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Biosynthetic Thiolase from *Zoogloea ramigera*

EVIDENCE FOR A MECHANISM INVOLVING CYS-378 AS THE ACTIVE SITE BASE*

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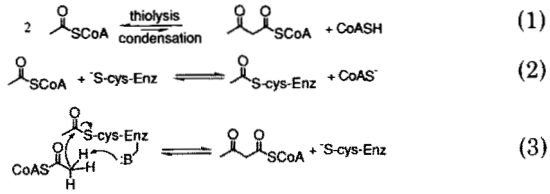
Biosynthetic thiolase from *Zoogloea ramigera* was inactivated with a mechanism-based inactivator, 3-pentynoyl-*S*-pantetheine-11-pivalate (3-pentynoyl-SPP) where K_I = 1.25 mM and k_{inact} = 0.26 min^{-1}, 2,3-pentadienoyl-SPP obtained from nonenzymatic rearrangement of 3-pentynoyl-SPP where K_I = 1.54 mM and k_{inact} = 1.9 min^{-1} and an affinity labeling reagent, acryl-SPP. The results obtained with the alkynoyl and allenoyl inactivators are taken as evidence that thiolase from *Z. ramigera* is able to catalyze proton abstraction uncoupled from carbon–carbon bond formation. The inactivator, 3-pentynoyl-SPP and the affinity labeling reagent, acryl-SPP, trap the same active site cysteine residue, Cys-378. To assess if Cys-378 is the active site residue involved in deprotonation of the second molecule of acetyl-CoA, a Gly-378 mutant enzyme was studied. In the thiolysis direction the Gly-378 mutant was more than 50,000-fold slower than wild type and over 100,000-fold slower in the condensation direction. However, the mutant enzyme was still capable of forming the acetyl-enzyme intermediate and incorporated 0.81 equivalents of ^{14}C-label after incubation with [^{14}C]Ac-CoA for 60 min. The reversible exchange of ^{32}P-label from [^{32}P]CoASH into Ac-CoA, catalyzed by the Gly-378 mutant enzyme, proceeded with a V_{max} (exchange) 8,000-fold less than the wild type enzyme but at least 10-fold faster than the overall condensation reaction. These data provide evidence that Cys-378 is the active site base.

Biosynthetic thiolase from *Zoogloea ramigera* catalyzes the condensation of two acetyl-CoA molecules (Equation 1), utilizing one as an electrophile at C-1 by acetyl-*S*(cys)-enzyme formation (Equation 2), and the other as a C-2 carbanion equivalent (Equation 3). Three important issues to be addressed in understanding the catalytic mechanism of thiolase are identification of the amino acid residue acylated in the first half-reaction, the relationship of the rate of proton abstraction with respect to carbon–carbon bond formation, and identification of the amino acid residue which acts as a base to remove a proton from the second acetyl-CoA molecule prior to or concomitant with carbon–carbon bond formation. In earlier work (1, 2) we presented evidence which indicated



(1)

(2)

(3)

that Cys-89 was involved in acetyl-enzyme formation. Subsequently this covalent intermediate has been characterized and Cys-89 identified as an essential catalytic residue by means of several techniques including site-directed mutagenesis (3). Three strategies (group selective modification, affinity labeling, and mechanism-based inactivation) have been used in efforts directed toward the identification of the active site base responsible for C-2 deprotonation of the second bound molecule of Ac-CoA.[1] Detailed studies using 5,5′-dithiobis(2-nitrobenzoate) are complex and experiments with diethyl pyrocarbonate proved to be ambiguous.[2]

Mechanism-based inactivation using suicide substrates has been successfully used to investigate acyl-CoA and acyl-ACP enzymes following the observation that 3-decynoyl-*N*-acetylcysteamine efficiently inactivates β-hydroxythioester dehydratase by labeling a nucleophilic histidine residue (4, 5). Subsequently, inactivation of butyryl-CoA dehydrogenase with 3-pentynoylpantetheine led Fendrich and Abeles (6) to conclude that the γ-carboxyl group of a glutamate residue had been alkylated. The reaction of (degradative) pig heart thiolase with 3-butynoyl- and 3-pentynoyl-CoA has also been reported (7). In each case the mechanism of inactivation involves an isomerization of the 3-alkyne to the 2,3-allene (by α-deprotonation and γ-protonation) with subsequent inactivation by Michael addition of an active site nucleophile to the allene (8). Support for this base-catalyzed mechanism is based on a kinetic deuterium isotope effect observed with an α,α-dideuterated inactivator (9, 10).

In this paper we describe inactivation studies of *Z. ramigera* thiolase with the mechanism-based inactivator, 3-pentynoyl-SPP, and with a new affinity labeling reagent, acryl-SPP. Both reagents trap the same active site cysteine residue Cys-378. Site-directed mutagenesis of this residue to glycine provides a mutant enzyme which can no longer catalyze the deprotonation step but can still form the acetyl-*S*-enzyme intermediate, effecting the first half-reaction. Evidence pre-

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[1] The abbreviations used are: Ac-CoA, acetyl-coenzyme A; SPP, pantetheine-11-pivalate; AcAc-CoA, acetoacetyl-CoA; DTT, dithiothreitol; ME, β-mercaptoethanol; DCC, *N,N′*-dicyclohexylcarbodiimide; BrAc-SPP, 3′-bromoacetyl-S-pantetheine-11-pivalate; DMSI, dimethyl suberimidate; ACP, acyl camer protein; AcAcOPP, acetoacetyl-pantetheine 11-pivalate *O*-ester.

[2] S. Masamune, unpublished results.

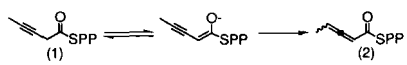
sented below strongly indicates that this second essential cysteine residue acts as the catalytic base in this homotetrameric enzyme. The timing of proton abstraction from the second acetyl-CoA molecule with respect to carbon–carbon bond formation is discussed.

EXPERIMENTAL PROCEDURES[3]

RESULTS AND DISCUSSION

The second half-reaction of the thiolase-catalyzed Claisen condensation involves the deprotonation of a second bound Ac-CoA molecule followed by nucleophilic attack on the acetyl-enzyme intermediate (Equation 3). In an effort to identify the active site basic residue responsible for deprotonation, our initial studies focused on the affinity labeled inactivator bromoacetyl-*S*-pantetheine-11-pivalate (BrAcSPP) (11). This inactivator incorporates the SPP moiety as an effective CoA substitute and two electrophilic functionalities which have the potential to capture the active site basic residue by addition to Cys-89 (the site of acylation). In practice the enzyme underwent two competitive reactions, acylation and alkylation. While alkylation proceeded irreversibly, the acylated enzyme was subject to hydrolytic cleavage to regenerate active enzyme. Eventually all the thiolase was alkylated at Cys-89, and no intramolecular alkylation of the acylated enzyme occurred. In response to the exceptional reactivity of Cys-89, we turned our attention toward the mechanism-based inactivator 3-pentynoyl-SPP and the affinity labeling reagent acryl-SPP.

Activated by two functionalities the α-hydrogens of 3-pentynoyl-SPP are highly acidic with pK_a values comparable to an acetoacetyl ester (12). Under mild nonenzymatic conditions, these thioesters undergo rapid deuterium exchange at the α-position (α-protonation) as well as isomerization to 2,3-pentadienyl thioesters (γ-protonation) (Equation 4).



(4)

Since thiolase cannot form a C_3 or C_4 acyl-enzyme intermediate in the condensation reaction (1), this inactivator provides an opportunity to determine whether this enzyme can cleave a carbon–hydrogen bond in a transition state unconnected from carbon–carbon bond formation.

Appropriate conditions under which to study the enzyme inactivation were examined and the inactivator 3-pentynoyl-SPP was found to be stable enough ($t_{0.5}$ = 29 h) in 0.01 M KP_i, 1 mM EDTA, pH 7.0, to comfortably observe the enzymatic process. Incubation of thiolase with 3-pentynoyl-SPP (at 250, 500, and 1,000 μM) at pH 7 in 0.01 M KP_i, 1 mM EDTA buffer resulted in a loss of activity. Extrapolation from a plot of the half-life for inactivation *versus* 1/[inhibitor] gave K_I = 1.25 mM and k_{inact} = 0.26 min^{-1}, and under similar conditions, the allene **2** inactivated thiolase 7-fold faster with K_I = 1.54 mM and k_{inact} = 1.9 min^{-1} (13). Comparison of k_{inact}/K_I for alkyne **1** and allene **2** indicated that if the alkyne was not responsible for inactivation, approximately 17% of the allene would be required as a contaminant to achieve inactivation. Since UV (see "Experimental Procedures") and NMR measurements indicate that the isomeric purity of 3-pentynoyl-SPP was greater than 99%, mechanism-based inactivation by 3-pentynoyl-SPP (**1**) must have occurred.

[3] Portions of this paper (including "Experimental Procedures" and Figure 1 of "Results and Discussion") are included in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size copies are included in the microfilm edition of the Journal available from Waverly Press.

If inactivation by the acetylenic inhibitor (**1**) was initiated by enzymatic C_2-H cleavage at the active site, followed by γ-protonation to give the allene (**2**), then the natural substrates (Ac-CoA and AcAc-CoA) and substrate analog AcAc-OPP (1) would be expected to exhibit kinetic protection for inactivation. Interestingly, the rate of inactivation (at an inactivator concentration of 2 mM) was largely dependent on the concentration of the substrate used for preincubation (Fig. 2). At high concentrations of AcAc-CoA (>2 K_M) a slight protection for inactivation was observed; however, lowering the concentration of AcAc-CoA resulted in an increase in the inactivation rate, with a maximum at 50 μM. At this concentration of AcAc-CoA the half-life for inactivation was determined for a range of inactivator concentrations (0.5–2 mM data not shown). By plotting $t_{1/2}$ against 1/[inactivator], we obtained k_{inact} = 7 min^{-1}, which is at least a 30-fold increase in the rate of inactivation. These results may be interpreted in the following manner. Thiolase cleaves AcAc-CoA to yield the acetyl-*S*-enzyme intermediate which enhances the enzyme's reactivity toward proton abstraction. However, at high concentrations of AcAc-CoA competitive inhibition protects for the inactivator. This interpretation is supported by the fact that AcAc-OPP, a competitive inactivator, which is not cleaved by thiolase and is therefore unable to form an acetyl-enzyme intermediate (1), protected (rather than activated) thiolase from inactivation (Fig. 2). These results are consistent with Bloxham's observations (7) on pig heart thiolase. Inactivation with 3-butynoyl- and 3-pentynoyl-CoA at high concentrations of AcAc-CoA afforded kinetic protection, whereas in certain cases an increase in the inactivation rate in the presence of Ac-CoA was observed.

Inactivation of thiolase with 3-pentynoyl-SPP (**1**), after preincubation with AcAc-CoA (2 mM), resulted in a loss of activity (10% residual thiolysis activity after 18 min). No activity was recovered when partially inactivated thiolase was gel filtered or incubated with a nucleophile (CoASH), which would cleave an acyl-*S*-enzyme species. This indicated that the linkage between inactivator and enzyme was not a thioes-

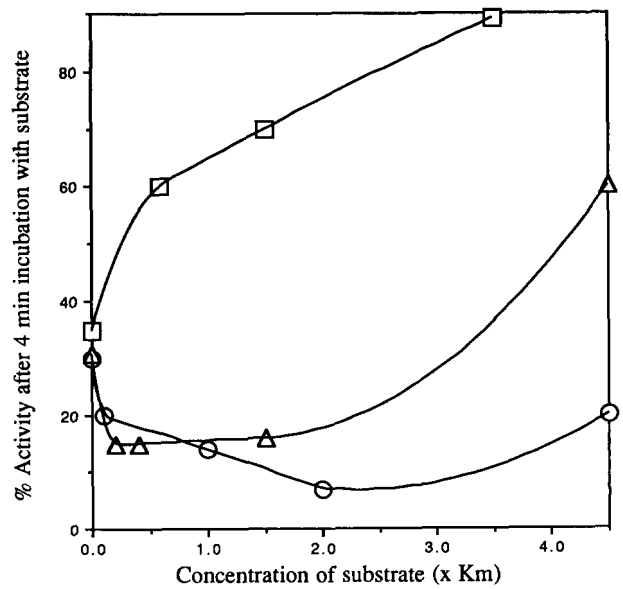
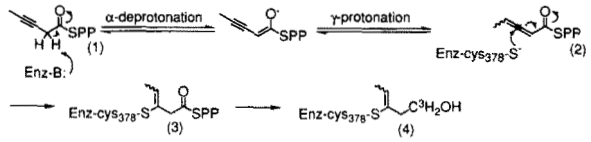


FIG. 2. **Rate of inactivation of thiolase (1 μM) by 3-pentynoyl-SPP (1) in the presence of Ac-CoA (△), AcAc-CoA (○) or AcAc-OPP (□).** Thiolase was preincubated with increasing amounts of substrate and the % activity remaining after addition of 3-pentynoyl-SPP (2 mM final concentration) plotted against substrate concentration [K_M = 330 μM (Ac-CoA), 25 μM (AcAc-CoA), and ~80 μM (AcAc-OPP)].

ter bond (due to acylation at Cys-89). Initial studies with [^{14}C]3-pentynoyl-SPP (specific activity 1 nCi/nmol) indicated that the inactivated enzyme was stoichiometrically labeled after gel filtration (labeled enzyme specific activity 1.1 nCi/nmol), but when the protein was denatured and/or digested the linkage of labeled inactivator to enzyme was found to be labile. It is possible that the basic conditions used in the isolation resulted in this loss of label by a retro-Michael reaction. The stability of the adduct was, however, increased sufficiently by borohydride reduction to allow isolation of the modified peptide. Thus, unlabeled **1** was used to inactivate the enzyme, and after gel filtration the enzyme was denatured and reduced with [^{3}H]$NaBH_4$ solution and a radiolabeled tryptic peptide was isolated, sequenced, and Cys-378 identified as the modified residue (see "Experimental Procedures").

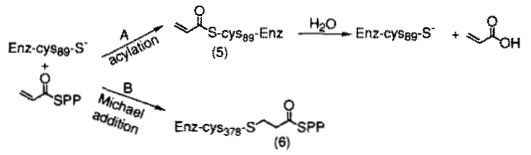
The proposed mechanism of inactivation is depicted in Scheme 1. Deprotonation at the α-position of 3-pentynoyl-



SCHEME 1

SPP **1** is partially rate limiting (with AcAc-CoA preincubation), generating the allenoyl ester **2** by γ-protonation. The latter subsequently reacts with Cys-378 to give a Michael adduct **3**, resulting in inactivation of thiolase catalysis. Initial formation of an alkynoyl thioester intermediate by acylation of Cys-89 by the inactivator is excluded since thiolase cannot form a C_4 acyl-enzyme intermediate (1). [^{3}H]$NaBH_4$ reduces **3** to provide **4**. It is interesting to note that while γ-protonation of the inactivator catalyzed by thiolase (as shown in Scheme 1) must proceed at least at the net inactivation rate of 0.26 min^{-1} (or 650-fold faster than the uncatalyzed isomerization), the rate of the enzyme catalyzed exchange of the α-protons with solvent is less than 0.015 min^{-1} (see "Experimental Procedures"). The enzyme, therefore, favors selective reprotonation of the delocalized carbanion at the γ-site by a factor of 0.26:0.015 ~ 17 (compared to less than 1:100 in solution) and forms the inactivating allenic thioester. These experiments indicate that thiolase, like malate (14) and citrate synthase, is able to catalyze proton abstraction uncoupled from carbon–carbon bond formation. This evidence is supported by recent findings that thiolase can catalyze rapid exchange of the α-hydrogens of acetyldithio-CoA, a kinetically competent nucleophilic substrate (15).

Acryl-SPP, like BrAcSPP, also has two electrophilic groups in addition to the SPP moiety. Small amounts of this inactivator (1.8–4.0 equivalents/monomer) did not completely inactivate thiolase. After an initial rapid drop in activity the enzyme regenerated 53–89% of its initial activity depending on the amount of inactivator used (Fig. 3). These results indicated that acryl-SPP was inactivating thiolase by partitioning between acylation and Michael reaction (Scheme 2),



SCHEME 2

similar processes to those previously observed for BrAcSPP (11). While hydrolysis of the acylated enzyme **5** can regener-

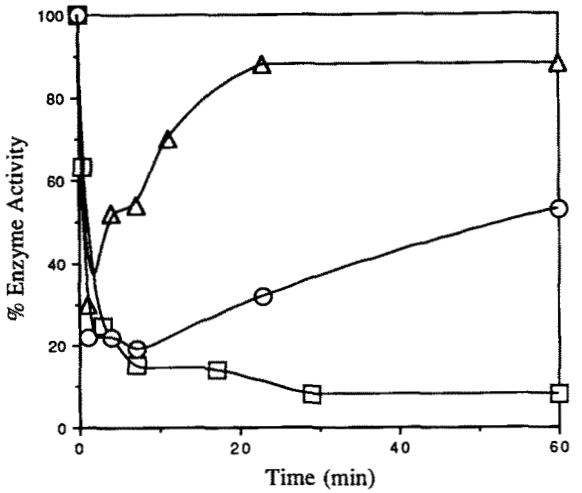


FIG. 3. **Inactivation of *Z. ramigera* thiolase by acryl-SPP.** Thiolase (21 μM) was inactivated at three concentrations of inactivator, (△, 1.8 eq; ○ 4.0 eq; and □, 20 eq).

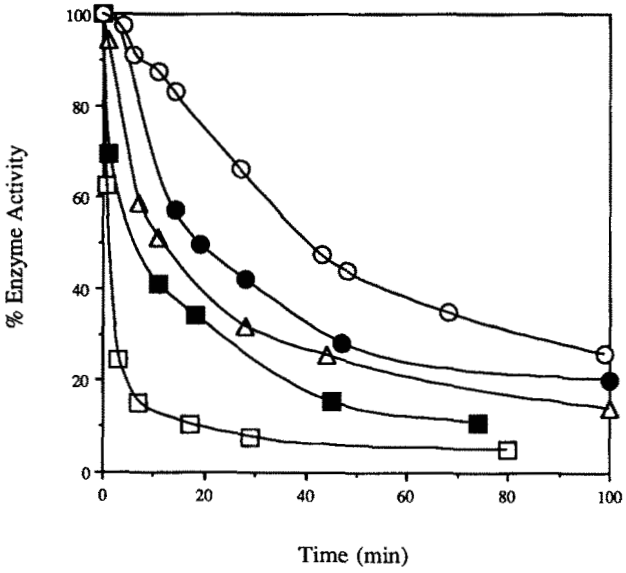


FIG. 4. **Inactivation of thiolase by acryl-SPP after preincubation with AcAc-CoA.** Thiolase (1 μM) was inactivated by acryl-SPP (20 eq) after the addition of varying amounts of AcAc-CoA (□, 0 μM; ■, 5 μM; △, 50 μM; ●, 100 μM; and ○, 500 μM).

ate the active enzyme and acrylic acid, Michael addition to give **6** proceeds more slowly than pathway **A** but irreversibly. Thus, at low concentrations (<4 equivalents) of acryl-SPP, most of the inactivator was consumed by hydrolysis (pathway **A**) and only small amounts (0.11–0.47 equivalents/subunit) caused permanent inactivation via pathway **B**. Higher concentrations of inactivator (20 equivalents/subunit) resulted in almost exclusive inactivation after 60 min (Fig. 3), and studies with [1-^{14}C]acryl-SPP indicated that one equivalent of label was covalently bound/monomer subunit (data not shown). These results suggested that thiolase was reacting with acryl-SPP at the active site Cys-89, as observed for iodoacetamide and BrAc-SPP, before irreversible inactivation occurred. To suppress acylation at Cys-89, the enzyme was preincubated with various amounts of AcAc-CoA to form the acetyl-*S*-enzyme intermediate at this residue, prior to the addition of inactivator. As expected the rate of inactivation decreased as more AcAc-CoA was added (Fig. 4), the most dramatic change in the rate of inactivation occurred between 0 and 50 μM AcAc-CoA (K_M(AcAc-CoA) = 25 μM) (Fig. 5),

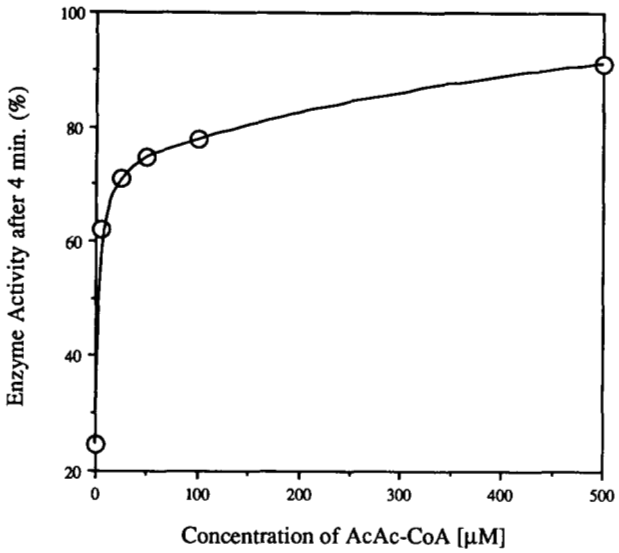


FIG. 5. **Rate of inactivation of *Z. ramigera* thiolase with acryl-SPP in the presence of AcAc-CoA.** Thiolase (1 μM) was preincubated with increasing concentrations of AcAc-CoA and inactivated with acryl-SPP (20 eq).

ZRAT (373)	- G L A T L	C I - G G G M G V A M
AEAT (374)	- G L A S L	C I - G G G M G V A L
SUAT (379)	- G V A A I	C N - G G G G A S S V
RMAT (375)	- G L A S I	C N - G G G G A S A V
ECKT (368)	- G L A D G	C V S G L G Q G I A T
RMKT (376)	- A V G S A	C I - G G G Q G I S L
RPKT (376)	- G V V S M	C I - G T G M G A A A
HPKT (404)	- G V V S M	C I - G T G M G A A A

FIG. 6. **Conservation of residues corresponding to *Z. ramigera* thiolase Cys-378.** The amino acid sequences in the region corresponding to the *Z. ramigera* thiolase putative active site base Cys-378 of all thiolases reported to date were compared. Both type I and type II sequences are shown. Data were obtained from the following sources: *ZRAT*, *Z. ramigera* acetoacetyl-CoA thiolase (2); *AEAT*, *A. Alcaligenes eutrophus* acetoacetyl-CoA thiolase (18); *SUAT*, *Saccharomyces uvarum* acetoacetyl-CoA thiolase (19); *RMAT*, rat mitochondrial acetoacetyl-CoA thiolase (20); *ECKT*, *Escherichia coli* β-ketothiolase (21); *RMKT*, rat mitochondrial β-ketothiolase (22); *RPKT*, rat peroxisomal β-ketothiolase (23); and *HPKT*, human peroxisomal β-ketothiolase (24). Amino acids conserved in all thiolase sequences including the putative active site base cysteine ⊛ are *highlighted*.

above 50 μM the rate reaches a limiting value. Furthermore, the level of enzyme activity obtained 50 min after cleavage of adducts of type **5** (Scheme 2) by a large excess of CoASH decreased as the amount of AcAc-CoA used in preincubation was increased. Since less inactivator will be consumed by path **A** when AcAc-CoA competes with the inactivator for Cys-89, path **B** must become more competitive indicating that another essential residue, other than Cys-89, may have been labeled. This Michael reaction was then found also to involve Cys-378 (see "Experimental Procedures"), the same active site cysteine residue modified by 3-pentynoyl-SPP. A favorable disposition of the acryl functionality for Cys-378 and/or a decreased energy requirement for the Michael reaction compared with the S_N2 type reaction (as in the case of BrAc-SPP) may account for the successful capture of this new residue.

Similar experiments with the Ser-89 mutant (3) resulted in inactivation of the mutant enzyme in less than 5 min with only one equivalent of acryl-SPP. In this mutant the acetyl-*O*-enzyme intermediate forms much more slowly than the wild type thioester adduct **5** (Scheme 2) and is therefore a much less competitive step compared to Michael alkylation which results in inactivation. Purification of a single labeled tryptic peptide and amino acid sequence analysis again identified Cys-378 as the modified residue (data not shown). The nucleophilic capture of Cys-378 by both the allenic and acryl inactivators and the conservation of this residue for all seven thiolases whose structural genes have been sequenced (Fig. 6) suggested that this residue might be responsible for the catalytic deprotonation of the second molecule of acetyl-CoA in the second half-reaction (Equation 3). These observations led us to prepare a Gly-378 mutant enzyme using site-directed mutagenesis.

The Gly-378 mutant was shown to exist as a homotetrameric enzyme by dimethyl suberimidate cross-linking (data not shown), a result consistent with the mutant enzyme assembling in the same three-dimensional conformation as wild-type. However, the Gly-378 mutant enzyme could not be assayed in the standard fashion (1) due to its very low activity. The assay in the thiolysis direction was performed by monitoring the consumption of AcAc-CoA over 100 min, using a concentration of mutant enzyme which was 50,000-fold that of wild type (50 μg *versus* 1 ng). Under these conditions only background activity was observed, and it was concluded that the mutant was at least 50,000 times slower than wild type in the thiolysis direction. To assay the mutant enzyme in the condensation direction, the mutant enzyme was used at a concentration which was 100,000-fold that normally used for the assay of wild type. The consumption of Ac-CoA was followed in a coupled assay in which the product of the condensation, AcAc-CoA, was immediately reduced to β-hydroxybutyryl-CoA. Again the mutant enzyme displayed only background activity, and it was concluded that the Gly-378 mutant was at least 100,000 times slower than wild type in the condensation direction. Clearly the Cys to Gly mutation at residue 378 profoundly depresses carbon–carbon bond formation and cleavage activity.

If Cys-378 acts as the catalytic base in the second half-reaction, the Gly-378 mutant enzyme should still be capable of effecting the first half-reaction, which involves the formation of the acetyl-enzyme intermediate (see Equations 1–3). Formation of this intermediate was followed over a period of 60 min by incubating the Gly-378 mutant enzyme with [^{14}C] Ac-CoA and precipitating the protein with acetone/HCl at 0 °C. After periods of 15 and 20 min, the mutant enzyme had incorporated 0.41 and 0.51 equivalents of ^{14}C label, respectively; the rate of incorporation then leveled off at 60 min with 0.81 equivalent/subunit bound. In comparison, wild type incorporates 0.86 equivalent of ^{14}C/subunit in less than 1 min (1) which means that the rate of formation of the mutant acetyl-enzyme intermediate was at least 60-fold slower than wild type. A more accurate estimate of this rate difference has been made by following the rate of exchange of [^{32}P] CoASH with Ac-CoA, catalyzed by the mutant enzyme (see below). Attempts to isolate the native mutant acetyl-enzyme species (0.81 equivalents/monomer) by gel filtration (Bio-Gel P-4) at 4 °C resulted in the loss of 99% of the bound radioactivity. The half-life of the native mutant acyl-enzyme intermediate was estimated to be less than 5 min compared to 2 min for the wild type acetyl-enzyme species (1).

Studies on the first half-reaction involving the reversible formation of the acetyl-enzyme intermediate can be monitored by determining the ability of the enzyme to catalyze the exchange of ^{32}P label from [^{32}P]CoASH into Ac-CoA (3, 16). Thus, although the Gly-378 mutant cannot detectably cata-

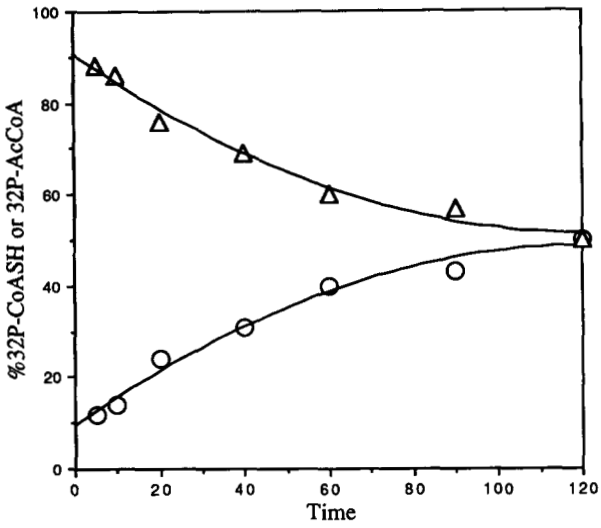


FIG. 7. **[^{32}P]CoASH-Ac-CoA exchange catalyzed by the Gly-378 mutant enzyme.** The Gly-378 mutant (1.2 μM) was added to a solution of 50 μM Ac-CoA and 50 μM [^{32}P]CoASH (△) and [^{32}P]Ac-CoA (○) determined by high performance liquid chromatography.

lyze the overall condensation, this experiment provides an opportunity to obtain kinetic parameters for the first half-reaction, enzyme acetylation at Cys-89. The acetyl-exchange reaction catalyzed by wild type thiolase proceeds with a V_{max} (exchange) of 163 units/mg, 2.8-fold faster than the overall forward reaction, and the K_M for the first molecule of Ac-CoA was found to be 350 μM (3). To observe a reasonable exchange rate for the Gly-378 mutant with 50 μM Ac-CoA and CoASH, a 1,000-fold excess of mutant enzyme (1.2 μM) over the normal wild type level was required to attain isotopic equilibrium in 110 min (Fig. 7). At this concentration V (exchange) was 0.01 unit/mg, but still the background rate accounted for less than 2% of the Gly-378 enzyme-mediated rate. Concentrations of Ac-CoA greater than 0.1 mM resulted in inhibition, and this compares with a value of 1 mM for wild type (3). Over a concentration range of 15 to 100 μM Ac-CoA, the K_M for Ac-CoA was found to be 35 μM. The Gly-378 enzyme has a 16,000-fold lower V_{max} for exchange than wild type Cys-378 and V/K criteria is 1,600-fold less efficient as a catalyst. While substantially impaired, the Gly-378 enzyme is nonetheless competent to carry out reversible self-acylation at least 6- (V_{max}) fold more effectively than the (nondetectable) carbon–carbon-bond forming step in the Claisen condensation process. The large decrease in the rate of acetyl-exchange may be due to the absence of base assistance in this reaction or a conformational change at the active site. Cys-378 is flanked by the amino acids leucine and isoleucine, so although a mutation to glycine should not cause a large steric difference some disruption at the active site due to a change in hydrophilicity cannot be ruled out.

In conclusion, mutation of Cys-378 to a glycine residue eliminated the ability of thiolase to catalyze proton abstraction from C_2 of acetyl-CoA but not its ability to form the acetyl-enzyme intermediate. The Gly-378 enzyme can still process acetyl-CoA as an electrophilic substrate but not as a nucleophilic substrate in the Claisen condensation. The experimental evidence gathered is consistent with the interpretation that the Cys-378 residue is acting as a catalytic base in the carbanion-generating half-reaction. It has been suggested that a cysteine residue acts as a base in proline racemase (17), although no direct experimental evidence other than isotope fractionation factor studies exists to support this hypothesis. Our search for the catalytic base in thiolase catalysis (Cys-378) and the catalytic nucleophile (Cys-89) appear to have been successful using a combination of approaches described in this paper and in our previous publications where both site-directed chemical modification and mutagenesis were complementary probes of mechanism. Confirmation of these functional assignments by x-ray analysis is underway.

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Supplemental Material to
Biosynthetic Thiolase from *Zoogloea ramigera*. Evidence for a Stepwise Mechanism Involving Cys-378 as the Active Site Base
by
M.A.J. Palmer, E. Differding, R. Gamboni, S.F. Williams, O.P. Peoples, C. T. Walsh, A.J. Sinskey and S. Masamune

EXPERIMENTAL PROCEDURES

DNA Manipulations

Routine DNA manipulations including plasmid DNA isolation and enzymatic treatments were performed as described by Sambrook et al. (25). Plasmid pUCDBK1 containing the *Z. ramigera* thiolase gene was reported previously (2). The lac promoter vector pKK223-2 and host strain *E. coli* JM105 were obtained from Pharmacia and modified to remove the BamH1 site and 5ph1 site located outside the polylinker region. This derivative, pKK226, was used to construct the thiolase overproduction plasmids. Site directed mutagenesis experiments were performed using restriction fragments cloned into the M13 vectors mp18 and mp19 and the T7 polymerase/gene 32 protein method of (26) as described previously (3). All mutations were confirmed by DNA sequence analysis following reconstruction of the complete gene.

Purification of Recombinant Thiolase

Z. ramigera thiolase was purified from *E. coli* JM105 containing the overproduction plasmid pZTH (Figure 1a) as follows: An overnight culture of *E. coli* JM105/pZTH was grown in 2XTY medium (16 g Tryptone 10 g yeast extract and 5 g NaCl in 1 l dH_2O) containing 100 µg/ml ampicillin. A 5 ml aliquot of overnight culture was used to inoculated 1 l of fresh 2 X TY medium containing ampicillin (100 µg/ml) and the culture incubated at 37°C until an A_{600} 0.6-0.7 was obtained. Isopropyl-ß-D-thiogalactoside was added to a final concentration of 2 mM followed by a 16 hour incubation at 37°C. Cells were harvested by centrifugation (6000 rpm, 10 min, 4°C, GSA rotor) resuspended in 15 mL of lysis buffer (20 mM Tris Cl, pH 8.2, 1 mM EDTA, 5 mM ß-mercaptoethanol, 5% (v/v) glycerol, 0.2 mM phenylmethylsulfonyl fluoride PMSF) and lysed by sonication. Cell debris was removed by centrifugation (15000 rpm, 15 mm, 4°C, SS34 rotor). The clarified extract (15-20 ml) was loaded onto a DEAE Sepharose CL6B column (1.7 x 20 cm) previously equilibrated in lysis buffer and washed with 20 ml lysis buffer to remove unbound proteins. The column was then eluted with a 180 ml linear 75-200 mM NaCl gradient. Fractions (9 ml) were collected and those having thiolase activity greater than 200 U/mg combined and loaded directly onto a Red A Sepharose (Sigma) column (1.5 x 50 cm) equilibrated with lysis buffer minus PMSF but containing 250 mM NaCl. Again the column was washed with 2 volumes of this buffer and the proteins eluted with a 300 ml linear 250 mM-2M NaCl gradient. Fractions (9 ml) having a specific activity greater than 500 U/mg protein were combined and dialyzed against lysis buffer (minus PMSF) at 4°C. The purified enzyme was stored in lysis buffer containing 50% glycerol at -20°C.

Chemicals

3-Pentynoic acid was prepared by oxidation of the corresponding alcohol (27), [1-^{14}C]3-Bromopropionic acid was purchased from Pathfinder Laboratories Inc., all other substrates and reagents used in the synthesis of inhibitors were purchased from either Aldrich or Sigma. [^{32}P]CoASH was prepared as previously described (3). [1-^{14}C]Ac-CoA was purchased from ICN and [γ-^{32}P]ATP was purchased from Amersham. Enzyme assays for thiolase, ultrafiltration, UV measurements, tryptic digestion of labelled thiolase, HPLC separation of peptides and amino acid analysis of peptides were carried out as previously reported (1). NMR spectra were recorded on either a Bruker 250 MHz or Varian 400 MHz spectrometer.

Synthesis of Inactivators

Pantetheine-11-pivalate was prepared as described by Davis et al. (1) with the following modification for reduction of pantethine dipivalate. Dithiothreitol (1.15 g, 7.4 mmol) and triethylamine (130 µL, 0.1 mmol) were added to a stirred solution of pantethine dipivalate (0.9 g, 1.24 mmol) in CH_2Cl_2 (25 mL) at room temperature. After 2 h the reaction mixture was washed with water (4 x 50 mL), the organic layer dried over Na_2SO_4 and concentrated in vacuo. Flash column chromatography (silica gel, CH_2Cl_2 : MeOH, 97.5 : 2.5) gave 0.75 g (83 %) of pantetheine-11-pivalate as a colorless oil.

3'-Pentynoyl-S-pantetheine-11-pivalate, (3'-pentynoyl-SPP **1**). DCC (0.21 g, 1 mmol) and pantetheine-11-pivalate (90 mg, 0.25 mmol in THF 4 mL) were added sequentially to a stirred solution of 3-pentynoic acid (0.1 g, 1 mmol) in THF (3 mL) at 0°C, under argon. After 5.5 h at 0°C, the reaction mixture was poured into a half saturated solution of $NaHCO_3$ (20 mL), stirred at room temperature for 2 min, extracted with CH_2Cl_2 (3 x 5 mL), the combined organic layers dried over anhydrous $MgSO_4$ and concentrated in vacuo. Insoluble dicyclohexylurea was removed by filtration after dissolving the colorless oil in a small amount of $CHCl_3$. The crude product was purified by flash column chromatography (deactivated silica gel, $CHCl_3$: MeOH, 20 : 1) to yield 56 mg (50%) of the thioester. The thioester, 3-pentynoyl-SPP was dissolved in CH_3CN to give a 10 mM solution and stored at -78°C. UV (CH_3CN) λ_{max}=235 nm (ε = 3.9 x 10^3 M^{-1}) ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 3 H), 1.1 (s, 3 H), 1.25 (s, 9 H), 1.9 (t, 3 H), 2.4 (t, 2 H), 3.05 (dt, 2 H), 3.4-3.65 (m, 7 H), 3.75 (d, 1 H), 3.8 (d, 1 H), 4.2 (d, 1 H), 6.05-6.15 (m, 1 H), 7.15-7.25 (m, 1 H).

2',3'-Pentadienoyl-S-pantetheine-11-pivalate, (2',3'-pentadienoyl-SPP **2**). Triethylamine (45 mg, 320 µmol) was added to a stirred solution of 3'-pentynoyl-SPP (14 mg, 32 µmol) in CH_3CN (16 mL) at room temperature. After 3.5 h the solution was concentrated in vacuo and the resulting oil filtered through deactivated silica gel ($CHCl_3$: MeOH, 20:1) to give a mixture of the allene (85%) and the alkyne (15%), as determined by UV (see above). The allene (**2**) was stored in CH_3CN (100 mM) at -78 C. UV (CH_3CN) λ_{max}=263 nm (ε = 3.8 x 10^3 M^{-1}) ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 3 H), 1.1 (s, 3 H), 1.25 (s, 9 H), 1.85 (dt, 3 H), 2.4 (t, 2 H), 3.05 (dt, 2 H), 3.4-3.65 (m, 5 H), 3.75 (d, 1 H), 3.8 (d, 1 H), 4.2 (d, 1 H), 5.7-5.8 (m, 1 H), 5.8-5.85 (m, 1 H), 6.05-6.15 (m, 1 H), 7.15-7.25 (m, 1 H).

Determination of the alkyne (**1**)/allene (**2**) ratio. The isomerization of the alkyne to the allene in solution was followed by UV. 3'-Pentynoyl-SPP or 2,3-pentadienoyl-SPP (1 µL of a 100 mM CH_3CN solution) was added to 1 mL of a given solution. The absorption spectrum of this solution was recorded, the absorbances at two wavelengths measured, and the relative amounts of allene and alkyne calculated from the expressions:

Abs_{263} = [Alkyne] x ε_{263}(alkyne) + [Allene] x ε_{263}(allene)

and

Abs_{235} = [Alkyne] x ε_{235}(alkyne) + [Allene] x ε_{235}(allene)

where, ε(alkyne, 235 nm) = 3,970 M^{-1}, ε (alkyne, 263nm) = 255 M^{-1}, ε (allene, 235 nm)= 2,500 M^{-1}, and ε (allene, 263 nm)= 3,800 M^{-1} are the absorption coefficients obtained from a solution of pure alkyne and also extrapolated from a 85 / 15 mixture of allene/alkyne. The calculated values are in good agreement with values determined by NMR for a set of 7 representative mixtures of alkyne/allene.

3'-Bromopropionyl-S-pantetheine-11-pivalate, (3'-Bromopropionyl-SPP). DCC (31 mg, 0.15 mmol) was added to a solution of pantetheine-11-pivalate (48 mg, 0.13 mmol) and 3-bromopropionic acid (15.3 mg, 0.1 mmol) in CH_2Cl_2 (2.5 mL) at 0°C . After 2 h at 0°C the temperature was raised to room temperature for 1h. Additional DCC (27 mg, 0.13 mmol) was added and the reaction mixture left at 5-10°C for 20 h. The white suspension was then diluted with ethyl acetate (10 mL) and filtered through cotton. The filtrate was concentrated *in vacuo* and purified by flash chromatography (SiO_2, CH_2Cl_2: MeOH, 97.5 : 2.5) to give 38 mg (78%) of the desired product. UV (CH_3CN) λ_{max}=235 nm (ε = 4.0 x 10^3 M^{-1}). ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.9 (s, 3H), 1.05 (s, 3H), 1.2 (s, 9H), 2.4 (t, 2H), 3.0-3.2 (m, 4H), 3.35-3.6 (m, 7H), 3.7 (d, 1H), 3.8 (d, 1H), 4.2 (d, 1H), 6.0-6.1 (m, 1H), 7.1-7.2 (m, 1H).

[1'-^{14}C]-3'-Bromopropionyl-S-pantetheine-11-pivalate ([1'-^{14}C]-3'-Bromo propionyl-SPP). [1-^{14}C]-3-Bromopropionic acid (3.7 mg, 4.1 mCi/mmol) was diluted with cold 3-bromopropionic acid (11 mg, 0.07 mmol) and the synthesis performed as described above to yield the labelled compound (50% yield) with a specific activity of 0.78 mCi/mmol.

Acryl-S-pantetheine-11-pivalate, (Acryl-SPP). Diisopropylethylamine (19 µL, 0.11 mmol) was added to a stirred solution of 3'-bromopropionyl-SPP (36 mg, 0.07 mmol) in CH_2Cl_2 (3.5 mL) at room temperature. After 4 h the reaction mixture was concentrated *in vacuo* and purified by flash chromatography (SiO_2, CH_2Cl_2 : MeOH, 97.5 : 2.5) to give 28 mg (95%) of the inactivator. UV (CH_3CN) λ_{max}=266 nm (ε= 4.3 x 10^3 M^{-1}). ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.9 (s, 3H), 1.05 (s, 3H), 1.2 (s, 9H), 2.4 (t, 2H), 3.1 (dt, 2H), 3.4-3.6 (m, 5H), 3.7 (d, 1H), 3.8 (d, 1H), 4.2 (d, 1H), 5.7(dd, 1H), 6.0-6.1 (m, 1H), 6.25-6.4 (m, 1H), 7.1-7.2 (m, 1H).

[1-^{14}C]-Acryl-S-pantetheine-11-pivalate, ([1'-^{14}C]-Acryl-SPP). The ^{14}C-labelled compound was prepared as described above in 100% yield with a specific activity of 0.69 mCi/mmol.

Biochemical Analysis

Inactivation of Thiolase by 3'-pentynoyl-SPP (**1**) and 2',3'-pentadienoyl-SPP (**2**). All inactivation assays were carried out with an inactivator/enzyme ratio of ~10^3 at pH 7 in 0.01 M KPi buffer containing 1 mM EDTA, where the observed rate constant for non-enzymatic isomerization is k = 4 x 10^{-4} min^{-1} which corresponds to a half-life of 1720 min for the alkyne. Under these conditions, incubation of thiolase with (**1**) or (**2**) results in a decreasing activity. With (**1**), a plot of log % activity vs time was linear to 50 % activity and then slowly leveled off. A plot of the half-life for inactivation vs 1 / [inhibitor] for (**1**), with extrapolation to infinite inhibitor concentration gave K_i and k_{inact}. Similar plots were made for (**2**) to determine K_i and k_{inact}.

Inactivation of Thiolase by 3'-pentynoyl-SPP (**1**) after preincubation with a substrate. Thiolase was incubated for 2 min with increasing amounts of substrate (AcAc-CoA, Ac-CoA or AcAc-OPP) and the initial activity determined. 3'-Pentynoyl-SPP (**1**) was added (2 mM final concentration) and the % activity remaining after 4 min (timed from the addition of 3'-pentynoyl-SPP) plotted against substrate concentration.

Incubation of 3'-Pentynoyl-SPP (**1**) in D_2O with thiolase. In two parallel runs, 3-pentynoyl-SPP (**1**) was dissolved in buffered D_2O (final concentration 2.2 mM) and the exchange of the α-protons for deuterium monitored as a function of time by NMR, in the absence and presence of thiolase (25 µM). Under these conditions the half-life of inactivation was > 30 min. Although a rigorous integration of the NMR signals was not possible due to the presence of EDTA signals, their intensities and shapes revealed no substantial difference between the two runs. It was estimated that at least a 30 % increase in exchange rate would have been detected, therefore the exchange rate due to the enzyme catalyzed proton abstraction (k_{exch} in D_2O) must be < 0.015 min^{-1}.

Labelling of thiolase with 3'-pentynoyl-SPP (**1**) and reduction with [^{3}H]-$NaBH_4$. The enzyme stock solution was washed by ultrafiltration at 4°C with degassed 0.01M KPi, 1 mM EDTA, (pH 7.0). *Z. ramigera* thiolase (4 mg, 100 nmol) in 0.01M KPi, 1 mM EDTA, (pH 7.0) (3 mL) was preincubated with AcAc-CoA (5 mg) for 3 min, then 3'-pentynoyl-SPP (20 µmol, 109 mM in CH_3CN) was added. After 18 min the residual enzyme activity was 10 % (thiolysis direction). Excess inhibitor was removed by gel filtration (Sephadex P6-DG) at 4°C with 0.1 M KPi, 1 mM EDTA, (pH 7.0), and the protein containing fractions combined and concentrated to 240 µL by ultrafiltration. The enzyme was denatured by treatment with 8 M urea (1.5 mL) for 3 h, reduced with [^{3}H]-$NaBH_4$ solution (31 µmol, 0.3 M in diglyme, 60 mCi/mmol) at 0°C for 1 h, followed by 1 h at room temperature and the reduction quenched with 4 drops of 1 N HCl. After dialysis against water (4 x 2 L) for 39 h, the enzyme had partially precipitated in the dialysis bag. The dialyzed solution (3 mL) was removed and the bag rinsed with 8 M urea (400 µL) to redissolve the precipitated protein. The protein concentration of the combined fractions was determined by the Bradford assay (66 nmol) and the radioactivity counted (1815 nCi) to give a specific activity of 27.5 nCi/nmol. Tryptic digestion (trypsin 1.1 mg in water 100 µL) of the protein for 26 h was followed by HPLC purification. First separation: Vydac-phenyl column; gradient 0 min 100 % A, 60 min 50 %A / 50 %B, A = H_2O, 0.1 % TFA, B = 94.9 % CH_3CN, 5 % H_2O, 0.1 % TFA; flow 1.5 mL min^{-1} (228 nCi isolated). Second separation: C18 semipreparative column; gradient 0 min 100 % A, 90 min 50 % A / 50 % B, A and B as before; flow 2.8 mL min^{-1} (153 nCi isolated). Third separation: Vydac-phenyl column; gradient 0 min 100 % A, 70 min 50 % A / 50 % B, A = 94.9 % H_2O, 5 % CH_3CN, 0.1 % TFA, B = 94.9 % CH_3CN, 5 % H_2O, 0.1 % TFA; flow 1.5 mL min^{-1} (76 nCi isolated). Fourth separation: the conditions used for the second separation were repeated and this resulted in the isolation of a pure radiolabelled tryptic peptide (53 nCi isolated). Peptide sequence analysis (from 36 nCi) was performed at the Whitehead Institute, and fractions counted for radioactivity. The following sequence was obtained: unknown, Ile, Gly, Gly, Gly, Met, Gly, Val, Ala, Met. By comparison to the known thiolase primary structure (Peoples et al., 1987), an identical match was found at residues 379-387 (Ile - Met) of thiolase identifying the unknown residue of this peptide as Cys-378. Greater than 90 % of the radioactivity was found in this residue (Cys-378).

Labelling of thiolase with [1'-^{14}C]-acryl-SPP in the presence of AcAc-CoA. Thiolase (1.1 mg, 27.5 nmol) in 0.01 M KPi (pH 7.0), 1 mM EDTA (0.8 mL) [thiolase was concentrated by ultrafiltration and washed with 0.01 M KPi buffer] was preincubated with AcAc-CoA (2.1 mg, 2.4 µmol) for 3 min. Then 50µL of [1'-^{14}C]-acryl-SPP (10 mM in CH_3CN, 0.5 µmol, 0.69 nCi/nmol) was added and the mixture allowed to incubate for 40 min. The excess inhibitor was removed by gel filtration (Sephadex P6-DG) at 4°C eluting with 0.1 M KPi (pH 7.0), 1 mM EDTA to give 1.1 mg of protein with a specific activity of 0.7 nCi/nmol. The reaction mixture was concentrated by ultrafiltration (Amicon Centricon 30) and the enzyme was denatured in 5 M guanidine solution (0.75 mL) for 1 h. The denatured enzyme was treated with iodoacetamide (1.2 mg in 0.6 mL of water), β-mercaptoethanol (0.1 µL) was added 30 min later and the enzyme concentrated after a further 10 min and washed with 2.5 M guanidine solution (87% of the radioactivity was lost during this process). The enzyme was diluted by adding NH_4HCO_3 (450 µL of 0.1 M). Radiolabelled peptide was purified (in low yield) following tryptic digestion as described above. Following purification by HPLC a single polypeptide was found to contain 33 % of the injected radioactivity. The sequence of this single labelled peptide was determined as described above: unknown, Ile, Gly, Gly, Gly, Met, Gly, Val which matches residues 379-385 of the thiolase amino acid sequence. Again Cys-378 was found to be radioactively labelled.

Assay of the Gly-378 mutant enzyme in the Thiolysis and Condensation Direction. In the thiolysis direction the extinction coefficient used for AcAc-CoA in the assay buffer (67 mM Tris-HCl (pH 8.1), 5 mM $MgCl_2$) was ε_{303} = 16.9 mM^{-1} (28). The thiolysis assay mixture (total volume 0.75 mL) contained 50 µmol of Tris-HCl (pH 8.1), 40 mmol of $MgCl_2$, 0.05 µmol CoASH, 0.05 µmol of AcAc-CoA and wildtype ß-ketothiolase (1 ng, 0.02 pmol) or Gly-378 mutant enzyme (50 ng, 1.2 nmol). The reaction was initiated by the addition of enzyme and the decrease in AcAc-CoA concentration was then measured at 303 nm every 30 s over 100 min to determine the initial rate.

The assay mixture for the condensation reaction (total volume 0.75 mL) contained 50 µmol of Tris-HCl (pH 7.4), 0.375 µmol of DTT, 0.3 µmol of NADH, 0.31 units of ß-hydroxyacyl-CoA dehydrogenase from pig heart, 1.88 mmol of Ac-CoA and wildtype ß-ketothiolase (1 ng, 0.02 pmol) or Gly-378 mutant enzyme (50 µg, 1.2 nmol). The assay mixture, minus thiolase, was incubated for 2 min at 30°C. The reaction was initiated by the addition of thiolase and the rate of decrease of NADH was then measured at 340 nm every 30 s for 100 min to determine the initial rate (ε_{340} = 6.22 mM^{-1} for NADH).

Formation and isolation of the ^{14}C-labelled acetyl-mutant enzyme. Gly-378 mutant enzyme (9.1 µM) in 64 µL of 0.1 M KP, (pH 7.0), 1 mM EDTA and 1 mM ME was incubated with [1-^{14}C]AcCoA (0.47 mM, 10.6 nCi/nmol) for a given time interval (t = 15, 30, 60 min) at 0°C. The reaction mixture was diluted with 2 mL of chilled acetone-HCl (0.5% v/v), with 45 mg of bovine serum albumin added as a carrier. The precipitated enzyme was isolated by centrifugation (10 min, 15,000 rpm). The protein pellet was washed with cold acetone (1 mL) and cold ethanol (3 x 1 mL). The protein was air-dried and then redissolved in 200 mL of 8 M urea in 0.2 M Tris (pH 8.1). The radioactivity was then counted.

The attempt to isolate the labelled native acetyl-enzyme was carried out as follows. The formation of the acetyl-enzyme was performed as described above. The precipitated enzyme was redissolved in 200 µL of 0.1 M KP, (pH 7.0), 1 mM EDTA , 1 mM ME instead of 8 M urea. The solution was then placed on a gel filtration column (Sephadex G-10, 1 x 15 cm) and eluted with 0.1 M KP, (pH 7.0), 1 mM EDTA , 1 mM ME at 4°C. The fraction containing protein was then counted.

The [^{32}P]CoASH, Ac-CoA exchange reaction with the Gly-378 mutant enzyme. Stock solutions of Ac-CoA (5.4 mM), CoASH [0.58 mM with DTT(1 mM)] and [^{32}P]CoASH (8.3 µCi/nmol) were prepared. A solution of Ac-CoA (15, 25, 35, 50 or 100 µM) and [^{32}P]CoASH (50 µM, 16 nCi/nmol) in 950 µL of 0.1 M KP, buffer (pH 7.4), was temperature equilibrated at 25°C. A 100 µL aliquot was removed for use as a blank (quenched and injected onto the HPLC column at t = 120 min). The exchange reaction was initiated by the addition of the Gly-378 mutant (50 µg, 1.2 nmol). At time intervals 5, 10, 20, 40, 60, 90 and 120 min, 100 µL aliquots were removed and added to 20 µL of 0.3 M HCl, and then placed on dry ice immediately. Ac-CoA and CoASH (25 µL injections) were separated by HPLC on a reverse phase column (Vydac C_{18} Protein Peptide Column, 0.4 x 25 cm) eluting with 50 mM KH_2PO_4 (pH 4.5), 13% (v/v) methanol (flow rate 1.0 mL/min). The fractions containing CoASH and Ac-CoA were counted. The velocity (V) of the exchange reaction was calculated using the relation,

$$V = - \frac{[CoA][AcCoA]}{[AcCoA]+[CoA]} \times \frac{2.3}{t} \times \log(1-F)$$

where F is the fraction of the isotopic equilibrium attained at time t (16). In the absence of the enzyme there was no significant (<2%) incorporation of radioactivity into Ac-CoA.

RESULTS AND DISCUSSION

We previously demonstrated that the 1.5 Kb *Eco*R1-*Sal*1 fragment of plasmid pUCDBK1 contains the complete Z. *ramigera* thiolase structural gene plus 283 bp of 5'-flanking DNA (2). To provide a readily available supply of this enzyme for mechanistic and site-directed mutagenesis studies, we used a *tac* promoter vector (pKK223-3) to express this enzyme in *E. coli*. A series of thiolase expression plasmids were constructed by using *Bal*31-nuclease to generate deletions in the 5'-flanking DNA of the thiolase structural gene. From the analysis of a number of constructs, plasmid pZTH (Figure 1a) having 34 bp of 5'-flanking DNA was identified as having the highest level of thiolase expression, 178 U/mg protein in crude lysate in *E. coli*. Plasmid pZTH was also modified to remove the *Bam*H1 and *Sph*1 sites located in the vector DNA. This enabled us to isolate specific fragments of the thiolase gene using the unique restriction sites for *Eco*R1, *Sph*1, *Bam*H1 and *Hind*III for insertion into the M13 vectors (as indicated in Figure 1a) and subsequent site directed mutagenesis studies.

A rapid two step procedure was developed for purification of the overproduced thiolase enzyme from *E. coli* (see Experimental Procedures). Following induction, sonication and preliminary purification on a DEAE-CL6B column, the thiolase was finally purified by affinity chromatography on Red gel agarose. This affinity matrix had previously been used for the purification of another CoA-binding enzyme, citrate synthase from *E. coli* (29). Figure 1b shows an SDS-polyacrylamide gel analysis of the crude lysate (lane 1), pooled DEAE-CL6B fractions (lane 2) and Red gel fraction (lane 3). Routinely 150 mg of pure thiolase enzyme was obtained from 1 L of induced cells. The enzyme had a specific activity in the range 500-800 U/mg and was physically and kinetically identical to the enzyme purified from *Z. ramigera*.

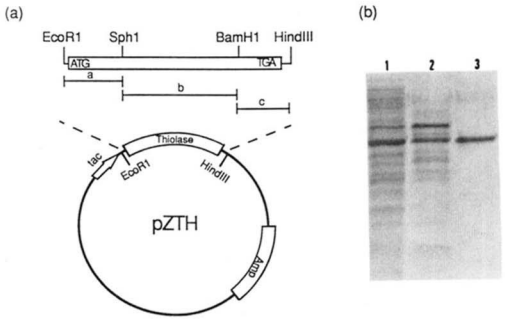

FIGURE 1: a A restriction map of the Z. *ramigera* thiolase overproduction plasmid pZTH, constructed as described in Experimental Procedures, is shown. The locations of unique restriction sites for EcoR1, Sph1, BamH1 and HindIII are indicated enabling fragments a,b or c to be excised for mutagenesis studies. b Results of SDS-PAGE analysis of protein samples from each stage of the purification (see Experimental Procedures). Lanes contained the following: lane 1, 20 μg of protein from crude lysate; lane 2, 20 μg of protein from pooled DEAE CL6B fractions; lane 3, 10 μg of purified thiolase eluted from Red Gel agarose. The gel was stained with Coomassie brilliant blue.