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Oct-2-en-4-ynoyl-CoA as a Specific Inhibitor of Acyl-CoA Oxidase

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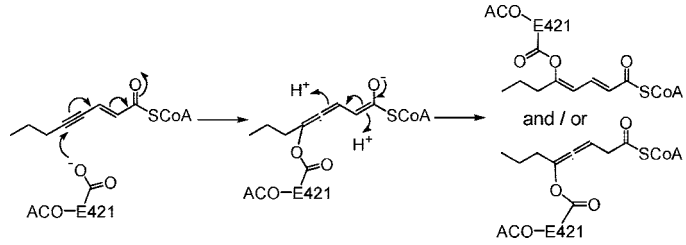
ABSTRACT



Oct-2-en-4-ynoyl-CoA was found to be a specific inhibitor of acyl-CoA oxidase in fatty acid oxidation in peroxisomes that has no inhibitory effect on acyl-CoA dehydrogenase in mitochondria. The inhibition reaction involves a nucleophilic attack of Glu421 to the δ carbon of the inhibitor. The result indicates that acyl-CoA oxidase and acyl-CoA dehydrogenase have certain differences in active-site structure, which makes it possible to control fatty acid oxidation selectively in either mitochondria or peroxisomes with different enzyme inhibitors.

Fatty acid degradation in most organisms occurs primarily via the β-oxidation cycle. In mammals, β-oxidation occurs in both mitochondria and peroxisomes, whereas plants and most fungi harbor the β-oxidation cycle only in the peroxisomes.[1] Fatty acid oxidation in mitochondria is 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 at times 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.[2] The peroxisomal β-oxidation cycle consists principally of the same four reactions as the mitochondrial pathway: dehydrogenation/oxidation, hydration, another dehydrogenation, and thiolytic cleavage. The main differences lie in the substrate and stereospecificities of these pathways.[3] Peroxisomes degrade fatty acids and fatty acid derivatives that cannot be oxidized by mitochondrial enzymes. The main role of peroxisomal β-oxidation is to shorten or otherwise convert fatty acids into a form that can be accepted by the mitochondrial enzymes. The substrates of peroxisomal β-oxidation include very-long-chain fatty acids, dicarboxylic fatty acids, branched-chain fatty acids (e.g., pristanic acid), intermediates of C27 bile acid synthesis (di- and trihydroxycholestanoic acid), prostaglandins, leucotrienes, and some xenobiotics. After being chain-shortened or sufficiently modified in peroxisomes, the acyl moieties are linked to carnitine inside peroxisomes and transported into mitochondria for complete oxidation. The acetyl-CoA molecules formed in peroxisomes after every β-oxidation cycle are also taken into mitochondria for oxidation in the citric acid cycle.

One major difference in mitochondrial and peroxisomal β-oxidation is in their initial and rate-determining oxidation

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(1) Poirier, Y.; Antonenkov, V. D.; Glumoff, T.; Hiltunen, J. K. *Biochim. Biophys. Acta* **2006**, *1763*, 1413–1426.
(2) Ghisla, S. *Eur. J. Biochem.* **2004**, *271*, 459–461.
(3) Hiltunen, J. K.; Qin, Y. *Biochim. Biophys. Acta* **2000**, *1484*, 117–128.

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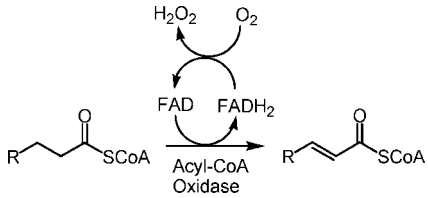

Figure 1. ACO-catalyzed reaction (R, aliphatic carbon chain).

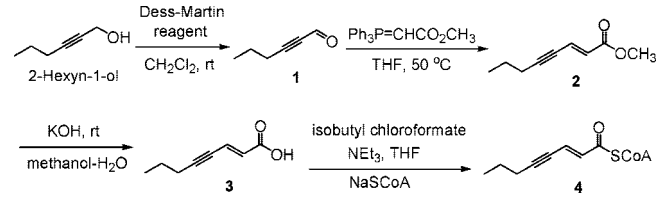

Figure 2. Organic synthesis of oct-2-en-4-ynoyl-CoA.

step. The reaction is catalyzed by acyl-CoA dehydrogenase (ACD) in mitochondria, while it is catalyzed by acyl-CoA oxidase (ACO) in peroxisomes.[4] It has been suggested that these two enzymes have similar active site cavity, while they differ in subtle aspects.[5] ACO has a more open active site, which might facilitate physical access of dioxygen functioning as electron acceptor. In comparison, access of solvent and dioxygen to the active site of ACD appears to be repressed. ACO (EC. 1.3.3.6) is a flavoenzyme that contains one molecule of noncovalently bound flavin adenine dinucleotide (FAD) per subunit. In the reductive half-reaction of ACO, the substrate acyl-CoA is α,β-dehydrogenated into the corresponding *trans*-2-enoyl-CoA by FAD, which becomes reduced, while in the oxidative half-reaction, reduced FAD is reoxidized by molecular oxygen, thereby closing the catalytic cycle (Figure 1). Although a number of inhibitors for the enzymes in mitochondrial β-oxidation have been developed previously, an irreversible enzyme inhibitor targeting specifically a rate-limiting step in peroxisomal β-oxidation has not been found so far.

Acetylenic acids are widespread in nature and are found in many organisms.[6] Over 600 naturally occurring acetylenic compounds are known, and some of them exhibit diverse bioactivities. Previously, we have cloned and purified rat ACO and rat medium-chain acyl-CoA dehydrogenase (MCAD),[7] which were used in the present study. We have synthesized and tested a variety of acetylenic acids as potential enzyme inhibitors in fatty acid oxidation. We have reported that oct-2-yn-4-enoyl-CoA is an irreversible inhibitor of MCAD.[8] In the present study, we found that oct-2-en-4-ynoyl-CoA was a specific irreversible inhibitor of ACO in peroxisomes without inhibitory effect on MCAD. The preparation of oct-2-en-4-ynoic acid has been previously reported.[9] In the present research, we synthesized oct-2-en-4-ynoyl-CoA following a synthetic scheme as shown in Figure 2. 2-Hexyn-1-ol was purchased from Aldrich and oxidized with Dess−Martin reagent to give compound **1**, which was then reacted through Wittig reaction to generate compound **2**. Ester **2** was hydrolyzed and coupled with coenzyme A to produce oct-2-en-4-ynoyl-CoA.

The effect of oct-2-en-4-ynoyl-CoA on the catalytic activity of the ACO was investigated through the incubation of the ACO with 5 molar equiv of oct-2-en-4-ynoyl-CoA at room temperature. Time-dependent loss of the oxidase activity was noted during the incubation as shown in Figure 3. The same inhibition can also occur under anaerobic conditions, indicating the oxygen is not required for the inactivation process. The flavin chromophore (λ_{max} 450 nm) was not affected during the incubation process, which indicates the inactivation does not involve modification of the FAD coenzyme. The ACO was protected by 2-octenoyl-CoA (product of ACO catalyzed reaction) from inactivation by oct-2-en-4-ynoyl-CoA, which indicates the inactivation is active-site directed. 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-2-en-4-ynoyl-CoA with an amino acid residue in the active site of the ACO. The competence of oct-2-en-4-ynoyl-CoA to inactivate the ACO was further studied using kinetic analysis. The K_I and k_{inact} of oct-2-en-4-ynoyl-CoA were determined to be 45 μM and 3.7 min^{-1}, respectively.

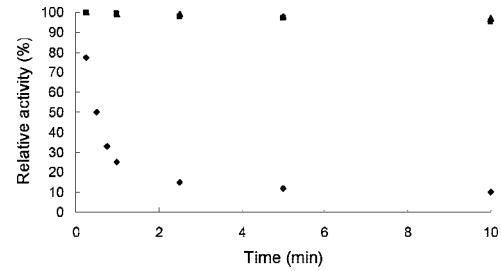

Figure 3. Incubation of rat ACO (5.0 μM) with oct-2-en-4-ynoyl-CoA (◆, 25 μM), oct-2-yn-4-enoyl-CoA (▲, 250 μM), and *trans*-2,*trans*-4-octadienoyl-CoA (■, 250 μM) at 25 °C at different times.

For comparison, we also tested the effect of *trans*-2,*trans*-4-octadienoyl-CoA and oct-2-yn-4-enoyl-CoA on the activity of ACO as shown in Figure 3, but no inhibition of ACO activity was noticed. We also tested the effect of these three compounds on the activity of the MCAD as shown in Figure 4. Only oct-2-yn-4-enoyl-CoA can inhibit the MCAD, and the other two compounds have no inhibition on this enzyme.

(4) Zeng, J.; Liu, Y.; Wu, L.; Li, D. *Biochim. Biophys. Acta* **2007**, *1774*, 1628–1634.
(5) Kim, J. J.; Miura, R. *Eur. J. Biochem.* **2004**, *271*, 483–493.
(6) Zhou, Z.-Y.; Wang, F.; Tang, J.-G.; Fang, L.-Z.; Dong, Z.-J.; Liu, J.-K. *J. Nat. Prod.* **2008**, *71*, 223–226.
(7) (a) Zeng, J.; Li, D. *Protein Expression Purif.* **2004**, *37*, 472–478. (b) Zeng, J.; Li, D. *Protein Expression Purif.* **2004**, *38*, 153–160.
(8) (a) Zeng, J.; Deng, G.; Yu, W.; Li, D. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1445–1448. (b) Wu, L.; Liu, X.; Li, D. *Org. Lett.* **2008**, *10*, 2235–2238.
(9) Ogawa, Y.; Maruno, M.; Wakamatsu, T. *Heterocycles* **1995**, *41*, 2587–2599.

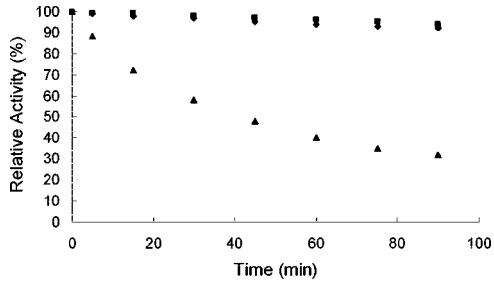

Figure 4. Incubation of rat MCAD (5.0 μM) with oct-2-en-4-ynoyl-CoA (◆, 250 μM), oct-2-yn-4-enoyl-CoA (▲, 50 μM), and *trans*-2,*trans*-4-octadienoyl-CoA (■, 250 μM) at 25 °C at different time.

We also tested the effect of oct-2-en-4-ynoyl-CoA on the activity of other enzymes in fatty acid oxidation, including enoyl-CoA hydratase 1, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, mitochondrial trifunctional protein, and enoyl-CoA hydratase 2, but no inhibition of any above enzyme was noticed. Therefore, oct-2-en-4-ynoyl-CoA is a specific inhibitor for ACO.

In another experiment, ACO was inactivated with oct-2-en-4-ynoyl-CoA, and the incubation mixture was digested with chymotrypsin. The resulting peptide mixture was separated with an 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 in both 220 and 260 nm wavelengths because the inhibitor contains coenzyme A. In a control experiment, nonlabeled ACO was also digested with chymotrypsin, and the resulting peptide mixture was separated with HPLC reversed-phase column for comparison. Only one new peak was isolated, which may contain the peptide labeled by the inhibitor.

The labeled peptide was purified and analyzed with MS/MS as shown in Figure 5. Oct-2-en-4-ynoyl-CoA degrades into a 381 Da fragment that was still attached to the peptide, as shown in Figure 5. The degradation pattern of oct-2-en-4-ynoyl-CoA is the same as those for the degradation of (methylenecyclopropyl)-formyl-CoA and 2-octynoyl-CoA.[10] The result indicated that the catalytic residue Glu421 was covalently labeled by the inhibitor. This result clearly demonstrated that oct-2-en-4-ynoyl-CoA is an irreversible inhibitor of ACO. Our sequence alignment indicates that ACOs from various mammalian sources have very high sequence homology, and Glu421 is a highly conserved residue among ACOs and ACDs from various sources.

Since *trans*-2,*trans*-4-octadienoyl-CoA and oct-2-yn-4-enoyl-CoA have no inhibition on the ACO as shown in Figure 3, it may indicate that the triple bond between C-4 and C-5 is essential for the inhibitory activity of oct-2-en-4-ynoyl-CoA on the ACO. It should be noted that inactivation

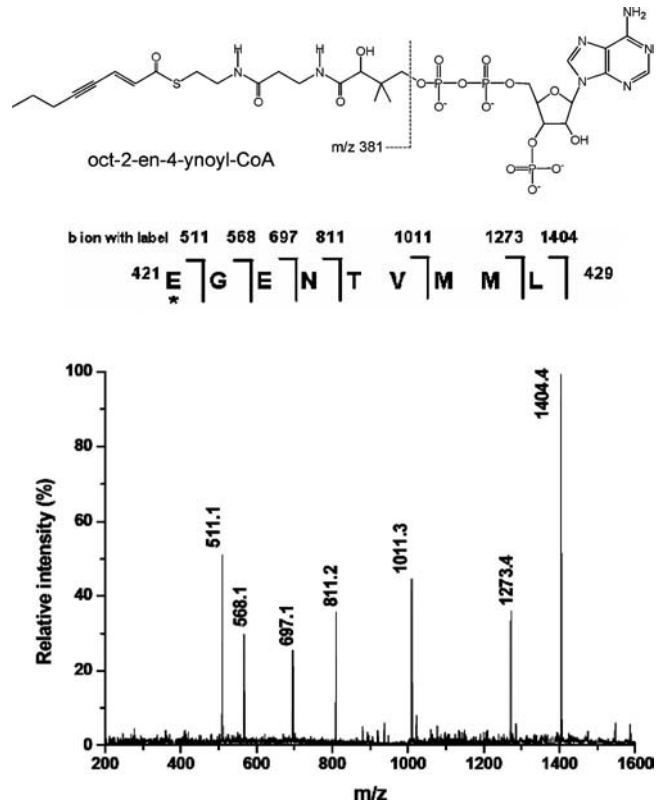

Figure 5. MS/MS spectrum analysis of a 1404 Da peptide showing covalent modification of rat ACO by oct-2-en-4-ynoyl-CoA.

of ACD by 2-alkynoyl-CoA and oct-2-yn-4-enoyl-CoA have been studied in the past.[11] However, these compounds have no inhibitory activity for ACO, although above two enzymes belong to the same superfamily. This result indicates ACO and ACD have certain differences in active-site structure.

Further molecular modeling experiment was carried out to explain the inactivation of the ACO by oct-2-en-4-ynoyl-CoA. After docking the inhibitor oct-2-en-4-ynoyl-CoA into the active site of the ACO, it was found that Glu421 of the enzyme is close to carbons 4 and 5 of oct-2-en-4-ynoyl-CoA as shown in Figure 6, indicating that Glu421 of ACO can serve as a nucleophile to attack the δ carbon of oct-2-en-4-ynoyl-CoA. It is possible that the binding of oct-2-en-4-ynoyl-CoA causes an induced fitting or conformational change of the ACO, which makes Glu421 close to the δ carbon of the inhibitor. The same modeling experiment was carried out to dock oct-2-en-4-ynoyl-CoA into the active site of the MCAD, and the result indicates the catalytic residue Glu376 is far from the δ carbon of oct-2-en-4-ynoyl-CoA (data not shown).

In another experiment, it was found that the inactivation rate was pH dependent and was higher under basic condition

(10) (a) Agnihotri, G.; He, S.; Hong, L.; Dakoji, S.; Withers, S. G.; Liu, H. W. *Biochemistry* **2002**, *41*, 1843–1852. (b) Wu, L.; Zeng, J.; Deng, G.; Guo, F.; Li, N.; Liu, X.; Chu, X.; Li, D. *Org. Lett.* **2007**, *9*, 3877–3880. (c) Wu, L.; Lin, S.; Li, D. *Org. Lett.* **2008**, *10*, 3355–3358.

(11) (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.

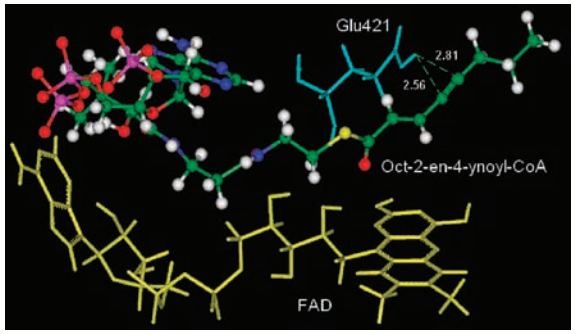

Figure 6. Docking experimental result showing interaction of ACO with oct-2-en-4-ynoyl-CoA.

as shown in Figure 7 with an apparent p*K* of about 6.8. It should be noted that the rate for the inactivation of MCAD by 2-alkynoyl-CoA has been found to be pH dependent as well in a previous report with an apparent p*K* of 7.9,[11b] and the inactivation rate was higher with increasing pH value. The trend of pH dependence in our study is consistent with that for the inactivation of MCAD by 2-octynoyl-CoA reported previously, but with different p*K* values. In both cases, catalytic glutamate residue was involved in nucleophilic attack to the inhibitor, and glutamate residue is more nucleophilic for Michael addition reaction under basic conditions resulting in increased inactivation rate. The different p*K* values may be related with different microenvironment of the enzyme active sites.

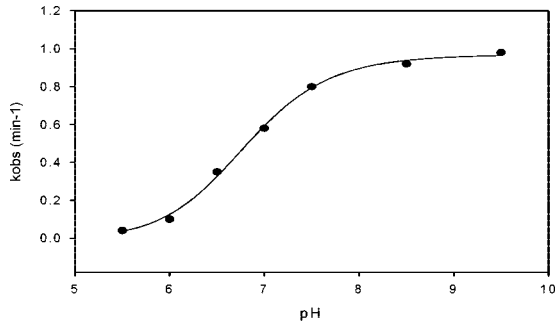

Figure 7. pH dependence of apparent rate for inactivation of ACO by oct-2-en-4-ynoyl-CoA.

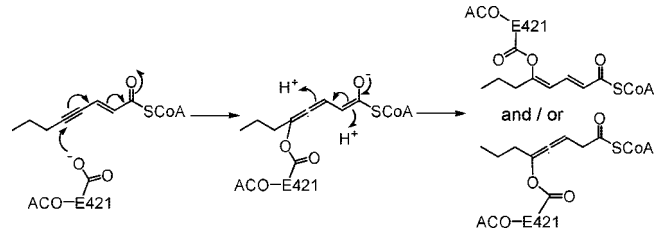

Figure 8. Proposed mechanism for the inactivation of ACO by oct-2-en-4-ynoyl-CoA.

Based on these experimental results, a possible mechanism for the inactivation of ACO by oct-2-en-4-ynoyl-CoA is proposed as shown in Figure 8. The nucleophilic attack of Glu421 of ACO to the δ carbon of oct-2-en-4-ynoyl-CoA gives a relatively stable conjugated enolate intermediate, which subsequently attract a proton to generate a covalently linked enzyme−inhibitor adduct. Normally, conjugate addition or Michael addition reaction happens at β carbon of the inhibitor or the substrate, and our study indicates the conjugate addition reaction can also occur at δ carbon. This result might be applied to organic synthesis of conjugated structures with substitution at δ carbon, which might be further extended to the synthesis of natural products with large-ring system. Our above result also indicates that ACO and ACD have certain differences in active-site structure, which makes it possible to control fatty acid oxidation selectively in either mitochondria or peroxisomes with different enzyme inhibitors. As we know, oct-2-en-4-ynoyl-CoA is the first irreversible and selective inhibitor of ACO.

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Supporting Information Available: Supporting experimental data for inactivation studies of ACO with oct-2-en-4-ynoyl-CoA, sequence alignment, and docking experimental conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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