ORGANIC LETTERS

2008
Vol. 10, No. 11
2235−2238

Oct-2-yn-4-enoyl-CoA as a Multifunctional Enzyme Inhibitor in Fatty Acid Oxidation

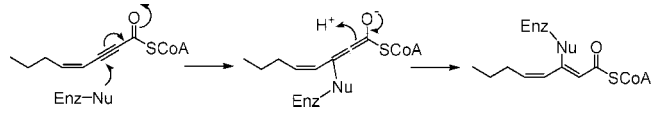
Long Wu, Xiaojun Liu, and Ding Li*

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

bhdingli@cityu.edu.hk

Received March 16, 2008

ABSTRACT



Oct-2-yn-4-enoyl-CoA was found to be a multifunctional irreversible enzyme inhibitor in fatty acid oxidation mainly targeting mitochondrial trifunctional protein β-subunit. It can also inactivate enoyl-CoA hydratase 2 and medium-chain acyl-CoA dehydrogenase. This study increased our understanding for the effect of acetylenic acids on fatty acid oxidation.

Fatty acid oxidation in mitochondria is an essential energy generation system for cells (Figure 1). Mitochondrial trifunctional protein (MTP) catalyzes three consecutive step reactions in the β-oxidation of long-chain fatty acids and plays important roles in control and regulation of β-oxidation.[1] It is a membrane-bound multienzyme complex composed of α-subunits and β-subunits.[2] The α-subunit catalyzes two consecutive steps in the oxidation of long-chain fatty acids, namely hydration of enoyl-CoA and dehydrogenation of 3-hydroxyacyl-CoA, while the β-subunit has long-chain 3-ketoacyl-CoA thiolase activity. The partial fatty acid oxidation (pFOX) inhibition through inactivation of the β-subunit has been reported as a therapy for chronic stable angina.[3]

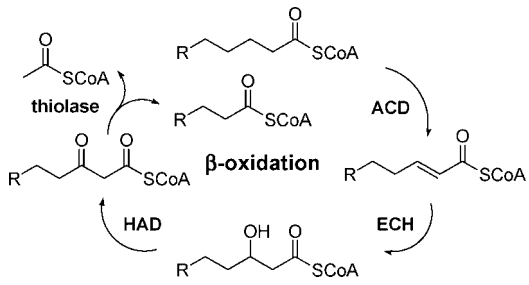


Figure 1. β-Oxidation of fatty acyl-CoA: R, aliphatic carbon chain; ACD, acyl-CoA dehydrogenase; ECH, enoyl-CoA hydratase; HAD, 3-hydroxyacyl-CoA dehydrogenase.

Acetylenic acids are widespread in nature and are found in many organisms.[4] Over 600 naturally occurring acetylenic compounds are known, and some of them exhibit diverse bioactivities. In our screening of acetylenic acids for enzyme inhibitors in fatty acid oxidation, we found that oct-2-yn-4-enoyl-CoA was a strong irreversible inhibitor of the MTP β-subunit. The effect of oct-2-yn-4-enoyl-CoA on the catalytic activity of the MTP β subunit was investigated through the incubation of the MTP β subunit with 5 molar equiv of oct-2-yn-4-enoyl-CoA at room temperature. Time-

(1) (a) Eaton, S.; Bursby, T.; Middleton, B.; Pourfarzam, M.; Mills, K.; Johnson, A. W.; Bartlett, K. *Biochem. Soc. Trans.* **2000**, *28*, 177–182. (b) Eaton, S.; Middleton, B.; Bartlett, K. *Biochim. Biophys. Acta* **1998**, *1429*, 230–238.

(2) (a) Kamijo, T.; Aoyama, T.; Miyazaki, J.; Hashimoto, T. *J. Biol. Chem.* **1993**, *268*, 26452–26460. (b) Carpenter, K.; Pollitt, R. J.; Middleton, B. *Biochem. Biophys. Res. Commun.* **1992**, *183*, 443–448. (c) Uchida, Y.; Izai, K.; Orii, T.; Hashimoto, T. *J. Biol. Chem.* **1992**, *267*, 1034–1041.

(3) (a) Fragasso, G.; Palloshi, A.; Puccetti, P.; Silipigni, C.; Rossodivita, A.; Pala, M.; Calori, G.; Alfieri, O.; Margonato, A. *J. Am. Coll. Cardiol.* **2006**, *48*, 992–998. (b) Conti, C. R. *Clin. Cardiol.* **2003**, *26*, 161–162.

(4) Zhou, Z.-Y.; Wang, F.; Tang, J.-G.; Fang, L.-Z.; Dong, Z.-J.; Liu, J.-K. *J. Nat. Prod.* **2008**, *71*, 223–226.

10.1021/ol800606r CCC: $40.75
Published on Web 04/29/2008

dependent loss of the thiolase activity was noted during the incubation as shown in Figure 2. Since the activity of the

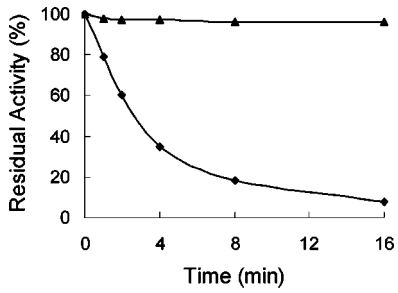


Figure 2. Inhibition of rat MTP β-subunit by oct-2-yn-4-enoyl-CoA. The MTP β-subunit (5.0 μM) was incubated with oct-2-yn-4-enoyl-CoA (▲, 0 μM; ◆, 25 μM) at 25 °C at different times.

inactivated enzyme remained unchanged after prolonged dialysis, the inactivation is clearly irreversible and most likely involves covalent linkage of oct-2-yn-4-enoyl-CoA with an amino acid residue in the active site of the MTP β subunit. The competence of oct-2-yn-4-enoyl-CoA to inactivate the MTP β-subunit was further studied using kinetic analysis. The K_I and k_{inact} of oct-2-yn-4-enoyl-CoA were determined to be 16 ± 2 μM and 1.05 ± 0.07 min^{-1}, respectively. For comparison, our study demonstrates that a widely used antianginal agent trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting the MTP β-subunit irreversiblly, which shows K_I and k_{inact} of 16.7 ± 1.2 μM and 0.60 ± 0.01 min^{-1}, respectively. This result indicates oct-2-yn-4-enoyl-CoA (or oct-2-yn-4-enoic acid for in vivo study) is a better inhibitor for the MTP β-subunit than trimetazidine, and its corresponding acid or ester could become a lead compound for further development for antianginal agent.

In another experiment, the MTP β-subunit was inactivated with oct-2-yn-4-enoyl-CoA, and the incubation mixture was digested with pepsin. The resulting peptide mixture was separated with a HPLC reversed-phase column, and the eluent 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 3. Oct-2-yn-4-enoyl-CoA degrades into a 381 Da fragment that was still attached to the peptide, as shown in Figure 3. The degradation pattern of oct-2-yn-4-enoyl-CoA is same as that for the degradation of (methylenecyclopropyl)formyl-CoA.[5] The result indicated that the catalytic residue Cys425 was covalently labeled by the inhibitor. This result clearly demonstrated that oct-2-yn-4-enoyl-CoA is an irreversible inhibitor of the MTP β-subunit.

In order to further understand the effect of oct-2-yn-4-enoyl-CoA on fatty acid oxidation, we carried out incubation

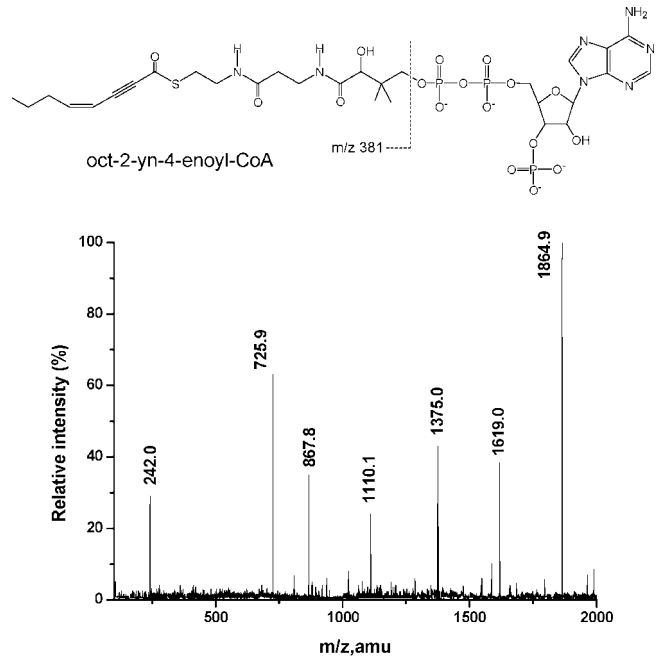


Figure 3. MS/MS spectrum analysis of a 1865 Da peptide showing covalent modification of rat MTP β-subunit peptide (422 VAA-CAAGGQGHAMIVE 437) by oct-2-yn-4-enoyl-CoA. Molecular mass of peptide fragments: M1 = V + A + A (242 Da); M2 = M1 + C + inhibitor (726 Da); M3 = M2 + A + A (868 Da); M4 = M3 + G + G + Q (1110 Da); M5 = M4 + G + H + A (1375 Da); M6 = M5 + M + I (1619 Da); M7 = M6 + V + E + H_2O (1865 Da).

of the compound with other enzymes in both mitochondrial and peroxisomal fatty acid oxidation. Then oct-2-yn-4-enoyl-CoA was also found to be an irreversible inhibitor of mammalian multifunctional protein-2 (MFP-2, also called multifunctional enzyme 2, D-bifunctional enzyme, or 17-β-estradiol dehydrogenase type IV) for its hydratase activity. The MFP-2 plays a central role in peroxisomal β-oxidation as it handles most peroxisomal β-oxidation substrates.[6] The MFP-2 can be divided into three domains: an *N*-terminal domain displaying 3*R*-hydroxyacyl-CoA dehydrogenase activity, a central domain displaying 2-enoyl-CoA hydratase activity, and an *C*-terminal domain that is similar to sterol carrier protein-2. 2-Enoyl-CoA hydratase 2 is the middle part of the MFP-2, which catalyzes the (*R*)-specific hydration of 2-enoyl-CoA thioesters. Its crystal structure indicates the enzyme is a homodimer,[7] unlike the mitochondrial 2-enoyl-CoA hydratase 1 that is a hexamer. The amino acid sequence similarity between hydratase 1 and hydratase 2 are low. The hydratase reaction of 2-enoyl-CoA hydratase 2 requires two protic residues (Glu366 and Asp510 for human MFP-2), suggesting that the reaction follows a process of acid−base catalysis.

The truncated rat 2-enoyl-CoA hydratase 2 (ECH2) was cloned and purified following a previously reported proce-

(5) Agnihotri, G.; He, S.; Hong, L.; Dakoji, S.; Withers, S. G.; Liu, H. W. *Biochemistry* **2002**, *41*, 1843–1852.

(6) Huyghe, S.; Mannaerts, G. P.; Baes, M.; Veldhoven, P. P. V. *Biochim. Biophys. Acta* **2006**, *1761*, 973–994.

(7) Koski, M. K.; Haapalainen, A. M.; Hiltunen, J. K.; Glumoff, T. *J. Mol. Biol.* **2005**, *345*, 1157–1169.

dure[7] with addition of hexaHistag to its *N*-terminus. The effect of oct-2-yn-4-enoyl-CoA on the catalytic activity of the ECH2 was investigated through the incubation of the ECH2 with 5 molar equiv of oct-2-yn-4-enoyl-CoA at room temperature. Time-dependent loss of the hydratase activity was noted during the incubation as shown in Figure 4. Since

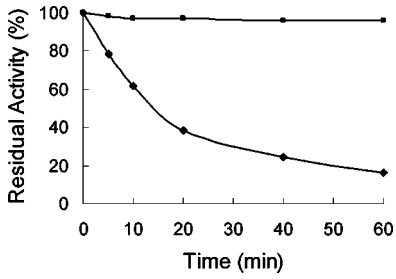


Figure 4. Inhibition of rat ECH2 by oct-2-yn-4-enoyl-CoA. The ECH2 (5.0 μM) was incubated with oct-2-yn-4-enoyl-CoA (□, 0 μM; ◆, 25 μM) at 25 °C at different times.

the activity of the inactivated enzyme remained unchanged after prolonged dialysis, the inactivation is clearly irreversible and most likely involves covalent linkage of oct-2-yn-4-enoyl-CoA with an amino acid residue in the active site of the ECH2. The competence of oct-2-yn-4-enoyl-CoA to inactivate the ECH2 was further studied using kinetic analysis. The K_I and k_{inact} of oct-2-yn-4-enoyl-CoA were determined to be 25 $\pm$ 2 μM and 0.060 $\pm$ 0.011 min^{-1}, respectively.

The identification of labeled residue was carried out in another experiment. The ECH2 was inactivated with oct-2-yn-4-enoyl-CoA, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with an HPLC reversed-phase column with a similar method. The labeled peptide was isolated, purified, and analyzed with MS/MS as shown in Figure 5. Oct-2-yn-4-enoyl-CoA degrades into a 381 Da fragment that was still attached to the peptide. The result indicated that the catalytic residue Glu47 was covalently labeled by the inhibitor, which clearly demonstrated that oct-2-yn-4-enoyl-CoA is an irreversible inhibitor of the ECH2. It should be mentioned that oct-2-yn-4-enoyl-CoA is only a weak reversible inhibitor of enoyl-CoA hydratase 1 (ECH1). This might be related to the binding conformations of the inhibitor in these two different enzymes. The triple bond of the inhibitor might be more close to the catalytic residue in the active site of ECH2 than ECH1.

It should be mentioned that oct-2-yn-4-enoyl-CoA has been identified as an irreversible inhibitor of medium-chain acyl-CoA dehydrogenase (MCAD, EC 1.3.99.3) in our earlier study,[8] and the kinetic parameters K_I and k_{inact} have been determined to be 11 μM and 0.025 min^{-1}, respectively. Previously, we thought oct-2-yn-4-enoyl-CoA was a specific inhibitor of MCAD because we did not have purified MTP

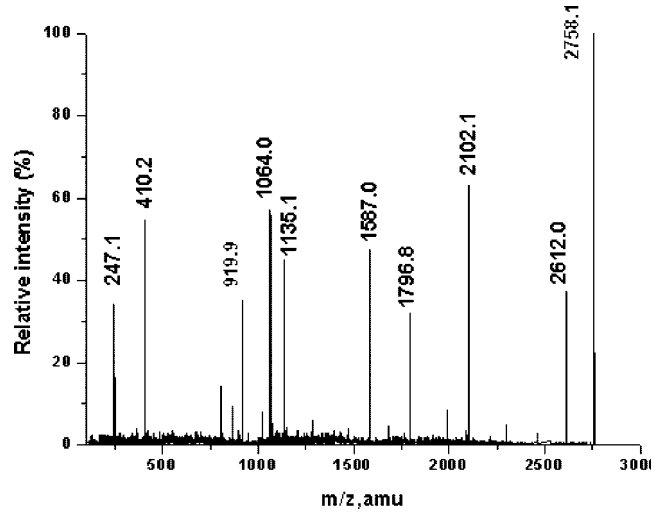


Figure 5. MS/MS spectrum analysis of a 2758 Da peptide showing covalent modification of rat ECH2 peptide (44 FVY**E**GSADFS-CLPTFGVIVAQK 65) by oct-2-yn-4-enoyl-CoA. Molecular mass of peptide fragments: M1 = F + V (247 Da); M2 = M1 + Y (410 Da); M3 = M2 + E + inhibitor (920 Da); M4 = M3 + G + S (1064 Da); M5 = M4 + A (1135 Da); M6 = M5 + D + F + S + C (1587 Da); M7 = M6 + L + P (1797 Da); M8 = M7 + T + F + G (2102 Da) ; M9 = M8 + V + I + V + A + Q (2612 Da); M10 = M9 + K + H_2O (2758 Da).

β-subunit and ECH2 for incubation study at that time. 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.[9] There are nine known members in the acyl-CoA dehydrogenase family, and five members are involved in fatty acid β-oxidation. MCAD acted on C4−C16 acyl-CoAs with its peak activity toward medium-chain (C6−C12) substrates.

In the present study, we further identified the amino acid labeled by the inhibitor as catalytic residue Glu376. The MCAD was inactivated with oct-2-yn-4-enoyl-CoA, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with HPLC reverse phase column with a similar method. The labeled peptide was isolated, purified, and analyzed with MS/MS as shown in Figure 6. Oct-2-yn-4-enoyl-CoA degrades into a 381 Da fragment that was still attached to the peptide. The result indicated that the catalytic residue Glu376 was covalently labeled by the inhibitor. This result clearly demonstrated that oct-2-yn-4-enoyl-CoA is an irreversible inhibitor of the MCAD.

Incubation of the MTP β-subunit, ECH2, and MCAD with 50 molar equiv of *trans*-2,*trans*-4-octadienoyl-CoA were also carried out respectively, but no inhibition of any enzyme was observed. This result indicates that the triple bond between C-2 and C-3 is mainly responsible for the inhibitory activity of oct-2-yn-4-enoyl-CoA. It should be noted that inactivation of acyl-CoA dehydrogenase by 2-alkynoyl-CoA

(8) Zeng, J.; Deng, G.; Yu, W.; Li, D. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1445–1448.

(9) Kim, J.-J.; Miura, R. *Eur. J. Biochem.* **2004**, *271*, 494–508.

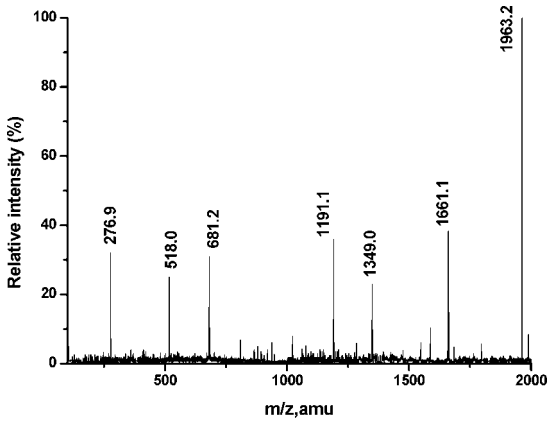


Figure 6. MS/MS spectrum analysis of a 1,963 Da peptide showing covalent modification of rat MCAD peptide (371 IYQIY**E**GTA-QIQR 383) by oct-2-yn-4-enoyl-CoA. Molecular mass of peptide fragments: M1 = I + Y (277 Da); M2 = M1 + Q + I (518 Da); M3 = M2 + Y (681 Da); M4 = M3 + E + inhibitor (1191 Da); M5 = M4 + G + T (1349 Da); M6 = M5 + A + Q + I (1661 Da); M7 = M6 + Q + R + H_2O (1963 Da).

has been well studied in the past.[10] It has been well demonstrated that the inactivation process involves an initial abstraction of the γ-proton followed by protonation at the α-carbon resulting in formation of 2,3-alkadienoyl-CoA as an active intermediate, which subsequently traps a nucleophile at the active-site resulting in enzyme inactivation. Although the structure of oct-2-yn-4-enoyl-CoA is similar to that of 2-octynoyl-CoA, it is impossible for oct-2-yn-4-enoyl-CoA to go through a pathway involving abstraction of the γ-proton. It is possible that the inactivation process involves a nucleophilic addition of a catalytic residue to the triple bond between C2 and C3 of oct-2-yn-4-enoyl-CoA known as Michael addition as shown in Figure 7.

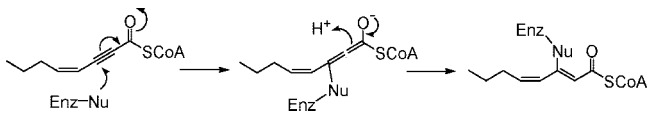


Figure 7. Proposed mechanism for inactivation of the enzymes by oct-2-yn-4-enoyl-CoA.

The inactivation kinetic parameters and labeled residues are summarized as shown in Table 1, which indicates oct-2-yn-4-enoyl-CoA mainly targets MTP β-subunit since its k_{inact} value is significantly higher than those of other two enzymes. One major reason is probably because C425 of the MTP β-subunit is much more nucleophilic than E47 of ECH2 and E376 of MCAD. K_I values of these three inactivations are at a comparable range, which indicates the binding affinities of the inhibitor to these three enzymes are not significantly different.

Table 1. Kinetic Parameters and Labeled Residues for Inactivation of Enzymes by Oct-2-yn-4-enoyl-CoA

inactivated enzyme	K_I (μM)	k_{inact} (min^{-1})	labeled residue
MTP β-subunit	16 ± 2	1.05 ± 0.07	C425
ECH 2	25 ± 2	0.060 ± 0.011	E47
MCAD	11	0.025	E376

In summary, oct-2-yn-4-enoyl-CoA was found to be a very good irreversible enzyme inhibitor of the MTP β-subunit, which is a stronger inhibitor than a well-known antianginal agent trimetazidine. Besides, oct-2-yn-4-enoyl-CoA can also covalently inactivate ECH2 and MCAD effectively. Therefore, oct-2-yn-4-enoyl-CoA is a multifunctional enzyme inhibitor in fatty acid oxidation. As far as we know, oct-2-yn-4-enoyl-CoA is the first multifunctional irreversible enzyme inhibitor of fatty acid oxidation, which can inactivate long-chain fatty acid metabolism in both mitochondria and peroxisomes. The corresponding oct-2-yn-4-enoic acid or its esters could be used for further in vivo studies for lead compound discovery. This study increased our understanding for the effect of acetylenic acids on fatty acid oxidation.

Acknowledgment. The work described in this paper was supported by grants from City University of Hong Kong (Strategic Research Grant, Project No. 7001836).

Supporting Information Available: Supporting experimental data for cloning, expression, and purification of MTP β-subunit and ECH2. Inactivation studies of the enzymes with oct-2-yn-4-enoyl-CoA. This material is available free of charge via the Internet at http://pubs.acs.org.

OL800606R

(10) (a) Fendrich, G.; Abeles, R. H. *Biochemistry* **1982**, *21*, 6685–6695. (b) Freund, K.; Mizzer, J.; Dick, W.; Thorpe, C. *Biochemistry* **1985**, *24*, 5996–6002. (c) Powell, P. J.; Thorpe, C. *Biochemistry* **1988**, *27*, 8022–8028. (d) Zhou, J. Z.; Thorpe, C. *Arch. Biochem. Biophys.* **1989**, *271*, 261–269. (e) Lundberg, N. N.; Thorpe, C. *Arch. Biochem. Biophys.* **1993**, *305*, 454–459. (f) Schaller, R. A.; Mohsen, A. W.; Vockley, J.; Thorpe, C. *Biochemistry* **1997**, *36*, 7761–7768. (g) Wu, L.; Zeng, J.; Deng, G.; Guo, F.; Li, N.; Liu, X.; Chu, X.; Li, D. *Org. Lett.* **2007**, *9*, 3877–3880.