

Supporting Information for

Inactivation of thiolase by 2-alkynoyl-CoA  
via its intrinsic isomerase activity

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*Materials*

A HiTrap chelating metal affinity column was purchased from Amersham Pharmacia Biotech. Coenzyme A and acetoacetyl-CoA were purchased from Sigma. HB101 competent cells, *E. coli* strain BL21(DE3) competent cells and agarose came from Invitrogen Life Technologies. The Plasmid Mini kit and synthesized oligonucleotides were obtained from Tech Dragon Company of Hong Kong. Restriction enzymes came from MBI Fermentas of Germany. All other reagents were of research grade or better and were obtained from commercial sources. 2-Octynoic acid was purchased from Aldrich. Various *cis*- and *trans*-alkenoic acids were obtained as described previously.<sup>1</sup> The rat liver thiolase was purified as previously described.<sup>2</sup> We incorporated six histidine amino acids on the *N*-terminus of the enzyme considering it is much easier to be purified to apparent homogeneity without possible contamination of authentic enoyl-CoA isomerase, which can give reproducible and reliable result.

*Construction of Rat Liver Thiolase Mutants C382S, C382A and C92S*

A QuikChange mutagenesis kit (Stratagene) was applied for constructing the pThiolase(C382S), pThiolase(C382A), and pThiolase(C92S) mutant expression plasmids. The plasmid pLM1::Thiolase was used as a template for constructing

mutant plasmids through PCR reaction. The following primers and their antisense primers were synthesized to introduce the mutated sequence:

1. C382S, 5' c gca gtg gga tca gct **agc** att gga ggt ggc c 3', codon tgc for cysteine (C) was changed to codon agc for serine (S);
2. C382A, 5' c gca gtg gga tca gct **gcc** att gga ggt ggc caa g 3', codon tgc for cysteine (C) was changed to codon gcc for alanine (A);
3. C92S, 5' c acc ctc aac aga ctc **agt** ggc tct ggt ttc cag 3', codon tgt for cysteine (C) was changed to codon agt for serine (S).

PCR amplification was performed using *Pfu* DNA polymerase and samples were subjected to 13 cycles of 0.5 minute of denaturation at 95 °C, 1 minute of annealing at 60-63 °C, and 12 minutes of elongation at 72 °C in a Mastercycler (Eppendorf). The mutant-carrying plasmid was transformed into *E. coli* HB101 competent cells (Novagen) by electroporation (Bio-Rad) for screening purposes. Positive clones were identified and the DNA sequenced to verify the presence of the desired mutations and the absence of any PCR-generated random mutations. Plasmids were then transformed in *E. coli* strain BL21(DE3) cells for expression purposes. All constructs and mutations were confirmed by sequencing with the dye-terminator cycle sequencing kit employing specific oligonucleotides.

#### *Expression and Purification of Rat Liver Thiolase Mutants C382S, C382A and C92S*

Established methods were used to prepare the samples.<sup>2</sup> The expressions of rat liver thiolase and its variant proteins in *E. coli* BL21(DE3) were carried out at different temperatures and IPTG concentrations, and proteins were obtained for all tested conditions. It was found that the protein expressed very well even without the addition of IPTG and that it resulted in a higher yield of soluble protein at room temperature than at 37 °C. Nickel metal-affinity resin columns were used for

single-step purifications of His-tagged rat liver thiolase wild-type and variant proteins. The wild-type and variant proteins C382S, C382A and C92S were obtained as soluble proteins that can be stored in a  $-80^{\circ}\text{C}$  freezer and were stable for at least 6 months as tested on the basis of their activity. The proteins, all observed to be apparent homogeneity by SDS-PAGE as shown in Figure 1, were stored at  $-80^{\circ}\text{C}$  in 50 mM potassium phosphate buffer, pH 7.5, 5% glycerol, and 5 mM  $\beta$ -mercaptoethanol.

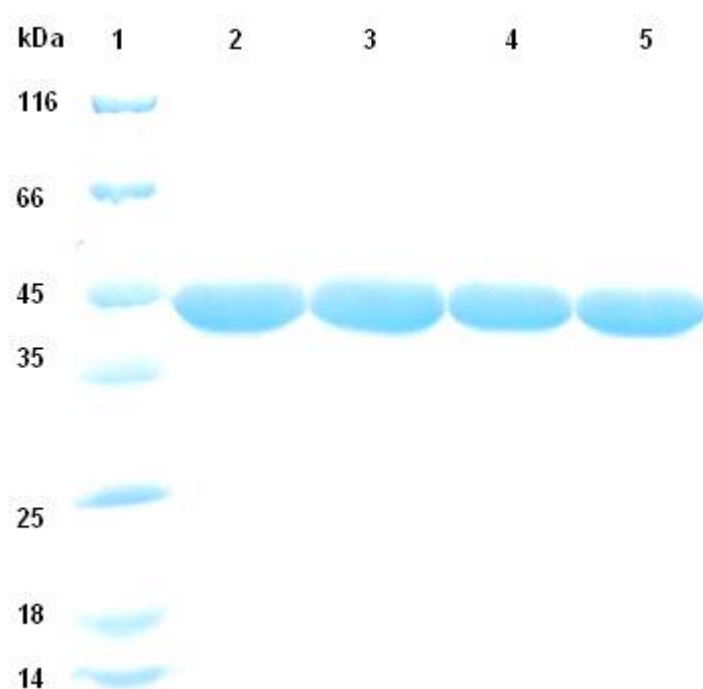


Figure 1. Coomassie blue-stained SDS-PAGE of the purified rat liver thiolase wild-type and variant enzymes. Lane 1, molecular mass standards; lane 2, purified rat liver thiolase wild-type enzyme; lane 3, purified rat liver thiolase mutant C382A; lane 4, purified rat liver thiolase mutant C382S; lane 5, purified rat liver thiolase mutant C92S.

#### *Activity Assay for Rat Liver Thiolase*

Rat liver thiolase activity was assayed by following spectrophotometrically the decrease in absorbance at 303 nm due to the disappearance of the  $\text{Mg}^{2+}$ -enolate complex of acetoacetyl-CoA ( $\epsilon_{303} = 16.5 \text{ mM}^{-1}\text{cm}^{-1}$ ), as described previously.<sup>2</sup> The reaction progress curves were recorded for 1 min on a Hitachi U-2000 UV-visible

spectrophotometer. A standard assay mixture contained 0.1 mM Tris-HCl (pH 8.2), 25 mM MgCl<sub>2</sub>, 33  $\mu$ M acetoacetyl-CoA, 90  $\mu$ M CoASH, and the reaction was started by adding 5  $\mu$ L of appropriate diluted enzyme in the reaction mixture. Unit of thiolase activity was defined as the amount of thiolase that catalyzes the cleavage of 1  $\mu$ mol of acetoacetyl-CoA to acetyl-CoA per minute.

#### *Kinetic Studies of Thiolase Activity for Rat Thiolase Wild-Type and Variant Proteins*

For the kinetic characterization of rat thiolase wild-type and variant proteins, rates were measured at five substrate concentrations and averages of two assays were used for each point.  $K_M$  of  $46 \pm 14$   $\mu$ M for acetoacetyl-CoA and  $k_{cat}$  of  $39 \pm 3$  s<sup>-1</sup> were obtained for rat thiolase wild-type enzyme by non-linear curve fitting using the SigmaPlot 8.0 program (Table 1). Cys382 is the catalytic residue responsible for deprotonation of  $\alpha$ -proton of acetyl-CoA in biosynthetic direction. In this study, we mutated Cys382 into serine and alanine. The  $k_{cat}/K_M$  values of the mutants C382S and C382A are 400 times and 1,200 times lower than that of the wild-type enzyme respectively, which confirmed that Cys382 is the catalytic residue for rat thiolase. Cys92 is the catalytic residue responsible for attacking carbonyl carbon of acyl-CoA in both degradative and biosynthetic directions. In the present study, we mutated Cys92 into serine. The  $k_{cat}/K_M$  value of the mutant C92S is 800 times lower than that of the wild-type enzyme, which confirmed that Cys92 is also the catalytic residue for rat thiolase. It should be noted that mutation of Cys382 and Cys92 into other residues could affect the redox properties of the enzyme <sup>3</sup>, which also affected the activity of the thiolase.

TABLE 1

Kinetic parameters for the thiolase activity of rat thiolase wild-type  
and variant proteins using acetoacetyl-CoA as the substrate

	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_{\text{M}}$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_{\text{M}}$ ( $\text{s}^{-1} \mu\text{M}^{-1}$ )
WT	$39 \pm 3$	$46 \pm 14$	0.85
C382S	$0.07 \pm 0.01$	$29 \pm 5$	$2 \times 10^{-3}$
C382A	$0.06 \pm 0.01$	$86 \pm 32$	$7 \times 10^{-4}$
C92S	$0.08 \pm 0.01$	$73 \pm 12$	$1 \times 10^{-3}$

*Activity Assay for Isomerase Activity of Rat Liver Thiolase*

3-Enoyl-CoA thiolester was prepared from the corresponding free acid and coenzyme A by the mixed anhydride method<sup>4</sup> and subsequently purified by reverse-phase HPLC. *cis*-3-Enoyl-CoAs were made from corresponding *cis*-3-enoic acids, which showed different retention time on HPLC profiles from those of corresponding *trans*-3-enoyl-CoAs. A previously described method was used for assaying the activity of enoyl-CoA isomerase.<sup>5,6</sup> The assay mixture contained 50 mM of potassium phosphate (pH 7.5), 100  $\mu\text{M}$  EDTA, 50  $\mu\text{M}$  3-enoyl-CoA, and appropriate amount of enzyme in a final volume of 1.0 mL. The reaction mixtures were preincubated for 1 minute at 25 °C and were initiated by the addition of the enzyme. The increase in absorbance at 263 nm was monitored, which is characteristic for the conjugated  $\alpha,\beta$ -unsaturated thioester group ( $\epsilon_{263} = 6.7 \text{ mM}^{-1} \text{ cm}^{-1}$ ). One enzyme unit (U) was defined as the activity that converts 1  $\mu\text{mol}$  of 3-enoyl-CoA to 2-enoyl-CoA per minute. Determination of the  $K_{\text{M}}$  and the  $V_{\text{max}}$  was performed using the same assay buffer with varying substrate concentrations.

### *Analysis of Enzymatic Incubation Mixture with HPLC*

In order to further confirm that rat liver thiolase has intrinsic enoyl-CoA isomerase activity, we carried out incubation studies of the thiolase with *trans*-3-hexenoyl-CoA and analyzed the metabolites of the incubation mixture with HPLC, as shown in Figure 2. Enzymatic incubations were carried out at room temperature and the incubation reactions were terminated by filtering through a Microcon YM-10 filter (Millipore) with centrifugation. The filtrate was applied to a HPLC C<sub>18</sub> reverse-phase column (4.6×250mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 260 nm. Separation was achieved by linearly increasing the methanol/H<sub>2</sub>O (85%, v/v) content of 25 mM potassium phosphate elution buffer (pH 5.9) from 20% to 80% in 35 minutes at a flow rate of 0.8 ml/min.

*trans*-3-Hexenoyl-CoA showed a single peak **a** in our HPLC profile with a retention time of 22.8 minutes (Fig. 2A). After incubating with rat liver thiolase, *trans*-3-hexenoyl-CoA was partially transformed to peak **b**, which may be *trans*-2-hexenoyl-CoA with a retention time of 23.2 minutes (Fig. 2B). However, rat liver thiolase variant proteins C382S and C382A cannot transform *trans*-3-hexenoyl-CoA to *trans*-2-hexenoyl-CoA as shown in Fig. 2C and Fig. 2D. The incubation mixture of rat liver thiolase and *trans*-3-hexenoyl-CoA was co-injected into HPLC with *trans*-2-hexenoyl-CoA, and the height of peak **b** increases as shown in Fig. 2E, which indicates peak **b** is indeed *trans*-2-hexenoyl-CoA. Peak **b** of Fig. 2B was mostly converted to peak **c** of Fig. 2F when incubating with enoyl-CoA hydratase, which further confirmed that peak **b** is *trans*-2-hexenoyl-CoA and peak **c** is 3-hydroxyhexanoyl-CoA. This result confirmed that Cys382 is the catalytic residue for the isomerase activity of rat liver thiolase.

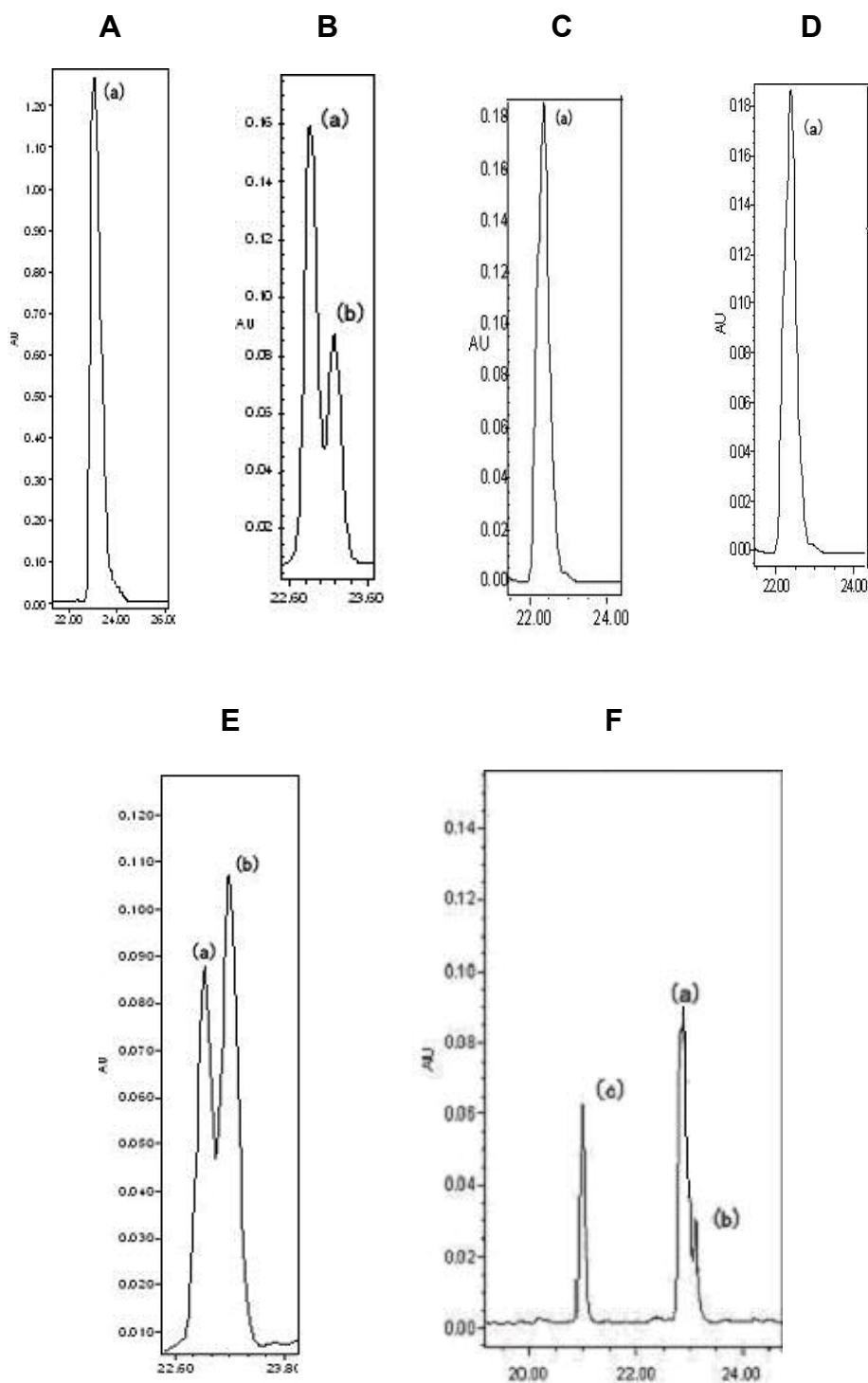


Figure 2. Analysis of incubation mixture using HPLC. A. *trans*-3-hexenoyl-CoA; B. incubation of *trans*-3-hexenoyl-CoA with rat liver thiolase; C. incubation of *trans*-3-hexenoyl-CoA with rat liver thiolase mutant C382S; D. incubation of *trans*-3-hexenoyl-CoA with rat liver thiolase mutant C382A; E. incubation of *trans*-3-hexenoyl-CoA with rat liver thiolase, and the resulting mixture was co-injected with *trans*-2-hexenoyl-CoA; F. incubation of *trans*-3-hexenoyl-CoA with rat liver thiolase and enoyl-CoA hydratase (add enoyl-CoA hydratase to mixture B). Possible identity of substrate or product peaks: a. *trans*-3-hexenoyl-CoA; b. *trans*-2-hexenoyl-CoA; c. 3-hydroxyhexanoyl-CoA

### *pH Dependence of Thiolase and Isomerase Activities for Rat Liver Thiolase*

Both thiolase and isomerase activities of rat liver thiolase were measured at different pH, and the results were shown in Figure 3. It was found that both activities have similar pH dependence curves with maximum activity at pH 8.5. This may further indicate that both thiolase and isomerase activities are associated with same active site of the enzyme, and Cys382 is the catalytic residue of the enzyme for both thiolase and isomerase activities.

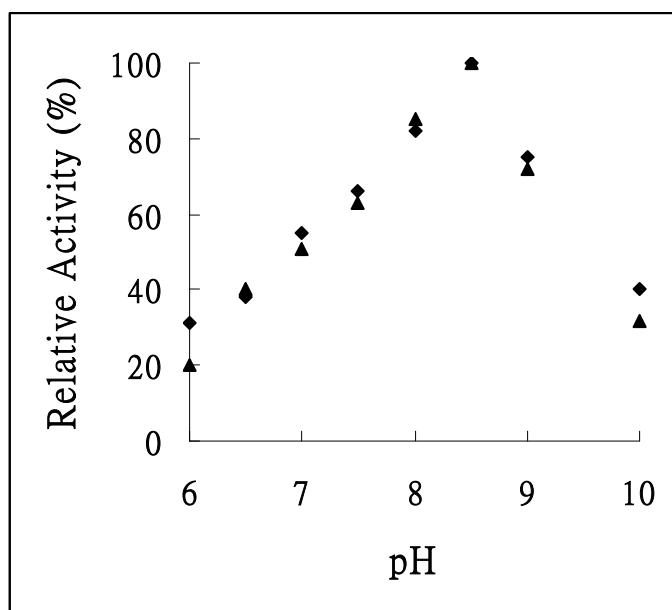


Figure 3. Effect of pH on thiolase (◆) and isomerase (▲) activities of rat liver thiolase

### *Comparison of Rat Liver Thiolase with Other Enzymes for their Isomerase Activities*

The  $k_{\text{cat}}$  values of rat liver thiolase variant enzymes C382S and C382A decreased 524 and 157 times for their isomerase activities comparing with that of the wild-type enzyme, which confirmed that Cys382 is the catalytic residue for its isomerase activity. In comparison, C92S has comparable isomerase activity to the wild-type enzyme, although its thiolase activity decreased significantly. This result may indicate only one catalytic base is required for thiolase catalyzed isomerization reaction. After deprotonation of  $\alpha$ -proton, the isomerization step is a thermodynamic favorable



process since the product is a more stable conjugated  $\alpha,\beta$ -unsaturated molecule. The isomerases usually have two bases, one for deprotonation and another for protonation. Since the isomerase activity of the thiolase is a side activity of the enzyme, its isomerase activity seems not optimized during its evolution. The normal function of the thiolase is to deprotonate  $\alpha$ -proton of one acyl-CoA for the subsequent nucleophilic attack to the carbonyl group of another acyl-CoA, which does not need the participation of another base.

So far, seven mammalian enzymes with enoyl-CoA isomerase activities have been described. They are mitochondrial enoyl-CoA isomerase,<sup>7,8</sup> mitochondrial long-chain enoyl-CoA isomerase,<sup>9</sup> mitochondrial enoyl-CoA hydratase or crotonase,<sup>10</sup> acyl-CoA dehydrogenase,<sup>6</sup> peroxisomal enoyl-CoA isomerase,<sup>11</sup> multifunctional enzyme 1,<sup>12,13</sup> and acyl-CoA oxidase.<sup>1</sup> The first four enoyl-CoA isomerase activities are present in mitochondria, and the last three enzymes are associated with peroxisomes. These enzymes have different substrate specificities, which compensate each other so that isomerase reaction can occur on all spectra of substrates with different chain-length. Thiolase is another class of enzyme, which has intrinsic enoyl-CoA isomerase activity. The comparison of kinetic properties for enoyl-CoA isomerase activities of enzymes from various sources is listed in Table 2. The  $k_{cat}/K_M$  value of the thiolase for isomerization reaction is comparable to that of rat mitochondrial medium-chain acyl-CoA dehydrogenase.

TABLE 2  
Kinetic properties of enoyl-CoA isomerase activities of enzymes from various sources

Enzyme	Substrate	$K_M$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_M$ ( $\mu\text{M}^{-1}\text{s}^{-1}$ )	Reference
enoyl-CoA isomerase	<i>cis</i> -3-hexenoyl-CoA	$1200 \pm 157$	$58.0 \pm 5.6$	0.049	8
multifunctional enzyme 1	<i>cis</i> -3-hexenoyl-CoA	$31 \pm 4$	$1.60 \pm 0.13$	0.052	8
medium-chain acyl-CoA dehydrogenase	<i>cis</i> -3-hexenoyl-CoA	$20 \pm 3$	$0.11 \pm 0.01$	$5.5 \times 10^{-3}$	6
acyl-CoA oxidase I	<i>cis</i> -3-hexenoyl-CoA	$63 \pm 13$	$21 \pm 3$	0.33	1
Rat liver thiolase	<i>cis</i> -3-hexenoyl-CoA	$52 \pm 18$	$0.33 \pm 0.01$	$6.4 \times 10^{-3}$	this report
enoyl-CoA isomerase	<i>cis</i> -3-octenoyl-CoA	$100 \pm 7$	$47.0 \pm 1.4$	0.45	8
multifunctional enzyme 1	<i>cis</i> -3-octenoyl-CoA	$32.0 \pm 6.3$	$2.4 \pm 0.2$	0.075	8
medium-chain acyl-CoA dehydrogenase	<i>cis</i> -3-octenoyl-CoA	$30 \pm 9$	$(1.6 \pm 0.2) \times 10^{-2}$	$5.3 \times 10^{-4}$	6
acyl-CoA oxidase I	<i>cis</i> -3-octenoyl-CoA	$57 \pm 7$	$4.5 \pm 0.3$	0.073	1
Rat liver thiolase	<i>cis</i> -3-octenoyl-CoA	$76 \pm 14$	$0.21 \pm 0.02$	$2.7 \times 10^{-3}$	this report
enoyl-CoA isomerase	<i>trans</i> -3-hexenoyl-CoA	$1600 \pm 79$	$100 \pm 39$	0.063	8
multifunctional enzyme 1	<i>trans</i> -3-hexenoyl-CoA	$38 \pm 1$	$1.8 \pm 0.1$	0.047	8
medium-chain acyl-CoA dehydrogenase	<i>trans</i> -3-hexenoyl-CoA	$15 \pm 3$	$(3.2 \pm 0.2) \times 10^{-2}$	$2.1 \times 10^{-3}$	6
acyl-CoA oxidase I	<i>trans</i> -3-hexenoyl-CoA	$63 \pm 17$	$3.0 \pm 0.5$	0.048	1
Rat liver thiolase	<i>trans</i> -3-hexenoyl-CoA	$55 \pm 16$	$(5.7 \pm 0.4) \times 10^{-2}$	$1.0 \times 10^{-3}$	this report
enoyl-CoA isomerase	<i>trans</i> -3-octenoyl-CoA	$120 \pm 12$	$210 \pm 11$	1.8	8



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Thiolase_ZR 166 QLSRDEQDAFAVASQNKAEEAAQKDGFRKDEIVPFIVKGRKGDITVDADEYIRHGATLDSM 225
Thiolase_Hs 179 QVSREDQDKVAVLSQNRTEAQAQAGHFDKEIVPVLVSTRRLIEVKTDEFPRHGSNIEAM 238
Thiolase_Rn 173 NISREDCDRYALQSQRWKAANEAGYFNEEMAPIEVKTKKGKQTMQVDEHARPQTTLLEQL 232
      ::***: * *: ***: : *: * *. *..*. *. *: *   ...**. * :.: :

Thiolase_ZR 226 AKLRPAFDKE—GTVTAGNASGLNDGAAAALLMSEAEASRRGIQPLGRIVSWATVGVDPK 283
Thiolase_Hs 239 SKLKPYFLTDGTGTVTPANASGINDGAAAVLMKKSEADKRGLTPLARIVSWSQVGVEPS 298
Thiolase_Rn 233 QNLPPVFKKE—GTVTAGNASGMSDGAGVVIASEDAVKKHNF TPLARVVG YFVSGCDPA 290
      :* * * .:  ****..*****.***...: :  ..::: **.*.*:  * :*

Thiolase_ZR 284 VMGTGPIPASRKALERAGWKIGDLDLVEANEAFAAQACAVNKDLGWDPSIVNVNGGAIAI 343
Thiolase_Hs 299 IMGIGPIPAIKQAVTKAGWSLEDVDIFEINEAFAAVSAAIVKELGLNPEKVNIEGGAIAL 358
Thiolase_Rn 291 IMGIGPVPAITGALKKAGLSLKMDLIDVNEAFAPQFLAVQKSLDLDPSTNVSGGAIAL 350
      **: **:**  *: **: .: *: :. : *****.  *: *. *. :*. .*: *****:

Thiolase_ZR 344 GHPIGASGARILNTLLFEMKRRGARKGLATLCIGGGMGVAMCIESL— 389
Thiolase_Hs 359 GHPLGASGCRILVTLLHTLERMGRSRGVAALCIGGGMGIAMCVQRE— 404
Thiolase_Rn 351 GHPLGGSGSRITAHLVHELRRRGKYAVGSACIGGGQGISLIIQNTA 397
      ***:*. **.*  *: .: * *  .: : ***** *: : :

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Figure 4. The sequences of thiolases from various sources. Thiolase\_ZR: Zoogloea ramigera biosynthetic thiolase; Thiolase\_Hs: Homo sapiens cytosolic thiolase; Rn: Rattus norvegicus thiolase. Residues conserved in all sequences are marked with \*. Residues not conserved in all sequences but conserved in some sequences are marked with : or . based on the degree of conservation. The sequence alignment was made using Clustal X.<sup>17,18</sup>

### *Protection of the Thiolase from the Inactivation by 2-Octynoyl-CoA*

The thiolase was protected by acetoacetyl-CoA (substrate of the thiolase catalyzed reaction) from inactivation by 2-octynoyl-CoA as shown in Figure 5, which indicates the inactivation is active-site directed. The enzyme (100 mM potassium phosphate buffer, pH 7.4) was preincubated with appropriate amount of protecting compound for 10 min at room temperature, followed by the addition of inhibitor. The residual enzyme activity was then assayed.

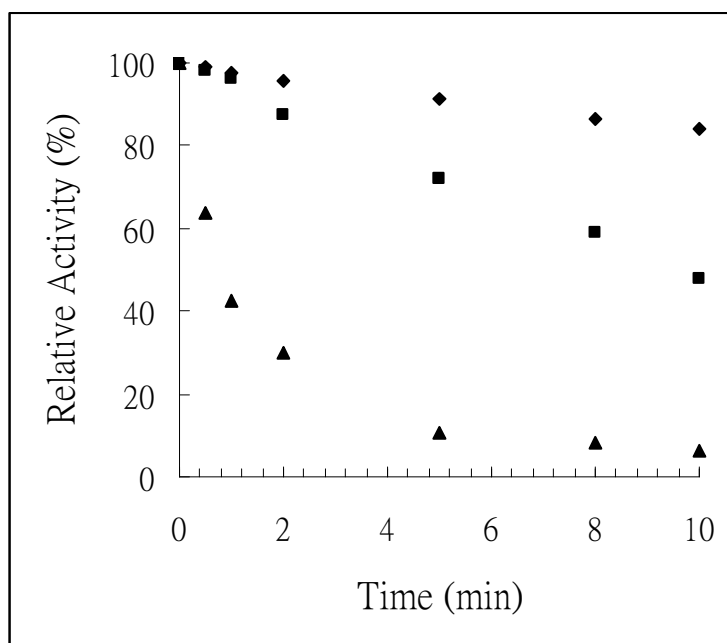


Figure 5. Protection of the thiolase by acetoacetyl-CoA from inactivation by 2-octynoyl-CoA. The thiolase (5.0  $\mu\text{M}$ ) was inactivated with 5.0 equivalents of 2-octynoyl-CoA in the presence of 0 ( $\blacktriangle$ ), 200  $\mu\text{M}$  ( $\blacksquare$ ) and 400  $\mu\text{M}$  ( $\blacklozenge$ ) acetoacetyl-CoA.

#### *Test for the Reversibility of the Inactivation*

A sample containing appropriate amount of enzyme and inhibitor was incubated for 60 min at room temperature to ensure complete inactivation of the enzyme. The inactivated enzyme was dialyzed against 100 mM potassium phosphate buffer (pH 7.4) over two days at 4 °C with seven buffer changes (100 mM potassium phosphate buffer, pH 7.4). The residual enzyme activity was determined before and after dialysis. A control experiment was performed under identical conditions without inhibitor.

#### *Kinetic Analysis of Inactivation*

The competence of 2-octynoyl-CoA to inactivate the thiolase was further studied by kinetic analysis. The loss of activity follows saturation kinetics and also exhibits dependence on inhibitor concentration. In a typical inactivation experiment to study the kinetics for inactivation, an appropriate amount of inhibitor dissolved in water was

incubated with the enzyme solution (100 mM potassium phosphate buffer, pH 7.4) at 25 °C. At various time intervals, aliquots (50 µL) of the incubation mixture were taken and diluted into the standard assay cocktail, and then the remaining enzyme activities were determined. The apparent dissociation constant ( $K_I$ ) and the inactivation rate constant ( $k_{inact}$ ) were deduced using SigmaPlot 8.0 program from first-order rate constants ( $k_{app}$ ) versus values of inhibitor concentration. As shown in Figure 6, the  $K_I$  and  $k_{inact}$  were determined to be 38 µM and 2.6 min<sup>-1</sup>, respectively, from this analysis.

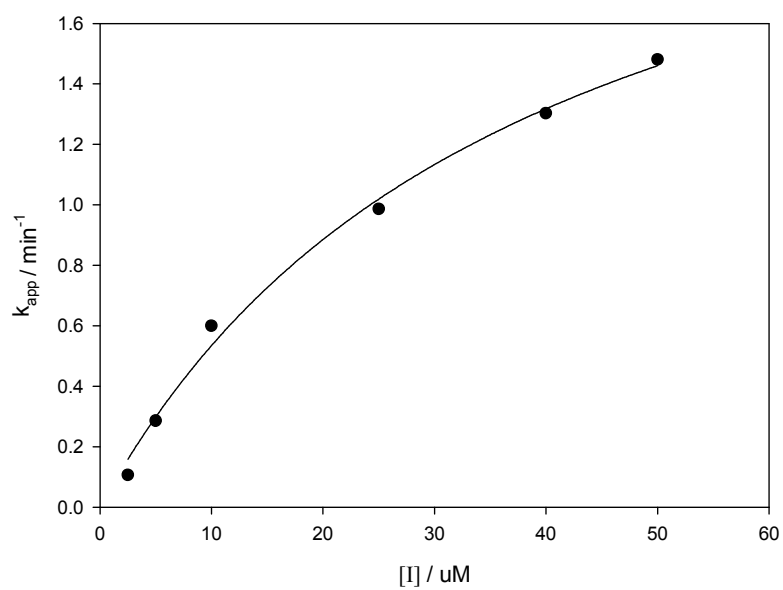
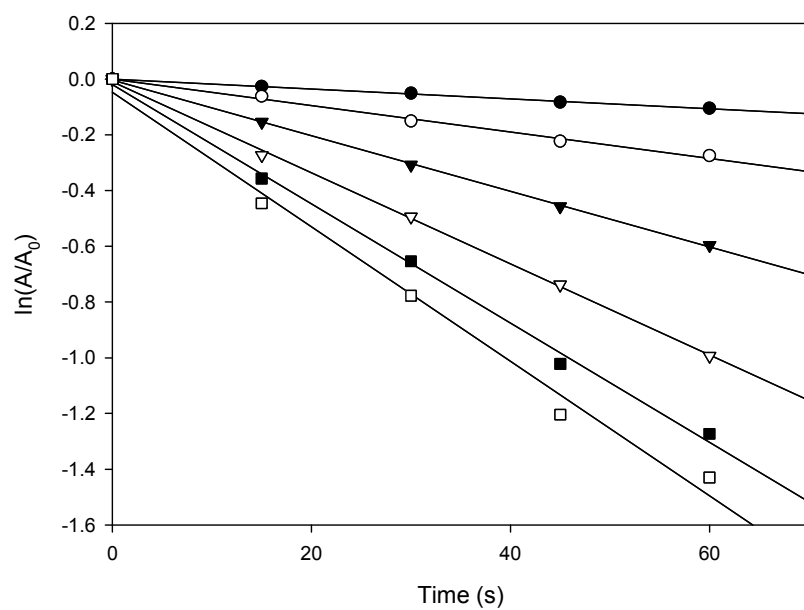
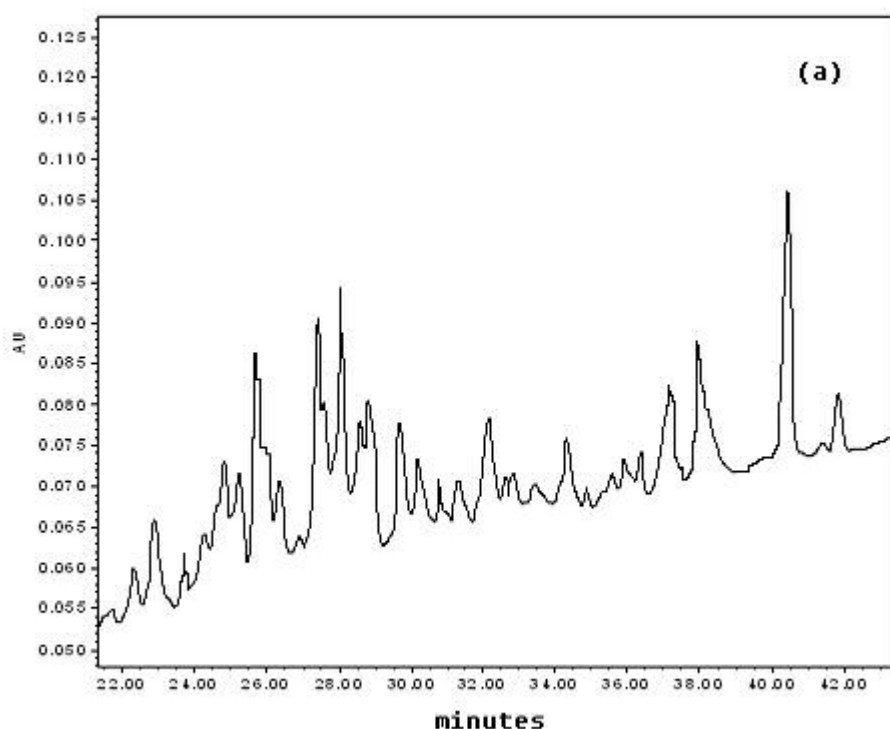


Figure 6. Time and concentration-dependent inactivation of the thiolase by 2-octynoyl-CoA (●, 0.75  $\mu\text{M}$  2-octynoyl-CoA; ○, 3.8  $\mu\text{M}$ ; ▼, 11.3  $\mu\text{M}$ ; ▽, 22.5  $\mu\text{M}$ ; ■, 45  $\mu\text{M}$ ; □, 90  $\mu\text{M}$ )

### *Trypsin Digestion of Incubation Mixture, and HPLC Separation of Labeled Peptide*

The thiolase was inactivated with 2-octynoyl-CoA, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with HPLC reverse phase column as shown in Figure 7b, and the eluent was monitored with UV detector at 220 nm wavelength. In a control experiment, non-labeled thiolase was also digested with trypsin, and the resulting peptide mixture was separated with HPLC reverse phase column as shown in Figure 7a for comparison. A new peak at 39 minutes was shown in Figure 7b, but not shown in Figure 7a. This peak may contain the peptide labeled by the inhibitor, which was subsequently further purified and analyzed with mass instrument. Rat liver thiolase amino acid sequence is shown in Figure 8.





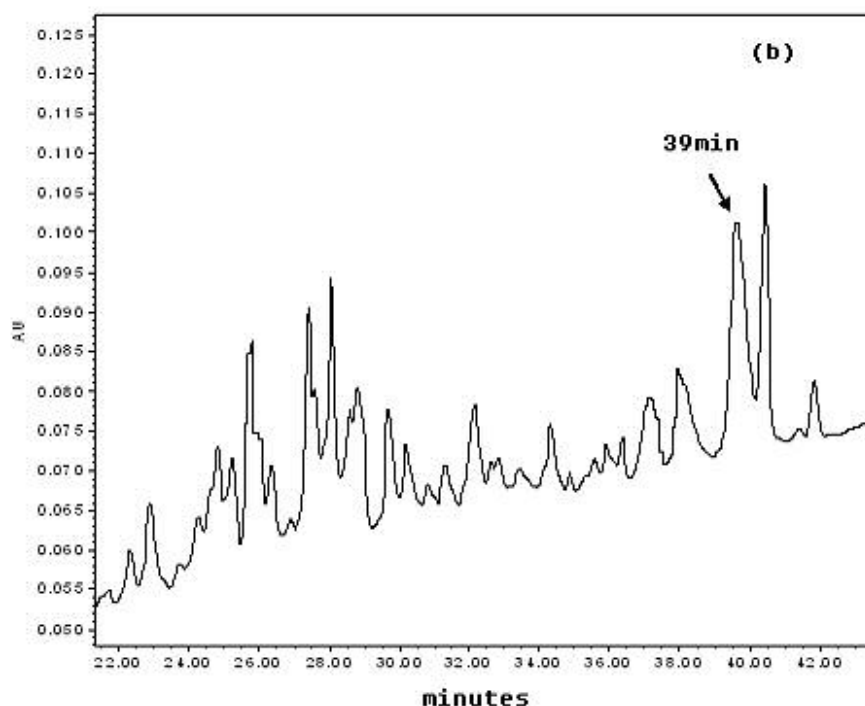


Figure 7. HPLC profile of tryptic digests of rat liver thiolase (a) and inactivated thiolase (b).

#### Rat Liver Thiolase Protein Sequence:

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1  MALLRGVFIVAARKRTPFGAYGGLLKDFTATDLTEFAARAALSAG  44
45  KVPETIDSVIVGNVMQSSDAAYLARHVGLRVGVPTETGALTNR  90
91  LCGSGFQSIIVSGCQEICK  109
110  DAEVVLCCGTESMSQSPYSVRNVRFGTKFGLDLKLEDTLWAGLTD  154
155  QHVKLPMGMTAENLAAKYNISREDCDRYALQSQQRWKAANEAGY  198
199  FNEEMAPIEVKTKKGKQTMQVDEHARPQTTLQLQNLPPVFKKEGT  244
245  VTAGNASGMSDGAGVVIIASEDAVKKHNFPLARVVG YFVSGCDPA  290
291  IMGIGPVPAITGALKKAGLSLKDMDLIDVNEAFAPQFLAVQKSLDLD  337
338  PSKTNVSGGAIALGHPLGGSGSRITAHLVHELRRRGGK  375
376  YAVGSACIGGGQGISLIQNTA  397

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Figure 8. Rat liver thiolase amino acid sequence

*Trypsin Digestion of Incubation Mixture of Rat Liver Thiolase Variant Enzyme C382A and 2-Octynoyl-CoA, and HPLC Separation of the Peptide Mixture*

The thiolase C382A variant enzyme was incubated with 2-octynoyl-CoA for 2 hours, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with HPLC reverse phase column as shown in Figure 9b, and the eluent was monitored with UV detector at 220 nm wavelength. In a control experiment, thiolase C382A variant enzyme was also digested with trypsin, and the resulting peptide mixture was separated with HPLC reverse phase column as shown in Figure 9a for comparison. Two HPLC profiles are almost identical, and the new peak at 39 minutes shown in Figure 7b is not shown in both Figure 9a and Figure 9b. This result indicates 2-octynoyl-CoA cannot label Cys92 of rat thiolase C382A variant enzyme, which further indicates Cys382 is required for covalent labeling of Cys92 of rat thiolase wild-type enzyme with 2-octynoyl-CoA. Since Cys382 catalyzes isomerization of 2-octynoyl-CoA into 2,3-octadienoyl-CoA, the result indicates the isomerization of 2-octynoyl-CoA to 2,3-octadienoyl-CoA is a required step for inactivation of rat thiolase wild-type enzyme by 2-octynoyl-CoA. It should be noted that Michael addition has been proposed for inactivation of medium-chain acyl-CoA dehydrogenase by oct-4-en-2-ynoyl-CoA,<sup>19</sup> which involves nucleophilic addition to the beta-carbon of the inhibitor. Therefore, another possible mechanism for inactivation of the thiolase involves Michael addition of Cys92 to the beta-carbon of 2-octynoyl-CoA. This mechanism does not need isomerization step, which is not likely based on above result.

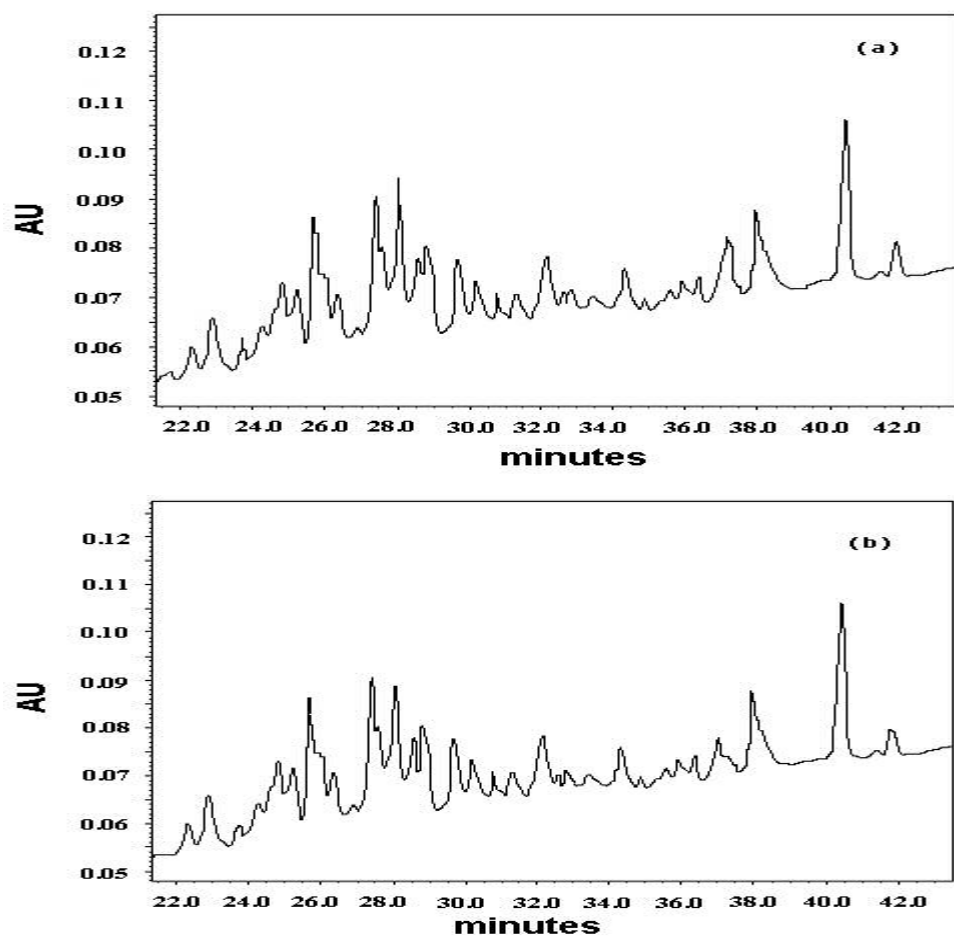


Figure 9. HPLC profile of tryptic digests of rat liver thiolase C382A variant enzyme (a) and the incubation mixture of rat liver thiolase C382A variant enzyme with 2-octynoyl-CoA (b).

Reference:

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