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Poly-(*R*)-3-hydroxybutyrate (PHB) biosynthesis: mechanistic studies on the biological Claisen condensation catalyzed by β-ketoacyl thiolase

S. Masamune, C.T. Walsh†, A.J. Sinskey, and O.P. Peoples

Massachusetts Institute of Technology, Departments of Chemistry and Applied Biological Sciences, Cambridge, Massachusetts 02139.
† Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, U.S.A.

Abstract – The organisms *Zoogloea ramigera* and *Alcaligenes eutrophus*, like many other bacteria, produce poly-(R)-3-hydroxybutyrate (PHB) from acetyl-CoA through the three-enzyme pathway involving thiolase, reductase and PHB synthase. Thiolase catalyzes condensation of two acetyl-CoA molecules to acetoacetyl-CoA via two steps: In the first half reaction the active site cysteine attacks acetyl-CoA to form an acetyl-S-enzyme intermediate which reacts, in the second half reaction, with the anion formerly formed from the second acetyl-CoA molecule by enzymic deprotonation to complete the condensation. An adequate supply of thiolase from either organism now made available through isolation of the encoding structural gene and construction of an overproduction vector has permitted extensive studies on the mechanism of the thiolase-catalyzed reaction. This article attempts to answer several basic questions of or comments on: (1) thiolase substrate specificity, (2) identification of the active site cysteine involved in acetyl enzyme formation in the first half reaction, (3) identification of the active site basic residue responsible for deprotonation of the second acetyl-CoA molecule, (4) timing of deprotonation vs. C-C bond formation both involved in the second half reactions, and (5) the energy profile of the forward and reverse reactions.

INTRODUCTION

In the development of our macrolide project over many years, we witnessed a major breakthrough, the synthesis of 6-deoxyerythronolide B in 1981 (ref. 1). The synthesis led to the establishment of a general strategy for acyclic stereoselection, based on the rule of double asymmetric synthesis (ref. 2). While this synthetic field continued to grow and mature through the efforts of many investigators and numerous macrolides and related compounds became amenable to organic synthesis, our interests were irresistibly expanded to include studies on enzyme-catalyzed carbon-carbon bond-forming and breaking reactions. A long ranged project was thus launched several years ago with the hope that uncovering and understanding enzyme "tricks" would someday be utilized for the design of man-made catalysts. This article outlines our initial efforts directed towards the mechanistic elucidation of the bio-Claisen condensation of acetyl CoA (C_2 unit) to form acetoacetyl CoA (C_4 unit), a step that is utilized to initiate several biosynthetic processes.

A variety of carbon-carbon bond forming reactions proceed enzymically and the C_1, C_2, C_3, and C_5 fragment transfers shown in Table 1 represent carboxylation, a Claisen condensation, aldol reaction, and prenyl transfer reaction, respectively. Of particular interest are the C_2 fragment transfer

Table 1 **Biological Routes of Carbon-Carbon Formation**

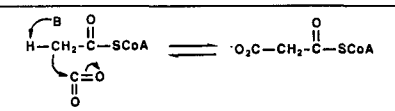
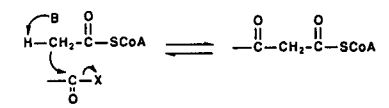
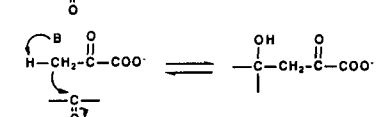
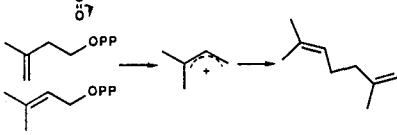
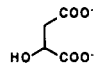
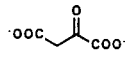
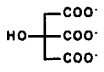
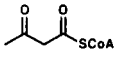
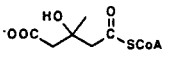
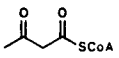
A. C_1 Fragments :		Carboxylation
B. C_2 Fragments :		Claisen condensation
C. C_3 Fragments :		Aldol reaction
D. C_5 Fragments :		Prenyl transfer

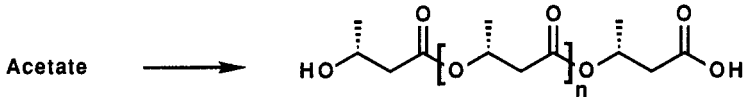
Table 2 **Some Enzymes Utilizing Acetyl CoA for Claisen Condensations**

Enzyme	Nucleophilic Substrate	Electrophilic Substrate	Product
Malate Synthase			
Citrate Synthase			
HMG CoA Synthase			
Thiolase			

reactions which in many cases use acetyl CoA as a substrate. Four different enzymes of this type are listed in Table 2.

In all of these condensation reactions the anion formally derived from acetyl CoA acts as a nucleophile, which reacts with an aldehyde (in the case of malate synthase), on ketones (citrate, HMG-CoA synthase), and on a thiol ester. This last reaction is catalyzed by thiolase which is so named as its reverse reaction effects thiolytic cleavage of acetoacetyl-CoA (AcAcSCoA) with the cosubstrate CoASH to form 2 molecules of acetyl-CoA (AcSCoA).

There are multiple isozymes of thiolase both in mammalian cells, yeast, and prokaryotes. They may be classified into two groups. Isozymes of one group show broad specificity for thiolysis of β–ketoacyl CoAs at C_4 to C_{16} chain length and are clearly involved in the β-oxidation of long chain fatty acids. These enzymes, located in mitochondria and peroxisomes of mammalian cells and prokaryotes, are referred to as degradative thiolases. In contrast, isozymes of the other group, called biosynthetic thiolases, have narrow specificity for short chain CoA esters, particularly for AcAcSCoA. Each biosynthetic thiolase has a different role; either in (a) ketone body utilization, (b) generating AcAcSCoA as substrate for the HMG-CoA synthase reaction in steroid and isoprenoid biosynthesis, or (c) initiating a three-enzyme pathway leading to polyhydroxybutyrate (PHB).



PHB n ≅ 500 - 15,000
MW $5x10^4$ - $1.5x10^6$

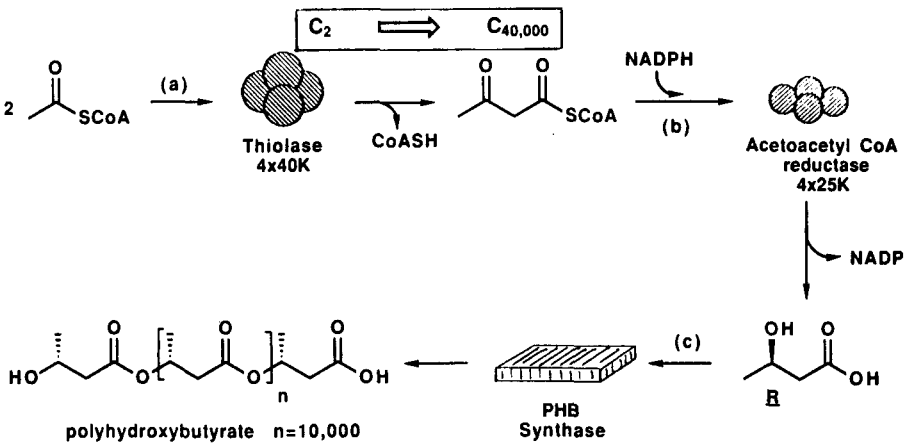
Numerous microorganisms have the ability to accumulate intracellular reserves of polyhydroxybutyrate (PHB) (ref. 3), which is a straight chain polyester of (R)-3-hydroxybutyrate with a molecular weight of 5×10^4 - 1.5×10^6. PHB serves as an energy reserve and can be accumulated to large quantities by growth limitations (induced for example by limiting nitrogen, oxygen, phosphate or sulfur sources) up to 80% of the dry cell weight of the organism (ref. 4). PHB is of commercial value, being an essentially non-toxic (LD_{50} > 5,000 mg/Kg) and biodegradable polyester. From this thermoplastic material many useful products, both consumer and medical, can be made without the environmental adversity that plastic has. Among the species that biosynthesize and metabolize PHB are *Zoogloea ramigera* (ref. 5) and *Alcaligenes eutrophus* (ref. 6). The former is a floc-forming bacterium isolated from sludge in wastewater treatment plants and the latter is a species ICI has used to develop PHB ("BIOPOL") as a biodegradable plastic. The hydrolytic end-product, (R)-3-hydroxybutyrate is a normal consistuent of human blood. In addition, it is widely used in organic synthesis serving as a chiral C_4-unit.

PHB is not the sole polyester produced by bacteria. Feeding acetate and propionate to whole cells of *A. eutrophus* for instance leads to the formation of copolymers of 3-hydroxybutyryl (C_4) and 3-hydroxyvaleryl (C_5) units. Pure C_5 polyhydroxyvaleryl polymer (PHV) has also been isolated from activated sewage sludge (ref. 7).

The PHB synthesis involves three enzymes as shown in Scheme 1. The first enzyme thiolase functions biosynthetically and is coupled to acetoacetyl CoA reductase and PHB synthase. For several reasons we have selected this metabolic pathway as an object of our investigation. (1) The carbon-carbon bond forming reaction is a process to which our original interest in enzymology was directed. (2) This metabolic pathway is direct and relatively simple. (3) The three enzymes are likely to be overproduced through gene cloning to supply an adequate amount of each enzyme for studies. (4) PHB synthase will likely open up a hitherto unknown field of investigation in the study of biosynthesis and mechanism of formation of polyesters.

Three Enzyme Pathway : Acetyl-CoA ⟹ poly-D-β-hydroxybutyrate

Scheme 1



(a) C-C bond forming step
(b) chiral reduction step
(c) head to tail polymerization (initiation, elongation, termination)

PHB Synthesis (*Z. ramigera* and *A. eutrophus*)

Although the metabolic pathway is direct, very little had been known about the nature of these three enzyme catalysts or about the molecular modes of regulation when our project was initiated. Tomita and colleagues used *Z. ramigera* as a source for purification and preliminary characterization of the PHB-biosynthetic thiolase (ref. 8) and the reductase (ref. 9) and for activity detection and partial purification of PHB synthase. The majority of the ICI work on *A. eutrophus* concerns whole cell studies and provides no mechanistic information on this metabolic pathway (ref 6). Our work began with the isolation of thiolase from *Z. ramigera* and has expanded to cover all three enzymes from both *Z. ramigera* and *A. eutrophus* for comparative studies.

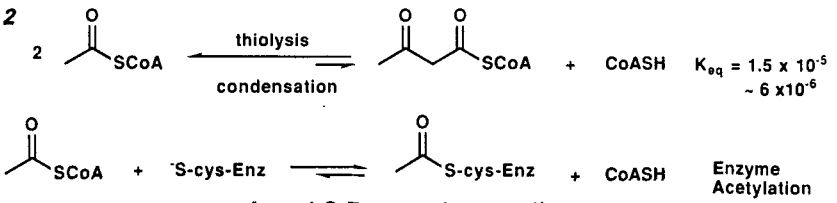
ZOOGLOEAL AND *ALCALIGENES* THIOLASE

According to the procedure reported by Tomita, *et al.* for the *Zoogloeal* thiolase (ref. 8), we isolated a limited amount of the enzyme. This allowed us to (1) determine N-terminal sequence, (2) isolate and sequence a putative active site tryptic peptide containing an iodoacetamide-modified cysteine residue (see below) and (3) prepare anti-thiolase antibodies. The two amino acid sequences ultimately served to align the encoding DNA sequence of the cloned gene. We used antibody screening methods for the isolation of the gene whose DNA sequence was determined for the first time for any thiolase. Construction of an overproduction vector by placing the gene behind the *tac* promoter led to a 200-fold overproduction to the point where 29% of the soluble cell protein in the *E. coli* host is the *Zoogloeal* thiolase. One liter of cell growth generates 140 mg of thiolase purifiable to homogeneity in two chromatographic steps (ref 10). The gene contains 1,176 nucleotides, encoding a 392-residue subunit of M_r = 40.8 Kd. The native enzyme is a homotetramer of 163 Kd. The cloned *Zoogloeal* thiolase gene was used as a hybridization probe to identify the location of the *A. eutrophus* thiolase gene (ref. 11). The sequence of the latter reveals that 248 out of 392 residues are identical for a 63% absolute homology. Recently the two rat thiolase gene sequences have been determined (ref. 12). A four-way comparison for the conserved residues helps identify candidates for functionally important residues. The gene cloning technique we used will be elaborated upon in some detail toward the end of this article.

MECHANISTIC STUDIES

Lynen was the first to notice inactivation of thiolase activity in the presence of SH-blocking reagents (ref. 13). This type of inactivation, a universal property of all thiolase isozymes, prompted him to propose that a thiol group, intimately involved in inactivation, was present in the active site. He proposed the two step mechanism for condensation as shown in Scheme 2. The first half reaction involves nucleophilic attack of the active site thiol on the carbonyl group of AcSCoA to provide CoASH and an acetyl-S-enzyme intermediate (enzyme acetylation). In fact Gehring and Lynen in a later work isolated

Scheme 2



Acetyl-S-Enzyme Intermediate

the acetyl enzyme and identified this active site cysteine by peptide mapping techniques (ref. 14). In the second half reaction (acetyl-enzyme deacetylation) another molecule of AcSCoA reacts with acetyl-S-enzyme, yielding the product, AcAcSCoA and regenerating the enzyme species. In this way thiolase treats two identical acetyl CoA molecules differentially, utilizing one as a C-2-carbanion equivalent and the other as an electrophile at C-1, to effect the classic head-to-tail condensation. It should be stressed that the thermodynamics of the thiolase reactions strongly favors thiolytic cleavage of AcAcSCoA. The Keq value has been estimated to be between 1.5×10^{-5} and 6×10^{-6}. Despite the unfavorable thermodynamics, the AcAcSCoA is drawn off biosynthetically with reductase for PHB accumulation.

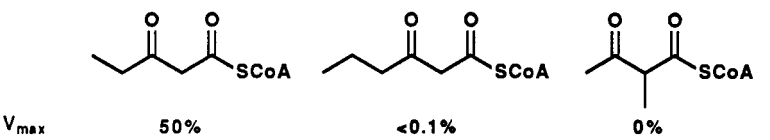
The above two-step reaction mechanism is a classic example of a Bi Bi ping-pong mechanism. Bi Bi ping-pong mechanisms are common in enzyme-catalyzed functional group transfer reactions and require that one of the products is released between the addition of the first and second reactant, resulting in an oscillation between two enzyme species. Analysis of the Zoogloeal thiolase reaction showed that the kinetic data were consistent with this mechanism and provided the following parameters: for the forward (synthetic) direction, $k_{cat} = 71\ s^{-1}$, K_M (AcSCoA) = 1.2 mM, $k_{cat}/K_M = 6.0 \times 10^4\ M^{-1}s^{-1}$; for the reverse (cleavage) direction, $k_{cat} = 810\ s^{-1}$, K_M (AcAcSCoA) = 15.3 μM, K_M (HSCoA) = 8.8 μM, $k_{cat}/K_M = 5.3 \times 10^7\ M^{-1}s^{-1}$. Note that (1) the k_{cat} value for the forward reaction is 11-fold smaller than that for reverse reaction and (2) the k_{cat}/K_M for the reverse reaction is approaching a value expected for diffusion-controlled encounter of substrate and enzyme (ref. 8, 15). The *Zoogloeal* thiolase is indeed a highly efficient catalyst in the cleavage direction.

With the thiolase primary sequence now available, acceptance of the two step mechanism and preliminary characterization of the enzyme raise several basic questions or comments concerning: (1) thiolase substrate specificity, (2) identification of the active site cysteine involved in acetyl enzyme formation in the first half reaction, (3) identification of the active site basic residue capable of deprotonating a second AcSCoA, (4) timing of deprotonation vs C-C bond formation both involved in the second half reaction, and (5) the energy profile of the thiolase forward and reverse reaction. These items will be discussed below in sequence.

1. Thiolase substrate specificity

It has already been described that biosynthetic thiolases have narrow specificity for AcAcSCoA. In order to define the types of monomer units that might be incorporated in the three enzyme pathway to make PHB analogs, we have further assessed some features of substrate specificity and recognition in substrate processing. Since AcSCoA molecules serve as both electrophilic and nucleophilic partners in the condensation, we have separately assessed what tolerance the enzyme displays for the first AcSCoA molecule that acts as an electrophile and makes the acetyl-S-enzyme intermediate and also what tolerance the enzyme displays for the second AcSCoA acting as nucleophile and carbanion generator. Also of interest are the two questions: (1) Is a thiol ester necessary and (2) how much of the CoA portion can be dispensed with?

The enzyme cleaves the C_5-3-oxoacyl-CoA at 50% V_{max} of AcAcCoA and the C_6-3-oxoacyl-CoA at 6% V_{max}. The branched chain 2-methylacetoacetyl-CoA is <u>not</u> detectably cleaved (< 0.1% V_{max}). These data show the enzyme will make a linear C_3 and C_4 acyl enzyme intermediate, allowing some bulk tolerance in the electrophilic acyl component, but will not make a carbanion equivalent with propionyl-CoA. In support of this conclusion, no exchange of propionyl-CoA C_2 hydrogens by the enzyme occurred in D_2O long after all three methyl hydrogens of acetyl-CoA were exchanged. The rejection of

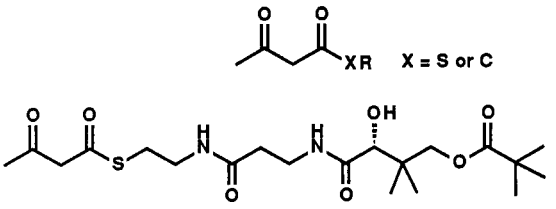


longer acyl-CoAs as C_2 carbanion precursors by thiolase ensures it will not make <u>branched</u> chain 3-oxo-acyl-CoA products.

The *Zoogloeal* thiolase will not accept the oxo ester analog of acetyl-CoA either as electrophilic or nucleophilic partner nor will it use the corresponding amide analog. The pK_a for the methyl hydrogens of acetyl-CoA are estimated to be ca. 20 while for the oxo ester a pK_a of 26 is estimated, so perhaps this exceeds the kinetic acidity limit that the enzyme can deal with for facile enolate generation. The pK_a of the methylketone analog of acetyl-CoA should also be ca. 20 and we have shown that thiolase will use this analog as nucleophilic partner in Claisen condensation with acetyl-CoA as electrophilic partner (ref. 15). The thiolase takes advantage of the enhanced acidity of AcSCoA and also the known high reactivity towards thiolate and enolate anions compared with its oxygen analog.

We have prepared and tested several synthetic analogs of AcAcSCoA, modified in the CoA moiety and have found that AcAcS-pantetheine-11-pivalate has a k_{cat}/K_m only three fold smaller than that of

AcAcSCoA, indicating that the triphosphoadenesine portion is not essential for binding (ref. 15). This is in contrast to the situation for another AcAcSCoA-utilizing enzyme, succinyl-SCoA: 3-ketoacid-SCoA transferase (ref. 16). The pantetheine pivalate group facilitates non-aqueous synthetic manipulations and has been used throughout this work.



2. Acetyl-S-enzyme

As described earlier, ^{14}C-iodoacetamide inactivated enzyme in a substrate-protectable manner to provide a stoichiometric amount of an enzyme-X-CH_2COOH species. A labelled peptide obtained by tryptic digestion was sequenced to show that the reactive nucleophile was Cys-89. This residue has now been shown more directly to be the acetylated active site nucleophile. Incubation of thiolase with *$CH_3CoSCoA$ to yield acetyl enzyme, followed by acid precipitation, denaturation, tryptic digestion, radioactive peptide isolation, and sequencing has identified the same residue, Cys-89, as the active site nucleophile. The active site sequence including this cysteine is highly conserved in the four cognate thiolases referred to above. In native form Ac-S-enzyme is hydrolytically labile and attempts to isolate it in that form have invariably resulted in a substantial loss of labelled enzyme as indicated by the decrease of radioactivity from 0.86 equiv to 0.072 equiv of ^{14}C/subunit during 10 min gel filtration. The half life of Ac-S-enzyme is estimated to be ca. 2 min. While the relative reactivity of Ac-S-enzyme towards hydrolysis ($k = 5 \times 10^{-3} s^{-1}$) is 20,000 fold compared to AcSCoA substrate ($2 \times 10^{-7} s^{-1}$) (ref. 17) and comparable to the acyl enzymes Ac-O-(Ser-195)-chymotrypsin (ref. 18) and Bz-S-(Cys-125)-papain (ref. 19), the rates of C-C bond formation and thiol exchange reaction with Ac-S-enzyme are estimated to be higher than or equal to 71 s^{-1} and 810 s^{-1}, respectively. These rates are much larger (14×10^3 and 16×10^4 fold) than that of hydrolysis, therefore the reactions of Ac-S-enzyme in both the forward and reverse directions proceed virtually without competition with its hydrolysis to acetate. The key intermediate in the thiolysis reactions is well "protected" against water.

3. Search for active site base

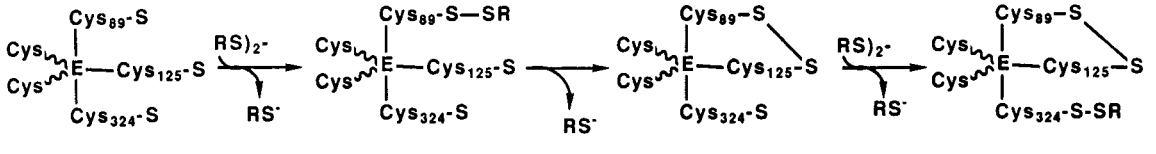
After acetyl-S-enzyme has formed, the enzyme must execute, at least formally, deprotonation of the second bound AcSCoA to generate the enolate anion, the nucleophilic partner of the condensation. In an effort to identify this active site basic residue responsible for the deprotonation we have undertaken studies using three different approaches: (1) group selective inactivation using dithionitrobenzoate (DTNB) specific for cysteine residues, and diethyl pyrocarbonate (DEPC) a histidine-selective reagent, (2) affinity labelling of active site residues with the pantetheine 11-pivalate thiol esters of α-haloacetic and acrylic acid, and (3) mechanism-based inactivations using classical "suicide" substrates.

a. 5,5'-Dithiobis(2-nitrobenzoate) (DTNB) Titration. This disulfide-forming reagent DTNB selectively reacts with enzyme cysteine residues to form mixed disulfide linkages with liberation of a highly chromophoric thionitrobenzoate (TNB) anion ($e_{412} = 14{,}120$).

Zoogloeal thiolase has five cysteines per subunit at the residues 89, 125, 324, 378, and 388 and *Alcaligenes* thiolase has three cysteines at 89, 358, and 378, two of which (89 and 378) are conserved. Cys-89 has already been shown to be a highly reactive active site residue.

Incubation of the native *Zoogloeal* thiolase with excess DTNB liberated 3.08 equivalents of TNB/subunit, two cysteines reacting very rapidly and the third 4-5 times more slowly. Isolation of the modified enzyme, followed by addition of excess β–mercaptoethanol resulted in the release of 1 equivalent of the TNB anion and restoration of enzyme activity. These and other results indicate that DTNB first reacts with a highly reactive Cys-89 to form the mixed disulfide which is converted to the intramolecular disulfide through the reaction with a cysteine located in proximity of Cys-89 (with the release of 1 equivalent of TNB) and then another cysteine undergoes modification. Three equivalents of TNB are liberated and the modified enzyme contains only one mixed disulfide linkage per subunit.

Repetitions of these labelling experiments with *Alcaligenes* thiolase produced interesting results. Only one of the three cysteines was titrated to form an intermolecular disulfide (with no intramolecular disulfide bond). The Cys-125, -324, and -388 residues of the *Zoogloeal* thiolase which are absent in



Alcaligenes thiolase are candidates for the proximal cysteine involved in the intramolecular disulfide bond formation. We have changed the Cys-125 to a serine residue by employing site directed mutagenesis following the standard procedure devised by Zoller and Smith (ref. 20). Incubation of the resulting Ser-125 mutant under native conditions yielded incorporation of 2 equivalent of TNB/subunit. Therefore, we conclude that the proximal cysteine is Cys-125. The third cysteine of *Zoogloeal* thiolase referred to above is probably Cys-324, as prolonged exposure of native *Zoogloeal* thiolase to a large excess of iodoacetamide led to modification of two cysteine residues at 89 and at 324. The Ser-125 is 47% as active toward thiolysis as the wild type in terms of k_{cat}/K_M. Cys-125 is in or near the active site, but certainly not essential. Its exact role remains unknown. Conserved Cys-378 is not titrated with DTNB, but has been trapped in another experiment as will be demonstrated shortly.

b. Diethyl Pyrocarbonate (DEPC) Inactivation. Under certain sets of conditions, DEPC reacts quite selectively with histidines to provide the corresponding ethoxyformyl derivatives, although other residues such as cysteine are also modified with this reagent. Our studies have demonstrated that (1) the reagent rapidly inactivates *Zoogloeal* thiolase in a substrate-protected manner to form the Cys-89-ethoxyformyl enzyme and (2) it modifies His-348 if Cys-89 is preprotected in the form of MeS-S(Cys-89). Mercaptoethanol-mediated regeneration of the Cys-89 functionality of the inactivated enzyme in the latter experiment does not restore the enzyme activity, indicating His-348 may be involved in catalytic deprotonation. To assess this possibility, we have prepared Asn-348 mutant which is shown to retain 7% of the activity of the wild type. Clearly His-348 is not the essential base responsible for deprotonation, because, if it were, the mutant would have lost all or virtually all catalytic activity. The interpretation of this ambiguous result requires further investigation, but we note the primary sequence in the region containing His-348 is highly conserved in all four thiolases as shown.

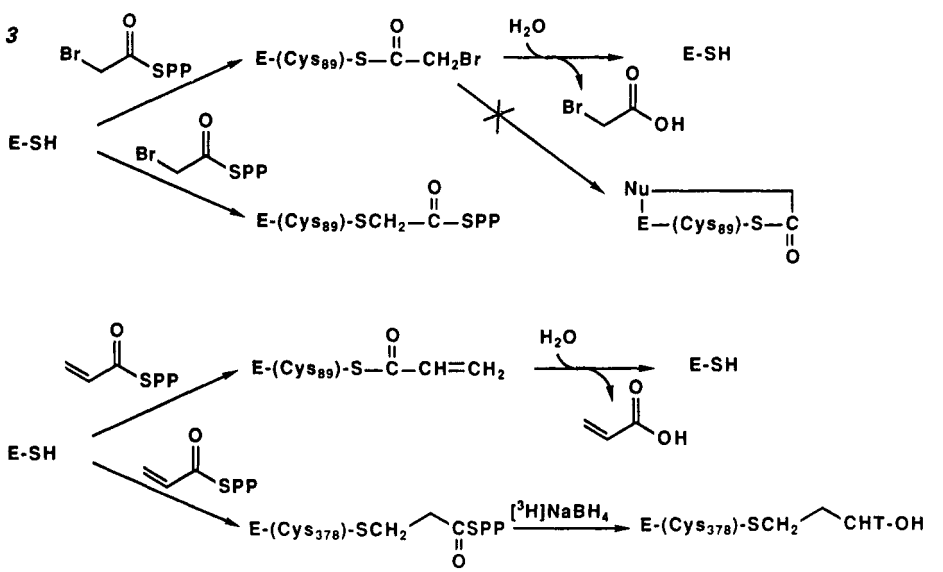
$$\text{MeS-S(Cys}_{89}\text{)-E} \xrightarrow{\text{DEPC}} \text{MeS-S(Cys}_{89}\text{)-E-(His}_{348}\text{)-SCOOEt}$$

GGAIAIGH*(348)PIGA (*Zoogloeal, Alcaligenes*)

GGAIALGH*(348)PLGG (rat mitochondrial and peroxisomal)

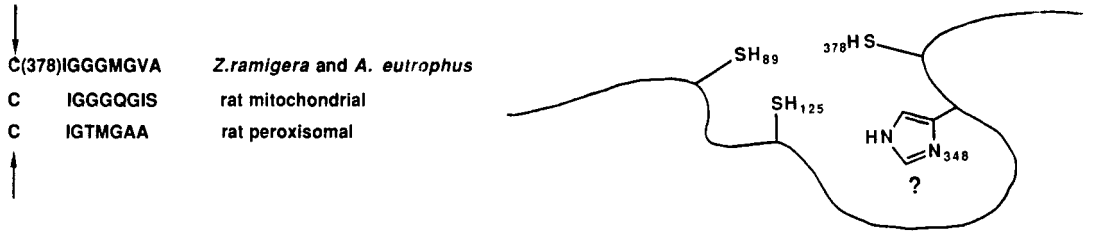
c. Affinity Labelled Inactivators: Bromoacetyl-S-Pantetheine 11-Pivalate and Acryloyl-S-Pantetheine 11-Pivalate. Although the alkylating reagent iodoacetamide demonstrated its kinetic specificity towards Cys-89 of thiolases, it bears no structural similarity to AcSCoA. Therefore, the affinity labelled inactivators, bromoacetyl-SPP and acryl-SPP, have attracted our interest, as each of them incorporates the SPP moiety, an effective CoA substitute, and also two electrophilic functionalities which not only react with Cys-89 but also may capture a "hidden" nucleophilic residue that has thus far eluded our attempts at modification. As it turns out, *Zoogloeal* thiolase undergoes two competitive reactions to provide acylated and alkylated enzymes. While the alkylation proceeds irreversibly, the acylated enzyme is subject to hydrolytic cleavage to regenerate active enzymes for recycling and eventually all the thiolase is alkylated at Cys-89. Hoped-for intramolecular alkylation of the acylated enzyme did not occur. Inactivation of enzyme with acryl-SPP followed very similar courses, again partitioning between acylation and Michael type alkylation. Our enzyme modifications thus far appeared to prove nothing but the exceptional reactivity of Cys-89. Therefore, it was to our surprise we discovered, after [^{3}H]-$NaBH_4$ reduction of inactivated enzyme followed by tyrptic digestion, that the Michael addition with

Scheme 3



Affinity-labelled Inactivators : Bromoacetyl-S-Pantetheine 11-Pivalate and Acryl-S-Pantetheine 11-Pivalate

acryl-SPP involved Cys-378 rather than Cys-89. The decreased energy required for the Michael reaction compared with the S_N2 type replacement (of bromoacetyl-SPP) together with a favorable disposition of the acryl functionality for Cys-378 may account for this successful capture of a new residue. The region starting with Cys-378 is highly conserved for all four thiolases. In order to assess if nucleophilic Cys-378 functions as the essential base, we are at present preparing Gly (or Ala)-378 thiolase by site-directed mutagenesis and expect the results to be available shortly. In summary, our search for the essential residues has shown that there are three cysteine residues and possibly a histidine residue in or near the active site.



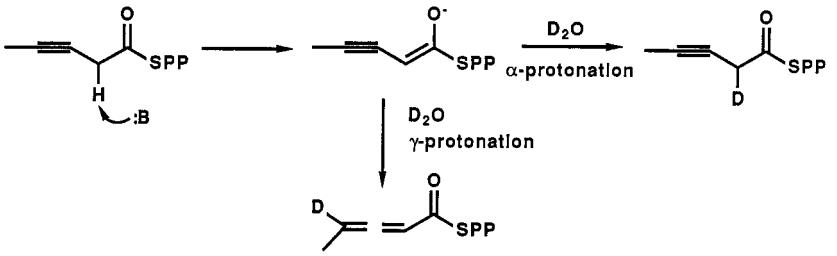
4. Search for deprotonation separate from C–C bond formation using 3-pentynoyl pantetheine 11-pivalate

The second half reaction of thiolase-catalyzed acetoacetyl ester condensation involves deprotonation of a second, bound AcSCoA molecule and nucleophilic attack of the resulting AcCoA anion on the acetyl group of acetyl-enzyme to complete the condensation.

Citrate synthase and maleate synthase, both of which effect aldol reactions, are capable of exchanging AcSCoA methyl hydrogens with solvent protons in the absence of C-C bond formation. In order to assess if thiolase possesses this capability we have used 3-pentynoyl-SPP as a mechanism-based inactivator, a strategy pioneered by Bloch and colleagues (ref. 21) and since then having been applied in many cases by others.

Doubly activated by the two functionalities the α-hydrogens of pentynoyl-SPP are highly acidic (pKa estimated to be between 12 and 15). Under mild conditions this thiol ester undergoes rapid deuterium exchange at the α position (α-protonation) as well as isomerization to the 2,3-pentadienyl thiol ester (g–protonation), the former process being much faster than the latter. We have found even the isomerization proceeds fast. However, since it is general base-catalyzed, the decrease in buffer concentration lowers its rate. Thus, in 0.01M KPi, pH 7.0, 1 mM EDTA, the alkynoyl ester has a half-life of 29 hours, long enough to permit one to comfortably observe the enzymic process. We have found that both the alkynoyl and allenoyl esters inactivate *Zoogloeal* thiolase and the latter (Ki = 1 mM, k_{inact} = 0.23 min^{-1}) does so 6-fold as efficiently as the former. The observed enzymic inactivation with the alkynoyl ester, in all likelihood, proceeds through enzymic abstraction of a C_2-proton from the alkynoyl ester as the rate-determining step to generate the allenoyl ester which subsequently reacts with a nucleophile in the active site. This result can be taken as a definite piece of evidence to show that thiolase can cleave a C-H bond in a transition state unconnected from C-C bond formation or cleavage.

Scheme 4



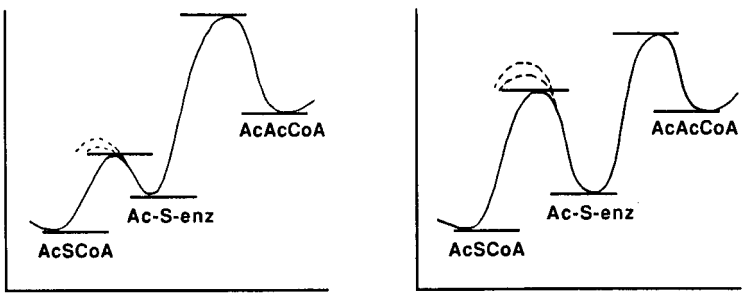
Several interesting observations have been made. The nucleophile referred to above has been identified as Cys-378, the residue that underwent a Michael addition to the inactivator acryl-SPP. Protection studies with AcAcOPP which bind in the active site as reversible inactivator have substantiated the kinetic protection anticipated for an active site process, but interestingly enough preincubation with the substrate AcAcSCoA in a concentration equal to 1 to 2 K_M led to a 30 fold increase in enzyme inactivation rate. This acceleration probably reflects prior formation of the acetyl-S-enzyme intermediate in which the active site base becomes more active towards protons and carbon centers.

Finally the deuterium isotope effect has been examined using 2-[$^2H_2$]-3-pentynoyl-SPP. While the inactivation without preincubation with AcAcSCoA showed no isotope effect, thiolase preincubated with the substrate displayed k_H/k_D = 1.8 on inactivation rate. The interpretation of this small primary isotope effect must be made with caution, but the result can be corroborated into data presented in the next section.

5. Energy profile of the *Zoogloeal* thiolase catalyzed reactions

It has already been shown that the thiolase catalyzed reactions involve the acetyl enzyme intermediate and the equilibrium of the overall reactions highly favor thiolytic cleavage of AcAcSCoA. In order to define the rate-limiting step in this thiolytic (reverse) direction we have conducted V_{max} analysis of the thiolysis using cosubstrates (RSH) of structural dissimilarity. If formation of acetyl-enzyme were the rate-limiting step (Case 1 below), then thiolysis V_{max} ratios, V_{max} (RSH)/V_{max} (CoASH), should be insensitive to the identity of the thiol used regardless of its affinity. Conversely, if the rate-limiting process were the acetyl transfer step from the acetyl enzyme to a thiol acceptor (Case 2), the V_{max} ratios would vary. We have found that the ratios change with thiols and typically, the V_{max} (CoASH)/V_{max} (mercaptol) = 2.3. This indicates that the acyl transfer half reaction is at least partially rate-limiting in the cleavage direction.

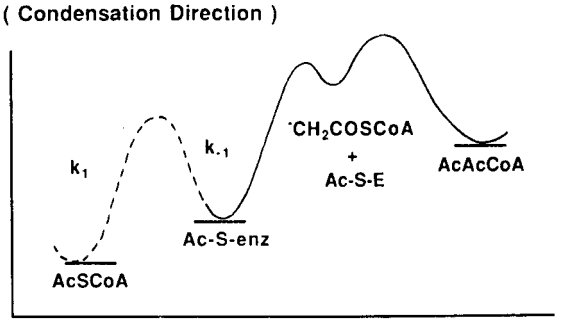
Rate-Determining Step in the AcAcCoA Cleavage Reaction



Case 1 Case 2

The acetyl enzyme intermediate is isolable, but its hydrolytic lability has slowed our studies on the first half reaction of the bio-Claisen condensation. We have not yet obtained a reliable value for K_1 = [E-Ac][CoASH]/[AcSCoA], nor k_1 and k_{-1} (see diagram below), the two kinetic parameters associated with this acyl transfer reaction to form the intermediate, although these k_1 and k_{-1} value will be in hand shortly.

Deuterium Isotope Effect Studies with $CD_3COSCoA$
(Condensation Direction)



The effect of deuterium substitution at the α–carbon of AcSCoA on the kinetics of the overall reaction in the synthesis direction has been determined to be V_H/V_D = 2.4. Since the first half reaction would not be expected to exhibit an extraordinary large secondary kinetic isotope effect (ref. 22), the observed deuterium isotope effect is, in all likelihood, displayed by the deprotonation reaction, a step necessarily involved in the second half, enzyme-deacylation reaction. If this inference is correct, it implies that in the forward condensation direction the second half reaction proceeds more slowly than the first half. Furthermore it tends to favor the interpretation that deprotonation of the second AcSCoA provides an, at least partially, discrete anion through a transition state separate from the C-C bond forming reaction, consistent with the view expressed earlier on the basis of the inactivation experiment with 3-pentynoyl SCoA (see above). These interpretations will be critically examined with the expected completion of our kinetic studies on the first half reaction.

Thiolase I and II have been kinetically characterized by Gilbert (ref. 17) and Gehring (ref. 14), respectively. Both catalyze the same reaction through an acetyl enzyme intermediate, but there are distinct kinetic differences between the two enzymes, with respect to the location of the rate-limiting step. For Thiolase I, the AcAcSCoA carbon-carbon cleavage step is rate-limiting in the thiolytic (reverse) process and enzyme acylation is rate-limiting in the acyl transfer (forward) direction. This is just the opposite from Thiolase II which is rate-limiting in the deacylation half reaction in the thiolytic direction and rate determining in the carbon-carbon bond forming half reaction in the C-C bond forming forward direction. Our studies show that thiolase from *Zoogloea ramigera* is similar to Thiolase II in that it appears to have the same qualitative type of free energy profile. This is consistent

with the fact that both Thiolase I and *Z. ramigera* are biosynthetic enzymes and short chain (AcAcSCoA) specific. Thiolase II has broad specificity for long chain β–ketoacyl CoA substrates and is involved in degradative fatty acid β-oxidation. With *Zoogloeal* thiolase we have succeeded for the first time in identifying the essential residues in the complete amino acid sequence of the thiolase and will likely succeed in securing decisive information as to the timing of deprotonation and C-C bond coupling. An obvious next step is a crystallographic analysis of the enzyme which is now underway.

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APPENDIX: SUMMARY OF MOLECULAR BIOLOGY STUDIES ON PHB BIOSYNTHESIS

1. Isolation of the PHB Biosynthetic Thiolase Gene from *Zoogloea ramigera*

The purified thiolase protein from *Z. ramigera* was used to raise antibodies in New Zealand White rabbits. An expression library of random 4 Kb (DNase 1 digestion) fragments of *Z. ramigera* chromosomal DNA was constructed in the vector λgt11 and screened using the anti-thiolase antibodies. Two different clones giving a positive signal were identified in the initial screen. A combination of enzyme assays and Western blot experiments identified clone LDBK1 as containing the thiolase gene. Nucleotide sequence analysis located the structural gene region and the complete sequence of this region was determined. Experimentally determined amino acid sequence data from the N-terminus and active site regions was used to correctly align the gene sequence. The structural gene is 1176 nucleotides long encoding a polypeptide of 392 amino acids. These data are described in more detail by Peoples *et. al.* (1987).

2. Overproduction of the *Z. ramigera* Thiolase in *E. coli*

In order to express high levels of the *Z. ramigera* thiolase enzyme in *E. coli,* the structural gene was inserted in front of the highly efficient synthetic *E. coli tac* promoter in the vector pKK223-3 (Pharmacia). After deleting regions of the *Z. ramigera* sequence located upstream from the thiolase structural gene, a plasmid, pZT3.5 was identified which yields around 25% of total cellular protein as soluble active thiolase after IPTG induction for 15 hours. Thiolase was purified by a rapid purification method, first on 1 DEAECL6B column and then affinity chromatography on Red gel agarose. Typically 150 mg of pure thiolase can be obtained from 1 liter of induced *E. coli* cells containing plasmid pZT3.5. A derivative of pZT3.5 in which an SphI site in the vector was deleted enables the thiolase structural gene to be cut into three fragments for site-directed mutagenesis experiments.

3. *Z. ramigera* NADP-Specific Acetoacetyl-CoA Reductase Gene

Enzyme assays of lysates of *E. coli* cells containing a series of expression plasmids constructed from the insert of plasmid pUCDBK1 (Peoples *et al.*, 1987) identified the gene encoding NADP-specific Acetoacetyl-CoA reductase in the 2.3 kb of *Z. ramigera* DNA located downstream from the thiolase gene in this clone. SDS-PAGE analysis revealed a Mr 25,000 polypeptide in those lysates which had high levels of reductase activity. This polypeptide was purified from the lysate of *E. coli*/PZR14, which has the highest level of activity, by electroelution and the N-terminal sequence determined. Nucleotide sequence analysis of the entire 2.3 kb fragment identified a single open reading frame starting 88 bp downstream from the thiolase stop codon. Residues 2-6 predicted from translation of this open reading frame are identical to the sequence obtained by Edman degradation of the purified Mr 25,000 polypeptide. Hence the NADP-specific acetoacetyl-CoA reductase gene encodes a 241 residue polypeptide. From these data it appears that in *Z. ramigera* at least the first two enzymes of the PHB biosynthetic pathway are organized as an operon.

4. Isolation and Characterization of the Genes Encoding Thiolase and NADP-Specific Acetoacetyl-CoA Reductase from *Alcaligenes eutrophus*

The PHB biosynthetic thiolase gene from *Z. ramigera* was used as a hybridization probe to screen restriction digests of *A. eutrophus* H16 DNA. Specific hybridization signals were obtained and two fragments, a 2.3 kb PstI fragment and a 15 kb EcoR1 fragment cloned into the *E. coli* plasmid vector pUC8 (clones pAET3/10 and pAET29, respectively). Enzyme assays carried out on lysates of *E. coli* containing each plasmid identified thiolase and NADP-specific acetoacetyl-CoA reductase activity encoded by plasmids pAET10 and pAET29 but not pAET3. As pAET3 and pAET10 contain the same insert but in reverse orientation with respect to the lacZ promoter in the vector pUC8, it is likely that the expression of thiolase and reductase activity by pAET10 is driven by this promoter. This would also account for the 10-fold lower level of both activities expressed by pAET10 as compared to pAET29. The complete nucleotide sequence of the 2.3 kb Pst1 insert is plasmid pAET3/10 was determined. Two open reading frames were identified extending from nucleotide 28 to 1219 and from 1149 to 2034. This is very similar to the organization of the thiolase and reductase genes in *Z. ramigera.* The nucleotide sequence of the *Z. ramigera* thiolase structural gene shares 69% identity to the first open reading frame. Similarly, comparing *Z. ramigera* reductase gene sequence with the second open reading frame revealed 72% identity over the last two thirds of each sequence. The first 160 bp of each reductase sequence were not significantly homologous indicating that these two genes may have evolved independently. These findings were further substantiated when the protein sequences from the two species were compared. The thiolase amino acid sequences were 86% identical whereas the identity was only 55% for the reductases.

In order to carry out kinetic and substrate specificity studies on *A. eutrophus* thiolase and reductase, overproduction vectors were constructed with the tac vector pKK223-3. Using *E. coli* JM105 containing pAT5 (thiolase) or pAR1 (reductase) large quantities of these two enzymes are readily purified for investigation. The *A. eutrophus* reductase is currently being assayed for its ability to use a range of alternate substrates. These experiments have already been completed for the thiolase (Michelle Palmer).

5. Isolation of PHB Polymerase Gene(s)

The complete absence of either pure polymerase with which to raise antibodies or obtain amino acid sequence data for the design of oligonucleotide probes led us to take a more traditional genetic approach to identifying the gene(s) for PHB polymerase. A series of mutants defective in PHB biosynthesis were constructed using the transposable genetic element Tn5 as the mutagen. A library of *A. eutrophus* Tn5-containing, i.e., kanamycin resistant strains was constructed and mutants defective in PHB biosynthesis identified by the color of the colonies they form when grown on nitrogen-limited mineral media agar plates. Three types of PHB-negative mutant classes were identified based on the chromosomal location of the Tn5 inserted in each case. This was based on the results of Southern hydribization experiments using ^{32}P-labelled Tn5 DNA as a probe. Enzymatic analysis of each of these strains identified thiolase and reductase enzyme activities but no PHB polymerase activity. Complementation of the mutants back to the PHB positive phenotype using a pLAFR3 (broad host range vector) gene library of *A. eutrophus* wild type DNA identified clones containing the PHB polymerase gene(s). The location of the PHB polymerase structural gene(s) is currently being mapped by subcloning experiments.