Communication

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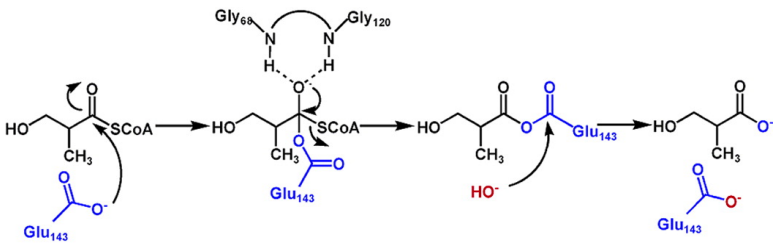

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Divergent Function in the Crotonase Superfamily: An Anhydride Intermediate in the Reaction Catalyzed by 3-Hydroxyisobutyryl-CoA Hydrolase

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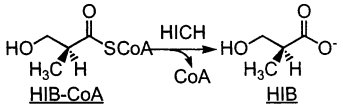
The members of the mechanistically diverse enoyl-CoA hydratase (crotonase) superfamily catalyze reactions that usually involve a thioester enolate anion intermediate stabilized by hydrogen bonding with two peptidic NH groups in an oxyanion hole. For example, in the dehydration reaction catalyzed by the paradigm rat mitochondrial crotonase, Glu164 abstracts the α proton of the 3-hydroxybutyryl-CoA substrate to generate a thioester enolate intermediate stabilized by hydrogen bonding with the NH groups of Ala98 and Gly141.[1] Without this stabilization, the intermediate would be too unstable to be kinetically competent. The intermediate is directed to product with the assistance of Glu144.

3-Hydroxyisobutyryl-CoA hydrolase (HICH) catalyzes the hydrolysis of 3-hydroxyisobutyryl-CoA (HIB-CoA) to form 3-hydroxyisobutyrate (HIB) and coenzyme A.[2,3]

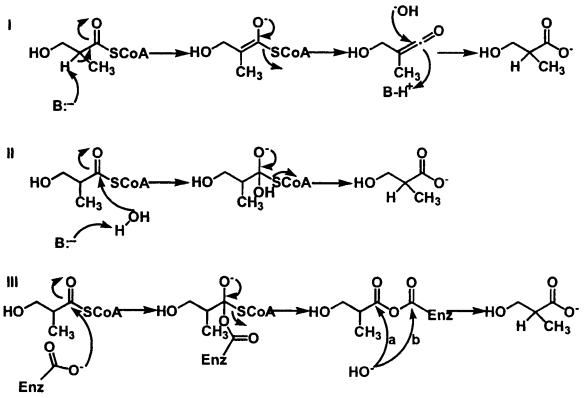


HICH is a member of the crotonase superfamily: its sequence contains the consensus for the oxyanion hole as well as a homologue of Glu164 in crotonase. However, this reaction may not involve a thioester enolate intermediate.[1,2] We have explored the reaction catalyzed by HICH from *Pseudomonas aeruginosa* and discovered that it involves the formation of an anhydride intermediate with a conserved homologue of the general base Glu164 in crotonase. The conserved oxyanion hole stabilizes one or more anionic tetrahedral intermediates.

A priori, three mechanisms can be envisaged for the HICH-catalyzed reaction (Scheme 1). The first is base-catalyzed abstraction of the α-proton of the thioester to form an enolate anion that decomposes to product via a ketene intermediate (Path I). Although ketene formation is unfavorable, the hydrolysis of thioesters in aqueous solution occurs by this mechanism, and the enolate anion intermediate could be stabilized by the oxyanion hole.[4] The second mechanism involves general base-catalyzed attack of water on the thioester carbon to form an anionic tetrahedral intermediate that also could be stabilized by the oxyanion hole (Path II). A third mechanism involves nucleophilic attack of an active-site carboxylate group on the thioester carbon to generate an anhydride intermediate (Path III). Hydrolysis occurs *via* attack of water on the carbonyl carbon of either the substrate (Path IIIa) or the nucleophile (Path IIIb).

Path I can be eliminated by the observation that the reaction can occur in the absence of an α proton: although reduced, the kinetic constants for hydroxypivalyl-CoA ($k_{cat} = 1.1\ s^{-1}$; $k_{cat}/K_m = 1.2 \times 10^4\ M^{-1}\ s^{-1}$) are significant relative to those for HIB-CoA ($k_{cat} = 89\ s^{-1}$; $k_{cat}/K_m = 1.5 \times 10^7\ M^{-1}\ s^{-1}$).[5,6] Furthermore, a negligible substrate deuterium isotope effect accompanies the reaction: the hydrolysis of [2-^{2}H]-HIB-CoA is

Scheme 1. Three Possible Mechanisms for HICH



accompanied by isotope effects of 1.2 ± 0.1 and 1.1 ± 0.3, respectively, for k_{cat} and k_{cat}/K_m. Neither hydrolysis of protiated substrate in D_2O nor hydrolysis of deuterated substrate in H_2O is accompanied by incorporation of solvent deuterium or hydrogen into HIB.

The mechanisms involving nucleophilic attack (Paths II and III) were studied by performing experiments in $H_2{}^{18}O$.[7] Using an excess of enzyme to substrate (single-turnover conditions), solvent-derived oxygen would be incorporated into HIB if the reaction involved base-catalyzed attack of water (Path II) or an anhydride intermediate were hydrolyzed by attack of water at the substrate carboxyl carbon (Path IIIa). No ^{18}O would be incorporated into HIB if an anhydride intermediate were formed and hydrolysis occurred at the enzyme carboxyl carbon (Path IIIb). In the latter pathway, the probability of ^{18}O incorporation into HIB would be 50% in a subsequent single-turnover experiment because of the rotational symmetry of the carboxylate group. However, if both carboxylate oxygens of the enzyme carboxylate were fully labeled in $H_2{}^{18}O$ with an excess of substrate to enzyme (multiple-turnover conditions), the probability of ^{18}O incorporation would be 100%.

A control reaction performed in $H_2{}^{16}O$ under single-turnover conditions yielded HIB that contained negligible ^{18}O (Table 1, entry 1). While hydrolysis using multiple-turnover conditions gave the expected incorporation of ^{18}O in HIB (Table 1, entry 2), hydrolysis in $H_2{}^{18}O$ under single-turnover conditions gave a level of ^{18}O incorporation similar to that of the control (Table 1, entries 3A, 3B).[8] As explained previously, the absence of ^{18}O in HIB in the single-turnover experiment suggests a mechanism involving an anhydride intermediate that is hydrolyzed by attack at the enzyme carboxyl group. This mechanism was further investigated by isolating the enzyme from a single-turnover experiment and performing a second single-turnover experiment in $H_2{}^{16}O$; the incorporation of ^{18}O was ∼35% of the isotopic enrichment of the solvent, consistent with labeling of the rotationally symmetric carboxylate group (Table 1, entries 4A, 4B). We also performed a

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Table 1. Incorporation of Solvent Oxygen into HIB under Single- and Multiple-Turnover Conditions

	enzyme (μM)	substrate (μM)	turnovers	% ^{18}O solvent	% ^{18}O product[a]
1	280.0	65.0	0.23	−	2.7
2	1.0	500.0	500.0	49.0	47.5
3A	350.0	65.0	0.19	45.8	1.9
3B	275.0	65.0	0.24	45.8	1.7
4A[b]	69.0	65.0	0.94	−	14.2
4B[b]	114.0	110.0	0.96	−	18.2
5A[c]	62.4	43.0	0.69	−	36.5
5B[c]	55.6	43.8	0.79	−	38.2

[a] Isotopic composition of the product was determined by chemical ionization mass spectrometry using a 70-VSE mass spectrometer with methane as the reagent gas at an ionization voltage of 70 eV. Incorporation of ^{18}O into the carboxylate group of the product was estimated by comparing the intensities of the molecular ion peaks at 105 $(M + H)^+$ and 107 amu. [b] ^{18}O-labeled enzyme derived from single-turnover reaction in 44% $H_2{}^{18}O$. [c] ^{18}O-labeled enzyme derived from multiple-turnover reaction in 47.5% $H_2{}^{18}O$.

multiple-turnover experiment in $H_2{}^{18}O$, isolated the labeled enzyme, and performed a single-turnover experiment in $H_2{}^{16}O$; the isotopic enrichment of the product was ~80% of the solvent (Table 1, entries 5A, 5B).[9] We attribute the less than quantitative incorporation of ^{18}O in both of these experiments to a modest exchange of the enzyme carboxylate group oxygens during the isolation of the labeled enzyme. Taken together, these results point to an anhydride mechanism in which an active-site carboxylate is the initial nucleophile and the anhydride intermediate is hydrolyzed by attack of water on the carbonyl carbon.

The sequences of orthologous HICH's contain two conserved acidic groups expected to be in the active site: a homologue of Glu164 in crotonase (Glu143) and a homologue of Asp145 in 4-chlorobenzoyl dehalogenase (4-CBD; Asp151). In the crotonase-catalyzed reaction, Glu164 is proximal to the thioester carboxyl carbon and mediates abstraction of the α-proton; in the 4-CBD-catalyzed reaction, Asp145 is the nucleophile that attacks C4 of the distal aromatic ring of the substrate to form a covalent Meisenheimer complex.[10,11] Mutants of Glu143 and Asp151 were constructed and assayed. The E143D, E143Q, and E143A mutants had no detectable activity ($k_{cat} \leq 2.8 \times 10^{-4}$ s^{-1}, $\leq 9.2 \times 10^{-4}$ s^{-1}, and $\leq 3.5 \times 10^{-5}$ s^{-1}, respectively), but the D151N mutant retained significant activity ($k_{cat} = 1$ s^{-1}; $k_{cat}/K_m = 1.8 \times 10^4$ M^{-1} s^{-1}). The importance of the oxyanion hole, formed by Gly68 and Gly120, is confirmed by the lack of detectable activity for the G120P mutant ($k_{cat} \leq 2.9 \times 10^{-5}$ s^{-1}).[12] Although a structure is not available for HICH, Glu143 is expected to be spatially proximal to the carboxyl carbon of the thioester substrate and, based on the observations described in this communication, the nucleophile in the hydrolysis reaction.

The mechanism of the HICH-catalyzed reaction can be compared with that of the reaction catalyzed by the Clp protease, a highly divergent member of the crotonase superfamily. The latter reaction employs a Ser-His-Asp catalytic triad and involves tetrahedral intermediates likely stabilized by the conserved oxyanion hole.[13] Therefore, the HICH-catalyzed hydrolysis reaction is particularly noteworthy because the mechanism utilizes as a nucleophile a conserved Glu that is a homologue of the general basic catalysts in many other reactions in the crotonase superfamily.

The nucleophilic mechanism for the HICH-catalyzed reaction is in contrast to those established for other members of the crotonase superfamily but demonstrates that stabilization of an anionic intermediate by a conserved oxyanion hole, either a thioester enolate anion intermediate or a thioester tetrahedral intermediate as in the HICH-catalyzed reaction, guides the pathway for divergence of function in the crotonase superfamily.

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(6) Assays (1 mL at 25 °C) contained one of the three substrates, 20 mM phosphate buffer, pH 7.9 and 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The increase in absorbance at 412 nm due to the reduction of DTNB was monitored. The kinetic parameters were determined by varying the concentrations of each of the three substrates.
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(8) Samples for a single-turnover experiment were prepared by concentrating HICH in a Millipore Ultrafree-0.5 centrifugal filter to 200 μL (>250 μM enzyme). The enzyme was then diluted with an equal volume of $H_2{}^{18}O$ (97.9% ^{18}O) and concentrated to 200 μL to achieve ~50% ^{18}O enrichment. Small aliquots were withdrawn for quantitation. Sample reactions (200 μL) contained ^{18}O-labeled HICH, 1 mM Tris-HCl, pH 7.9, and HIB-CoA. After incubation at room temperature for 20 min, the reaction was stopped with 3 M HCl (pH ≈ 1), and HICH was removed using a Millipore Ultrafree-0.5 centrifugal filter. The flow-through was saturated with ammonium sulfate and extracted with 3 × 200 μL of THF. The THF extracts were combined and concentrated to ca. 10 μL for mass spectroscopic analysis.
(9) The labeling of HICH with ^{18}O was performed by carrying out a single- or multiple-turnover reaction in $H_2{}^{18}O$ as described above. Upon completion, the reaction was concentrated to 125 μL using a Millipore Ultrafree-0.5 centrifugal filter. The remaining enzyme was diluted with 400 μL $H_2{}^{16}O$ and concentrated to 200 μL. The process was repeated three times to ensure that the solvent was >90% enriched in ^{16}O. The absorbance at 260 nm of the flow-through was measured to ensure that neither coenzyme A nor the free acid remained in the enzyme solution. After concentration to 200 μL, the enzyme was quantitated and used to conduct a single-turnover reaction in $H_2{}^{16}O$ as described above.
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