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Comparative Inhibition Studies of Enoyl-CoA Hydratase 1 and Enoyl-CoA Hydratase 2 in Long-Chain Fatty Acid Oxidation

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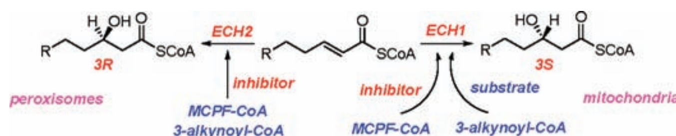
ABSTRACT



Enoyl-CoA hydratase 1 and enoyl-CoA hydratase 2 in long-chain fatty acid oxidation were comparatively investigated through mechanistic studies for inactivation of the enzymes with methylenecyclopropylformyl-CoA and 3-octynoyl-CoA. Methylenecyclopropylformyl-CoA can inactivate both enzymes, while 3-octynoyl-CoA inactivates enoyl-CoA hydratase 2 only. The study increased our understanding of these two enzymes in fatty acid oxidation.

Fatty acid degradation in most organisms occurs primarily via the β-oxidation cycle. In mammals, β-oxidation occurs in both mitochondria and peroxisomes.[1] The main differences lie in the substrate and stereospecificities of these pathways.[2] 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 β-oxidation in mitochondria involves a 3*S*-hydroxyacyl-CoA intermediate, while the β-oxidation in peroxisomes has a 3*R*-hydroxyacyl-CoA intermediate (Figure 1). The enzymes responsible for the formation of these two different intermediates are enoyl-CoA hydratase 1 (ECH1) in mitochondria and enoyl-CoA hydratase 2 (ECH2) in peroxisomes. It has been suggested that these two enzymes

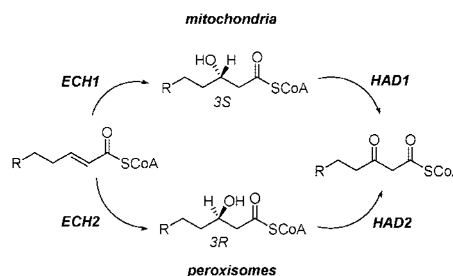


Figure 1. Conversion of 2-enoyl-CoA to 3-ketoacyl-CoA through β-oxidation in mitochondria and peroxisomes. R, aliphatic carbon chain; ECH1, enoyl-CoA hydratase 1; ECH2, enoyl-CoA hydratase 2; HAD1, 3-hydroxyacyl-CoA dehydrogenase converting 3*S* substrate; HAD2, 3-hydroxyacyl-CoA dehydrogenase converting 3*R* substrate.

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have similar active site geometry, but in a mirror image fashion.[3] To further understand the functional differences of these two enzymes, we carried out comparative inhibition studies of these two enzymes with methylenecyclopropylformyl-CoA (MCPF-CoA) and 3-octynoyl-CoA.

In eukaryotes, ECH2 is a ~31 kDa integral part of multifunctional protein-2 (MFP-2, also called multifunctional enzyme 2, D-bifunctional enzyme or 17-β-estradiol dehydrogenase type IV). The MFP-2 plays a central role in peroxisomal β-oxidation as it handles most peroxisomal β-oxidation substrates.[4] ECH2 exists as a homodimer in its crystal structure,[5] unlike the mitochondrial ECH1 that exists as a hexamer.[6] The amino acid sequence similarity between hydratase 1 and hydratase 2 is 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. In our study, the truncated rat ECH2 was cloned and purified following a previously reported procedure with addition of hexaHistag to its N-terminus.[7]

MCPF-CoA is a metabolite derived from a natural amino acid, (methylenecyclopropyl)glycine.[8] MCPF-CoA has been found to be an irreversible inhibitor of bovine mitochondrial enoyl-CoA hydratase, and the mechanism of this inactivation has been well studied.[9] We further tested the activity of MCPF-CoA on the ECH2 through the incubation of the ECH2 with 5 mol equiv of (*R*)- and (*S*)-MCPF-CoA at room temperature as shown in Figure 2. Time-dependent loss of the hydratase activity was noted during both incubations. Since the activity of the inactivated enzyme remained unchanged after prolonged dialysis, the inactivation is clearly irreversible and most likely involves covalent linkage of MCPF-CoA with an amino acid residue in the active site of the ECH2. The competence of (*R*)- and (*S*)-MCPF-CoA to inactivate the ECH2 was further studied using kinetic analysis. The K_I and k_{inact} of (*R*)-MCPF-CoA were determined to be 41 ± 6 μM and 0.082 ± 0.005 min^{-1}, respectively. The K_I and k_{inact} of (*S*)-MCPF-CoA were determined to be 53 ± 8 μM and 0.010 ± 0.001 min^{-1}, respectively. This result indicates MCPF-CoA is a better inhibitor for ECH2 rather than monofunctional mitochondrial ECH1. (The K_I and k_{inact} for (*R*)-MCPF-CoA have been determined to be 49.2 μM and 0.00336 min^{-1}, respectively. The K_I and k_{inact} for (*S*)-MCPF-CoA have been determined to be 57.1 μM and 0.00265 min^{-1}, respectively.) Besides, (*R*)-MCPF-CoA is a significantly better inhibitor for ECH2 than (*S*)-MCPF-CoA. In comparison, both (*R*)- and (*S*)-MCPF-CoA have a similar inhibitory effect for ECH1.

The identification of the labeled residue was carried out in another experiment. The ECH2 was inactivated with MCPF-CoA, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with an HPLC reverse phase column, and the eluent was monitored with a UV detector at both 220 and 260 nm wavelengths. 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. Only one peptide was found to be labeled by the inhibitor, which was isolated, purified, and analyzed with MS/MS as shown in Figure 3. MCPF-CoA degrades into a 341 Da fragment that was still attached to the peptide in mass analysis. This degradation pattern of MCPF-CoA is the same as that reported previously.[9a] The result indicated that the catalytic residue

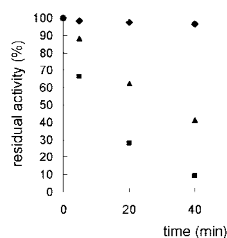

Figure 2. Inhibition of rat ECH2 by (*R*)- and (*S*)-MCPF-CoA. The ECH2 (5.0 μM) was incubated with (*R*)-MCPF-CoA (■, 25 μM), (*S*)-MCPF-CoA (▲, 25 μM), and the same volume of water (◆) at 25 °C at different times.

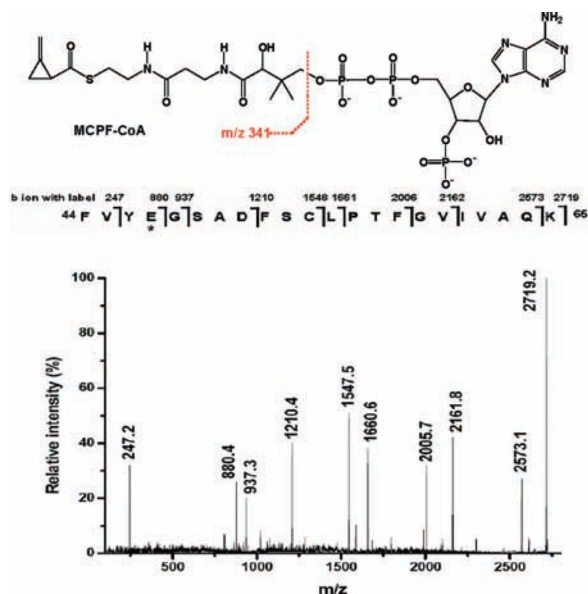

Figure 3. MS/MS spectrum analysis of a 2719 Da peptide showing covalent modification of rat ECH2 by MCPF-CoA.

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Glu47 was covalently labeled by the inhibitor, which clearly demonstrated that MCPF-CoA is an irreversible inhibitor of the ECH2. Both ECH1 and ECH2 are inhibited by MCPF-CoA through modification of the catalytic glutamate residue, which may indicate inactivation of ECH2 by MCPF-CoA follows the same mechanism for inactivation of ECH1 by MCPF-CoA. This result indicates MCPF-CoA can affect β-oxidation in both mitochondria and peroxisomes through the inactivation of their enoyl-CoA hydratase activities.

Although MCPF-CoA has been identified as an irreversible inhibitor of monofunctional mitochondrial ECH1, its activity has not been studied for the mitochondrial trifunctional protein (MTP) responsible for long-chain fatty acid oxidation. 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.[10] It is a membrane-bound multienzyme complex composed of α-subunits and β-subunits.[11] 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.

Our incubation study indicates MCPF-CoA is also an irreversible inhibitor of the MTP α subunit, and time-dependent loss of the enoyl-CoA hydratase activity was noted during the incubation. The competence of (*R*)- and (*S*)-MCPF-CoA to inactivate the MTP α-subunit was further studied. The K_I and k_{inact} for (*R*)-MCPF-CoA were determined to be 60 ± 7 μM and 0.053 ± 0.004 min^{-1}, respectively. The K_I and k_{inact} for (*S*)-MCPF-CoA were determined to be 66 ± 8 μM and 0.049 ± 0.003 min^{-1}, respectively. This result indicates MCPF-CoA is a better inhibitor for the MTP α-subunit rather than the monofunctional ECH1.

In another experiment, the MTP α-subunit was inactivated with MCPF-CoA, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with an HPLC reverse phase column. Only one peptide was found to be labeled by the inhibitor, which was isolated, purified, and analyzed with MS/MS as shown in Figure 4.

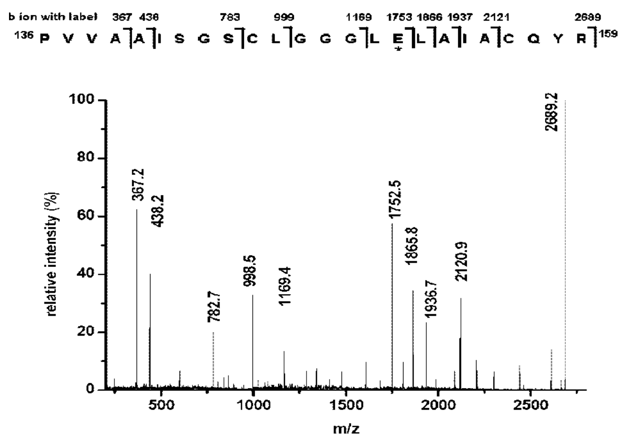

Figure 4. MS/MS spectrum analysis of a 2689 Da peptide showing covalent modification of the MTP α-subunit by MCPF-CoA.

MCPF-CoA degrades into a 341 Da fragment that was still attached to the peptide in mass analysis. The result indicated that the catalytic residue Glu151 was covalently labeled by the inhibitor. It should be mentioned that MTP has been previously suggested as a drug target for treating cancer, heart disease, and diabetes.[12] Therefore, our identification of MCPF-CoA as an irreversible inhibitor of the MTP α-subunit may have practical significance in providing a useful lead compound for treating related diseases. It should be mentioned that we have also identified oct-2-yn-4-enoyl-CoA as an irreversible inhibitor of both ECH1 and ECH2 recently.[7] All these results are consistent with the early notion that ECH1 and ECH2 have similar active site geometry, but in a mirror image fashion.

To compare and further understand the catalytic properties of ECH1 and ECH2, we studied the activity of 3-octynoyl-CoA on both ECH1 and ECH2. It should be mentioned that 3-octynoyl-CoA has been identified as a substrate of ECH1,[13] and our experimental data are consistent with previously reported results for ECH1. In comparison, our results indicated that 3-octynoyl-CoA is an irreversible inhibitor instead of a substrate of ECH2.

The effect of 3-octynoyl-CoA on the catalytic activity of the ECH2 was investigated through the incubation of the ECH2 with 5 mol equiv of 3-octynoyl-CoA at room temperature as shown in Figure 5. Time-dependent loss of

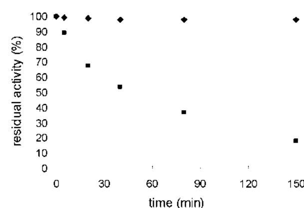

Figure 5. Inhibition of rat ECH2 by 3-octynoyl-CoA. The ECH2 (5.0 μM) was incubated with 3-octynoyl-CoA (■, 25 μM; ◆, 0 μM) at 25 °C at different times.

the enoyl-CoA hydratase activity was noted during the incubation. Since the activity of the inactivated enzyme remained unchanged after prolonged dialysis, the inactivation is clearly irreversible and most likely involves covalent linkage of 3-octynoyl-CoA with an amino acid residue in the active site of the ECH2. The competence of 3-octynoyl-

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CoA to inactivate the ECH2 was further studied using kinetic analysis. The K_I and k_{inact} of 3-octynoyl-CoA were determined to be 65 ± 7 μM and 0.024 ± 0.001 min^{-1}, respectively.

The identification of the labeled residue was carried out in another experiment. The ECH2 was inactivated with 3-octynoyl-CoA, and the incubation mixture was digested with trypsin. The resulting peptide mixture was separated with an HPLC reverse phase column with a similar method. Only one peptide was found to be labeled by the inhibitor, which was isolated, purified, and analyzed with MS/MS as shown in Figure 6. 3-Octynoyl-CoA degrades into a 269 Da

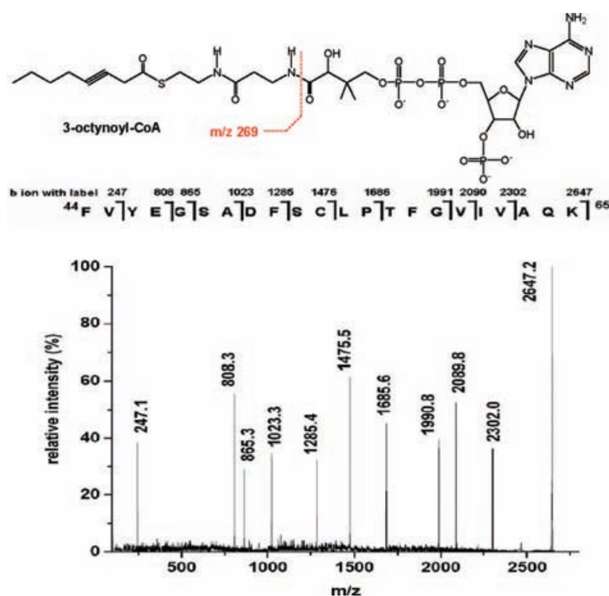

Figure 6. MS/MS spectrum analysis of a 2647 Da peptide showing covalent modification of rat ECH2 by 3-octynoyl-CoA.

fragment that was still attached to the peptide in mass analysis. The result indicated that the catalytic residue Glu47 was covalently labeled by the inhibitor, which clearly demonstrated that 3-octynoyl-CoA is an irreversible inhibitor of the ECH2.

It should be noted that the 3-alkynoyl group has been found to be an inhibitor of several other enzymes.[14] It has been proposed that the 3-alkynoyl group is first isomerized to an allene, which is then attacked by an enzyme nucleophile resulting in enzyme inactivation. A similar mechanism is proposed for the inactivation of the ECH2 by 3-octynoyl-CoA as shown in Figure 7. It is possible that 3-octynoyl-

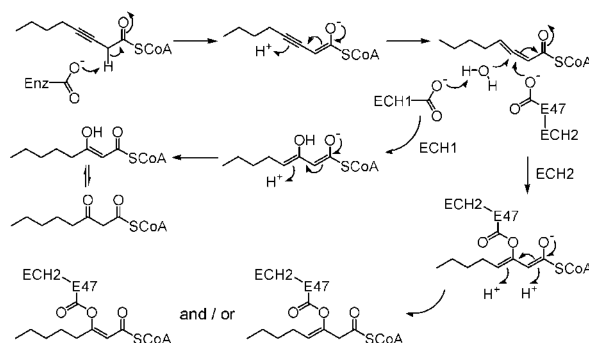

Figure 7. Proposed mechanisms for the conversion of the 3-octynoyl-CoA to 3-ketooctanoyl-CoA product by ECH1 and the inactivation of the ECH2 by 3-octynoyl-CoA.

CoA is isomerized to reactive 2,3-octadienoyl-CoA through deprotonation of the α-proton at first. The catalytic Glu47 may be close to the β-carbon of this reactive allene intermediate, and its conjugate addition to 2,3-octadienoyl-CoA takes place in a following step to form a covalent linkage between the ECH2 and the inhibitor. As mentioned earlier, 3-octynoyl-CoA has been found to be a substrate of ECH1. It is possible that the catalytic residue of ECH1 is a little far away from the β-carbon of the reactive allene intermediate, and therefore an activated water molecule attacks the β-carbon through a conjugate addition reaction instead. The resulting intermediate is then converted to the 3-ketoacyl-CoA product through isomerization and tautomerization steps (Figure 7).

This result indicates ECH1 and ECH2 have certain difference in active site geometry. Our further modeling experiment indicates the active sites of these two enzymes seem not entirely in a mirror image fashion. 3-Octynoyl-CoA or its acid derivatives may selectively inactivate the β-oxidation in peroxisomes without significant effect on the β-oxidation in mitochondria. This comparative study increased our understanding of these two enzymes, which is a prerequisite for controlling or mimicking their physiological roles.

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Supporting Information Available: Supporting experimental data for inactivation and modeling studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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