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Formation of an enolate intermediate is required for the reaction catalyzed by 3-hydroxyacyl-CoA dehydrogenase

Xiaojun Liu, Guisheng Deng, Xiusheng Chu, Nan Li, Long Wu and Ding Li*

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

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Abstract—Fluorinated substrate analogs were synthesized and incubated with rat liver 3-hydroxyacyl-CoA dehydrogenase, which reveals that the formation of an enolate intermediate is required for the reaction catalyzed by the enzyme.

Numerous diseases have been reported in relation to fatty acids, such as cardiovascular disease,[1] cancer[2], and diabetes.[3] The degradation of saturated fatty acids occurs in a sequence of four reactions referred to as the β-oxidation cycle.[4] Fatty acid oxidation in mitochondria is an essential energy generation system for cells. The partial fatty acid oxidation (pFOX) inhibition has been reported as a therapy for non-insulin dependent diabetes mellitus (NIDDM) and chronic stable angina.[5] The third step of the mitochondrial β-oxidation cycle is catalyzed by 3-hydroxyacyl-CoA dehydrogenase (HAD; EC 1.1.1.35), a membrane-associated long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), and short chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD).[6] SCHAD is also known as type 10 17β-hydroxysteroid dehydrogenase (HSD10),[7] which is a potential target for intervention of Alzheimer's disease (AD), Parkinson's disease (PD), infantile neurodegeneration, and other neural disorders.[8] Therefore, mechanistic studies of the reactions catalyzed by the HAD are important not only for the understanding of this essential enzyme but also for the design of enzyme inhibitors targeting above-mentioned diseases.

Rat HAD catalyzes the reversible oxidation of the hydroxyl group of 3-hydroxyacyl-CoA to a keto group, concomitant with the reduction of NAD to NADH (Fig. 1). The dimeric enzyme displays broad substrate specificity, utilizing substrates with 4–16 carbons in the acyl chain.[9] The enzyme is a 'B-side'-specific dehydrogenase with hydride transfer occurring on the *si* face of the nicotinamide ring.[10] The crystal structures have been solved for the apoenzyme, binary complexes of the enzyme with reduced cofactor or 3-hydroxybutyryl-CoA substrate, and an abortive ternary complex of the enzyme with NAD^+ and acetoacetyl-CoA.[11] Several residues have been confirmed to play essential roles in the function of the enzyme.[12]

In our previous study, we have cloned His-tagged rat mitochondrial HAD, and purified both wild-type and Ser137Ala variant proteins.[12e] In this study, we further constructed Asn208Ala mutant plasmid, and the variant protein was subsequently obtained through overexpression in *E. coli* BL21::DE3 and purification with nickel metal affinity column chromatography. These wild-type and variant proteins were used in this study for investigating enzymatic reaction mechanism.

It has been noted that a charge transfer complex is formed when incubating HAD with acetoacetyl-CoA in a reverse reaction mixture, which shows a broad peak centered in the 410–420 nm range in UV/vis spectrum.[11a,12a] The enolate species of acetoacetyl-CoA serves as electron-donor, and NAD^+ is suggested as

Abbreviations: AD, Alzheimer's disease; HAD, 3-hydroxyacyl-CoA dehydrogenase; HSD10, type 10 17β-hydroxysteroid dehydrogenase; LCHAD, long chain 3-hydroxyacyl-CoA dehydrogenase; NIDDM, non-insulin dependent diabetes mellitus; PD, Parkinson's disease; pFOX inhibition, partial fatty acid oxidation inhibition; SCHAD, short chain 3-hydroxyacyl-CoA dehydrogenase; UV/vis, ultraviolet–visible spectroscopy.

Keywords: 3-Hydroxyacyl-CoA dehydrogenase; Enolate; Fatty acid oxidation; Chronic stable angina; Diabetes; Alzheimer's disease.

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

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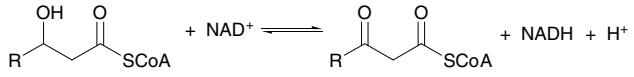

Figure 1. HAD catalyzes the reversible oxidoreduction reaction.

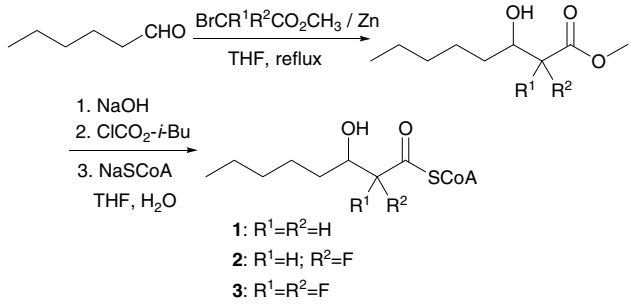

Figure 2. Organic syntheses of HAD substrate and substrate analogs.

the electron acceptor. The resultant negative charge on O3 of the substrate is stabilized by interactions with the side-chain amide of Asn208 and the hydroxyl group of Ser137.

Interestingly, we found the charge transfer complex existed not only in the reverse reaction mixture, but also in the forward reaction mixture when we incubated HAD with 3-hydroxyacyl-CoA substrate and NAD^+ cofactor. This suggests that the formation of an enolate intermediate may be a prerequisite for the subsequent oxidation reaction. In order to further study the mechanism of HAD catalyzed reaction, we synthesized a variety of 3-hydroxyacyl-CoA substrate analogs with fluorine substitution at carbon-2. The incubation studies of HAD with these analogs were carried out, which gave us interesting results.

3-Hydroxyoctanoyl-CoA (**1**), 2-fluoro-3-hydroxyoctanoyl-CoA (**2**), and 2,2-difluoro-3-hydroxyoctanoyl-CoA (**3**) were conveniently synthesized using Reformatsky reaction followed by hydrolysis and coupling with coenzyme A,[13] as shown in Figure 2. These substrate and analogs were incubated with HAD wild-type and variant enzymes, and kinetic studies were carried out. The results of kinetic studies are shown in Table 1. 3-Hydroxyoctanoyl-CoA (**1**) was still a substrate of Asn208Ala variant enzyme with significantly decreased V_{max} value, while it was not a substrate of Ser137Ala variant enzyme at all. This result indicated that both Asn208 and Ser137 can stabilize the enolate intermediate of the forward reaction through interaction with O3 of the enolate intermediate. Ser137 may play more significant role than Asn208, since Ser137Ala completely prevented the formation of the enolate intermediate, resulting in an inactive enzyme for 3-hydroxyoctanoyl-CoA (**1**). 2-Fluoro-3-hydroxyoctanoyl-CoA (**2**) was still a substrate of HAD wild-type enzyme with significantly lower V_{max} value. This may be because the deprotonation at carbon-2 is stereospecific and only half of the analog can be deprotonated at carbon-2 to form the enolate intermediate. The strong electron-withdrawing property of fluorine atom might also decrease the p orbital overlap between carbon-2 and -3. 2-Fluoro-3-hydroxyoctanoyl-CoA (**2**) is not a substrate for both variant enzymes, which is then understandable because the enolate intermediate cannot be stabilized by either Ser137 or Asn208 besides the effect of fluorine substitution at carbon-2 of the analog. 2,2-Difluoro-3-hydroxyoctanoyl-CoA (**3**) was no longer a substrate of the wild-type and variant enzymes. Since compound **3** cannot form an enolate intermediate, it supports that the formation of an enolate intermediate may be a prerequisite for subsequent oxidation reaction.

We hypothesized that the formation of an enolate intermediate can facilitate the oxidation reaction through formation of a more stable conjugated oxidation reaction product, as shown in Figure 3. In order to test this hypothesis, we synthesized 3-hydroxy-4-octenoyl-CoA (**4**), 2-fluoro-3-hydroxy-4-octenoyl-CoA (**5**), and 2,2-difluoro-3-hydroxy-4-octenoyl-CoA (**6**) using the same synthetic strategy as that for the syntheses of compounds **1**–**3** (Fig. 4).[14] These substrate analogs were incubated with HAD wild-type and variant enzymes, and the results are shown in Table 1. Apparently, the introduction of a double bond at carbon-4 of compounds **4** and **5** increased the reaction rate for the wild type enzyme. Compounds **4** and **5** became the substrates

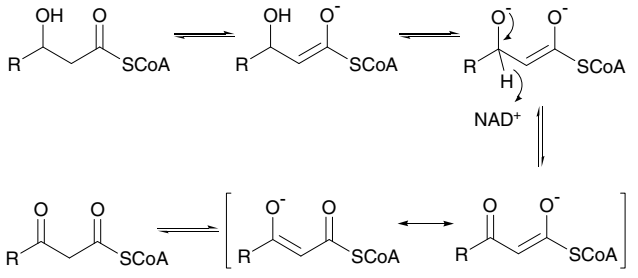

Figure 3. Proposed mechanism for oxidation reaction catalyzed by HAD.

Table 1. Incubation of HAD with substrate and analogs

Compound	Wild-type		N208A		S137A	
	K_M	V_{max}	K_M	V_{max}	K_M	V_{max}
1	34.6 ± 13.9	10.5 ± 1.7	46.8 ± 7.4	0.28 ± 0.02	No detectable activity	
2	69.3 ± 16.1	1.04 ± 0.15	No detectable activity		No detectable activity	
3	No detectable activity		No detectable activity		No detectable activity	
4	20.0 ± 0.8	21.8 ± 0.3	49.6 ± 16.8	1.08 ± 0.18	53.2 ± 14.3	0.46 ± 0.06
5	46.8 ± 5.1	11.7 ± 0.7	44.9 ± 13.1	0.10 ± 0.01	55.7 ± 13.1	0.17 ± 0.02
6	No detectable activity		No detectable activity		No detectable activity	

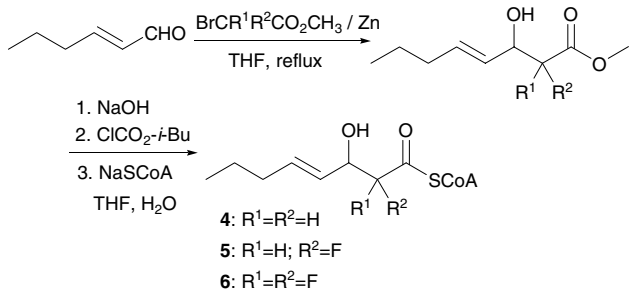

Figure 4. Organic syntheses of HAD substrate analogs.

of the HAD variant enzymes, which indicates that the loss of the stabilization of the enolate intermediate by Ser137 or Asn208 can be partially compensated by the introduction of a double bond at carbon-4. This result supports that the formation of a more stable conjugated oxidation reaction product can facilitate the oxidation reaction. The result indicates the introduction of a double bond at carbon-4 cannot compensate the complete loss of the enolate intermediate, since compound **6** cannot form an enolate intermediate and was not a substrate of the enzyme. Further incubation studies showed that compound **6** was a competitive inhibitor of the wild-type and variant enzymes, which confirm that it can bind with the enzyme active site although it cannot be turned over into the product.

In summary, our results from the incubation of HAD with the fluorinated substrate analogs strongly supports that an enolate intermediate is present in the HAD catalyzed forward reaction. The enolate can be effectively stabilized by residues Ser137 and Asn208. This mechanistic study of the HAD catalyzed reaction suggests that enolate-like organic analogs may be effective inhibitors of the HAD.

Since enolate intermediates have been found for the reactions catalyzed by several other enzymes involved in fatty acid oxidation such as acyl-CoA dehydrogenase, enoyl-CoA hydratase, 2,4-dienoyl-CoA reductase, enoyl-CoA isomerase, and dienoyl-CoA isomerase,[15] the analogs of enolate intermediates may become multifunctional enzyme inhibitors in fatty acid oxidation. Inactivation of only one specific enzyme involved in fatty acid oxidation can result in accumulation of the substrate, which could generate various side effects. The use of several enzyme inhibitors simultaneously targeting several enzymes could produce side effect due to drug interactions.[16] Therefore, the better solution is to use multifunctional enzyme inhibitors for partial inhibition of fatty acid oxidation in treating non-insulin dependent diabetes mellitus (NIDDM) and chronic stable angina.

SCHAD is a multifunctional enzyme with its major function as HAD in fatty acid oxidation, which has been shown as a drug target for intervention of Alzheimer's disease (AD), Parkinson's disease (PD), infantile neurodegeneration, and other neural disorders.[7,8] Therefore, this research may also shed light on the design of enzyme inhibitors treating these important diseases.

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13. (a) The spectra data of 3-hydroxyoctanoic acid (for coupling with coenzyme A in the synthesis of compound **1**) are shown as the following: ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 0.89 (t, J = 6.9 Hz, 3H), 1.40–1.59 (m, 6 H), 2.40 (dd, J = 16.5, 8.7 Hz, 1H), 2.51 (dd, J = 16.5, 3.6 Hz, 1H), 3.95–4.03 (m, 1H), 6.27 (br s, 2H); (b) The spectra data of 3-hydroxy-2-fluorooctanoic acid (for coupling with coenzyme A in the synthesis of compound **2**) are shown as the following: ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 0.86 (t, J = 6.3 Hz, 3H), 1.28–1.44 (m, 6 H), 1.53–1.63 (m, 2H), 4.05 (dt, J = 27.6, 6.6 Hz, 1H), 4.85 (d, J = 49.2 Hz, 1H), 6.55 (s, 1H); (c) The spectra data of 3-hydroxy-2,2-difluorooctanoic acid (for coupling with coenzyme A in the synthesis of compound **3**) are shown as the following: ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 0.88 (t, J = 6.9 Hz, 3H), 1.24–1.37 (m, 6 H), 1.49–1.70 (m, 2H), 3.99–4.10 (m, 1H), 5.41 (br s, 2H).
14. (a) The spectra data of 3-hydroxy-4-octenoic acid (for coupling with coenzyme A in the synthesis of compound

4) are shown as the following: ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 0.85 (t, J = 7.5 Hz, 3H), 1.33–1.42 (m, 2H), 1.94–2.01 (m, 2H), 2.54 (m, 2H), 4.34–4.49 (m, 1H), 5.45 (ddt, J = 15.3, 6.6, 1.4 Hz, 1H), 5.69 (dt, J = 15.3, 7.2 Hz, 1H), 6.33 (br s, 2H); (b) The spectra data of 3-hydroxy-2-fluoro-4-octenoic acid (for coupling with coenzyme A in the synthesis of compound **5**) are shown as the following: ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 0.87 (t, J = 7.2 Hz, 3H), 1.35–1.44 (m, 2H), 2.03–2.11 (m, 2H), 4.51–4.59 (m, 1H), 4.83 (dd, J = 18.6, 3.0 Hz, 1H), 5.02 (br s, 2H), 5.55 (dt, J = 15.3, 6.3 Hz, 1H), 5.84 (dd, J = 15.3, 5.1 Hz, 1H); (c) The spectra data of 3-hydroxy-2,2-difluoro-4-octenoic acid (for coupling with coenzyme A in the synthesis of compound **6**) are shown as the following: ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 0.91 (t, J = 7.2 Hz, 3H), 1.37–1.49 (m, 2H), 2.06–2.14 (m, 2H), 4.56 (dd, J = 15.6, 7.5 Hz, 1H), 6.90 (dt, J = 15.6, 7.2 Hz, 1H), 6.96 (br s, 2H).

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