Lipases Provide a New Mechanistic Model for Polyhydroxybutyrate (PHB) Synthases: Characterization of the Functional Residues in *Chromatium vinosum* PHB Synthase†

Yong Jia,‡ T. Joseph Kappock,‡ Tobias Frick,‡ Anthony J. Sinskey,§ and JoAnne Stubbe*,‡,§

Departments of Chemistry and Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139

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ABSTRACT: Polyhydroxybutyrate (PHB) synthases catalyze the conversion of β-hydroxybutyryl coenzyme A (HBCoA) to PHB. These enzymes require an active site cysteine nucleophile for covalent catalysis. A protein BLASTp search using the Class III *Chromatium vinosum* synthase sequence reveals high homology to prokaryotic lipases whose crystal structures are known. The homology is very convincing in the α-β-elbow (with the active site nucleophile)-α-β structure, residues 131−175 of the synthase. A conserved histidine of the Class III PHB synthases aligns with the active site histidine of the lipases using the ClustalW algorithm. This is intriguing as this histidine is approximately 200 amino acids removed in sequence space from the catalytic nucleophile. Different threading algorithms suggest that the Class III synthases belong to the α/β hydrolase superfamily which includes prokaryotic lipases. Mutagenesis studies were carried out on *C. vinosum* synthase C149, H331, H303, D302, and C130 residues. These studies reveal that H331 is the general base catalyst that activates the nucleophile, C149, for covalent catalysis. The model indicates that C130 is not involved in catalysis as previously proposed [Müh, U., Sinskey, A. J., Kirby, D. P., Lane, W. S., and Stubbe, J. (1999) *Biochemistry 38*, 826−837]. Studies with D302 mutants suggest D302 functions as a general base catalyst in activation of the 3-hydroxyl of HBCoA (or a hydroxybutyrate acyl enzyme) for nucleophilic attack on the covalently linked thiol ester intermediate. The relationship of the lipase model to previous models based on fatty acid synthases is discussed.

Polyhydroxyalkanoates (PHAs,[1] Figure 1) are polyoxoesters produced intracellularly by a wide variety of bacteria for the purpose of carbon and energy storage when the bacteria find themselves in limiting nutrient environments (*1*, *2*). The copolymers derived from 3-hydroxybutyrate (HB) and 3-hydroxyvalerates (HV) (Figure 1, R = CH_3 (PHB) and R = CH_3CH_2 (PHV)) are interesting because they possess properties of thermoplastics which are suitable for certain applications and are biodegradable (*3*, *4*). These properties have prompted interest in commercial production of these copolymers (*4*, *5*).[2] At present, the possibility that these PHAs could replace some of the widely used polypropylenes for use in plastic materials is being debated (*3*). A key issue is whether the PHAs can be produced in an economically competitive fashion (*6*−*8*). An understanding of the mechanism by which these biodegradable polymers are formed, the factors that determine their chain length and polydispersity, and the basis of their specificities will play an important role in determining the feasibility of these materials for industrial use. In the present paper we report that the *Chromatium vinosum* PHA synthase is a member of the prokaryotic lipase superfamily which in turn is a member of the α/β hydrolase family (*9*, *10*). A new

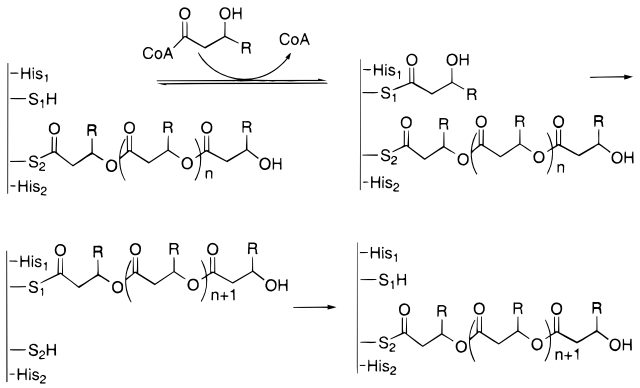

FIGURE 1: Proposed mechanistic model for *C. vinosum* PHA synthase based on fatty acid synthases (*19*). Two thiol groups are postulated to participate in covalent catalysis. S_1H is the loading site, and S_2H is the priming and elongation site. S_1H and S_2H were previously proposed to be C149 and C130 (*18*).

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* To whom correspondence should be addressed: telephone (617) 253-1814, fax (617) 258-7247, e-mail stubbe@mit.edu.

‡ Department of Chemistry.

§ Department of Biology.

[1] Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); FAS, fatty acid synthase; HB, 3-hydroxybutyrate; HBCoA, 3-hydroxybutyryl CoA, HV, 3-hydroxyvalerate; HVCoA, 3-hydroxyvaleryl CoA; PCR, polymerase chain reaction; PDB, protein data bank; PHA, polyhydroxyalkanoate acid; PhaEC, PhaE and PhaC coexpressed and copurified; PHB, polyhydroxybutyrate; PHV, polyhydroxyvalerate; sT, a trimer of 3-hydroxybutyrate in which the terminal hydroxyl is replaced with a hydrogen; sT-CoA, saturated trimer-CoA; wt, wild-type; UD, undetectable.

[2] Holmes, P. A., Collins, S. H., and Wright, F. (1984) 3-Hydroxybutyrate Polymers, U.S. Patent 4,477,654.

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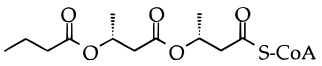
mechanistic model is presented to accommodate site-directed mutagenesis studies and our recent mechanistic studies based on the structural and functional homologies to the lipases.

The PHA synthases have been classified based on their molecular weights and the side chain (R) of their substrates (Figure 1) (*11*). The Class I and III enzymes possess many common features as well as many important differences. In common, both classes of enzymes use β-hydroxybutyryl coenzyme A (HBCoA) or β-hydroxyvaleryl coenzyme A (HV-CoA) as substrates. Both classes use the same biosynthetic pathway involving a thiolase (*phaA*), a reductase (*phaB*), and the synthase (*phaC*) (*12*−*16*). Both classes require a cysteine that is essential for covalent catalysis (*17*, *18*).

In contrast, the subunits and native molecular weights of the Class I and Class III PHA synthases and the kinetics of polymer formation are distinct. The best studied Class I enzyme, from *Ralstonia eutropha*, has a subunit molecular weight of 64 KDa and exists as a monomer in equilibrium with its dimer in solution. The best studied Class III enzyme, from *C. vinosum*, is composed of two different gene products, PhaE and PhaC, each with a subunit molecular weight of 40 KDa. PhaC has recently been shown to be the synthase and forms a 1:1 complex with PhaE (*18*). This 80 KDa complex further aggregates to form trimers and hexamers in solution.[3] The kinetics of the polymerization process in the Class I and III PHA synthases are distinct (*17*, *18*). While both show biphasic kinetics of coenzyme A (CoA) release, with the Class I enzymes there is a lag phase followed by a more rapid linear phase, while with the Class III enzymes there is a rapid phase, followed by a slower phase. Studies reported herein suggest a common mechanism for these two classes of PHA synthases, despite the apparent structural and kinetic differences.

The mechanism of fatty acid synthases (FASs) (*19*) has been used to develop a hypothesis by which PHA synthases might function (Figure 1; (*20*−*23*)) In this model, two thiol groups are proposed to play key roles in covalent catalysis. One thiol group (S_1H) serves as the loading site for HBCoA, and the second thiol group (S_2H) serves as the priming and elongation site. As noted above, in the case of both the *C. vinosum* and the *R. eutropha* PHA synthases, a cysteine in similar sequence context (C319 of *R. eutropha* PHA synthase and C149 of *C. vinosum* PHA synthase) has been demonstrated to be involved in covalent catalysis (*17*, *18*). These cysteines were identified using a terminally saturated CoA analogue of a trimer of hydroxybutyrate (HB) units (sT-CoA, **1**, Chart 1) as a probe of the priming process. Sequence

Chart 1



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alignments of all PHA synthases further indicate that this is the only conserved cysteine residue among all classes of PHA synthases. In FASs a second thiolate is generated by posttranslational modification by pantetheinylation with coenzyme A (CoA) using a phosphopantetheinyl transferase (*24*). However, recent studies from our labs (K. Snell, U. Müh, A. J. Sinskey, and J. Stubbe, unpublished data) and those from Steinbüchel's lab (*25*) suggest that this is not the case for either Class I or III PHA synthase. Thus if the postulated mechanism (Figure 1) is valid, each monomeric unit must contribute one cysteine. The active form of the PHA synthase would be a dimer with its active site at the interface of two monomers.

The present paper presents a mechanistic study of the class III PHA synthase from *C. vinosum*. Evidence suggests that the *C. vinosum* PHA synthase is a member of the lipase superfamily and not a β-ketoacyl acyl carrier protein (ACP) synthase such as that involved in FAS (*26*). Evidence presented suggests that PhaC and the prokaryotic lipases share a similar fold in their catalytic domain containing the active site nucleophile and perhaps throughout the entire sequence. Lipases contain a catalytic triad of a serine, histidine, and aspartate at their active site (*27*−*29*). The serine is involved in covalent catalysis, and the histidine activates the serine for nucleophilic attack. The function of aspartate is open to debate. Furthermore, lipases bind to membranes (*30*, *31*) in a fashion reminiscent of PHB synthase's interaction with granules inside cells (*32*, *33*). Prokaryotic lipases are thus proposed to provide a structural and functional model for studies of *C. vinosum* PHA synthase (*34*). Site-directed mutagenesis studies on PhaC indicate that H331 forms a catalytic dyad with C149, activating it toward nucleophilic attack and covalent catalysis. We have further demonstrated, in contrast to our initial reports (*18*), that C130 does not provide the second thiol group involved in catalysis. In addition, studies with sT-CoA (**1**) provide the first evidence that *C. vinosum* PHA synthase may possess a functional dimer as previously suggested for the Class I *R. eutropha* synthase.

MATERIALS AND METHODS

Materials. The expression of plasmid pET-UM4, carrying the *C. vinosum* PHA synthase gene, *phaEC*, under a T7 promoter, has been reported (*18*). Syntheses of HBCoA, sT-CoA, and [^{3}H]sT-CoA[4] have been described (*18*). DNA sequencing and amino acid analysis were carried out by the MIT Biopolymers Laboratory. Oligonucleotides were purchased from BRL Life Technology. DNA modification enzymes and restriction enzymes were purchased from New England Biolabs.

Construction of PhaC Site-Directed Mutants. Site-directed mutagenesis was carried out using the four-primer PCR technique and protocol as described (*35*, *36*) using plasmid pET-UM4 (*18*) as a template. PCR was used to amplify DNA cassettes that include the target residues with two unique restriction sites on each end of the cassettes. Amplified DNA fragments containing the desired mutations were used to replace the original segment of pET-UM4, yielding plasmid pET-UM22(C149S), pET-YJ30 (H303Q), pET-YJ32 (H331Q), pET-YJ33 (D302A), pET-YJ36 (D302N), pET-YJ37 (C130S), and pET-YJ39 (C130A). The C149S/H331Q double mutant was constructed by placing a fragment containing the H331Q mutation from plasmid pET-YJ32 into plasmid pET-UM22, yielding plasmid pET-YJ38 (C149S/H331Q). Mutant plasmid DNA isolated from JM109 *Escherichia coli* host cells was sequenced to confirm that all the mutations were as expected. Mutants were introduced using the primers listed in Table 1.

[3] Jia, Y., and Stubbe, J. unpublished data.

[4] Yuan, W., and Stubbe, J. (1999) manuscript in preparation.

Table 1: Primers Used in Mutagenesis Studies

mutant	forward primer with mutation site underlined
C149S	5′-CAATCTGCTCGGTATCTCTCAGGGCGGCGCCTTC-3′
H303Q	5′-CAGGATCAGCTGGTGCCGCC-3′
H331Q	5′-CGGCGGTCAGATCGGCATCTA-3′
D302A	5′-CTTCGCGCTCCAGGCTCACC-3′
D302N	5′-CGCGCTCCAGAATCACCTG-3′
C130S	5′-CATCGACCGCTCAGTGGACTATC-3′
C130A	5′-CATCGACCGCGCCGTGGACTATC-3′

Expression and Purification of C. vinosum PHA Synthase and Mutants. PHA synthase was overexpressed using the expression vector that contains the genes for both *phaC* and *phaE* transformed into *E. coli* BL21(DE3) cells (Novagen). Typically, 6 L of culture gave ~25 g of cells. All of the mutants were purified using the procedures previously reported for the wt protein (*18*). The proteins were judged to be homogeneous by SDS−PAGE (*37*). All the mutants isolated are comprised of a 1:1 mixture of mutated PhaC and wt PhaE. Typically 35 mg of protein were isolated, and the proteins were stored at −80 °C.

Determination of the Extinction Coefficient for C. vinosum PHA Synthase. The A_{280} nm of purified PHA synthase in doubly distilled H_2O was determined. A portion of the same sample was sent to the MIT Biopolymers Laboratory, and quantitative amino acid analysis showed that the protein concentration was 3 pmol/mL. From this information PhaEC ϵ_{280} was calculated to be 138 500 M^{-1} cm^{-1}.

The ϵ_{280} for PhaEC was also calculated by the method of Lohman and Mascotti (*38*) using eq 1

$$\epsilon_{\text{Native}} = A_{\text{Native}}\epsilon_{\text{GuHCl}}/A_{\text{GuHCl}} \quad (1)$$

in which ϵ_{Native} and A_{Native} are the native protein extinction coefficient and A_{280} nm, respectively; while $\epsilon_{\text{Gu-HCl}}$ and $A_{\text{Gu-HCl}}$ are the denatured protein extinction coefficient (in 6 M Gu-HCl) and A_{280} nm, respectively. The calculations gave an ϵ_{Native} of 134 249 M^{-1} cm^{-1}, comparable with the experimentally determined value. An ϵ_{280} of 138 500 M^{-1} cm^{-1} for PhaEC is used in all of our calculations.

Enzyme Assay. All assays were carried out at 30 °C in a final volume of 300 μL containing: 20 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM HBCoA; and enzyme (19 nM wt PHA synthase, 127 nM C149S, 21 nM H303Q, 11.4 μM H331Q, 10.6 μM C149S/H331Q, 8.9 μM D302A, 840 nM D302N, 29 nM C130S, or 41 nM C130A). At defined time points, 20 μL aliquots were removed from the reaction mixture and quenched with 50 μL of 10% trichloroacetic acid. After brief centrifugation to remove the precipitated protein, 68 μL of the quenched reaction mixture was added to 282 μL of 0.25 mM DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) in 0.5 M potassium phosphate, pH 7.8, and A_{412} was measured. One unit is defined as 1 μmol of substrate consumed per minute (detection limit $\geq 1 \times 10^{-3}$ U/mg).

3-Hydroxybutyrate (HB) Assay. The concentration of 3-hydroxybutyric acid (HB) was determined using the previously described HB dehydrogenase/hydrazine assay (*39*). The dehydrogenase oxidizes HB to acetoacetate with concomitant reduction of NAD, resulting in a change in A_{340}.

Kinetics of PHA Synthase Reaction with sT-CoA. All reaction mixtures were contained in a final volume of 150 μL: 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and wt or mutant PhaEC and sT-CoA in a molar ratio of 1:10. The protein and sT-CoA concentrations were 34.6 μM wt PhaC and 406 μM sT-CoA; 28.4 μM D302A PhaC and 364 μM sT-CoA; 34.3 μM D302N PhaC and 343 μM sT-CoA; and 16.9 μM H331Q PhaC and 170 μM sT-CoA. The reaction was initiated by the addition of enzyme and carried out at 30 °C. At designated time points, an aliquot of the reaction mixture was removed and assayed for CoA release by the discontinuous assay method described above.

Labeling of wt and D302N Mutant with [^{3}H]sT-CoA. PHA synthase (wt, 54 μM; D302N mutant, 51.4 μM) was incubated with 220 μM [^{3}H]sT-CoA (4.97×10^5 cpm/μmol) in 20 mM Tris-HCl, pH 7.5; 50 mM NaCl in a final volume of 50 μL. The [^{3}H]sT-CoA was purified directly before use. The reaction was allowed to proceed at 30 °C for 5 min. The reaction product was loaded onto a Nanosep 10K filter system (Pall Filtron) and centrifuged at 14 000 rpm for 4 min in a benchtop centrifuge. The filtrate was collected. To remove radiolabel not bound to PhaC, the protein fraction was washed with additional buffer (100 μL of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl) and the centrifugation step was repeated until no radioactivity was detected in the filtrate. At this point, the amount of radioactivity attached to the protein was determined by scintillation counting.

Sequence Homology Search and Protein Modeling. The *C. vinosum* PhaC amino acid sequence was used as the entry to search the protein database using the basic BLAST Sequence Similarity Searching program from NCBI (*40*). Sequence alignments were performed using ClustalW (version 1.7) (*41*) from the BCM (Baylor College of Medicine) Search Launcher, and output was formatted with the ESPript program (version 1.4; Gouet, P., Courcelle, E., Stuart, D., and Metoz, F., unpublished program). The *C. vinosum* PhaC sequence was threaded onto bacterial lipase structures using BLAST alignments and the SWISS-MODEL facility (*42*, *43*) at the EXPASY server (http://www.expasy.ch).

A second threading model was constructed using ClustalW alignments and the Swiss PDB Viewer (*42*) version 3.5 for the SGI. Gaps were inserted into all six sequences where required by the alignment (Figure 2). The edited sequence alignment, together with the five structural templates (*44*−*46*),[5] was then submitted to SWISS-Model for refinement. DSSP (*47*) was used to make secondary structure assignments in the threading model.

RESULTS

PHA Synthases and Lipases Belong to the Same Enzyme Superfamily. In 1992 the Steinbüchel lab noted that all PhaCs

[5] Lang, D. A., Stadler, P., Kovacs, A., Paltauf, B. W., and Dijkstra, B. W., unpublished data.

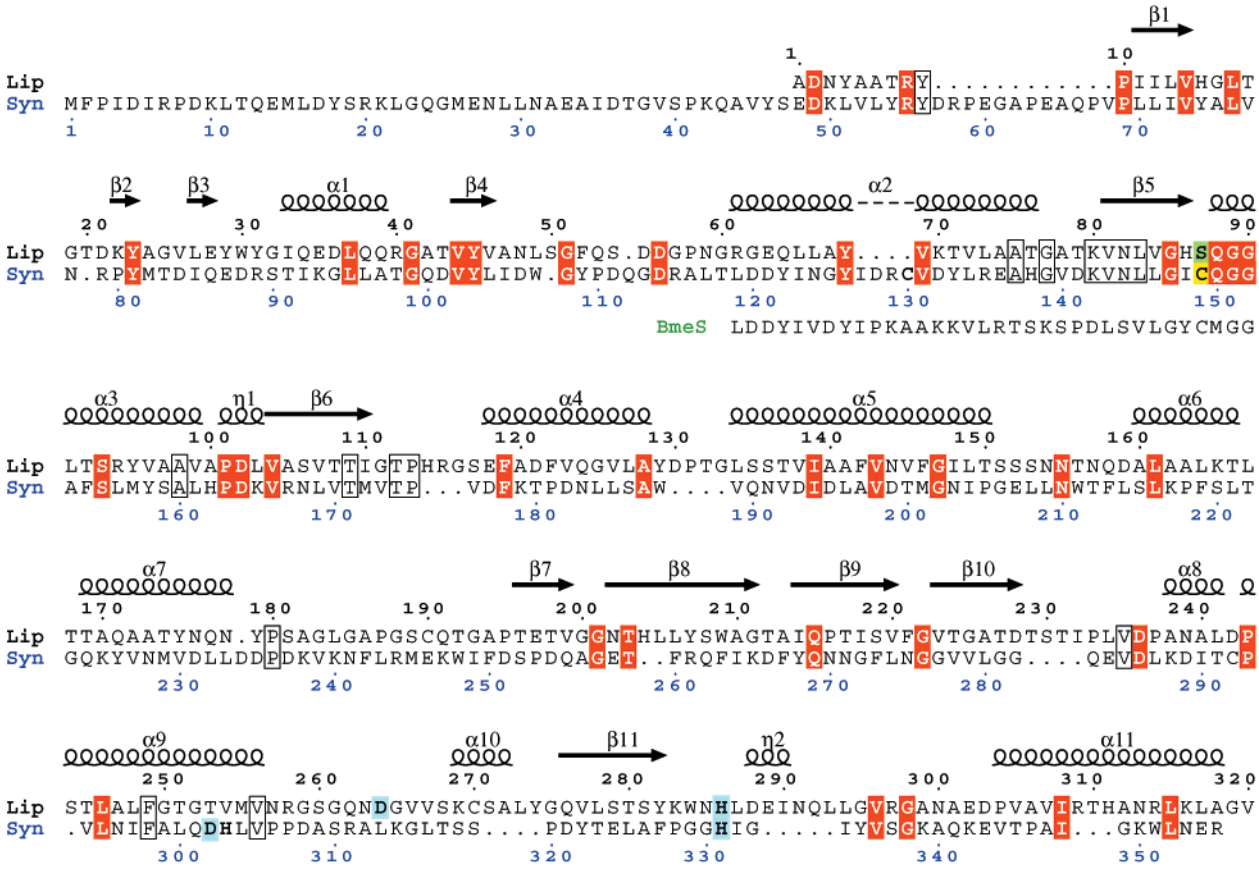

FIGURE 2: ClustalW sequence alignment of Class III PHA synthases and three *Pseudomonas* lipases. The sequences used for the alignment calculation are lipases from *Pseudomonas cepacia* (accession no. 1942114), *Pseudomonas sp. KWI-56* (accession no. P25275), and *Pseudomonas luteola* (accession no. AAC05510); PHA synthases from *Chromatium vinosum* (accession no. L01112), *Thiocystis violacea* (accession no. L01113), and *Synechocystis sp.* (accession no. BAA17430). The sequences shown are *P. cepacia* (Lip), *C. vinosum* PHA synthase (Syn), and a portion of the core region sequence of the putative Class III PHA synthase from *Bacillus megaterium* (BmeS; accession no. AF109909; not included in the sequence alignment calculation). Strictly conserved residues in all six sequences are in red boxes. Strictly conserved residues between *P. cepacia* lipase and *C. vinosum* PHA synthase are in open boxes. The *P. cepacia* lipase catalytic triad residues (S87-H286-D264) and the proposed *C. vinosum* PHA synthase dyad residues (C149 and H331) and D302 are in colored boxes. Residue C130 in *C. vinosum* PHA synthase sequence is in bold. The secondary structure elements of *P. cepacia* lipase (PDB identifier 5LIP) are shown above the alignment.

contain a lipase box (G-X-[S/C]-X-G) in which the essential active site serine of lipase is replaced with a cysteine in PhaC (*15*). At the time of this report the functional implications of this homology remained unknown. Recently, however, we have determined that C149 of the *C. vinosum* PhaC is essential for covalent catalysis (*18*). Furthermore, a BLAST sequence homology search using the *C. vinosum* PhaC protein sequence revealed that this cysteine aligns with the essential serine in bacterial lipases from *Pseudomonas cepacia* (*44*), *Pseudomonas sp. KWI-56* (*48*), and *Pseudomonas luteola* (Litthauer, D., Ginster, A., and Skein, E., direct submission). These alignments revealed that in a span of 45 residues (131−175) in the PhaC sequence without sequence gaps there is 42% sequence identity between *C. vinosum* PhaC and *P. cepacia* lipase. This region includes the lipase box. The *P. cepacia* lipase has been characterized crystallographically (*44*, *45*), and hence it is likely that PhaC will possess a similar structure in this region.

To determine if there are greater similarities than apparent from the BLAST search, ClustalW was used to align the three full-length lipase sequences with Class III PhaCs from *C. vinosum*, *Thiocystis violacea* (*49*), and *Synechocystis sp.* (*50*, *51*). Figure 2 presents a comparison of the *P. cepacia* lipase and *C. vinosum* PhaC sequences shown below the secondary structure elements derived from the *P. cepacia* lipase crystal structure. Highlighted in red are the conserved residues from all six sequences. The boxed residues are conserved between the two indicated proteins. As found by the BLAST search, the active site nucleophile of the lipases, S87 (colored green in Figure 2), which is within the lipase box, aligns to the modified lipase box in PhaC where the key serine is replaced by a cysteine (C149 for *C. vinosum* PHA synthase, colored yellow in Figure 2). The ClustalW alignment gives 19% sequence identity and requires the insertion of several gaps between residues 49 and 355 of *C. vinosum* PhaC. The alignment between the C-termini of these proteins is less compelling than the lipase box region. However, it is interesting that H286 (blue, Figure 2), an essential member of the catalytic triad of lipases, aligns with H331, conserved among all PHA synthases.

Structural Model of PHA Synthase. Structures of lipases have been determined in closed and open conformations (*44*−*46*, *52*, *53*). In the open, enzymatically active form, the structures have an inhibitor covalently bound to the active site serine (Figure 3). Many open-form *Pseudomonas* lipase structures are available from the PDB. Using five structures (*44*−*46*)[5] and the BLAST and ClustalW alignments as input for the SWISS-MODEL protein threading algorithm (*42*),

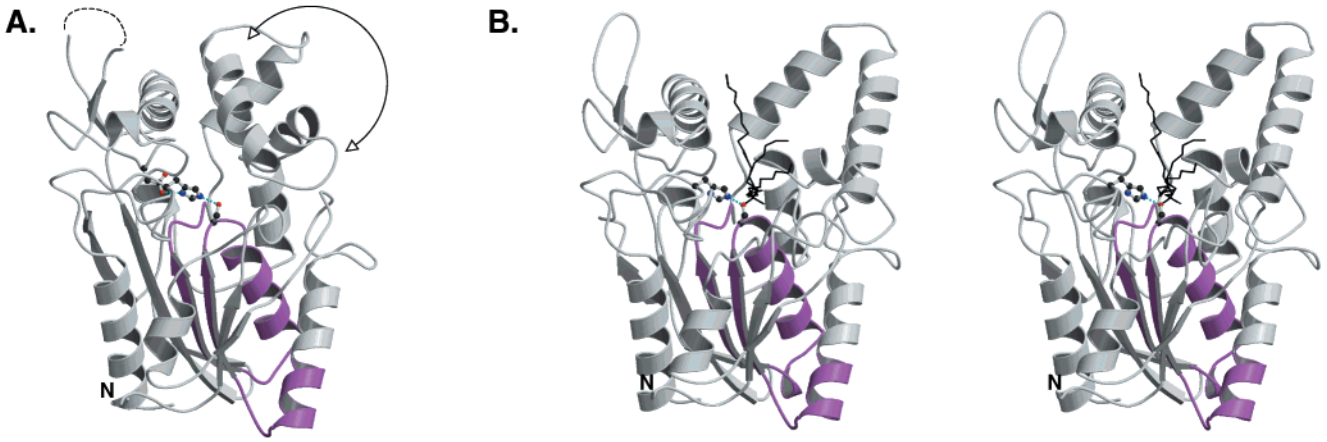

FIGURE 3: Lipase open and closed forms and homology with Class III PHA synthases. (A) Inactive, closed form of *Pseudomonas* lipase (PDB id 1CVL; (*53*)). The catalytic triad residues (Ser-His-Asp) are shown in ball-and-stick representations. S87 is at the center, located below a helix that moves out of the active site pocket when substrate is bound (motion is indicated by the arrow). The region showing high sequence homology between lipases and Class III PHA synthases is in purple. (B) Stereoview of the active, open form of *Pseudomonas* lipase (PDB id 5LIP, (*45*)). The complexed phosphonate lipid substrate analogue [R_C-(R_P,S_P)-1,2-dioctylcarbamoylglycero-3-*O*-octylphosphonate] is depicted in thick black lines. Two of the catalytic triad residues (H286 and S87) are shown in ball-and-stick representations. The region showing high sequence homology between lipases and Class III PHA synthases is in purple. The structure in panel B is presented in the same orientation as in panel A based upon a DALI alignment (*80*) of the two structures. This figure and Figure 4 were prepared using Molscript (*81*) and Raster3D (*82*).

we have attempted to construct structural models for PhaC. Both the BLAST and ClustalW inputs generated excellent models between residues 131 to 175 of PhaC (purple in Figure 3A,B), corresponding to α2-β5-α3-β6 in *P. cepacia* lipase. This region includes S87 of lipase, located on an elbow between β5 and α3. The results strongly suggest that PhaC will have a similar fold in its active site with C149 located on an elbow between a β stand and an α helix.

A full-length threading model (residues 49−355) was also generated using the ClustalW alignments, Swiss PDB viewer and SWISS-MODEL, as described in Materials and Methods. The resulting model suggests that PhaC will be a member of the protein family possessing an α/β hydrolase fold as in the case of the prokaryotic lipases. Additionally, submission of the *C. vinosum* PhaC sequence to three other algorithms that search the structural databases (SAM-T98 (*54*), 3D-PSSM (*55*), and the UCLA Foldserver (*56*)) also gave fits to enzymes in the α/β hydrolase fold family with high confidence levels. Inspection of the resulting models in all four cases revealed that H331, D302, and H303 of PhaC are adjacent to the core structure (Figure 4). These residues are conserved in all PHA synthases and are thought to be important in catalysis.

Recently we proposed that C130 plays an important role in catalysis in the Class III PHA synthases (*18*). The position of this residue within the modeled structure, relative to the active site C149, was thus also of interest to determine. On the basis of our threading model, C130 is at the edge of helix α2 (Figure 4) and is likely on the outside of the protein, far removed from the active site C149. Our modeling thus suggests that C130 should not be involved in catalysis. A recent addition to the protein database further supports this conclusion. The *Bacillus megaterium* (*57*) Class III PhaC has an Ala residue in this position (Figure 2). These observations prompted a reinvestigation of this residue.

Characterization of C. vinosum PHA Synthase PhaC Point Mutants. The mechanistic and suggested structural similarities between PHA synthases and lipases have provided the basis for mutagenesis studies to understand catalysis. As

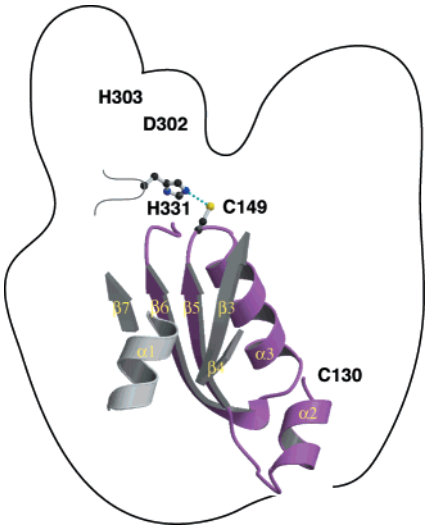

FIGURE 4: Schematic model for Class III PHA synthases. The threaded region showing highest homology to bacterial lipases is in purple. H331 and C149, the proposed catalytic dyad, are shown in ball-and-stick representations. Minimal α/β hydrolase secondary structure elements (*9*), including the core β sheet, are shown. The remainder of the ClustalW-based threading model is shown in outline, and the approximate positions of C130, D302, and H303 are indicated. This figure is presented in the same orientation as Figure 3.

noted above, the location of C130 seems incompatible with our previous proposal for its function (*18*), suggesting our experiments needed to be reexamined. In addition, the ClustalW alignment and similarities of PhaC to α/β hydrolase fold family proteins (*10*) suggested that H331 could be the general base catalyst required to generate the thiolate on the active site C149. Finally, the conservation and location of the conserved H303 and D302 residues, near a well-defined structural core containing C149 (Figure 4), suggested that the function of these residues might be elucidated by mutagenesis studies as well.

PhaC mutants C130A, C130S, C149S, H303Q, H331Q, C149S/H331Q, D302A, and D302N were constructed. All

Table 2: PHA Synthase Catalyzed Reaction Rates by wt Enzyme and Mutants

	initial phase		second phase	
PHA synthase	rate (U/mg)	% of wt	rate (U/mg)	% of wt
wild-type	140	100	34	100
C149A (*18*)	UD[a]		UD	
C149S	0.14	0.10	0.032	0.094
D302A	0.017	0.012	0.10	0.29
D302N	0.35	0.25	0.55	1.6
H303Q	71.0	51	24.8	73
H331Q	0.014	0.010	0.0058	0.017
C149S/H331Q	UD		UD	
C130A	125	89	13.2	39
C130S	170	121	5.5	16

[a] UD: undetectable.

mutants and wt enzymes were purified to apparent homogeneity, as judged by SDS−PAGE, with a yield ranging from 15 to 20%. During the purification processes, all PHA synthases showed similar chromatographic properties, consistent with there being no drastic changes in their protein folds. The activities of purified PHA synthases were assayed by measuring the release of CoA from HBCoA. The wt PHA synthase catalyzes CoA release in a biphasic fashion, with the initial phase being more rapid than the second, slow phase. The kinetic properties of these proteins, including the rates of the first phase and second phase relative to wt PHA synthase, are summarized in Table 2. The assay used detects only the consumption of the reactant; however, CoA release can result from both polymerization and a hydrolytic process. We previously established that the wt PHA synthase does not catalyze formation of HB (*18*). A similar assay using 3-hydroxybutyrate dehydrogenase revealed that none of the mutants catalyzed formation of HB, ruling out the possibility that the mutant PHA synthases catalyze an aberrant hydrolysis reaction.

C130 Is Not Involved in Catalysis. Our previous work suggested that C130 could potentially be the second thiol group involved in the catalytic mechanism shown in Figure 1. We reported that the C130A mutant exhibited very low activity (0.0028% of that of the wt enzyme) (*18*). At the time, this residue was also conserved among all Class III PHA synthases (*18*). The recent identification of a Class III PHA synthase from *B. megaterium* (*57*) that possesses an Ala in place of this Cys, and the likelihood from the structural model that C130 is not adjacent to C149, prompted us to reexamine this mutant. Resequencing of the C130A PhaC gene construct revealed an additional mutation at the essential C149. The C130A mutant was originally constructed in a low copy number plasmid which was determined to have only the desired mutation by complete sequencing. A fragment of this plasmid containing the C130A mutation was then subcloned into the expression vector pET-UM4 (*18*) and transformed into *E. coli* DH5α cells. During this process an undesired second mutation, C149S, arose. Thus, for reasons that remain unknown, our previously reported study of C130A PhaC was actually of a C130A/C149S double mutant. In the present study, we reconstructed the C130A mutant and constructed, in addition, a C130S mutant. As revealed in Table 2, both C130A and C130S mutants retained ≥90% activity. These results clearly demonstrate that C130 is not involved in catalysis. This observation is also consistent with our threading model (Figure 4).

H331 Is Essential for Catalysis. There are two conserved histidines in all PHA synthases: H303 and H331. One of these histidines is likely to function as the general base catalyst that deprotonates C149, the active site nucleophile involved in covalent catalysis. The ClustalW alignment (Figure 2) and structural modeling (Figure 4) suggested that H331 is the best candidate. To test this hypothesis, both histidines were mutated to Gln. As shown in Table 2, only the H331Q mutant exhibits a dramatically decreased rate of turn over, 0.014 U/mg or 0.01% of the wt enzyme activity. The H331Q protein is well-behaved in solution, suggesting that the mutation induces a catalytic defect, rather than a protein structure defect. This decreased activity is consistent with its postulated role in catalysis.

We had previously reported that the C149A mutation totally abolished PHA synthase activity (*18*), consistent with its role in catalysis. Given that lipases possess an active site serine, we generated the C149S mutation. While this mutant has greatly diminished activity, it still can turn over substrate at a rate of 0.1% that of the wt enzyme (Table 2). The double mutant H331Q/C149S has no catalytic activity. The observation that the C149S mutant is active is interesting, given that this enzyme appears to lack an aspartate, proposed to be an essential component of the catalytic triad of lipases. Given the ambiguity of the role of D264 in lipase, that is its replacement with Ala results in 25% activity (*29*), this result may not be so surprising.[6]

Any proposed mechanism for polyoxoester formation requires a second base outside the catalytic dyad to deprotonate the hydroxyl group of HBCoA or a covalently bound HB, activating it for nucleophilic attack on the active site bound thioester (Figure 1). The α/β hydrolase fold models suggest that the strictly conserved residues H303 or D302 might reside in a location to be able to participate in this function. When H303 was converted into a Gln, 50% of wt activity was retained (Table 2), suggesting that H303 is not this second base. D302 mutants proved substantially more interesting and will be discussed subsequently (Table 2). The role of H303 may be to hold D302 in position to function as the general base catalyst. Q303 could conceivably function in a similar capacity.

The Role of D302. The ClustalW sequence alignment (Figure 2) places D302, conserved in all PHA synthases, near in primary sequence to the triad Asp in the lipase active site. Given the differences in chemical reactivity between Cys and Ser, an aspartate as part of a catalytic triad would not expected to be involved in Cys deprotonation (*67*−*72*). As noted above, D302 could play a role as a general base catalyst to deprotonate the hydroxyl of HBCoA. To test this hypothesis, two mutants were constructed: D302A and D302N. Both mutants showed greatly decreased enzyme activity (0.012% and 0.25% of wt, respectively; Table 2), suggesting that this residue plays an important role in catalysis. Furthermore, both mutants exhibit kinetics that are very different from the wt PHA synthase (Figure 5): a lag phase, followed by a more rapid phase.

Use of sT-CoA To Help Define the Function of the H331 and D302 Mutants. We have previously reported that sT-

[6] The catalytic role(s) of Asp in α/β hydrolase family proteins appears to vary widely and needs to be examined in each protein (*27*−*29*, *58*−*66*).

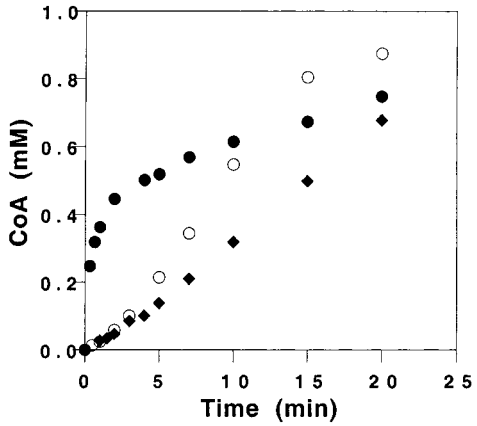

FIGURE 5: Time course of CoA release from HBCoA catalyzed by wt, D302A, and D302N PHA synthases at 30 °C. The reaction mixtures contain 1 mM HBCoA, 40 nM wt enzyme (solid circles), 8.9 μM D302A (open circles), 0.84 μM D302N (solid diamonds).

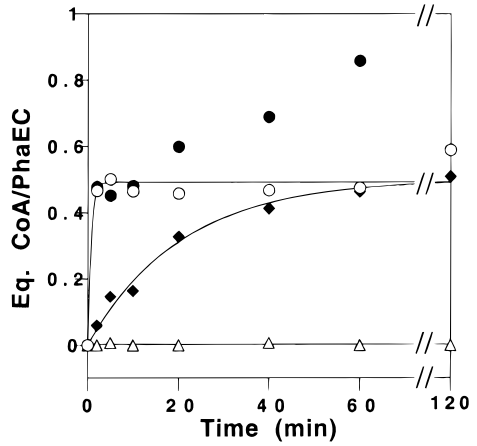

FIGURE 6: Time course of CoA release from sT-CoA catalyzed by PHA synthases at 30 °C. The reaction mixtures contain 34.6 μM wt enzyme and 406 μM of sT-CoA (solid circles); 28.4 μM D302A and 364 μM sT-CoA (solid diamonds); 34.3 μM D302N and 343 μM sT-CoA (open circles); and 16.9 μM H331Q and 170 μM sT-CoA (open triangles).

CoA can covalently label C149 and then be chased into polymer by addition of HBCoA by a mechanism proposed to bypass the priming process (*18*). This study provided the first direct evidence for covalent catalysis and the importance of C149. We now report the kinetics of the interaction of sT-CoA with wt and mutant PHA synthases, based on monitoring CoA release. Studies with the wt enzyme reveal a "burst" of 0.5 equiv of CoA per equivalent of PhaEC (Figure 6). Incubation of wt PHA synthase with [^{3}H]sT-CoA also results in 0.5 equiv of bound radiolabel per PhaEC. These results are very similar to radiolabeling experiments previously performed with the Class I PHA synthase (*17*). They suggest that the active form of the Class III enzyme might also be a dimer of PhaEC subunits in analogy with the model for the Class I PHA synthases. Figure 6 also shows that the burst is followed by slow release of CoA. The product of this reaction has recently been identified as sT-acid.

When the same experiment is repeated with H331Q PHA synthase, no CoA release is detected (Figure 6). This result supports our earlier conclusion that the function of this residue is to activate C149 for nucleophilic attack, in this case on the sT-CoA.

A similar experiment has been performed with the two D302 mutant proteins, and the results are also shown in Figure 6. In each case, 0.5 equiv of CoA per 1 equiv of PhaEC is liberated. In the case of the D302N mutation, this reaction occurs rapidly and is not followed by a slow release of CoA. Incubation of D302N with [^{3}H]sT-CoA results in 0.5 equiv of bound radiolabel per PhaEC, consistent with the CoA release data. These results again support our conclusion that Asp is not involved in the initial priming step and is not part of a catalytic triad. They also could be interpreted to support a model in which this Asp functions as a general base catalyst to activate the hydroxyl group of HBCoA or a covalently bound HB for nucleophilic attack during the priming and elongation steps. When HBCoA is not present, D302 could instead activate a water molecule that hydrolyzes the covalent thioester linkage at C149 and allows release of additional CoA as observed with wt enzyme (Figure 6).

DISCUSSION

The paradigm for thinking about a mechanism for polyoxoester polymerizations has been the β-ketoacyl ACP synthase (*26*) involved in FAS. A variant of this mechanism is shown in Figure 1. The choice of FAS as a model for PHB synthases was based on the observations that the substrate, a thiolester of HB, is also an intermediate in fatty acid synthesis. Furthermore, studies of Merrick in 1968 (*20*) using group-selective reagents implicated the importance of thiols in this reaction. However, recent studies on a β-ketoacyl ACP synthase (*26*) and a chalcone synthase (*73*), the lack of any apparent sequence homology between these enzymes and PhaCs, and inability to chemically characterize a pantetheinylation site, have caused us to reexamine the appropriateness of FAS as a mechanistic model for PHA synthase.

In 1992 a putative lipase box, N-x-x-G-x-C-x-G-G, was identified in PhaC (*15*). A recent review has again suggested that lipase might be a useful model for PHA synthases (*34*). A BLAST search using the *C. vinosum* PhaC as the query sequence gave surprising results. The sequence identities between residues 131 and 175 of PhaC and several *Pseudomonas* lipases (*44*, *48*) are high (~40%) (Figure 2). The lipase box, including the active site serine, is contained within this region. Remarkably, the active site cysteine (C149) of PhaC aligns with this serine. Both enzymes use an active site nucleophile for covalent catalysis.

The lipases are members of the α/β hydrolase superfamily of proteins (*10*, *74*). Enzymes within this superfamily have markedly different substrate specificities and include thioesterases (*75*), dienelactone hydrolases (*76*, *77*), and cholesterol esterase (*78*). Many of substrates for these enzymes use long chain fatty acid derivatives. The hydrolases are all proposed to possess catalytic triads with the nucleophile (Ser, Cys or Asp), acid, and His always being in this order in the primary sequence space. In addition, the nucleophile in all enzymes is found in a strand-elbow-helix motif with the nucleophile sitting at the elbow (*10*). In many ways the lipases are confronted with problems similar to those encountered by the PHA synthases. Bacterial lipases function as interfacial catalysts by acting at the lipid-water interface of a micellar or emulsified substrate (*79*). In the

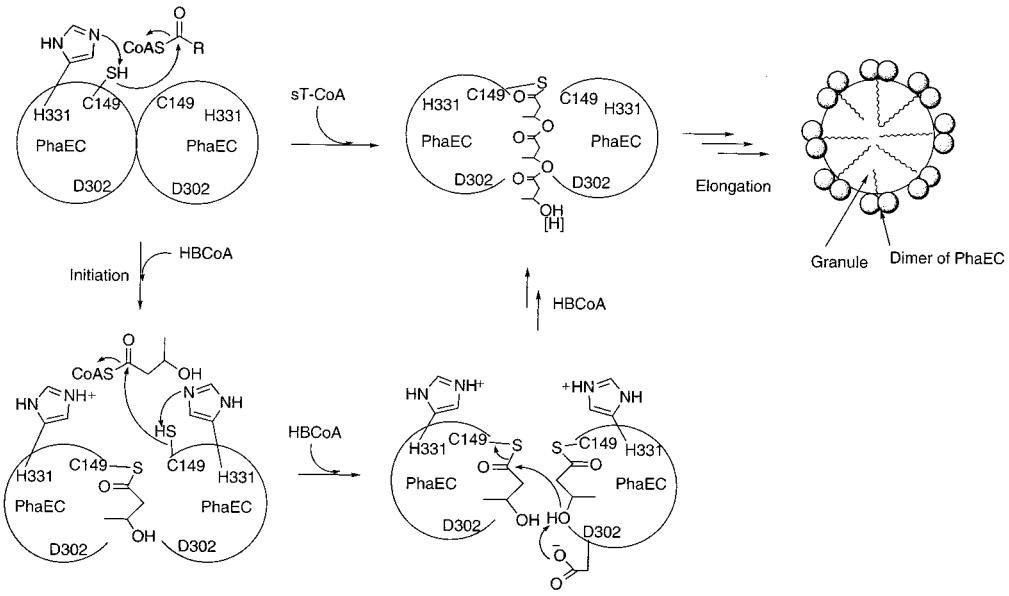

FIGURE 7: Modified model for the PHA synthase catalyzed reaction. The active site is at the subunit interface with each monomer providing one thiol group (C149). H331 functions as a general base to activate the nucleophile C149. D302 functions as a general base to activate the 3-hydroxyl group of HBCoA or a covalently bound HB for nucleophilic attack. sT-CoA is proposed to bypass the initiation process while synthase is proposed to be a dimer of trimers. For simplicity, only the active dimeric form of PhaEC is shown.

case of the PHA synthases, as the polymerization process proceeds, the enzymes find themselves attached to the surfaces of the insoluble granules. Thus they also function as interfacial catalysts.

Structures of the *P. cepacia* lipase in open and closed forms may be instructive as to the function of the PHB synthases. The closed form of this lipase is inactive (Figure 3A), and the open form (Figure 3B) possesses a covalently bound phosphonate inhibitor. The length of the hydrophobic side chain of this inhibitor, ~17 Å, is remarkably similar to that of the sT-CoA analogue (~18 Å) we have used to investigate the priming process. Whether this observation is significant requires a structure of the PHB synthases. This sT-CoA when covalently attached to the D302N PhaEC, which cannot convert sT into polymer, may be of use in obtaining crystals appropriate for structure determination.

The lipase structures and the conserved sequences between all PHA synthases (Classes I, II, and III) provided the starting point for our mutagenesis studies. In the lipase structure, the His that functions as a general base catalyst to activate the active site serine is 200 amino acids removed in primary sequence space and is located in the C terminal region of the protein. Thus the alignment of PHA synthase PhaC H331 with the lipase H286 using ClustalW and our mutagenesis studies suggest that the lipase structure may provide a good framework for thinking about the PHA synthase's structure. The activity of H331Q mutant is 10^5 lower than that of the wt enzyme, and furthermore sT-CoA is unable to load onto the active site C149 with this mutant. These studies strongly support the hypothesis that this histidine is required to activate the active site cysteine for catalysis.

In all of the lipases, there appears to be an aspartate or glutamate that is part of the putative catalytic triad. The same catalytic triad appears in almost all α/β hydrolases.[6] However, when the triad Asp of the *P. glumae* lipase was mutated to alanine, the mutant retained 25% of the wt enzyme activity (*29*). The proposal was made, based on a structural argument, that the Asp could be replaced by a nearby Glu (*53*), but this has not been unambiguously verified. In PHA synthase there is no need chemically for an Asp in a position corresponding to that observed in the lipases, as the catalytic dyad of His and Cys has long been established in cysteine proteases to be sufficient for nucleophilic activation (*67*−*69*, *71*).

D302, conserved in all PHA synthases, and close to the putative active site based on the model structure, is the closest in primary sequence space to the lipase Asp and was therefore mutated. The mutant has activity 10^3 lower than that of wt enzyme, suggesting that it is important in catalysis. Given the structural model, the kinetic behavior (Figures 5 and 6), and the fact that PHA synthases utilize cysteine as an active site nucleophile, it is unlikely that this Asp plays a role similar to that proposed for the lipases.

When thinking about the function of PHA synthases, one still needs a general base catalyst to activate the 3-hydroxyl of HBCoA or a covalently bound HB for nucleophilic attack on the acylated enzyme. The results of the interaction of sT-CoA with D302N PHA synthase suggest that D302 might be this base and lend further support to the proposed mechanism. As with wt PHA synthase, the active site Cys of the D302N mutant can be acylated (based on CoA release), and to the same extent as the wt PHA synthase. In wt enzyme, however, the acylated enzyme is unstable, and additional CoA is slowly released (Figure 6). In the D302N mutant, no or very slow turnover of the acylated enzyme appears to occur. Furthermore, studies with [^{3}H]sT-CoA show that 0.5 equiv are bound per equivalent of PhaEC. An explanation for these results consistent with the above model is that D302 functions, in the absence of HBCoA, as a general base catalyst to activate water to attack the acylated enzyme and allow deacylation. The identification of the sT-acid provides strong support for this hypothesis.

A structural model constructed from sequence alignments and lipase structures (Figure 4) suggested that the function of C130, which we originally proposed to be the second thiolate involved in catalysis (*18*), should be reexamined.

Our previously reported low activity for the C130A mutant, as noted in Results, actually corresponded to the C130A/C149S double mutant. We therefore reconstructed both serine and alanine mutants of C130 and found them both to be active (Table 2). Thus, both Class I and III PHA synthases possess only a single thiolate per monomer that can be involved in catalysis.

Finally, besides the conserved and functionally important Cys, His, and Asp of all PHA synthases, an additional piece of evidence links the mechanisms of the Class I and III enzymes. We have recently shown, using size exclusion chromatography, that the *C. vinosum* enzyme appears to be composed of an equilibrium mixture of trimers and hexamers of a 1:1 complex of PhaC and PhaE.[3] Acylation of the wt and D302N PHA synthase with 0.5 equiv of [^{3}H]sT per 1 equiv of PhaEC is remarkably similar to our previous studies on the *R. eutropha* enzyme (*17*). In the case of this Class I enzyme, we proposed that the dimer is the active form of the enzyme with one cysteine from each monomer forming the active site at the subunit interface (*17*). One could now propose a similar scenario for the *C. vinosum* enzyme, in which the active enzyme would now be a dimer of trimers. A model for this proposal is shown in Figure 7. Whether the priming facilitates dimer formation by conversion of the trimer to the hexamer or occurs prior to dimer formation (within the trimer) is actively being investigated.

One can now ask, given the arguments in this paper for structural homology with lipases, are two cysteines utilized in covalent catalysis, a model based on FAS mechanisms? Alternatively, could it be that a single active site cysteine is sufficient and that there is a second binding site for HBCoA that does not require covalent attachment to the enzyme? A chalcone synthase (*73*), which catalyzes multiple steps involving multiple CoA substrates in a single active site, has been proposed, based on the structure, to use only a single covalent nucleophile. At present, we cannot distinguish mechanistically between these models. However, if the active form of both Class I and III PHA synthases is in fact a dimer, then the two-thiolate model, despite the structural homology with lipases, must still be given serious consideration. The question then can be posed, how could a dimerization event occur to bring the active site cysteines together?

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REFERENCES

1. Anderson, A. J., and Dawes, E. A. (1990) *Microbial. Rev. 54*, 450−472.
2. Steinbüchel, A. (1991) *Biomaterials. Novel Materials and Biological Sources* (Byrom, D., Ed.), Macmillan Publishers Ltd., Basingstoke, U.K.
3. 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., Padgette, S. R., Kishore, G., and Gruys, K. J. (1999) *Nat. Biotechnol. 17*, 1011−1016.
4. Müller, H.-M., and Seebach, D. (1993) *Angew. Chem., Int. Ed. Engl. 32*, 477−502.
5. Byrom, D. (1992) *FEMS Microbiol. Rev. 103*, 247−250.
6. Marchessault, R. H. (1996) *Trends Polym. Sci. 4*, 163−168.
7. Poirier, Y., Nawrath, C., and Somerville, C. (1995) *Bio/Technology 13*, 142−150.
8. Gerngross, T. U. (1999) *Nat. Biotechnol. 17*, 541−544.
9. Heikinheimo, P., Goldman, A., Jeffries, C., and Ollis, D. L. (1999) *Structure 7*, 141−146.
10. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) *Protein Eng. 5*, 197−211.
11. Steinbüchel, A., Hustede, M., Liebergesell, M., Pieper, U., Timm, A., and Valentin, H. (1992) *FEMS Microbiol. Rev. 103*, 217−230.
12. Slater, S. C., Voige, W. H., and Dennis, D. E. (1988) *J. Bacteriol. 170*, 4431−4436.
13. Peoples, O. P., and Sinskey, A. J. (1989) *J. Biol. Chem. 264*, 15293−15297.
14. Peoples, O. P., and Sinskey, A. J. (1989) *J. Biol. Chem. 264*, 15298−15303.
15. Liebergesell, M., and Steinbüchel, A. (1992) *Eur. J. Biochem. 209*, 135−150.
16. Schubert, P., Steinbüchel, A., and Schlegel, H. G. (1988) *J. Bacteriol. 170*, 5837−5847.
17. Wodzinska, J., Snell, K. D., Rhomberg, A., Sinskey, A. J., Biemann, K., and Stubbe, J. (1996) *J. Am. Chem. Soc. 118*, 6319−6320.
18. Müh, U., Sinskey, A. J., Kirby, D. P., Lane, W. S., and Stubbe, J. (1999) *Biochemistry 38*, 826−837.
19. Smith, S. (1994) *FASEB J. 8*, 1248−1259.
20. Griebel, R., Smith, Z., and Merrick, J. M. (1968) *Biochemistry 7*, 3676−3681.
21. Griebel, R. J., and Merrick, J. M. (1971) *J. Bacteriol. 108*, 782−789.
22. Ballard, D. G., Holmes, P. A., and Senior, P. J. (1987) *Recent Advances in Mechanistic and Synthetic Aspects of Polymerization* (Fontanille, M., & Guyot, A., Eds.), D. Reidel Publishing Company, Lancaster, U.K.
23. Gerngross, T. U., Snell, K. D., Peoples, O. P., Sinskey, A. J., Cushai, E., Masamune, S., and Stubbe, J. (1994) *Biochemistry 33*, 9311−9320.
24. Walsh, C. T., Gehring, A. M., Weinreb, P. H., Quadri, L. E., and Flugel, R. S. (1997) *Curr. Opin. Chem. Biol. 1*, 309−315.
25. Hoppensack, A., Rehm, B. H., and Steinbüchel, A. (1999) *J. Bacteriol. 181*, 1429−1435.
26. Huang, W., Jia, J., Edwards, P., Dehesh, K., Schneider, G., and Lindqvist, Y. (1998) *EMBO J. 17*, 1183−1191.
27. Karlsson, M., Contreras, J. A., Hellman, U., Tornqvist, H., and Holm, C. (1997) *J. Biol. Chem. 272*, 27218−27223.
28. Brumlik, M. J., and Buckley, J. T. (1996) *J. Bacteriol. 178*, 2060−2064.
29. 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.
30. Hermoso, J., Pignol, D., Penel, S., Roth, M., Chapus, C., and Fontecilla-Camps, J. C. (1997) *EMBO J. 16*, 5531−5536.
31. van Tilbeurgh, H., Egloff, M. P., Martinez, C., Rugani, N., Verger, R., and Cambillau, C. (1993) *Nature 362*, 814−820.
32. Liebergesell, M., Sonomoto, K., Madkour, M. H., Mayer, F., and Steinbüchel, A. (1994) *Eur. J. Biochem. 226*, 71−80.
33. Gerngross, T. U., Reilly, P., Stubbe, J., Sinskey, A. J., and Peoples, O. P. (1993) *J. Bacteriol. 175*, 5289−5293.
34. Madison, L. L., and Huisman, G. W. (1999) *Microbiol. Mol. Biol. Rev. 63*, 21−53.
35. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
36. Higuchi, R. (1990) *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., & White, T. J., Eds.), Academic Press, San Diego, CA.
37. Laemmli, U. K. (1970) *Nature 227*, 680−685.
38. Lohman, T. M., and Mascotti, D. P. (1992) *Methods Enzymol. 212*, 424−458.
39. Williamson, D. H., and Mellanby, J. (1974) *Methods Enzymol. 4*, 1836−1840.

40. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.
41. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res. 22*, 4673−4680.
42. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis 18*, 2714−2723.
43. Guex, N., Diemand, A., and Peitsch, M. C. (1999) *Trends Biochem. Sci. 24*, 364−367.
44. Schrag, J. D., Li, Y., Cygler, M., Lang, D., Burgdorf, T., Hecht, H. J., Schmid, R., Schomburg, D., Rydel, T. J., Oliver, J. D., Strickland, L. C., Dunaway, C. M., Larson, S. B., Day, J., and McPherson, A. (1997) *Structure 5*, 187−202.
45. Lang, D. A., Mannesse, M. L., de Haas, G. H., Verheij, H. M., and Dijkstra, B. W. (1998) *Eur. J. Biochem. 254*, 333−340.
46. Kim, K. K., Song, H. K., Shin, D. H., Hwang, K. Y., and Suh, S. W. (1997) *Structure 5*, 173−185.
47. Kabsch, W., and Sander, C. (1983) *Biopolymers 22*, 2577−2637.
48. Iizumi, T., Nakamura, K., Shimada, Y., Sugihara, A., Tominaga, Y., and Fukase, T. (1991) *Agric. Biol. Chem. 55*, 2349−2357.
49. Liebergesell, M., and Steinbüchel, A. (1993) *Appl. Microbiol. Biotechnol. 38*, 493−501.
50. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) *DNA Res. 3*, 109−136.
51. Hein, S., Tran, H., and Steinbüchel, A. (1998) *Arch. Microbiol. 170*, 162−170.
52. Noble, M. E., Cleasby, A., Johnson, L. N., Egmond, M. R., and Frenken, L. G. (1993) *FEBS Lett. 331*, 123−128.
53. Lang, D., Hofmann, B., Haalck, L., Hecht, H. J., Spener, F., Schmid, R. D., and Schomburg, D. (1996) *J. Mol. Biol. 259*, 704−717.
54. Karplus, K., Barrett, C., and Hughey, R. (1998) *Bioinformatics 14*, 846−856.
55. Kelley, L. A., Maccallum, R., and Sternberg, M. J. E. (1999) *Proceedings of the Third Annual Conference on Computational Molecular Biology* (Istrail, S., Pevzner, P., & Waterman, M., Eds.) The Association for Computing Machinery, New York.
56. Fischer, D., and Eisenberg, D. (1996) *Protein Sci. 5*, 947−955.
57. McCool, G. J., and Cannon, M. C. (1999) *J. Bacteriol. 181*, 585−592.
58. Tai, M. H., Chirala, S. S., and Wakil, S. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1852−1856.
59. Liao, D. I., Breddam, K., Sweet, R. M., Bullock, T., and Remington, S. J. (1992) *Biochemistry 31*, 9796−9812.
60. Hynkova, K., Nagata, Y., Takagi, M., and Damborsky, J. (1999) *FEBS Lett. 446*, 177−181.
61. DiPersio, L. P., and Hui, D. Y. (1993) *J. Biol. Chem. 268*, 300−304.
62. Shafferman, A., Kronman, C., Flashner, Y., Leitner, M., Grosfeld, H., Ordentlich, A., Gozes, Y., Cohen, S., Ariel, N., Barak, D., and et al. (1992) *J. Biol. Chem. 267*, 17640−17648.
63. Krooshof, G. H., Kwant, E. M., Damborsky, J., Koca, J., and Janssen, D. B. (1997) *Biochemistry 36*, 9571−9580.
64. Vernet, T., Tessier, D. C., Chatellier, J., Plouffe, C., Lee, T. S., Thomas, D. Y., Storer, A. C., and Menard, R. (1995) *J. Biol. Chem. 270*, 16645−16652.
65. Osterlund, T., Contreras, J. A., and Holm, C. (1997) *FEBS Lett. 403*, 259−262.
66. Emmerich, J., Beg, O. U., Peterson, J., Previato, L., Brunzell, J. D., Brewer, H. B., Jr., and Santamarina-Fojo, S. (1992) *J. Biol. Chem. 267*, 4161−4165.
67. Storer, A. C., and Menard, R. (1994) *Methods Enzymol. 244*, 486−500.
68. Roberts, P. J., and Belsham, G. J. (1995) *Virology 213*, 140−146.
69. Chen, J. P., Strauss, J. H., Strauss, E. G., and Frey, T. K. (1996) *J. Virology 70*, 4707−4713.
70. Schmidt, G., Selzer, J., Lerm, M., and Aktories, K. (1998) *J. Biol. Chem. 273*, 13669−13674.
71. Chen, J. M., Rawlings, N. D., Stevens, R. A. E., and Barrett, A. J. (1998) *FEBS Lett. 441*, 361−365.
72. Huang, X., and Raushel, F. M. (1999) *Biochemistry 38*, 15909−15914.
73. Ferrer, J. L., Jez, J. M., Bowman, M. E., Dixon, R. A., and Noel, J. P. (1999) *Nat. Struct. Biol. 6*, 775−784.
74. Schrag, J. D., and Cygler, M. (1997) *Methods Enzymol. 284*, 85−107.
75. Lawson, D. M., Derewenda, U., Serre, L., Ferri, S., Szittner, R., Wei, Y., Meighen, E. A., and Derewenda, Z. S. (1994) *Biochemistry 33*, 9382−9398.
76. Pathak, D., Ngai, K. L., and Ollis, D. (1988) *J. Mol. Biol. 204*, 435−445.
77. Pathak, D., and Ollis, D. (1990) *J. Mol. Biol. 214*, 497−525.
78. Ghosh, D., Wawrzak, Z., Pletnev, V. Z., Li, N., Kaiser, R., Pangborn, W., Jörnvall, H., Erman, M., and Duax, W. L. (1995) *Structure 3*, 279−288.
79. Desnuelle, P. (1972) *The Enzymes* (Boyer, P. D., Eds.), Academic Press, New York.
80. Holm, L., and Sander, C. (1993) *J. Mol. Biol. 233*, 123−138.
81. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.
82. Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol. 277*, 505−524.

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