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Investigation of the Catalytic Mechanism of the Hotdog-Fold Enzyme Superfamily *Pseudomonas* sp. Strain CBS3 4-Hydroxybenzoyl-CoA Thioesterase

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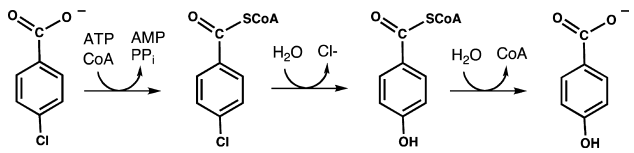
ABSTRACT: The 4-hydroxybenzoyl-CoA (4-HB-CoA) thioesterase from *Pseudomonas* sp. strain CBS3 catalyzes the final step of the 4-chlorobenzoate degradation pathway, which is the hydrolysis of 4-HB-CoA to coenzyme A (CoA) and 4-hydroxybenzoate (4-HB). In previous work, X-ray structural analysis of the substrate-bound thioesterase provided evidence of the role of an active site Asp17 in nucleophilic catalysis [Thoden, J. B., Holden, H. M., Zhuang, Z., and Dunaway-Mariano, D. (2002) X-ray crystallographic analyses of inhibitor and substrate complexes of wild-type and mutant 4-hydroxybenzoyl-CoA thioesterase. *J. Biol. Chem. 277*, 27468−27476]. In the study presented here, kinetic techniques were used to test the catalytic mechanism that was suggested by the X-ray structural data. The time course for the multiple-turnover reaction of 50 μM [^{14}C]-4-HB-CoA catalyzed by 10 μM thioesterase supported a two-step pathway in which the second step is rate-limiting. Steady-state product inhibition studies revealed that binding of CoA (K_{is} = 250 ± 70 μM; K_{ii} = 900 ± 300 μM) and 4-HB (K_{is} = 1.2 ± 0.2 mM) is weak, suggesting that product release is not rate-limiting. A substantial D_2O solvent kinetic isotope effect (3.8) on the steady-state k_{cat} value (18 s^{-1}) provided evidence that a chemical step involving proton transfer is the rate-limiting step. Taken together, the kinetic results support a two-chemical pathway. The microscopic rate constants governing the formation and consumption of the putative aspartyl 17-(4-hydroxybenzoyl)anhydride intermediate were determined by simulation-based fitting of a kinetic model to time courses for the substrate binding reaction (5.0 μM 4-HB-CoA and 0.54 μM thioesterase), single-turnover reaction (5 μM [^{14}C]-4-HB-CoA catalyzed by 50 μM thioesterase), steady-state reaction (5.2 μM 4-HB-CoA catalyzed by 0.003 μM thioesterase), and transient-state multiple-turnover reaction (50 μM [^{14}C]-4-HB-CoA catalyzed by 10 μM thioesterase). Together with the results obtained from solvent ^{18}O labeling experiments, the findings are interpreted as evidence of the formation of an aspartyl 17-(4-hydroxybenzoyl)anhydride intermediate that undergoes rate-limiting hydrolytic cleavage at the hydroxybenzoyl carbonyl carbon atom.

Cellular carboxylic acids are activated for acyl transfers, Michael additions, and Claisen condensations by conversion to the corresponding thioester. The thiol unit is typically coenzyme A (CoA) or an acyl carrier protein (ACP) modified at a serine residue with a pantetheine phosphate unit. Following the conversion of the thioester, the CoA or holo-ACP is released via hydrolysis, a reaction that is catalyzed by a thioesterase. The majority of these thioesterases belong to the hotdog-fold enzyme superfamily.[1] The 4-hydroxybenzoyl-CoA (4-HB-CoA) thioesterase from *Pseudomonas* sp. strain CBS3 was the first hotdog-fold thioesterase to be characterized.[2] This particular thioesterase is used in the three-step pathway that converts 4-chlorobenzoate to the metabolite 4-hydroxybenzoate (4-HB) (Scheme 1).[3]

The X-ray structure of the apo *Pseudomonas* 4-HB-CoA thioesterase revealed a homotetrameric structure comprised of a dimer of dimers.[4] The subunits consist of a five-stranded antiparallel sheet that wraps an elongated α-helix. The ensuing atomic-resolution X-ray structure determinations of the tetrameric thioesterase, bound with substrate (4-HB-CoA) or a substrate analogue (4-hydroxybenzyl-CoA or 4-hydroxyphenacyl-CoA (4-HP-CoA)) [Protein Data Bank (PDB) entries 1LO7, 1LO8, and 1LO9], showed that the active sites are located at the subunit−subunit interfaces (Figure 1A).[5] The structure of the active site suggested a unique catalytic mechanism for thioester hydrolysis, as well as a strict order for product release.

Scheme 1. Three Chemical Steps of the 4-Chlorobenzoate Degradation Pathway Catalyzed by 4-Chlorobenzoate (CoA ligase, 4-chlorobenzoyl-CoA dehalogenase, and 4-hydrobenzoyl-CoA thioesterase, respectively)



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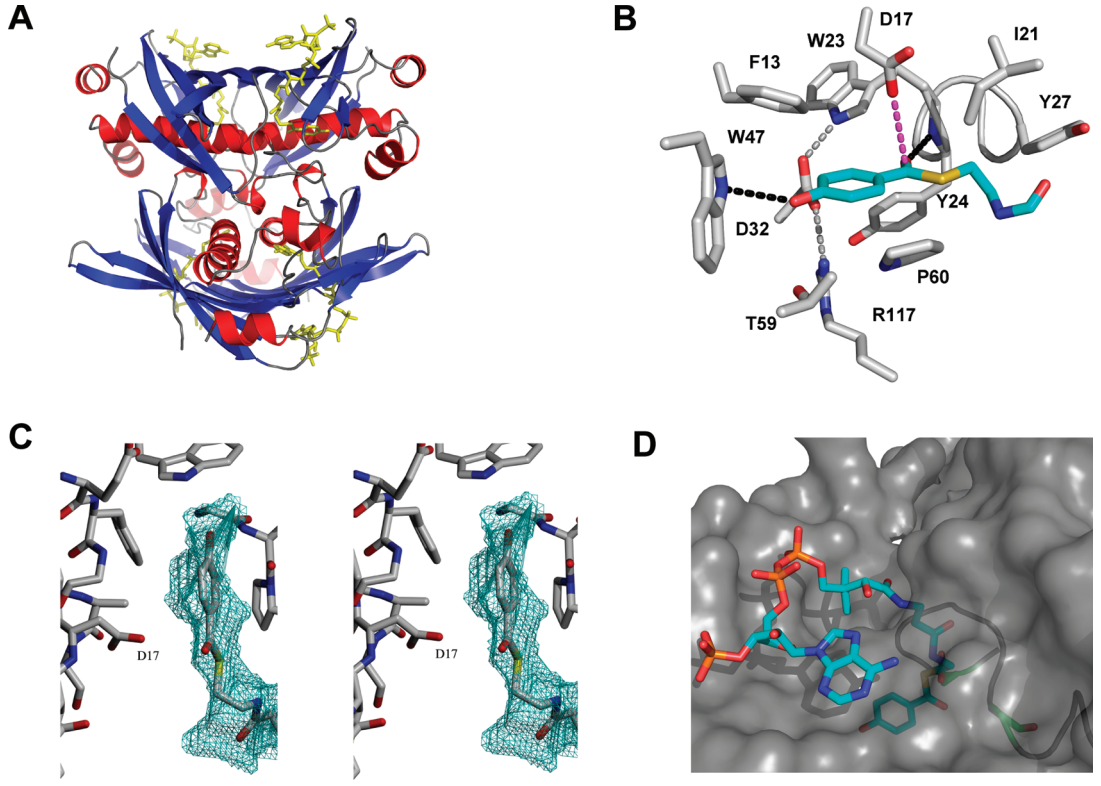

Figure 1. Structure of D17N *Pseudomonas* sp. strain CBS3 4-HB-CoA thioesterase bound with 4-HB-CoA (PDB entry 1LO9). (A) The tetramer shown as a Pymol cartoon with the 4-HB-CoA ligands shown as yellow sticks. (B) Active site structure wherein the 4-HB-CoA has been truncated for better viewing and Asn17 has been mutated in silico to Asp. Hydrogen bonds are represented as gray or black dashed lines. The distance of 3.7 Å between the Asp17 carboxylate group oxygen atom and the 4-HB-CoA ring carbonyl carbon atom is represented by the magenta dashed line. The nitrogen atoms are colored blue and the oxygen atoms red. The sulfur atom is colored yellow, and the enzyme carbons atoms are colored gray and the 4-HB-CoA carbon atoms cyan. (C) Stereoview of the active site solvent cage generated from the empty active site cavity produced in VOIDOO (teal-colored mesh). The 4-HB-CoA ligand fills the cage. Carbon atoms are colored gray, oxygen atoms red, nitrogen atoms blue, and sulfur atoms yellow. (D) Surface representation of the thioesterase substrate-binding site. The 4-HB-CoA ligand is shown as sticks with carbon atoms colored cyan. The Asp17 side chain and Tyr24 amide unit are shown as sticks with carbon atoms colored green.

The catalytic scaffold, which is quite minimal, consists of the N-terminal region of the elongated α-helix and its connecting loop (Figure 1B). The loop Asp17 carboxylate oxygen atom (3.7 Å from the thioester carbon atom of the C═O bond) is optimally positioned for nucleophilic addition to the substrate. Notably, there is no room for a water molecule to bind between the Asp17 carboxylate group and thioester moiety (Figure 1C). The amide NH group of the α-helix N-terminal residue Tyr24 is within hydrogen bonding distance (3.2 Å) of the thioester oxygen atom of the C═O bond (Figure 1B). Also, the positive pole of the α-helix macrodipole is directed at the thioester group. Raman spectral analysis has shown that these electrostatic interactions orient and polarize the thioester C═O bond for nucleophilic attack.[6]

There are no electropositive side chains located near the substrate thioester sulfur atom. Thus, the CoA leaving group appears to be displaced by the Asp17 as a "naked" thiolate anion, stabilized only by the electrostatic field emanating from the α-helix macrodipole. Notably, the catalytic site is devoid of a basic amino acid side chain for performing the role of a base in the activation of the water molecule for hydrolysis of the anhydride intermediate. It is conceivable that the CoA thiolate serves this function, and once protonated, it dissociates from its binding site allowing the exit of the 4-HB, which is otherwise trapped at the bottom of the active site (Figure 1D).

The kinetic studies reported herein were conducted to test and explore the catalytic mechanism of thioesterase-catalyzed 4-HB-CoA hydrolysis suggested by the structural data and to define the microscopic rate constants that govern the kinetic mechanism. Below we report and interpret the results from steady-state and transient-state kinetic analyses, solvent kinetic isotope effect measurements, and solvent ^{18}O labeling experiments.

MATERIALS AND METHODS

Materials. Recombinant wild-type *Pseudomonas* sp. strain CBS3 4-HB-CoA thioesterase was prepared from the *Escherichia coli* clone as previously described.[7] 4-HP-CoA and 4-HB-CoA were prepared according to published procedures.[8] 4-Methoxybenzoyl-CoA was synthesized from the corresponding benzoyl chloride derivatives and the CoA lithium salt according to the procedures described by Liang et al.[9] [^{14}C]-4-HB-CoA was prepared as previously described.[10]

Determination of Steady-State Kinetic Constants from Initial Velocity Data. 4-HB-CoA thioesterase-catalyzed hydrolysis of 4-HB-CoA ($\Delta\varepsilon$ = 11.8 mM^{-1} cm^{-1}) was monitored using a direct spectrophotometric assay in which the decrease in absorbance at 300 nm associated with the hydrolysis of the thioester substituent is monitored. Reaction solutions initially contained thioesterase, 4-HB-CoA, KCl (0.2 M), and 50 mM K^+HEPES (pH 7.5, 25 °C). Initial velocity data

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were measured as a function of substrate concentration (K_m to $10K_m$) and were fitted to eq 1 using KinetAsyst (IntelliKinetics) to determine the V_{max} and K_m values. The k_{cat} was calculated from $V_{max}/[E]$, where [E] is the total enzyme active site concentration (determined using the Bradford method[11]).

$$V = V_{max}[S]/([S] + K_m) \quad (1)$$

where V is the initial velocity, V_{max} the maximal velocity, [S] the substrate concentration, and K_m the Michaelis constant.

Initial velocity data were measured in the absence or presence (K_i, $2K_i$, and $3K_i$) of 4-HB or CoA and were fitted to eq 2 (to define the competitive inhibition constant K_{is}) or eq 3 (to define the kinetic constants for noncompetitive inhibition, K_{ii} and K_{is}), respectively.

$$V = V_{max}[S]/[K_m(1 + [I]/K_{is}) + [S]] \quad (2)$$

$$V = V_{max}[S](1 + [I]/K_{ii})/[K_m(1 + [I]/K_{is}) + [S]] \quad (3)$$

where [I] is the inhibitor concentration, K_{is} the slope inhibition constant, and K_{ii} the intercept inhibition constant.

Steady-State Multiple-Turnover Time Courses. The full time courses for 0.003 μM thioesterase-catalyzed 4-HB-CoA hydrolysis of 4-HB-CoA (1.4, 3.5, and 5.2 μM) in 50 mM K^+HEPES (pH 7.5, 25 °C) were monitored at 300 nm ($\Delta\varepsilon$ = 11.8 mM^{-1} cm^{-1}). To determine the k_{cat} and K_m values, the data were fitted by curve simulation using the kinetic model

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

and the definitions $k_{cat} = k_2$ and $K_m = (k_{-1} + k_2)/k_1$.

The KinTek Corp. Global Kinetic Explorer (http://www.kintek-corp.com/KGExplorer/index.php) was used to generate the simulations.[12]

Determination of the Solvent Kinetic Isotope Effect on k_{cat}. The pH−rate profiles for thioesterase-catalyzed hydrolysis of 4-HB-CoA in H_2O and D_2O solvent were measured using a dual-buffer system consisting of 50 mM acetate and 50 mM MES (pH 4.0−5.6), 50 mM MES and 50 mM HEPES (pH 5.6−8.0), 50 mM HEPES and 50 mM TAPS (pH 8.0−9.1), or 50 mM TAPS and 50 mM CAPSO (pH 9.1−9.6). The enzyme activity at overlapping pH values was measured to ensure equal enzyme activity in the different buffer systems. The stability of the thioesterase at the acidic and basic pH values was verified by conducting preincubation tests. The k_{cat}−pH profile was fitted using eq 4.

$$\log Y = \log[C(1 + H/K_{a1})/(1 + H/K_{a2})] \quad (4)$$

where Y is the steady-state kinetic constant k_{cat}, H is the proton concentration of the reaction solution, K_1 and K_2 are the apparent ionization constants and C is the constant value of Y where it does not change with solution pH.

Determination of Rapid Quench Transient Kinetics. A rapid-quench instrument from KinTek Instruments was used to combine buffered wild-type thioesterase with buffered [^{14}C]-4-HB-CoA to initiate the reaction and then to add 0.2 M HCl to terminate the reaction. For the multiple-turnover reactions, mixtures initially contained 10 μM enzyme and 50 μM [^{14}C]-4-HB-CoA in a 0.15 M KCl/50 mM K^+HEPES mixture (pH 7.5, 25 °C), whereas for the single-turnover reactions, the concentrations of enzyme and [^{14}C]-4-HB-CoA were 50 and 5 μM, respectively. The enzyme was precipitated from the quenched reaction solution by vigorous mixing with 200 μL of CCl_4 using a Vortex mixer and then pelleted by (micro)centrifugation of the mixture (14000 rpm for 1 min). The water phase was injected into a high-performance liquid chromatography (HPLC) system for the separation of [^{14}C]-4-HB-CoA and [^{14}C]-4-HB as described previously.[13] The eluent was collected using a fraction collector and analyzed by liquid scintillation counting. Control reactions (lacking enzyme) were conducted in parallel to establish the background radioactivity associated with the [^{14}C]-4-HB fraction. The background (~2%) was taken into account when calculating the concentrations of substrate and product remaining in each quenched reaction mixture. The amounts of radioactivity [in counts per minute (cpm)] contained in the [^{14}C]-4-HB-CoA and [^{14}C]-4-HB HPLC fractions were used in conjunction with the specific activity (cpm per micromole) of the substrate to calculate the concentration of substrate remaining and product formed in the reaction mixture for each time point. The time courses for [^{14}C]-4-HB-CoA consumption and [^{14}C]-4-HB formation were plotted and then fitted by simulation using the KinTek Corp. Global Kinetic Explorer. The initial estimate of an apparent rate constant governing a single turnover of substrate was determined by fitting the single-turnover time course data to eq 5.

$$\ln[S] = -kt + \ln[S]_o \quad (5)$$

where [S] is the substrate concentration at time t, $[S]_o$ the initial substrate concentration, and k the apparent first-order rate constant.

Binding of 4-HB-CoA and 4-HP-CoA to Wild-Type Thioesterase Measured by Stopped-Flow Fluorescence Spectroscopy. Kinetic experiments were performed with an Applied Photophysics DX.17MV sequential stopped-flow spectrophotometer (optical path length of 1 cm, mixing time of ~3 ms) fitted with a 340 nm cutoff filter. The excitation wavelength was 280 nm. The measurement of the time-dependent quenching of the fluorescence of the wild-type thioesterase (0.54 μM) by 4-HP-CoA (5.3 μM) or 4-HB-CoA (5.0 μM) was conducted under pseudo-first-order conditions. The observed pseudo-first-order rate constant, k_{obs}, was obtained by fitting the data to eq 6 using the software package provided by Applied Photophysics.

$$\ln(F_0 - F_t)/(F_0 - F_\infty) = -k_{obs}t \quad (6)$$

where F_t is the fluorescence at time t, F_0 the initial fluorescence, F_∞ the final fluorescence, and k_{obs} the observed first-order rate constant.

To measure the association rate constant (k_{on}) and dissociation rate constant (k_{off}) of binding of 4-HP-CoA or 4-HB-CoA to wild-type thioesterase, time courses for fluorescence quenching were measured as a function of ligand concentration at a fixed enzyme concentration. The resulting k_{obs} values were plotted against the ligand concentrations ([L]), and the data were fit to eq 7 to define the k_{on} and k_{off} values.

$$k_{obs} = k_{on}[L] + k_{off} \quad (7)$$

Incorporation of ^{18}O into 4-Hydroxybenzoate during Wild-Type Thioesterase-Catalyzed Hydrolysis in ^{18}O-Enriched Water. A 320 μL solution of 1.9 mM wild-type thioesterase in 10 mM K^+HEPES (pH 7.5) containing 0.2 M KCl and 1 mM DTT was lyophilized. To the resulting powder was added 230 μL of 95% $H_2{}^{18}O$ (Aldrich). After a 30 min incubation at 25 °C, the enzyme solution in $H_2{}^{18}O$ buffer was assayed to ensure that catalytic activity was retained. Next,

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20 μL of $H_2{}^{18}O$ was added to a sample of 4-HB-CoA powder obtained by lyophilization of 20 μL of 3.0 mM 4-HB-CoA in natural isotopic abundance water. A 5 μL aliquot of the 4-HB-CoA in $H_2{}^{18}O$ solution was mixed with the enzyme solution at room temperature. After ~1 min, a second 5 μL aliquot was added. This was repeated twice more. The final enzyme concentration in the reaction solution was 2.4 mM, whereas the final substrate concentration was 0.24 mM ([E]:[S] = 10:1). After incubation at room temperature for 15 min, the reaction solution was filtered using a 10 kDa micro separation filter (Pall Filtron). The filtrate was acidified by addition of 5 μL of 6 N HCl and extracted four times with 1 mL portions of ethyl acetate. The ethyl acetate extracts were combined and dried over anhydrous sodium sulfate, and the solvent was evaporated in vacuo. The solid was dissolved in 100 μL of absolute methanol and subjected to gas chromatography−mass spectrometry (GC−MS) analysis using a Saturn GC−MS spectrometer equipped with a Restek XTI-5 column.

RESULTS AND DISCUSSION

Detection of the Anhydride Intermediate. *Experimental Design.* Enzyme covalent intermediates can usually be captured by using a fast quench technique or a steady-state chemical trap.[14,15] However, in view of the intrinsic chemical reactivity of anhydrides, it was not surprising that our attempts to isolate the putative aspartyl 17-(4-hydroxybenzoyl)anhydride intermediate by using ^{14}C-labeled 4-HB-CoA, in conjunction with rapid quench techniques and enzyme separation, failed. An attempt to trap the anhydride intermediate with the nucleophilic reagent hydroxylamine was also unsuccessful.

Stopped-flow spectroscopy provides a noninvasive method for observing enzyme intermediates provided that their spectral properties are easily distinguished from those of the bound substrate and product. Although stopped-flow UV absorbance proved to be an effective method for monitoring the consumption of 4-HB-CoA (at 300 nm, $\Delta\varepsilon$ = 11.8 mM^{-1} cm^{-1}), the signal from the anhydride intermediate was found to be obscured by those emanating from the substrate and product complexes. Ultimately, we relied on the accurate measurement of radiolabeled substrate and product to reveal, via kinetic analyses, the anhydride intermediate. On the basis of this strategy, the kinetic experiments reported below were designed and executed.

Kinetic "Burst" Experiment. The time course for the multiple turnovers of [^{14}C]-4-HB-CoA (50 μM) catalyzed by wild-type thioesterase (10 μM) was measured using rapid (acid) quench techniques coupled with HPLC separation and quantitation of the [^{14}C]-4-HB-CoA and [^{14}C]-4-HB present in the quenched reaction mixtures (Figure 2). The acid quench denatures the enzyme, thereby releasing the bound substrate and product. The anhydride is hydrolyzed to form [^{14}C]-4-HB, which contributes to the "product pool" of [^{14}C]-4-HB {i.e., $[4\text{-HB}]_{observed}$ = [enzyme anhydride intermediate] + [enzyme-(CoA)(4-HB)] + $[4\text{-HB}]_{free}$}. If the anhydride intermediate is formed at a rate that exceeds the rate of its hydrolysis, the first catalytic turnover of substrate will be observed to be faster than the subsequent turnovers. Indeed, the measured time course for the first few catalytic turnovers shows an initial "burst phase" of [^{14}C]-4-HB-CoA consumption, which roughly corresponds to one-half of the active sites initially present in the reaction solution. The burst phase is followed by a slower, linear phase of substrate consumption that takes place during the subsequent catalytic turnovers (Figure 2).

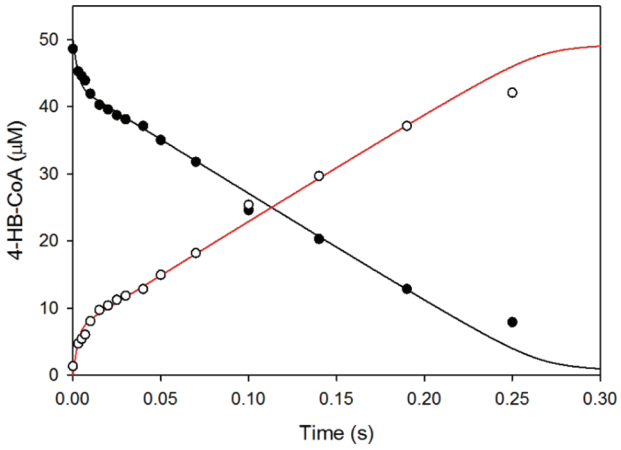

Figure 2. Plot of the experimental and simulated time courses for the transient-state multiple-turnover reaction of 10 μM wild-type thioesterase and 50 μM [^{14}C]-4-HB-CoA in 50 mM K^+HEPES (pH 7.5, 25 °C). The time point data [[^{14}C]-4-HB-CoA (●) and [^{14}C]-4-HB (○)] were calculated from the fraction of [^{14}C]-4-HB-CoA or [^{14}C]-4-HB present in the acid-quenched reaction mixture multiplied by the initial concentration of [^{14}C]-4-HB-CoA present in the reaction mixture. The simulated curves were generated using the kinetic model shown in Scheme 2 and the following set of rate constants: k_1 = 71 μM^{-1} s^{-1}, k_{-1} = 12 s^{-1}, k_2 = 290 s^{-1}, k_{-2} = 67 s^{-1}, k_3 = 23 s^{-1}, k_{-3} = 0 s^{-1}, k_4/k_{-4} = 300 μM, and k_5/k_{-5} = 1200 μM. The simulated curve coloring scheme is as follows: red for E-I(CoA) + E(4-HB)(CoA) + E(4-HB) + 4-HB and black for E(4-HB-CoA) + 4-HB-CoA.

Although the observed time course is consistent with the intermediacy of the anhydride intermediate as proposed, so too is a single-step reaction pathway that proceeds with rate-limiting CoA and/or 4-HB product release. Consequently, further experimentation was undertaken to distinguish between rate-limiting anhydride intermediate hydrolysis and rate-limiting product release. To this end, the D_2O solvent kinetic isotope effect (SKIE) on k_{cat} was measured. Product release is typically isotope insensitive, whereas a rate-limiting hydrolysis step is expected to give rise to a significant SKIE. Accordingly, the k_{cat}−pH profile (Figure 3) was measured in D_2O solvent as well as in H_2O solvent so that the SKIE could be accurately determined. The pH profile shows a double wave, indicative of slowly exchanging substrate and proton.[16] The kinetic data were fitted to eq 4 to define a break at 5.15 ± 0.09 and a C_1 of 5.0 ± 0.1 and a break at 7.70 ± 0.08 and a C_2 of 55 ± 4 for catalysis in buffered H_2O, and a break at 5.1 ± 0.1 and a C_1 of 1.3 ± 0.2 and a break at 8.0 ± 0.1 and a C_2 of 15 ± 1 for catalysis in buffered D_2O. The pH-independent isotope effect was calculated from the ratio of the C_1 or C_2 value to be 3.8 or 3.7, respectively.

Together, the substantial SKIE of 3.7−3.8 and the observed burst-phase kinetic time course (Figure 2) constitute strong evidence of the proposed two-step chemical pathway. Our next step was to define the microscopic rate constants governing thioesterase catalysis by fitting these and additional kinetic data to a kinetic model representing a two-step chemical pathway.

Substrate Binding and Product Release. The X-ray structure of the D17N thioesterase(4-HB-CoA) complex reveals a "wormhole"-like active site in which the CoA pantetheine arm fills the tunnel leading to a tight pocket that accommodates the 4-hydroxybenzoyl unit, while the CoA nucleotide unit sits at the entrance to this tunnel (Figure 1D).[5] In earlier work, we

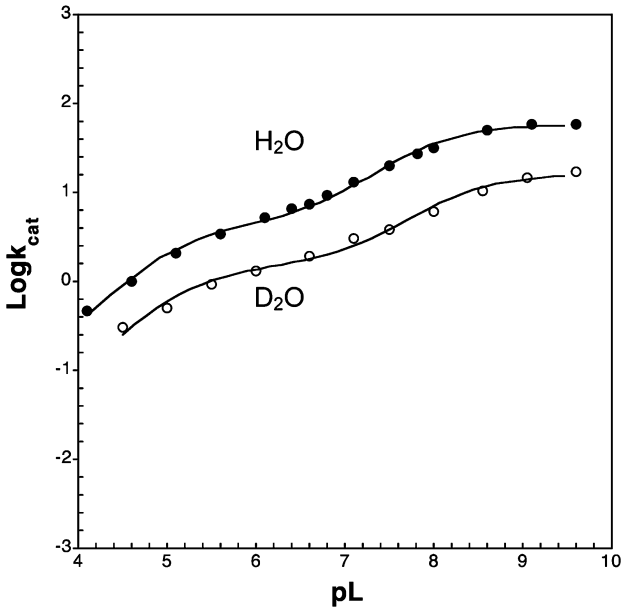

Figure 3. Plot of log k_{cat} vs reaction solution pH measured for wild *Pseudomonas* sp. strain CBS3 4-HB-CoA thioesterase-catalyzed hydrolysis of 4-HB-CoA in H_2O solvent (●) or D_2O solvent (○). The pL represents the pH value measured for the H_2O reaction solution or the pD value measured for the D_2O solution (= pH reading + 0.4).

measured the K_d values for the wild-type thioesterase(4-hydroxyphenacyl-CoA) inhibitor complex and the inert D17N thioesterase(4-HB-CoA) complex to be 0.33 and 0.14 μM, respectively, by using steady-state fluorescence quenching techniques.[5] In the present study, stopped-flow fluorescence quenching techniques were used to measure the corresponding binding rates. In Figure 4A, the time course for the binding reaction of 5.3 μM 4-hydroxyphenacyl-CoA with 0.54 μM thioesterase is shown, whereas Figure 4B shows the time course for the binding reaction of 5.0 μM 4-HB-CoA with 0.54 μM thioesterase. The values of the respective apparent pseudo-first-order rate constants (k_{obs} = 350 ± 4 and 280 ± 30 s^{-1}) were obtained by data fitting (eq 6). The plots of k_{obs} versus ligand concentration, shown as insets of panels A and B of Figure 4, defined a linear correlation. The association (k_{on}) and dissociation (k_{off}) rate constants were determined by fitting the data to eq 7. For 4-hydroxyphenacyl-CoA, k_{on} = 59 ± 1 μM^{-1} s^{-1} and k_{off} = 20 ± 5 s^{-1}, which define the microscopic rate constants for substrate binding and dissociation, respectively. Indeed, the k_{off}/k_{on} ratio of 0.33 μM is in perfect agreement with the independently measured K_d of 0.33 μM.[5] For 4-HB-CoA, we note that only if the substrate binding step is in rapid equilibrium do the k_{on} (65 ± 2 μM^{-1} s^{-1}) and k_{off} (13 ± 5 s^{-1}) values accurately define the microscopic rate constants for substrate binding and dissociation, respectively. The k_{off}/k_{on} ratio of 0.20 μM falls halfway between the K_m for 4-HB-CoA of 0.26 μM and the independently measured K_d of 0.14 μM.[5]

The quantitative analysis of product binding was limited (for technical reasons) to the determination of the steady-state inhibition constants for thioesterase-catalyzed hydrolysis of 4-HB-CoA. CoA proved to be a weak noncompetitive inhibitor (K_{is} = 250 ± 70 μM; K_{ii} = 900 ± 300 μM) and 4-HB a weak competitive inhibitor (K_{is} = 1.2 ± 0.2 mM). The competitive inhibition observed for 4-HB (binds only to the free enzyme) and the noncompetitive inhibition [binds to the free enzyme (slope effect) and to the enzyme−anhydride intermediate and/or enzyme(4-HB) complex (intercept effect)] observed for CoA are consistent with CoA departure preceding that of 4-HB, as was predicted from the X-ray structure the D17N thioesterase(4-HB-CoA) complex.

Reaction Time Course Analysis. To define the microscopic rate constants for thioesterase catalysis, the measurement of time courses, which would supplement the transient-state, multiple-turnover time course (Figure 2) and the time course for substrate binding (Figure 4), was pursued. First, the time course for a single-turnover reaction was measured by using 50 μM wild-type thioesterase to catalyze the hydrolysis of 5 μM [^{14}C]-4-HB-CoA (Figure 5). Second, steady-state multiple-turnover time courses were measured

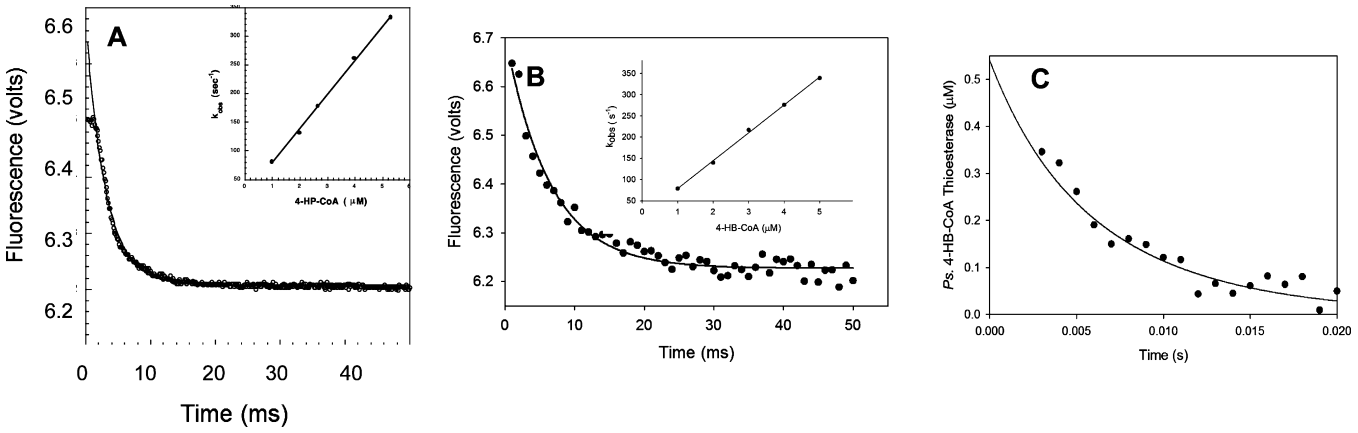

Figure 4. (A) Time-dependent fluorescence change associated with the binding reaction between 5.3 μM 4-HP-CoA and 0.54 μM thioesterase in 50 mM K^+HEPES (pH 7.5, 25 °C). The data were fit to eq 6 to define the observed rate constant k_{obs} as 350 ± 4 s^{-1}. The inset is a plot of k_{obs} vs 4-HP-CoA concentration (1.0−5.3 μM). The data were fit to eq 7 to define the association rate constant k_{on} as 59 ± 1 μM^{-1} s^{-1} and the dissociation rate constant k_{off} as 20 ± 5 s^{-1}. (B) Time-dependent fluorescence change associated with the binding reaction between 5.0 μM 4-HB-CoA and 0.54 μM thioesterase in 50 mM K^+HEPES (pH 7.5, 25 °C). The data were fit to eq 6 to define the observed rate constant k_{obs} as 280 ± 30 s^{-1}. The inset is a plot of k_{obs} vs 4-HB-CoA concentration (1.0−5.0 μM). The data were fit to eq 7 to define the association rate constant k_{on} as 65 ± 2 μM^{-1} s^{-1} and the dissociation rate constant k_{off} as 13 ± 5 s^{-1}. (C) A plot of the concentration of unbound thioesterase vs time for the time course for the binding reaction between 3.0 μM 4-HB-CoA and 0.54 μM thioesterase in 50 mM K^+HEPES (pH 7.5, 25 °C). The simulated curve was generated using the kinetic model shown in Scheme 2 and rate constants k_1 (70 μM^{-1} s^{-1}) and k_{-1} (15 s^{-1}).

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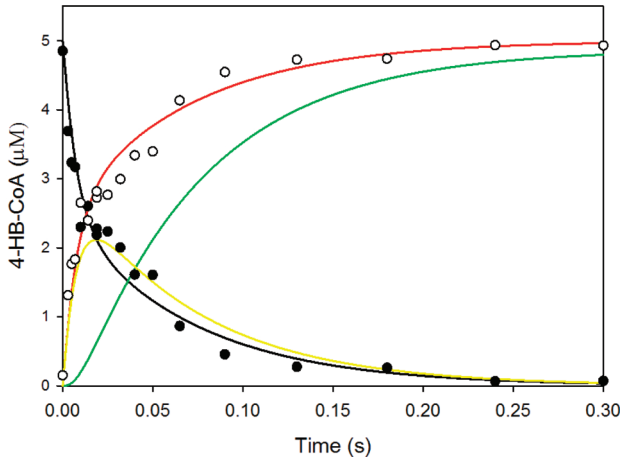

Figure 5. Experimental and simulated time courses for the single-turnover reaction of 50 μM wild-type thioesterase and 5 μM [^{14}C]-4-HB-CoA in 50 mM K$^+$HEPES (pH 7.5, 25 °C). The time point data [[^{14}C]-4-HB-CoA (●) and [^{14}C]-4-HB (○)] were calculated from the fraction of [^{14}C]-4-HB-CoA or [^{14}C]-4-HB present in the acid-quenched reaction mixture multiplied by the initial concentration of [^{14}C]-4-HB-CoA present in the reaction mixture. The simulated curves were generated using the kinetic model shown in Scheme 2 and the following set of rate constants: k_1 = 79 μM^{-1} s^{-1}, k_{-1} = 20 s^{-1}, k_2 = 76 s^{-1}, k_{-2} = 50 s^{-1}, k_3 = 26 s^{-1}, k_{-3} = 0 s^{-1}, k_4/k_{-4} = 300 μM, and k_5/k_{-5} = 1200 μM. The simulated curve coloring scheme is as follows: red for E-I(CoA) + E(4-HB)(CoA) + E(4-HB) + 4-HB, black for E(4-HB-CoA) + 4-HB-CoA, yellow for E-I(CoA), and green for E(4-HB)(CoA) + E(4-HB) + 4-HB.

using 1.4, 3.5, or 5.2 μM 4-HB-CoA and 0.003 μM thioesterase (Figure 6).

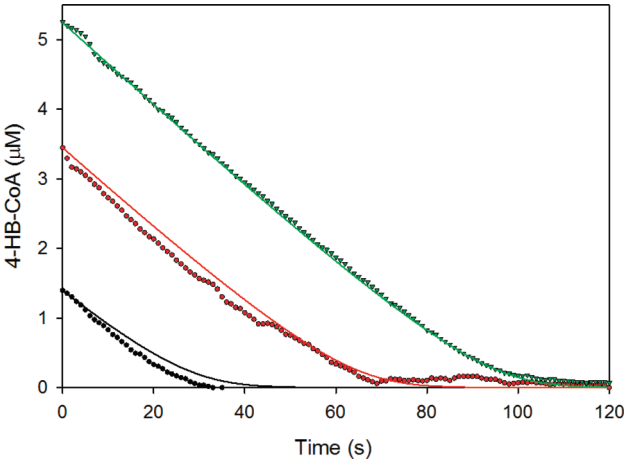

Figure 6. Time courses for the steady-state multiple-turnover reactions of 1.4 (black), 3.5 (red), or 5.2 μM 4-HB-CoA (green) catalyzed by 0.003 μM thioesterase in 50 mM K$^+$HEPES (pH 7.5, 25 °C). The 300 nm absorbance traces (dotted lines) are shown in the context of the calculated concentration of 4-HB-CoA (ε = 11.8 mM^{-1} cm^{-1}) present in the reaction mixture (y-axis) as a function of reaction time (x-axis). The simulated curves (solid lines) were generated using the kinetic model shown in Scheme 2 and the following rate constants: k_1 = 71 μM^{-1} s^{-1}, k_{-1} = 12 s^{-1}, k_2 = 290 s^{-1}, k_{-2} = 67 s^{-1}, k_3 = 23 s^{-1}, k_{-3} = 0 s^{-1}, k_4/k_{-4} = 300 μM, and k_5/k_{-5} = 1200 μM.

The simulation-based fitting of the four sets of time course data was conducted using the kinetic model shown in Scheme 2. This model includes the substrate binding step, the two chemical steps, and the two (ordered) product release steps. A more complex model in which CoA dissociates prior to the hydrolysis of the anhydride intermediate was also considered. However, the divergence observed in the sets of microscopic rate constants used to generate optimal fits to the respective time courses was comparatively large. For this reason, the model was not carried forward.

Scheme 2. Kinetic Model Representing 4-HB-CoA Thioesterase Catalysis[a]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI{\cdot}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E{\cdot}Q{\cdot}P \underset{k_{-4}\ (P)}{\overset{k_4}{\rightleftharpoons}} E{\cdot}Q \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + Q$$

[a]Abbreviations: E, thioesterase; EI, thioesterase−anhydride intermediate; S, 4-HB-CoA; P, CoA; Q, 4-HB.

To initiate the simulation process, estimates of the microscopic rate constants were obtained by conventional fitting of the individual data sets (see Materials and Methods). The values of the microscopic rate constants were varied individually, and in combination, until the simulated curve matched the experimental time course. The simulated curves are shown in Figures 2 and 4−6 along with the values of the microscopic rate constants used in the simulation. During the process of fitting the four time courses, we discovered, as one might anticipate, that there is some variation in the extent to which the allowed range of a given microscopic rate constant can be confined. For instance, the value of k_3 (governs the rate-limiting anhydride hydrolysis step) was inconsequential to the simulation of the substrate binding time course, whereas it could not deviate more than 10% without noticeably compromising the respective fits of the simulated curves to the other time courses. On the other hand, the values of the microscopic rate constants governing the product release steps had a limited impact on the simulation process.

Everything considered, the microscopic rate constants governing the substrate binding step and the two chemical steps are reasonably well-defined. We show in Figure 5, along with the simulated single-turnover time course data, the simulated curves representing the anhydride intermediate and the 4-HB product. At 25 ms, the level of the anhydride intermediate peaks at ~40% and then slowly declines. The slow onset of 4-HB formation (i.e., initial lag in the simulated time course colored green) is the reflection of the rate-limiting hydrolysis step. In the section that follows, we report the ^{18}O solvent labeling experiment that was used to determine the regiochemistry of hydrolysis of the anhydride intermediate.

Regiochemistry of the Hydrolysis of the Anhydride Intermediate. The attack of the water nucleophile at the anhydride intermediate formed in the first step of thioesterase catalysis can potentially occur at either the benzoyl carbonyl carbon or the aspartyl carbonyl carbon. To determine the regiochemistry of the hydrolysis step, single-turnover reactions were conducted in a 95% $H_2{}^{18}O$ solvent. If the Asp17 carbonyl carbon is attacked, the 4-HB product will not contain the solvent-derived ^{18}O atom in its carboxylate group but instead will contain the Asp17-derived ^{16}O atom. On the other hand, if the benzoyl carbonyl carbon is attacked, the 4-hydroxybenzoate product will contain the ^{18}O atom derived from solvent. The mass spectrum of the 4-hydroxybenzoic acid standard (see Figure 7A) contains the parent ion (M*) peak at m/z 138, the parent ion fragment (M* − OH) peak at m/z 121 (note that the OH group lost is from the carboxylic acid group, not the aromatic ring), the (M* − COOH) fragment at m/z 93, and, finally, a fragment at m/z 65 (not assigned). The parent ion (M*) and parent ion fragment

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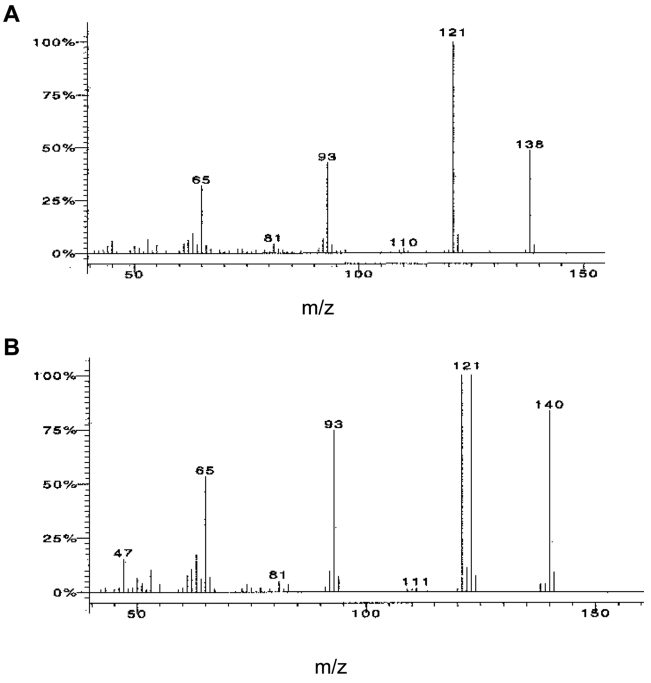

Figure 7. GC–MS analysis of the 4-HB product generated in the single-turnover reaction of 4-HB-CoA (0.24 mM) catalyzed by the wild-type thioesterase (2.4 mM) in 95% $H_2{}^{18}O$ containing 50 mM K^+HEPES (pH 7.5, 25 °C) (A) GC–MS spectrum of the 4-hydroxybenzoic acid standard. (B) GC–MS spectrum of the 4-hydroxybenzoic acid isolated from the quenched reaction mixture.

(M* − OH) contain oxygen from the carboxylic acid group and can thus be used to measure the level of incorporation of ^{18}O.

The mass spectrum of the 4-hydroxybenzoic acid isolated from the single-turnover reaction of 4-HB-CoA (0.24 mM) catalyzed by the wild-type thioesterase (2.4 mM) in 95% $H_2{}^{18}O$ revealed that >90% of the parent ion peak occurs at m/z 140 (see Figure 7B), which means that the vast majority of the 4-hydroxybenzoic acid is labeled at the carboxylic acid group with one ^{18}O atom and one ^{16}O atom. The equal probability of losing either carboxylic acid oxygen atom in the parent ion resulted in an equal amount of (M* − OH) species at m/z 121 and 123. These findings demonstrate that hydrolysis occurs at the benzoyl carbonyl carbon of the Asp17-(4-hydroxybenzoyl)anhydride intermediate.

CONCLUSIONS

In Figure 8 we propose a reaction mechanism for 4-HB-CoA catalysis, which integrates the findings from earlier investigations[5,6] with those presented here. The reaction begins with very tight substrate binding. The catalytic site is located at the end of a narrow tunnel that leads from the solvent to an enclosed, largely hydrophobic pocket (Figure 1D). The conformation of the thioesterase, and the substrate-binding site in particular, is unchanged by substrate binding. Thus, a "lock-in-key" model of substrate binding is applicable. The substrate binding rate ($k_1 \sim 70\ \mu M^{-1}\ s^{-1}$) is about average; however, the dissociation rate is slow ($k_{-1} \sim 15\ s^{-1}$), and therefore, the K_d of 0.14 μM is quite small. The substrate 4-hydroxybenzoyl unit is immobilized by tight packing with the surrounding side chains, several of which are aromatic. The formation of a single hydrogen bond with the benzoyl ring hydroxyl group involves the indole NH group of Trp47 (Figure 1B).

At the catalytic site, the substrate thioester C═O group is oriented and activated for nucleophilic attack by the Asp17 carboxylate group. The range for a pre-transition-state conformation (i.e., "near-attack conformation") of a carboxylate oxygen anion and the carbonyl carbon electrophile, posited by

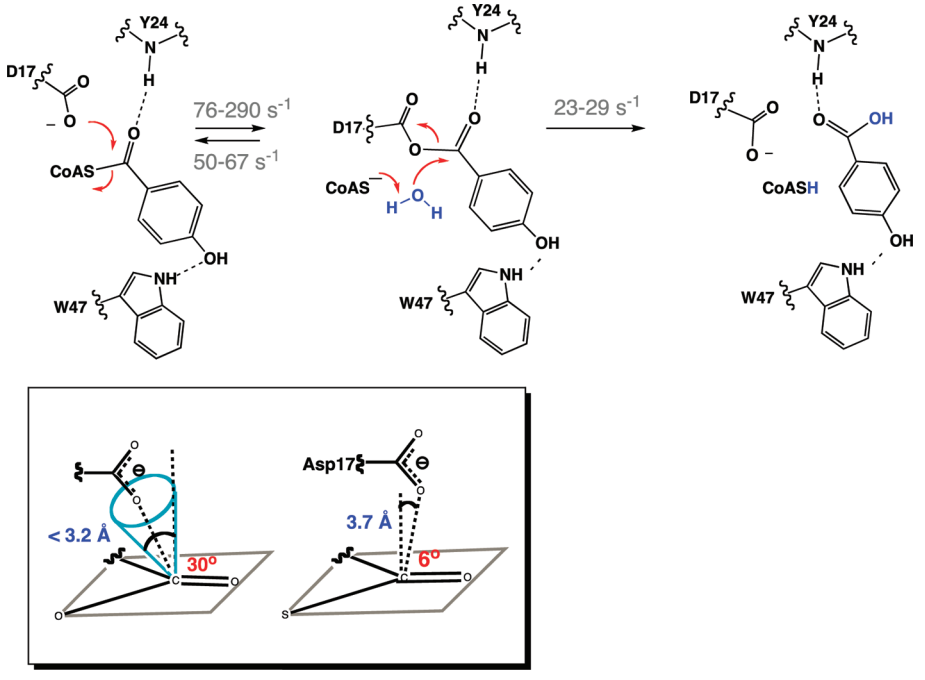

Figure 8. Cartoon representation of the proposed mechanism of 4-HB-CoA thioesterase catalysis. The rates shown over the arrows correspond to the range of values obtained for the microscopic rate constants via the time course simulations. In the box are (left) an adaptation of a figure from ref 17, which depicts the ranges of optimal positions for nucleophilic attack of a carboxylate oxygen anion at a carbonyl carbon as posited by Lightstone and Bruice, and (right) the distance and angle of attack of the Asp17 carboxylate oxygen atom at the 4-HB-CoA carbonyl carbon as inferred from the X-ray structure of the D17N thioesterase(4-HB-CoA) complex (PDB entry 1LO9).

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Lightstone and Bruice,[17] is depicted in Figure 8 alongside the orientation of the Asp17 observed in the thioesterase X-ray structures.[5] The Asp17 thus appears to be in a near-attack conformation. Substitution of the Asp17 with a Glu extends the side chain by one methylene group, which we expect would offset the alignment of the carboxylate oxygen anion with the 4-HB-CoA thioester C═O group. Indeed, the magnitude of the rate reduction observed for the D17E thioesterase is ∼1000-fold,[6] which is consistent with a significant perturbation of the ground state.

The displacement of the CoA thiolate anion is assumed to be a concerted reaction. This is because the pK_a of the conjugate acid (9.7) is low enough to fall well within the limit prescribed for concerted ester hydrolysis.[18] The CoA thiolate anion is not within reach of an active site residue, yet it could favorably interact with the electropositive field generated by the N-terminus of the α-helix. If the CoA does not depart before the ensuing hydrolysis step, it might serve to orient and activate the water molecule for attack at the 4-hydroxybenzoyl carbonyl carbon. This scenario is depicted in Figure 8, with the caveat that we have provided little in the way of supporting evidence. Additional work will be required to define the mechanism of the rate-limiting step of thioesterase catalysis.

The proposed mechanism of the *Pseudomonas* 4-HB-CoA thioesterase catalysis deviates from the mechanisms employed by the thioesterases of the α/β-fold hydrolase enzyme superfamily, which are centered on covalent catalysis by the Ser of the Ser-His-Asp/Glu catalytic triad and on electrophilic catalysis by an oxyanion hole.[19] In contrast, the thioesterase of the crotonase enzyme superfamily (3-hydroxyisobutyryl-CoA thioesterase) shares with the 4-HB-CoA thioesterase the use of a carboxylate residue (Glu143) in nucleophilic catalysis and the use of the N-terminus (positive pole) of an α-helix in electrophilic catalysis.[20] A detailed comparison of the catalytic mechanisms of hotdog-fold thioesterases, including the 4-HB-CoA thioesterase, is planned for a separate publication. Nevertheless, to date, the 4-HB-CoA thioesterase is the only member of the hotdog-fold family known to use the catalytic carboxylate group in nucleophilic catalysis.

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ABBREVIATIONS

CoA, coenzyme A; 4-HB-CoA, 4-hydroxybenzoyl-CoA; 4-HB, 4-hydroxybenzoate; 4-HP-CoA, 4-hydroxyphenacyl-CoA; MES, 2-(*N*-morpholino)ethanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperzine-*N*′-2-ethanesulfonate; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonate.

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