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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)1 thioesterase from *Pseudomonas* sp strain CBS3 catalyzes the final step of the 4-chlorobenzoate degradation pathway, which is the hydrolysis of 4-hydroxybenzoyl-CoA (4-HB-CoA) to coenzyme A (CoA) and 4-hydroxybenzoate. In previous work, X-ray structural analysis of the substrate-bound thioesterase provided evidence for the role of an active site Asp17 in nucleophilic catalysis (Thoden, J. B., Holden, H. M., Zhuang, Z., 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 present study, 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 μ M [¹⁴C]4-HB-CoA catalyzed by 10 μ M thioesterase supported a two-step pathway in which the second step is rate-limiting. Steady-state product inhibition studies revealed that CoA (K_{is} = 250 $\pm$ 70 μ M, K_{ii} = 900 $\pm$ 300 μ M) and 4-HB (K_{is} = 1.2 $\pm$ 0.2 mM) binding is weak, suggesting that product release is not rate-limiting. A substantial D₂O 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 aspartyl17-(4-hydroxybenzoyl)anhydride intermediate were determined by simulation-based fitting of a kinetic model to time courses for the substrate binding reaction (5.0 μ M 4-HB-CoA and 0.54 μ M thioesterase), single-turnover reaction (5 μ M [¹⁴C]4-HB-CoA catalyzed by 50 μ M thioesterase), steady-state reaction (5.2 μ M 4-HB-CoA catalyzed by 0.003 μ M thioesterase) and transient-state multiple-turnover reaction (50 μ M [¹⁴C]4-HB-CoA catalyzed by 10 μ M thioesterase). Together with the results obtained from solvent ¹⁸O-labeling experiments, the findings are interpreted as evidence for the formation of an aspartyl17-(4-hydroxybenzoyl)anhydride intermediate that undergoes rate-limiting hydrolytic cleavage at the hydroxybenzoyl carbonyl carbon atom.

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¹Abbreviations used are: coenzyme A (CoA), 4-hydroxybenzoyl-CoA (4-HB-CoA), 4-hydroxybenzoyl-3'-dephospho-CoA (4-HB-3'-dephospho-CoA), 4-hydroxyphenacyl-CoA (4-HP-CoA), 2-(N-morpholino)ethanesulfonate (MES); N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate (HEPES); N-tris(hydroxymethyl)methyl-3-aminopropane sulfonate (TAPS); 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonate (CAPSO).

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Keywords

4-Hydroxybenzoyl-CoA thioesterase; nucleophilic catalysis; base catalysis; solvent kinetic isotope effects; transient kinetics; thioester hydrolysis; hotdog-fold family; divergent evolution; enzyme mechanism

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 (Scheme 1) (3).

The X-ray structure of the *apo* *Pseudomonas* 4-HB-CoA thioesterase revealed a homotetrameric structure comprised of a dimer of dimer (4). The subunits consist of a five-stranded anti-parallel 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 analog (4-hydroxybenzyl-CoA; 4-hydroxyphenacyl-CoA) (PDB accession codes 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.

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 (at 3.7 Å from the thioester C=O carbon atom) is optimally positioned for nucleophilic addition to the substrate. Notably, there is no room for a water molecule to bind in between the Asp17 carboxylate group and thioester moiety (Figure 1C). The amide NH of the α -helix N-terminal residue Tyr24 is within hydrogen bond distance (3.2 Å) of the thioester C=O oxygen atom (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 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 to perform the role of base in the activation 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 carried out to test and explore the catalytic mechanism of thioesterase-catalyzed 4-HB-CoA hydrolysis suggested by the structural data,

¹Abbreviations used are: coenzyme A (CoA), 4-hydroxybenzoyl-CoA (4-HB-CoA), 4-hydroxybenzoyl-3'-dephospho-CoA (4-HB-3'-dephospho-CoA), 4-hydroxyphenacyl-CoA (4-HP-CoA), 2-(N-morpholino)ethanesulfonate (MES); N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate (HEPES); N-tris(hydroxymethyl)methyl-3-aminopropane sulfonate (TAPS); 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonate (CAPSO).

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 *E. coli* clone as previously described (7). 4-HP-CoA, 4-HB-3'-dephospho-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 (1993) (9). $[^{14}\text{C}]$ 4-HB-CoA was prepared as previously described (10).

Steady-state kinetic constant determinations from initial velocity data

4-HB-CoA thioesterase-catalyzed hydrolysis of 4-HB-CoA ($\Delta\epsilon = 11.8 \text{ mM}^{-1}\text{cm}^{-1}$) 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 were measured as a function of substrate concentration (K_m to $10K_m$) and were fitted to equation 1 using KinetAsyst (IntelliKinetics, PA) to determine the $V_{\max}$ and K_m values. The k_{cat} was calculated from the $V_{\max}/[E]$ where $[E]$ is the total enzyme active site concentration (determined using the Bradford method (11)).

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

Where V = initial velocity, $V_{\max}$ = maximum velocity, $[S]$ = substrate concentration and K_m = Michaelis constant.

Initial velocity data were measured in the absence or presence ($1K_i$, $2K_i$ and $3K_i$) of 4-HB or CoA and were fitted to equation 2 (to define the competitive inhibition constant K_{is}) or to equation 3 (to define the kinetic constants for noncompetitive inhibition K_{ii} and K_{is}), respectively.

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

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

Where $[I]$ = 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 ($\Delta\epsilon = 11.8 \text{ mM}^{-1}\text{s}^{-1}$). To determine the k_{cat} and K_m values, the data were fitted by curve simulation using the kinetic model:

and the definitions: $k_{\text{cat}} = k_2$ and $K_m = (k_{-1} + k_2)/k_1$. The KinTek Corporation Global Kinetic Explorer (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 H₂O and D₂O 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); 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 carrying out pre-incubation tests. The k_{cat} pH profile was fitted using equation 4.

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

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

Rapid quench transient kinetic determinations

A rapid-quench instrument from KinTek Instruments was used to combine buffered wild-type thioesterase with buffered [¹⁴C]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 [¹⁴C]4-HB-CoA in 0.15 M KCl/50 mM K⁺HEPES (pH 7.5, 25 °C), whereas for the single turnover reactions, enzyme and [¹⁴C]4-HB-CoA concentrations were 50 μM and 5 μM, respectively. The enzyme was precipitated from the quenched reaction solution by vigorous mixing with 200 μL CCl₄ using a Vortex mixer, and then pelleted by (micro)centrifugation of the mixture (14,000 rpm for 1 min). The water phase was injected into an HPLC for separation of the [¹⁴C]4-HB-CoA and [¹⁴C]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 carried out in parallel to establish the background radioactivity associated with the [¹⁴C]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 CPM) contained in the [¹⁴C]4-HB-CoA and [¹⁴C]4-HB HPLC fractions were used in conjunction with the specific activity (CPM/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 [¹⁴C]4-HB-CoA consumption and [¹⁴C]4-HB formation were plotted and then fitted by simulation using the KinTek Corporation 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 equation 5.

$$\ln [S] = -k t + \ln [S]_0 \quad \text{eq. 5}$$

where [S] is the substrate concentration at time “t”, [S]₀ is the initial substrate concentration and k is the apparent first order rate constant.

4-HB-CoA and 4-HP-CoA binding 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 1 cm, mixing time ~3 ms) fitted with a 340 nm cutoff filter. The excitation wavelength was 280 nm. The measurement of the time-dependent quenching of the wild-type thioesterase (0.54 μ M) fluorescence by 4-HP-CoA (5.3 μ M) or 4-HB-CoA (5.0 μ M) was carried out under pseudo-first-order conditions. The observed pseudo-first-order rate constant, k_{obs} , was obtained by fitting the data to equation 6 using the software package provided by Applied Photophysics.

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

Where F_t = the fluorescence at time “t”, F_0 = initial fluorescence, F_∞ = final fluorescence, k_{obs} = the observed first-order rate constant.

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

$$k_{\text{obs}} = k_{\text{on}} [L] + k_{\text{off}} \quad \text{eq. 7}$$

^{18}O -Incorporation into 4-hydroxybenzoate (4-HBA) during wild-type thioesterase catalyzed hydrolysis in ^{18}O -enriched water

A 320 μ L 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 230 μ L of 95% H_2^{18}O (Aldrich) was added. After a 30 min incubation period at 25 $^\circ\text{C}$, the enzyme solution in H_2^{18}O buffer was assayed to ensure that catalytic activity was retained. Next, 20 μ L of H_2^{18}O was added to a sample of 4-HB-CoA powder obtained by lyophilization of 20 μ L of 3.0 mM 4-HB-CoA in natural isotopic abundance water. A 5 μ L 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 μ L 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 ($[\text{E}]:[\text{S}]=10:1$). 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 adding 5 μ L of 6 N HCl and extracted four times with 1 mL portions of ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous sodium sulfate and the solvent was evaporated *in vacuo*. The solid was dissolved in 100 μ L of absolute methanol and subjected to 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 aspartyl117-(4-hydroxybenzoyl)anhydride intermediate by using C-14 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 to observe 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 to monitor the consumption of 4-HB-CoA (at 300 nm $\Delta\epsilon = 11.8 \text{ mM}^{-1} \text{ cm}^{-1}$), 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. Based on this strategy, the kinetic experiments reported below were designed and executed.

The kinetic “burst” experiment—The time course for the multiple-turnover 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., $[\text{4-HB}]_{\text{observed}} = [\text{enzyme anhydride intermediate}] + [\text{enzyme}(\text{CoA})(\text{4-HB})] + [\text{4-HB}]_{\text{free}}$). Providing that 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 take place faster than will 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).

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 ratelimiting 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 equation (4) to define a break at 5.15 ± 0.09 and $C_1 = 5.0 \pm 0.1$ and a break at 7.70 ± 0.08 and $C_2 = 55 \pm 4$ for catalysis in buffered H_2O , and a break at 5.1 ± 0.1 and $C_1 = 1.3 \pm 0.2$ and a break at 8.0 ± 0.1 and $C_2 = 15 \pm 1$ for catalysis in buffered D_2O . The pH independent isotope effect was calculated from the ratio of the respective C_1 or C_2 values to be 3.8 or 3.7.

Together, the substantial SKIE of 3.7–3.8 and the observed burst-phase kinetic time course (Figure 2), constitute strong evidence for 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 “worm-hole”-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 units sits at the entrance to this tunnel

(Figure 1D) (5). In earlier work, we 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 4.0 μM 4-HB-CoA with 0.54 μM thioesterase. The values of the respective apparent pseudo-first order rate constants $k_{\text{obs}} = 350 \pm 4 \text{ s}^{-1}$ and $280 \pm 30 \text{ s}^{-1}$ were obtained by data fitting (equation 6). The plots of k_{obs} vs ligand concentration, shown as insets to Figures 4A and 4B, defined a linear correlation. The association (k_{on}) and dissociation (k_{off}) rate constants were determined by fitting the data to equation 7. For 4-hydroxyphenacyl-CoA, $k_{\text{on}} = \pm 1 \mu\text{M}^{-1}\text{s}^{-1}$ and $k_{\text{off}} = 20 \pm 5 \text{ s}^{-1}$ define the microscopic rate constants for substrate binding and dissociation. Indeed, the ratio $k_{\text{off}}/k_{\text{on}} = 0.33 \mu\text{M}$ is in perfect agreement with the independently measured $K_d = 0.33 \mu\text{M}$ (5). For 4-HB-CoA, we note that only if the substrate binding step is in rapid equilibrium do the $k_{\text{on}} = 65 \pm 2 \mu\text{M}^{-1}\text{s}^{-1}$ and the $k_{\text{off}} = 13 \pm 5 \text{ s}^{-1}$ values accurately define the microscopic rate constants for substrate binding and dissociation. The ratio $k_{\text{off}}/k_{\text{on}} = 0.20 \mu\text{M}$ falls halfway in between the 4-HB-CoA $K_m = 0.26 \mu\text{M}$ (*vide infra*) and the independently measured $K_d = 0.14 \mu\text{M}$ (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 ($K_{\text{is}} = 250 \pm 70 \mu\text{M}$, $K_{\text{ii}} = 900 \pm 300 \mu\text{M}$) and 4-HB, a weak competitive inhibitor ($K_{\text{is}} = 1.2 \pm 0.2 \text{ mM}$). 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—In order 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. Firstly, 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). Secondly, steady-state multiple-turnover time courses were measured using 1.4, 3.5 or 5.2 μM 4-HB-CoA and 0.003 μM thioesterase (Figure 6).

The simulation-based fitting of the four sets of time-course data was carried out using the kinetic model shown in Scheme 2. This model assumes 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.

To initiate the simulation process, estimates of the microscopic rate constants were obtained by conventional fitting of the individual data sets (see Material 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, 4, 5 and 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 rate 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 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. In order to determine the regiochemistry of the hydrolysis step, single turnover reactions were carried out in 95% $\text{H}_2\ ^{18}\text{O}$ 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 it 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 138 m/z , parent ion fragment (M^*-OH) peak at 121 m/z (note that the OH group lost is from the carboxylic acid group, not the aromatic ring), a fragment (M^*-COOH) at 93 m/z and lastly, a fragment at 65 m/z (not assigned). The parent ion (M^*) and parent ion fragment (M^*-OH) contain oxygen from the carboxylic acid group, and can thus be used to measure the oxygen-18 incorporation. 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% $\text{H}_2\ ^{18}\text{O}$ revealed that >90% of the parent ion peak occurs at 140 m/z (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) specie at 121 and 123 m/z . 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 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 ($k_1 \sim 70\ \mu\text{M}^{-1}\text{s}^{-1}$) is about average, however the dissociation rate is slow ($k_{-1} \sim 15\ \text{s}^{-1}$) and therefore the $K_d = 0.14\ \mu\text{M}$ is quite small. The substrate 4-hydroxybenzoyl unit is immobilized by tight packing with the surrounding side chains,

several of which are aromatic. The single hydrogen bond formation with the benzoyl ring hydroxyl group involves the indole NH of Trp47 (Figure 1B).

At the catalytic site, the substrate thioester C=O 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 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. Indeed, the magnitude of the rate reduction observed for the D17E thioesterase is ~1000-fold (6), 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 electropositive field generated by the α -helix N-terminus. 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 in order 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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Figure 1.

The structure of D17N *Pseudomonas* sp. strain CBS3 4-HB-CoA thioesterase bound with 4-HB-CoA (PDB accession code 1LO9). (A.) The tetramer shown in Pymol cartoon with the 4-HB-CoA ligands shown in yellow stick. (B.) The active site structure wherein the 4-HB-CoA has been truncated for better viewing and the Asn17 has been mutated *in silico* to Asp17. Hydrogen bonds are indicated in gray or black dashed lines. The 3.7 Å distance 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, the oxygen atoms red, the sulfur atom yellow and the enzyme carbon atoms are colored gray whereas the 4-HB-CoA carbon atoms are colored cyan. (C.) Stereo figure 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 shown in gray, oxygen atoms in red, nitrogen atoms in blue and sulfur atoms in yellow. (D) The surface representation of the thioesterase substrate-binding site. The 4-HB-CoA ligand is shown in stick with carbon atoms colored cyan. The Asp17 side chain and Tyr24 amide unit are shown in stick with carbon atoms colored green.

Figure 2.

A 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 $^{\circ}\text{C}$). 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. The simulated curves were generated using the kinetic model shown in Scheme 2 and the following set of rate constants: $k_1 = 71 \mu\text{M}^{-1} \text{s}^{-1}$, $k_{-1} = 12 \text{s}^{-1}$, $k_2 = 290 \text{s}^{-1}$, $k_{-2} = 67 \text{s}^{-1}$, $k_3 = 23 \text{s}^{-1}$, $k_{-3} = 0 \text{s}^{-1}$, $k_4/k_{-4} = 300 \mu\text{M}$ and $k_5/k_{-5} = 1200 \mu\text{M}$. The simulated curve-coloring scheme is as follows: E-I(CoA) + E(4-HB)(CoA) + E(4-HB) + 4-HB (red); E(4-HB-CoA) + 4-HB-CoA (black).

Figure 3.

A plot of $\log k_{\text{cat}}$ 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 (○).

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 $^{\circ}\text{C}$). The data were fitted to equation 6 to define the observed rate constant k_{obs} as $350 \pm 4 \text{ s}^{-1}$. Inset: a plot of the k_{obs} vs 4-HP-CoA concentration (1.0 – 5.3 μM). The data were fitted to equation 7 to define the association rate constant k_{on} as $59 \pm 1 \mu\text{M}^{-1}\text{s}^{-1}$ and the dissociation rate constant k_{off} as $20 \pm 5 \text{ s}^{-1}$. (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 $^{\circ}\text{C}$). The data were fitted to equation 6 to define the observed rate constant k_{obs} as $280 \pm 30 \text{ s}^{-1}$. Inset: a plot of the k_{obs} vs 4-HB-CoA concentration (1.0 – 5.0 μM). The

data were fitted to equation 7 to define the association rate constant k_{on} as $65 \pm 2 \mu\text{M}^{-1}\text{s}^{-1}$ and the dissociation rate constant k_{off} as $13 \pm 5 \text{s}^{-1}$. (C) The time course for the binding reaction between $3.0 \mu\text{M}$ 4-HB-CoA and $0.54 \mu\text{M}$ thioesterase in $50 \text{ mM K}^+\text{HEPES}$ (pH 7.5, 25°C). The simulated curved was generated using the kinetic model shown in Scheme 2 and the rate constants $k_1 = 70 \mu\text{M}^{-1}\text{s}^{-1}$ and $k_{-1} = 15 \text{s}^{-1}$.

Figure 5.

The 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 $^\circ\text{C}$). 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. The simulated curves were generated using the kinetic model shown in Scheme 2 and the following set of rate constants: $k_1 = 79 \mu\text{M}^{-1} \text{s}^{-1}$, $k_{-1} = 20 \text{s}^{-1}$, $k_2 = 76 \text{s}^{-1}$, $k_{-2} = 50 \text{s}^{-1}$, $k_3 = 26 \text{s}^{-1}$, $k_{-3} = 0 \text{s}^{-1}$, $k_4/k_{-4} = 300 \mu\text{M}$ and $k_5/k_{-5} = 1200 \mu\text{M}$. The simulated curve-coloring scheme is as follows: E-I(CoA) + E(4-HB)(CoA) + E(4-HB) + 4-HB (red); E(4-HB-CoA) + 4-HB-CoA (black); E-I(CoA) (yellow); E(4-HB)(CoA) + E(4-HB) + 4-HB (green).

Figure 6.

The time courses for the steady-state multiple-turnover reactions of 1.4 (black), 3.5 (red) or 5.2 (green) μM 4-HB-CoA catalyzed by 0.003 μM thioesterase in 50 mM K^+HEPES (pH 7.5, 25 $^\circ\text{C}$). The 300 nm absorbance traces (dotted lines) are shown in the context of the calculated concentration of 4-HB-CoA ($\epsilon = 11.8 \text{ mM}^{-1} \text{ cm}^{-1}$) 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 rate constants: $k_1 = 71 \text{ } \mu\text{M}^{-1} \text{ s}^{-1}$, $k_{-1} = 12 \text{ s}^{-1}$, $k_2 = 290 \text{ s}^{-1}$, $k_{-2} = 67 \text{ s}^{-1}$, $k_3 = 23 \text{ s}^{-1}$, $k_{-3} = 0 \text{ s}^{-1}$, $k_4/k_{-4} = 300 \text{ } \mu\text{M}$ and $k_5/k_{-5} = 1200 \text{ } \mu\text{M}$.

A **B** **Figure 7.**

The 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 the quenched reaction mixture.

Figure 8.

A 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. The boxed content: (left) an adaptation of a figure from reference (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 accession code 1LO9).

Scheme 1.

The three chemical steps of the 4-chlorobenzoate degradation pathway catalyzed by 4-chlorobenzoate: CoA ligase, 4-chlorobenzoyl-CoA dehalogenase and 4-hydroxybenzoyl-CoA thioesterase, respectively.

Scheme 2.

The kinetic model representing 4-HB-CoA thioesterase catalysis. Abbreviations: E, thioesterase; EI, thioesterase-anhydride intermediate; S, 4-HB-CoA; P, CoA; Q, 4-HB.