Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 1445–1448

Inactivation of medium-chain acyl-CoA dehydrogenase by oct-4-en-2-ynoyl-CoA

Jia Zeng, Guisheng Deng, Wenhua Yu and Ding Li*

Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong SAR, PR China

Received 1 September 2005; revised 18 October 2005; accepted 31 October 2005
Available online 15 November 2005

Abstract—Mitochondrial medium-chain acyl-CoA dehydrogenase is a key enzyme for the β-oxidation of fatty acids, which catalyzes the FAD-dependent oxidation of a variety of acyl-CoA substrates to the corresponding *trans*-2-enoyl-CoA thioesters. Oct-4-en-2-ynoyl-CoA was identified as a new irreversible inhibitor of acyl-CoA dehydrogenase, and kinetic parameters K_I and k_{inact} were determined to be 11 μM and 0.025 min^{-1}, respectively. Triple bond between C2 and C3 of the inhibitor was identified as the functional group responsible for enzyme inactivation, and Michael addition is proposed as the mechanism for this inactivation, which is a new pathway for inactivation of MCAD by inhibitors. The inhibitor may become a lead for further development for treating non-insulin dependent diabetes mellitus.

Numerous diseases have been reported in relation to fatty acids, such as cardiovascular disease,[1] cancer,[2] and diabetes.[3] The regulation of fatty acid oxidation has been reported as a potential method of treating non-insulin dependent diabetes mellitus (NIDDM),[4] and inhibitors of enzymes involved in the metabolism of fatty acids have been synthesized and studied as potential medicines by the Sandoz Research Institute.[5] It has been observed that rates of fatty acid oxidation are greater in NIDDM, and it has been hypothesized that elevated free fatty acids are the cause of increased endogenous glucose production and the resultant hyperglycemia in NIDDM.[4] A rational treatment would therefore be to inhibit the abnormally high rate of fatty acid oxidation. Fatty acid oxidation in mitochondria is also an essential energy generation system for cells. During prolonged fasting and starvation, fatty acids are the precursors of ketone bodies, which are an important alternate fuel in extrahepatic tissues when the supply of glucose is limited.

The degradation of saturated fatty acids occurs in mitochondria in a sequence of four reactions referred to as the β-oxidation cycle.[6] Acyl-CoA dehydrogenases (ACDs) catalyze the first and rate-limiting step reaction of the β-oxidation cycle, which involves conversion of saturated acyl-CoA substrate to unsaturated acyl-CoA (Fig. 1). There are nine known members in the acyl-CoA dehydrogenase family, and five members are involved in fatty acid β-oxidation. Medium-chain acyl-CoA dehydrogenase (MCAD, EC 1.3.99.3) acted on C4-C16 acyl-CoAs with its peak activity toward medium-chain (C6-C12) substrates. The enzyme is a soluble homotetramer with a subunit mass of approximately 43 kDa, with each subunit containing one FAD. The MCAD purified from pig liver has been solved at 2.0 Å resolution,[7–9] and Glu-376 has been confirmed as the catalytic residue.

Various mechanism-based or irreversible inhibitors of acyl-CoA dehydrogenase have been reported and

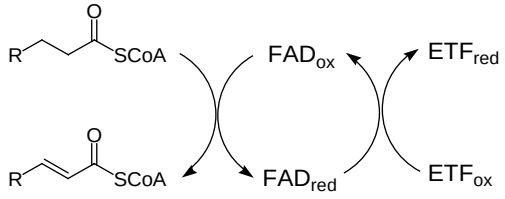


Figure 1. Acyl-CoA dehydrogenase catalyzed reaction.

Abbreviations: ACD, acyl-CoA dehydrogenase; DCPIP, 2,6-dichlorophenolindophenol; FAD, flavin adenine dinucleotide; MCAD, medium-chain acyl-CoA dehydrogenase; MCPA-CoA, methylenecyclopropylacetyl-CoA; NIDDM, non-insulin dependent diabetes mellitus; PMS, phenazine methosulfate; SPA-CoA, spiropentylacetyl-CoA; UV–vis, ultraviolet–visible spectroscopy.

Keywords: Acyl-CoA dehydrogenase; Oct-4-en-2-ynoyl-CoA; Michael addition; Diabetes.

* Corresponding author. Tel.: +852 2788 7824; fax: +852 2788 7406; e-mail: bhdingli@cityu.edu.hk

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.10.106

reviewed recently,[10] which can be grouped into two general classes. The first type of inhibitors attacks the FAD cofactor of acyl-CoA dehydrogenase, which causes bleaching of FAD absorbance at 450 nm. This type of inhibitor includes methylenecyclopropylacetyl-CoA (MCPA-CoA), spiropentylacetyl-CoA (SPA-CoA), 3-methyleneoctanoyl-CoA, 3-methyl-2-octenoyl-CoA, and 3,4-alkadienoyl-CoA. The second type of inhibitor attacks a nucleophile in the active site (glutamate catalytic residue in most cases) of acyl-CoA dehydrogenase, resulting in the formation of a covalent linkage between inhibitor and apoprotein. This type of inhibitor includes 3-alkynoyl-CoA, 2-alkynoyl-CoA, 2,3-alkadienoyl-CoA, 2-halogen alkanoyl-CoA, and 2,3-epoxyalkanoyl-CoA.

In the present study, we report a new irreversible inhibitor of the MCAD, oct-4-en-2-ynoyl-CoA, which is a chain-shortened analog and a potential metabolite of natural product polyacetylenic acid. A wide variety of polyacetylenic acids, esters or amides have been isolated from natural sources.[11] Polyacetylenic hydrocarbons were distributed in the microsomes obtained from leaves and roots of various mature plants, while polyacetylenic epoxide was found in chloroplasts. The polyacetylenes rapidly increased during germination, but were not detected in mature seeds. In a screening of potential metabolites or chain-shortened analogs of polyacetylenic acids for inhibitors of enzymes involved in fatty acid oxidation, we found that oct-4-en-2-ynoyl-CoA is an irreversible inhibitor of the MCAD, which was characterized in this study.

Octanoyl-CoA was purchased from Sigma, and coenzyme A was from ICN Biochemicals. All other chemical and biochemical reagents were of research grade or better and were obtained from commercial sources. The wild-type rat mitochondrial MCAD was purified as previously described.[12,13]

The activity of MCAD was assayed spectrophotometrically following the decrease in absorbance at 600 nm using phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCPIP) as intermediate and terminal electron acceptor, respectively (ε_{600nm} = 21 mM^{-1} cm^{-1}), as described previously.[14] The reaction progress curves were recorded for 1 min on a Hitachi U-2000 UV–vis spectrophotometer. A standard assay mixture contained 33 μM octanoyl-CoA, 1.5 mM PMS, 48 μM DCPIP, 20 mM phosphate buffer, pH 7.4, and 30 μM EDTA, and the final volume was 0.7 mL. The reaction was started by adding 5 μL of appropriately diluted enzyme to the reaction mixture. Unit of enzyme activity was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per minute. Determination of the K_M and the V_{max} was performed using the same assay buffer with varying substrate concentrations.

Oct-4-en-2-ynoyl-CoA was prepared from the corresponding free acid (**1**) (Fig. 2) and coenzyme A by the mixed anhydride method[15] and subsequently purified by reverse-phase HPLC as described previously. Oct-4-en-2-ynoic acid (**1**) was prepared following synthetic Scheme 1. 2-Butyne-1,4-diol was protected using 1 equiv 3,4-dihydro-2*H*-pyran, which was subsequently oxidized to aldehyde (**3**) using the Dess–Martin reagent. Wittig reaction was carried out for compound **3** and *n*-butyl triphenylphosphine to generate product **4** with *cis* configuration. Deprotection followed by oxidations with mild oxidizing reagents including the Dess–Martin reagent and sodium chlorite gave the desired product oct-4-en-2-ynoic acid (**1**) in good yield. The product was analyzed and identified as oct-4-en-2-ynoic acid (**1**).[16]

The effect of oct-4-en-2-ynoyl-CoA on the catalytic activity of MCAD was investigated by the incubation of MCAD with 10 mol equiv of oct-4-en-2-ynoyl-CoA at room temperature. Time-dependent loss of activity of MCAD was noted during the incubation. The MCAD was protected by 2-octenoyl-CoA (product of MCAD catalyzed reaction) from inactivation by oct-4-en-2-ynoyl-CoA, which indicates that the inactivation is active-site directed. For the 'protection' experiment, the enzyme (5 μM in 100 mM potassium phosphate

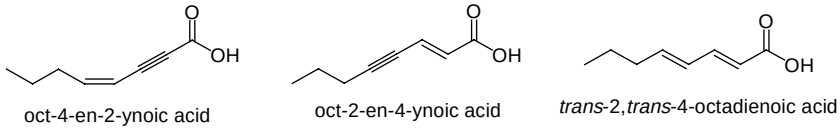
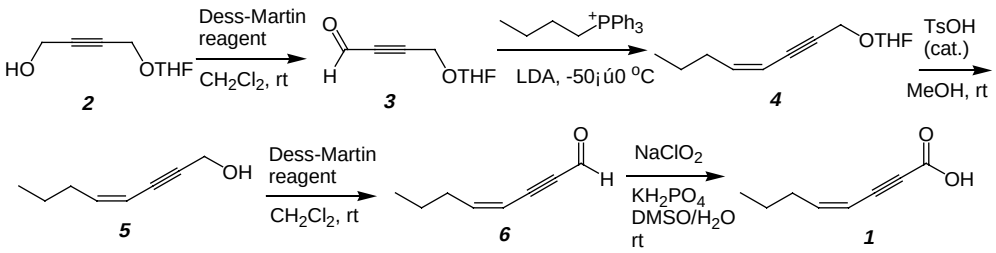


Figure 2. Chain-shortened analogs of polyacetylenic acids, oct-4-en-2-ynoic acid, oct-2-en-4-ynoic acid, and *trans*-2, *trans*-4-octadienoic acid.



Scheme 1. Organic synthesis of oct-4-en-2-ynoic acid (**1**).

buffer, pH 7.6) was preincubated with 100 μM 2-octenoyl-CoA for 10 min at room temperature, followed by the addition of 50 μM oct-4-en-2-ynoyl-CoA. The residual enzyme activity was monitored over time as described above.

The yellow flavin chromophore (λ_{max} 450 nm) was not bleached during the incubation process, which indicates that the inactivation does not involve modification of the FAD coenzyme. The FAD cofactor was removed from the inactivated enzyme using a method described previously,[17] followed by the addition of a new FAD cofactor. The experiment could not regenerate any active enzyme, further indicating that FAD was not the target of the inhibitor.

A sample containing wild-type MCAD (30 μM) 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 7.6) over two days at 4 °C with seven buffer changes. The residual enzyme activity was determined before and after dialysis. Since the activity of the inactivated enzyme remained unchanged after prolonged dialysis, the inactivation is clearly irreversible and most likely involves covalent linkage of oct-4-en-2-ynoyl-CoA with an amino acid residue in the active site of the MCAD. A control experiment was performed under identical conditions without oct-4-en-2-ynoyl-CoA.

Incubation of MCAD with 50 mol equiv of *trans*-2, *trans*-4-octadienoyl-CoA and 50 mol equiv of oct-2-en-4-ynoyl-CoA was also carried out, respectively, but no inhibition of MCAD was observed. This result indicates that the triple bond between C2 and C3 is mainly responsible for the inhibitory activity of oct-4-en-2-ynoyl-CoA on the MCAD.

The competence of oct-4-en-2-ynoyl-CoA to inactivate MCAD was further studied by kinetic analysis. In a typical inactivation experiment, an appropriate amount of oct-4-en-2-ynoyl-CoA dissolved in water was incubated with the enzyme solution (30 μM in 100 mM potassium phosphate buffer, pH 7.6) at 25 °C. At various time intervals, aliquots (50 μL) of the incubation mixture were taken and diluted into the standard assay cocktail (0.7 mL), and the remaining enzyme activity was determined as described above. The inactivation of MCAD by oct-4-en-2-ynoyl-CoA 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 SigmaPlot 8.0 program from first-order rate constants (k_{app}) versus values of inhibitor concentration. As shown in Figure 3, the K_I and k_{inact} were determined to be 11 μM and 0.025 min^{-1}, respectively.

It should be noted that inactivation of acyl-CoA dehydrogenase by 2-alkynoyl-CoA has been well studied in the past.[18–24] It is well demonstrated that the inactivation process involves an initial abstraction of the γ-proton followed by protonation at the α-carbon, resulting in the formation of 2,3-alkadienoyl-CoA as an active

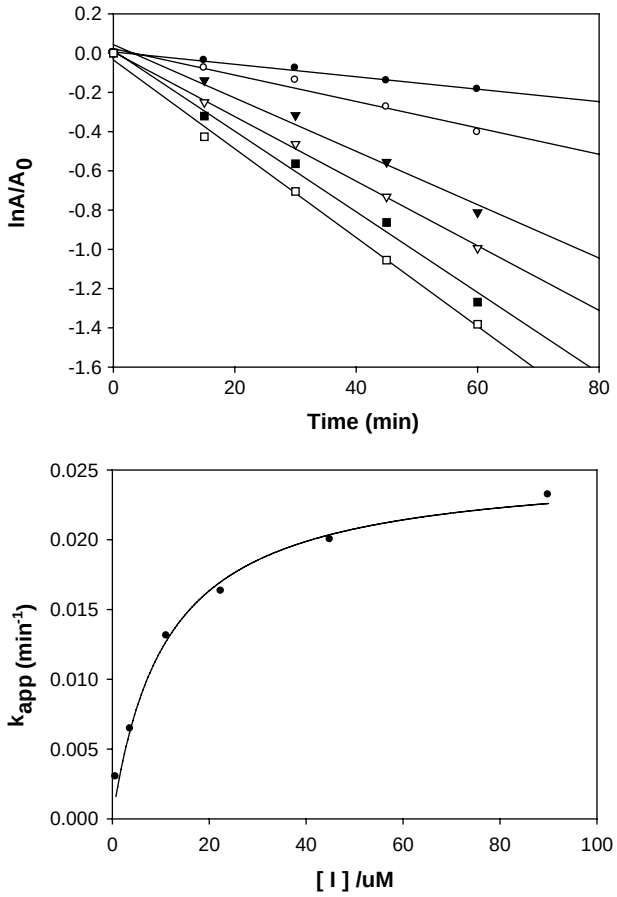


Figure 3. Time- and concentration-dependent inactivation of MCAD (30 μM) by oct-4-en-2-ynoyl-CoA (●, 0.75 μM oct-4-en-2-ynoyl-CoA; ○, 3.8 μM; ▼, 11.3 μM; ▽, 22.5 μM; ■, 45 μM; □, 90 μM).

intermediate, which subsequently traps a nucleophile at the active-site of the enzyme, resulting in enzyme inactivation. Incubation of MCAD and 2-alkynoyl-CoA with deuterium labeling at the γ-position shows an apparent primary kinetic isotope effect, which strongly supports the above mechanism.[21] Although the structure of oct-4-en-2-ynoyl-CoA is similar to that of 2-octynoyl-CoA, it is impossible for oct-4-en-2-ynoyl-CoA to go through a pathway involving abstraction of the γ-proton. Crystal structure of MCAD with bound substrate analog showed that catalytic Glu-376 is too far from the carbon 6 proton of the substrate.[7–9] Therefore, it is impossible for the MCAD to deprotonate proton attached to carbon 6, and inactivation of MCAD by oct-4-en-2-ynoyl-CoA should go through a different mechanism. It is possible that the inactivation process involves a nucleophilic addition of an amino acid of the active site to triple bond between C2 and C3 of oct-4-en-2-ynoyl-CoA known as Michael addition as shown in Figure 4. Similar inactivation mechanism has been proposed for inactivation of thiolase by 2-alkenoyl analog.[25] However, Michael addition has not been reported as a pathway for inactivation of MCAD by any known MCAD inhibitors so far. The nucleophilic amino acid in the active site of MCAD responsible for Michael addition is probably the catalytic glutamate residue responsible for deprotonation of substrate α

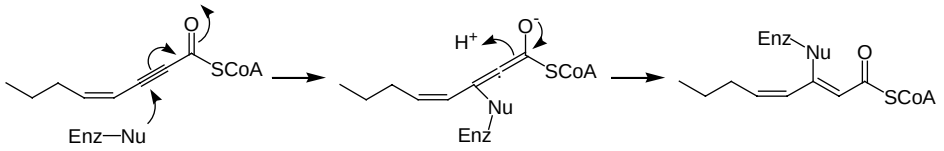


Figure 4. Proposed mechanism for inactivation of MCAD by oct-4-en-2-ynoyl-CoA.

proton. It has been demonstrated that the catalytic glutamate residue of MCAD was covalently attached to inhibitor 2,3-alkadienoyl-CoA.[21]

It should also be noted that 2-octynoyl-CoA can go through a detoxification process in vivo by conversion into 3-ketoacyl-CoA catalyzed by crotonase.[26] However, our experimental results indicate that oct-4-en-2-ynoyl-CoA cannot be converted to the corresponding 3-ketoacyl-CoA by crotonase. Crotonase also cannot catalyze the hydration of *trans*-2, *trans*-4-octadienoyl-CoA and oct-2-en-4-ynoyl-CoA. This may be due to highly conjugated structures of these molecules, which cannot be converted into less conjugated structures by crotonase because of the thermodynamic barrier. These highly stable conjugated structures may have a much higher activation energy for hydration.

Oct-4-en-2-ynoyl-CoA is neither a substrate nor an irreversible inhibitor for 2,4-dienoyl-CoA reductase, although the enzyme can convert *trans*-2, *trans*-4-octadienoyl-CoA to *trans*-3-octenoyl-CoA effectively. Oct-4-en-2-ynoyl-CoA is also not an irreversible inhibitor for Δ^3-Δ^2-enoyl-CoA isomerase and enoyl-CoA hydratase, too. It should be noted that oct-4-en-2-ynoyl-CoA has no irreversible inhibition for acyl-CoA oxidase, which might be due to the slightly different binding orientation of oct-4-en-2-ynoyl-CoA to the enzyme.[6] Therefore, in vivo oct-4-en-2-ynoyl-CoA may specifically target mitochondria without a significant effect on acyl-CoA oxidases in the peroxisomes. Since oct-4-en-2-ynoyl-CoA is a relatively mild inhibitor of MCAD, the corresponding acid could be a lead compound for further development for treating NIDDM without severe adverse effects on fatty acid oxidation caused by over-inhibition of the enzyme. It also indicates that polyacetylenic acids with a similar conjugated structure may be used for treating NIDDM.

Acknowledgment

The work described in this paper was substantially supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China [Project No. 9040660, CityU 1110/01 M].

References and notes

1. Nelson, G. *Nutr. Rev.* **1998**, *56*, 250.
2. Ip, C.; Marshall, J. *Nutr. Rev.* **1996**, *54*, 138.
3. Salmeron, J.; Hu, F.; Manson, J.; Stampfer, M. *Am. J. Clin. Nutr.* **2001**, *73*, 1019.
4. Foley, J. E. *Diabetes Care* **1992**, *15*, 773.
5. Bell, P.; Cheon, S.; Fillers, S.; Foley, J.; Fraser, J.; Smith, H.; Young, D.; Revesz, L. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1007.
6. Kim, J.-J.; Miura, R. *Eur. J. Biochem.* **2004**, *271*, 483.
7. Kim, J.-J.; Wang, M.; Paschke, R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7523.
8. Satoh, A.; Nakajima, Y.; Miyahara, I.; Hirotsu, K.; Tanaka, T.; Nishina, Y.; Shiga, K.; Tamaoki, H.; Setoyama, C.; Miura, R. *J. Biochem.* **2003**, *143*, 297.
9. Wang, W.; Fu, Z.; Zhou, J. Z.; Kim, J.-J.; Thorpe, C. *Biochemistry* **2001**, *40*, 12266.
10. Ghisla, S.; Thorpe, C. *Eur. J. Biochem.* **2004**, *271*, 494.
11. Ichihara, K. I.; Noda, M. *Biochim. Biophys. Acta* **1977**, *487*, 249.
12. Zeng, J.; Li, D. *Protein Expr. Purif.* **2004**, *37*, 472.
13. Zeng, J.; Li, D. *Biochemistry* **2005**, *44*, 6715.
14. Thorpe, C.; Matthews, R. G.; Williams, C. H. *Biochemistry* **1979**, *18*, 331.
15. Li, D.; Agnihotri, G.; Dakoji, S.; Oh, E.; Lantz, M.; Liu, H.-W. *J. Am. Chem. Soc.* **1999**, *121*, 9034.
16. The spectral data of oct-4-en-2-ynoic acid (**1**) are shown as the follows: ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 0.95 (t, J = 7.2 Hz, 3H), 1.48 (q, J = 7.2 Hz, 2H), 2.38 (dq, J = 7.5, 1.5 Hz, 2H), 5.59 (dt, J = 10.8, 1.5 Hz, 1H), 6.32 (dt, J = 10.8, 7.5 Hz, 1 H), 6.98 (br s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.4, 21.7, 35.3, 84.1, 85.6, 106.4, 152.2, 157.7. HRMS (EI): 139.0758 (139.0759 calcd for $C_8H_{10}O_2$+H^+).
17. Mayer, E. J.; Thorpe, C. *Anal. Biochem.* **1981**, *116*, 227.
18. Frerman, F. E.; Miziorko, H. M.; Beckmann, J. D. *J. Biol. Chem.* **1980**, *255*, 11192.
19. Fendrich, G.; Abeles, R. H. *Biochemistry* **1982**, *21*, 6685.
20. Freund, K.; Mizzer, J.; Dick, W.; Thorpe, C. *Biochemistry* **1985**, *24*, 5996.
21. Powell, P. J.; Thorpe, C. *Biochemistry* **1988**, *27*, 8022.
22. Zhou, J. Z.; Thorpe, C. *Arch. Biochem. Biophys.* **1989**, *271*, 261.
23. Lundberg, N. N.; Thorpe, C. *Arch. Biochem. Biophys.* **1993**, *305*, 454.
24. Schaller, R. A.; Mohsen, A. W.; Vockley, J.; Thorpe, C. *Biochemistry* **1997**, *36*, 7761.
25. Palmer, M. A.; Differding, E.; Gamboni, R.; Williams, S. F.; Peoples, O. P.; Walsh, C. T.; Sinskey, A. J.; Masamune, S. *J. Biol. Chem.* **1991**, *266*, 8369.
26. Thorpe, C. *Anal. Biochem.* **1986**, *155*, 391.