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Evolution of Function in the Crotonase Superfamily: The Stereochemical Course of the Reaction Catalyzed by 2-Ketocyclohexanecarboxyl-CoA Hydrolase

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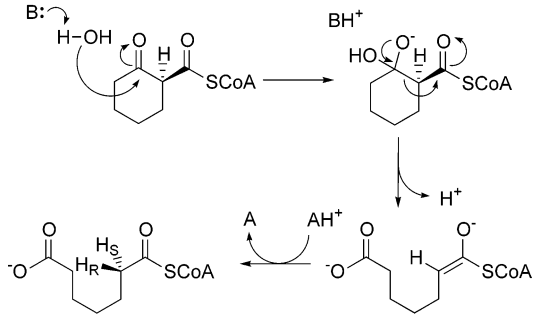
Thioester enolate anion intermediates are the hallmark of reactions catalyzed by members of the mechanistically diverse enoyl-CoA hydratase (crotonase) superfamily.[1] Many reactions involve either hydration of α,β-enoyl-CoA thioesters or isomerization of β,γ-enoyl-CoA thioesters. However, Dieckmann reactions are catalyzed by certain members of the superfamily: the anaerobic catabolism of benzoate by *Rhodopseudomonas palustris* includes hydrolysis of 2-ketocyclohexanecarboxyl-CoA (KC-CoA) to pimelyl-CoA catalyzed by KC-CoA hydrolase (BadI).[2] This reverse Dieckmann reaction is expected to involve addition of water to form a hydrated ketone that decomposes to product via stereospecific protonation of a thioester enolate anion intermediate (Scheme 1). We now report that the reaction proceeds with *inversion* of configuration, thereby restricting the functions of conserved active-site functional groups.

Scheme 1

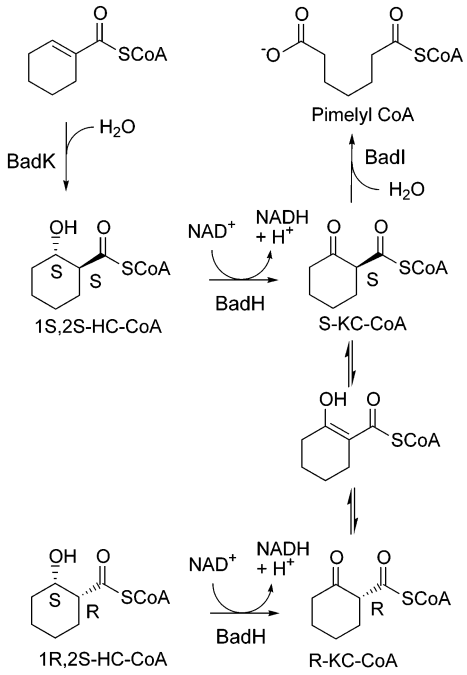


Relevant steps in benzoate catabolism[3] (Scheme 2) are (1) hydration of cyclohex-1-enecarboxyl-CoA by BadK, a homologue of rat mitochondrial crotonase, (2) oxidation of the resulting 2-hydroxycyclohexanecarboxyl-CoA (HC-CoA) by BadH,[4] and (3) hydrolysis of the resulting KC-CoA by BadI. KC-CoA is configurationally labile at the α-carbon; in fact, the spectrophotometric assay for BadI is based on the absorbance of the enolized substrate.[2]

We generated the possible substrates for BadI in situ by the BadH-catalyzed oxidation of the separated, configurationally stable 1*S*,2*S*- and 1*R*,2*S*-diastereomers of HC-CoA. The carboxylic acids were obtained by separation of racemic *cis*- and *trans*-2-hydroxycyclohexanecarboxylic acids[5] followed by their resolution with brucine;[6,7] the configurations of the brucine salts were assigned by X-ray crystallography. The 1*S*,2*S*-HC-CoA was identical to the product of the BadK-catalyzed reaction.[8] Fortuitously, both 1*S*,2*S*- and 1*R*,2*S*-HC-CoA are substrates for BadH, permitting the transient preparation of the *S*- and *R*-diastereomers of KC-CoA, one of which is expected to be the substrate for BadI.

The identity of the substrate was determined by incubating each diastereomer of HC-CoA with a constant amount of BadH and increasing amounts of BadI in ^2H_2O. Starting with 1*R*,2*S*-HC-CoA,

Scheme 2



the pimelyl-CoA *always* contained two deuteria (Table 1), consistent with required configurational inversion of *R*-KC-CoA via enolization/deuteration prior to the BadI-catalyzed reaction that incorporates a single atom of deuterium (Scheme 2). In contrast, starting with 1*S*,2*S*-HC-CoA, the pimelyl-CoA contained two deuteria with low amounts of BadI but only one deuterium with large amounts of BadI, consistent with interception of *S*-KC-CoA by BadI prior to enolization. Thus, *S*-KC-CoA is the substrate.

Table 1. Incorporation of Deuterium[a] into Pimelyl-CoA from 1*R*,2*S*- and 1*S*,2*S*-HC-CoA

	1*R*,2*S*-HC-CoA			1*S*,2*S*-HC-CoA		
BadH:BadI[b]	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_0$	$^2H_1$	$^2H_2$
1:0.1	1	nd[c]	99	nd	nd	100
1:1	nd	nd	100	nd	28	72
1:10	nd	nd	100	nd	76	24

[a] Deuterium content of the product was determined using Micromass Quattro mass spectrometer, equipped with an electrospray ion source, and comparing intensities of the molecular ion peaks at $(M - H)^-$ 908 ($^2H_0$), 909 ($^2H_1$), and 910 ($^2H_2$) amu. [b] Molar ratios of BadH:BadI. [c] Not detected.

The β-oxidation of pimelyl-CoA in *R. palustris* is initiated by pimelyl-CoA dehydrogenase (PCDH),[3] a member of the FAD-dependent acyl-CoA dehydrogenase superfamily in which oxidation occurs with abstraction of the 2-pro*R*-hydrogen.[9] Our purified PCDH was contaminated with dehydropimelyl-CoA hydratase so

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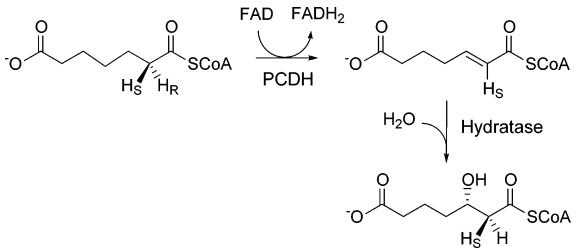
that pimelyl-CoA is converted to 3-hydroxypimelyl-CoA (Scheme 3); however, this further reaction does not preclude the use of this enzyme preparation to determine the configurations of samples of 2-[$^2H_1$]-pimelyl-CoA.

Scheme 3



The 2-methylene group of 3-hydroxypimelyl-CoA is associated with an ABX multiplet at 2.58 ppm in the ^1H NMR spectrum (Figure 1A). When 2-[$^2H_2$]-pimelyl-CoA, obtained from 1*R*,2*S*-HC-CoA, BadH, and BadI in ^2H_2O, is incubated with the PCDH−hydratase mixture in H_2O, the multiplet is simplified to an upfield doublet, establishing that the PCDH−hydratase mixture does not catalyze nonstereospecific exchange of the 2-hydrogens with solvent (Figure 1B). The predicted stereospecificity of PCDH was verified using pimelyl-CoAs synthesized from enantiomers of 2-[$^2H_1$]-pimelic acid,[10−13] yielding thioesters that are equimolar mixtures of chiral 2-[$^2H_1$]- and achiral 2-[$^1H_2$]-pimelyl-CoAs. 3-Hydroxypimelyl-CoA derived from 2*R*-[$^2H_1$]-pimelyl-CoA did not retain deuterium (Figure 1C), but that obtained from 2*S*-[$^2H_1$]-pimelyl CoA did (Figure 1D); for reference, Figure 1E is the spectrum of an equimolar mixture of 2*S*-[$^2H_1$]-3-hydroxypimelyl-CoA (Figure 1B) and 2-[$^1H_2$]-3-hydroxypimelyl-CoA (Figure 1A).

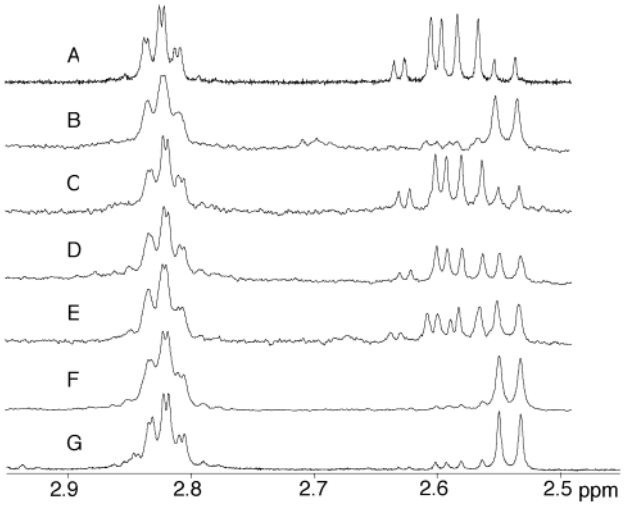


Figure 1. ^1H NMR spectra at 500 MHz of samples of 3-hydroxypimelyl-CoA: A, from pimelyl CoA; B, from 2-[$^2H_2$]-pimelyl CoA; C, from 2*R*-[$^2H_1$]-pimelyl CoA; D, from 2*S*-[$^2H_1$]-pimelyl CoA; E, an equimolar mixture of 2-[$^1H_2$]- and 2*S*-[$^2H_1$]-3-hydroxypimelyl-CoA; F, from 2-[$^2H_1$]-pimelyl CoA obtained from the BadH−BadI coupled reaction in ^2H_2O; and G, from 2-[$^2H_1$]-pimelyl CoA obtained from BadI-catalyzed exchange in ^2H_2O. The triplet at 2.82 ppm is associated with a methylene group in the coenzyme A moiety.

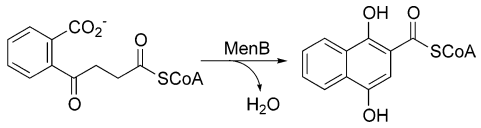
A sample of pimelyl-CoA obtained from 1*S*,2*S*-HC-CoA by the coupled actions of BadH and BadI in ^2H_2O was 60% monodeuterated and 40% dideuterated, as quantitated by ESI-MS. Presumably, one prochiral hydrogen on carbon-2 was 40% deuterated because of the competing enolization of the *S*-KC substrate, and the other was 100% deuterated because it contains the deuteron delivered to the enolate anion intermediate by BadI. The 3-hydroxypimelyl-CoA resulting from incubation of this enzymatically deuterated pimelyl CoA with the PCDH−hydratase mixture was 100% monodeuterated at carbon-2 (Figure 1F), indicating that the enzymatically delivered deuteron was retained by the action of PCDH. Thus, the deuteron was located in the 2-pro*S* position of pimelyl-CoA, establishing that BadI delivers a solvent-derived proton to the *si*-face of the carbon of the enolate anion.

In a parallel experiment, protiated pimelyl-CoA was incubated with BadI in ^2H_2O, and one hydrogen on carbon-2 was exchanged, as quantitated by both ^1H NMR spectroscopy and ESI-MS. When the monodeuterated pimelyl-CoA was analyzed with the PCDH−hydratase mixture, the deuterium was retained, again indicating incorporation into the 2-pro*S* position (Figure 1G).

We conclude that BadI (1) catalyzes the hydrolysis of 2*S*-KC-CoA and (2) incorporates hydrogen into the 2-pro*S* position of pimelyl-CoA. Therefore, the stereochemical course of the reaction is inversion of configuration.

Homologues of BadI include 1,4-dihydroxynaphthoyl-CoA synthase (MenB) that catalyzes a Dieckmann condensation in bacterial menaquinone biosynthesis using the aliphatic CoA ester of *o*-succinylbenzoate[14,15] (Scheme 4). Despite the different structures of the substrates and products for the MenB- and BadI-catalyzed reactions, the enzymes share as much as 53% sequence identity. We expect that the stereochemical course of the BadI-catalyzed reaction will be important not only in understanding the roles of its active-site functional groups, including Ser 138, Asp 140, and Tyr 235, but also the roles of strictly conserved homologues of these residues in the Dieckmann condensation catalyzed by MenB.

Scheme 4



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