Mechanistic Studies on β-Ketoacyl Thiolase from *Zoogloea ramigera*: Identification of the Active-Site Nucleophile as Cys_{89}, Its Mutation to Ser_{89}, and Kinetic and Thermodynamic Characterization of Wild-Type and Mutant Enzymes†

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ABSTRACT: Thiolase proceeds via covalent catalysis involving an acetyl–S–enzyme. The active-site thiol nucleophile is identified as Cys_{89} by acetylation with [^{14}C]acetyl-CoA, rapid denaturation, tryptic digestion, and sequencing of the labeled peptide. The native acetyl enzyme is labile to hydrolytic decomposition with $t_{1/2}$ of 2 min at pH 7, 25 °C. Cys_{89} has been converted to the alternate nucleophile Ser_{89} by mutagenesis and the C89S enzyme overproduced, purified, and assessed for activity. The Ser_{89} enzyme retains 1% of the V_{max} of the Cys_{89} enzyme in the direction of acetoacetyl-CoA thiolytic cleavage and 0.05% of the V_{max} in the condensation of two acetyl-CoA molecules. A covalent acetyl–O–enzyme intermediate is detected on incubation with [^{14}C]acetyl-CoA and isolation of the labeled Ser_{89}-containing tryptic peptide. Comparisons of the Cys_{89} and Ser_{89} enzymes have been made for kinetic and thermodynamic stability of the acetyl enzyme intermediates both by isolation and by analysis of [^{32}P]CoASH/acetyl-CoA partial reactions and for rate-limiting steps in catalysis with trideuterioacetyl-CoA.

The β-ketoacyl thiolases catalyze the thermodynamically favored thiolytic cleavage of β-ketoacyl-CoA molecules in the presence of CoASH as cosubstrate (Gehring & Lynen, 1972). Isozymes with specificity for long-chain β-oxoacyl-CoAs are key catalysts in fatty acid β-oxidation cycles, and in eukaryotes these are thiolases distinct in mitochondria and in peroxisomes which have this function (Staak et al., 1978; Lazarow, 1978). On the other hand, short chain specific thiolases tend to function in a biosynthetic, carbon–carbon bond forming condensation step, at the start of isoprenoid biogenetic pathways, such as steroidogenesis where two successive Claisen condensations (involving three acetyl-CoA molecules) by thiolase and HMGCoA synthase yield the six-carbon β-(hydroxymethyl)glutaryl-CoA (Gehring & Lynen, 1972). In many species of bacteria that accumulate the intracellular lipid energy storage polymer poly(hydroxybutyrate) (PHB), thiolase is at the start of a three-enzyme pathway converting acetyl-CoA to this high molecular weight polyester (Tomita et al., 1983; Walsh et al., 1988).

We have been investigating the catalytic mechanism of this acetyl-CoA-specific thiolase in the PHB-forming bacterium *Zoogloea ramigera* and have recently reported cloning and sequencing of the thiolase gene (Peoples et al., 1987), overproduction and purification of thiolase to homogeneity, and initial study of mechanism via substrate specificity (Davis et al., 1987a) and susceptibility to affinity-labeling reagents (Davis et al., 1987b). The classical studies of Gehring and Lynen (1972) and the subsequent careful kinetic studies of Gilbert (1981) have shown that thiolases engage in covalent catalysis and form acetyl–S–enzyme intermediates. However, the specific cysteine residue had not been placed in any thiolase primary sequence. In this paper we identify the active-site cysteine in the *Z. ramigera* thiolase as Cys_{89} by isolation of the hydrolytically labile acetyl enzyme and sequencing of the acetylated tryptic peptide. To assess further the role of Cys_{89} in catalysis, we have mutated it to a less potent nucleophile, Ser_{89}, and have overproduced the C89S enzyme for assessment of overall Claisen condensation and Claisen cleavage, for formation of acetyl–O–enzyme intermediate, and for kinetic and thermodynamic comparisons of the reactivity of the native acetyl–S–enzyme and the mutant acetyl–O–enzyme species.

Experimental Procedures

Materials

[γ-^{32}P]ATP was purchased from Amersham; all other radiolabeled materials were obtained from ICN. Perdeuterioacetic anhydride was obtained from MSD. Reagents for mutagenesis were purchased from New England Biolabs except for T4 gene 32 protein, which was purchased from Pharmacia. All other reagents used were from Aldrich or Sigma.

Methods

Enzyme Purification. The thiolase gene from *Zoogloea ramigera* had been previously cloned and expressed (Peoples et al., 1987). A single colony of this strain was used to inoculate 10 mL of 2× TY (16 g of bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter) containing 50 μg/mL ampicillin. This culture was grown overnight at 37 °C and used to inoculate 1 L of 2× TY containing 50 μg/mL ampicillin. When the culture reached an A_{600} of 0.6, 0.5 g of isopropylthiogalactoside was added, and the culture was grown overnight at 37 °C for a total of 18 h. The overnight culture was centrifuged in a GS-3 rotor at 5000 rpm, the cell pellet was resuspended in lysis buffer (10 mM Tris·HCl, 1 mM EDTA, 5% glycerol, 0.1% β-mercaptoethanol, and 0.5% PMSF, pH 8.1), and the cells were lysed at 4 °C by sonication. The crude cell lysate was centrifuged in an SS34 rotor at 10000 rpm, and the supernatant was column chromatographed

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on DEAE-Sepharose (1.5 × 20 cm) in lysis buffer with a gradient of 75–300 mM NaCl. Fractions containing thiolase activity were pooled and further purified by Red Agarose gel chromatography (2.5 × 1.6 cm), elution being with a linear gradient of 0.25–2.0 M NaCl. Purity was assessed by SDS–PAGE and was regularly greater than 95%. A total of 50–100 mg of protein with a specific activity of ca. 300–400 units/mg could be isolated from 1 L of an overnight cell culture.

Determination of Substrate Concentration. The concentrations of AcSCoA and AcAcSCoA were calculated by reacting the substrate with hydroxylamine at neutral pH and determining the amount of released CoASH with DTNB. In this way we found that our preparations were essentially free of hydrolyzed AcSCoA so that we were also able to accurately determine the concentration of the AcSCoA from the UV absorbance of the CoASH adenosine group, which has a molar extinction coefficient of 16 $mM^{-1}\cdot cm^{-1}$ (Bergmeyer, 1974). Stock solutions of CoASH and AcSCoA were also checked periodically by HPLC (see method below). Alternatively, the AcSCoA concentration could be determined enzymatically. In a 1-mL total volume containing 1 mM NAD, 1.5 mM malic acid, 5 μL each (from stock solutions as supplied by Sigma) of citrate synthase [from porcine heart, suspension in 2.2 M $(NH_4)_2SO_4$] and malate dehydrogenase [from porcine heart, suspension in 2.8 M $(NH_4)_2SO_4$], 50 mM NaP_i, pH 8, and acetyl-CoA, the concentration of acetyl-CoA was determined from the change in absorbance at 340 nm.

The molar extinction coefficient of the enolate form of AcAcSCoA was calculated by determining the optical density of the substrate at 302 nm in 67 mM Tris·HCl containing 5 mM $MgCl_2$, pH 8.1, at a known concentration of substrate. Under these conditions the value was determined to be 14.3 $mM^{-1}\cdot cm^{-1}$.

Steady-State Kinetics and Hydrolytic Activity of Wild-Type and Ser_{89} Thiolase. Thiolase (Cys_{89} wild type and Ser_{89} mutant) was assayed in both the thiolytic and acetyl-transfer directions. For the acetyl-transfer reaction, appropriate amounts of acetyl-CoA were added to a solution of 0.4 mM NADH and 0.4 unit of 3-hydroxyacyl-CoA dehydrogenase in 0.1 M NaP_i, pH 7.4, and the reaction was initiated by addition of enzyme. The rate of NADH disappearance was then monitored at 340 nm.

Enzyme activity in the direction of thiolytic cleavage was determined by addition of 5 μL of appropriately diluted enzyme to 0.75 mL of 66 μM CoASH and 66 μM AcAcSCoA in 67 mM Tris·HCl containing 5 mM $MgCl_2$, pH 8.1. The loss of AcAcSCoA enolate chromophore was followed at 302 nm. The specific activity of the enzyme was then calculated as $\mu mol \times min^{-1} \times mg^{-1}$. From the subunit molecular mass of 42 kDa, k_{cat} values could be determined as collected in Table II.

The thiolytic reaction was also conducted without added thiol to determine the ability of wild-type and Ser_{89} thiolase to hydrolyze acetoacetyl-CoA. These experiments were carried out in the same fashion except that the nonenzymatic rate of AcAcSCoA breakdown was measured and subtracted from the enzymatic rate. This was not necessary when thiol acceptor has been added because the nonenzymatic rate was an insignificant portion of the overall rate. The observed hydrolysis rates (0.04 and 0.12 min^{-1} for Ser_{89} and Cys_{89} enzyme) were interpreted as rates of decomposition of acetyl–X–enzyme intermediate.

[^{32}P]CoASH/Acetyl-CoA Exchange. The reversible formation of the acetyl–X–enzyme intermediate could be monitored in both Cys_{89} wild-type and Ser_{89} mutant thiolase by analysis of the radioisotope exchange reaction (Gilbert, 1981) that monitored enzymic exchange of radiolabeled CoASH into acetyl-CoA. Because several (greater than six) commercial preparations of [^{3}H]CoASH were observed to contain most of the tritium in a peak distinguishable from those of CoASH or CoA disulfide by HPLC analysis, we turned to preparation and use of 3′-^{32}P-labeled CoASH (Miziorko & Behnke, 1985), available from incubation of dephospho-CoA and [γ-^{32}P]ATP with dephospho-CoA kinase, partially purified from fresh pig liver according to the method of Worrall and Tubbs (1983). Dephospho-CoA kinase was also provided as a gift by P-L Biochemicals, Milwaukee, WI. [^{32}P]CoASH product was separated from reactants by HPLC (25 cm × 4.6 mm Vydac C-18 protein/peptide column; gradient was 0 min 95% A/5% B, 6 min 95% A/5% B, and 7 min 85% A/15% B; A = 10 mM KP_i, pH 5.5; B = MeOH; 254 nm) and was available at a specific activity of 3 nCi/pmol. For radiolabeled exchange assays, incubations contained ca. 0.2 μCi of labeled CoASH, 100 mM KP_i, pH 7.4, 200 μM DTT, and thiolase [ca. 3 milliunits (forward assay)/mL Cys_{89} enzyme and 0.3 milliunits (forward assay)/mL Ser_{89} enzyme]. For wild-type enzyme acetyl-CoA was varied from 20 to 2000 μM while CoASH was held at 50 μM. In the case of the Ser_{89} enzyme, the range of acetyl-CoA was 50–1000 μM at 250 μM CoASH. Aliquots were removed at time intervals (1, 3, 5, 7, and 9 min), and enzymatic activity was stopped by lowering the pH to 4 and freezing on dry ice. Peaks of acetyl-CoA and CoASH were separated by HPLC (conditions as above, except for isocratic elution with 87% 50 mM KH_2PO_4/13% MeOH) and counted. K_M and V_{max} values for exchange were obtained from data analysis by a hyperbolic fit (Cleland, 1979).

Acetylation of the Active-Site Nucleophile. For the wild-type enzyme, 1.25 μmol (50 equiv) of [^{14}C]acetyl-CoA in 0.25 mL of 10 mM NaOAc, pH 6.8 (10 nCi/nmol), was added to 25 nmol of enzyme in 0.075 mL of 20 mM Tris·HCl containing 1 mM EDTA, pH 8.1 at 4 °C. The mixture was incubated on ice for 10 min. The protein was precipitated with 1.3 mL of 0.5% HCl in acetone and centrifuged. The pellet was dissolved in 0.2 mL of 8 M urea and diluted with 0.5 M KP_i, pH 6.8, and 0.3 mg of trypsin. The solution was incubated 6–8 h at room temperature and the digestion mixture chromatographed by RP-HPLC on a Vydac phenyl column (4.3 × 250 mm) with a 45-min acetonitrile gradient of 10–60% with 0.1% TFA. Peptides were collected, and the radioactivity of a portion of each was determined. The radiolabeled peptide was rechromatographed with the same gradient on a Vydac C_{18} column. The purified peptide (obtained in low yield) was sequenced on an ABI 470A sequenator and yielded the sequence shown commencing at Ala_{74} (Peoples et al., 1987): AGVPQEATAWGMNQLCGSGL. The Ser_{89} enzyme was digested analogously and yielded two radiolabeled peptides, one of which was a chymotryptic contaminant of the same tryptic peptide as above (a different batch of trypsin was used in this experiment). Thus both Cys_{89} and Ser_{89} thiolases yielded the same radiolabeled active-site peptide on acylation with [^{14}C]acetyl-CoA.

Preparation of Protioacetyl-CoA and Deuterioacetyl-CoA. A total of 30 mg of CoASH was dissoved in 2 mL of H_2O with a 3 molar excess of pyridine. A 2.5 molar excess of acetic anhydride or perdeuterioacetic anhydride was added and stirred at room temperature until the reaction was complete (approximately 2 min), as determined by addition of a small aliquot of the reaction mixture to a solution of Ellman's reagent. The solution was extracted three times with 4 mL of diethyl ether and acidified with 1 N HCl. The NMR

Table I: Kinetic Destabilization of Acetyl-X-Enzymes Relative to Acetyl-S-CoA

species	k(hydrol) (s^{-1})	k(rel)	$t_{1/2}$ for acetyl enzyme (min)	citation
acetyl-S-CoA	$\leq 2 \times 10^{-7}$	1.0		Gilbert et al., 1981
acetyl-S-thiolase I (pig heart)	2.6×10^{-4}	1.3×10^{3}	44	Gilbert et al., 1981
acetyl-S-HMGCoA synthase (pig heart)	1.6×10^{-3}	8.0×10^{4}	nd	Lowe & Tubbs, 1985
acetyl-S-papain	1.0×10^{-2}	5.0×10^{4}	nd	Street et al., 1985
acetyl-S-thiolase (*Z. ramigera*)	5.2×10^{-3}	2.6×10^{4}	~2	this work; Davis, 1987
acetyl-O-thiolase (*Z. ramigera*)	1.4×10^{-3}	9×10^{3}	~6	this work

spectra of the two compounds were identical and consistent with the spectra of acetyl-CoA (Patel & Walt, 1987) except that the deuterated compound lacked acetyl protons.

Iodoacetamide Inactivation of Enzyme. Protein in 0.1 M NaP_i, pH 7.0, containing 1 mM EDTA was concentrated to 0.05 mM; 5 μL of a 5 mM solution of iodoacetamide or buffer (as the no inhibitor control) was added to 0.05 mL of protein and the mixture incubated on ice. Aliquots were removed at various times and diluted as necessary. Remaining enzyme activity was determined in the AcAcSCoA cleavage direction at saturating concentration of substrate.

Acetyl Enzyme Formation from [^{14}C]Acetyl-CoA. In a microcentrifuge tube, 0.95 nmol of wild-type enzyme or 1.23 nmol of Ser_{89} mutant in 5 μL of 0.1 M NaP_i containing 1 mM EDTA, pH 6, was added to 95 μL of 0.1 M NaP_i containing 1 mM EDTA, pH 6, that had a final concentration of 1–100 μM [^{14}C]acetyl-CoA (41 nCi/nmol). The solution was incubated for 0.5 min at 25 °C and then quenched with 0.9 mL of 10% trichloroacetic acid at 4 °C. After 2 min on ice, this mixture was loaded with suction onto a glass fiber filter on a Büchner funnel. The filter was washed with 20 mL of 10% TCA, 20 mL of 0.1 M NaP_i, pH 5, and 10 mL of EtOH. All washes were at 4 °C. The filter was air-dried, and the radioactivity was counted. The radioactivity on the filter was determined as a fraction of the theoretical maximum.

Site-Directed Mutagenesis. The preparation of the cysteine 89 to serine 89 mutant of thiolase was carried out essentially by the procedure of Zoller and Smith as modified by Craik et al. (1985). The oligonucleotide primer used for mutagenesis was ATG-AAC-CAG-CTG-TCC-GGC-TCG, which encodes for a serine at TCC and a *Pvu*II restriction site at CAG-CTG. The mutagenesis procedure was conducted on a 300-bp fragment of the thiolase gene located between the *Eco*RI and *Sph*I restriction sites (Peoples et al., 1987). The mutation was then identified by dideoxy sequencing. The mutant thiolase gene was reconstructed back into the original overproducer plasmid and transformed into JM105. The mutation was confirmed by subcloning back into M13 mp18 for dideoxy sequencing.

The C89S mutant was expressed and purified by the same method as the wild-type enzyme. The amount of mutant protein that could be purified from a 1-L culture was approximately two-thirds of that of the native enzyme.

Cultures of 500 mL of JM105 and JM105 transformed with the C89S plasmid were prepared as described above, and after centrifugation, the crude lysates were checked for thiolytic activity.

Results and Discussion

Identification of the Active-Site Nucleophile. Thiolase enzymes catalyze reactions by a ping-pong bi-bi kinetic mechanism consistent with the existence of an acetyl enzyme intermediate (Gilbert, 1981; Gilbert et al., 1981). In our previous work this residue had been tentatively identified as cysteine 89 after enzyme inhibition with [^{14}C]iodoacetamide in a substrate-protectable fashion and sequencing of the radiolabeled tryptic fragment (Davis et al., 1987a). To establish more directly that this residue is in fact the acetylated active-site nucleophile, the acylated peptide fragment has now been directly identified following a modification of the procedure of Gehring and Lynen (1972). Incubation of pure thiolase with [^{14}C]acetyl-CoA followed by rapid precipitation at low Ph (acetyl thioesters are stable in acid) trapped the [^{14}C]acetyl enzyme intermediate. This denatured protein could be unfolded in urea and then returned to neutral pH in a now kinetically stable acetylated form and digested with trypsin. The subsequent peptide map of the tryptic digest produced only one radiolabeled fragment by HPLC analysis (no quantitation of yield was made). The peptide after rechromatography and purification was sequenced and observed to be the same peptide labeled with iodoacetamide (data not shown), confirming that the peptide did contain cysteine 89. Therefore, we take this as strong evidence for cysteine 89 as the active-site nucleophile responsible for the formation of the covalent acetyl enzyme intermediate. The active-site sequence including this cysteine is highly conserved in the cognate thiolase from *Alcaligenes eutrophus* (Peoples and Sinskey, unpublished results), and in eukaryotic thiolases from mitochondria and peroxisomes (Hijikata et al., 1987). There is also some detectable homology to the cysteine acetylated in the long-chain β-ketoacyl thiolase from pig heart (Gehring & Lynen, 1972). There is little doubt that Cys_{89} is the active-site nucleophile attacking the first of the two acetyl-CoA molecules bound in the active site and marking it as the electrophilic acetyl fragment in the carbon–carbon bond forming process.

Mutagenesis of the Active-Site Nucleophile. The acetyl-S-enzyme intermediate appeared to be hydrolytically very labile on the basis of gel-filtration experiments. Initial experiments suggested that the half-life for hydrolysis of the native acetyl-S-enzyme was approximately 2 min (Davis et al., 1987a; Davis, 1987). This is some 20-fold less stable kinetically than the pig heart thiolase acetyl-S-enzyme, whose hydrolytic half-life is 44 min under comparable conditions (Gilbert et al., 1981). As shown in Table I, the *Z. ramigera* acetyl-S-enzyme is about 26 000-fold more labile hydrolytically than the substrate acetyl thioester acetyl-CoA. The hydrolytic half-lives for two other acyl-S-enzymes, acetyl-S-HMGCoA synthase from pig heart and the protease acetyl-S-papain, are about equivalent to that of the zoogloeal thiolase covalent enzyme intermediate. This kinetic instability has precluded attempts to characterize the reactivity of the *isolated* acetyl-S-enzyme intermediate in either the forward or backward direction and thereby to assess rate-limiting half-reactions. If the cysteine nucleophile were converted to a serine residue and the enzyme were still active, one might be able to obtain a more stable acetyl enzyme intermediate, kinetically and thermodynamically. Furthermore, such a mutant form of the enzyme would permit direct comparison of how a thioester intermediate effects catalysis relative to an oxoester enzyme intermediate in Claisen condensation and Claisen cleavage.

To convert cysteine 89 to a serine residue, oligonucleotide site-directed mutagenesis according to the method of Zoller and Smith (1983) was first used with minor modifications. In

Table II: Kinetic Constants for Cys_{89} Thiolase and Ser_{89} Thiolase in Cleavage and Condensation Directions

parameter	Cys_{89} enzyme	Ser_{89} enzyme	Cys_{89}/Ser_{89} activities
$k_{f\,cat}$ (s^{-1})	71	0.03	2300/1
$k_{r\,cat}$ (s^{-1})	810	8.4	96/1
K_M(AcSCoA) (mM)	1.2	1.1	1.1/1
K_M(AcAcSCoA) (μM)	15	10	1.5/1
K_M(CoASH) (μM)	9	7	1.4/1
K_M(ex AcSCoA) (μM)	~350	~100	~3.5/1
K_M(ex CoASH) (μM)	~40	~50	~1/1
$k_{f\,cat}/K_M$ ($M^{-1}\cdot s^{-1}$)	60×10^3	27	2200/1
$k_{r\,cat}/K_M$ ($M^{-1}\cdot s^{-1}$)	53×10^6	0.80×10^6	66/1

[a] $k_{f\,cat}$ is the k_{cat} in the Claisen condensation direction (at pH 7.4) to produce acetoacetyl-CoA, $k_{r\,cat}$ is the k_{cat} in the acetoacetyl-CoA thiolysis direction (at pH 8.1). Assays were conducted as described under Experimental Procedures. $k_{f\,cat}/K_M$ uses the K_M for acetyl-CoA while $k_{r\,cat}/K_M$ uses the K_M for acetoacetyl-CoA. K_M(CoASH) applies to acetoacetyl-CoA thiolysis and K_M(ex) to the first half-reaction of enzyme acetylation. All parameters of the table were determined in this work.

our earlier work the gene for thiolase has been previously cloned into pKK223 (Peoples et al., 1987), and a 300 base pair fragment has been subcloned into M13mp19 for mutagenesis. We were unable to obtain mutants by this procedure, apparently due to a failure in the ligation step. From dideoxy sequencing, a strong stop region was observed just upstream from the mutant oligonucleotide annealing site. This suggested that the ligation failure could be the result of secondary structures in the single-stranded DNA, which would prevent the DNA polymerase from completely extending the new DNA strand around the M13 plasmid. To resolve this problem, we used the modification recommended by Craik (1985), which uses T4 DNA polymerase and T4 gene 32 protein. This method did provide the desired mutant in the 300-bp fragment of the gene, which was reconstructed back into the overproducer plasmid. The Ser_{89} mutation was confirmed by dideoxy sequencing of a fragment of the mutant gene which had been subcloned into M13mp18. The mutant protein could be expressed and purified in the same manner as the wild type (Davis et al., 1987a; Peoples et al., 1987) in 50–100-mg quantities and was homogeneous by gel electrophoresis.

Steady-State Kinetics of the C89S Thiolase Mutant. The C89S mutant of thiolase retained detectable catalytic activity. The activities of the mutant and the wild-type enzyme in the Claisen condensation and in the Claisen cleavage directions are shown in Table II. All three substrates for the enzymes (acetoacetyl-S-CoA, acetyl-S-CoA, and CoASH) showed only minor changes in K_M values while the catalytic rate constants for both the forward (acetyl transfer) and reverse (thiolytic) reactions were dramatically affected. The thiolytic rate constant decreased by a factor of 90 while the acetyl-transfer rate constant for C–C bond formation decreased by more than 2000-fold (2300 times). The change from the cysteine sulfur nucleophile to the serine 89 oxygen nucleophile is affecting catalysis rather than the ability of the substrates to bind to the enzyme, an effect that would be expected for a residue involved in catalysis. The 20-fold differential effects on condensation (down to 0.05%) versus cleavage (down to 1%) suggest different energy barriers from the acetyl–O–enzyme intermediate onto product in each direction. Lastly, while the relative rates are low, the k_{cat} of 8 s^{-1} for the C89S mutant is in absolute terms still an appreciable turnover number.

To exclude the possibility that the serine mutant was completely inactive and that the low level of activity was due to

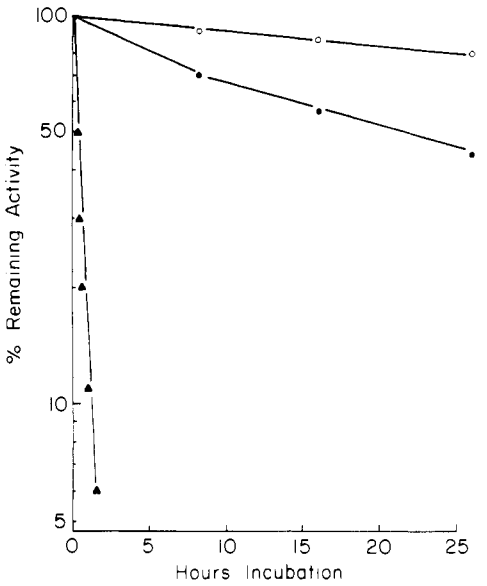


FIGURE 1: Differential susceptibility of thiolase and Ser_{89} thiolase mutant to alkylating inactivation by iodoacetamide (0.45 mM). Incubations were conducted as described under Experimental Procedures. (▲) Wild-type enzyme; (●) Ser_{89} mutant enzyme; (○) wild-type enzyme without iodoacetamide.

a copurified thiolase activity native to *Escherichia coli* JM105, we compared thiolase activities in JM105 before and after its transformation with the C89S plasmid. When the thiolytic activities of the crude lysates were compared, the specific activity of the C89S preparation was more than 20-fold greater than that of the preparation lacking the recombinant enzyme. Therefore in a worst case scenario, at least 95% of the activity must be from the mutant thiolase even if another thiolytic enzyme was contaminating the preparation.

Covalent Derivatization of Ser_{89} Thiolase. To confirm that Ser_{89} was behaving as a catalytic nucleophile in the C89S thiolase and to begin to compare its reactivity to Cys_{89}, two experiments were conducted. First, we examined the ability of iodoacetamide to inhibit catalysis. This reagent has already been established to be an inhibitor of the wild-type *Z. ramigera* thiolase (Davis et al., 1987a). When this reagent (0.45 mM) was incubated with both wild-type thiolase and the C89S mutant, the wild-type enzyme was quickly inhibited in a pseudo-first-order fashion ($t_{1/2}$ = 20 min) while the mutant was only poorly inhibited over a period of several hours (Figure 1). This accords with the known differential reactivity of cysteine–SH versus serine–OH toward S- versus O-alkylation by iodoacetamide.

To eliminate the possibility that the mutant enzyme is catalyzing Claisen cleavage or condensative turnover by a mechanism that does not use the serine hydroxyl as a nucleophile, the enzyme was incubated with [^{14}C]acetyl CoA, the ^{14}C-labeled acetylated enzyme isolated by acid precipitation and subjected to digestion by trypsin. Separation and purification of tryptic fragments by HPLC furnished two labeled peptides. On sequencing one was a smaller fragment of the other (due to possible chymotrypsin contamination in the trypsin samples, seen occasionally in other digestions), both of which started at Ala_{74} as for the wild type and contained serine 89 as noted. Therefore the C89S mutant does undergo covalent acetylation on residue 89, this time on the serine hydroxyl rather than cysteine sulfhydryl, and this argues that the same chemical mechanism involving an acetyl–X–enzyme intermediate is operant.

Characterization of Reaction Intermediates in Wild-Type and C89S Thiolases. Given that the C89S thiolase mutant

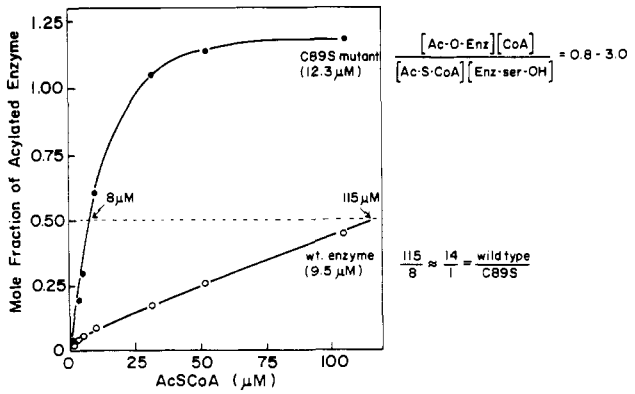


FIGURE 2: Mole fraction of acetyl enzyme intermediate accumulating after incubation of wild-type thiolase or the C89S mutant thiolase with varying amounts of [^{14}C]acetyl-CoA. The incubations were conducted as described under Experimental Procedures. (●) C89S mutant enzyme (12.3 μM); (○) wild-type enzyme (9.5 μM).

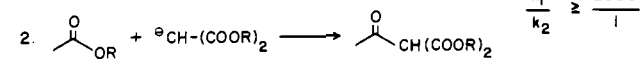
retains measurable catalytic activity and that Ser_{89} functions as a nucleophile in catalysis, we turned to the study of the kinetic and thermodynamic activation of the acetyl-O-Ser enzyme versus the acetyl-S-Cys enzyme species and their behavior in the two catalytic half-reactions, enzyme acetylation and deacetylation. In the acetylation half-reaction the altered nucleophilicity of Ser-OH versus Cys-SH and the thermodynamic activation of acetyl oxoester versus acetyl thioester enzyme will be relevant. The thermodynamic stability of covalent intermediates are equally relevant in deacetylation as are the behavior of Ser-O^- versus Cys-S^- as leaving groups.

Accumulation of Acetyl Enzyme Species in Wild-Type and C89S Thiolases. Attempts to determine the equilibrium constant for the acetyl enzyme intermediate have met with marginal success to date. Incubation of a fixed amount of C89S enzyme with different amounts of radiolabeled acetyl-CoA followed by rapid acid precipitation and counting of the acetyl enzyme species resulted in progressive accumulation of the reaction intermediate. At acetyl-CoA concentrations of 50 μM or more, a stoichiometric amount of thiolase Ser_{89}-OH acetylation is achieved. A K_M of 8 μM for acetyl-CoA in this acetylation half-reaction is crudely approximated by the data in Figure 2, but a more reliable value of ~100 μM was obtained from the [^{32}P]CoASH/acetyl-CoA exchange data (vide infra). Note this is substantially lower than the K_M (1100 μM) measured for this mutant enzyme in the overall condensation to acetoacetyl-CoA (Table II).

Also shown in Figure 2 are comparable data for wild-type Cys_{89} thiolase to determine the mole fractions of acetyl enzyme accumulating at varying acetyl-CoA/enzyme ratios. It is clear that at any given ratio of acetyl-CoA/enzyme the wild-type thiolase gives a lower mole fraction than C89S enzyme. Thus only 0.45 molar equiv of acetyl-S-enzyme were accumulated at 100 μM acetyl-CoA. The K_M for acetyl-CoA in acetylation of wild-type enzyme was estimated crudely at 115 μM from these data and refined to ~350 μM by [^{32}P]CoASH/acetyl-CoA exchange studies noted later.

The data of Figure 2 support the hypothesis that the acetyl-S-Cys_{89} enzyme intermediate appears to be more thermodynamically activated than the acetyl-O-Ser_{89} enzymic species. By simple comparison of the molar excess of acetyl-CoA required to give 50% of the acetyl enzyme and correction for differences in enzyme concentration (wild-type enzyme, 9.5 μM; C89S enzyme, 12.3 μM), the Ser_{89} enzyme is 17-fold more readily acetylated. This trend is in general accord with the chemical expectation on properties of acetyl oxoesters versus acetyl thioesters (Bruice & Benkovic, 1966; Jencks, 1969). A particularly useful model is from Jencks and colleagues (Jencks et al., 1960), who observed the intramolecular partition of an acetyl group between the S and O atoms of mercaptoethanol, i.e., from *S*-acetylmercaptoethanol to *O*-acetylmercaptoethanol. There is a 50-fold predominance of oxoester to thiolester for a ΔG of 2.3 kcal/mol.

Scheme I



Hydrolytic Stability of Acetyl-X-Thiolase. One of the practical goals in construction of the C89S thiolase mutant was an attempt to extend the lifetime of the acetyl-O-enzyme species significantly beyond the ca. 2-min half-life of the acetyl-S-enzyme species for further kinetic characterization in acylation and deacylation half-reactions and eventually for crystallization of the acetyl-O-enzyme intermediate. Crystals of both wild-type and C89S thiolase have been grown, and X-ray studies are under way (unpublished data).

Given the increased thermodynamic stability of acetyl-O-enzyme versus acetyl-S-enzyme observed in Figure 2, comparable kinetic trends in hydrolytic stability were anticipated. In the event, however, this was not borne out. The acetyl-O-Ser_{89} enzyme appears only about 3 times more stable to hydrolysis than the acetyl-S-Cys_{89} enzyme (0.04 min^{-1} versus 0.12 min^{-1} for hydrolysis under a comparable set of conditions as noted under Methods). This selectively heightened hydrolytic lability of acetyl-O-Ser_{89} enzyme is not presently understood, but our hopes of putting that species conveniently on the shelf have been unrealized. A comparison of the partition of acetyl-S-Cys_{89} thiolase between thiolysis and hydrolysis gives a ratio of ~800 s^{-1}/~0.002 s^{-1} = 4 × 10^5/1 for capture by CoASH versus H_2O. For the Ser_{89} acetyl-O-enzyme the ratio of ~8 s^{-1}/~0.0008 s^{-1} = 10^4/1 for capture by CoASH versus H_2O is down 10-fold but reflects still a high specificity for acetyl transfer to the specific cosubstrate.

Reactivity of Acetyl-S-Thiolase and Acetyl-O-Thiolase with the Carbon Nucleophile in Catalysis. To date the inability to isolate the acetyl-O-thiolase and acetyl-S-thiolase intermediates in high yield in native conformation, due to hydrolytic lability, has hampered efforts to compare directly the reactivity of the isolated acetyl-O-enzyme and the acetyl-S-enzyme species as the electrophilic acetyl donor species in the C-C bond forming transition state of this enzymatic Claisen condensation. One precedent may be helpful in setting a boundary expectation for reactivity comparisons. Thus, Jencks and co-workers (Lienhard & Jencks, 1963) have reported comparison of reactivity of acetyl oxoesters and acetyl thioesters with the carbanions of malonic ester and malononitrile as shown in Scheme I. The acetyl-SR reacted 2500-fold faster than acetyl-OR ester. The k_{cat} values for Cys_{89}-SH and Ser_{89}-OH thiolases differ by an analogous 2300-fold (Table II) in reaction with the C_2 carbanion equivalent of acetyl-CoA to make acetoacetyl-CoA. It remained to be determined then to what extent enzymic deacylation of the acetyl-O-enzyme and acetyl-S-enzyme intermediates are rate limiting in the condensation direction, and this was approached by the study of isotope exchange to obtain rates of acetyl-X-enzyme formation.

Rates of Formation of Acetyl-S-Thiolase and Acetyl-O-Thiolase from Acetyl-CoA. To assess separately the half reaction between acetyl-CoA and enzyme to yield the acetyl-X-enzyme intermediate, acetyl-S-Cys_{89} thiolase or acetyl-O-Ser_{89} thiolase, the ability of each enzyme to catalyze

the reversible exchange of [^{32}P]-label from [^{32}P]-CoASH into acetyl-CoA was monitored. The exchange monitors reversible formation of covalent acetyl enzyme intermediate and allows V_{max} determinations ($V_{max}^{exchange}$) for the reactions involving acetyl-S-enzyme or acetyl-O-enzyme formation.

The V_{max} for exchange on Cys_{89} enzyme was determined with a preparation of enzyme that had a specific activity at the time of assay of 332 units/mg in the thiolytic cleavage direction and 58 units/mg in the condensation direction. The V_{max} for [^{32}P]CoASH/acetyl-CoA exchange was 163 units/mg, 2.8-fold faster than the overall forward direction. Thus the acetyl-S-Cys_{89} enzyme formation in the condensation direction, as reflected by $V_{max}^{exchange}$ is kinetically competent. It is not yet known whether enzyme acylation or deacylation limits exchange, but at a minimum the relative partition of acetyl-S-enzyme is 2.8 times back for thiolytic capture by the thiolate of $CoAS^-$ for every one time it is captured by C_2 carbanion of acetyl-CoA and partitions forward.

When the Ser_{89} enzyme was assayed for [^{32}P]CoASH/acetyl-CoA exchange, it was also active and thus validated the reversible formation of the covalent acetyl oxoester enzyme intermediate. For a particular batch of Ser_{89} enzyme with a specific activity of 1.7 units/mg in the cleavage direction and 0.065 unit/mg in the condensation direction, at the time of assay, the V_{max} for the exchange half-reaction was 4 units/mg. Thus the exchange is ~62-fold faster than the overall condensation reaction, and the partition of the acetyl oxoester enzyme in this intermediate is altered to ca. 62 captures by $CoAS^-$ per forward capture by acetyl-CoA carbanion. The C-C bond forming step is substantially more rate limiting in the acetyl oxoester enzyme intermediate than in the acetyl thioester enzyme intermediate.

The above exchange experiments also provided K_M values for the first half-reaction of enzyme acetylation. For wild-type Cys_{89} enzyme (Figure 3A) K_M(AcCoA) was ~350 μM and K_M(CoASH) was ~40 μM. The Ser_{89} mutant (Figure 3B) exhibited K_M(AcCoA) of ~100 μM and K_M(CoA) of ~50 μM. Both enzymes have significantly lower K_Ms for acetyl-CoA in enzyme acetylation when compared to the overall K_M of ~1 mM (Table II), which is an aggregate for both acetyl-CoA molecules. The difference suggests a higher K_M for the second acetyl-CoA that reacts as carbanion equivalent with acetyl enzyme.

Deuterium Isotope Effect Studies with Trideuterioacetyl-CoA. As enzymic precedent, the thorough kinetic analysis of Gilbert et al. (1981) on pig heart thiolase (where the acetyl enzyme is stable enough for half-reaction study) shows that there is no deuterium kinetic isotope effect ($V_H/V_D = 1$, nor was there any preequilibrium washout) on condensation V_{max}, with trideuterioacetyl-CoA. Our studies with acetyl-CoA and trideuterioacetyl-CoA for wild-type zoogleoal and C89S thiolase in contrast reveal detectable V_{max} deuterium isotope effects in the condensation direction. The V_H/V_D values were 2.4 and 2.2, respectively, for wild-type and C89S thiolase. To eliminate the possibility that the deuterium isotope effect might stem from impurity or inhibitor in the trideuterioacetyl-CoA, the triprotio control species was synthesized and characterized identically with the trideuterio sample.

Two issues arise from these 2-fold V_{max} deuterium isotope effects. First in earlier studies using [2-^{3}H]acetyl-CoA as a probe for ^{3}H washout or for detectable $(V/K)_H/(V/K)_T$ in the condensation direction with this thiolase, we noted neither washout nor detectable selection against tritium in the condensation (Davis et al., 1987a). By contrast with the deuterated substrates both V_H/V_D and $(V/K)_H/(V/K)_D$ were

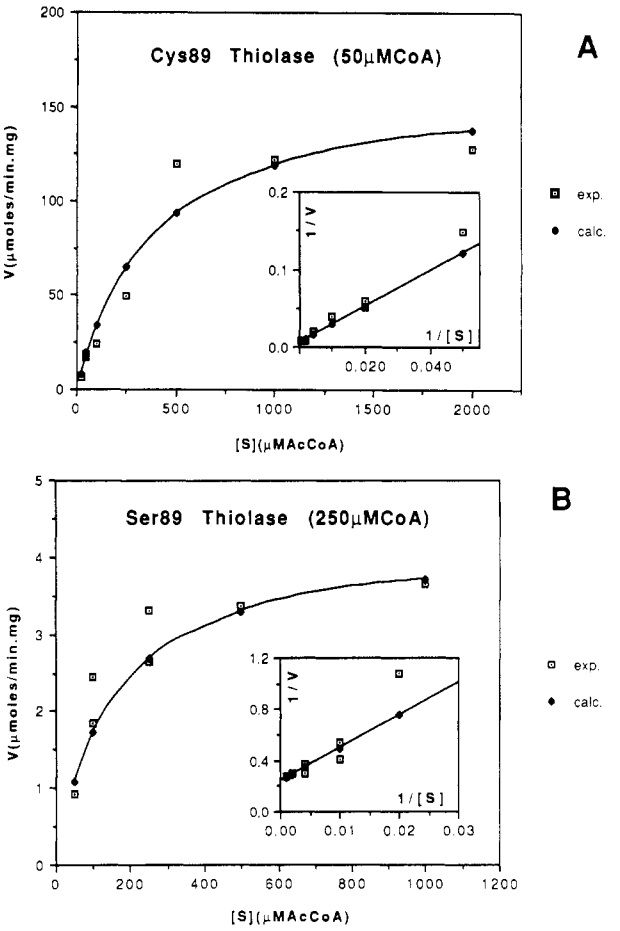


FIGURE 3: Plots of velocities (V) of exchange reactions as a function of the concentration of acetyl-CoA ([S]) for Cys_{89} (A) and Ser_{89} (B) thiolases. Inserts show corresponding $1/V$ vs $1/[S]$ plots.

identically 2.4 and 2.2 for the wild type and the C89S mutant, respectively. This discrepancy is yet to be resolved, but the deuterium isotope effects are highly reproducible. The second issue is why and how the wild-type enzyme and C89S enzyme show equivalent deuterium isotope effects on V_{max}, suggesting equivalent kinetic significance to each C-H(^{2}H) cleavage transition state even though the absolute value of the V_{max} for condensation differs by 2300-fold (Table II)! Given that enzyme acetylation occurs prior to enolization of the second acetyl-CoA molecule, then one wonders if the large V_{max} differences can arise from small differences in bond lengths (fractions of angstroms) and in bond angles for orientation of the acetyl-S-Cys_{89} enzyme vs acetyl-O-Ser_{89} enzyme with the closely bound acetyl-CoA molecule about to undergo C_2-H cleavage. An equivalent V_H/V_D value may mean the C-H cleavage step is equivalently rate limiting, but the different geometry of acetyl-S-enzyme vs acetyl-O-enzyme and anticipated close packing of bound acetyl-CoA, combined with the heightened thermodynamic stability of the acetyl-O-enzyme, could slow its absolute condensation rate dramatically. Lastly, the observed kinetic isotope effect may possibly reflect equilibrium isotope effects between enzyme-bound intermediates. We do not address here the issue of whether C-C bond formation and C-H cleavage are in fact stepwise (C-H cleavage first) but note that for other Claisen condensing enzymes evidence for stepwise routes is accumulating (Clark et al., 1988).

Alternate Thiol Acceptors in Acetoacetyl-CoA Cleavage Direction. To search for the rate-determining step in the thiolytic direction, the cleavage reaction was conducted in the presence of either CoASH (K_M = 8.8 and 6.5 μM) or 2-

mercaptoethanol (K_M = 50 and 51 mM) with wild-type and C89S thiolase. If the carbon–carbon bond cleavage step were rate limiting, we would expect that there should be no effect on the maximum catalytic rate regardless of the affinity of the acceptor thiol for the enzyme. Conversely, if the rate-determining process were the acyl-transfer step from the acetyl enzyme intermediate to thiol acceptor, one would expect to see a change in the k_{cat}. As it turns out, the CoASH/mercaptoethanol V_{max} ratio for wild-type thiolase is 2.2 (data not shown), indicating that the enzyme deacetylation acyl-transfer half-reaction is at least partially rate determining in the cleavage direction. This is consistent with porcine heart thiolase II as reported by Gehring and Lynen (1972). For both of these thiolases, acetyl enzyme thiolysis is at least partially rate determining in overall cleavage. Both of these enzymes have a strict substrate specificity for acetoacetyl-CoA as opposed to longer chain 3-oxoacyl CoAs and are involved in cellular biosynthetic processes.

In contrast to the wild-type enzyme, the C89S mutant thiolase showed k_{CoASH}/k_{ME} of 1.1. This indicates that the acetyl–O–enzyme deacylation step in the cleavage direction is not rate limiting, suggesting that the slow step is the carbon–carbon bond cleaving, acetyl enzyme forming half-reaction. When the wild-type and mutant enzymes are assayed with acetoacetyl-CoA in the absence of CoASH, any detectable turnover (monitored by loss of 302-nm absorbance of the β-ketoester group of acetoacetyl-CoA) occurs by acetyl–X–enzyme intermediate hydrolysis since there is no thiol acceptor species for discharge of the acetyl enzyme. Since the turnover rate is much slower for both enzymes in such acetyl enzyme *hydrolysis* conditions (0.04 and 0.12 s^{-1}) as compared to *thiolysis* conditions (8.4 and 813 s^{-1}) for C89S and wild type, respectively, the hydrolysis step must be the slowest step in this pathway.

Comments on Reaction Energetics of Wild-Type and C89S Thiolases. Thiolase I and thiolase II from pig heart are reported to have rate-determining steps that differ according to the direction of the reaction being catalyzed (Gehring & Lynen, 1972). For pig heart thiolase I, the acetoacetyl-CoA carbon–carbon bond cleavage step is rate limiting in the thiolytic process, and enzyme acylation is rate limiting in the acetyl-transfer direction (Gilbert, 1981; Gilbert et al., 1981). This is just the opposite from pig heart thiolase II, which was reported to be rate limiting in the deacylation half-reaction in the thiolytic direction and rate determining in the carbon–carbon bond forming half-reaction in the C–C bond forming condensation direction (Gehring & Lynen, 1972). Our experiments show that thiolase from *Z. ramigera* appears similar to type II thiolase in that it has the same qualitative type of free energy profile, consistent with the fact that both thiolase II and *Z. ramigera* thiolase are *biosynthetic* enzymes and are short chain (AcAcSCoA) specific. Thiolase I is specific for long-chain β-ketoacyl CoA compounds and is involved in *degradative* fatty acid oxidation.

A qualitative diagram for the free energy changes in the reaction coordinate for both wild-type and C89S thiolase can now be delineated (Figure 4). The Claisen cleavage reaction (left to right) is thermodynamically favored ($K_{eq} = 10^6$). In the formation of the covalent acetyl enzyme at the end of the carbon–carbon cleavage step, it is likely there is a higher barrier in the Ser_{89} mutant since for equivalent pK_a's ROH species are poorer nucleophiles than RSH by a factor of 10 or more (Hupe & Jencks, 1977). While the pK_a values of both Cys_{89} and Ser_{89} residues are not yet determined, it may be that the expected solution ΔpK_a of ca. 7 between ROH (pK_a ~ 14) and RSH (pK_a ~ 7) is maintained in the enzyme. The covalent acetyl–O–enzyme oxoester appears less activated thermodynamically than the corresponding acetyl–S–enzyme thiolester, as suggested by the data of Figure 2. The thiolytic decomposition of acetyl–S–enzyme is a kinetically significant transition state as assessed by alternate thiol acceptor V_{max} data but is not so in the acetyl–O–enzyme species, even though the absolute thiolysis rate is down ca. 100-fold. Jencks and coworkers in model studies (Hupe & Jencks, 1977) have determined that RS^- thiolate anions are 10–100-fold more reactive with acyl thiolesters than with acyl oxoesters, so a higher net barrier for thiolysis of acetyl–O–enzyme formation is anticipated. But this relatively higher barrier is not substantially rate determining in turnover, suggesting then that acetyl–O–enzyme formation is rate limiting. Thus in this Claisen cleavage direction one would postulate that the Cys_{89} to Ser_{89} change, in addition to altering the stability of the acetyl enzyme intermediate and raising barriers for both enzyme acetylation and deacetylation, selectively slows enzyme acetylation making it much more rate determining than in wild-type enzyme.

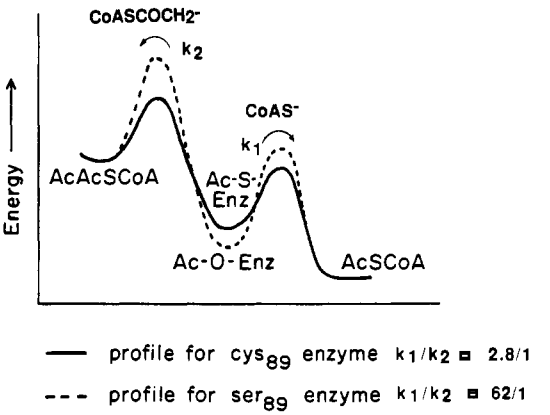


FIGURE 4: Schematic for energy profiles of the wild-type Cys_{89} thiolase (solid line) and the mutant Ser_{89} thiolase (dashed line). Partition of acetyl–S–enzyme and acetyl–O–enzyme between capture by the thiolate anion of coenzyme A to return to acetyl-CoA starting material and capture by the C_2 carbanion of acetyl-CoA to go forward to acetoacetyl-CoA product. Partition ratios were obtained by comparing V_{max} rates of acetyl–S–enzyme and acetyl–O–enzyme formation from [^{32}P]CoASH/acetyl-CoA exchange data with V_{max} rates for overall condensation as noted under Experimental Procedures. The energy barrier for acyl enzyme formation from both C_4 and C_2 substrates is higher in the Ser_{89} enzyme, and the covalent acetyl–O–enzyme intermediate is more stable.

In the Claisen condensation direction (right to left in Figure 4) the formation of acetyl–O–enzyme versus acetyl–S–enzyme should again be ca. 10 times slower by reference to the poorer nucleophilicity of ROH versus RSH as noted above. A factor of 41 between V_{max} for [^{32}P]CoASH/acetyl-CoA exchanges was observed between Cys_{89} and Ser_{89} enzymes, indicating acetyl–S–enzyme may be formed some 41 times faster than acetyl–O–enzyme. On the other hand, the acetyl-CoA concentration dependence studies for acetyl–X–enzyme accumulation (Figure 2) suggest a 17-fold preference for acetyl–O–enzyme accumulation; so while acetyl–O–enzyme is formed by a ~3 kcal/mol higher barrier, the acetyl–O–enzyme is ca. 1.5–2 kcal/mol more stable than acetyl–S–enzyme. The 2300-fold-lessened V_{max} in condensation for the Ser_{89} mutant probably stems both from the deeper well in which the acetyl–O–enzyme is situated and from the selectively higher barrier for C–H cleavage, C–C formation (treated here as a single step). The acetyl–O–enzyme now partitions some 40-fold less often in the forward direction (1 in 62 events) compared to acetyl–S–enzyme (1 in 2.8 events). The fairly close

partition backward/forward in wild-type acetyl–S–enzyme is converted to a more disparate partition and a poorer Claisen condensation catalyst by selective slowing of reaction of the acetyl oxoester enzyme with the C_2 carbanion of acetyl-CoA, in line with precedents in model chemistry.

These studies document the catalytic consequences in altering the covalent acetyl enzyme intermediate in thiolase catalysis from an acetyl thiolester to an acetyl oxoester grouping. In thiolase catalysis the acetyl–X–enzyme intermediate is utilized exclusively as the electrophilic species (at C_1 of the acetyl fragment) in condensation. In the very next enzyme in isoprenoid biogenesis, β-(hydroxymethyl)-glutaryl-CoA synthase, the same acetyl–S–enzyme type of intermediate is used exclusively as the nucleophilic species (at C_2 of the acetyl fragment) in condensation, and it may be of use to compare the functional consequences of Cys to Ser mutation in that enzyme as well. We have previously noted (Davis et al., 1987a) that when acetyl–S–pantetheine is replaced by acetyl–O–pantetheine as a thiolase substrate, the acetyl oxoester is not accepted either as a electrophilic or as a nucleophilic partner in thiolase catalysis, suggesting that the oxoester has insufficient thermodynamic activation for acetyl transfer and that the C_2 carbanion of the acetyl oxoester (pK_a of ca. 26 vs ca. 20 for acetyl thioester) is out of reach of the enzymatic reaction manifold.

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