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Mechanistic Studies on Class I Polyhydroxybutyrate (PHB) Synthase from *Ralstonia eutropha*: Class I and III Synthases Share a Similar Catalytic Mechanism[†]

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Received September 20, 2000

ABSTRACT: The Class I and III polyhydroxybutyrate (PHB) synthases from *Ralstonia eutropha* and *Chromatium vinosum*, respectively, catalyze the polymerization of β -hydroxybutyryl-coenzyme A (HBCoA) to generate PHB. These synthases have different molecular weights, subunit composition, and kinetic properties. Recent studies with the *C. vinosum* synthase suggested that it is structurally homologous to bacterial lipases and allowed identification of active site residues important for catalysis [Jia, Y., Kappock, T. J., Frick, T., Sinskey, A. J., and Stubbe, J. (2000) *Biochemistry* 39, 3927–3936]. Sequence alignments between the Class I and III synthases revealed similar residues in the *R. eutropha* synthase. Site-directed mutants of these residues were prepared and examined using HBCoA and a terminally saturated trimer of HBCoA (sT-CoA) as probes. These studies reveal that the *R. eutropha* synthase possesses an essential catalytic dyad (C319–H508) in which the C319 is involved in covalent catalysis. A conserved Asp, D480, was shown not to be required for acylation of C319 by sT-CoA and is proposed to function as a general base catalyst to activate the hydroxyl of HBCoA for ester formation. Studies of the [³H]sT-CoA with wild-type and mutant synthases reveal that 0.5 equiv of radiolabel is covalently bound per monomer of synthase, suggesting that a dimeric form of the enzyme is involved in elongation. These studies, in conjunction with search algorithms for secondary structure, suggest that the Class I and III synthases are mechanistically similar and structurally homologous, despite their physical and kinetic differences.

Polyhydroxyalkanoate (PHA)¹ synthase catalyzes the polymerization of 3-(*R*)-hydroxyalkanoyl-CoA derivatives to polyoxoesters, PHAs. More than 30 microorganisms have been identified that can synthesize these polymers as an energy and carbon storage material under nutrient-limited growth conditions when a carbon source is readily available (1–3). These polyesters have gained attention because they are thermoplastics and/or elastomers and are biodegradable (4, 5). The copolymer of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) has properties of a thermoplastic similar to the polypropylenes, the prime material of conventional plastics (5). Unfortunately, the PHAs cannot yet

be produced in an economically competitive fashion with polypropylenes (6). They are being used commercially, however, for specialty purposes (7–10). To make these biopolymers a viable material source, a basic understanding of the polymerization process is required. The genes for the PHB synthase from *Ralstonia eutropha* were first discovered in 1988 (11–13). Since then there have been several reports of purification of the recombinant *R. eutropha* synthase and several mechanistic studies (14–19). This paper uses a terminally saturated trimer of β -hydroxybutyryl-coenzyme A (sT-CoA, 5, Scheme 1)—a primer which functions in the polymerization process in vitro, and site-directed mutagenesis studies to identify the *R. eutropha* synthase as a lipase superfamily member which requires a Cys for covalent catalysis.

R. eutropha and *Chromatium vinosum* synthases utilize similar substrates, β -hydroxybutyryl-CoA and β -hydroxyvaleryl-CoA, to make PHB/PHV polyoxoesters. The synthases have been divided into three classes based on their substrate specificities and subunit compositions (20). The *R. eutropha* synthase is a Class I enzyme, and the *C. vinosum* synthase is a Class III enzyme. The *R. eutropha* enzyme has a subunit molecular mass of 64 kDa and exists in equilibrium with its dimeric form in solution (14, 17). The *C. vinosum* enzyme is composed of two different polypeptides, each ~40 kDa. The polypeptide with synthase activity has been designated PhaC. The second protein has been designated

[†] This work was supported by NIH Grant GM49171 to J.S. and A.J.S.

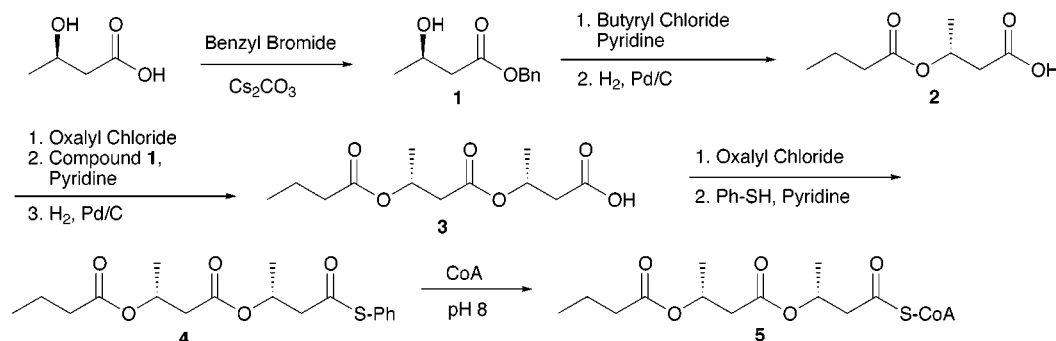
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¹ Abbreviations: DCC, dicyclohexylcarbodiimide; DMAP, (dimethylamino)pyridine; DMF, dimethylformamide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); eq, equivalent; 3-(*R*)-HB, 3-(*R*)-hydroxybutyric acid; HBCoA, β -hydroxybutyryl-CoA; HF, hydrofluoric acid; ¹H NMR, proton nuclear magnetic resonance; IPTG, isopropyl- β -D-thiogalactopyranoside; KPi, potassium phosphate; PCR, polymerase chain reaction; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; PHV, polyhydroxyvalerate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sT-CoA, a terminally saturated trimer of β -hydroxybutyryl-coenzyme A; TBDMS, *tert*-butyldimethylsilyl; TBDMSCl, *tert*-butyldimethylsilyl chloride; THF, tetrahydrofuran; TLC, thin-layer chromatography; wt, wild-type.

Scheme 1



PhaE and shows no homology to any proteins in the protein database (21, 22). We have recently demonstrated that this enzyme exists as two large molecular aggregates which may be in equilibrium with each other. Size exclusion chromatography analyses suggest that one of the aggregates is a hexamer composed of six PhaE/PhaC subunits and that the other is a trimer composed of three PhaE/PhaC subunits (23). The *R. eutropha* and *C. vinosum* synthases also exhibit different kinetics of polymer formation in vitro (15, 22).

Our recent studies on *C. vinosum* PHB synthase using protein homology modeling and site-directed mutagenesis studies suggested that this enzyme is a member of the lipase superfamily (23) and not a β -ketosynthase family member (fatty acid synthase family member) as previously proposed (24, 25). The mechanistic paradigm for thinking about the polymerization process has thus changed. The *C. vinosum* PHB synthase possesses an α/β hydrolase fold and like its structural counterpart, the lipase, functions at water-membrane interfaces. Lipases have been proposed to require a catalytic triad, Ser-His-Asp, for triacylglycerol hydrolysis (26–28). The serine is involved in covalent catalysis. The function of the Asp, however, has not been firmly established (28–30). We have recently shown using a saturated trimer of β -hydroxybutyryl-coenzyme A (sT-CoA, **5**, Scheme 1), a putative primer of the polymerization process in vitro, and site-directed mutagenesis that the *C. vinosum* PHB synthase requires the catalytic dyad of C149–H331 for covalent catalysis at C149 (23). The completely conserved D302, the closest Asp in primary sequence space to the Asp in the putative lipase catalytic triad, has also been demonstrated to be important for catalysis. The proposal was made that it could function as a general base to deprotonate the hydroxyl group of the HBCoA for nucleophilic attack in the chain elongation step. The similarities between the Class I and III enzymes, given this change in mechanistic paradigm, thus remain to be established.

Sequence alignments of the Class I and III synthases reveal only 24 completely conserved residues. In *R. eutropha* PHB synthase, residues C319, H508, and D480 correspond to C149, H331, and D302 in the *C. vinosum* PHB synthase. Mutagenesis studies of these and additional residues in conjunction with studies using the sT-CoA (**5**, Scheme 1) demonstrate the mechanistic similarities between the Class I and III enzymes. Studies with sT-CoA provide additional insight into our original model that the dimer of the synthase is the active form of the enzyme in the elongation process (15, 23). The Class I synthases, in analogy with the Class III enzymes, are proposed to be a member of the α/β

hydrolase family which involve covalent catalysis with a Cys nucleophile.

MATERIALS AND METHODS

Materials. pHAS in BL21(DE3) or BL21(DE3)/pLysS was induced with isopropyl- β -D-thiogalactopyranoside (IPTG, Boehringer Mannheim), resulting in the production of an N-terminal (His)₆-tagged *R. eutropha* PHB synthase.² Hecameg [6-*O*-(*N*-heptylcarbamoyl)methyl- α -D-glucopyranoside] was obtained from Vegatec, Villejuif, France. 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. DNA sequencing was carried out by the MIT Biopolymers Laboratory. Proton nuclear magnetic resonance (¹H NMR) spectra were obtained on a Bruker-AC250 or AC300. All NMR chemical shift values were reported in δ units (ppm) using tetramethylsilane (TMS) as a reference. 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).

Synthesis of a Terminally Saturated Trimer of β -Hydroxybutyryl-CoA (sT-CoA)

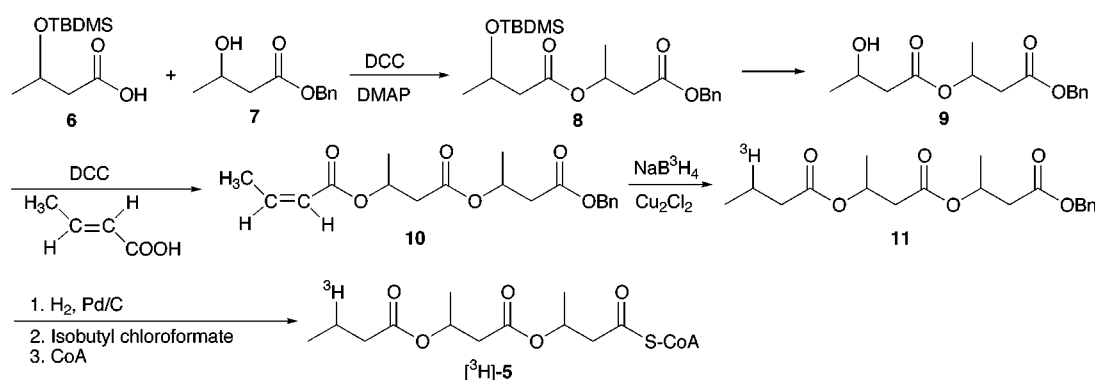
The synthesis of sT-CoA (**5**) is shown in Scheme 1.

(A) *Benzyl (R)-3-Hydroxybutyrate (1)*. To a solution of 3-(*R*)-hydroxybutyric acid (1.5 g, 14.4 mmol, Fluka) and benzyl bromide (2.46 g, 14.4 mmol, 1.0 eq) in 10 mL of dry DMF was added cesium carbonate (Cs₂CO₃, 5.16 g, 15.8 mmol, 1.1 eq) at 22 °C. The reaction was stirred at 22 °C for 12 h. The reaction mixture was diluted with ether (100 mL), and the organic layer was washed with water (3 \times 30 mL), dried over MgSO₄, and concentrated in vacuo to afford the product as a pale yellow liquid (2.7 g, 99%): TLC (*R*_f 0.40, 25% ethyl acetate in hexanes, UV); ¹H NMR (CDCl₃, 250 MHz) δ 1.23 (d, *J* = 6.3 Hz, 3H), 2.5 (m, 2H), 4.2 (m, 1H), 5.15 (s, 2H), 7.35 (s, 5H).

(B) *Compound 3*. **1** (0.97 g, 5 mmol) was reacted with butyryl chloride (0.75 g, 7 mmol, 0.73 mL, Aldrich) in 10 mL of dry methylene chloride with anhydrous pyridine (1 mL, 12 mmol) and (dimethylamino)pyridine (DMAP, 20 mg,

² Yuan, W., Jia, Y., Snell, K. D., Müh, U., Park, C., Tian, J., Lambalot, R. H., Walsh, C. T., Sinskey, A. J., and Stubbe, J., manuscript in preparation.

Scheme 2



0.16 mmol). The reaction was stirred at 22 °C for 12 h and, after purification on a silica gel flash column (10% ethyl acetate in hexanes), gave an oil of the benzyl ester dimer (1.2 g, 91%): TLC (R_f 0.72, 25% ethyl acetate in hexanes, UV); ^1H NMR (CDCl_3 , 250 MHz) δ 0.90 (t, J = 7.4 Hz, 3H), 1.29 (d, J = 6.3 Hz, 3H), 1.58 (m, 2H), 2.19 (t, J = 7.3 Hz, 2H), 2.5–2.75 (m, 2H), 5.11 (s, 2H), 5.30 (m, 1H), 7.34 (s, 5H).

The dimer (1.2 g) was dissolved in 30 mL of ethyl acetate and hydrogenated in the presence of 0.2 g of 10% Pd-C (Aldrich) at 22 °C and atmospheric pressure for 5 h. The free acid (**2**) was obtained as a clear oil (0.78 g, 99%): ^1H NMR (CDCl_3 , 250 MHz) δ 0.92 (t, J = 7.3 Hz, 3H), 1.31 (d, J = 6.4 Hz, 3H), 1.52 (m, 2H), 2.25 (t, J = 7.3, 2H), 2.62 (m, 2H), 5.38 (m, 1H).

2 (0.78 g, 4.5 mmol) was dissolved in 10 mL of dry methylene chloride at 22 °C. Oxalyl chloride (1.27 g, 11.5 mmol, 2.6 eq, Aldrich) was added. The reaction mixture was stirred at 22 °C for 2 h. Three drops of dry DMF were then added, and the reaction mixture was stirred for an additional 2 h. The solvent was then removed in vacuo, and the residue was dried under high vacuum for 1 h. To the resulting acyl chloride was added by syringe a solution of **1** (0.9 g, 4.5 mmol) in 10 mL of methylene chloride, followed by dry pyridine (1 mL, 12 mmol, 2.7 eq). After stirring for 4 h at 22 °C under argon, the reaction was diluted with ether (50 mL), and the organic layer was washed with 0.1 N HCl, dried over MgSO_4 , and concentrated. The trimer benzyl ester was obtained as a clear oil (1.4 g, 88%) after purification on a silica gel flash column (10% ethyl acetate in hexanes, then 15% ethyl acetate in hexanes): TLC (R_f 0.70, 25% ethyl acetate in hexanes, UV); ^1H NMR (CDCl_3 , 250 MHz) δ 0.93 (t, J = 7.3 Hz, 3H), 1.24 and 1.26 (two d, J = 6.4 Hz, 6H), 1.6 (m, 2H), 2.22 (t, J = 7.4, 2H), 2.35–2.70 (m, 2H), 5.12 (s, 2H), 5.25 (m, 2H), 7.35 (s, 5H).

The hydrogenation of trimer benzyl ester to **3** was accomplished in the same manner as described above. Starting with trimer benzyl ester (2.0 g, 5.6 mmol) and 0.2 g of 10% Pd-C in 30 mL of ethyl acetate, **3** was obtained as a clear oil (1.40 g, 96%): ^1H NMR (CDCl_3 , 250 MHz) δ 0.93 (t, J = 7.4 Hz, 3H), 1.27 and 1.30 (two d, J = 6.6 Hz, 6H), 1.63 (m, 2H), 2.23 (t, J = 7.4, 2H), 2.4–2.7 (m, 4H), 5.28 (m, 2H).

(C) **Compound 4**. To a solution of **3** (0.4 g, 1.5 mmol) in 4 mL of dry methylene chloride in a 50 mL round-bottom flask was added 0.5 mL of oxalyl chloride under argon at 22 °C. The reaction mixture was stirred at 22 °C for 2 h.

Three drops of dry DMF were then added, and the stirring was continued for another 2 h. The solvent was removed in vacuo, and the residue was dried under high vacuum for 1 h. To the residue under argon was added 6 mL of dry methylene chloride through a syringe, followed by dry pyridine (0.15 g, 150 μL , 1.85 mmol, 1.2 eq) and benzenethiol (204 mg, 190 μL , 1.85 mmol, 1.2 eq). After stirring at 22 °C for 6 h, the solvent was removed in vacuo, and the residue was purified on a silica gel flash column (10% ethyl acetate in hexanes, then 15% ethyl acetate in hexanes) to obtain **4** as a pale yellow oil (0.46 g, 85%): TLC (R_f 0.67, 25% ethyl acetate in hexanes, UV); ^1H NMR (CDCl_3 , 250 MHz) δ 0.92 (t, J = 7.3 Hz, 3H), 1.28 and 1.31 (two d, J = 6.3 Hz, 6H), 1.62 (m, 2H), 2.23 (t, J = 7.4, 2H), 2.56 (m, 2H), 2.90 (m, 2H), 5.30 (m, 2H), 7.41 (s, 5H).

(D) *sT*-CoA (**5**). CoA (80 mg, 0.1 mmol) was dissolved in 2.0 mL of 50 mM potassium phosphate (KPi), pH 7.8 (saturated with nitrogen), in a 7 mL scintillation vial with screw-cap. The pH of the solution was adjusted to 7.8 by addition of approximately 100 μL of 1 N NaOH. To this solution was added **4** (180 mg, 0.5 mmol, 5 eq) in 300 μL of acetonitrile. The vial was sealed, and the reaction mixture was stirred vigorously at 22 °C for 20 h (efficient stirring is necessary because the reaction is biphasic). About 60% of the CoA was converted to product as judged by HPLC. The reaction mixture was extracted with ether (5 \times 3 mL) to remove benzenethiol and excess **4**. The aqueous phase was loaded into a 5 mL syringe and passed through a 0.45 μm Acrodisc (Gelman Science) filter. The filtrate was purified by preparative HPLC (C-18 reversed phase column, Econosil 10 μm , 22 mm \times 250 mm) chromatography (\sim 0.5 mL per loading). Ten percent methanol in water was used to elute unreacted CoA and other polar impurities. The product was eluted using a linear gradient from 10 to 60% methanol (8 mL/min) over 30 min. The fractions containing product were pooled, concentrated in vacuo to 10 mL, and then lyophilized to give a white powder (32 mg, 32%): HPLC (R_t 27 min, 10–60% methanol in 50 mM KPi, pH 4.7, over 30 min, 1.0 mL/min, detected at 260 nm); ^1H NMR (D_2O , 250 MHz) δ 0.72 (s, 3H), 0.86 (s, 3H), 0.86 (t, J = 7.5 Hz, 3H), 1.22 (d, J = 6.3 Hz, 6H), 1.55 (m, 2H), 2.28 (t, J = 7.4, 2H), 2.40 (t, J = 6.3 Hz, 2H), 2.62 (m, 2H), 2.86 (m, 2H), 2.95 (t, J = 6.4 Hz, 2H), 3.29 (t, J = 6.4 Hz, 2H), 3.43 (t, J = 6.3, 2H), 3.52 (m, 1H), 3.78 (m, 1H), 4.0 (s, 1H), 4.20 (s, 2H), 4.54 (s, 1H), 5.18 (m, 2H), 6.14 (d, J = 6.8 Hz, 1H), 8.24 (s, 1H), 8.54 (s, 1H).

The synthesis of $[^3\text{H}]\text{-5}$ is shown in Scheme 2.

(E) *tert*-Butyldimethylsilyl (TBDMS) Ether of 3-(R)-Hydroxybutyric Acid (TBDMS)HB (**6**). Imidazole (3.4 g, 50 mmol) was added to a solution of *tert*-butyldimethylsilyl chloride (TBDMSCl) (2.53 g, 16.8 mmol) in dry DMF (8 mL). The solution was stirred in an ice bath under argon for 15 min, followed by addition of 3-(R)-HB (0.5 g, 4.8 mmol) in DMF (2 mL). The reaction was stirred overnight at room temperature. The mixture was then poured into a saturated solution of NaCl (60 mL) and extracted with a 1:3 mixture of diethyl ether and petroleum ether (5 × 30 mL). The combined organic layers were dried over MgSO₄, filtered, and concentrated to give the crude bis-silylated material (1.9 g). This material was dissolved in a mixture of methanol (60 mL) and tetrahydrofuran (THF) (30 mL). Potassium carbonate (1.5 g) dissolved in water (10 mL) was added, and the mixture was stirred overnight at room temperature. The solution was diluted with a saturated solution of NaCl (20 mL) and acidified to pH 3.0 with 1 M H₂SO₄. The solution was then extracted with 1:3 diethyl ether/petroleum ether (5 × 20 mL), and the combined extracts were dried over MgSO₄. The solvent and TBDMS alcohol were removed in vacuo (2 mmHg, overnight). The product, **6**, is a colorless oil (0.95 g, 91%): ¹H NMR (300 MHz, CDCl₃) δ 0.12 (s, 3H), 0.87 (s, 9H), 1.22 (d, *J* = 6.1 Hz, 3H), 2.05–2.45 (m, 2H), 4.26–4.34 (m, 1H).

(F) *Benzyl Ester of 3-(R)-HB* (**7**). 3-(R)-HB (3.5 g, 33.6 mmol), freshly distilled benzyl alcohol (34 g, 314 mmol), and the lithium salt of *p*-toluenesulfonic acid (400 mg, 2.5 mmol) were refluxed in dry benzene (200 mL) for 5 h with an azeotrope trap. Benzene was removed in vacuo, and diethyl ether (150 mL) was added. The mixture was washed with a 10% solution of sodium bicarbonate (3 × 30 mL) and a saturated solution of NaCl (3 × 25 mL) and dried over MgSO₄, and the ether was evaporated in vacuo. Distillation (12 mmHg, 91–100 °C) of the residual oil gave **7** (4.8 g, 73%): ¹H NMR (300 MHz, CDCl₃) δ 1.98 (d, *J* = 6.9 Hz, 3H), 2.52 (m, 2H), 4.18–4.28 (m, 1H), 5.25 (s, 2H), 7.28–7.37 (m, 5H).

(G) *Compound 8*. Dicyclohexylcarbodiimide (DCC) (0.14 g, 0.66 mmol) in dry dichloromethane (2 mL) was added to a mixture of **6** (112 mg, 0.51 mmol) and **7** (107 mg, 0.55 mmol) in dichloromethane (3 mL), followed by DMAP (7 mg), and the mixture was stirred at room temperature overnight. The reaction mixture was cooled to 0 °C, and cold diethyl ether (10 mL) was added. After 15 min, a white precipitate of dicyclohexylurea was removed by filtration. The organic layer was washed with 0.5 M HCl (2 × 2 mL) and then with a saturated solution of NaCl (4 × 2 mL), dried over MgSO₄, and concentrated in vacuo. Chromatography on a silica gel column (40 × 2.5 cm) using a mixture of petroleum ether and diethyl ether (4:1) gave **8** (103 mg, 51%): ¹H NMR (300 MHz, CDCl₃) δ 0.07 (d, *J* = 3.8 Hz, 6H), 0.88 (s, 9H), 1.18 (d, *J* = 6.1 Hz, 3H), 1.31 (d, *J* = 6.4 Hz, 3H), 2.30 (dd, *J*₁ = 5.8 Hz, *J*₂ = 14.7 Hz, 1H), 2.44 (dd, *J*₁ = 6.9 Hz, *J*₂ = 14.6 Hz, 1H), 2.55 (dd, *J*₁ = 5.6 Hz, *J*₂ = 15.8 Hz, 1H), 2.72 (dd, *J*₁ = 6.5 Hz, *J*₂ = 15.7 Hz, 1H), 4.23 (sex, *J* = 6.3 Hz, 1H), 5.15 (s, 2H), 5.28 (sex, *J* = 6.4 Hz, 1H), 7.28–7.37 (m, 5H).

(H) *Compound 9*. To a solution of **8** (0.33 g, 0.838 mmol) in acetonitrile (2 mL) was added a 5% solution of HF in acetonitrile (6 mL). The progress of the reaction was monitored by TLC. After 20 min, a saturated solution of

NaHCO₃ was added until no more CO₂ evolved. The mixture was extracted with diethyl ether (3 × 20 mL). The combined organic layers were washed with a saturated solution of NaCl, dried over MgSO₄, and concentrated under reduced pressure to yield **9** (0.227 g, 97%): ¹H NMR (300 MHz, CDCl₃) δ 1.21 (d, *J* = 6.4 Hz, 3H), 1.32 (d, *J* = 6.34 Hz, 3H), 2.28–2.45 (m, 2H), 2.55–2.74 (m, 2H), 4.14–4.18 (m, 1H), 5.13 (s, 2H), 5.32–5.39 (m, 1H), 7.37 (s, 5H).

(I) *Compound 10*. The same DCC coupling procedure (see **8** above) was used to synthesize **10** (0.208 g, 74%) from 0.227 g of **9** (0.811 mmol): ¹H NMR (300 MHz, CDCl₃) δ 1.26 (d, *J* = 5.44 Hz, 3H), 1.28 (d, *J* = 6.23 Hz, 3H), 1.85–1.88 (m, 2H), 2.40–2.73 (m, 4H), 5.12 (s, 2H), 5.25–5.32 (m, 2H), 5.77–5.83 (m, 2H), 6.82–7.00 (m, 2H), 7.32–7.37 (m, 5H).

(J) [³H]B(HB)₂COOBn (**11**). To a solution of **10** (50 mg, 0.144 mmol) in anhydrous methanol (2.5 mL) was added solid Cu₂Cl₂ (23 mg, 0.112 mmol), and the mixture was cooled to 0 °C using an ice bath. NaB³H₄ (200 mCi, 1000 mCi/mmol) was added in methanol (3 mL), and the reaction was stirred for 25 min. Then additional NaBH₄ (15 mg, 0.4 mmol) was added in methanol (40 μL), and the reaction was allowed to proceed for another 10 min, at which time the catalyst was removed by filtration through a glass wool plug. The reaction mixture was acidified by the careful addition of 0.5 N HCl (~6 mL). **11** was extracted with diethyl ether (4 × 25 mL). The extract was washed with a saturated solution of NaCl (2 × 10 mL), dried over MgSO₄, and filtered, and the solvent was evaporated in vacuo. Yield: 98%, 50 mg. The specific activity of the product was 3.7 × 10⁷ cpm/μmol.

(K) [³H]-5. To a solution of **11** (50 mg, 0.142 mmol) in absolute ethanol (25 mL) was added 10% Pd-C catalyst (30 mg), and the reaction was stirred at room temperature under H₂ overnight. The catalyst was removed by filtration over Celite. Concentration under reduced pressure gave the product [³H]sT-acid (36 mg, 96%).

To a solution of [³H]sT-acid (36 mg, 0.137 mmol) in methylene chloride (5 mL) at 0 °C was added freshly distilled trimethylamine (21 μL, 0.138 mmol). After stirring for 10 min at 0 °C, isobutyl chloroformate (18.5 μL, 0.140 mmol) was added, and the reaction was stirred for 60 min at room temperature. Methylene chloride was removed by evaporation in vacuo, and the residue was resuspended in dry THF (5 mL). This suspension was immediately added to a solution of 60 mg of CoA (0.078 mmol) in 4 mL of water (pH adjusted to 8.0, degassed). During the course of the reaction, the pH was maintained at 8.0 with 1 M NaOH. The reaction was complete after 20 min [negative nitroprusside assay (31)]. The pH of the solution was adjusted to 5.5, and it was extracted with diethyl ether (3 × 4 mL). Volatile solvents were evaporated from the aqueous solution in vacuo. [³H]-5 was purified on a preparative C-18 reversed phase column (Econosil 10 μm, 10 mm × 250 mm) using a linear gradient of 0–60% methanol in 50 mM KPi (pH 4.8) over a period of 30 min. The [³H]-5 was desalted by chromatography on the same column equilibrated with water and eluted with water. [³H]-5 had a specific activity of 5.0 × 10⁷ cpm/μmol, and its ¹H NMR characterization was same as **5**.

Construction of R. eutropha PHB Synthase Site-Directed Mutants. Site-directed mutagenesis was carried out using the 4 primer PCR technique previously described (32, 33). pHAS

was used as the template.² PCR was used to amplify DNA cassettes that included the target residue and in addition contained two unique restriction sites on each end of the cassette. The amplified DNA fragments were used to replace the original segment within pHAS, yielding plasmids pHAS-D480N, pHAS-H481Q, and pHAS-H508Q. The following primers were used to introduce the mutation sites (underlined):

D480N: 5'-GGCTCGCGCGAAAAACCATATC-3'

H481Q: 5'-TCGCGCGAAGACCAAAATCGTG-3'

H508Q: 5'-TCGGGCCAGATCGCCGGTGTGATCAA-3'

Plasmid pHAS-C319A was constructed by subcloning a restriction fragment of plasmid pKAS4-C319A (14) (containing the C319A mutation) into plasmid pHAS. All mutant plasmid DNAs isolated from XL1-Blue *Escherichia coli* host cells (Stratagene) were sequenced by the MIT Biopolymer Lab to confirm the constructs.

Expression and Purification of *R. eutropha* PHB Synthase and Mutants. N-terminal (His)₆-tagged wt PHB synthase and mutants C319A and H481Q were overexpressed in *E. coli* BL21(DE3)/pLysS cells (Novagen) by 0.1 mM IPTG induction at an $A_{600\text{nm}}$ of 0.5–0.6. The cells were grown for 3 h at 30 °C. D480N and H508Q synthases were overexpressed in *E. coli* BL21(DE3) cells (Novagen). The cells were grown at 30 °C until an $A_{600\text{nm}}$ of 0.5–0.6 was reached; the temperature was then lowered to 15 °C, and IPTG was added to a final concentration of 0.1 mM. The cells were grown for ~12 h at 15 °C and harvested. Typically, 4 L of culture yielded ~8 g of cells. All the mutants were purified by the procedure described by Yuan et al.² The proteins were judged to be ≥95% homogeneous by SDS–PAGE (34). Typically ~1–1.5 mg of protein was isolated from 1 g of cells. The proteins were stored at –80 °C.

Enzyme Assay. All assays were carried out at 25 °C in a final volume of 300 μL containing 150 mM KPi (pH 7.2), 0.2% glycerol, 0.05% Hecameg, 1.2 mM HBCoA, and enzyme (0.1 μM wt PHB synthase, 7.6 μM C319A, 16.7 μM D480N, 0.21 μM H481Q, or 18.7 μM H508Q). At defined time points, 20 μL aliquots were removed from the reaction mixture and quenched with 50 μL of 10% trichloroacetic acid. Each sample was centrifuged to remove the precipitated protein, and then 68 μL of the quenched reaction mixture was added to 282 μL of 0.25 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.5 M KPi (pH 7.8), and $A_{412\text{nm}}$ was measured. One unit is defined as 1 μmol of substrate consumed per minute (detection limit $\geq 1 \times 10^{-4}$ unit/mg). In all the studies described in this paper, the enzyme concentrations were determined by $\epsilon = 162\,000\text{ M}^{-1}\text{ cm}^{-1}$.²

Kinetics of Wild-Type and Mutant PHB Synthases with sT-CoA. All reactions were carried out at 25 °C in a final volume of 160 μL : 150 mM KPi (pH 7.2), 0.2% glycerol, 0.05% Hecameg, and wt synthase or mutants and sT-CoA in a molar ratio of ~1:10. The synthase and sT-CoA concentrations were 60.7 μM wt synthase and 490 μM sT-CoA, 40.6 μM D480N and 439 μM sT-CoA, or 44.8 μM H508Q and 409 μM sT-CoA. The reaction was initiated by the addition of enzyme, and at designated time points, 20 μL aliquots were removed and quenched with 40 μL of 10% trichloroacetic acid. Fifty-eight microliters of the quenched

reaction mixture was added to 242 μL of 0.25 mM DTNB in 0.5 M KPi (pH 7.8), and $A_{412\text{nm}}$ was measured.

Analysis of the Reaction Product Generated from Incubation of Wild-Type PHB Synthase with [³H]sT-CoA. The reaction mixture was the same as described above except [³H]sT-CoA (1577 cpm/nmol) was used. The reaction was allowed to proceed at 25 °C for 20 min. The reaction product was loaded onto a Nanosep 10K filter system (Pall Filtron) and centrifuged at 14 000 rpm for 10–15 min in a benchtop centrifuge. The filtrate was collected. To remove radiolabel not bound to the protein, the protein fraction was washed with additional buffer (100 μL of 150 mM KPi, pH 7.2, 0.2% glycerol, 0.05% Hecameg), and the centrifugation step was repeated until the radioactivity detected in the filtrate was <200 cpm. At this point, the collected filtrates were combined and injected into a HPLC reverse-phase C18 column (10 μm , 250 \times 4.6 mm; Alltech). The column was eluted with a linear gradient from 20 to 90% methanol in 50 mM KPi, (pH 7.5) over a period of 45 min at 25 °C. The fractions were collected every 30 s and analyzed for radioactivity in a scintillation counter.

Stoichiometry Labeling of PHB Synthase with [³H]sT-CoA. The reaction mixture contained 105 μM PHB synthase, 2.2 mM [³H]sT-CoA (1860 cpm/nmol) in a final volume of 15 μL . The reaction was allowed to proceed at 25 °C for 20 min, and the product was injected into a Bio-Silect SEC250 size exclusion column (BioRad). The column was eluted with 50 mM KPi (pH 7.5) and 75 mM NaCl at a flow rate of 1 mL/min over 20 min at 4 °C on a Bio-CAD Sprint system (PerSeptive Biosystems, Inc.). The fractions were collected every 30 s. The $A_{280\text{nm}}$ of the protein-containing fractions was monitored and analyzed for radioactivity.

RESULTS

Our previous studies with both the *R. eutropha* and *C. vinosum* synthases have revealed that sT-CoA (5, Scheme 1) can function as a primer of PHB formation in the test tube (15, 22). In a preliminary study, we reported that the *R. eutropha* synthase was covalently modified by [³H]sT-CoA and that the addition of HBCoA to the labeled enzyme resulted in [³H]-PHB (15). More recently, the [³H]sT-CoA was shown to be instrumental in identifying the importance of C149 in covalent catalysis with the *C. vinosum* synthase (22). In this study, a more detailed analysis of sT-CoA with the wt *R. eutropha* synthase and a number of site-directed mutants is reported. In all cases, the synthase contains a (His)₆-tag N-terminus which has greatly facilitated purification of the protein.

Syntheses of sT-CoA and [³H]sT-CoA. The syntheses of the unlabeled and labeled sT-CoA have not been previously reported and are outlined in Schemes 1 and 2. Synthesis of the unlabeled sT-CoA differs from synthesis of the labeled material and is briefly summarized. The trimeric acid 3 (Scheme 1) was synthesized by acylation of 1 with butyryl chloride, followed by debenzoylation by hydrogenation. The resulting acid was activated as the acid chloride and then coupled to a second molecule of 1 to give 2. The removal of the benzyl group, activation, followed by addition of a third molecule of 1 resulted in 3. The thioester of 3 was efficiently prepared by coupling the acyl chloride of 3 with benzenethiol. Direct coupling of 3 with benzenethiol using

Table 1: Rates of (His)₆-Tagged wt and Mutant PHB Synthase-Catalyzed Reactions with HBCoA

(His) ₆ -PHB synthase ^a	rate (units/mg)	% of wt
wt	20 ^b	100
C319A	<1 × 10 ⁻⁴	<5 × 10 ⁻⁴
H508Q	<1 × 10 ⁻⁴	<5 × 10 ⁻⁴
D480N	0.0008	0.004
H481Q	4	20

^a All mutants contain a (His)₆-tag in the N-terminus.² ^b All mutant activities are reported relative to the (His)₆-tag wt protein.

DCC resulted in more side products and a lower yield. The desired sT-CoA (**5**) was prepared by thioltransesterification of **4** with CoA. The thioltransesterification reaction is slow due to the solubility properties of **4**.

Synthesis of [³H]-**5** is outlined in Scheme 2. The synthetic scheme is a variation of Scheme 1 using TBDMS and benzyl blocking groups for the alcohol and acid groups of HB. In addition, DCC coupling was used in place of the acid chloride to activate the carboxylate for ester formation. To attach the ³H label, crotonic acid replaced butyrate in Scheme 1, and the corresponding trimer **10** was made. Reduction with NaB³H₄ in the presence of Cu₂Cl₂ is the key step in the synthesis. Previous studies have shown that this reagent affords 1,4 conjugate addition to esters and **11** was generated in high yield (35). After removal of the benzyl blocking group by catalytic hydrogenation, the resulting acid was activated with the mixed anhydride, isobutylchloroformate, followed by addition of CoA (36). Both the unlabeled and labeled sT-CoAs were homogeneous by analysis using reverse phase HPLC. They each displayed a ¹H NMR spectrum and electrospray mass spectrum (calculated: 1009.5, found: 1009.3; Mariner Biospectrometry Workstation from PerSeptive Biosystems) consistent with the desired products.

Characterization of *R. eutropha* PHB Synthase Site-Directed Mutants. The choice of mutants to be examined was based on our previous studies with the *C. vinosum* synthase and on sequence alignments between the Class I and III synthases identifying conserved residues. *R. eutropha* PHB synthase site-directed mutants (C319A, D480N, H481Q, and H508Q) with a (His)₆ N-terminal tag were constructed. They were overexpressed and purified using a Ni affinity column.² Large molecular weight aggregates were observed in variable amounts and were removed by gel filtration on a Sephacryl S200 column (Pharmacia Biotech). The synthases were judged to be ≥95% pure by SDS-PAGE. The activity of each mutant was determined by monitoring CoA release from HBCoA using the discontinuous DTNB assay (22). The results are summarized in Table 1.

In preliminary studies, C319 of the *R. eutropha* synthase was identified as the active site nucleophile involved in covalent catalysis (14, 15). It is not surprising, therefore, that the Ala mutation gave activity <10⁻⁴ unit/mg. This number is the lower limit of detection of the assay.

H508 is the equivalent of H331 in the *C. vinosum* synthase. This residue is completely conserved and was shown with the *C. vinosum* enzyme to be the general base catalyst to generate the Cys thiolate required for covalent catalysis. The mutant H508Q synthase showed no detectable activity, supporting the proposed role for this residue as a general base to form a catalytic dyad with nucleophile C319.

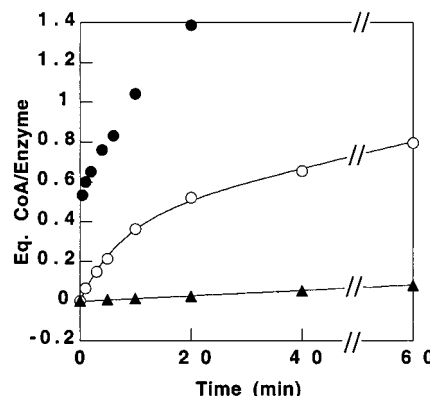


FIGURE 1: Time course of CoA release from sT-CoA catalyzed by PHB synthases at 25 °C. The reaction mixtures contain 60.7 μM wt synthase and 490 μM sT-CoA (solid circles); 40.6 μM D480N and 439 μM sT-CoA (open circles); and 44.8 μM H508Q and 409 μM sT-CoA (solid triangles).

Residue D480 corresponds to D302 in the *C. vinosum* synthase. Our studies with the *C. vinosum* synthase D302A mutant showed activity of 1/10⁴ that of the wt enzyme (23). Residue D302 was proposed to function as the general base catalyst to activate the 3-hydroxyl group of HBCoA for nucleophilic attack on the thiol ester to form the oxygen ester bond. Mutation of D480 to an Asn decreased the *R. eutropha* synthase activity by 4 × 10⁵, suggesting that this residue has an important function in catalysis.

Finally, residue H481, a second conserved His corresponding to H303 in the *C. vinosum* synthase, was changed to a Gln. In both enzymes, the Gln mutant has excellent activity. Thus, the reason for the conservation of this residue and its function remains unknown.

Overall, the mutagenesis studies support the similarities in the catalytic mechanisms between the Class I and III enzymes, despite their limited sequence homology and their differences in physical and kinetic properties.

Interaction of the Wild-Type and Mutant Synthases with sT-CoA. Our preliminary studies with the *R. eutropha* synthase showed that sT-CoA provided insight into the priming step of the polymerization in vitro (15). Specifically, incubation of the synthase with sT-CoA resulted in a number of interesting observations which have now been examined in more detail. In addition, these results can now be compared with similar studies carried out on the *C. vinosum* synthase (23) as a mechanistic probe of the similarities and differences between these two classes of enzymes.

Incubation of wt PHB synthase with sT-CoA resulted in a “burst” of CoA release as monitored by the DTNB assay (Figure 1). The “burst” corresponds to 0.5 equiv of CoA per monomer of synthase. This result agrees with our previous radiolabeling experiments using [³H]sT-CoA (15), providing support for the model that the dimer of *R. eutropha* synthase is the active form of the enzyme in the elongation process. The “burst” of CoA is followed by a much slower release of CoA (Figure 1 and Table 2). A similar observation was recently made with the *C. vinosum* enzyme (23). In the case of the *C. vinosum* enzyme, the saturated trimer acid, the result of slow hydrolysis of the covalently labeled active site nucleophile, was identified. A similar experiment with the *R. eutropha* synthase to that described in Figure 1 using [³H]sT-CoA was carried out. At the end of a 20 min

Table 2: Rates of sT-CoA Acylation and Deacylation of wt and Mutant PHB Synthases

PHB synthase	acylation rate (units/mg)	deacylation rate (units/mg)
wt	2×10^{-2}	7×10^{-4}
D480N	4×10^{-4}	1×10^{-4}
H508Q	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$

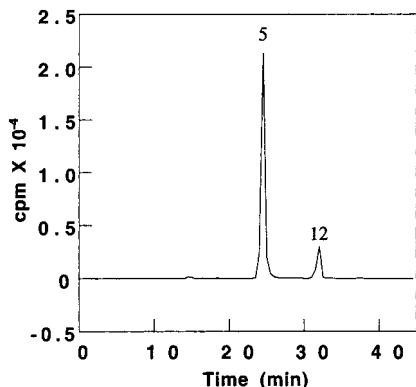


FIGURE 2: HPLC separation of the radiolabeled products generated by incubation of PHB synthase with [^3H]sT-CoA at 25 °C for 20 min. The reaction mixture contains 60.7 μM synthase and 490 μM [^3H]sT-CoA. Unreacted substrate [^3H]sT-CoA (**5**) and the product [^3H]sT-acid (**12**) are indicated.

incubation, the small molecules were separated from the protein using a membrane filtration device, and the radiolabeled product(s) was (were) analyzed by HPLC. The results are shown in Figure 2. The major peak of radiolabeled material is **5**, unreacted sT-CoA. Compound **12** is the corresponding acid (sT-acid) which migrates with an identical retention time to the authentic sT-acid prepared synthetically. The ^3H -label attached to protein in addition to that observed in the sT-acid (**12**) quantitatively accounts for the amount of CoA release observed independently. These observations, in conjunction with our earlier identification of the labeled C319-containing peptide (**15**), indicate that the initial “burst” corresponds to the acylation of the C319 by sT-CoA and that the label is not stable and undergoes slow hydrolysis. A similar result has been observed with the Class III synthase (**23**), although the initial covalent linkage with this enzyme is less stable (**22**).

If H508 is the general base catalyst that generates the thiolate of C319, then one would expect that incubation of the sT-CoA with this mutant protein would result in no CoA release. The experimental results show that this is the case (Figure 1 and Table 2). These results provide further support for the function of this residue in catalysis. An identical result has been observed with the *C. vinosum* synthase (**23**).

The function of D480, while important based on its low catalytic activity, still remains to be elucidated. In *C. vinosum* synthase, the corresponding mutant D302N, when reacted with sT-CoA, exhibited a “burst” phase of 0.5 equiv of CoA per PhaE/PhaC, followed by no detectable release of CoA for up to 2 h (**23**). The rapid acylation by the sT-CoA suggested that this Asp does not play an essential role in the acylation process. These observations elicited the proposal that this residue could function as a general base catalyst to deprotonate the 3-hydroxyl group of HBCoA. The slow release of saturated trimer acid from the active site Cys

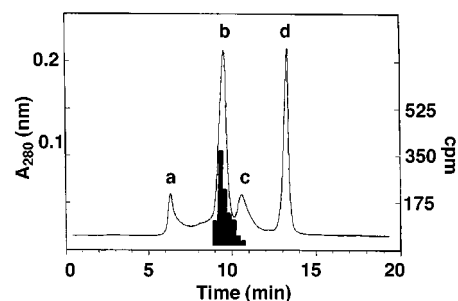


FIGURE 3: Bio-Silex SEC250 size exclusion chromatography analysis of the interaction of *R. eutropha* PHB synthase with [^3H]sT-CoA. Peak a is protein aggregate, peaks b and c are synthase dimer and monomer, respectively, and peak d is [^3H]sT-CoA. Black bars indicate the radioactivity. Note, only the protein-containing fractions were counted for radioactivity.

in the wt synthase was proposed to result from the activation of water for nucleophilic attack by this Asp. In a similar experiment performed with the D480N synthase of the *R. eutropha* enzyme, the initial release of CoA was very slow and biphasic (Figure 1 and Table 2). Extrapolation of the slow phase to zero time shows that ~ 0.4 equiv of CoA is released in the first phase. However, the relative rate of deacylation to acylation (1:4, Table 2) suggests that ~ 0.5 equiv of CoA release per monomer of synthase occurred. The stoichiometry of the D480N mutant is similar to that observed with the wt synthase, but the rate of acylation is much slower (Table 2). Radiolabeling experiments with [^3H]sT-CoA and D480N synthase (incubated for 50 min) gave ~ 0.5 equiv of label per monomeric subunit (data not shown). Thus, while the kinetics contrast with those observed with the *C. vinosum* synthase, the stoichiometries are similar. The function of this residue needs further investigation.

*Is the Dimer the Active Form of the *R. eutropha* Synthase in Elongation?* We have previously shown that the *R. eutropha* PHB synthase under conditions of size exclusion chromatography exists as monomer in equilibrium with dimer (**14, 15**). Acylation of the synthase with sT-CoA resulted in a shift from the monomeric to the dimeric form of the enzyme, a reduction of the lag phase observed in the CoA release assay, and an increase in the observed specific activity of the synthase (**15**). These observations led us to propose that the dimer is the active form of the enzyme. To further test this hypothesis, synthase was incubated with [^3H]sT-CoA and the reaction mixture examined by size exclusion chromatography at 4 °C. As shown in Figure 3, the majority of the counts are associated with the dimeric form of the protein, and the label corresponds to about 0.6 equiv of [^3H]sT per equivalent of monomer. A similar experiment carried out under identical conditions with the C319S synthase showed no shift in the monomer/dimer equilibrium and no radioactivity associated with the protein (data not shown). One possible interpretation of these results is that the active form of the enzyme for elongation is the dimer with each subunit providing a Cys, C319, that plays a role in catalysis. With this batch of (His) $_6$ -tagged wt synthase, the size exclusion chromatography analyses reveal a large molecular weight aggregate (a, Figure 3). There is no activity associated with this aggregate, nor is any radiolabel attached. Once the aggregate is removed, no additional aggregate is generated (data not shown), indicating it is not in equilibrium with the active synthase.

DISCUSSION

The primary and quaternary structures of the Class I and III PHB synthases are distinct. Furthermore, the kinetics of the polymerization processes are also distinct. Both enzymes catalyze biphasic release of CoA: the *R. eutropha* enzyme exhibits a lag phase, while the *C. vinosum* enzyme exhibits a burst phase. In the present paper, we address the mechanistic and structural relationships between the Class I and Class III synthases.

We have recently shown that the *C. vinosum* enzyme is a member of the α/β hydrolase superfamily of proteins and is structurally homologous to bacterial lipases (23). Sequence alignments, the availability of structures of lipases, and threading models allowed us to conclude that the Class III enzyme exhibits an α/β structure with an elbow loop between a β strand and an α helix which contains the Cys involved in covalent catalysis. Initial efforts to obtain a model of the *R. eutropha* synthase using the SWISS-MODEL facility at EXPASY server (<http://www.expasy.ch>) have been unsuccessful. Sequence alignments between the Class I and III enzymes reveal that the N-terminal 150 amino acids of the Class I enzymes share no sequence in common with the Class III enzymes. We therefore focused our attention on the C-terminal ~450 amino acids of the Class I enzymes. Sequence alignments between the Class III and truncated Class I enzymes reveal only 6.7% sequence identity. The program of UCLA-DOE Fold Recognition Server (<http://fold.doe-mbi.ucla.edu>), using the sequence of the truncated *R. eutropha* synthase as the probe, suggests that this protein will contain α/β structure, with the bacterial lipases and a TIM barrel aldolase giving the highest scores. Thus, with the available information, the argument that the Class I enzymes are structurally homologous to lipases and are members of the α/β hydrolase superfamily is much less compelling than that for the Class III enzymes.

The structural homology of the Class III synthases with the lipases and site-directed mutagenesis studies were very informative. We have now used the sequence alignments between the Class I and III enzymes to compare and contrast the results of similar mutagenesis studies. The results provide strong support that both classes of enzymes possess a Cys-His catalytic dyad and that the Cys is involved in covalent catalysis. The sT-CoA analogue has been a useful probe, establishing the importance of C319 (15). The inability of C319 to be acylated when H508 is changed to a Gln strongly supports our proposal that H508 functions as a general base catalyst to activate C319 for nucleophilic attack on HBCoA. The stoichiometry of acylation of C319 by sT-CoA, 0.5 equiv/equiv of monomer, is also observed with the Class III synthases, despite the differences in their quaternary structures. We suggest from these results that a dimeric form of the synthases will be important in their catalytic function. In the case of both enzyme systems, the active site Cys acylated with sT-CoA undergoes slow hydrolysis, generating the sT-acid. While the stabilities of the acylated enzymes are different, in both cases the hydrolysis is very slow.

The third mutation generated was D480N. As we noted previously, lipases have been proposed to require a catalytic triad including an active site nucleophile, Ser, a His, and an Asp (26–28). The role of the Asp in catalysis is unclear from published data (28–30). The requirement for such a

residue in ester hydrolysis, in contrast with amide hydrolysis, remains to be established. The Asp mutant in the *R. eutropha* enzyme behaves qualitatively in a fashion similar to that in the *C. vinosum* enzyme. The synthase becomes acylated with sT-CoA to ~0.5 equiv per monomer, suggesting that this residue is not playing an essential role in activating C319 for acylation. In the case of the *C. vinosum* enzyme, no deacylation is detectable with the D302N mutant (23). In the case of the *R. eutropha* synthase, deacylation occurs, but at a very slow rate. Whether this Asp can actually function as the general base catalyst to deprotonate the hydroxyl group of HBCoA in either enzyme remains to be investigated further.

The studies of the *R. eutropha* site-directed mutants with HBCoA and sT-CoA support the proposal of mechanistic congruity between the two classes of synthases. We further propose that these proteins will be structurally homologous. The evidence in support of this proposal is the predicted α/β structure, the sequence comparisons, and the mutagenesis results.

Recently, Zhang et al. (17) claimed to have demonstrated that dimer is the active form of the *R. eutropha* PHB synthase. However, data in Figure 5 of their paper contradict their conclusion. Defining the active form of the protein requires a knowledge of the subunit equilibrium and the rate constants for association of the monomers and dissociation of the dimer. These numbers can be determined experimentally, and studies with a 1:1 mixture of the wt enzyme and the (His)₆-tagged C319A synthase should define the importance of the dimer in catalysis, specially in elongation, as suggested by the studies with the sT-CoA.

ACKNOWLEDGMENT

We thank Dr. Gregory York for helpful discussions.

REFERENCES

- Anderson, A. J., and Dawes, E. A. (1990) *Microbial. Rev.* 54, 450–472.
- Steinbüchel, A. (1991) in *Biomaterials. Novel Materials and Biological Sources* (Byrom, D., Ed.) pp 123–213, Macmillan Publishers Ltd., Basingstoke, U.K.
- Rehm, B. H., and Steinbüchel, A. (1999) *Int. J. Biol. Macromol.* 25, 3–19.
- Slater, S., Mitsky, T. A., Houmiel, K. L., Hao, M., Reiser, S. E., Taylor, N. B., Tran, M., Valentin, H. E., Rodriguez, D. J., Stone, D. A., Padgett, S. R., Kishore, G., and Gruys, K. J. (1999) *Nat. Biotechnol.* 17, 1011–1016.
- Müller, H.-M., and Seebach, D. (1993) *Angew. Chem., Int. Ed. Engl.* 32, 477–502.
- Gerngross, T. U. (1999) *Nat. Biotechnol.* 17, 541–544.
- Holmes, P. A. (1985) *Phys. Technol.* 16, 32–36.
- Byrom, D. (1987) *Trends Biotechnol.* 5, 246–250.
- Williams, S. F., Martin, D. P., Horowitz, D. M., and Peoples, O. P. (1999) *Int. J. Biol. Macromol.* 25, 111–121.
- Sodian, R., Hoerstrup, S. P., Sperling, J. S., Martin, D. P., Daebritz, S., Mayer, J. E., Jr., and Vacanti, J. P. (2000) *ASAIO J.* 46, 107–110.
- Schubert, P., Steinbüchel, A., and Schlegel, H. G. (1988) *J. Bacteriol.* 170, 5837–5847.
- Slater, S. C., Voige, W. H., and Dennis, D. E. (1988) *J. Bacteriol.* 170, 4431–4436.
- Peoples, O. P., and Sinskey, A. J. (1989) *J. Biol. Chem.* 264, 15298–15303.
- Gerngross, T. U., Snell, K. D., Peoples, O. P., Sinskey, A. J., Cushai, E., Masamune, S., and Stubbe, J. (1994) *Biochemistry* 33, 9311–9320.

15. Wodzinska, J., Snell, K. D., Rhomberg, A., Sinskey, A. J., Biemann, K., and Stubbe, J. (1996) *J. Am. Chem. Soc.* **118**, 6319–6320.
16. Su, L., Lenz, R. W., Takagi, Y., Zhang, S., Goodwin, S., Zhong, L., and Martin, D. (2000) *Macromolecules* **33**, 229–231.
17. Zhang, S., Yasuo, T., Lenz, R. W., and Goodwin, S. (2000) *Biomacromolecules* **1**, 244–251.
18. Hoppensack, A., Rehm, B. H., and Steinbüchel, A. (1999) *J. Bacteriol.* **181**, 1429–1435.
19. Williams, M. D., Fieno, A. M., Grant, R. A., and Sherman, D. H. (1996) *Protein Expression Purif.* **7**, 203–211.
20. Steinbüchel, A., Hustede, M., Liebergesell, M., Pieper, U., Timm, A., and Valentin, H. (1992) *FEMS Microbiol. Rev.* **103**, 217–230.
21. Liebergesell, M., and Steinbüchel, A. (1992) *Eur. J. Biochem.* **209**, 135–150.
22. Müh, U., Sinskey, A. J., Kirby, D. P., Lane, W. S., and Stubbe, J. (1999) *Biochemistry* **38**, 826–837.
23. Jia, Y., Kappock, T. J., Frick, T., Sinskey, A. J., and Stubbe, J. (2000) *Biochemistry* **39**, 3927–3936.
24. Griebel, R. J., and Merrick, J. M. (1971) *J. Bacteriol.* **108**, 782–789.
25. Ballard, D. G., Holmes, P. A., and Senior, P. J. (1987) in *Recent Advances in Mechanistic and Synthetic Aspects of Polymerization* (Fontanille, M., and Guyot, A., Eds.) pp 293–314, D. Reidel Publishing Co., Lancaster, U.K.
26. Karlsson, M., Contreras, J. A., Hellman, U., Tornqvist, H., and Holm, C. (1997) *J. Biol. Chem.* **272**, 27218–27223.
27. Brumlik, M. J., and Buckley, J. T. (1996) *J. Bacteriol.* **178**, 2060–2064.
28. Frenken, L. G., Egmond, M. R., Batenburg, A. M., Bos, J. W., Visser, C., and Verrips, C. T. (1992) *Appl. Environ. Microbiol.* **58**, 3787–3791.
29. Kingma, R. L., Fragiathaki, M., Snijder, H. J., Dijkstra, B. W., Verheij, H. M., Dekker, N., and Egmond, M. R. (2000) *Biochemistry* **39**, 10017–10022.
30. Lang, D., Hofmann, B., Haalck, L., Hecht, H. J., Spener, F., Schmid, R. D., and Schomburg, D. (1996) *J. Mol. Biol.* **259**, 704–717.
31. Nachmansohn, D., and Wilson, I. B. (1955) *Methods Enzymol.* **1**, 619–624.
32. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77**, 51–59.
33. Higuchi, R. (1990) in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds.) pp 177–183, Academic Press, San Diego, CA.
34. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
35. Narisada, M., Horibe, I., Watanabe, F., and Takeda, K. (1989) *J. Org. Chem.* **54**, 5308–5313.
36. Lai, M., Liu, L., and Liu, H. (1991) *J. Am. Chem. Soc.* **113**, 7388–7397.

BI002219W