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Biosynthetic Thiolase from *Zoogloea ramigera*

I. PRELIMINARY CHARACTERIZATION AND ANALYSIS OF PROTON TRANSFER REACTION*

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The biosynthetic thiolase, from *Zoogloea ramigera*, involved in generation of acetoacetyl-CoA for poly-β-hydroxybutyrate synthesis, has been prepared pure in quantity for initial structural characterization of this homotetrameric enzyme. Edman degradation provided the sequence of the NH_2 terminal 25 residues and an active site cysteine-containing nonapeptide labeled on stoichiometric inactivation by iodoacetamide. Both sequences were used to align the encoding DNA sequence of the cloned gene as described in an accompanying paper. Synthetic analogs of acetoacetyl-*S*-CoA, modified in the CoA moiety, were prepared and tested, and acetoacetyl-*S*-pantetheine 11-pivalate 1 was shown to have a k_{cat}/K_m of 6.4×10^6 M^{-1} s^{-1}, comparable to the k_{cat}/K_m of 2×10^7 M^{-1} s^{-1} for acetoacetyl-*S*-CoA. The pantetheine pivalate group facilitates nonaqueous synthetic manipulations and may be generally useful as a CoA replacement. We have also prepared the carba analog of 1, with CH_2 replacing *S*, to yield a β-diketone analog 10 of acetoacetyl-*S*-CoA and the corresponding methyl ketone analog 9 of acetyl-*S*-CoA. These analogs have been used to prove the ability of *Z. ramigera* thiolase to catalyze proton abstraction from the C-2 methyl group of the acetyl portion of substrate in a transition state separate from C–C bond formation. NMR studies in D_2O show exchange only when condensation is possible. Further studies with [2-^{3}H]acetyl-CoA show there is neither pre-equilibrium washout nor detectable k_H/k_T expressed in turnover and provide no evidence for a discrete acetyl-CoA C-2 carbanion or a nonconcerted reaction.

The enzyme acetoacetyl-CoA thiolase (acetyl-CoA:acetyl-CoA *C*-acetyltransferase EC 2.3.1.9) (1) is a ubiquitous enzyme (2, 3). Although the physiological equilibrium is far in the direction of CoASH-mediated thiolysis of acetoacetyl-CoA (AcAc-CoA[1], or AcAc-*S*-CoA when emphasis is placed on the thioester moiety), analysis of the condensation direction shows that this enzyme can generate a carbon-carbon bond in a biological Claisen condensation at the initiation of terpenoid, steroid, macrolide, and other biosynthetic pathways. Thiolases treat the two identical acetyl-CoA (Ac-CoA or Ac-*S*-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. Although this is one of the fundamental categories of carbon skeletal assembly patterns in biological systems, surprisingly little is known about details of mechanism or on rate acceleration over nonenzymic cases. Stereochemical studies via chiral methyl group methodology on this class of acyl-CoA ligases uniformly show inversion of configuration at the nucleophilic carbon center (4), but that fact and a lack of detectable hydrogen isotope exchange or significant kinetic rate effects are among the few mechanistic constraints tabulated (5).

There are multiple isozymes of thiolase both in mammalian cells, yeast, and prokaryotes. One isozyme shows broad specificity for CoASH-initiated thiolysis of β-ketoacyl-CoAs from C_4 to C_{16} chain length and is clearly involved in the β-oxidation of long chain fatty acid (6, 7). This isozyme (EC 2.3.1.16) is referred to as degradative thiolase. A second isozyme has narrow specificity for AcAc-CoA and its role is in ketone body utilization (6). A third isozyme, located in the cytoplasm of eukaryotic cells, is also specific for AcAc-CoA and is likely the biosynthetic enzyme generating this C_4-β-ketoacyl-CoA as substrate for the β-hydroxymethylglutaryl-CoA synthase reaction in steroid biogenesis (8). These second and third isozymes are grouped as biosynthetic thiolases.

As part of an ongoing effort in these laboratories to define the mechanisms of this class of enzymes, we have turned our attention to a thiolase from the bacterium *Zoogloea ramigera*, an organism that accumulates large amounts of poly-β-hydroxybutyrate (PHB) and is also of substantial importance in inducing flocculation of particles in waste water treatment. A large flux of cell carbon to PHB involves a highly active thiolase functioning biosynthetically, coupled to β-hydroxyacyl-CoA dehydrogenase and the polymerization enzyme(s) (9). This thiolase was purified earlier to homogeneity (9) and is the enzyme we have focused on for structure/function studies to analyze the C–C bond-forming sequence. This paper describes characterization of several properties of this biosynthetic *Z. ramigera* enzyme including amino acid composition, NH_2-terminal sequence by Edman analysis, susceptibility to stoichiometric inactivation by iodoacetamide, and isolation and sequencing of an active site tryptic peptide containing the derivatized cysteine residue. These data are utilized in a companion paper which describes cloning of the thiolase gene

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[1] The abbreviations used are: AcAc-CoA or AcAc-S-CoA, acetoacetyl-CoA; Ac-CoA or Ac-S-CoA, acetyl-CoA; ADH, alcohol dehydrogenase; DCC, dicyclohexylcarbodiimide; DMAP, *N,N*-dimethylaminopyridine; DMSI, dimethylsuberimidate; HPLC, high performance liquid chromatography; KP_i, $KH_2PO_4 + K_2HPO_4$; ME, mercaptoethanol; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PHB, poly-β-hydroxybutyrate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; THF, tetrahydrofuran.

by antibody-screening methods along with the DNA sequence and encoded primary sequence of this protein (10). Also included are our initial mechanistic studies on the process of deprotonation at the C-2 site of Ac-CoA by thiolase, a prerequisite to the carbon-carbon bond-forming reaction involved in the enzymatic process. The paper (11) immediately following this describes the detection of an acetyl-enzyme thought to be a crucial catalytic intermediate in both thiolysis and condensation reactions as well as the inactivation of thiolase with a variety of haloacetyl-CoA analogs.

EXPERIMENTAL PROCEDURES[2]

RESULTS AND DISCUSSION

Enzyme Properties—With minor modifications of the published procedure (2), the *Z. ramigera* thiolase was purified to homogeneity, as judged by electrophoretic analysis. Typically 20–40 mg of pure enzyme was obtained from 600 g (wet weight) of cells, but the phosphocellulose step was often troublesomely irreproducible. Our best results, summarized in Table I (Miniprint), were obtained when the protease inactivator PMSF (0.1 mM) was included up to step 4, and phosphocellulose purchased from Schleicher and Schuell was used in step 5. A much shorter enzyme purification with higher yield is being developed from an overproduction with the cloned gene.[3]

Although the physiological role of this thiolase is thought to be the synthesis of AcAc-CoA for PHB synthesis, the enzyme is most active in the cleavage direction. A maximum specific activity of 280 units/mg was reported (9) for thiolysis of AcAc-CoA, on the basis of a millimolar extinction coefficient of 12.9 as quoted by Huth *et al.* (13). However, this value is for the extinction coefficient at 313 nm for a AcAc-CoA solution in 0.1 M Tris (pH 8.9). A more accurate value for the pH-dependent extinction coefficient at 303 nm is that quoted by Middleton (14): $A_{303} = 16.9\ mM^{-1}$ at pH 8.1 in 0.1 M Tris containing $MgCl_2$ (25 mM). Using this value a recalculation of the earlier value gives their maximum specific activity to be 214 units/mg, whereas our preparations typically showed values in the 300–400 range with a maximal value of 412 units/mg, *i.e.* approximately 2-fold higher. The enzyme is, as other thiolases, a homotetramer with subunits of M_r 42,000 (Fig. 1, Miniprint) estimated from SDS-PAGE, a value somewhat smaller than that reported earlier (9), but comparable to M_r of 40,000 from the encoding DNA sequence (10). The tetramer then has a calculated M_r of 162,000.

Kinetic assays indicated that there was no detectable cooperativity between subunits (data not shown). In the thiolysis direction this thiolase has a k_{cat} of 465 s^{-1} (per subunit). The K_m for AcAc-CoA is 24 μM for a k_{cat}/K_m of $2.0 \times 10^7\ s^{-1}\ M^{-1}$ (Table II).

The velocity in the condensation direction from two Ac-CoA molecules can be determined by *in situ* coupling to β-hydroxyacyl-CoA dehydrogenase with oxidation of NADH. This yields a specific activity of 0.16 units/mg and a K_m for Ac-CoA of 330 μM. The ratio of cleavage to condensation k_{cat} values is 4100:1. Temperature dependence analysis of k_{cat}/K_m in the cleavage direction allowed determination of $\Delta H^{\neq} = 12.3 \pm 0.8$ Kcal/mol and $\Delta S^{\neq} = -4.75 \pm 2.95$ cal/mol °K.

The stability of enzyme activity in urea was found to be substantial at low urea concentrations, 17 h for 50% loss in 2.5 M urea, 45 min at 5 M urea, but rapid ($t_{1/2}$ = 1 min) at 7 M urea. Enzyme that had lost 99% activity on prolonged exposure to 5 M urea regained 45% activity on dilution and 40 min preincubation before assay, whereas exposure to 7 M urea led to no regain of subsequent activity on dilution (data not shown). Likewise, pig heart biosynthetic thiolase dissociates reversibly into inactive monomers in 5 M urea (15).

The pI of native *Z. ramigera* thiolase, determined by isoelectric focusing gels, was about 4.4, comparable to the major short-chain thiolase of the butyrate producing *Clostridium pasteurianum* (pI = 4.5) (16), the cytoplasmic biosynthetic thiolase from ox liver (pI = 5.2) (17), and the major biosynthetic isozyme from yeast (pI = 5.3) (18). This low pI is in contrast with that from pig heart biosynthetic thiolase (pI = 7.2–7.3) (15) and rat liver mitochondrial thiolase (pI = 7.7) (7). On denaturation the pI of *Z. ramigera* thiolase rose to 9.4, consistent with basic groups having been involved in subunit interaction.

The amino acid composition was determined and is shown in Table III (Miniprint). A comparison of the amino acid composition with the composition predicted for the DNA sequence is presented in an accompanying paper (10). The NH_2-terminal sequence up to residue 25 was determined by Edman sequenator analysis to be Ser-Thr-Pro-Ser-Ile-Val-Ile-Ala-Ser-Ala-Thr-Ala-Val-Gly-Ser-Phe-Asn-Gly-Ala-Phe-Ala-Asn-Thr-Pro.

Inactivation with Iodoacetamide and Diethyl Pyrocarbonate—One anticipates that the *Z. ramigera* thiolase operates via acetyl-*S*(Cys)-enzyme intermediacy as precedented for other thiolases (3), and we undertook the classical iodoacetamide inactivation experiments, reported by Gehring and coworkers (15, 19) on pig heart thiolase, to determine susceptibility and stoichiometry of any active site cysteine modification and, more consequentially, to enable isolation and sequencing of an active site peptide for comparison to the pig heart enzyme and to place the iodoacetamide-labeled peptide in the thiolase-predicted primary sequence (10).

Thiolase was rapidly inactivated by a 125 molar excess of iodoacetamide, following pseudo-first order kinetics ($t_{1/2}$ = 6.5 min, Fig. 2*A*, Miniprint). Gel filtration of enzyme inactivated by iodo[^{14}C]acetamide indicated 95% inactivation with incorporation of 1.2 molar equivalents of radioactivity per subunit (see Fig. 2*B*, Miniprint). Tryptic digestion and peptide separation by HPLC permitted isolation of a single radioactive peptide which on Edman degradation, had the sequence NH_2-Gly-Met-Asn-Gln-Leu-Cys-Gly-Ser-Gly-somewhat analogous to the corresponding peptide from pig heart thiolase (NH_2-Val-Cys-Ala-Ser-Gly-Met-Lys) (19).

Analysis of the peptide sequence obtained from iodoacetamide labeling shows that it is identical to that for amino acids 84–93 predicted by the DNA sequence. (This amino acid numbering starts with Met[1], Ser[2], and Thr[3]. See above for NH_2 terminal.) Also during the Edman sequencing, the radioactivity (>90%) was associated with the cysteine residue indicating that alkylation had in fact occurred at this position. It was also noted that the rate of inactivation by iodoacetamide was retarded by AcAc-CoA, as expected if the susceptible cysteine is in the active site. Diethyl pyrocarbonate caused rapid inactivation, *e.g.* 350 μM pyrocarbonate and 2.4 μM thiolase in 0.2 M MES (pH 5.95, 25 °C) showed a $t_{1/2}$ of 8.75 min for inactivation. From experiments at three concentrations (0.7, 0.275, and 0.07 mM) a second order rate constant

[2] Portions of this paper (including "Experimental Procedures," Figs. 1–3, Tables I and III, and Schemes 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-311, cite the authors, and include a check or money order for $7.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

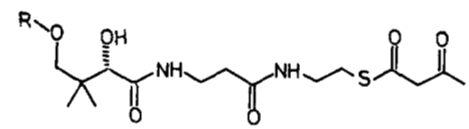
[3] O. P. Peoples and A. J. Sinskey, unpublished data.

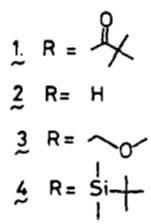
TABLE II

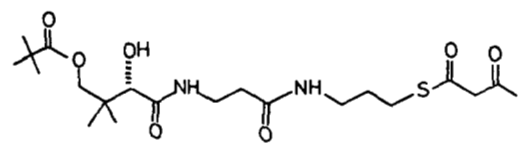
Kinetic parameters for thiolytic cleavage of AcAc-S-CoA and its analogs with Z. ramigera thiolase

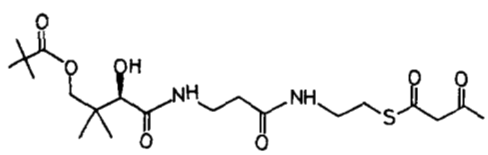
Entry	Substrate	K_m	V_{max}	k_{cat}	k_{cat}/K_m
		M	$M^{-1}\ s^{-1}/mg$	s^{-1}	$M^{-1}\ s^{-1}$
1	AcAc-CoA	2.4×10^{-5}	1.07×10^{-5}	465	2.0×10^{7}
2	AcAc-*S*-pantetheine (**2**)[a]	4.6×10^{-4}	4.00×10^{-6}	174	3.8×10^{5}
3	AcAc-*S*-(11-methoxymethyl)pantetheine (**3**)	1.2×10^{-4}	8.1×10^{-6}	353	2.9×10^{6}
4	AcAc-*S*-(11-*t*-butyldimethylsilyl)pantetheine (**4**)	7.4×10^{-5}	1.0×10^{-5}	434	5.9×10^{6}
5	AcAc-*S*-(D-pantetheine) 11-pivalate (**1**)	7.3×10^{-5}	1.08×10^{-5}	469	6.9×10^{6}
6	AcAc-*S*-(L-pantetheine) 11-pivalate (**5**)	6.7×10^{-4}	5.9×10^{-6}	256	3.8×10^{5}
7	AcAc-*S*-homopantetheine 12-pivalate (**6**)	2.5×10^{-4}	4.0×10^{-6}	177	7.0×10^{5}
8	AcAc-10-bis-demethylpantetheine 11-pivaloate (**7**)	2.1×10^{-4}	6.1×10^{-6}	266	1.2×10^{6}

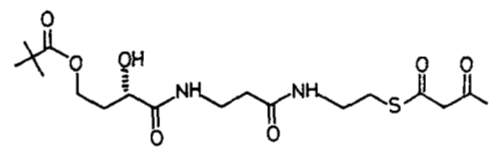
[a] The structures of **1**–**7** are











of 230 M^{-1} min^{-1} was obtained, consistent with literature values for histidine residue modification (20). Modifying reagents targeted for lysine, arginine, serine, glutamate, or tyrosine had no effect on enzyme activity, suggesting, at this level of probing, the lack of crucial involvement (or lack of accessibility) of such residues in catalysis.

Substrate Specificity—As an initial step in probing substrate structural requirements, changes were made in the CoASH moiety of AcAc-*S*-CoA. Classically acyl-CoA-utilizing enzymes have been studied with acyl-*S*-pantetheine or acyl-*S*-*N*-blocked cystamines for reasons of ease of preparation of those thiol moieties. In this investigation we have set out to systematically vary both the length of the pantetheine moiety and the nature of the atom (S,O,N,C) connecting the acyl fragment to the pantetheine group. A set of pantetheine derivatives was prepared where the primary 11-OH group was blocked to facilitate nonaqueous synthesis. Collected in Table II are k_{cat} and K_m data for the acetoacetyl-*S*-pantetheine analogs, including acetoacetyl-*S*-pantetheine (**2**) itself with free 11-OH group, its 11-methoxymethyl and 11-*t*-butyldimethylsilyl ethers (**3** and **4**) and 11-pivalate ester (**1**).

The k_{cat} for **2** is about 40% that for AcAc-*S*-CoA while the K_m is up 20-fold. As the 11-OH of pantetheine is blocked, the K_m decreases and the k_{cat} approaches that of AcAc-CoA. Thus, the 11-pivalate **1** (referred to earlier as AcAc-*S*-Pan analog **1**) showed a $k_{cat} = 469\ s^{-1}$ and a $K_m = 73\ \mu M$ for a $k_{cat}/K_m = 6.4 \times 10^{-6}\ M^{-1}\ s^{-1}$ (a respectable 33% of the k_{cat}/K_m for AcAc-*S*-CoA).

To probe additional elements of enzymic recognition of the pantetheine portion of substrate, the (unnatural) L-isomer of **1** was prepared. In terms of k_{cat}/K_m this isomer (**5**) displays 2% reactivity toward thiolase, as opposed to 32% for the natural counterpart **1**. The effect of elongating the pantetheine moiety by one methylene group was tested with acetoacetyl-*S*-homopantetheine 12-pivalate (**6**), and 3.5% activity was observed. So there is some sensitivity to placement of the acetoacetyl group within the active site. Finally, we note that deletion of the dimethyl group (**7**) at the 10 position leads to a 3-fold increase in K_m as compared with **1**. Pantetheine 11-pivalate has been chosen for synthetic accessibility and enzymic affinity as a good compromise for a CoA-like moiety. Thus, most of the substrates described below and the inhibitor work in the following paper has involved this moiety.

Catalysis of Proton Transfer from C-2 Methyl of Ac-CoA to Solvent by Thiolase—The *Z. ramigera* thiolase catalyzed reaction, viewed in the cleavage direction, can be formally divided into the three steps shown by analogy with formulations for other thiolases (1, 2).

$$CH_3COCH_2COSCoA + E\text{-}S^- \rightleftarrows CH_3COS\text{-}E + {}^-CH_2COSCoA \quad (1)$$

$${}^-CH_2COSCoA + E\text{-}BH^+ \rightleftarrows CH_3COSCoA + E\text{-}B \quad (2)$$

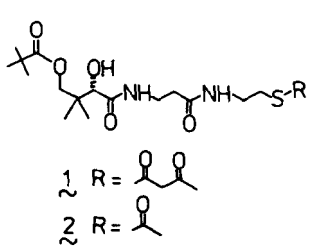
$$CH_3COS\text{-}E + CoASH \rightleftarrows CH_3COSCoA + E\text{-}SH \quad (3)$$

In step 1 the enzyme bound AcAc-CoA reacts with a nucleophilic active site cysteine to undergo the C–C bond cleavage step and so generate the covalent acetyl-*S*-enzyme and the C-2 carbanion of Ac-CoA. Initial evidence for the existence of the acetyl-*S*-enzyme for *Z. thiolase* is presented in the accompanying paper (11). The acetyl-CoA carbanion still in the active site is protonated with a proton in equilibrium with bulk water and with inversion of configuration at C-2 (as assayed by chiral methyl group methodology (4)). We will return to the question of whether steps 1 and 2 are in fact a single, concerted process. The last chemical step in catalysis is thiolysis of the covalent acetyl-*S*-enzyme intermediate.

If the enzyme were able to catalyze reaction 2 very rapidly relative to reaction 1 or 3, then more than stoichiometric amounts of tritium should "wash out" to solvent from the three torsiosymmetric C-2-methyl hydrogens. As a first probe we used (*RS*)-[2-^{3}H]acetoacetyl-*S*-pantetheine pivalate (**1a**), prepared by prior nonenzymic incubation of **1** with [^{3}H]OH in 0.1 M KP_i to inquire whether tritium wash-out occurred during thiolysis. That no detectable loss of tritium occurs could be determined by isolation of radioactive acetyl-*S*-pantetheine pivalate product (**8a**), purified as described under "Methods" (Miniprint). The specific radioactivity was compared with that of [2-^{3}H]3-hydroxybutyryl pantetheine 11-pivalate generated by reduction of **1a** by NADH and β-hydroxy-acyl-CoA dehydrogenase. No tritium was lost so that two hydrogens at C-2 of **1a** are preserved at that locus during thiolysis.

The fate of the C-2 hydrogens could also be probed by following the enzymic reaction in the condensation direction using doubly labeled [2-^{3}H,1-^{14}C]Ac-CoA and drawing off the AcAc-CoA product in *in situ* reduction to β-hydroxyacyl-CoA. HPLC analysis allowed quantitation of radioactivity remaining in Ac-CoA substrate and in hydroxybutyryl-CoA product, and tritium released as HTO (HO^3H). The ^{14}C content allows quantitation of amount of substrate reacted and product generated while the ^{3}H ratio of product/substrate reveals either a pre-equilibrium washout and/or a significant kinetic isotopic discrimination (k_H/k_T) against C-2-T bond cleavage. As shown in Fig. 3 (Miniprint) the ^{3}H ratio of product/substrate is 0.84 and holds constant from 3% conversion to 12% conversion. As two molecules of Ac-CoA are condensed to AcAc-CoA (and reduced *in situ* to prevent nonenzymic enolization of product) six hydrogens at C-2 of substrates yield five hydrogens at C-4 and C-2 of product. In the absence of any expressed k_H/k_T or pre-equilibrium washout, a ^{3}H product/^{3}H substrate ratio of 5:6 or 0.833 is expected. The observed value of 0.84 is in satisfyingly good agreement. The lack of change as a function of substrate conversion also argues against any significant expression of an intrinsic k_H/k_T. Thus, the C-2-H cleavage step is neither fast nor even partially rate-limiting in catalysis. Essentially identical data have been accumulated by Gilbert *et al.* (3, 5) on the degradative thiolase from pig heart. We note that since C–H cleavage does not occur in a kinetically significant transition state, and if C–H cleavage and C–C coupling are in fact concerted, neither facet of a combined step 1,2 can be rate-determining.

We tested whether thiolase inactivated at Cys^{89} by iodoacetamide (via alkylation) or by formation of an *E*-Cys-*S*-*S*-CH_3 linkage after inactivation with Kenyon's reagent could catalyze deprotonation at C-2 of acetyl-CoA even though condensation was precluded. In these assays, the alkylated enzyme used had 8% residual activity, the Cys-*S*-SCH_3 modified enzyme 1% residual activity, and therefore the low rates of tritium washout from [2-^{3}H]Ac-CoA observed (data not shown) could have derived from residual native activity. In neither case was rapid deprotonation attributable to enzyme with a modified cysteine SH group detected.



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*Ketone Analogs **9** and **10** of Ac-CoA and AcAc-CoA: Studies of Enzymic Proton Exchange in D_2O by NMR Analysis*— Since the experiments with Cys^{89} blocked enzyme showed no tendency of this enzyme form to catalyze C-2-H cleavage of Ac-CoA disconnected from C–C condensation, we turned to ketone analogs of Ac and AcAc-CoA, where the sulfur atom of the acyl thioesters was replaced with a carbon atom. The resulting methyl ketone **9** is an Ac-CoA analog which has potentially acidic methyl hydrogens available for enzyme-catalyzed exchange with D_2O if the enzyme could catalyze deprotonation. No C–C bond formation would be possible nor can an acetyl-*S*-enzyme form, therefore proton exchange, assayable by NMR, would be unambiguous evidence for reversible discrete carbanion formation.

In fact *Z. ramigera* thiolase did *not* catalyze exchange of D^+ from D_2O into the methyl protons (δ2.19 ppm in D_2O) of **9** even after 36 h of incubation at a molar ratio of **9** to enzyme of 120:1. However, if 12 equivalents (to enzyme) of Ac-*S*-pan analog **8** was included in such a mixture, then the enzyme catalyzed complete exchange of all three CH_3-protons of **9** (and the C-2-methyl protons of **8**) in this 36-h interval. Fig. 4*A* shows a series of 400 MHz [^{1}H]NMR spectra of **9** deuterium incorporation over a 24-h incubation and the time course for decrease in that methyl signal is shown in Fig. 4*B*.

Our interpretation of these NMR experiments is that substrate **8** can react to acetylate the enzyme's active site cysteine and only then can one detect catalytic deprotonation of methyl ketone **9**. These H/D exchange data are indirect evidence for the existence of the acetyl-enzyme. They raise the question of whether the acetyl-*S*-enzyme is the catalyst for discrete carbanion formation on methyl ketone **9** or whether **9** functions as specific nucleophilic cosubstrate and undergoes C–C bond formation. The condensation product between a carbanion equivalent generated from **9** and acetyl-enzyme would be the β-diketone **10** along with free enzyme. By microscopic reversibility **10** must be a substrate for C–C cleavage. The synthesis of this novel β-diketone, **10**, the carba analog of AcAc-*S*-pan **1**, is described under "Experimental Procedures" (Miniprint). β-Diketone **10** was indeed found to be cleaved thiolytically by *Z. ramigera* thiolase. From the measured A_{294} of 15 mM^{-1}, **10** shows a k_{cat} of 1.4 s^{-1}, a K_m of 200 μM (3-fold larger than 1) and so a k_{cat}/K_m of 7×10^3 M^{-1} s^{-1}. This value is 10^{-3} that of the 6.5×10^6 M^{-1} s^{-1} ratio for **1**. Most of the lessened catalytic efficiency of the β-diketone is in a 300-fold smaller k_{cat} value, despite the fact that the pKa of the CH_2 group protons between the diketone group in **10** and **1** are of comparable acidity.

The k_{cat} of 1.4 s^{-1} for cleavage of **10** was determined at pH 7.5 and should be approximately 2-fold higher at pH 8.1. In either case, the thiolytic cleavage of **10** proceeds 1.5–2 times faster than the observed H/D exchange at the methyl ketone group of **9**. Therefore, the exchange of methyl hydrogens of **9** catalyzed by acetyl-enzyme can be accommodated kinetically by condensation to **10** and thermodynamically favored recleavage. The proton exchange would signal a turnover event and need not signify a discrete carbanion of **9** separate from a C–C forming transition state.

All the results reported here for *Z. ramigera* thiolase failing to detect C-2-H methyl exchange separate from C–C bond formation focus attention on the question of how fast an active site basic group, for example, with a pKa in the range of 5–8, can deprotonate the weak carbon acid C-2 of Ac-CoA with an estimated pKa of 20 (5). This question has previously been considered carefully by Gilbert (5) in his studies on the pig heart degradative thiolase. He accumulated essentially

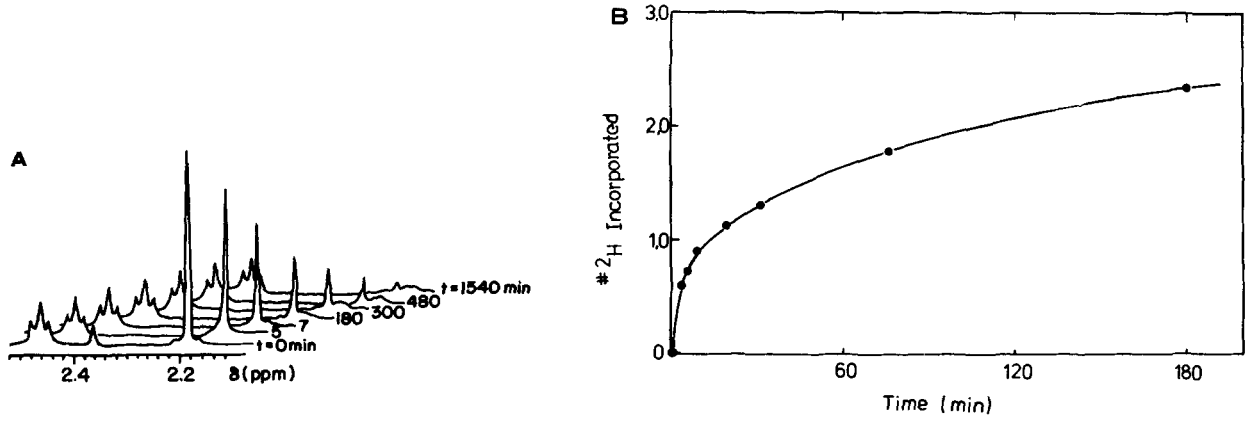

FIG. 4. **Deprotonation of methylketone 9 by thiolase and kinetics of ^2H incorporation into 9 during the deprotonation.** *A*, a solution of **9** (2.19 mg, 7.1 mM) and Ac-*S*-pan analog **8** (0.26 mg, 0.12 mM) in 0.1 M KP_i (0.8 ml, D_2O, pD = 7.5) was incubated at room temperature with thiolase (2.1 mg, 0.06 mM subunit). The methyl resonance of **9** at 2.19 ppm was monitored by 400 MHz NMR over a 36-h period (see "Methods" (Miniprint) for more details). *B*, ^2H incorporation into **9** as a function of time. The rate of exchange has a half-time of about 2.5 min. Taking a tangent to this curve which proceeds monophasically for 2.5–3.0 half-lives corresponds to a rate of approximately 0.4 substrate molecules exchanged per minute per molecule of enzyme active site.

identical results to those here and has pointed out that even if the reverse process, protonation of the corresponding conjugate base, proceeds on the bond vibrational time scale, 10^{13} s^{-1}, the maximal rate of deprotonation is considerably slower than turnover (which must be at least as slow as deprotonation). This reasoning supports a concerted Ac-CoA C-2 deprotonation and C–C bond formation. This is in contrast to Ac-CoA ligases which use ketone or aldehyde carbonyl groups as electrophiles for cosubstrates, *e.g.* citrate and malate with citrate synthase (21) and malate synthase (22) enzymes. For these two enzymes there is clear evidence that deprotonation of acetyl-CoA at C-2 is indeed uncoupled from carbon-carbon bond formation. Citrate synthase catalyzes a slow ^3H incorporation into Ac-CoA when the noncondensable substrate analog (2*S*)-malate is included in an incubation mixture in place of oxalacetate, and incorporation which does not proceed with enzyme and Ac-CoA alone. Malate synthase does catalyze the slow, but detectable, deprotonation of Ac-CoA, even in the absence of cosubstrate or analogs. Thiolase differs from citrate synthase and malate synthase in the generation of covalent acetyl-enzyme intermediate. We have yet to make an analog of covalent acetyl-*S*-enzyme that is noncondensable, but will permit deprotonation of bound acetyl-*S*-CoA, and so cannot yet distinguish unambiguously between concerted *versus* stepwise processes in this enzyme.

Finally, the *Z. ramigera* thiolase with k_{cat}/K_m of 2×10^7 M^{-1} s^{-1} is a highly efficient catalyst in the favored thiolytic C–C cleavage direction. Fersht (23) has tabulated several cases of enzymes where k_{cat}/K_m approaches the diffusional upper limit for encounter of enzyme and substrate with ranges from 1.5×10^7 to 3×10^8 M^{-1} s^{-1}. Thus, the *Z. ramigera* thiolase may have physical association steps rate-limiting in catalysis, consistent with lack of detectable deuterium or tritium isotope effects for this and other (5) thiolases.

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Supplemental Material to

Biosynthetic Thiolase from Zoogloea ramigera.
1. Preliminary Characterization and Analysis of Proton Transfer Reaction

by

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Experimental Procedures[2]

Materials

Hydroxyapatite was prepared according to the method of Main et al. (24) or purchased from Biorad. The phosphocellulose that proved the most satisfactory was from Schleicher and Schuell. Coenzyme A (CoA or CoASH), Ac-CoA, and AcAc-CoA were purchased from Sigma and PL Biochemicals. Kenyon's reagent was prepared according to Bloxham (25). [2-^{3}H]Ac-CoA, [1-^{14}C]Ac-CoA, and [1-^{14}C]iodoacetamide, were obtained from New England Nuclear. All organic reagents used in the synthesis of substrates and inhibitors were purchased from either Aldrich or Sigma, and purified by standard methods.

Methods

General

UV measurements were obtained using a Hewlett-Packard 8450A diode UV spectrophotometer. Radioactive samples were counted on a Beckman LS-1800 scintillation counter equipped with a dual labeling program using automatic quench correction. High field NMR spectra were obtained on either a 250 MHz Bruker spectrometer or a 400 MHz Varian spectrometer.

Peptide sequencing and amino acid analysis were done by Sequemat, Inc., Watertown, MA. Amino acid analysis was also done in the laboratories of Prof. Robert Sauer, Biology Department, Massachusetts Institute of Technology.

Enzyme Purification

Thiolase from Z. ramigera was purified according to the published procedure (2) but with some modifications. These include the incorporation of hydroxyapatite chromatography and the substitution of Blue Sepharose chromatography for Sephadex G-200, as summarized in Table I. The protease inhibitor PMSF (0.1 mM) was added to the buffer solutions up to the stage of the hydroxyapatite chromatography and all operations were carried out at 0-4°C.

Table I

Purification of Biosynthetic Thiolase from Zoogloea ramigera

Purification Step	Protein mg	Activity units	Activity units/mg	Purification fold	Yield %
1. Crude extract	25,600	29,400	1.15	1.0	100
2. Ammonium Sulfate (35-70%)	16,100	24,600	1.5	1.3	83
3. DEAE Sepharose	1,000	23,100	23.0	20	79
4. Hydroxyapatite	200	19,300	95.6	83	66
5. Phosphocellulose	43	16,700	389	338	57
6. Blue Sepharose	24	9,900	412	358	34

The first three steps are basically those described by Nishimura et al. (2). In step 4, the DEAE fraction (1.01 g of protein), obtained from 600 g of frozen cells from Z. ramigera was applied to a hydroxyapatite column (41 x 116 cm) equilibrated with 5 mM KP_i buffer (pH 7.0), 10% glycerol, 5 mM ME, 1 mM EDTA. The column was first washed with 200 ml of the same buffer and then the adsorbed protein was eluted with 800 ml of a linear gradient of the starting buffer and a 0.25 M KP_i buffer. The elution rate was 0.5 ml/min. The sample solution (202 mg of protein) was concentrated using polyethylene glycol and dialyzed against 5 mM Tris (pH 7.5), 3% glycerol, 5 mM ME.

The pH of the sample was adjusted to 6.5 using 1 M KH_2PO_4 and the sample was divided into two equal parts, each of which was applied to a column of phosphocellulose (5.8 x 9.0 cm) equilibrated with 10 mM KP_i (pH 6.5), 10% glycerol, 5 mM ME, and 1 mM EDTA. The adsorbed protein was eluted with 600 ml of 0.25 M KP_i (pH 6.5) at a flow rate of 5 ml/min. Fractions of 15 ml were collected into tubes containing 2.5 ml of 1 M Tris (pH 7.7). The pH of combined fractions was adjusted to 7.0 with 1 M Tris. The resultant mixture from the two columns was concentrated to 15 ml with an Amicon concentrator.

The sample solution (43 mg of protein) was applied to a Blue Sepharose CL-6B column equilibrated with 10 mM Tris (pH 7.5), 10% glycerol, 5 mM ME and 1 mM EDTA. The enzyme was eluted with a linear gradient of buffers containing the starting buffer and 0.5 M KCl at a flow rate at 0.5 ml/min. The active fraction (24 mg) was homogeneous by disc electrophoresis. After concentration the pure thiolase was stored at -20°C in 50% glycerol, 10 mM Tris (pH 7.5), 5 mM ME, and 1 mM EDTA. Stored under these conditions thiolase retained full activity for at least 24 months.

Enzyme Assays

Thiolase activities for both the cleavage (thiolysis) and condensation directions were measured according to the procedures described earlier (2) with one distinct exception. In the thiolysis direction, A_{303} = 16.9 mM^{-1} (14) was used for AcAc-CoA in 0.2 M Tris (pH 8.1) and 50 mM $MgCl_2$, instead of A_{303} = 12.9 mM^{-1} as quoted by Nishimura et al. (2). Protein concentrations were assayed according to the method of Bradford (26) or Lowry (27), using bovine serum albumin as standard. A unit of thiolase activity corresponds to that amount of enzyme which catalyzes the reaction of 1 μmol substrate per minute.

Determination of Subunit Molecular Weight

The subunit molecular weight of purified thiolase was determined by SDS-PAGE in 6% slab gels by the method of Weber and Osborn (28).

Cross-Linking of Thiolase

According to the method described by Davis and Stark (29), thiolase or yeast ADH (1 mg/ml) was incubated with 1 mg/ml DMSI solution in 0.134 M Tris (pH 8.5) at 37°C for 3 h, and then was treated with 1% SDS-1% ME at room temperature overnight. The resulting cross-linked thiolase was subjected to PAGE in the standard fashion (28).

Amino Acid Composition

Thiolase (2.4 nmol subunit) was exhaustively dialyzed against 0.1 N HOAc (5 x 100 ml). A portion of the protein solution (20%) was saved for Bradford protein analysis. The remainder of the protein sample was oxidized with performic acid before acid hydrolysis in order to determine the cysteine content via cysteic acid according to Hirs (30). In another run, a protein sample was hydrolyzed directly with 1% thioglycolate to suppress the decomposition of tryptophan (31).

N-Terminal Amino Acid Sequence Determination

Thiolase (0.2 mg, sp. act. = 384 U/mg) was exhaustively dialyzed against 0.1 M HOAc, and this sample was submitted to the Biology Department, MIT, for automated sequencing using a Beckman 890C liquid phase sequencer (Edman-Begg type).

Inactivation of Thiolase with [1-^{14}C]Iodoacetamide and its Stoichiometry

Thiolase (2.5 nmol subunit) was incubated at 25°C in 200 mM Tris (pH 8.1) for 95 min in the presence of [1-^{14}C]iodoacetamide (240 nmol, 53 nCi/nmol)). All enzyme activity was lost during this incubation. The final volume of the reaction mixture was 1 ml. The reaction mixture was concentrated to 300 μl via ultrafiltration (3000 g, 20 min). The sample was applied to a Sephadex G-25 column (20 x 1 cm) and eluted with 10 mM Tris (pH 7.5) at 10 ml/h; 0.85 ml fractions were collected. The protein containing peak eluted well before the peak containing iodoacetamide. The specific radioactivity of this protein was determined.

Tryptic Peptide Isolation

Thiolase (4 mg, 100 nmol subunit) was incubated in the dark at 25°C in 0.2 mM Tris (pH 8.1) for 3.5 h in the presence of [1-^{14}C]iodoacetamide (1 μmol, 2.0 nCi/nmol). The final volume was 4 ml.

The protein solution (50 μl), after removal of [1-^{14}C]iodoacetamide by ultrafiltration, was diluted with 0.2 ml of 0.2 M Tris (pH 8.1), 0.8 ml of 8 M guanidine and 50 μl of 100 mM iodoacetamide in a dark, inert atmosphere. The solution was kept at room temperature for 1 h, and then an additional 100 μl of iodoacetamide was added. The reaction was terminated after 1 h by the addition of 0.2 ml of 0.1 M ME. Concentration to 0.2 ml, followed by dilution with 0.8 ml of 0.5 M $(NH_4)_2CO_3$ (pH 8.1) gave a sample ready for tryptic digestion.

S-Carbamoylmethylated thiolase was treated with 0.1 mg of trypsin and the digestion allowed to proceed for 24 h at room temperature.

The radiolabeled peptide was purified by reverse-phase HPLC. The separations were performed on a Waters HPLC consisting of two Model M-45 pumps, a UK6 injector, a Waters 660 gradient controller, and a Waters 441 absorbance detector fitted with a 214 nm filter. A phenyl 5 μm bondapak reverse phase column (4.6 x 30 cm) was fitted with a Rainin precolumn containing Whatmann C-18 RP precolumn gel. Separation was obtained at 2 ml/min with a 60 min linear gradient running from 10 to 60% acetonitrile in H_2O or a 60 min linear gradient running from 10 to 60% isopropanol in H_2O. Both solvent systems contained 0.08% trifluoroacetic acid. Fractions were collected every 1 min and aliquots were counted for ^{14}C radioactivity. Peptides were detected by their absorbance at 214 nm. Two columns were required to give a pure peptide and this peptide's purity was checked by reinjection on the column. The solution of the desired peptide was concentrated for sequencing, using a Savant speed vac concentrator at 25°C with a pressure of 100 mm Hg.

Diethyl Pyrocarbonate Inactivation

Thiolase (0.6 μM subunit concentration) was incubated at 25°C in 20 mM MES (pH 6.0) in the presence of varying concentrations (70-700 μM) of diethyl pyrocarbonate (20). At various time intervals aliquots were withdrawn and assayed for thiolase activity. The inactivation was also measured as a function of pH.

Other Group Specific Inactivations

Inactivation studies of thiolase using various group specific reagents were carried out in a similar manner as described for the above procedures. Thus, 1,2-cyclohexanedione (arginine specific), phenylmethylsulphonyl fluoride (serine), KCNO (amine), N-ethyl-5-phenyl-isooxazolium-3'-sulfonate, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (carboxyl group) were all tested for their inactivation rates.

Cleavage of (R,S)-[2-^{3}H]Acetoacetyl-S-Pantetheine 11-Pivalate ([2-^{3}H]AcAc-S-Pan Analog, 1a), by Thiolase and Excess CoASH and Specific Activity of [2-^{3}H]Acetyl-S-Pantetheine 11-Pivalate ([2-^{3}H]Ac-S-Pan Analog, 8a) See the caption Table 2 for the structures of 1a and 8a which are isotopically labeled 1 and 2, respectively.

A solution of [2-^{3}H]AcAc-S-Pan Analog 1a was prepared by incubating "cold" AcAc-S-Pan Analog 1 (75 mM) with [^{3}H]OH (1 mCi/ml) for 3 h at room temperature. The cleavage of 1a was initiated by adding 50 μl of the stock solution (3.75 mM) to 1 ml of 0.1 M KP_i (pH 6.8), containing CoASH (27 mM) and thiolase (1.1 mg, 500 units). After incubation for 1 min at room temperature, the reaction was quenched by extracting the aqueous phase with $CHCl_3$ (3 x 1 ml). The combined organic phase was washed with sat. NaCl (3 x 1 ml) and concentrated after drying over Na_2SO_4. The residue was dissolved in toluene (2 x 1 ml) and azeotroped to dryness. A UV spectra of the residue showed it to be the Ac-S-Pan Analog 8a (λ_{max} = 232 nm, ϵ = 3.3 x 10^3). The isolated [2-^{3}H]Ac-S-Pan Analog 8a was found to have a specific activity of 15.2 nCi/μmol (96% of control; see below).

A control experiment was performed with [2-^{3}H]AcAc-S-Pan Analog (3.75 mM) being incubated with NADH (15 mM) and 3-hydroxyacyl-CoA dehydrogenase (3 units) in 1 ml of 0.1 M KP_i (pH 6.8). The corresponding 8-hydroxybutyryl thioester isolated in a similar manner to that described above had a specific activity of 15.8 nCi/μmol.

Tritium Isotope Effect in Deprotonation of Ac-CoA

An 11.3 mM solution of [2-^{3}H, 1-^{14}C]Ac-CoA was prepared by the addition of [2-^{3}H]Ac-CoA (10 μCi) and [1-^{14}C]Ac-CoA (2.4 μCi) to 5 mg of Ac-CoA in 250 μl of 100 mM MES (pH 6.8). This solution contained 4% CoASH as an impurity. A 29 mM NADH solution was prepared by the addition of 5 mg of NADH to 250 μl of 100 mM MES (pH 6.8). The standard 8-hydroxybutyryl-CoA dehydrogenase was prepared by the dilution of 10 μl of stock enzyme solution (0.9 μg/μl) to 500 μl with 10 mM MES (pH 6.8).

An aliquot (0-40 μl) of thiolase solution (0.02 μg/μl) was added at room temperature to 70 μl of 80 mM MES (pH 6.8) containing 5 mM dithiothreitol, 15 μl of [2-^{3}H,1-^{14}C] Ac-CoA, 15 μl of NADH, and 3 μl of the dehydrogenase. The reaction was terminated by injection of the sample (100 μl) into the HPLC system after 5 min to determine the tritium (dpm)/carbon (dpm) ratios for Ac-CoA and 3-hydroxybutyryl-CoA as well as the amount of T$_2$O washout. A Waters C-18 RP μbondapak reverse phase column (4.6 x 30 cm) equipped with Rainin precolumn containing Whatman C-18 precolumn gel was used. A solvent system containing 87% 0.1 M KP_i (pH 5.8) and 13% methanol was used for isocratic elution (2 ml/min). Fractions were collected into 20 ml poly vials containing 17 ml of National Diagnostics Liquiscint in 1.5 min intervals.

Enzyme-Catalyzed Deuterium Incorporation into Methyl Ketone 9 by D_2O (see Scheme 2 for the Structure of 9)

A concentrated solution of thiolase (28 mg/ml) in 0.1 M MOPS (pH 7.2) was diluted with 0.1 M MOPS in D_2O (pD 7.2) and concentrated via ultrafiltration (3 x 1 ml) to ensure removal of H_2O.

Three experiments were performed. i) Control: A solution of methyl ketone 9 (3.45 mg, 8.9 μmol) in 0.1 M KP_i (D_2O, 800 μl, pD = 7.5) was monitored for $H^/D^$ exchange by measuring the integration of the methyl ketone CH_3 resonance of 9 at 2.19 ppm using a 400 MHz Varian NMR. ii) Thiolase addition: To a solution of 9 (3.45 mg, 8.9 μmol) in 0.1 M KP_i (D_2O, 700 μl, pD = 7.5) was added 50 μl of thiolase in 0.1 M MOPS (28 mg/ml) and the $H^/D^$ exchange was monitored for 19 h at room temperature. After 19 h an additional 100 μl of thiolase was added and the reaction was monitored an additional 17 h. iii) Thiolase and Ac-S-Pan Analog 8: To a solution of methyl ketone 9 (2.19 mg, 5.66 μmol) and Analog 8 (0.27 mg, 0.66 μmol, 12% molar equiv.) in 700 μl of 0.1 M KP_i (D_2O, pD = 7.5) was added 75 μl of thiolase (28 mg/ml, 38 nmols subunit) solution. The kinetics of $H^/D^$ exchange were monitored for 36 h at room temperature. A packaged water suppression routine from Varian (12) was used to minimize the HDO peak at 4.8 ppm.

Substrate Affinity of 8-Diketone 10 in Thiolysis Reaction: Kinetic Parameters and Product Identification (see Scheme 3 for the Structure of 10)

A solution of 8-diketone 10 (5 μmol) and CoA (5 μmol) in 800 μl of 0.1 M KP_i (pH 7.5) was incubated with thiolase (30 nmol). After the reaction was complete, as monitored by the decrease in absorbance at 294 nm in 0.2 M Tris (pH 8.1) with 50 mM $MgCl_2$, the reaction products were separated from thiolase by ultrafiltration. The filtrate was extracted with $CDCl_3$ (3 x 1 ml). The organic layer was concentrated after drying over Na_2SO_4. The structure of the product was determined by comparison of its 250 MHz ^{1}H NMR and tlc behavior with a sample of authentic methyl ketone 9.

Steady state kinetic parameters were determined in the usual manner with the absorbance at 294 nm (ϵ = 1.5 x 10^4) for the 8-diketone 10 used in place of the usual 303 nm for AcAc-S-CoA and its analogs.

Syntheses of Substrates and Analogs*

Synthesis of AcAc-S-Pan Analog 1

The synthesis of 1 is representative of the syntheses of all substrates listed in Table 2.

(a) Pantetheine 11-Pivalate

To a stirred solution of commercial D-pantethine (1.00 g, 1.8 mmol, 99% pure from Sigma) in THF (30 ml) at room temperature was added dry pyridine (1 ml, 12.4 mmol). After stirring for 5 min, pivaloyl chloride (0.55 ml, 5.0 mmol) was added. The reaction mixture was stirred at room temperature for 24 h and then poured into a saturated aqueous NH_4Cl solution (50 ml) and extracted with ethyl acetate (5 x 30 ml). The extracts were dried over anhydrous $MgSO_4$ and concentrated in vacuo. Flash chromatography (SiO_2, $CHCl_3$-MeOH 10:1) gave 1.1 g (86%) of pantethine dipivalate as an amorphous white powder: ^1H NMR (250 MHz, $CDCl_3$) δ 7.47 (s, J=5.0 Hz, 2H), 7.20 (s, J=5.0 Hz, 2H), 4.61 (br.d, J=4.0 Hz, 2H), 4.13 (d, J=10.9 Hz, 2H), 3.89 (d, J=4.0 Hz, 2H), 3.80 (d, J=10.9 Hz, 2H), 3.70-3.39 (m, 8H), 2.80 (s, J=6.0 Hz, 4H), 2.51 (dt, J=4.0, 5.0 Hz, 4H), 1.21 (s, 18H), 1.05 (s, 6H), 0.95 (s, 6H).

To a stirred solution of the above powder (47 mg, 0.066 mmol) in methanol-aqueous $NaHCO_3$ (2 ml, 0.5 ml of 0.25 M) was added sodium borohydride in 10 mg portions (40 mg, 1.06 mmol) over a 1 h period. Glacial acetic acid was added to quench the reaction and the reaction mixture was concentrated in vacuo. Flash chromatography (SiO_2, $CHCl_3$-MeOH 20:1) gave 38 mg (81%) of pantetheine 11-pivalate as a colorless oil: ^1H NMR (250 MHz, $CDCl_3$) δ 7.20 (bs, 1 H), 6.24 (bs, 1 H), 4.23 (d, J=11.0 Hz, 1 H), 3.81 (bs, 1 H), 3.73 (d, J=11.0 Hz, 1 H), 3.35-3.66 (m, 4 H), 2.68 (dt, J=7.0,7.7 Hz, 2 H), 2.48 (t, J=6.2 Hz, 2 H), 1.68 (br.s, 1 H), 1.23 (s, 9 H), 1.08 (s, 3 H), 0.94 (s, 3 H).

(b) AcAc-S-Pan Analog 1

To a solution of pantetheine 11-pivalate (100 mg, 0.276 mmol) in ethyl acetate (8 ml) and 0.2 M aqueous $KHCO_3$ (2 ml) was added diketene (0.023 ml, 0.29 mmol). The resultant reaction mixture was stirred at room temperature for 40 min.

Saturated NH_4Cl solution (10 ml) was added and the aqueous phase was extracted with ethyl acetate (5x10 ml). The extracts were dried over anhydrous $MgSO_4$, filtered, concentrated in vacuo to give a yellow oil. Analog 1 (93 mg, 64%) was obtained after flash chromatography (SiO_2, $CHCl_3$-MeOH 15:1) UV (0.2 M Tris, pH 8.1 50 mM $MgCl_2$) λ_{max} = 303 (ε = 1.69 x 10^4) [α]$_D$ +13.8 (c = 1.5, MeOH); ^1H NMR (250 MHz, $CDCl_3$) 7.16 (br.s, 1H), 6.28 (br.s, 1H) 5.43 (s, 0.33 H), 4.15 (d, J=11.0 Hz, 1H), 3.75 (m, 2H), 3.69 (s, 1.33 H), 3.55 (m, 2H), 3.44 (m, 2H), 3.07 (t, J = 6.2 Hz, 2H), 2.40 (t, J = 6.0 Hz, 2H), 2.25 (s, 2H), 1.93 (s, 1H), 1.20 (s, 3H), 1.04 (s, 3H), 0.95 (s, 3H). 33% enol content as shown by NMR.

Syntheses of Methyl Ketone 9, and 8-Diketone 10

Both syntheses of 9 and 10 (Scheme 2 and 3) use 8-pivaloyl pantothenic acid 11 (Scheme 1) as a common intermediate as outlined below.

(a) Tert-Butyldiphenylsilyl ester 12 of 8-pivaloylpantothenic acid (see Scheme 1)

To a solution of pantothenic acid (13) (1.1 g, 5.15 mmol), prepared from its calcium salt (Sigma) in THF (50 ml) at room temperature was added imidazole (0.71 g, 10.3 mmol). The solution was cooled to 0°C and tert-butyldiphenylchlorosilane (1.35 ml, 5.2 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 2 h and then poured into water (50 ml). The aqueous solution was extracted with ethyl acetate (5 x 30 ml). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered and concentrated in vacuo to give a yellow oil. Chromatography (SiO_2, $CHCl_3$-MeOH 10:1) gave the silyl ester 14 (1.88 g, 82%) as a colorless oil which solidified upon storage at 0°C.

To a solution of 14 (0.883 g, 1.93 mmol) in CH_2Cl_2 (15 ml) at 0°C was added pyridine (0.311 ml, 3.86 mmol). To this mixture was added pivaloyl chloride dropwise (0.218 ml, 197 mmol) and the reaction was stirred at room temperature for 24 h. The reaction mixture was concentrated and the residue dissolved in ethyl acetate (50 ml) and washed with dilute HCl (50 ml), saturated $CuSO_4$ solution (50 ml), and brine (50 ml). The organic layer was dried over anhydrous $MgSO_4$, concentrated in vacuo, and the residue was purified by flash chromatography (SiO_2, $CHCl_3$-MeOH 20:1) to give compound 12 (0.945 g, 88%) as a colorless oil which crystallized upon storage at 0°C: ^1H NMR (250 MHz, $CDCl_3$): δ 7.63 (d, J=9.0 Hz, 2 H), 7.30-7.47 (m, 3H), 7.00 (s, J=5.0 Hz, 1.H), 4.26 (d, J=11.0 Hz, 1 H), 3.75 (br.s, 1H), 3.67 (d, J=11.0 Hz, 1H), 3.03 (dd, J=7.0 Hz, 2 H), 2.72 (t, J=7.0 Hz, 2H), 1.33 (s, 9H), 1.08 (s, 9H), 1.03 (s, 3H), 0.86 (s, 3 H).

(b) 8-Pivaloyl Pantothenic Acid (11)

A solution of 12 (0.785 g, 1.7 mmol) in 2% HF-CH_3CN (20 ml) was allowed to stand at room temperature for 30 min. Solid Na_2CO_3 was added to the reaction mixture until CO_2 bubbling subsided. The reaction mixture was filtered and concentrated in vacuo. The residue was dissolved in $NaHCO_3$ solution (10 ml, pH 9). The aqueous phase was extracted with diethyl ether (2 x 50 ml) and then its pH adjusted to 2.0. The aqueous phase was extracted with ethyl acetate (2 x 30 ml) to give compound 11 (0.375 g, 72%): ^1H NMR (250 MHz, $CDCl_3$) δ 7.35 (br.s, 1H), 7.14 (br.s, 1H), 4.27 (d, J=11.0 Hz, H), 3.81 (s, 1H), 3.70 (d, J=11.0 Hz, 1H), 3.56 (m, 2H), 2.59 (t, J=7.0 Hz, 2H), 1.20 (s, 1H), 1.04 (s, 3H), 0.93 (s, 3H).

(c) Amide 15 (see Scheme 2)

To a 250 ml round-bottom flask equipped with a Dean-Stark trap, a condenser, and a magnetic stirrer was added methyl levulinate (3.33 g, 25.6 mmol), ethylene glycol (1.91 g, 30.7 mmol), p-toluenesulfonic acid (500 mg), and benzene (80 ml). After heating at reflux for 3 h the reaction mixture was cooled and quenched with saturated $NaHCO_3$ solution. The resultant mixture was extracted with ether, washed with saturated $NaHCO_3$, and dried over Na_2SO_4. Flash chromatography gave the ketal ester in 82% yield.

The ketal ester (1.40 g) was dissolved in MeOH (20 ml) and concentrated NH_4OH (20 ml) was added. The reaction mixture was stirred under N_2 for 48 h. After removing the methanol in vacuo the aqueous solution was extracted with ethyl acetate (6 x 10 ml). Flash chromatography (SiO_2, CH_2Cl_2-MeOH 10:1) of the residue of the organic extracts gave the amide 15 in 86% yield as a colorless oil: ^1H NMR (250 MHz, $CDCl_3$) δ 5.80 (br.s, 2H), 3.95 (m, 4H), 2.34 (t, J=7.0 Hz, 2H), 2.03 (t, J=7.0 Hz, 2H), 1.33 (s, 3H).

(d) Methyl Ketone 9

To a 0°C solution of lithium aluminum hydride (200 mg, 5.27 mmol) in THF (50 ml) was added dropwise a solution of the amide 15 (0.50 g, 3.14 mmol) in THF (5 ml). The mixture was allowed to warm up to room temperature and stirring continued for 6 h. The reaction was cooled to 0°C and quenched by the addition of 1 ml of H_2O in 10 ml of THF. The white precipitate was removed by filtration and the filtrate dried over Na_2SO_4. The solvent was removed in vacuo to give 0.43 g of a crude amine which was used in the next reaction without purification.

To a solution of 8-pivaloyl pantothenic acid (11) (1.14 g, 3.75 mmol) and the crude amine in CH_2Cl_2 (50 ml) was added DCC (0.851 g, 4.13 mmol). The reaction was allowed to stand at room temperature for 80 min. The precipitate was separated by filtration and the filtrate was dried over Na_2SO_4. Flash chromatography (Florisil, EtOAc-CH_2Cl_2 5:2) gave the ketal 16 in 82% yield as a colorless oil: ^1H NMR (250 MHz, $CDCl_3$) δ 7.21 (br.s, 1H), 6.01 (br.s, 1H), 4.14 (d, J=11.0 Hz, 1H), 3.92 (m, 4H), 3.79 (m, 2H), 3.73 (d, J=11.0 H, 2H), 3.54 (m, 2H), 3.31 (m, 1H), 3.14 (m, 1H), 2.89 (m, 2H), 1.6 (m, 4H), 1.28 (s, 3H), 1.20 (2, 9H), 1.05 (s, 3H), 0.90 (s, 3H).

To a solution of the above ketal 16 (0.724 g, 1.61 mmol) in acetone (30 ml) was added p-toluenesulfonic acid mohohydrate (10 mg, 0.05 mmol). The reaction was allowed to stir at room temperature for 10 h and quenched by the addition of triethylamine (7.3 µl). The solvent was removed in vacuo and the residue was separated by flash chromatography (SiO_2, EtOAc-MeOH 15:1) giving 9 in 80% yield as a colorless oil: ^1H NMR (250 MHz, $CDCl_3$) δ 7.19 (br.s, 1H), 6.13 (br.s, 1H), 4.17 (d, J=11.0 Hz, 1H), 3.81 (d, J=3.0 Hz, 1H), 3.75 (d, J=11.0 Hz, 1H), 3.54 (m, J=6.1 Hz, 2H), 3.20 (m, 2H), 2.48 (t, J=7.0 Hz, 2H), 2.39 (t, J=5.5 Hz, 2H), 2.13 (s, 3H), 1.74 (m, 2H), 1.19 (s, 9H), 1.05 (s, 3H), 0.90 (s, 3H); mass, 386.5 (M^+).

(e) Isoxazole Alcohol 17 (Scheme 3)

To a solution of 3,5-dimethylisoxazole (14.0 g, 0.145 mol) in THF (400 ml) at -78°C was added BuLi (55 ml, 0.145 mol) over a 10-min period. The resulting yellow solution was stirred at -78°C for 30 min. A solution of oxirane (7.25 ml, 0.145 mmol) in THF (100 ml) was added via cannula over a 10 min period at -78°C. The reaction mixture was stirred at -78°C for 30 min and then quenched with glacial acetic acid (10 ml). The reaction mixture was concentrated in vacuo and the residue was distilled under vacuum (140-145°C/ 0.5-1.0 mm) to give the alcohol 17 (13.0 g, 64%): ^1H NMR (60 MHz, $CDCl_3$) δ 5.78 (s, 1H), 3.63 (t, J=6 Hz, 2H), 3.15 (br.s, 1H), 2.80 (t, J=7 Hz, 2H), 2.20 (s, 3H), 1.92 (m, 2H).

(f) Phthalimide 18

To a solution of the isoxazole alcohol 17 (3.16 g, 22.4 mmol) and triphenylphosphine (5.90 g, 22.4 mmol) and phthalimide (3.31 g, 22.4 mmol) in THF (60 ml) was added diethyl azodicarboxylate (3.90 ml, 22.4 mmol) at 0°C. The orange reaction mixture was stirred at room temperature for 2.5 h. The reaction mixture was washed with brine (100 ml) and the organic portion was separated and dried over $MgSO_4$. The organic phase was concentrated in vacuo and the residue purified by flash chromatography (SiO_2, EtOAc-hexane 1:1) to give the desired product 18 (3.1 g, 55%): ^1H NMR (250 MHz, $CDCl_3$) δ 7.83 (m, 2H), 7.70 (m, 2H), 5.88 (s, 1H), 3.75 (s, J=7.1 Hz, 2H), 2.75 (t, J=7.5 Hz, 2H), 2.20 (s, 3H), 2.06 (m, J=7.0, 7.5 Hz, 2H).

(g) Isoxazolylpropylamide 19

An ethanol solution (60 ml) of the phthalamide 18 (3.0 g, 11.1 mmol) and hydrazine hydrate (1.08 ml, 22.2 mmol) was heated at reflux for 1 h. The reaction mixture was filtered and the filtrate was concentrated and the residue dissolved in ethyl acetate (100 ml) and washed with 1N NaOH (50 ml). The organic layer was dried over Na_2SO_4 and concentrated to give a crude amine which was dissolved in CH_2Cl_2 (60 ml).

To this CH_2Cl_2 solution of the amine was added 8-pivaloyl pantothenic acid (11) (1.2 g, 3.96 mmol), DCC (0.775 g, 3.75 mmol) and DMAP (0.036 mg, 0.3 mmol). The reaction mixture was stirred at room temperature for 30 min and then cooled to -78°C. The mixture was filtered and the filtrate was concentrated in vacuo to give a yellow oil. Flash chromatography (SiO_2, $CHCl_3$-MeOH 11:1) gave compound 19 (0.85 g, 57%) as a colorless oil: ^1H NMR (250 MHz, $CDCl_3$) δ 7.2 (br.s, 1 H), 6.3 (br.s, 1 H), 5.8 (s, 1 H), 4.20 (d, J=11.0 Hz, 1 H), 3.80 (s, 1 H), 3.70 (d, J=11.0 Hz, 1 H), 3.68 (m, 2 H), 3.38 (t, J=5.0 Hz, 2 H), 2.75 (t, J=7.0 Hz, 2 H), 2.41 (br.s, 2 H), 2.19 (s, 3 H), 1.90 (t, J=7.0 Hz, 2 H), 1.22 (s, 9 H) 1.07 (s, 3 H), 0.93 (s, 3 H); mass, 425 (M^+).

(h) 8-Diketone 10

A solution of the isoxazolylpropylamide 19 (0.214 g, 0.5 mmol) in ethanol (3 ml) was added to a pre-reduced suspension of PtO_2 (10 mg) in ethanol (3 ml) at room temperature. The stirred reaction mixture was hydrogenated at atmospheric pressure for 6 h. The reaction mixture was filtered and the filtrate was concentrated. Flash chromatography (SiO_2, $CHCl_3$-MeOH 10:1) of the residue gave the corresponding vinylogous amide (0.20 g, 93%) as a colorless oil: UV (EtOH) λ_{max} = 302 (ε = 13,150); ^1H NMR (250 MHz, $CDCl_3$) δ 9.68 (br.s, 1 H), 7.25 (br.s, 1 H), 6.46 (br.s, 1 H), 5.22 (br.s, 1 H), 5.01 (s, 1 H), 4.15 (d, J=11.0 Hz, 1 H), 3.85 (s, 1 H), 3.76 (d, J=11.0 Hz, 1 H), 3.70-3.00 (m, 4 H), 2.40 (dt, J=3.5, 7.0 Hz, 2 H), 2.29 (t, J=7.0 Hz, 2 H), 1.95 (s, 3 H), 1.77 (m, 2 H), 1.19 (s, 9 H), 1.05 (s, 3 H), 0.93 (s, 3 H); mass, 427 (M^+).

A solution of the above vinylogous amide (0.071 mg, 0.166 mmol) in ethanol (1.0 ml) was prepared and 2 N HCl (1 ml) was added. The reaction mixture was allowed to stand for 4 h and then neutralized with solid $NaHCO_3$. The reaction mixture was extracted with CH_2Cl_2 (3 x 5 ml). Flash chromatography (SiO_2, CH_2Cl_2-MeOH 10:1) gave 8-diketone 10 (40.6 mg, 58%) as a colorless oil: UV (EtOH) λ_{max} = 294 nm (ε = 1.5 x 10^4); ^1H NMR (250 MHz, $CDCl_3$) δ 7.24 (br.s, 1 H), 6.20 (s, 1 H), 5.49 (s, 0.63 H), 4.17 (d, J=11.0 Hz, 1 H), 3.80 (br.s, 1 H), 3.74 (d, J=11.0 Hz, 1 H), 3.59 (s, 0.74 H), 3.55 (t, J=5.0 Hz, 2 H), 3.25 (m, 2 H), 2.41 (t, J=5.0 Hz, 2H), 2.33 (t, J=7.0 Hz, 2 H), 2.04 (s, 2 H), 1.79 (t, J=6.7 Hz, 2 H), 1.20 (s, 9 H), 1.07 (s, 3 H), 0.91 (s, 3 H); mass, 428 (M^+). 67% enol content as shown by NMR.

Table III

Amino Acid Composition of Thiolase from *Z. ramigera* and Comparison with Other Thiolases

Amino Acid	No. Residues/Subunit: *Zoogloea ramigera* Biosynthetic	*E. coli*[a] Biosynthetic	Pig Heart[b] Biosynthetic
Cys	3[c]	4	5
Asp/Asn	33-34	28-29	37-38
Thr	16-17	15	21-22
Ser	20	17	22-23
Glu/Gln	36	34	32
Pro	14	11-12	21
Gly	50	36	37
Ala	62-63	55	48
Val	24-25	25	43
Ile	20-21	21-22	23
Leu	26	34	28
Tyr	3	6	8-9
Phe	11-12	10	7
His	6	5-6	5
Lys	20-21	19-20	33
Arg	18-19	13	10
Met	13-14	11	14-15
Tryp	5	3	3
Total	380-392	347-352	397-402
MW	43,000	41,500	42,500

[a] Duncombe, G.R. and Frerman, F.E. 1976. Arch. Biochem. Biophys. 176:159-170.
[b] Sehring, U. and Harris, J.I. 1970. Eur. J. Biochem. 16:487-491.
[c] In other runs where enzyme was exhaustively oxidized with performic acid, this number increased up to 6.

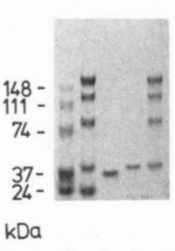


Figure 1. Slab SDS-PAGE of thiolase (see Materials and Methods for experimental detail): Lane i) cross-linked yeast alcohol dehydrogenase and trypsinogen; ii) cross-linked thiolase and trypsinogen; iii) ADH, iv) thiolase and v) cross-linked thiolase.

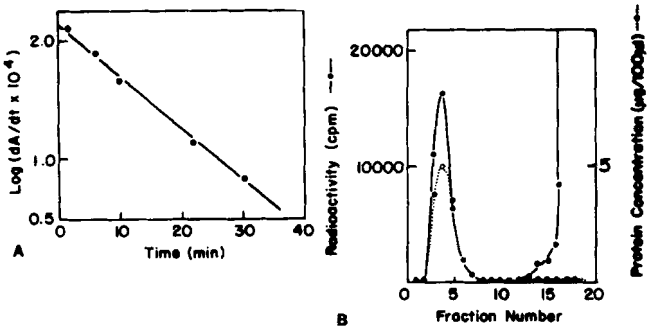


Figure 2. Inactivation of thiolase by iodoacetamide and gel filtration profile of thiolase inactivated by [^{14}C] iodoacetamide. A, inactivation of thiolase as a function of incubation time. Thiolase (0.1 μM subunit) and iodoacetamide (62.5 μM) were incubated at 25°C, aliquots of the incubation mixture were periodically removed and assayed for enzyme activity. B, gel filtration of thiolase inactivated by [^{14}C]iodoacetamide (see Methods and Materials for experimental details).

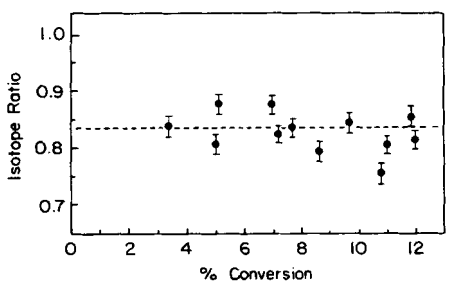
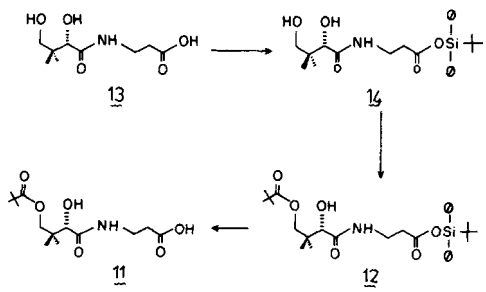
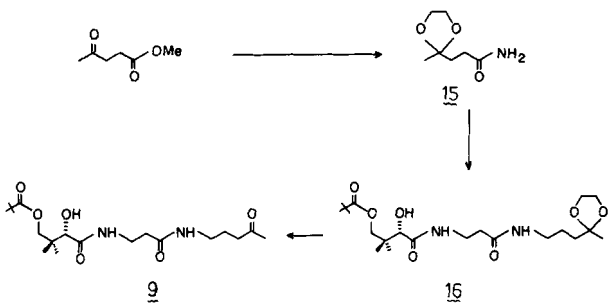


Figure 3. Plot of observed isotope ratio for product (3-hydroxybutyryl-CoA)/substrate (Ac-CoA) against % conversion to product. Doubly labeled [2-^{3}H, 1-^{14}C] Ac-CoA is self-condensed with thiolase and the resulting AcAc-CoA product is reduced *in situ* with NADH and 8-hydroxyacyl-CoA dehydrogenase.

Scheme 1



Scheme 2



Scheme 3