Mechanistic Studies of the Inactivation of Crotonase by (Methylenecyclopropyl)formyl-CoA

Ding Li, Zhihong Guo, and Hung-wen Liu*

Department of Chemistry, University of Minnesota
Minneapolis, Minnesota 55455

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Enoyl-CoA hydratase (crotonase, EC 4.2.1.17) catalyzes the reversible hydration of a $\Delta^{2,3}$-unsaturated enoyl-CoA substrate to the corresponding 3-hydroxyacyl-CoA product.[1] This reaction is the second step in the β-oxidation pathway of fatty acid metabolism and is also an important step in the catabolism of branched-chain amino acids. Crotonases of different origins are known[2] and a wide variety of enoyl-CoAs of varied chain length with or without substituents at the α- or β-C are accommodated as substrates.[3] Early studies revealed that bond formation/cleavage at α- and β-positions during the hydration/dehydration occurs in a concerted manner[4] and that the overall stereochemistry is syn.[5] The driving force of this catalysis has been attributed to the capability of crotonase to polarize the π-electrons of α,β-unsaturated double bond of the substrate in its active site, hence enhancing the electrophilicity of the β-C.[6] While the physiological significance of crotonase is well established and the mechanism of its catalysis has been well characterized, interestingly, very few inhibitors are known for this class of enzymes.[3,7,8]

In our quest for means to regulate fatty acid metabolism, we have noted that methylenecyclopropylglycine (**1**, MCPG), isolated from the kernels of litchi fruits, had been shown to cause hypoglycemia in mice and fasted rats.[8,9] It was suggested that **1** is first converted *in vivo* into a toxic metabolite, (methylenecyclopropyl)formyl-CoA (**2**, MCPF-CoA),[9] which then interrupts β-oxidation. Early experiments found that **1** is most inhibitory to 3-oxoacyl-CoA and acetoacetyl-CoA thiolases and has little effect on enoyl-CoA hydratase in fasted rats.[9] However, when **2** was used directly in the incubation with purified enzymes, it exhibited strong inhibition of crotonase from pig kidney[10] but less notably so for the bovine liver enzyme.[10b] The distinct effects of **1** and its metabolites on the activities of different enoyl-CoA hydratases is puzzling, and although this inhibition clearly offers an alternate mechanism in controlling the fatty acid metabolism, little is known about its molecular activity.

To gain insight into the toxicity of MCPF-CoA on crotonase, we have chemically prepared **2** in racemic form (Figure 1).[11] The crude product was purified and tested with crotonase

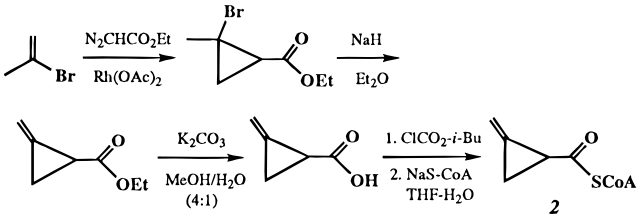

Figure 1.

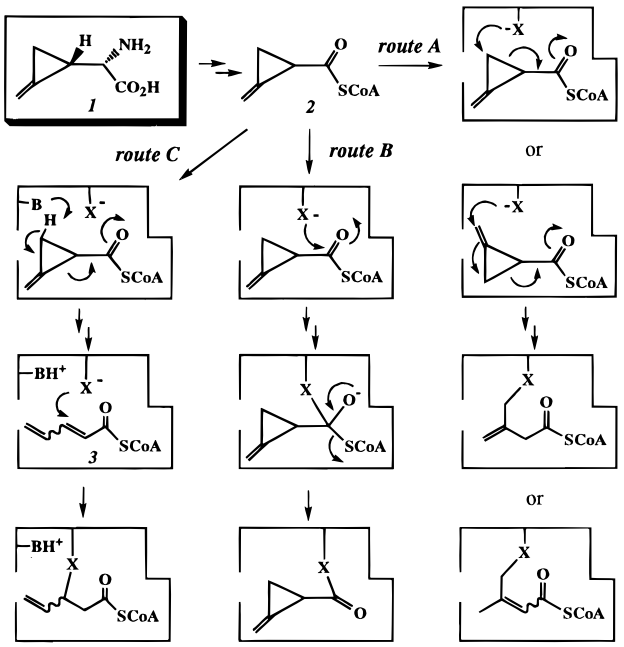

Figure 2.

isolated from pig kidney and bovine liver.[12] Interestingly, in contrast to an early report,[10b] **2** was found to be more inhibitory toward the bovine liver enzyme, with a K_I of 53 μM and a k_{inact} of 3.0×10^{-3} min^{-1}.[13] Since the enzyme activity was not recovered after extensive dialysis, the inhibition is clearly irreversible.[14] It should also be noted that the inhibition by **2** is likely active site directed, since its effect was suppressed by 40% in the presence of an equivalent of acetoacetyl-CoA, which is a known competitive inhibitor of crotonase.[3a] As delineated in Figure 2, the inactivation may be attributed to the electrophilic nature of the cyclopropane moiety, which, by virtue of its electron-withdrawing substituent, is readily susceptible to nucleophilic attack (route A).[15] While the initial attack, in principle, could occur at two distinct sites, both routes would lead to identical adduct(s). The covalent derivatization could also be achieved by direct attack of the thioester carbonyl, followed by releasing of CoA to form an acyl−enzyme adduct (route B). However, the tendency of **2** to release its structural strain by ring cleavage, generating a reactive intermediate (**3**) capable of trapping an enzyme nucleophile, may also account

(1) There are three types of enoyl-CoA hydratase in higher animals, two in mitochondria as short-chain enoyl-CoA hydratase (crotonase) and trifunctional protein (Uchida, Y.; Izai, K.; Orii, T.; Hashimoto, T. *J. Biol. Chem.* **1992**, *267*, 1034) and one in peroxisomes as bifunctional protein (Osumi, T.; Hashimoto, T. *Arch. Biochem. Biophys.* **1980**, *203*, 372).

(2) Crotonase has been purified from bovine liver,[3c] rat liver (Furuta, S.; Miyazawa, S.; Osumi, T.; Hashimoto, T.; Ui, N. *J. Biochem.* **1980**, *88*, 1059), pig heart (Fong, J. C.; Schulz, H. *Methods Enzymol.* **1981**, *71*, 390), and pig kidney (Buettner, H. Ph.D. Thesis, University of Konstanz, 1988).

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(12) The bovine liver enzyme was purchased from Sigma (St. Louis, MO). The pig kidney enzyme was purified to near homogeneity by a sequence similar to that used in the purification of pig heart crotonase.[2] The specific activity of the purified enzyme is 293 units/mg (1 unit = 1 μmol of product formation per minute).

(13) The kinetic parameters of inactivation were derived from plotting the apparent inactivation rate, obtained by incubating crotonase (9 μM, 6.3 nmol) with **2** of varied concentration (9−600 μM), versus inhibitor concentration. Unless otherwise specified, 50 mM potassium phosphate buffer, pH 7.5, was used in all experiments.

(14) Both covalent modification of the enzyme active site and formation of a very tight-binding complex had been proposed for the irreversible inactivation of pig kidney crotonase by **2**.[10b]

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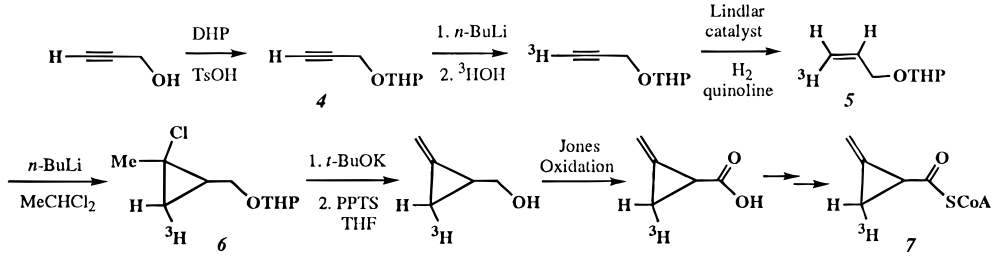

Figure 3.

for the eventual toxicity of **1** (route C). Routes B and C are less likely due to the facts that CoA is not a structural entity unique for **2** and the cyclopropyl ring hydrogen is not particularly acidic. Whether the molecular basis of the virulent consequences of **1** is caused by the nucleophilic trapping via an intact methylenecyclopropyl moiety or by the ring-opened form of the inhibitor can be discerned by scrutinizing the inactivation process using labeled **2**.

Preparation of the tritium-labeled MCPF-CoA (**7**) was effected by the sequence shown in Figure 3. The key steps involved the exchange of the acetylenic hydrogen of **4** with tritium by treatment of **4** with base and quenching with tritiated water, and the cyclization of **5** to **6** by *n*-butyllithium and dichloroethane.[16] The specific radioactivity of the final product **7** was 0.56 mCi/mmol. The covalent nature of this inactivation was substantiated by a 1:1 stoichiometry between **7** (10 molar equiv) and bovine liver crotonase (40 nmol), determined after extensive dialysis of the inactivated enzyme. The resulting inactivated enzyme was denatured by boiling, and precipitated protein was collected and redissolved in Tris-HCl buffer (0.1 M, pH 10) containing 6 M guanidine hydrochloride. Interestingly, >93% of the radioactivity was retained in the denatured protein after overnight incubation. This result, in conjunction with the failure to detect released CoA in the overnight incubation mixture with 5,5′-dithiobis(2-nitrobenzoic acid), provided strong evidence suggesting that modification of crotonase via an acyl−enzyme adduct is quite unlikely. Moreover, little tritium release (<1%) was detected when **7** (1 molar equiv) was incubated with crotonase, followed by quenching with a 10% charcoal solution.[17] On the basis of this observation, route C may also be excluded as a viable mechanism. Thus, these results are most consistent with route A, in which **2** inactivates crotonase via the intact methylenecyclopropane ring trapping of an active site nucleophile.

It is worth mentioning that examples of cyclopropane-containing, mechanism-based inactivators are known in which the target enzyme activates the cyclopropane for nucleophilic addition by oxidation or protonation of the appended groups that render them more electron-withdrawing.[15,18] However, no such catalysis-produced activation appears to be necessary for the inactivation of crotonase by **2**, as deduced from this study. Since in the normal catalysis the substrate has been shown to be polarized by an electrophile proximal to the C═O oxygen (either via a positive charge or strong H-bonds) and a nucleophile near the β-C in the active site of crotonase,[6] such a push−pull model may also explain the activation of **2** and facilitate the trapping of an active site nucleophile. The large ring strain associated with the methylenecyclopropyl system may have also contributed to the reactivity of **2**. Interestingly, while a glutamate residue (Glu[164] of the rat liver enzyme) conserved in enoyl-CoA hydratases[19] has been shown to be the base responsible for α-proton abstraction in the rat liver crotonase,[6b] the nucleophile trapped by MCPF-CoA in the bovine liver enzyme is unlikely a carboxylate residue, since the inhibitor−enzyme adduct is stable under denaturing alkaline conditions. Further characterization of the labeled protein will certainly provide valuable information about the active site of this important class of enzyme whose crystal structure is still lacking.

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