Cyclobutaneacetyl-CoA: A Janus-Faced Substrate for Acyl-CoA Dehydrogenases

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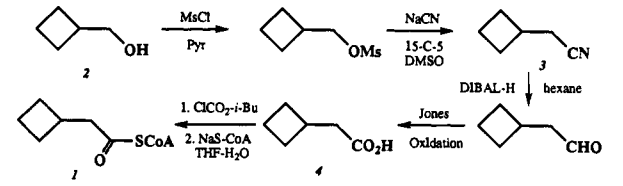
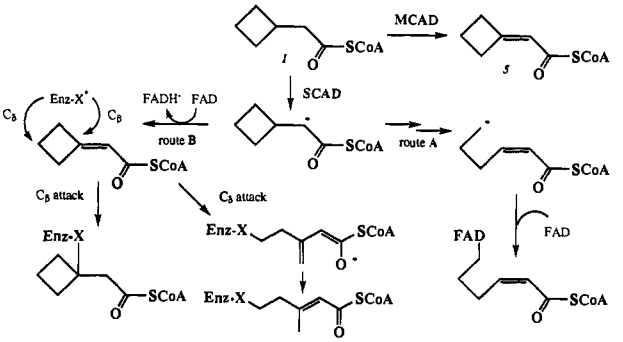
Acyl-CoA dehydrogenases are flavoproteins that catalyze the first step of β-oxidation, converting a fatty acyl thioester substrate to the corresponding α,β-enoyl-CoA product.[1] On the basis of the preferred chain length of the substrates, this class of enzymes can be further divided into three subgroups: long-chain (LCAD), medium-chain (MCAD), and short-chain (SCAD) acyl-CoA dehydrogenase.[1] Since these enzymes are closely related, having identical biochemical functions and similar physical properties, their general catalytic characteristics are also expected to be alike.[1,2] However, this hasty generalization has recently been called into question on the basis of the variance revealed in the studies of their inhibitory behaviors.[3] In an attempt to discern features causing such mechanistic disparities, we have prepared a variety of substrate analogues and compared their effects on MCAD and SCAD.[4] Among those, cyclobutaneacetyl-CoA (CBA-CoA, **1**) stands alone as a unique example due to the duality of its activity; while it is a substrate for MCAD, CBA-CoA acts as an inhibitor for SCAD.[5] Reported in this paper are the results and implications of this study.

Synthesis of the title compound from cyclobutanemethanol (**2**) was effected by the reactions delineated in Scheme 1. Chain elongation transforming **2** to cyclobutaneacetic acid (**4**) via the cyanide intermediate **3** was achieved in four steps with an overall yield of 29%.[6] Condensation of **4** with isobutyl chloroformate followed by coupling to coenzyme A in aqueous THF solution (pH 8–8.5)[6] gave the desired CBA-CoA, which after HPLC purification was obtained in 70% yield. Upon incubation with MCAD, **1** was converted to the corresponding enoyl-CoA product (Scheme 2, **5**), whose identity was substantiated by comparison with an authentic species prepared separately from cyclobutanone.[7] Since reoxidation of the reduced flavin coenzyme generated during turnover is extremely sluggish in the absence of artificial electron acceptors,[8] the k_{cat} of this catalysis was readily determined to be 0.8 min^{-1} by following the bleaching of the flavin chromophore at 446 nm. However, when SCAD was exposed to **1**, time-dependent inhibition occurred, along with

Scheme 1



Scheme 2



irreversible quenching of the FAD chromophore.[9] This inactivation exhibited Michaelis–Menten kinetics with a k_{inact} of 2.1 min^{-1} and K_I of 37.4 μM. A partition ratio of 0–1 could also be deduced from a plot of the residual activity observed versus total equivalents of **1** added.[10]

The molecular basis of this inactivation may proceed with an initial C_α anion formation, followed by ring fragmentation, and then covalent modification of the flavin coenzyme (Scheme 2, route A). It may also result from trapping an active-site nucleophile via its addition to C_δ or C_β of the enoyl-CoA product, while consequently hampers the reoxidation of the reduced flavin (Scheme 2, route B). Since the inhibitor-induced modification is predicted to occur at distinct sites among these mechanisms, incubation with tritiated CBA-CoA revealing the labeled sites should help to distinguish these possibilities. Because dehydrogenation prior to inactivation is a prerequisite for pathway B, analysis of the extent of tritium release from incubation of C_β-tritiated **1** would provide additional evidence to differentiate these inactivation mechanisms. Preparation of the multiple-labeled CBA-CoA was achieved by the procedure of Gaoni with minor alteration.[11] As shown in Scheme 3, the key intermediate, a γ,δ-epoxy sulfone **6**, was prepared by condensing ((phenylsulfonyl)methyl)magnesium bromide with allyl bromide followed by epoxidation. Sequential treatment with *n*-butyllithium, methanesulfonyl chloride, and *n*-butyllithium converted **6** to 1-(phenylsulfonyl)bicyclo[1.1.0]butane (**7**) in moderate yield (62%). The hydroxyethyl side chain was appended by coupling the anion of**7** with ethylene oxide, and the tritium labelings were

(1) (a) Beinert, H. *Enzymes, 2nd Ed.* **1963**, *7*, 447. (b) Engel, P. C. In *Chemistry* and *Biochemistry of Flavoenzymes*; Müller, F., Ed.; CRC Press: New York, 1991; p 597. (c) Schulz, H. In *Fatty Acid Oxidation: Clinical, Biochemical, and Molecular Aspects*; Tanaka, K., Coates, P. M., Eds.; Alan R. Liss: New York, 1990; p 23.

(2) (a) Ghisla, S.; Thorpe, C.; Massey, V. *Biochemistry* **1984**, *23*, 3154. (b) Dommes, V.; Kunau, W.-H. *J. Biol. Chem.* **1984**, *259*, 1789. (c) Ikeda, Y.; Tanaka, K. In *Fatty Acid Oxidation: Clinical, Biochemical, and Molecular Aspects*; Tanaka, K., Coates, P. M., Eds.; Alan R. Liss: New York, 1990; p 37.

(3) (a) Bell, P.; Cheon, S. H.; Fillers, S.; Foley, J.; Fraser, J.; Smith, H.; Young, D.; Revesz, L. *Bioorg. Med. Lett.* **1993**, *3*, 1007. (b) Tserng, K. Y.; Jin, S.-J.; Hoppel, C. L. *Biochemistry* **1991**, *30*, 10755.

(4) MCAD was purified from pig kidney according to the procedure of Thorpe (Thorpe, C. *Methods Enzymol.* **1981**, *71*, 366). The recombinant *Megasphaera elsdenii* SCAD was expressed and purified as previously described (Becker, D. F.; Fuchs, J. A.; Banfield, D. K.; Walter, F. D.; MacGillivray, R. T. A.; Stankovich, M. T. *Biochemistry* **1993**, *32*, 10736).

(5) Although the recombinant *M. elsdenii* SCAD is of bacterial origin, it shares many catalytic as well as physical characteristics with its mammalian counterpart. More importantly, the substrate recognition residues in the active site of *M. elsdenii* SCAD, identified by crystal structural analysis of the enzyme–acetoacetyl-CoA complex, are also conserved in mammalian SCAD (Professor Jung-Ja Kim, personal communication). Thus, the bacterial enzyme which is well characterized and readily available in large quantity is an ideal substitute for the mammalian SCAD and has been used in this study.

(6) Lai, M.-t.; Liu, L.-d.; Liu, H.-w. *J. Am. Chem. Soc.* **1991**, *113*, 7863.

(7) The key steps involved coupling of cyclobutanone with trimethyl phosphonoacetate in the presence of NaH (40% yield), followed by hydrolysis (1 N NaOH, dioxane; quantitative). ^{1}H NMR (D_2O, signals of methylenecyclobutyl moiety shown in italics): δ 8.69, 8.42 (1H each, s), 6.24 (1H, d, J = 5.3; ribose anomeric H), *6.09* (1H, br s, α-H), 4.92 (2H, m), 4.67 (1H, br s), 4.34 (2H, br s), 4.10 (1H, s), 3.93 (1H, m), 3.67 (1H, m), 3.52 (2H, m), 3.40 (2H, m), 3.07 (2H, m), *3.05* (2H, m, 2 β-H's), *2.87* (2H, m, 2 β-H's), 2.51 (2H, m), *2.14* (2H, m, 2 γ-H's), 1.00, 0.86 (3H each, s).

(8) Wang, R.; Thorpe, C. *Biochemistry* **1991**, *30*, 7895.

(9) While the reduced FAD of MCAD could be reoxidized by ferrocenium hexafluorophosphate (Lehman T. C.; Thorpe C. *Biochemistry* **1990**, *29*, 10594), the same procedure failed to regenerate the flavin coenzyme of SCAD. Furthermore, no activity was recovered after extensive dialysis.

(10) It should be noted that the apparent inactivation levels off at approximately 50% residual activity. While it is not obvious why the inactivation is not complete, there is no doubt that CBA-CoA is a specific inhibitor for SCAD. Interestingly, cyclobutaneformyl-CoA is a substrate of this SCAD (Williamson, G.; Engel, P. C. *Biochem. J.* **1984**, *218*, 521).

(11) (a) Gaoni, Y. *J. Org. Chem.* **1982**, *47*, 2564. (b) Gaoni, Y. *Tetrahedron* **1989**, *45*, 2819.

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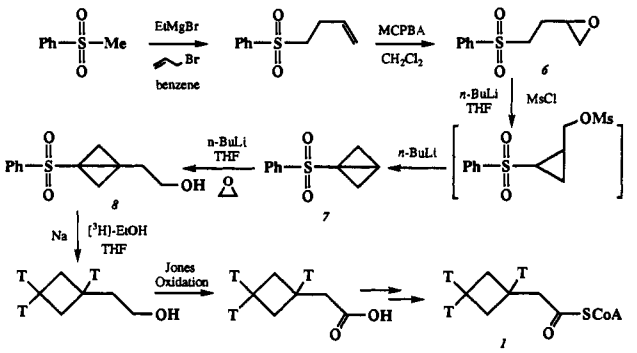
Scheme 3



then introduced via the alkaline metal catalyzed reduction of **8** in a solution of [^{3}H]ethanol and THF.[12] The final steps were analogous to those shown in Scheme 1.

As anticipated, tritium release (25%) was observed when CBA-CoA obtained from the above sequence (specific activity 0.84 mCi/mmol, 1 molar equiv) was incubated with MCAD (14.6 μmol) followed by quenching with charcoal (10% solution).[13] On the contrary, no significant tritium washout (5.4%) was noted when the incubaton was performed using SCAD (14.3 μmol) and **1** (1 molar equiv). A 1:1 stoichiometry between CBA-CoA and SCAD was determined after extensive dialysis of the inactivated enzyme (10 molar equiv of **1** was used in this case). The resulting inactivatd SCAD was treated with 90% methanol to denature the protein and release the flavin coenzyme.[13] Since less than 1% of radioactivity was detected in the denatured protein precipitates, modification of SCAD itself is clearly not a notable inactivation process. Instead, greater than 99% of the radioactivity which is expected to be associated with the modified FAD was found in the supernatant. The fact that the electronic absorption of the supernatant remained featureless further supported the presence of a modified FAD.[14] Thus, these results are most consistent with pathway A in which CBA-CoA inactivates SCAD via covalent flavin-adduct formation.[15]

Clearly, CBA-CoA represents a rare example for being a substrate and/or an inhibitor of two immediately related enzymes.[16] While its C_β-H is released during MCAD-mediated turnover, the cleavage of its C_β-C_γ linkage,[17] upon incubation with SCAD, appears to be the diverging point beyond which the reaction derails from its normal path leading to the modification of FAD. Thus, the Janus-faced nature of CBA-CoA may be ascribed to such selective bond scission, a consequence of the distinct steric and electronic demands imposed by the active site of MCAD and SCAD. This finding, in conjunction with the available three-dimensional structures of MCAD[18] and SCAD,[5] may allow us to better define the constraints associated with substrate/inhibitor binding with these enzymes. The identification of CBA-CoA as a selective inhibitor for different acyl-CoA dehydrogenases may also provide a new lead for developing more specific means for the treatment of non-insulin-dependent diabetes mellitus (NIDDM), a disease characterized by elevated hepatic glucose production, insulin resistance, and hyperglycemia.[19] Since inhibition of fatty acid oxidation results in the accumulation of fatty acyl-CoA's and the suppression of acetyl-CoA/NADH production, both of which contribute to the induction of hypoglycemia,[20] a class-selective inhibitor of acyl-CoA dehydrogenases can be used to regulate the metabolism of fatty acid and thus control the level of hyperglycemia.[21]

(12) [^{3}H]Ethanol was prepared by mixing [^{3}H]H_2O (specific activity 100 mCi/mL) with ethanol (v/v = 1/9) followed by drying and distillation. The [^{3}H]H_2 gas generated in the reduction step was trapped by palladium on charcoal and used to reduce olefinic compounds.

(13) Lai, M.-t.; Li, D.; Oh, E.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 1619.

(14) If the inactivation is due to the tight binding between CBA-CoA and SCAD, the flavin coenzyme released should be the intact FAD giving characteristic absorption features.

(15) It is also possible that the initially formed C_α-carbanion undergoes a single-electron transfer to FAD to generate the cyclobutylcarbinyl radical and the flavin semiquinone. Subsequent radical-induced ring opening followed by coupling with flavin semiquinone leads to inactivation. Such ring cleavage induced by cyclobutylcarbinyl radical is well precedented and has been used in the design of enzyme inhibitors (Silverman R. B.; Zieske, P. A. *Biochemistry* **1986**, *25*, 341).

(16) Another interesting example is 3-decynoyl thioester, which is an inhibitor for *E. coli* β-hydroxydecanoylthioester dehydrase (Helmkamp, G. M., Jr.; Rando, R. R.; Brock, D. J. H.; Block, K. *J. Biol. Chem.* **1968**, *243*, 3229) but is a substrate for a related hog liver isomerase (Miesowicz, F. M.; Block, K. *J. Biol. Chem.* **1979**, *254*, 5868).

(17) Since formation of a C_α anion is a prerequisite for catalysis, it is doubtful that the initially generated C_α anion is the nucleophile responsible for direct attacking and modifying FAD. This is supported by the fact that incubation of (2-methylcyclopropane)acetyl-CoA with SCAD in deuterated buffer did not result in inactivation but in the recovery of substrate with a deuterium incorporated at the α-C. Thus, a more likely candidate is the acyclic anion (or radical) resulting from C_β-C_γ ring cleavage.

(18) Kim, J. P.; Wang, M.; Paschke, R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7523.

(19) (a) DeFronzo, R. A. *Diabetes* **1988**, *37*, 667. (b) Olefsky, J. M.; Garvey, W. T.; Henry, R. R.; Matthaei, S. Freidenberg, G. R. *Am. J. Med.* **1988**, *85* (Suppl. 5A), 86.

(20) (a) Golay, S.; Swislocki, A. L. M.; Chen, Y.-D. I.; Reaven, G. M. *Metabolism* **1987**, *36*, 692. (b) Bressler, R.; Corredor, C. F.; Brendel, K. *Pharmacol. Rev.* **1969**, *21*, 105.

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