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Inactivation of Thiolase by 2-Alkynoyl-CoA via Its Intrinsic Isomerase Activity

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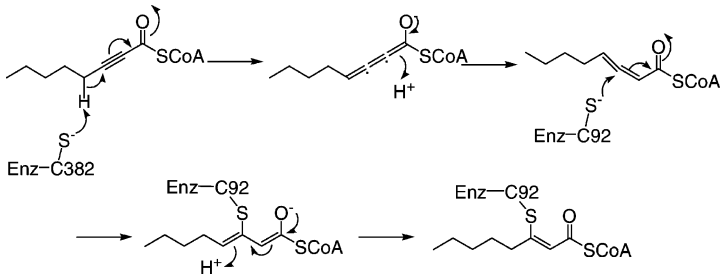
ABSTRACT



Selective inactivation of cytosolic thiolase by 2-alkynoyl-CoA via its intrinsic isomerase activity was studied, which provides an example for rationally developing mechanism-based inhibitors based on a side activity of the enzyme, and may become a supplemental method for better treatment of cardiovascular disease and cancer.

Cardiovascular disease and cancer are leading causes of death for people in western countries. The inactivation of enzymes involved in the mevalonate pathway (Figure 1) is one major method for treating the above diseases.[1] 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase catalyzes the main rate-limiting step in the mevalonate pathway for the synthesis of cholesterol and nonsteroid isoprenoid derivatives. Inhibition of HMG-CoA reductase by Lovastatin and related compounds has been used to block cholesterol synthesis in treating cardiovascular disease.[2] A variation on this approach has been suggested for cancer chemotherapy.[3] Extremely high levels of reductase inhibitors, given for a short period, could prevent farnesylation of Ras proteins, which inhibits the growth of Ras-dependent tumor cells.

However, this important medication has some side effects for patients, such as extreme muscle pain, serious liver problems,[4] erectile dysfunction, loss of memory, insomnia, personality changes and irritability, and even death.[5] Statin medications decrease the body's supply of coenzyme Q10,

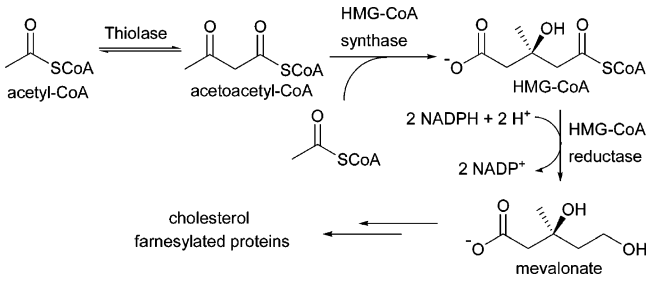


Figure 1. Mevalonate pathway in animal cells.

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which is an essential nutrient for heart strength and function.[6] The inhibition of one enzyme can cause an increase of the substrate concentration and a decrease of the product concentration, which may subsequently generate various side effects. New inhibitors targeting other enzymes in the mevalonate pathway need to be developed, which could be used together with statin in a drug combination strategy[7] to lower the dosage of statin.

The first step of the mevalonate pathway is catalyzed by cytosolic thiolase, which involves condensation of two acetyl-CoAs to give the acetoacetyl-CoA product.[8] We are interested in this enzyme because its substrate, acetyl-CoA, is a universal starting material for various biosynthetic pathways, and its accumulation may render less side effects for our bodies. The mitochondrial and peroxisomal thiolases are involved in fatty acid oxidation, which breaks 3-ketoacyl-CoA to produce two-carbon short acyl-CoA and acetyl-CoA. Although several bromine-labeled acyl-CoA analogues have been reported to be thiolase inhibitors,[9] they inactivate all thiolases without selectivity. In our study of the thiolase, we found that the enzyme has intrinsic isomerase activity as shown in Figure 2, which was thoroughly characterized in

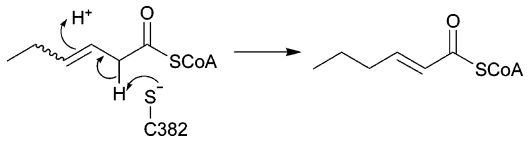

Figure 2. Isomerization reaction catalyzed by thiolase.

the present study. On the basis of this result, we synthesized 2-octynoyl-CoA as an isomerase mechanism-based inhibitor. It should be noted that 2-alkynoyl-CoA can be detoxified in mitochondria and peroxisomes in vivo by conversion into 3-ketoacyl-CoA catalyzed by enoyl-CoA hydratase.[10] Therefore, only cytosolic thiolase should be selectively inactivated by 2-alkynoyl-CoA in vivo.

The crystal structures of the thiolases from various sources have been solved,[11] and three amino acids including two cysteines and one histidine residue have been suggested as essential catalytic residues as shown in Figure 3. The mechanisms of thiolase-catalyzed reactions have been well

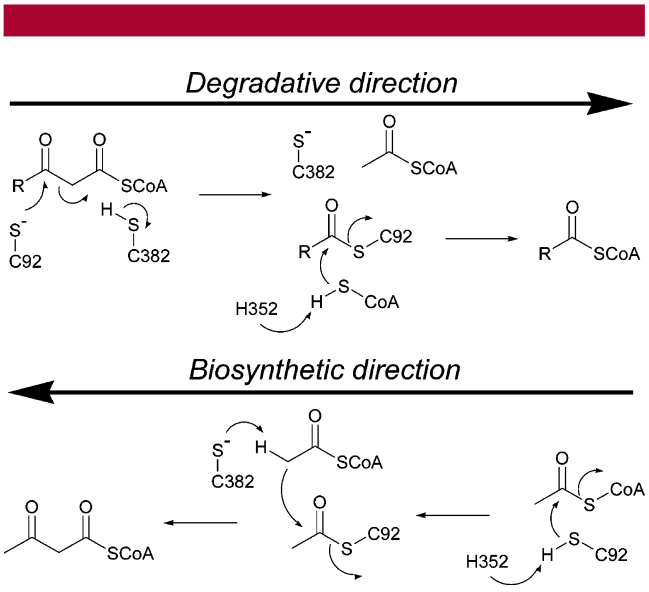

Figure 3. Cys92, His352, and Cys382 have been identified as catalytic residues for the reaction catalyzed by rat liver thiolase.

studied.[12] We have previously cloned and purified rat liver thiolase,[13] which has high sequence homology with well-studied *Homo sapiens* cytosolic thiolase and *Zoogloea ramigera* biosynthetic thiolase and was used in the present study. We think Cys382 may be the catalytic residue for the isomerase activity of the thiolase because it is responsible for α-proton abstraction in the biosynthetic direction. Therefore, we carried out site-directed mutagenesis and obtained variant proteins C382S and C382A. Kinetic studies were carried out for the wild-type and variant enzymes on their isomerase activities, and the result is shown in Table 1. The

Table 1. Kinetic Parameters for Isomerase Activity of Rat Thiolase Wild-Type and Variant Enzymes Using *cis*-3-Hexenoyl-CoA as Substrate[a]

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($s^{-1}/\mu M^{-1}$)
WT	0.33 ± 0.01	52 ± 18	6.3×10^{-3}
C382S	$(6.3 \pm 0.2) \times 10^{-4}$	ND	ND
C382A	$(2.1 \pm 0.1) \times 10^{-3}$	ND	ND
C92S	0.28 ± 0.01	61 ± 19	4.6×10^{-3}

[a] ND: Not determined.

k_{cat} values of C382S and C382A decreased 524 and 157 times for their isomerase activities compared with that of the wild-type enzyme, which confirmed that Cys382 is the catalytic residue for its isomerase activity. In comparison, C92S has

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isomerase activity comparable to the wild-type enzyme, although its thiolase activity decreased significantly. This result may indicate that only one catalytic base is required for thiolase-catalyzed isomerization reaction.

The substrate specificity of the thiolase for its isomerase activity was also studied, which is shown in Table 2. The

Table 2. Kinetic Parameters for Isomerase Activity of Rat Thiolase with Various Substrates

substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($s^{-1}/\mu M^{-1}$)
cis-3-hexenoyl-CoA	0.33 ± 0.01	52 ± 18	6.3×10^{-3}
cis-3-octenoyl-CoA	0.21 ± 0.02	76 ± 14	2.7×10^{-3}
cis-3-decenoyl-CoA	$(3.9 \pm 0.1) \times 10^{-2}$	80 ± 12	4.9×10^{-4}
trans-3-hexenoyl-CoA	$(5.7 \pm 0.4) \times 10^{-2}$	55 ± 16	1.0×10^{-3}
trans-3-octenoyl-CoA	$(4.6 \pm 0.1) \times 10^{-2}$	60 ± 20	7.7×10^{-4}
trans-3-decenoyl-CoA	$(3.0 \pm 0.2) \times 10^{-2}$	81 ± 39	3.7×10^{-4}

isomerase activity of the enzyme for the cis substrates is higher than that for the trans substrate. The isomerase activity of the enzyme for the shorter-chain substrate is higher than that for the longer-chain substrate. We carried out incubation studies of the thiolase with *trans*-3-hexenoyl-CoA and analyzed the metabolites of the incubation mixture with HPLC, which confirmed that 3-ketoacyl-CoA thiolase has intrinsic enoyl-CoA isomerase activity. No isomerization of *trans*-3-enoyl-CoA was detected in the presence of the C382S or C382A variant protein after incubation for several hours, and these variant proteins were purified following the same procedure as that for the purification of the wild-type rat liver thiolase. Both thiolase and isomerase activities of the thiolase were measured at different pH. 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 the same active site of the enzyme, and Cys382 is the catalytic residue for both thiolase and isomerase activities.

On the basis of the above result, we further synthesized 2-octynoyl-CoA as an isomerase mechanism-based inhibitor.

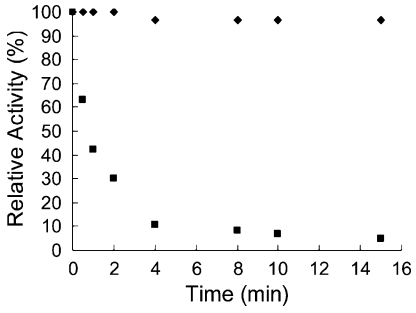

Figure 4. Inhibition of rat liver thiolase by 2-octynoyl-CoA. The thiolase (5.0 μM) was incubated with 2-octynoyl-CoA (◆, 0 μM; ■, 25 μM) at 25 °C at different times.

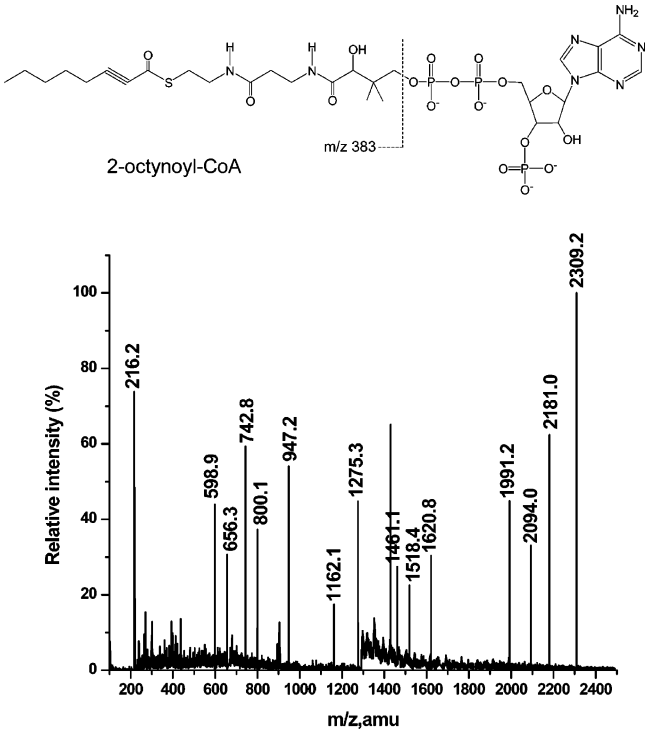

Figure 5. MS/MS spectrum analysis of a 2309 Da peptide showing covalent modification of rat liver thiolase peptide (91 L***C***GSGFQ-SIVSGCQEICSK 109) by 2-octynoyl-CoA. Molecular mass of peptide fragments: M1 = L + ***C*** (216 Da); M2 = M1 + Inhibitor (599 Da); M3 = M2 + G (656 Da); M4 = M3 + S (743 Da); M5 = M4 + G (800 Da); M6 = M5 + F (947 Da); M7 = M6 + Q + S (1162 Da); M8 = M7 + I (1275 Da); M9 = M8 + V + S (1461 Da); M10 = M9 + G (1518 Da); M11 = M10 + C (1621 Da); M12 = M11 + Q + E + I (1991 Da); M13 = M12 + C (2094 Da); M14 = M13 + S (2181 Da); M15 = M14 + K (2309 Da).

2-Octynoyl-CoA has been known as an irreversible inhibitor of MCAD.[14] The effect of 2-octynoyl-CoA on the catalytic activity of the thiolase was investigated by the incubation of the thiolase with 5 mol equiv of 2-octynoyl-CoA at room temperature. Time-dependent loss of the activity of the thiolase was noted during the incubation as shown in Figure 4. Because the activity of the inactivated enzyme remained unchanged after prolonged dialysis, the inactivation is clearly irreversible and most likely involves covalent linkage of 2-octynoyl-CoA with an amino acid residue in the active site of the thiolase. Incubation of the thiolase with 50 mol equiv of *trans*-2-octenoyl-CoA and 50 mol equiv of octanoyl-CoA was also carried out, respectively, but no inhibition of the thiolase was observed. This result indicates that the triple bond between C-2 and C-3 is mainly responsible for the inhibitory activity of 2-octynoyl-CoA on the thiolase. The thiolase was protected by acetoacetyl-CoA from inactivation by 2-octynoyl-CoA, which indicates the inactivation is active-site directed.

The competence of 2-octynoyl-CoA to inactivate the thiolase was further studied by kinetic analysis. The loss of

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activity follows saturation kinetics and also exhibits dependence on inhibitor concentration. The apparent dissociation constant (K_I) and the inactivation rate constant (k_{inact}) were deduced using the SigmaPlot 8.0 program from first-order rate constants (k_{app}) vs values of inhibitor concentration. The K_I and k_{inact} were determined to be 38 μM and 2.6 min^{-1}, respectively.

In another experiment, the thiolase was inactivated with 2-octynoyl-CoA, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with an HPLC reverse-phase column, and the elutent was monitored with a UV detector at both 220 and 260 nm wavelength. While nonlabeled peptide fragments only show peaks at 220 nm wavelength, the inhibitor-labeled peptide fragment shows a peak at both 220 and 260 nm wavelengths because the inhibitor contains coenzyme A. The labeled peptide was isolated, purified, and analyzed with MS/MS as shown in Figure 5. 2-Octynoyl-CoA degrades into a 383 Da fragment that was still attached to the peptide, as shown in Figure 5. The degradation pattern of 2-octynoyl-CoA is the same as that for the degradation of (methylenecyclopropyl)formyl-CoA.[15] The result indicated that Cys92 was covalently labeled by the inhibitor. This result clearly demonstrated that 2-octynoyl-CoA is an irreversible inhibitor of the thiolase.

The mechanism for the inactivation of the thiolase by 2-octynoyl-CoA is proposed as shown in Figure 6. 2-

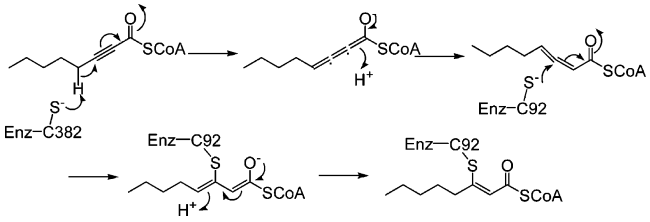

Figure 6. Proposed mechanism for the inactivation of the thiolase by 2-alkynoyl-CoA.

Octynoyl-CoA is first isomerized to reactive 2,3-octadienoyl-CoA following deprotonation of the γ-proton by Cys382 of the thiolase, and the resulting intermediate is attacked by Cys92 of the enzyme causing enzyme inactivation. In a thiolase-catalyzed normal reaction, Cys382 is responsible for protonation and deprotonation of the substrate, while Cys92 is responsible for nucleophilic attack of the carbonyl carbon. This is consistent with our experimental results. This proposed mechanism is similar to a well-established mechanism for inactivation of acyl-CoA dehydrogenase by 2-octynoyl-CoA.[16]

Another possible mechanism of the inactivation is direct nucleophilic attack of Cys92 to the β-carbon of the inhibitor through Michael addition as shown in Figure 7,[17] which does

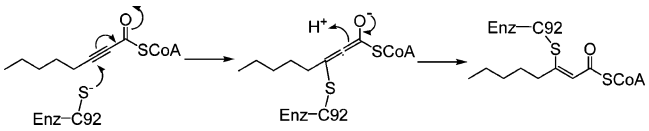

Figure 7. Proposed alternative mechanism for the inactivation of the thiolase by 2-alkynoyl-CoA through Michael addition.

not need an isomerization step. To test the possibility of this mechanism, we incubated the C382A variant enzyme with 2-octynoyl-CoA, followed with trypsin digestion of the incubation mixture. The labeled peptide peak was not found in the HPLC profile, which indicates that the C382A variant enzyme cannot react with 2-octynoyl-CoA. Because Cys382 catalyzes the isomerization reaction, it is possible that the isomerization is required for the inactivation of the wild-type enzyme by 2-octynoyl-CoA. Therefore, the mechanism shown in Figure 6 is more likely, whereas the inactivation through Michael addition shown in Figure 7 is less likely.

In summary, this research increased our understanding of thiolase-catalyzed chemical reactions. It not only proved the intrinsic isomerase activity in the thiolase but also provided an example for rationally designing a selective mechanism-based inhibitor according to a side activity of the enzyme. This might be further extended to become a general approach for developing a mechanism-based inhibitor because more and more enzymes have been found to be multifunctional, and inhibitor design could be based on any one of its functions.[18] This result may be used for selective inactivation of the mevalonate pathway as a supplemental method for treating cardiovascular disease and cancer without affecting fatty acid oxidation in mitochondria and peroxisomes.

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Supporting Information Available: Supporting experimental data for characterizing the isomerase activity of the thiolase and the study for the inactivation of the thiolase by 2-octynoyl-CoA. This material is available free of charge via the Internet at http://pubs.acs.org.

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