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# Structure–activity analysis of base and enzyme-catalyzed 4-hydroxybenzoyl coenzyme A hydrolysis

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**Abstract**

In this study, the second-order rate constant $k_2$ of base-catalyzed hydrolysis and the values of $k_{cat}$, $K_m$ and $k_{cat}/K_m$ of wild-type *Pseudomonas* sp. CBS3 4-hydroxybenzoyl coenzyme A (4-HBA-CoA) thioesterase-catalyzed hydrolysis of 4-HBA-CoA and its *para*-substituted analogs were measured. For the base-catalyzed hydrolysis, the plot of $\log k_2$ vs the $\sigma$ value of the *para*-substituents was linear with a slope ($\rho$) of 1.5. In the case of the enzyme-catalyzed hydrolysis, the $k_{cat}/K_m$ values measured for the *para*-substituted analogs defined substrate specificity. Asp32 was shown to play a key role in substrate recognition, and in particular, in the discrimination between the targeted substrate and other cellular benzoyl-CoA thioesters.


*Keywords:* Thioester hydrolysis; Specific base catalysis; Substrate specificity; 4-HBA-CoA; Thioesterase; Structure–activity; SAR; Enzyme catalysis

## 1. Introduction

Thioesterases are ubiquitous enzymes that catalyze the hydrolysis of acyl-thioesters in a variety of cellular contexts. Thioesterases play essential physiological roles in fatty acid

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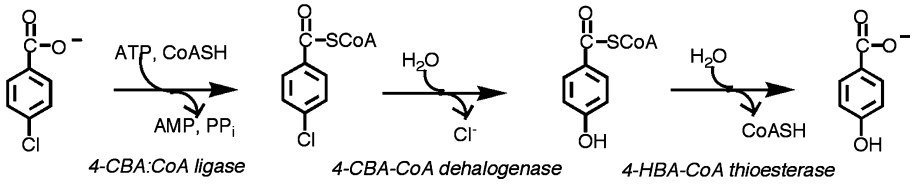


Fig. 1. The reaction steps of 4-chlorobenzoate degradation of *Pseudomonas* sp. Strain CBS3 [17].

and polyketide biosynthesis [1–3], aromatic compound degradation [4–6], acylated protein degradation [7,8], signal transduction [9] and lipid metabolism [10]. Most of thioesterases evolved within the α/β-hydrolase fold superfamily [11] or within the hotdog-fold enzyme superfamily [12]. The catalytic triad-based mechanism of amide, oxygen ester and thioester hydrolysis mediated by the enzymes of the α/β-hydrolase fold superfamily is well characterized as is the mechanism used for substrate recognition [13]. In contrast, little is known about the mechanisms of catalysis and substrate recognition employed by the hotdog-fold thioesterases.

The first hotdog-fold thioesterase structure reported was that of the 4-hydroxybenzoyl-CoA[2] (4-HBA-CoA) thioesterase [14,15]. This enzyme catalyzes the final step of 4-chlorobenzoate degradation pathway in the soil-dwelling bacterium *Pseudomonas* sp. strain CBS3 [4,16] (Fig. 1). The native structure is a dimer of dimers. Each monomer consists of a long α-helix wrapped by a 6-stranded β-sheet. Two monomers align to form a functional dimer having a 12-stranded sheet structure with the two α-helices aligned anti-parallel in a slight off set. The active site (Fig. 2) is located at the subunit interface. Two dimers associate to form the tetrameric structure. The nucleotide region of the substrate CoA moiety rests on the protein surface while the pantenoate arm extends into the active site crevice, desolvating the 4-hydroxybenzoyl thioester unit [15,17]. The substrate thioester C═O is positioned to engage in hydrogen bond interaction with the N-terminus of the monomer A α-helix, while the aromatic ring extends into the active site and approaches the α-helix of the opposing monomer B. The active site contains two carboxylate residues. Asp17, which functions in general base or nucleophilic catalysis, is located at the loop region that terminates in the N-terminus of α-helix A. Previous studies of site-directed mutants have shown that Asp17 is essential for catalysis [17]. Asp32, located on the α-helix B binds the ring C(4)OH via two bridging water molecules (Fig. 2).

In this paper, we report on the mechanism of action of the *Pseudomonas* sp. CBS3 4-HBA-CoA thioesterase with special focus on comparative substrate–activity relationships (SARs) in the specific base-catalyzed and enzyme-catalyzed hydrolysis of the C(4) substituted benzoyl-CoA substrates. In this manner, the role of the indirect interaction between Asp32 and the substrate benzoyl *para*-OH in substrate recognition is demonstrated.

[2] *Abbreviations used:* CoA, coenzyme A; 4-HBA-CoA, 4-hydroxybenzoyl-coenzyme A; SARs, substrate–activity relationships; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-galactopyranoside; DTT, dithiothreotal; 4-FBA-CoA, 4-fluorobenzoyl-CoA; 4-$CF_3$BA-CoA, 4-trifluorobenzoyl-CoA; 4-CBA-CoA, 4-chlorobenzoyl-CoA; 4-MBA-CoA, 4-methylbenzoyl-CoA; 4-MeOBA-CoA, 4-methoxybenzoyl-CoA; BA-CoA, benzoyl-CoA; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); $K^+$Hepes, potassium salt of *N*-(2-hydroxyethyl)piperzine-*N*′-2-ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonate; 4-HP-CoA, 4-hydroxyphenacyl-CoA; 4-CBA, 4-chlorobenzoate; 4-HBA, 4-hydroxybenzoate.

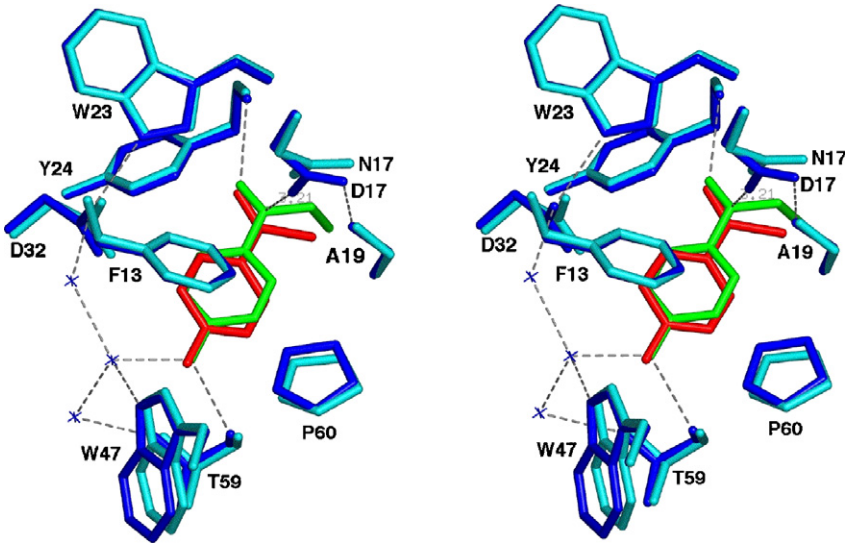


Fig. 2. Stereo active site picture of the complex of *Pseudomonas* sp. strain CBS3 4-HBA-CoA thioesterase (blue)/4-HP-CoA (green, truncated) superimposed with the complex of D17N (cyan)/4-HBA-CoA (red, truncated). The residue-residue and residue-substrate interactions are shown on the complex of WT/4-HP-CoA as black dash line [15]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

## 2. Materials and methods

### *2.1. Materials*

The recombinant *Pseudomonas* sp. strain CBS3 4-HBA-CoA thioesterase [15,17] was prepared from the *Escherichia coli* clone as described in ref 16. Mutagenesis was carried out using a PCR-based strategy [18] with the wild-type thioesterase gene as template, commercial primers from Invitrogen, the PCR kit supplied by Stratagene, and the Power Block IITM System thermal cycler manufactured by ERICOMP. PCR-amplified DNA was cloned into the pET-3a vector (Novagen) for expression in *E. coli* HMS174 (DE3). The mutated gene was verified by DNA sequencing. The mutant protein was purified to homogeneity (as judged by SDS–PAGE analysis) using the same procedure as used for purification of the wild-type thioesterase. 4-HBA-CoA, 4-fluorobenzoyl-CoA (4-FBA-CoA), 4-trifluorobenzoyl-CoA (4-$CF_3$BA-CoA), 4-chlorobenzoyl-CoA (4-CBA-CoA), 4-methylbenzoyl-CoA (4-MBA-CoA) and 4-methoxybenzoyl-CoA (4-MeOBA-CoA) were synthesized as reported in [15,19]. Benzoyl-CoA (BA-CoA) was purchased from Sigma.

### *2.2. Determination of steady-state kinetic constants for Pseudomonas thioesterase wild-type and D32S-catalyzed hydrolysis of substrate and analogs*

The $k_{cat}$ and $K_m$ values were determined from initial velocity data. The wild-type enzyme-catalyzed hydrolysis of 4-HBA-CoA was measured as described in ref 17. The enzyme-catalyzed hydrolysis of 4-HBA-CoA, 4-MeOBA-CoA, 4-MBA-CoA, BA-CoA,

4-FBA-CoA, 4-CBA-CoA, and 4-$CF_3$BA-CoA were monitored at 25 °C by measuring the absorbance of 5-thio-2-nitrobenzoate at 412 nm ($\Delta\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$), which was formed through the reaction of the 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) with the CoASH product. Each reaction mixture (200 μL) contained a catalytic amount of thioesterase (0.22 μM D32S for 4-HBA-CoA, 5.4 μM wild-type or 2.3 μM D32S for 4-MeOBA-CoA, 5.4 μM wild-type or 2.3 μM D32S for 4-MBA-CoA, 2.2 μM wild-type or 0.52 μM D32S for BA-CoA, 0.052 μM wild-type or 0.23 μM D32S for 4-FBA-CoA, 2.2 μM wild-type or 0.52 μM D32S for 4-CBA-CoA, and 21.6 μM wild-type or 2.3 μM D32S for 4-$CF_3$BA-CoA, respectively) and a varied concentration of substrate (0.5 $K_m \sim 5$ $K_m$), 2 mM DTNB, and 50 mM $K^+$Hepes, pH 7.5. The initial velocity data were analyzed using the equation $V = V_{max}[S]/([S] + K_m)$ ($V$ = initial velocity, $V_{max}$ = maximum velocity, $[S]$ = substrate concentration, $K_m$ = Michaelis constant). The $k_{cat}$ was calculated from $V_{max}/[E]$ where $[E]$ is the total enzyme active site concentration (protein concentration was determined using the Bradford method [20]) and the KinetAsyst program (IntelliKinetics, State College, PA).

### 2.3. Determination of the rate constant for base-catalyzed hydrolysis of the para-substituted benzoyl-CoA thioesters

A Beckman DU640 spectrophotometer was used to monitor the absorbance changes in the reaction solutions of the *para*-substituted benzoyl-CoA thioesters. Typically a 5 μL aliquot of the *para*-substituted benzoyl-CoA thioester stock solution was added to 195 μL of 50 mM Caps (for pH 11–12) or NaOH solution (for pH above and equal to 12) contained in a quartz cuvette (1 cm light path). The ionic strength of the reaction solution was adjusted to 3 M with NaCl. The molar extinction coefficients of 4-HBA-CoA and its analogs at 300 nm were determined as a function of reaction solution pH. The reaction was monitored at 300 nm at 25 °C and the first-order rate constant was obtained by fitting the data to the single exponential equation $A_t = A_0 + P\exp(-k_{obs}t)$, where $A_t$ is the absorbance at time $t$, $A_0$ is initial absorbance, $P$ is the total absorbance change, $k_{obs}$ is the observed rate constant. The $k_{obs}$ was used to calculate the second-order rate constant $k_2$ with the equation $k_2 = k_{obs}/[OH^-]$. The reported $k_2$ is the average of several measures at different pH values.

### 2.4. Calculation of $\Delta\Delta G_{bind}$ and $\Delta\Delta G^{\ddagger}$

Values for $\Delta\Delta G_{bind}$ (binding energy at the ground state) and $\Delta\Delta G^{\ddagger}$ (binding energy at the transition state) were calculated according to ref [21] using the equations:

$$\Delta\Delta G_{bind} = RT\ln(K_d^{\text{obs, without group present}}/K_d^{\text{obs, with group present}})$$

$$\Delta\Delta G^{\ddagger} = -RT\ln[(k_{cat}/K_m)^{\text{obs, without group present}}/(k_{cat}/K_m)^{\text{obs, with group present}}]$$

where $R$ is the gas constant (1.987 cal $mol^{-1}$ $K^{-1}$) and $T$ is temperature in Kelvin (298 K). $K_d^{\text{obs, without group present}}$ and $K_d^{\text{obs, with group present}}$ are the dissociation constants of the enzyme–ligand complex measured in the absence and in the presence of the functional group that contributes to substrate binding, respectively. For slow substrates, $K_m$ values were considered as an approximation of $K_d$ (when chemical steps are rate-limiting, $K_m = k_{-1}/k_1 = K_d$). $(k_{cat}/K_m)^{\text{obs, without group present}}$ and $(k_{cat}/K_m)^{\text{obs, with group present}}$

represent the $k_{cat}/K_m$ ratio of reactions in the absence of the functional group and in the presence of the functional group.

## 3. Results and discussion

### 3.1. Chemical model of 4-HBA-CoA catalysis

The chemical reaction catalyzed by 4-HBA-CoA thioesterase is rather simple: nucleophilic displacement of a thiolate by a water molecule (Fig. 3). Oxygen ester and thioester hydrolysis both occur through an addition–elimination pathway, but only the hydrolysis of the oxygen ester benefits from proton assisted departure of the leaving group [22–24]. The thioester hydrolysis in solution should occur faster at alkaline pH than at neutral pH because hydroxide is present to serve as the nucleophile in place of the water molecule. Indeed, whereas solution hydrolysis of 4-HBA-CoA and its analogs was too slow to detect at 25 °C, pH 7, the hydrolysis at >pH 10 was not. Thus, the values of first-order rate constant $k_{obs}$ for 4-HBA-CoA and the varied *para*-substituted benzoyl-CoA thioesters (MeO, Me, H, F, Cl and $CF_3$) were measured at pH 11.0–13.5. The second-order rate constant $k_2$ for each pH value was calculated by the equation $k_2 = k_{obs}/[OH^-]$ and the final $k_2$ values for the natural substrate and its analogs were derived from the average of the data measured at several pH values (Table 1).

The correlation between the rate constant ($\log k_2$) of base-catalyzed hydrolysis of benzoyl-CoA with various *para*-substituents and the *para*-substituent constant $\sigma$ [25], which

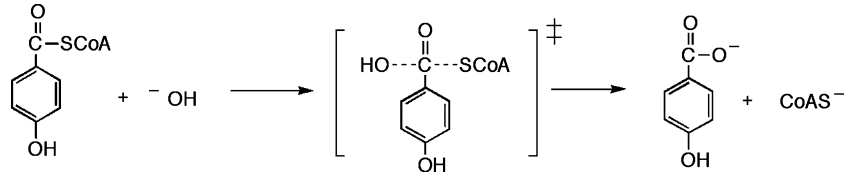


Fig. 3. The reaction pathway for hydroxide ion displacement of the CoA thiolate from 4-HBA-CoA.

Table 1
The steady-state kinetic constants determined for wild-type 4-HBA-CoA thioesterase-catalyzed hydrolysis of the natural substrate 4-HBA-CoA, and the substrate analogs 4-MeOBA-CoA, 4-MeBA-CoA, BA-CoA, 4-FBA-CoA, 4-CBA-CoA, and 4-$CF_3$BA-CoA measured at pH 7.5 and 25 °C

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($M^{-1}\,s^{-1}$) | $k_2$ ($M^{-1}\,s^{-1}$) | ASC ($\sigma_p$)[a] |
|---|---|---|---|---|---|
| 4-HBA-CoA | $(1.83 \pm 0.03) \times 10^1$ | $6.0 \pm 0.4$ | $3.1 \times 10^6$ | $1.8 \times 10^{-3}$ | −0.22 |
| 4-MeOBA-CoA | $(2.7 \pm 0.2) \times 10^{-1}$ | $(5.6 \pm 0.6) \times 10^1$ | $4.8 \times 10^3$ | $6.0 \times 10^{-2}$ | −0.27 |
| 4-MeBA-CoA | $(4.7 \pm 0.1) \times 10^{-2}$ | $(2.3 \pm 0.2) \times 10^2$ | $2.0 \times 10^2$ | $8.5 \times 10^{-2}$ | −0.14 |
| BA-CoA | $1.00 \pm 0.03$ | $(5.1 \pm 0.3) \times 10^2$ | $2.0 \times 10^3$ | $1.2 \times 10^{-1}$ | 0.00 |
| 4-FBA-CoA | $9.7 \pm 0.7$ | $(5.2 \pm 0.6) \times 10^2$ | $1.9 \times 10^4$ | $2.5 \times 10^{-1}$ | +0.15 |
| 4-CBA-CoA | $1.10 \pm 0.01$ | $(5.5 \pm 0.1) \times 10^2$ | $2.0 \times 10^3$ | $3.2 \times 10^{-1}$ | +0.24 |
| 4-$CF_3$BA-CoA | $(3.8 \pm 0.1) \times 10^{-2}$ | $(3.0 \pm 0.3) \times 10^2$ | $1.3 \times 10^2$ | $9.6 \times 10^{-1}$ | +0.53 |

See Section 2 for details.

[a] Average substituent constant for the substrate benzoyl ring C(4) substituent taken from Ref [25].

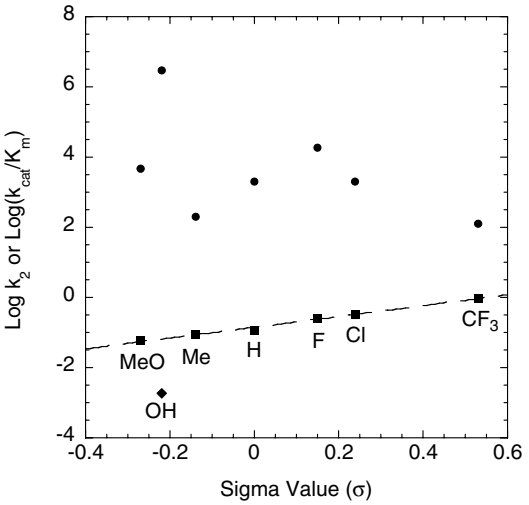


Fig. 4. The dependence of base-catalyzed hydrolysis rate constant (Log$k_2$ ($M^{-1}\,s^{-1}$), ■, (◆ is for OH)) or thioesterase-catalyzed hydrolysis rate constant (log($k_{cat}/K_m$) ($M^{-1}\,s^{-1}$), ●) of benzoyl-CoAs with different *para*-substituents upon the *para*-substituent $\sigma$ value.

was listed in Table 1, was measured. The plot of log$k_2$ vs the $\sigma$ value was linear with a slope ($\rho$) of 1.5 (see Fig. 4). The base-catalyzed hydrolysis of 4-HBA-CoA deviated from the fitted line when the $\sigma$ value of *para*-hydroxyl group was plotted. This is because at alkaline pH the C(4)OH is ionized (p$K_a$ of the 4-HBA-CoA hydroxyl group is 8.6 [26]). The $k_2$ of the unionized 4-HBA-CoA predicted by the log$k_2$ vs $\sigma$ plot of Fig. 4, is $6.5 \times 10^{-2}\,M^{-1}\,s^{-1}$.

The $\rho$ value is the reaction constant which measures the susceptibility of the reaction to the influence of the substituents and the extent to which negative charge builds in the rate-limiting transition state. The $\rho$ value measured for alkaline hydrolysis of *para*-substituted benzoyl-ethyl esters is 2.19 (measured in 75% methanol at 25 °C) [27]. The hydrolysis of the oxygen ester and the thioester proceeds via a rate limiting transition-state in which the build-up of negative charge at the reaction center is significant, yet not as large as observed with the oxygen ester.

### 3.2. Substituents effects in thioesterase-catalyzed hydrolysis of 4-HBA-CoA analogs

The requirement for precise steric and electronic complementation between substrate and enzyme active site as the complex proceeds along the reaction coordinate precludes the application of SAR analysis to define transition state charge. Instead, the SAR analysis provides information about the specificity of the reaction for the substrate (substituent varied in substrate analog) and for the catalytic site (amino acid replaced by site-directed mutagenesis). Examination of the active site region surrounding the *para*-hydroxyl in the X-ray structure of the wild-type thioesterase-4-HP-CoA complex (see Fig. 2) reveals that the hydroxyl group is involved in a network of H-bonds. The H-bond branches can serve to amplify the binding energy derived from the formation of the primary H-bond with the substrate in the ground state and/or in the transition state. From Fig. 2 we can see that the

nucleophile Asp17 is positioned close (3.2 Å) to the thioester C=O carbon, and that the thioester C=O is engaged in hydrogen bond formation with the backbone NH of Tyr24 located at the N-terminus of the active site α-helix. The benzoyl ring *para*-hydroxyl group is engaged in hydrogen bond formation with the Thr59 backbone NH, and with an active site-bound water molecule. If the *para*-benzoyl ring hydroxyl group was eliminated or replaced, the ring might shift in position, and the thioester C=O may no longer be optimally aligned with the Asp17 carboxylate.

To examine the importance of the hydrogen bond interaction, the $k_{cat}$ and $k_{cat}/K_m$ of the wild-type thioesterase-catalyzed hydrolysis of benzoyl-CoA substrates having the different *para*-substituents MeO, Me, H, F, Cl, and $CF_3$ were measured (see Table 1). The removal of the *para*-hydroxyl in the BA-CoA substrate resulted in an 18-fold and a 1500-fold decrease in $k_{cat}$ and $k_{cat}/K_m$ values, respectively. This observation agrees with the important role of *para*-hydroxyl in substrate binding. The loss of binding interaction with the *para*-hydroxyl substituent could allow the thioester C=O to shift slightly in position relative to the nucleophile, as the ring is given more freedom to move within the confines of the active site.

For 4-FBA-CoA, the $k_{cat}/K_m$ value is 160-fold smaller than that of 4-HBA-CoA, which suggests that the fluoride does not function as well as the hydroxyl group in facilitating substrate binding. Reduced hydrogen bond interaction between the *para*-F and the Thr59 backbone amide NH, and the active site bound water, might be responsible for this lowered binding affinity. The $k_{cat}$ for thioesterase-catalyzed hydrolysis of 4-CBA-CoA is 18-fold smaller in value than that of 4-HBA-CoA and the $k_{cat}/K_m$ value is 1500-fold smaller than that measured for 4-HBA-CoA. Apparently, both binding affinity and binding orientation are impaired by the inability of the Cl substituent to engage in hydrogen bond interaction with the Thr59 backbone amide NH, and with the bound water molecule.

The $CF_3$ and $CH_3$ *para*-substituted substrates were the least reactive. The $k_{cat}/K_m$ value for thioesterase-catalyzed 4-$CF_3$BA-CoA hydrolysis is 24,000-fold smaller than that measured for 4-HBA-CoA, while that for the 4-MBA-CoA is reduced 15,000-fold. Notably, the $k_{cat}/K_m$ value of 4-MeOBA-CoA is only 640-fold smaller than the $k_{cat}/K_m$ value of 4-HBA-CoA. This comparison of substituent effects suggests that interaction with the C(4)OH contributes to productive binding whereas the substitution of the C(4)OH proton with a methyl group impairs it.

The plot of $\log(k_{cat}/K_m)$ vs $\sigma$ (see Fig. 4) indicated that no correlation exists between electron withdrawing capacity of the ring substituent and substrate activity ($k_{cat}$ or $k_{cat}/K_m$). Thus, we can surmise that the electron withdrawing ability of the *para*-substituent does not determine the reactivity of the substrate. Instead, the interaction between this substituent and the enzyme active site seems to be the controlling factor.

The *para*-substituent of BA-CoA is a hydrogen atom. Unlike the *para*-hydroxyl of the 4-HBA-CoA substrate, the *para*-hydrogen of BA-CoA does not interact with water solvent in the unbound state nor with the polar active site groups in the enzyme complex. Because catalysis is slow with this substrate, the substrate binding step is likely to be at rapid equilibrium. According, the BA-CoA $K_m = 510$ μM is an estimate of its $K_d$ (the value proved to be too large to measure using fluoresence titration methods). Based on the $K_d = 0.15$ μM for 4-HBA-CoA bound with D17N mutant thioesterase, we can calculate that the *para*-hydroxyl contributes a $\Delta\Delta G_{bind} = 4.8$ kcal/mol for binding in the ground state. In view of the fact that this value represents the differential in H-bond interaction of the *para*-hydroxyl with enzyme and water solvent, it is impressively large. Based on

the ratio of $k_{cat}/K_m$ values, the *para*-hydroxyl contributes a $\Delta\Delta G = 4.3$ kcal/mol for transition state stabilization. Thus, the *para*-hydroxyl group functions in substrate binding and not in catalysis (as would be the case if it was bound more tightly at the transition state than at the ES complex).

### 3.3. The role of the active site Asp32 was revealed by site-directed mutagenesis

The active site Asp17, which is positioned 3.2 Å from the substrate thioester carbonyl carbon, is essential for catalysis as evidenced by the 100,000-fold decrease in activity that occurs upon replacement by Asn [17]. The Asp32 on the other hand, is positioned near the 4-hydroxybenzoyl ring where it binds to the *para*-hydroxyl through two bridging water molecules. The turnover rate of the D32S mutant is only 33-fold less than that of the wild-type thioesterase. This suggests that Asp32 plays only a supportive role in catalysis. In this work, the specificity of the D32S mutant towards the *para*-substituted substrate analogs was measured to determine if the Asp32 carboxylate contributes to substrate discrimination. The $k_{cat}$, $K_m$ and $k_{cat}/K_m$ values obtained are reported in Table 2. 4-HBA-CoA was found to be the best substrate for the D32S mutant. The $k_{cat}/K_m$ for D32S-catalyzed 4-HBA-CoA hydrolysis is reduced 220-fold compared to that of the wild-type enzyme. Although the wild-type thioesterase catalyzes the hydrolysis of each of the 4-HBA-CoA analogs faster than does the D32S mutant, the difference in the rate of catalysis is substantially less than it is when 4-HBA-CoA serves as substrate. For example, the decrease in the $k_{cat}/K_m$ value for the hydrolysis of 4-MeOBA-CoA is 37-fold, and the decrease in the $k_{cat}/K_m$ for the hydrolysis of 4-$CF_3$BA-CoA is only 3-fold. Thus, for the wild-type thioesterase-catalyzed hydrolysis of 4-HBA-CoA, the interaction between the *para*-benzoyl ring hydroxyl and the carboxyl group of the residue Asp32 is significant. If the substrate is modified to remove the C(4)OH for H-bond interaction, the contribution of Asp32 to catalysis is significantly reduced, as is the ability of the enzyme to discriminate substrates (Table 2).

### 3.4. Biological significance of substrate specificity

The purpose of *Pseudomonas* thioesterase is to function in the 4-chlorobenzoate (4-CBA) degradation pathway. In the first step of this pathway, 4-CBA-CoA is formed

Table 2
The steady-state kinetic constants determined for *Pseudomonas* 4-HBA-CoA thioesterase mutant D32S-catalyzed hydrolysis of the natural substrate 4-HBA-CoA, and the substrate analogs 4-MeOBA-CoA, 4-MeBA-CoA, BA-CoA, 4-FBA-CoA, 4-CBA-CoA and 4-$CF_3$-CoA measured at pH 7.5 and 25 °C

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($M^{-1}\,s^{-1}$) | ASC ($\sigma_p$) |
|---|---|---|---|---|
| 4-HBA-CoA | $(5.6 \pm 0.2) \times 10^{-1}$ | $(4.1 \pm 0.6) \times 10^{1}$ | $1.4 \times 10^{4}$ | −0.22 |
| 4-MeOBA-CoA | $(3.2 \pm 0.2) \times 10^{-2}$ | $(2.5 \pm 0.3) \times 10^{2}$ | $1.3 \times 10^{2}$ | −0.27 |
| 4-MeBA-CoA | $(1.4 \pm 0.1) \times 10^{-2}$ | $(7 \pm 1) \times 10^{2}$ | $2.0 \times 10$ | −0.14 |
| BA-CoA | $(5.1 \pm 0.2) \times 10^{-2}$ | $(3.7 \pm 0.4) \times 10^{2}$ | $1.4 \times 10^{2}$ | 0.00 |
| 4-FBA-CoA | $(2.38 \pm 0.04) \times 10^{-1}$ | $(2.8 \pm 0.1) \times 10^{2}$ | $8.6 \times 10^{2}$ | +0.15 |
| 4-CBA-CoA | $(1.35 \pm 0.05) \times 10^{-1}$ | $(2.1 \pm 0.2) \times 10^{2}$ | $1.3 \times 10^{2}$ | +0.24 |
| 4-$CF_3$BA-CoA | $(1.45 \pm 0.04) \times 10^{-2}$ | $(3.7 \pm 0.3) \times 10^{2}$ | $4.2 \times 10$ | +0.53 |

See Section 2 for details.

from 4-CBA and CoA at the expense of ATP (Fig. 1). Following dehalogenation of the 4-CBA-CoA to 4-HBA-CoA, the thioester linkage is hydrolyzed by the thioesterase releasing CoA and the pathway product, 4-HBA. The thioesterase must refrain from hydrolyzing the thioester unit of the 4-CBA-CoA in order to avoid a futile cycle of ATP hydrolysis. The *para*-benzoyl ring substituent might therefore, play an important role in substrate recognition. Indeed, the $k_{cat}/K_m$ value for benzoyl-CoA, and for each *para*-substituted benzoyl-CoA substrate tested (MeO, Me, H, F, Cl, and $CF_3$; Table 1), is significantly lower than that measured for 4-HBA-CoA. The $k_{cat}/K_m$ value of $2 \times 10^3\ M^{-1}\ s^{-1}$ for thioesterase-catalyzed 4-CBA-CoA hydrolysis is 1500-fold smaller than the $k_{cat}/K_m$ of $3.1 \times 10^6\ M^{-1}\ s^{-1}$ for thioesterase-catalyzed 4-HBA-CoA hydrolysis and 100-fold smaller than the $k_{cat}/K_m$ of $1.7 \times 10^5\ M^{-1}\ s^{-1}$ for dehalogenase-catalyzed 4-CBA-CoA dehalogenation [28] measured under the same conditions (pH 7.5, 25 °C). This rate difference prevents loss of the 4-CBA-CoA to thioesterase-catalyzed hydrolysis under physiological conditions, provided that the cellular dehalogenase concentration is equal to or greater than the cellular thioesterase concentration.

Recent studies have shown that benzoate is converted to benzoyl-CoA (at the expense of ATP) before degradation [5,29]. In bacteria that use 4-chlorobenzoate and benzoate as fuel, it is important that the benzoyl-CoA not be degraded by the 4-HBA-CoA thioesterase before it could be funneled in the benzoyl-CoA degradation pathway. Unless the 4-HBA-CoA thioesterase can discriminate between 4-HBA-CoA and benzoyl-CoA, the two pathways cannot coexist. The $k_{cat}/K_m$ benzoyl-CoA is low ($2 \times 10^3\ M^{-1}\ s^{-1}$), and much lower than the $k_{cat}/K_m$ ($3.1 \times 10^6\ M^{-1}\ s^{-1}$) for thioesterase-catalyzed hydrolysis of 4-HBA-CoA. Thus, at the enzyme concentration required to process the 4-HBA-CoA, the benzoyl-CoA pool should not be effected.

## 4. Conclusion

In conclusion, the reaction between hydroxide ion and the thioester moiety of 4-HBA-CoA proceeds through a transition state which accumulates less negative charge than does the rate limiting transition state formed in the reaction of hydroxide ion and the *para*-hydroxybenzoyl-ethyl ester. In the 4-HBA-CoA thioesterase-catalyzed reaction, the inductive effect of the substrate *para*-substituent is masked by steric and hydrogen bonding effects. Recognition of the C(4)OH in the targeted substrate 4-HBA-CoA is important for prevention of unwanted hydrolysis of the pathway substrate 4-CBA-CoA, as well as the metabolite benzoyl-CoA [5,29].

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