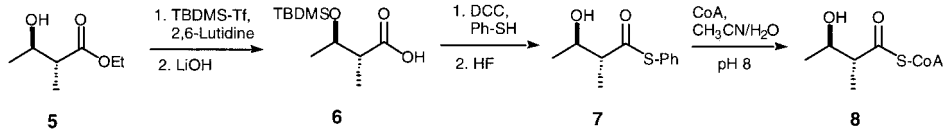
Removal of the (His)$_6$-tag from the PHA synthase. The (His)$_6$-tag can be removed by incubating the (His)$_6$-tagged PHA synthase with biotinylated thrombin (Novagen) at 0°C in a buffer containing 20 mM KPi (pH 7.5), 5% glycerol, and 0.05% Hecameg for 6 h at a ratio of 1.6 mg PHA synthase per unit of protease. The biotinylated thrombin is then removed by adding a biotin-specific affinity streptavidin resin (Novagen), and the protease-free PHA synthase is obtained by removal of the resin subsequent to centrifugation. The (His)$_6$-tag removed PHA synthase has three amino acids (GSH) attached on the N-terminus of the PHA synthase.

Determination of the Extinction Coefficient for R. eutropha PHA Synthase

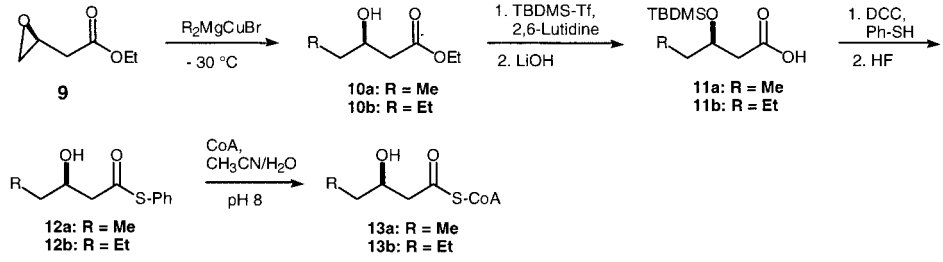
The A_{280} nm of purified $PhaC_{Re}$ in doubly distilled H_2O was determined. A portion of the same sample was submitted to the MIT Biopolymers Laboratory for quantitative amino acid analysis. The calculated ϵ_{280} for $PhaC_{Re}$ is 162,000 $M^{-1}cm^{-1}$ based on these data. All protein concentrations were determined using this value.

Syntheses and Characterization of Substrate and Substrate Analogs

*(R)-3-Hydroxybutyryl CoA (**4**).* The synthesis of **4** is shown in Scheme II. The preparation of 3-(*t*-butyldimethylsilylhydroxy)butyric acid (**2**) was previously described (28). To a stirring solution of **2** (870 mg, 4 mmol) and benzenethiol (451 mg, 4.1 mmol, 1.03 eq.) in 8 ml dry methylene chloride, at 0°C, was added dicyclohexylcarbodiimide (DCC) (845 mg, 4.1 mmol, 1.03 eq) under argon. The reaction



SCHEME III



SCHEME IV

was allowed to warm to room temperature and stirred at 22°C for 10 h. The reaction mixture was diluted by adding 20 ml ether and the white precipitate (*N,N*-dicyclohexylurea (DCU)) was removed by filtration. The solvent was removed *in vacuo* and the residue was purified by silica gel flash column chromatography (5% ethyl acetate in hexanes) to give protected thiophenyl (*R*)-3-hydroxybutyrate (**3**) as a clear liquid. The removal of the *t*-butyldimethylsilyl (TBDMS) protecting group as previously described (28) afforded **3** as a clear oil (0.72 g, 92%): TLC (R_f: 0.24, 25% ethyl acetate in hexanes, UV); ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 1.24 (d, J = 6.3 Hz, 3H), 2.82 (m, 2H), 4.28 (m, 1H), 7.42 (s, 5H); $[\alpha]_D^{20}$ = −43.8°C, (*c* 1.34, $CHCl_3$), *lit.* (25) −42.25°C, (*c* 1.42 $CHCl_3$); HRMS (EI) of $C_{10}H_{12}O_2S$: calculated: 196.0558 (M^+), observed: 196.0554.

Thiotransesterification of **3** with CoA was carried out as described for the synthesis of saturated trimer CoA (sT-CoA) (28) to give **4** as a white powder (204 mg, 83%). HPLC (R_t: 16.5 min); ^{1}H NMR (D_2O, 250 MHz) δ: 0.70 (s, 3H), 0.83 (s, 3H), 1.16 (d, J = 6.3 Hz, 3H), 2.38 (t, J = 6.4 Hz, 2H), 2.72 (d, J = 6.4 Hz, 3H), 2.96 (t, J = 6.2 Hz, 2H), 3.29 (t, J = 6.3 Hz, 2H), 3.40 (t, J = 6.3 Hz, 2H), 3.48 (m, 1H), 3.76 (m, 1H), 3.97 (s, 1H), 4.18 (m, overlapped, 1H and 2H), 4.54 (s, 1H), 6.13 (d, J = 6.1 Hz, 1H), 8.22 (s, 1H), 8.52 (s, 1H).

*(2R, 3R)-2-Methyl-3-hydroxylbutyryl CoA (**8**).* The synthesis of compound **8** is shown in Scheme III. Ethyl (2*R*, 3*R*)-2-methyl-3-hydroxylbutanoate (**5**) was prepared as previously described (30). The product (1.20 g, 82%) was purified on a silica gel flash column (30% ethyl acetate in hexanes). TLC (R_f: 0.78, 50% ethyl acetate in hexanes, KMO); ^{1}H NMR spectrum is identical to that reported (30). $[\alpha]_D^{20}$ = −26.3°C (*c* 1.84, $CHCl_3$), *lit.* (30) −28°C (*c* 1.59, $CHCl_3$).

To a solution of **5** (1.2 g, 8.2 mmol) under argon in 10 ml dry methylene chloride was added sequentially through a syringe, 2, 6-lutidine (1.28 g, 12 mmol, 1.5 eq, redistilled) and TBDMS triflate (2.83 g, 10.7 mmol, 1.3 eq) at 0°C. The solution was warmed to room temperature and stirred at 22°C for 2 h. The reaction mixture was then concentrated and the residue was purified on a silica gel column (10% ethyl acetate in hexanes) to afford the silylated **5** as a clear liquid (1.55 g, 74%). The clear liquid (0.75 g, 2.9 mmol) was dissolved in THF (2 ml) and methanol (10 ml), and lithium hydroxide (monohydrate, 250 mg, 6 mmol, 2 eq) and 1 ml water was added. The reaction was stirred at 50°C for 4 h, concentrated *in vacuo* and the residue was diluted with 10 ml water. The aqueous phase (pH >10) was extracted with ether (2 × 10 ml), acidified with 5 N H_2SO_4 to pH 3, and then extracted with ether (3 × 20 ml). The extracts were combined, dried over $MgSO_4$ and concentrated to give (2*R*, 3*R*)-2-methyl-3-(*t*-butyldimethylsilylhydroxyl)-butyric acid (**6**) as a pale yellow liquid (430 mg, 64%): ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 0.10 (s, 3H), 0.16 (s, 3H), 0.90 (s, 9H), 1.21 (d, J = 7.3 Hz, 2H), 1.22 (d, J = 6.3 Hz, 2H), 2.51 (m, 1H), 3.98 (m, 1H).

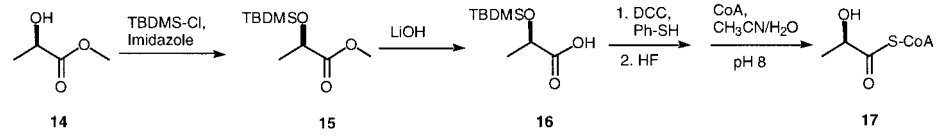
(2*R*, 3*R*)-2-Methyl-3-hydroxylbutyryl thiophenol ester (**7**) was prepared as a clear liquid (240 mg, 63%) in the same manner as described for **3** (Scheme II): TLC (R_f: 0.38, 25% ethyl acetate in hexanes, UV); ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 1.28 (t, J = 6.4 Hz, 3H), 1.31 (d, J = 7.1 Hz, 3H), 2.81 (m, 1H), 3.98 (m, 1H), 7.42 (s, 5H); $[\alpha]_D^{20}$ = −44.5°C (*c* 1.73, $CHCl_3$); HSMS (EI) of $C_{11}H_{14}O_2S$: calculated: 210.0714 (M^+), observed: 210.0717.

8 (48 mg, 69%) was prepared as described for **4:** HPLC (R_t: 22.2 min); ^{1}H NMR (D_2O, 250 MHz) δ: 0.68 (s, 3H), 0.83 (s 3H), 1.05 (d, J = 7 Hz, 3H), 1.14 (d, J = 6.3 Hz, 3H), 2.38 (t, J = 6.6 Hz, 2H), 2.70 (m, 1H), 2.97 (m, 2H), 3.29 (t, J = 6.5 Hz, 2H), 3.38 (t, J = 6.5 Hz, 2H), 3.48 (m, 1H), 3.78 (m, 1H), 3.9 (m, 1H), 3.97 (s, 1H), 4.19 (br s, 2H), 4.54 (br s, 1H), 6.14 (d, J = 6.9 Hz, 1H), 8.23 (s, 1H), 8.53 (s, 1H).

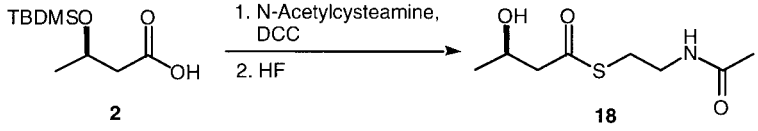
*(R)-3-Hydroxypentanoyl CoA (**13a**) and (R)-3-hydroxyhexanoyl CoA (3HHxCoA, **13b**).* The syntheses of compounds **13a** and **13b** are shown in Scheme IV. Ethyl (*S*)-3, 4-epoxybutanoate (**9**) was prepared as previously described (27). **9** was a clear liquid (3.9 g, 73%) after purification on a silica gel flash column (25% ethyl acetate in hexanes): TLC (R_f: 0.47, 25% ethyl acetate in hexanes, KMO); ^{1}H NMR is the same as that reported (27); $[\alpha]_D^{20}$ = −25.8°C (*c* 2.4, methanol), *lit.* (27) −25.3°C (*c* 3.7 methanol).

Ethyl (*R*)-3-hydroxyhexanoate (**10b**) was prepared as previously reported (31). Ethyl (*R*)-3-hydroxypentanoate (**10a**) was prepared using the same protocol. Both compounds were purified on a silica gel flash column (20% ethyl acetate in hexanes). **10a**: TLC (R_f: 0.25, 25% ethyl acetate in hexanes, KMO); ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 0.96 (t, J = 7.4, 3H), 1.27 (t, J = 7.1 Hz, 3H), 1.50 (m, 2H), 2.3 to 2.6 (m, 2H), 3.95 (m, 1H), 4.19 (q, J = 7.0 Hz, 2H). **10b:** TLC (R_f: 0.30, 25% ethyl acetate in hexanes, KMO); ^{1}H NMR is identical to that previously reported (31).

10a and **10b** were protected and saponified as described for **6** to give (*R*)-3-(*t*-butyldimethylsilylhydroxy)pentanoic acid (**11a**) and (*R*)-3-(*t*-butyldimethylsilylhydroxy)hexanoic acid (**11b**), respectively. **11a**: ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 0.09 (s, 3H), 0.10 (s, 3H), 0.89 (s, 9H), 0.91 (t, J = 7.2 Hz, 3H), 1.58 (m, 2H), 2.52 (m, 2H), 4.05 (m, 1H). **11b**: ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 0.09 (s, 3H), 0.10 (s, 3H), 0.89 (s, 9H), 0.91 (t, J = 7.2 Hz, 3H), 1.34 (m, 2H), 1.52 (m, 2H), 2.52 (m, 2H), 4.1 (q, J = 7.2 Hz, 1H).



SCHEME V



2 **18**

SCHEME VI

Thiophenyl-(*R*)-3-hydroxypentanoate (**12a**) and thiophenyl (*R*)-3-hydroxyhexanoate (**12b**) were prepared as described for **3**. **12a** (440 mg, 95%) or **12b** (497 mg, 94%) was obtained as a clear liquid after purification by silica gel flash chromatography (15% ethyl acetate in hexanes). **12a:** TLC (R_f: 0.36, 25% ethyl acetate in hexanes, UV); ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 0.98 (t, J = 7.4 Hz, 3H), 1.55 (m, 2H), 2.83 (m, 2H), 4.04 (m, 1H), 7.42 (s, 5H); $[\alpha]_D^{20} = -39.0°$C (c 1.91, $CHCl_3$); HRMS (EI) of $C_{11}H_{14}O_2S$: calculated: 210.0714 (M^+), observed: 210.0719. **12b**: TLC (R_f: 0.36, 25% ethyl acetate in hexanes, UV); ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 0.94 (t, J = 6.9 Hz, 3H), 1.3–1.6 (m, 4H), 2.83 (m, 2H), 4.1 (m, 1H), 7.42 (s, 5H); $[\alpha]_D^{20} = -27.4°$C (c 1.80, $CHCl_3$); HRMS (EI) of $C_{12}H_{16}O_2S$: calculated: 224.0871 (M^+), observed: 224.0869.

13a and **13b** were prepared as described for **4**. 13a (51 mg, 73%) or **13b** (51 mg, 73%) was obtained after purification on preparative HPLC. **13a**: HPLC (R_t: 23.4 min); ^{1}H NMR (D_2O, 250 MHz) δ: 0.72 (s, 3H), 0.85 (s + t, J = 9.8 Hz, 6H), 1.4–1.5 (m, 2H), 2.39 (t, J = 6.4 Hz, 2H), 2.65–2.7 (m, 2H), 2.97 (t, J = 6.3 Hz, 2H), 3.30 (t, J = 6.3 Hz, 2H), 3.42 (t, J = 6.3 Hz, 2H), 3.53 (m, 1H), 3.79 (m, 1H), 3.9–4.0 (m, 1H), 3.98 (s, 1H), 4.20 (br s, 2H), 4.56 (br s, 1H), 6.14 (d, J = 6.3 Hz, 1H), 8.23 (s, 1H), 8.52 (s, 1H). **13b**: HPLC (R_t: 28.5 min); ^{1}H NMR (D_2O, 250 MHz) δ: 0.80 (s, 3H), 0.85 (t, J = 7.0 Hz, 3H), 0.93 (s, 3H), 1.25–1.48 (m, 4H), 2.42 (t, J = 6.5 Hz, 2H), 2.65–2.85 (m, 2H), 3.0 (t, J = 6.3 Hz, 2H), 3.33 (t, J = 6.3 Hz, 2H), 3.44 (t, J = 6.5 Hz, 2H), 3.59 (m, 1H), 3.84 (m, 1H), 4.02 (s, 1H), 4.0–4.1 (m, 1H), 4.25 (br s, 2H), 4.59 (br s, 1H), 6.18 (d, J = 5.5 Hz, 1H), 8.38 (s, 1H), 8.64 (s, 1H).

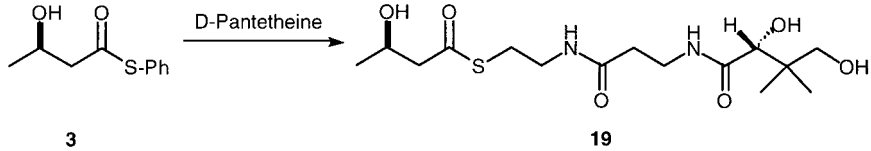
*(R)-Lactyl CoA (**17**).* The synthesis of **17** is shown in Scheme V. To a solution of **14** (96% ee, 5.0 g, 48 mmol) in 80 ml dry DMF was added imidazole (3.6 g, 53 mmol, 1.1 eq) and TBDMS chloride (7.24 g, 48 mmol). The solution was stirred at room temperature overnight. The reaction mixture was then diluted with water (50 ml)/ether (80 ml). The ether layer was washed with water (2 × 30 ml), dried over $MgSO_4$ and concentrated to give **15** as a clear oil (9.4 g, 90%): ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.069 (s, 3H), 0.096 (s, 3H), 0.90 (s, 9H), 1.40 (d, J = 6.7 Hz, 3H), 3.72 (s, 3H), 4.32 (m, 1H). **15** (3.6 g, 16.5 mmol) was dissolved in 50 ml 10% THF in methanol and lithium hydroxide (0.85 g LiOH · H_2O in 2 ml water, 20 mmol) was added. The reaction mixture was stirred at room temperature for 3.5 h, concentrated to ~20 ml and 30 ml of saturated sodium bicarbonate solution was added. The mixture was then extracted with ether (80 ml), acidified with 5 M H_2SO_4 to pH 2.9, and extracted with ether (2 × 80 ml). The ether extracts were combined, dried over $MgSO_4$ and concentrated to give **16** as a clear oil (2.9 g, 88%). **16** was converted to **17** (47 mg, 69%) as described for **4**. HPLC (R_t: 13.6 min); ^{1}H NMR (250 Hz, D_2O) δ: 0.70 (s, 3H), 0.83 (s, 6H), 1.32 (d, J = 6.9 Hz, 2H), 2.38 (t, J = 6.4 Hz, 2H), 2.95 (t, J = 6.4 Hz, 2H), 3.28 (t, J = 6.3 Hz, 2H), 3.41 (t, J = 6.3 Hz, 2H), 3.50 (m, 1H), 3.78 (m, 1H), 3.98 (s, 1H), 4.20 (br s, 2H), 4.34 (q, J = 6.9 Hz, 1H), 4.54 (br s, 1H), 6.14 (d, J = 6.8 Hz, 1H), 8.23 (s, 1H), 8.52 (s, 1H).

*(R)-3-Hydroxybutyryl N-acetylcysteamine thioester (**18**).* The synthesis of **18** is shown in Scheme VI. DCC (4.5 g, 21.9 mmol, 1.0 eq) was added to a solution of **2** (4.8 g, 21.9 mmol, 1.0 eq) and *N*-acetylcysteamine (2.6 g, 1.88 mmol, 1.0 eq) in 5 ml methylene chloride under argon, and the reaction mixture was stirred at 22°C for 12 h. The white solid was removed by filtration and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in 2 ml of 48% hydrofluoric acid (HF) (in water)/20 ml of acetonitrile, stirred at 22°C, and the reaction was monitored by TLC (10% methanol, 40% ethyl acetate in chloroform, R_f values for protected and deprotected products are 0.57 and 0.24, respectively, UV or KMO). The reaction was complete in 6 h. The solvent was removed *in vacuo* and the residue was purified on a silica gel flash column (4.8% methanol, 48% ethyl acetate in chloroform) to give **18** as a clear oil (3.9 g, 87%). TLC (R_f: 0.24, 10% methanol, 40% ethyl acetate in chloroform, UV or KMO); ^{1}H NMR (D_2O, 250 MHz) δ: 1.20 (d, J = 6.3 Hz, 3H), 1.92 (s, 3H), 2.77 (d, J = 6.5 Hz, 2H), 3.03 (t, J = 6.0, 2H), 3.35 (t, J = 6.2 Hz, 2H), 4.23 (m, 1H).

*(R)-3-Hydroxybutyryl (D)-pantetheine thioester (**19**).* The synthesis of **19** is shown in Scheme VII. To a solution of **3** (60 mg, 0.3 mmol) in 3.5 ml wet THF (0.5 ml water in 3 ml THF saturated with nitrogen) was added D-pantetheine (56 mg, 0.2 mmol), prepared as described (32). The reaction mixture was stirred for 20 h at 22°C. The product was purified using a preparative TLC plate (1.0 mm, Merck), developed by a solvent mixture of 25% methanol, 25% ethyl acetate in chloroform. **19** (pale yellow oil, 59 mg, 81%) was eluted from the plate with developing solvent and the silica gel was removed by filtration. TLC (R_f: 0.20, 20% methanol, 30% ethyl acetate in chloroform, UV); ^{1}H NMR (D_2O, 250 MHz) δ: 0.85 (s, 3H), 0.89 (s, 3H), 1.19 (d, J = 6.3 Hz, 3H), 2.44 (t, J = 6.5 Hz, 2H), 2.77 (d, J = 6.4 Hz, 2H), 3.03 (t, J = 6.2 Hz, 2H), 3.3–3.4 (m, 3H), 3.4–3.5 (m, 3H), 3.95 (s, 1H), 4.24 (m, 1H); HRMS (FAB) of $C_{15}H_{28}N_2O_6S$: calculated: 365.1746 (M^+), observed: 365.1747.

*3-Hydroxypropionyl CoA (3HPCoA, **20**).* **20** was prepared in 12% yield by reaction of propionlactone with CoA as reported (33–35). The product was purified by preparative HPLC (R_t: 25.5 min, 10% methanol in 50 mM KPi, pH 4.7, isocratic); ^{1}H NMR (D_2O, 250 MHz) δ: 0.65 (s, 3H), 0.77 (s, 3H), 2.23 (t, J = 6.6 Hz, 2H), 2.75 (t, J = 5.8 Hz, 2H), 2.90 (t, J = 6.3 Hz, 2H), 3.23 (t, J = 6.3 Hz, 2H), 3.35 (t, J = 6.6 Hz, 2H), 3.46 (m, 1H), 3.73 (m, 1H), 3.75 (t, J = 5.8 Hz, 2H), 3.91 (s, 1H), 4.14 (br s, 2H), 4.50 (br s, 1H), 6.08 (d, J = 6.9 Hz, 1H), 8.19 (s, 1H), 8.46 (s, 1H).

*(S)-3-Hydroxybutyryl CoA (**21**).* **21** was prepared from (*S*)-3-hydroxybutyryl acid as described for **4**.



3 **19**

SCHEME VII

TABLE I

Comparison of *R. eutropha* PHA Synthase Activities Reported in Literature

PHA Synthase[a]	Concentration determination	Reported rate (U/mg)	Normalized rate using ϵ^b (U/mg)
This study[c]	ϵ = 162,000 M^{-1} cm^{-1}	40	40
Gerngross (19)	Bradford/BSA	8	10.3
Gerngross[d] (21)	Bradford/BSA	1.4	1.8
Wodzinska (22)	Lowry/BSA	40	64
Williams (20)	Bradford/BSA	34	43.7
Zhang[d,e] (23)	Bradford/BSA	158	203
Song[c] (24)	Bradford/BSA	8.5	10.9

[a] Cell assays except for Zhang *et al.* (23) use 0.05% Hecameg in the reaction mixture.

[b] ϵ_{280} is determined in this study.

[c] In this study, the PHA synthase is N-terminal $(His)_6$-tagged and was purified as described under Materials and Methods.

[d] In these studies, the PHA synthase activity was assayed by a continuous spectrophotometric method monitoring A_{236} nm. In all other studies, the activity was assayed by a discontinuous method using DTNB to detect the $^-$SCoA released.

[e] In this study the PHA synthase is incubated in 50% fructose prior to assaying for activity.

*4-Hydroxybutyryl CoA (4HBCoA, **22**).* Synthesis of **22** was carried out as described for **4**. **22** was purified by preparative HPLC (R_t: 14.4 min) with a gradient of 10 to 70% methanol in 50 mM KPi (pH 4.7) over a period of 15 min. ^{1}H NMR (D_2O, 250 MHz) δ: 1.79 (tt, J = 6.2, 6.8 Hz; 2H), 2.61 (t, J = 6.8 Hz, 2H), 3.53 (t, J = 6.2 Hz, 2H). This compound is unstable, and stored at −80°C as a 4 mM to 10 mM solution in 50 mM KPi (pH 4.7).

Enzyme Assay

$PhaC_{Re}$ activity was assayed according to the procedure described by Wodzinska *et al.* (22) with minor modifications. The assay contained 150 mM KPi, pH 7.2, 0.2% glycerol, 0.05% Hecameg, 1.6 mM HBCoA (**4**) and enzyme. In most studies, $(His)_6$-tagged $PhaC_{Re}$ was used, as its activity is identical to wild-type PHA synthase purified independently and to $(His)_6$-tagged $PhaC_{Re}$ with the $(His)_6$ tag removed. The concentration of **8**, **13a**, **13b**, **17**, **20**, **21**, and **22** were all 1.6 mM while the concentration of **18** and **19** were 47 mM. The $(His)_6$-PHA synthase concentrations in the assay for each substrate were 0.12 μM (**4**), 0.56 μM (**13a**), 1.1 μM (**13b**), 1.3 μM (**20**), 10.5 μM (**17**), 11.8 μM (**22**), 17 μM (**8** and **21**), and 39 μM (**18** and **19**), respectively. **22** is chemically unstable as it rapidly cyclizes to generate lactone. Therefore, control experiments were run in parallel to correct for the contributions from the nonenzymatic hydrolysis.

To study the kinetics of the $PhaEC_{Av}$ catalyzed reactions with HBCoA and analogs, the assay was carried out at 30°C as described (29). The substrate concentrations were varied from ~0.2 to 5 × K_m and the concentrations of the enzyme were varied from 0.03 to 2.3 μM.

RESULTS AND DISCUSSION

Purification and Characterization of R. eutropha $(His)_6$-Tagged PHA Synthase

Since the initial report of $PhaC_{Re}$ purification, a wide range of SAs of protein that is 95% homogeneous based on SDS–PAGE have been reported (Table I). In an effort to obtain an enzyme preparation that results in reproducible SA, we generated an N-terminal $(His)_6$-tagged version of $PhaC_{Re}$. As shown in Fig. 1, the PHA synthase can be purified in a single step with a SA of 20 to 30 U/mg. The PHA synthase examined by size-exclusion chromatography (SEC) revealed in addition to monomer and dimer, variable amounts of larger molecular weight aggregates that elute at the void volume of the column. The combined aggregates are active with a SA of 10–20 U/mg. Once aggregates are removed, they do not reappear. The SA of $PhaC_{Re}$ with the aggregates removed is 40 U/mg. Removal of the $(His)_6$-tag using thrombin does not alter the SA of the protein. Recently, Song *et al.* also reported a one step purification of $PhaC_{Re}$ using a Methyl-HIC column (24). The SA of their $PhaC_{Re}$ was 8.5 U/mg. We have reproduced their procedure and determined the protein also contains high molecular weight aggregates. Removal of these aggregates gives $PhaC_{Re}$ with a SA of ~40 U/mg. Thus with a single exception (Table I), the SA of the enzyme has reached a reproducible plateau at 40 U/mg. In addition, the extinction coefficient for the protein has been determined and should now serve as the standard for comparison of SAs of $PhaC_{Re}$ between labs.

In a parallel set of experiments we considered an alternative explanation for the observed fluctuations in the SA of $PhaC_{Re}$. Based on our original report that [^{3}H]-β-alanine was incorporated into $PhaC_{Re}$ expressed in a heterologous system, *E. coli* SJ16 cells (auxotrophic for β-alanine) (19), we proposed that incomplete pantetheinylation was responsible for the variable activities observed (Fig. 2, lane 3). Our focus on the importance of pantetheinylation was intensified when we showed that [^{3}H]-β-alanine was also incorporated into the PhaC subunit of the Class III $PhaEC_{Av}$ (Fig. 2, lane 1). The control experiment (Fig. 2, lane 2), in

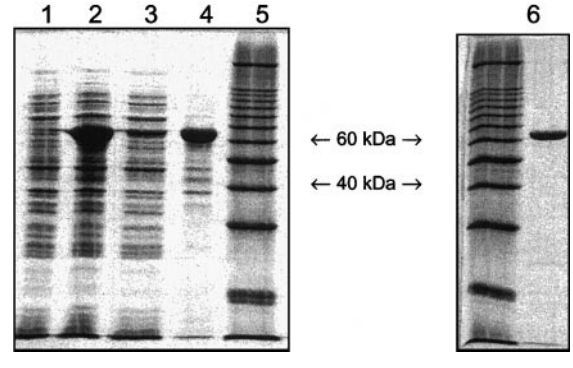


FIG. 1. SDS–PAGE of *R. eutropha* $(His)_6$-PHA synthase purification profile. Lane 1, uninduced cells; lane 2, induced cells; lane 3, soluble cell free extract; lane 4, insoluble cell suspension; lane 5, Molecular weight standards; lane 6, PHA synthase after Ni-NTA agarose column.

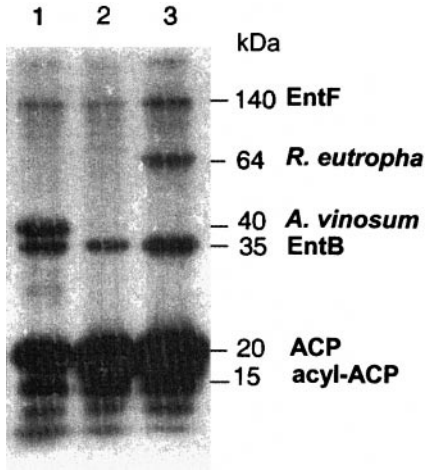


FIG. 2. Autoradiogram of a SDS–PAGE of crude extracts of *R. eutropha* and *A. vinosum* PHA synthases expressed in *E. coli* SJ16 cells grown on [^{3}H]-β-alanine: lane 1, SJ16/pTrc-UM7 (*A. vinosum*); lane 2: SJ16/pTrcN (control plasmid, no PHA synthase); lane 3: SJ16/pKAS4 (*R. eutropha*). The labeled proteins are identified as indicated. Acyl carrier protein (ACP) and acylated ACP (acyl-ACP) migrate aberrantly based on their molecular weight; EntF and EntB are proteins involved in enterobactin biosynthesis (for EntB see Ref. 54).

which a plasmid containing no PHA synthase gene was examined under identical experimental conditions, revealed no proteins labeled other than those previously identified to be pantetheinylated in *E. coli*. Finally, we have also shown that similar results are obtained with the *Zoogloea ramigera* PHA synthase (data not shown).

We focused on the *R. eutropha* PHA synthase in an effort to further characterize and quantitate the phosphopantetheine and its site of incorporation into the protein. In each enzyme that is pantetheinylated, a specific serine in a given sequence context is modified by CoA in a reaction catalyzed by a specific pantetheinyl transferase (25, 36–40). When each of the conserved serines in $PhaC_{Re}$ were mutated to alanines, [^{3}H]-β-alanine was still incorporated into each mutant protein in experiments similar to those shown in Fig. 2 (data not shown). Furthermore, several pantetheinyl transferases, ACPS (41), EntD (42), *o*195 (25), and Sfp (43) have been incubated with [^{3}H]-CoA and $PhaC_{Re}$ and $PhaEC_{Av}$ in an effort to find additional radiolabel incorporation. No labeling above background was detected even with Sfp transferase, which is known to be the least specific of pantetheinyl transferases.

Mass spectrometry has been an effective way to establish posttranslational modification and the site(s) of labeling. Efforts to use MALDI-TOF mass spectrometric methods on all of the peptides from proteolyzed $PhaC_{Re}$ failed to reveal any mass not accounted for based on the known sequence. Our observations that no serine residue is specifically required for labeling of $PhaC_{Re}$ and that less than 1% of $PhaC_{Re}$ (based on Western analysis and the SA of the [^{3}H]-β-alanine) is labeled in *E. coli* lead us to conclude that phosphopantetheinylation is unlikely to be important for PHA synthase activity. The clean labeling pattern observed (Fig. 2) and use of different sources of radiolabeled β-alanine suggest that impurity is not likely the explanation of our observed results. The basis of the observed radiolabeling thus remains a mystery.

The observation of large molecular weight aggregates in recombinant $PhaC_{Re}$ and the inability to detect stoichiometric pantetheinylation, suggests that aggregation provides the simplest explanation for the variable SA of this protein.

Synthesis of Substrate and Analogs

The synthesis of HBCoA and a variety of HBCoA analogs and their characterization are described in Schemes II to VII, Fig. 3, and Table II. The preparation of HBCoA (**4**) is outlined in Scheme II. Commercially available (*R*)-3-hydroxybutyric acid was protected with a TBDMS group and converted to the thioester using DCC and thiolbenzene. Desilylation of the blocked alcohol in the presence of HF in acetonitrile afforded the thiophenol of (*R*)-3-hydroxbutyrate (**3**). Compound **3** was converted to HBCoA (**4**) by thiotransesterification in H_2O/acetonitrile with >95% conversion of CoA to product. The success of the reaction requires that the pH be maintained at ~8 and that a solvent mixture be used to maximize solubility of the hydrophobic **3** and the hydrophilic CoA. HBCoA was purified by a preparative HPLC column and methanol/water elution.

Compound **8** was prepared as described in Scheme III. Ethyl (*R*)-3-hydroxybutanoate was stereospecifically methylated to give **5** (30, 44). The enantiomeric purity of **5** was established by comparing the optical rotation to that previously reported (30). Conversion of **5** to **8** was carried out by procedures analogous to those described for **4**.

The synthesis of **13a** and **13b** are outlined in Scheme IV. Epoxide **9** was made in enantiomerically pure form from ethyl (*S*)-4-bromo-3-hydroxybutanoate as described (31). Chemoselective opening of the epoxide with magnesium dimethyl (diethyl) cuprate gave (*R*)-3-hydroxyesters of **10a** and **10b.** Protection of the (*R*)-3-hydroxyl group with TBDMS group, followed by saponification gave the TBDMS-hydroxyl-protected free acids **11a** and **11b**. Conversion of **11a** and **11b** to desired products **13a** and **13b** were carried out as described above. Compounds **13a** and **13b** were purified by preparative HPLC and characterized by ^{1}H-NMR.

The synthesis of **17** is outlined in Scheme V. The synthetic route is similar to the procedure to synthe-

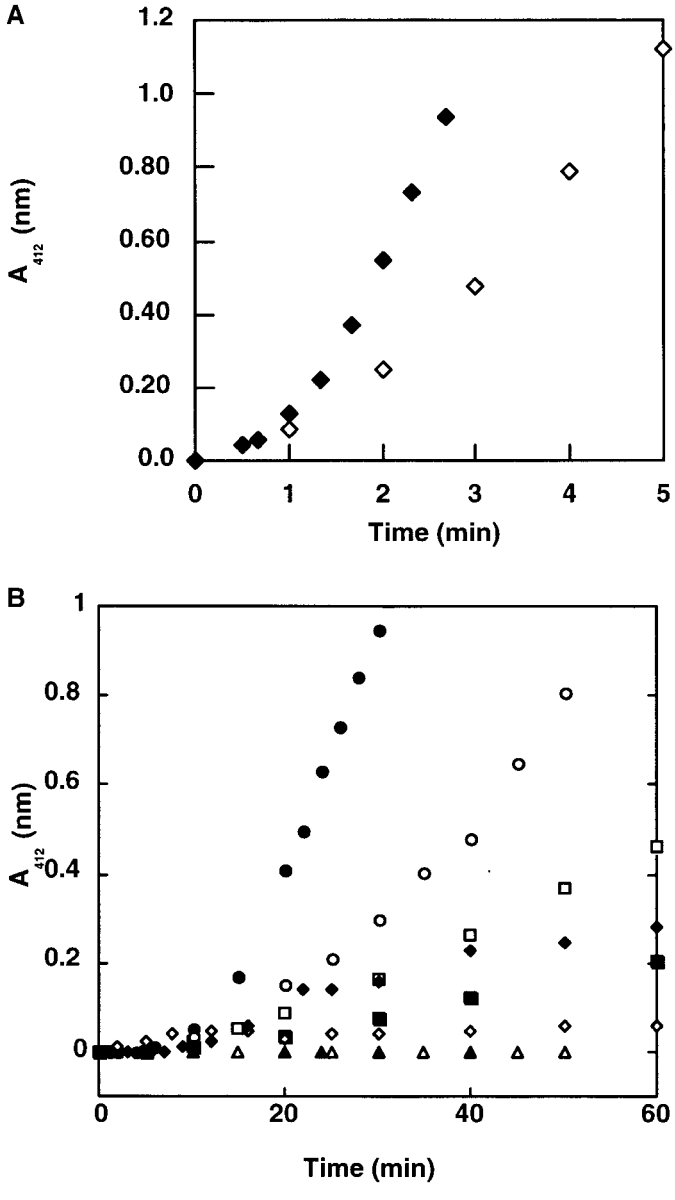


FIG. 3. (A) Time course of CoA release from HBCoA (solid diamonds) and HVCoA (open diamonds) catalyzed by *R. eutropha* PHA synthase at 25°C. The reaction mixtures contain 0.12 μM of PHA synthase and 1.6 mM of HBCoA **4**, or 0.56 μM of PHA synthase and 1.6 mM of HVCoA **13a**. (B) Time course of CoA release from HBCoA analogs catalyzed by *R. eutropha* PHA synthase at 25°C. The reaction mixtures contain 17 μM of PHA synthase and 1.6 mM of (2*R*, 3*R*)-2-methyl-HBCoA **8** (solid triangles); 1.1 μM of PHA synthase and 1.6 mM of 3HHxCoA **13b** (open squares); 10.5 μM of PHA synthase and 1.6 mM of (*R*)-lactyl CoA **17** (open diamonds); 39 μM of PHA synthase and 47 mM of 3-hydroxybutyryl *N*-acetylcysteamine **18** (open circles); 39 μM of PHA synthase and 47 mM of 3-hydroxybutyryl pantetheine **19** (solid squares); 1.3 μM of PHA synthase and 1.6 mM of 3HPCoA **20** (solid circles). 17 μM of PHA synthase and 1.6 mM of 3-(*S*)-hydroxybutyryl CoA **21** (open triangles); 11.8 μM of PHA synthase and 1.6 mM of 4HBCoA **22** (solid diamonds).

size **4** (Scheme II), with an additional saponification to convert **14** to **15**. **17** was purified by preparative HPLC and characterized by ^{1}H-NMR.

The syntheses of two truncated CoA substrate analogs **18** and **19** are shown in Schemes VI and VII, respectively. **2** was coupled directly with *N*-acetylcysteamine using DCC and then deprotection to give **18** in high yield. **19** was synthesized from **3** by thiotransesterification in wet THF with D-pantetheine (32). No reaction occurred if the thiotransesterification was done using anhydrous THF. The products were purified by flash chromatography and characterized by ^{1}H-NMR.

The synthesis of **22** was carried out in a similar fashion as described for **4**. This compound is not stable, and rapidly cyclizes to give lactone.

Substrate Specificity Studies

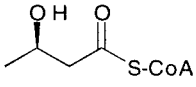
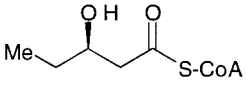
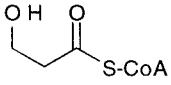
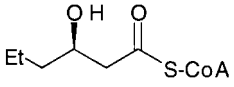
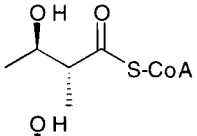
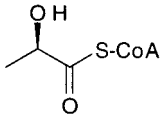
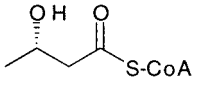
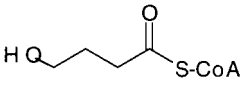
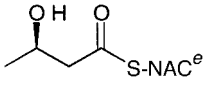
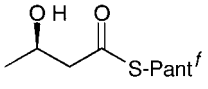
The ultimate goal of our research is to understand the polymerization mechanism and substrate specificity of the PHA synthases in sufficient detail to allow economically competitive production of biodegradable polymers with useful properties. Theoretically substrate accessibility should be much more flexible *in vitro* than *in vivo*, because substrates *in vivo* are limited by generation of appropriately hydroxylated CoA esters via complex metabolic pathways. Many substrate specificity studies have been carried out on both the *R. eutropha* and *A. vinosum* PHA synthase both *in vitro* and *in vivo* (21, 23, 24, 45–47). The primary focus of most studies has been *in vivo* using *R. eutropha* or *E. coli* containing the genes for the PHA biosynthetic pathway. In this study, we report the *in vitro* substrate specificity studies of the *R. eutropha* and *A. vinosum* PHA synthases, in comparison to other *in vitro* and *in vivo* studies.

The reactions with substrate analogs (Table II) have been monitored using a discontinuous CoA release assay (19). The disappearance of the starting material is not an optimal assay in that CoA release can result from polymer formation or from hydrolysis. In the case of even the slowest analog, polymer formation was apparent, however, by a phase change due to the insolubility of the polymer formed. Polymer size and properties are presently being investigated.

In the case of the $PhaC_{Re}$, the kinetics with HBCoA revealed an unexplained lag phase followed by a linear release of CoA (Fig. 3A). The activities of the analogs are reported as percentage of the activity of HBCoA using the linear portion of the curve following the lag phase. The PHA synthase activity varies dramatically depending on the CoA analog tested, and in all cases, the analogs have been tested at a single fixed concentration.

TABLE II

Substrate Specificity of $PhaC_{Re}$ and $PhaEC_{Av}$

Compound[a]		$PhaC_{Re}$	$PhaEC_{Av}$		
		Rel. activity (%)	Rel. activity (%)	k_{cat} (min^{-1})	K_m (mM)
	**4**	100	100	3920[b] 1120[c]	0.13[b] 0.45[c]
	**13a**	10	43	1680[b] 86[c]	0.18[b] 0.32[c]
	**20**	0.5	1.9	76	0.22
	**13b**	0.2	1	43	0.25
	**8**	na[d]	0.2	8.8	0.43
	**17**	na[d]	0.04	1.4	0.34
	**21**	na[d]			
	**22**	0.03			
	**18**	0.02	0.9	39	8.6
	**19**	0.002	0.5	19	12

[a] All analogs assayed at 1.6 mM, except *N*-acetylcysteamine and pantetheine derivatives (47 mM).
[b] First phase.
[c] Second phase.
[d] No detectable activity. Detection limit $\leq 1 \times 10^{-4}$ U/mg.
[e] NAC, *N*-acetylcysteamine.
[f] Pant, pantetheine.

PHB synthase polymerizes **4** and **13a** *in vivo* to yield PHBV, a plastic marketed under the trade name Biopol (48). As revealed in Table II for the $PhaC_{Re}$, **13a** is polymerized at 10% the rate of **4**. Increasing the chain length to 6 (**13b**) or decreasing it to 3 (**20**) resulted in a drop of activity to 0.2 and 0.5% that of **4**. Changing the location of the hydroxyl group to C4 (**22**) resulted in a dramatic loss of activity to 3×10^{-4} that

of **4**. This result is of interest as polyesters of this material appear to be readily made *in vivo* and are being tested for use in heart valves (11, 12). Moving the hydroxyl group to C2 (**17**) resulted in loss of all catalytic activity instead of the desired polylactides of current commerical interest (49). Changing the stereochemistry of the C3 hydroxyl group (**21**) or addition of a methyl group to C2 of HBCoA (**8**) resulted in complete loss of activity ($<10^{-4}$ U/mg). This latter number is our lower limit of detection.

Recent *in vitro* studies on $PhaC_{Re}$ reported by Zhang *et al.* (23) for **4**, **13a**, and **22** gave k_{cat}s of 339 s^{-1} (100%), 253 s^{-1} (75%), 287 s^{-1} (85%), respectively, and K_ms of 0.1 to 0.3 mM, dramatically different from our results (Table II). Unfortunately their exact experimental conditions were difficult to determine from their paper and our efforts to reproduce their results using 15–50% fructose and no Hecameg in our assay mixtures have been unsuccessful. Their rates were calculated by monitoring changes in A_{236} nm. With the coenzyme A moiety absorbing at 260 nm with a much larger extinction coefficient than the thioesters at 236 nm, this assay is not very sensitive. In another paper published by the same group (24), when using the same assay conditions as ours, they observed that the PHA synthase catalyzed reaction rate with 3HPCoA (**20**) was 3% that of HBCoA. This value is comparable with the one we observe (0.5% of that of HBCoA). In 1989, Haywood *et al.* demonstrated that *R. eutropha* PHA synthase, partially purified from PHA granules, was not capable of polymerizing monomers with side chains ranging in length from C6 to C10 (Scheme I) (13). Our results with soluble PHA synthase are similar to theirs on insoluble synthase, and suggest that this enzyme is very specific toward its substrate.

Previous studies *in vitro* reported that *N*-acetylcysteamine (NAC) and pantetheine analogs of HB were not substrates for the $PhaC_{Re}$ (21). In our hands, however, both **18** and **19** are substrates with rates 1/5,000 and 1/50,000 that of HBCoA, respectively. Control studies revealed that NAC release is enzyme dependent. Furthermore, studies using HB dehydrogenase revealed no hydroxybutyrate. NAC release is thus associated with polymerization. The turnover rate of **19** is probably limited by its low solubility. Failure of others to detect activity with these analogs resulted from the limitations of the assay conditions chosen (21). These *in vitro* studies suggest that $PhaC_{Re}$ is very specific for each component of its substrate structure: chain length, branching, hydroxyl group stereochemistry and location, and requirements for the entire CoA moiety.

Comparison of our results to studies *in vivo* is more challenging as in many cases copolymers of several monomers are obtained (45, 46). Contrary to the low substrate specificity observed from our *in vitro* studies, the *R. eutropha* PHA synthase has a much broader substrate specificity *in vivo* (46). Many labs have demonstrated that wild-type *R. eutropha* PHA synthase produces polymers having C3-C5 monomeric units (13, 15–17, 50). Recent studies have also demonstrated formation of copolymers of 3HB with 4-hydroxybutyrate, 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate, and 3-hydroxydodecanoate (46, 51, 52). These results indicate that *R. eutropha* is able to synthesize a large variety of polyesters from thioesters of short- and medium-chain-length hydroxyalkanoic acids *in vivo*, and that the composition of these copolyesters reflects the carbon source provided to the cells.

Recently, Fukui *et al.* reported that *Aeromonas punctata* strains, engineered to have extra copies of the PHA synthase and phasin genes and grown on alkanoates or oils, synthesize copolymers of poly(3HB-*co*-3HHx) in which 3HHx is 60 mol% (53). The wt organism gives copolymer with only 10% 3HHx. They demonstrated that the increase in 3HHx was unrelated to substrate biosynthesis. Furthermore, they showed that the SA of the PHA synthase for 3HHx increases 10-fold for reasons that are unclear. Since no one has isolated $PhaC_{Re}$ from its host cell, it is possible that recombinant $PhaC_{Re}$ differs structurally from the wild-type enzyme. Alternatively, the structure of the granules including phasin and PHA synthase could be altering substrate specificity. The basis for discrepancies between *in vitro* and *in vivo* experiments requires further investigation.

Our recent mechanistic studies on $PhaC_{Re}$ with HBCoA, a sT-CoA analog, and various site-directed mutants have demonstrated the mechanistic similarities between this Class I PHA synthase with a Class III PHA synthase, $PhaEC_{Av}$ (28). The substrate specificity of *A. vinosum* PHA synthase has thus also been examined using the same set of HBCoA analogs. The kinetics of the *A. vinosum* PHA synthase are also biphasic. In contrast to $PhaC_{Re}$, however, a rapid release of CoA is followed by a slower release of CoA. Given that the analogs have slower turnover numbers relative to HBCoA, the kinetic parameters were determined using the linear portion of the "first phase." The k_{cat} and K_m for each substrate analog have been determined and the results are summarized in Table II. In contrast with our studies on $PhaC_{Re}$, the analogs showed only a single phase of CoA release and consequently allowed a complete kinetic analysis of the substrate specificity. The chain length dependence of the rate (4 carbons $>$ 5 carbons $>$ 3 carbons $>$ 6 carbons) is the same as that observed for $PhaC_{Re}$. Addition of a methyl group at C2 (**8**), placement of the hydroxyl group at C2 (**17**), or alteration of the thiol ester (**18**, **19**) all result in a dramatic loss of activity. However, in contrast to Pha-

C_{Re}, polylactide can be made. This result confirms a study done by Steinbüchel's group, which reports polymerization of lactyl-CoA by extracts of *E. coli* expressing $PhaEC_{Av}$ (47). Only in the case of altered thiol esters are the K_ms dramatically increased. The relative rates of $PhaEC_{Av}$ (28), with each substrate analog, follow a similar trend as that observed for $PhaC_{Re}$. The similarities in substrate specificity observed with *R. eutropha* and *A. vinosum* PHA synthases *in vitro* provide further support that these two classes of enzymes share a similar catalytic mechanism and active site architecture despite their many differences in physical and kinetic properties (28).

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