Published in final edited form as:

Chembiochem. 2017 October 05; 18(19): 1935–1943. doi:10.1002/cbic.201700322.

The Hotdog-fold Thioesterase PA1618 Catalytic Mechanism Revealed by X-ray Structure Determination of the Substrate Oxygen Ester Analog Complex

John A. Latham^{1,#}, Tianyang Ji^{2,#}, Kaila Matthews³, Patrick S. Mariano³, Karen N. Allen^{2,*}, and Debra Dunaway-Mariano^{3,*}

¹Department of Chemistry & Biochemistry, University of Denver, Denver, CO 80208-0183,

²Department of Chemistry, Boston University, Boston, Massachusetts, USA 02215²

³Department of Chemistry & Chemical Biology, University of New Mexico, Albuquerque, NM USA 87131

Abstract

Thioesterase activity accounts for the majority of the activities in the hotdog-fold superfamily. The structure and mechanism of catalysis for many hotdog enzymes have been elucidated by X-ray crystallography and kinetics to probe the specific substrate usage and cellular functions. However, structures of hotdog thioesterases in complex with substrate analogs reported to date utilize ligands that comprise either truncations of the substrate or include additional atoms to prevent the hydrolysis. Herein, we present the synthesis of an isosteric and isoelectronic substrate analog, benzoyl-OdCoA, and the X-ray crystal structure of a complex of the analog with *P. aeruginosa* hotdog thioesterase, PA1618 (at 1.72 Å resolution). The complex is compared to that of the “imperfect” substrate analog phenacyl-CoA, refined to a resolution of 1.62 Å. Kinetic and structural results are consistent with Glu64 as the catalytic residue and with Gln49 in stabilization of the transition state. Structural comparison of the two ligand-bound structures revealed a crucial ordered water molecule coordinated in the active site of the benzoyl-OdCoA structure which is not present in the phenacyl-CoA bound structure. This suggests a general base mechanism of catalysis where Glu64 activates the coordinated water nucleophile. Together, our findings reveal the importance of a more indistinguishable substrate analog to determine proper substrate binding and catalytic mechanism.

Graphical Abstract

Hotdog-fold thioesterases play integral roles in cellular processes however, there remains a need or better inhibitors to aid in the mechanistic elucidation of these enzymes. Here, we provide a

* Address correspondence regarding X-ray structure determinations to Karen N. Allen, 590 Commonwealth Ave, Room 299, Boston, MA 02215. Tel. 617-358-5544; Fax. 617-353-6466; drkallen@bu.edu and biochemical analyses to Debra Dunaway Mariano, Department of Chemistry & Chemical Biology, University of New Mexico, Albuquerque, NM 87131. Tel. 505-277-3383; Fax. 505-277-2609; dd39@unm.edu.

[#]These two authors share first authorship. J.A.L. performed the biochemical and chemical work with assistance from K.M. and T.J. carried out the X-ray structure determinations. PSM directed the chemical synthesis, KNA the structure determination, and DDM the kinetic determinations. J.A.L. and T.J. prepared the initial draft of the manuscript.

The PDB accession codes for the *Pseudomonas Aeruginosa* thioesterase PA1618 X-ray structures are 4KUQ (*apo*), 4KUR (phenacyl-CoA complex), 4KUS (benzoyl-OdCoA complex), 4KUT (*apo* E64A variant) and 4KUU (*apo* Q49A variant).

synthetic route for benzoyl-CoA and the crystal structure of the *Pseudomonas aeruginosa* thioesterase PA1618 with benzoyl-CoA and phenacyl-CoA bound.

Introduction

Thioesters are required for a wide range of essential cellular processes including energy production, signal transduction, membrane biogenesis and gene regulation [1–3]. Thioesters are derived from, and function as activated forms of organic acids, and generally serve as acyl donors as well as substrates in Michael addition, Claisen condensation, and β -elimination reactions [4–6] in various metabolic processes. The formation of thioesters from coenzyme A (CoA) or pantetheine-charged acyl carrier protein (*holo*ACPs) and organic acids is catalyzed by ATP-dependent ligases or synthetases [7]. Thioester hydrolysis is catalyzed by thioesterases from the α/β hydrolase-fold and hotdog-fold enzyme superfamilies.

Hotdog-fold thioesterases are known to utilize an active site carboxylate residue (Glu or Asp) in catalysis. Mechanistic studies directed at the hotdog-fold thioesterases from evolutionary distant organisms [10–14] have revealed that the catalytic carboxylate functions as a nucleophile in a two-step pathway involving the intermediacy of a mixed anhydride intermediate (Scheme 1A) or alternatively in single-step process, as a general base, responsible for the activation of the water nucleophile (Scheme 1B). However, for any given thioesterase, the task of distinguishing between these two pathways can be quite difficult owing to the limitations of the mechanistic probes currently available. In our previous efforts to define mechanisms of hotdog-fold thioesterase catalysis a combination of X-ray structure determination of the thioesterase bound with an inert substrate analog, determination of the ratio of ^{18}O -transfer from ^{18}O -enriched water to the enzyme carboxylate group vs the product carboxylate group, and transient-state kinetic analysis was applied with mixed success [8–12]. This is because each of the three experimental methods has its limitations. Briefly, the mixed anhydride intermediate formed in the two-step mechanism cannot be detected by kinetic methods unless the second step (*viz.* hydrolysis) is rate-limiting. And,

the ^{18}O transfer from ^{18}O -enriched water is diagnostic of the two-step pathway only if the mixed anhydride intermediate undergoes hydrolysis at the thioesterase carbonyl carbon. The X-ray structural analysis, which is intended to distinguish the role of the catalytic carboxylate residue (general base vs nucleophile) by depicting the reaction site in the Michaelis complex, assumes that the inert substrate analog used in the structure determination binds in the exact fashion as the substrate. Herein lies the problem. To prevent catalytic turnover, the substrate analog is modified at the thioester moiety, which in principle and in practice severely limits its effectiveness as reporter of the arrangement of interacting groups at the reaction site. X-ray crystal structures of thioesterase-bound inert substrate analogs in which the $\text{O}=\text{C}$ of the thioester group is replaced with “ CH_2 ” or with “ $\text{O}=\text{C}-\text{CH}_2$ ” are compromised by the absence of noncovalent bonding interactions with the thioester carbonyl oxygen in the former case and in the latter, with the impact of the added steric bulk on electrostatic interactions with active site residues [12,13].

Because hotdog-fold thioesterases are known to play essential roles in human health and disease [14,15], and because effective inhibitors of these enzymes have yet to be discovered, there exists an urgent need for the development of a class of inert substrate analogs that can be used to unambiguously define the structure and mechanism of any hotdog-fold thioesterase targeted for inhibitor design. Herein, we report the synthesis of such an analog and demonstrate its successful application in defining the active site structure of a hotdog-fold thioesterase, PA1618, poised for catalytic turnover.

Materials and Methods

Materials

The synthetic protocols used in the preparations of the acyl-OdCoA analogs used in this study can be found in the supporting information (SI). The restriction enzymes, T4 DNA ligase, oligonucleotide primers, and the competent *E. coli* BL21(DE3) cells were purchased from Invitrogen. The *E. coli* BL21(DE3) RIPL competent cells were purchased from Stratagene. *Pfu Turbo* and *Deep Vent* DNA polymerases were purchased from Stratagene and New England Biolabs, respectively. The cloning vectors and the *E. coli* B834(DE3) competent cells were purchased from Novagen. DNA sequencing was performed by the DNA Sequencing Facility of the University of New Mexico. The thioester substrates 4-hydroxybenzoyl-CoA, DHNA-CoA and coumaroyl-CoA were synthesized as previously reported [16,17]. Phenacyl-CoA and undecanoyl-CoA were synthesized as previously reported [18,19]. All other chemicals were obtained from Sigma-Aldrich and used without further purification.

Preparation of recombinant PA1618.—Wild-type PA1618 (UniProt # Q9I3A4) was prepared by using a PCR-based strategy. Accordingly, the gene was amplified by PCR using genomic DNA from *P. aeruginosa* PAO1 (ATCC) as template, commercial oligonucleotides as primers, and *Deep Vent* as the polymerase. The PCR-product was digested by the restriction enzymes *NdeI* and *XhoI* and then purified by agarose gel electrophoresis. The gene was ligated to an *NdeI* and *XhoI*-digested pET23a vector using T4 DNA ligase. The cloned gene was verified by DNA sequencing and then used to transform competent *E. coli*

BL21(DE3) cells for gene expression. The PA1618/ pET23a-transformed *E. coli* BL21(DE3) cells were grown aerobically at 37 °C in LB media containing 50 µg/ml ampicillin. Production of C-terminal His₆-tagged PA1618 was induced with 0.4 mM isopropyl-β-D-galactopyranoside (IPTG) once the cell density had reached A₆₀₀ ~0.6. Following a 12 h induction period at 19 °C, the cells were harvested by centrifugation at 6,500 rpm for 10 min and then suspended in 100 mL of 50 mM Tris buffer (pH 8.4), 50 mM imidazole, and 500 mM NaCl (Lysis Buffer). The cells were lysed using a French press at 1,200 psi and the lysate was centrifuged at 20,000 rpm for 15 min. The supernatant was loaded onto a 5 mL HisTrap FF column (GE Life Sciences) and the column was washed at 4 °C with Lysis Buffer to remove non-tagged protein and then with 50 mM Tris (pH 8.4), 500 mM imidazole, and 500 mM NaCl (Elution Buffer) to elute the tagged protein. Column fractions were monitored by measuring solution absorbance at 280 nm and by carrying out SDS-PAGE analysis. The desired fractions were combined and dialyzed at 4 °C against three changes of 1 L of 50 mM Tris (pH 8.4)/50 mM NaCl. The homogeneity was confirmed by SDS-PAGE analysis. Yield: ~25 mg protein/g of wet cells.

Preparation of selenomethionine-substituted PA1618.—The PA1618 containing plasmid was transformed and expressed in methionine auxotroph B834(DE3) *E. coli* cells. The cells were grown in 1 of L PASM-5052 media containing 20X NPS (66 g (NH₄)₂SO₄, 136 g KH₂PO₄, 142 g Na₂HPO₄, 1 L of H₂O), 50X 5052 (250 g glycerol, 25 g glucose, 100 g lactose, 1 L of H₂O), 20 mL of amino acids mix at 10 mg/mL (all 20 standard amino acids except Cys, Tyr, Met), metals (2 mM CaCl₂, 5 mM FeCl₃, 1mM MnCl₂, 1 mM ZnSO₄, 0.2 mM CoCl₂, 0.2 mM CuCl₂, 0.2 mM NiCl₂, 0.2 mM Na₂MoO₄, 0.2 mM Na₂SeO₃, 0.2 mM H₃BO₄), vitamins (0.02 mM of nicotinic acid, pyroxidine, thiamine, p-aminobenzoic acid, pantothenate; 0.5 µM of folic acid, and riboflavin), 1 mM MgSO₄, 0.4 mL of L-methionine solution (1 g/40 mL H₂O) and 5.0 mL of L-SeMet solution (1 g/40 mL H₂O). Cell growth and protein preparation followed the same protocol as above. Purified protein was dialyzed in 50 mM sodium citrate buffer, pH 5.5, for crystallization, a buffer condition found to confer the greatest thermal stability based on a high-throughput fluorescence thermal shift assay.

Site-Directed Mutagenesis.—Site-directed mutagenesis was carried out using the Quick Change PCR strategy (Stratagene) on the *PA1618-His₆*/pET-23a as template with commercial primers and Pfu Turbo as the polymerase. The sequence of the mutated gene was confirmed by DNA sequencing. The recombinant mutated plasmids were used to transform competent *E. coli* BL21 (DE3) cells. The variant proteins were purified to >90% homogeneity (as determined by SDS-PAGE analysis) as described above in a yield of 25 mg protein/g wet cell paste.

PA1618 crystallization and X-ray data collection.—The five structures reported herein include wild-type PA1618 unliganded structure, liganded structures complexed with phenacyl-CoA and benzoyl-OdCoA and unliganded structures containing the single mutations Q49A and E64A. In all cases, purified proteins were concentrated to 15 mg/mL in 50 mM sodium citrate buffer, pH 5.5, before use. Initial crystallization conditions were identified using the Index Screen (Hampton Research). Crystals of PA1618 bound to

phenacyl-CoA were formed by co-crystallization using 1 mM ligand. The benzoyl-OdCoA bound structure was obtained by soaking crystals of apo PA1618 with 2 mM benzoyl-OdCoA dissolved in the mother liquor 3 hr prior to cryo-cooling. All crystals were obtained using the hanging-drop vapor diffusion method using 1.0 μ l protein at 10–15 mg/mL and equal volume of well solution (plus 2 mM substrate analogue when included). Crystals of all proteins except the E64A variant were grown in 40% polyethylene glycol 400 (PEG400) and 0.1 M Bis-Tris, pH 6.5. Crystals of the E64A variant were grown in 40% PEG200, 0.1 M sodium acetate and 8 mM β -mercaptoethanol. Crystals of liganded and unliganded PA1618 grew as thin sheets to the dimensions $0.7 \times 0.4 \times 0.02$ mm in 1–2 days at 17 °C. PA1618 crystals were cooled in liquid nitrogen directly without additional cryoprotection. PA1618 diffraction data were collected through remote access to SSRL beamline 12–2 and processed using autoXDS [20]. Data collection and processing statistics are provided in Table 1.

PA1618 complex structure refinement.—Phase information for the PA1618 structure was determined using single wavelength anomalous diffraction (SAD) from Se, with anomalous signal to 2.0 Å. Data was processed using autoXDS [20]. Thirty-one heavy atom selenium sites were found using PHENIX AutoSol [21], with a figure of merit of 0.44. An initial model was built using PHENIX AutoBuild [21] in which 813/912 amino acids and 579 water molecules were included, with an initial R-work and R-free of 0.22 and 0.23, respectively. Liganded and site-directed variant structures of PA1618 were determined by molecular replacement with unligand SeMet PA1618.

The models were refined in PHENIX [21] with the maximum-likelihood target and individual B-factor refinement. Ordered solvent molecules were added automatically in PHENIX and culled manually in the graphics program COOT [22]. The final models were validated using the RCSB Validation Server. The dihedral angles of all residues are within the allowed region of the Ramachandran plot. Refinement statistics and model geometry for all five structures are listed in Table 1. The final model of all PA1618 structures contain 6 subunits in the asymmetric unit to form two “back-to-back” dimers and two flanking monomers (Figure S3A). There are two active sites that lie at the opposing ends of the dimer interface, thus the asymmetric unit contains four “complete” active sites within the “back-to-back” dimers. The ligand was not modeled into the flanking monomers because the active sites are at a point of crystallographic symmetry, thereby interfering with interpretation of the density. The structure of PA1618 in complex with phenacyl-CoA includes four ligands- two bound at each dimer interface. The model of PA1618 in complex with benzoyl-OdCoA includes two ligands- one bound at each dimer interface. The PA1618 variants, E64A and Q49A, were co-crystallized with benzoyl-CoA and undecanone-CoA, respectively. However, ligands were excluded from the models due to the lack of continuous density for ligand at the active sites.

PA1618 Activity Assay.—Thioesterase activity was measured using the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) coupled assay. Reactions were monitored at 412 nm ($\epsilon = 13.6 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) using a Beckman 640U Spectrometer. Reactions were carried out at 25 °C with 0.5 mL solutions containing 50 mM K⁺HEPES (pH 7.5), 1 mM DNTB, a catalytic amount of thioesterase and varying concentrations of thioester ($0.5 - 5 \times K_m$). The enzyme-

catalyzed hydrolysis of 4-hydroxybenzoyl-CoA (4-HB-CoA) in 50 mM K⁺HEPES (pH 7.5) was directly monitored at 300 nm ($\epsilon = 11.8 \text{ mM}^{-1}\cdot\text{cm}^{-1}$).

Determination of Steady-state Kinetic Constants.—The initial rate data, measured as a function of substrate concentration, were analyzed using Enzyme Kinetics v 1.4 and equation 1:

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

where v is initial rate, $V_{\max}$ is maximum rate, $[S]$ is substrate concentration, and K_m is the Michaelis constant. The k_{cat} was calculated from $V_{\max}/[E]$ where $[E]$ is the total enzyme concentration.

Inhibition constants (K_i) were determined by measuring the initial rate at varying substrate concentration ($0.5 - 5 \times K_m$) and fixed, changing inhibitor concentration (0, K_i and $2 \times K_i$). The data analyzed using equation 2:

$$v = V_{\max}[S]/[K_m(1 + [I]/K_i) + [S]] \quad (2)$$

where $[I]$ is the concentration of inhibitor and K_i is the competitive inhibition constant.

Results and Discussion

Biochemical Characterization of the Target Thioesterase PA1618

PA1618 of *P. aeruginosa* is a hotdog-fold thioesterase which shares 52% sequence identity with the *E. coli* YdiI. In earlier work, we [9,23] and others [24] defined the biological function of YdiI as the 1,4-dihydroxynaphthoyl-CoA thioesterase of the menaquinone biosynthetic pathway. Bioinformatic analysis (see below) revealed that the *P. aeruginosa* genome does not encode the enzymes of the menaquinone pathway and therefore that PA1618 must perform a different biological function than its *E. coli* counterpart YdiI. On the other hand, a side-by-side comparison of the *in vitro* substrate specificity profile measured for PA1618 in this study with that of YdiI [9,23], shows that although these profiles are not identical they share notable likeness. Specifically, as indicated by the steady-state kinetic constants reported in Table 2 it is evident that although both thioesterases are somewhat promiscuous, each prefers aromatic thioester substrates, with benzoyl-CoA being an excellent *in-vitro* substrate for both enzymes. Our earlier mechanistic studies of *E. coli* YdiI employed benzoyl-CoA as substrate in ¹⁸O-solvent labeling experiments and transient kinetic analysis, and the inert ketomethylene benzoyl-CoA analog phenacyl-CoA in X-ray structure determinations, yet no clear distinction between nucleophilic catalysis and general-base catalysis was forthcoming [9]. This experience prompted us to invest in the development of a new effective mechanistic probe. To test this probe we selected PA1618 because it had not been previously characterized and because its host *P. aeruginosa* is an important human pathogen.

Inert Substrate Analog Design:

With rare exception, the replacement of the catalytic Glu or Asp with the isosteric Gln or Asn, does not remove all catalytic activity in thioesterases [9,10,12,25–27]. Indeed, the only example of an X-ray structure of a variant hotdog-fold thioesterase bound with substrate is that of the D17N variant of the 4-hydroxybenzoyl-CoA thioesterase from *Pseudomonas* sp strain CBS3 bound with 4-hydroxybenzoyl-CoA [28]. Thus, for structure determination an inert substrate analog is required. The two thioester analogs that have been used as crystallization ligands possess either a methylene ketone function ($\text{O}=\text{C}-\text{CH}_2-\text{SCoA}$) or simply a thiomethylene group (CH_2-SCoA) in place of the thioester moiety ($\text{O}=\text{C}-\text{SCoA}$). Both classes of analogs are inert to hydrolysis and both are relatively easy to synthesize. Unfortunately, neither analog constitutes the perfect catalytic-site probe. Unlike the native substrate, the thiomethylene adduct is unable to participate in the hydrogen bonding that takes place between the substrate thioester $\text{C}=\text{O}$ and the N-terminal residue of the conserved α -helix. This interaction has been shown to be essential to the orientation of the substrate for catalysis, as well as for generating binding energy [9,26,27,29]. Likewise, the use of the methylene ketone adduct introduces a CH_2 group to reaction center, and the accommodation of the added steric requirements in turn effects the alignment with the catalytic carboxylate residue, and the ability of the enzyme to bind the substrate water molecule.

The minimal structural change needed to render the hotdog-fold thioester substrate resistant to catalytic turnover is the substitution of the sulfur atom with an oxygen atom. The oxygen ester is intrinsically less susceptible to hydrolytic cleavage, and equally important, it is expected to conserve the space requirements and hydrogen bonding capabilities of the thioester. As a test case, we set out to synthesize the oxygen ester counterpart to the PA1618 substrate benzoyl-CoA and to carry out the structure determination of the liganded enzyme. This structure would be evaluated in a side-by-side comparison with the structure PA1618 bound with the methylene ketone analog, phenacyl-CoA.

Chemical Synthesis of OxydCoA Derivatives

Our first task was to develop an effective synthetic approach to the oxygen analog. Acyl-CoA analogs have been previously synthesized using a chemienzymatic approach. Martin *et al.* demonstrated that pantothenic kinase (PanK) and phosphopantetheine adenyltransferase (PPAT) could be readily used to synthesize acyl-CoA analogs featuring functionalization (methylene and nitro additions among others) in place of the thioester moiety [30,31]. Notably, although the pantothenic methyl ester was synthesized the corresponding CoA derivative, acetyl-OCOA was not. More recently, Dai *et al.* employed a chemienzymatic approach to synthesize crotonyl-OCOA from pantothenic acid [32]. In this approach, the pantothenic acid hydroxy groups were protected and ethanolamine was coupled to form oxypantethiene. Crotonic acid was used to acylate the terminal hydroxyl and the protecting groups were removed to form crotonyl-oxypantethiene. PanK, PPAT, and dephosphocoenzyme A kinase were used to make the final compound, crotonyl-OCOA [32].

Although the synthetic route used for crotonyl-OCOA synthesis is applicable to the synthesis of benzoyl-OCOA, we aimed to have a route with fewer steps and a commercially available precursor or at least one that is simple to prepare. Our synthesis, as outlined in Scheme 2,

provided compounds **4a-c** in quantities greater than 150 mg. To circumvent the need for protecting the pantothenic acid hydroxyl groups, we used *O*-acylated ethanolamines to couple to the pantothenic acid. *O*-benzylethanolamine is commercially available and *O*-acetyethanolamine can be synthesized from *N*-boc-ethanolamine and acetyl chloride. We used *O*-acetyethanolamine to show that the reaction can be used to make other derivatives. The yields of oxypantetheine, benzoyl-oxypantetheine, and acetyl-oxypantetheine ranged from 25–40%. The remaining reactions, catalyzed by PanK and PPAT, were carried out in the same manner used in the synthesis of crotonyl-OCOA^[32]. We chose to forego the final phosphorylation by dephosphocoenzyme A kinase because the phenacyl-CoA liganded structure of PA1618, which was determined first, indicated that the 3'-phosphate contributes minimally to binding (*vide infra*).

The X-ray Crystal Structure of Apo PA1618

The structure of the unliganded selenomethionine-substituted PA1618 was refined to a resolution of 1.72 Å in space group C222₁. The crystallographic asymmetric unit is comprised of six subunits- a dimer of dimers to form a tetramer assembled in a back-to-back manner (Figure 2A) and two monomers flanking the tetramer (Figure S3A). The structure of PA1618 represents a typical hotdog-fold in which each subunit is composed of six anti-parallel β-strands encompassing a five-turn α-helix with overall dimensions of ~22 Å × 22 Å × 40 Å. The anti-parallel β-sheets align in the order of β3- β4- β7- β8- β9- β6 as depicted in the secondary structure topology map (Figure S3B). The active sites of hotdog-fold enzymes are located at the interface of the homodimer, thus the dimer is the minimal catalytic unit. Typical of a hotdog-fold dimer, the two subunits associate along the edge of the β-sheet (β6 of one subunit to β6 of the adjacent subunit) to form an elongated 12-strand anti-parallel β-sheet. The intersubunit interactions involve mainly the α5-helix and β6-sheet as well as β2 and loop between β4-α5 which are held together by 18 hydrogen bonds and four salt bridges. The homotetrameric quaternary structure forms the biologically relevant assembly, as verified by FPLC size-exclusion chromatography and by assessment of buried surface area using PDBePISA^[33]. The tertiary and quaternary structures of PA1618 are typical for members of the AB clade to which PA1618 belongs, based on the prototypical member 4-hydroxybenzoyl-CoA thioesterase from *Arthrobacter sp.* strain AU (4HBT)^[34]. The structure of PA1618 is also conserved in comparison to the recently determined structures of *E. coli* homologues YdiI (4K4A) and YdbB (4K4C)^[9], where the biological unit is a tetramer and the dimer of dimers assemble in a back-to-back manner. A structural overlay of the dimers of the three homologs show similar overall structures as evidenced by the observed root mean square deviations (RMSDs) of 1.09 Å and 1.63 Å for PA1618 with YdiI and YdbB, respectively.

Liganded structures

Structures of PA1618 in complex with phenacyl-CoA and benzoyl-OdCoA were determined to a resolution of 1.6 Å and 1.72 Å, respectively. The crystals were isomorphous with one another and with the crystalline selenomethionine-substituted enzyme. Each shared the same arrangement and number of subunits in the asymmetric unit and thus, the same tetramer assembly. All four PA1618 active sites are occupied by ligand. For the benzoyl-OdCoA bound structure, there are two ligands clearly present in the tetramer, with one bound at each

dimer interface. Electron density for ligands in the remaining active sites was partially present but poor, thus the ligands were not modeled into these sites. For the purpose of simplicity, subunits C and D will be used for discussion for all structures. The electron density for the ligands in the liganded structures of phenacyl-CoA and benzoyl-OdCoA bound are shown in Figure 3A and 3B, respectively. Superimposition of the wild-type unliganded structure with the phenacyl-CoA and benzoyl-OdCoA bound structure showed a RMSD of 0.74 Å and the conservation of side chain positions and similar B-factors for residues located within 10 Å of the active-site (Figure S4). It should be noted that the low RMSD is biased by the fact that the unliganded structure was used as the isomorphous phasing model for the liganded structures.

In both liganded structures, the substrate analogs were positioned at the subunit interface of the functional dimer. The phenacyl-CoA bound structure was obtained to identify active site residues that contribute to the binding for substrate stabilization. Superposition of the structure of the PA1618 -phenacyl-CoA complex with that of the *E. coli* YdiI- phenacyl-CoA complex (PDB ID 4K4A), revealed perfect alignment of the phenacyl and pantetheine moieties. The residues involved in binding of the pantetheine arm include His91, Leu92, Arg93, Gly56, Leu53, Val83*, and Gly84* (* indicate residues of the opposing subunit). These substrate-binding residues are conserved in *E. coli* YdiI with the exception of Leu92, which is conservatively replaced by Val in YdiI. Backbone residues Arg93, Leu92, His91, Gly56, and Gly84* and side chains of Leu53, and Val83* form hydrogen bonds and van der Waals interactions with the pantetheine moiety. The pyrophosphate group aligned well with that of the YdiI phenacyl-CoA structure and makes favorable electrostatic interactions with the protein as indicated by the relatively low B-factors of the atoms. The pyrophosphate and nucleotide moiety lie at the surface of the protein located at the back-to-back dimer interface of the tetramer. Protein interactions with the pyrophosphate are made with the opposing dimer. The amide backbone of Arg111** and Thr112** and the side chain of His108** located at the loop between β5 and β6 form the noncovalent bonds that stabilize the pyrophosphate (** indicate residues of the opposing dimer). The ribosyl 3'-phosphate is also positioned near the same region in both structures and is within hydrogen bonding distance of His108** and a conserved Arg side chain (Arg93 in PA1618, Arg91 in YdiI) (Figure 3B). The adenosine nucleotide is located at the surface of the protein with no apparent binding interactions to the protein. Only one of the four liganded sites showed contiguous electron density for the nucleotide. An elongated loop (residues 41–64) between β4 and α9 serves as a lid over the substrate binding tunnel and provides solvent exclusion. The loop residues are highly conserved in YdiI with the exception of His48 (Lys47 in YdiI). However the loop is variable in conformation; a structure of YdiI in complex with undeca-2-one-CoA (PDB ID 4K4B) displayed a shift in the loop to accommodate the long acyl chain. The flexibility observed in this loop region is likely conserved in PA1618, thus allowing a range of substrates to bind to the active site, albeit at lower affinity compared to benzoyl-CoA. The PA1618 structure complexed with phenacyl-CoA was used to identify the catalytic residues responsible for the hydrolysis of the thioester. Like the prototypical 4HBT, PA1618 bears the homologous active-site carboxylate Glu64. The active-site variant PA1618 E64A was made and was shown to be catalytically inactive (Table 4). To insure that the mutation did not alter the structure, the X-ray crystal structure of the E64A variant was determined (Table 1) and

shown to be similar to that of the wild-type enzyme (RMSD 0.85 Å) with the exception of the replaced Glu64 side chain. In the 4HBT [27], a conserved glutamine is thought to stabilize the thiolate leaving group. Likewise, the corresponding Gln49 amide side chain in phenacyl-CoA bound PA1618 was positioned 3.6 Å from the sulfur atom. To examine the contribution to catalysis by the glutamine, the site directed mutant PA1618 Q49A was generated and shown to possess a 1000-fold reduced efficiency compared to wild type ($k_{\text{cat}}/K_M = 10^3 \text{ M}^{-1}\text{s}^{-1}$) (Table 4). The corresponding mutation made in the 4HBT abolished all activity [27]. The crystal structure of Q49A PA1618 was determined and it showed no global change in fold (RMSD 0.75 Å) compared to wild type. (Table 1). In PA1618, the His55 side chain and the backbone amide of Gly56 are positioned within hydrogen-bond distance (3.49 and 2.96 Å, respectively) of the substrate analog carbonyl oxygen a feature also characteristic of the AB clade thioesterase 4HBT.

Although the structure of PA1618 bound with phenacyl-CoA proved to be useful in the identification of substrate binding and catalytic residues, a “kinked” geometry is observed for the $-\text{CH}_2\text{-S-CH}_2\text{-C=O}$ bonds (Figure 3A), which may not provide an accurate picture of the reaction center in the enzyme-substrate complex. Therefore, the structure does not allow one to make the distinction between the role of the glutamate residue in general base catalysis vs. nucleophilic catalysis, using distance and relative orientation of the carboxylate group and ligand carbonyl carbon as criteria.

To circumvent the problem of having a spatially-distorted substrate configuration in the active site, the substrate mimic benzoyl-OdCoA was synthesized and used as a co-crystallization ligand in PA1618. Benzoyl-OdCoA was shown to be a competitive inhibitor (Figure S2) with a K_i that does not differ significantly from that of phenacyl-CoA (19 μM and 35 μM respectively). The PA1618 crystal structure with benzoyl-OdCoA was determined to 1.72 Å resolution (Table 1) (electron density map shown in Figure 3B). As expected, the configuration of the benzoyl-OdCoA near the ester moiety differed from that of phenacyl-CoA ester group in the corresponding complex.

The positions of the side chains of active-site residues are conserved in the two structures (Figure 3C left panel). The respective phenacyl-CoA and benzoyl-OdCoA pantetheine groups are similarly positioned and are held in place by the same hydrogen-bond network and set of van der Waals interactions. The positions of the benzene ring and carbonyl groups are also conserved (< 1.0 Å displacement). However, in the case of phenacyl-CoA the addition of the methylene group of the phenacyl-CoA ligand causes a “kink” with a *cis*-like configuration of the $-\text{CH}_2\text{-S-CH}_2\text{-C=O}$ as opposed to the geometry of the $-\text{CH}_2\text{-O-C=O-}$ group that is more representative of the planar geometry of the thioester. The relaxed/elongated conformation of the benzoyl-OdCoA allows the association of a water molecule (HOH171) 2.8 Å from the Glu64 carboxylate oxygen and ligand ester carbonyl carbon (Figure 3C right panel). The water molecule counterpart is not apparent in the structure of the phenacyl-CoA bound enzyme (Figure 3C middle panel).

In the case of the PA1618-phenacyl-CoA structure, the absence of the ordered water and the close juxtaposition of the Glu64 carboxylate and ligand carbonyl carbon could be interpreted as evidence for a role for Glu64 in nucleophilic catalysis. Indeed, the Glu64 carboxylate

oxygen atom is located within “striking distance” of the carbonyl carbon (3.7 Å) of the ligand and forms a 65.9° angle between the trigonal center of the phenacyl-CoA carbonyl and the O ϵ of the Glu64. The absence of an ordered water at the reaction site is probably due to steric hindrance by, and hydrophobicity of the phenacyl-CoA methylene group. Similarly, in the *E. coli* YdiI- and YbdB-phenacyl-CoA structures, the active-site Glu63 is positioned 3.9 Å and 4.1 Å, respectively, from the carbonyl carbon and an ordered water molecule is absent from the reaction site [9]. On the other hand, the results from ¹⁸O-labeling and transient kinetic experiments carried out earlier with YdiI neither supported or ruled-out Glu63 nucleophilic catalysis.

The structure of PA1618 bound to benzoyl-OdCoA and an ordered active-site water in the correct position, suggests that Glu64 serves as the general base, instead of as the catalytic nucleophile. The water, HOH171 (with a B-factor of 26.5 Å²), similar to that of surrounding active site residues (B-factors of 18 to 22 Å²) and the ester end of the ligand (B-factors of 22 to 31 Å²) (B-factors given for liganded chains C and D), is positioned with the correct geometry with respect to the carbonyl carbon of the CoASH, for a backside nucleophilic attack. The geometry of attack of the deprotonated water nucleophile, HOH171, to the trigonal carbonyl of the ligand is an angle of 95.5°. The involvement of a water nucleophile in the displacement of the thiolate-leaving group is consistent with the reduced dependence of the catalytic efficiency on the participation of the active-site Gln. As pointed out earlier the 4HBT, which is known to catalyze a two-step reaction in which the catalytic Glu displaces the thiolate, is inactivated by amino-acid replacement of the assisting Gln residue, compared to the partial loss of activity observed with the PA1618 Gln49 mutation. The inclusion of the water molecule in the transition state of the concerted general-base catalyzed thioester hydrolysis provides stabilizing hydrogen bond formation with the thiolate-leaving group.

In summary, we have shown that the hotdog fold thioesterase PA1618, orthologous to the *E. coli* DHNA-CoA thioesterase YdiI, catalyzes the hydrolysis of thioesters *in vitro* with preference for aromatic substrates. In addition we have provided a simple route, requiring little to no use of protecting groups, for the synthesis of acyl-OdCoA compounds. The benzoyl-OdCoA ester was used in the crystallization of PA1618 and was demonstrated to be superior as an isosteric ligand as compared to the thioether phenacyl-CoA. The structure with the ester analog afforded evidence for a general base mechanism of catalysis where Glu64 activates the water nucleophile. Acyl-OCOA analogs could enable the elucidation of mechanisms of other acyltransferases acting on thioester substrates e.g. as found in polyketide biosynthesis or fatty-acid biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

* This work was supported in part by NIH grant R01-GM028688 to DDM.

Abbreviations used include:

Listed in the order they appear:

4HBT	<i>Arthrobacter sp.</i> strain SU 4-HB-CoA thioesterase
CoA	coenzyme A
ACP	acyl carrier protein
PA1618	<i>Pseudomonas aeruginosa</i> 1618 thioesterase
Phenacyl-CoA	phenacyl-coenzyme A
Benzoyl-OdCoA	benzoyl-oxydephosphocoenzyme A
Benzoyl-CoA	benzoyl-coenzyme A
PCR	polymerase chain reaction
DNA	Deoxyribonucleic acid
LB media	Luria Broth media
IPTG	Isopropyl β -D-1-thiogalactopyranoside
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SeMet	selenomethionine
PEG400	polyethylene glycol 400
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)

References

- [1]. Hunt MC, Alexson SEH, Prog. Lipid Res 2002, 41, 99–130. [PubMed: 11755680]
- [2]. Resh MD, Cell. Signal 1996, 8, 403–12. [PubMed: 8958442]
- [3]. Hertz R, Magenheimer J, Berman I, Bar-tana J, Nature 1998, 392, 512–516. [PubMed: 9548258]
- [4]. Ordine RLD, Bahnson BJ, Tonge PJ, Anderson VE, Biochemistry 1994, 33, 14733–14742. [PubMed: 7993901]
- [5]. Heath RJ, Rock CO, Nat. Prod. Rep 2002, 19, 581–596. [PubMed: 12430724]
- [6]. Amyes TL, Richard JP, J. Am. Chem. Soc 1992, 114, 10297–10302.
- [7]. Schmelz S, Naismith JH, Curr. Opin. Struct. Biol 2009, 19, 666–71. [PubMed: 19836944]
- [8]. Song F, Thoden JB, Zhuang Z, Latham J, Trujillo M, Holden HM, Dunaway-Mariano D, Biochemistry 2012, 51, 7000–7016. [PubMed: 22873756]
- [9]. Wu R, Latham JA, Chen D, Farelli J, Zhao H, Matthews K, Allen KN, Dunaway-Mariano D, Biochemistry 2014, 53, 4788–805. [PubMed: 25010423]
- [10]. Song F, Zhuang Z, Finci L, Dunaway-Mariano D, Kniewel R, a Buglino J, Solorzano V, Wu J, Lima CD, J. Biol. Chem 2006, 281, 11028–38. [PubMed: 16464851]
- [11]. Zhuang Z, Latham J, Song F, Zhang W, Trujillo M, Dunaway-Mariano D, Biochemistry 2012, 51, 786–94. [PubMed: 22208697]
- [12]. Cao J, Xu H, Zhao H, Gong W, Dunaway-Mariano D, Biochemistry 2009, 48, 1293–304. [PubMed: 19170545]

- [13]. Thoden JB, Zhuang Z, Dunaway-Mariano D, Holden HM, J. Biol. Chem 2003, 278, 43709–43716. [PubMed: 12907670]
- [14]. Knobbe CB, Reifenger J, Blaschke B, Reifenger G, J. Natl. Cancer Inst 2004, 96, 483–6. [PubMed: 15026474]
- [15]. King KL, Young ME, Kerner J, Huang H, O'Shea KM, Alexson SEH, Hoppel CL, Stanley WC, J. Lipid Res 2007, 48, 1511–7. [PubMed: 17438340]
- [16]. Guo Z-F, Sun Y, Zheng S, Guo Z, Biochemistry 2009, 48, 1712–22. [PubMed: 19193103]
- [17]. Merkel SM, Eberhard a E., Gibson J, Harwood CS, J. Bacteriol 1989, 171, 1–7. [PubMed: 2914844]
- [18]. Luo L, Taylor KL, Xiang H, Wei Y, Zhang W, Dunaway-Mariano D, Biochemistry 2001, 40, 15684–15692. [PubMed: 11747444]
- [19]. Zhao H, Martin BM, Bisoffi M, Dunaway-Mariano D, Biochemistry 2009, 48, 5507–9. [PubMed: 19453107]
- [20]. Y. (2010) Gonzalez A and Tsai, “A Quick XDS Tutorial for SSRL,” n.d.
- [21]. Adams PD, V Afonine P, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung L-W, Kapral GJ, Grosse-Kunstleve RW, et al., Acta Crystallogr. D. Biol. Crystallogr 2010, 66, 213–21. [PubMed: 20124702]
- [22]. Emsley P, Lohkamp B, Scott WG, Cowtan K, “Features and development of Coot,” 2010.
- [23]. Latham JA, Chen D, Allen KN, Dunaway-Mariano D, Biochemistry 2014, 53, 4775–4787. [PubMed: 24992697]
- [24]. Chen M, Ma X, Chen X, Jiang M, Song H, Guo Z, J. Bacteriol 2013, 195, 2768–75. [PubMed: 23564174]
- [25]. Zhuang Z, Song F, Takami H, Dunaway-Mariano D, J. Bacteriol 2004, 186, DOI 10.1128/JB.186.2.393.
- [26]. Zhao H, Lim K, Choudry A, Latham JA, Pathak MC, Dominguez D, Luo L, Herzberg O, Dunaway-Mariano D, Biochemistry 2012, 51, 6490–2. [PubMed: 22871024]
- [27]. Song F, Thoden JB, Zhuang Z, Latham J, Trujillo M, Holden HM, Dunaway-Mariano D, Biochemistry 2012, 51, 7000–16. [PubMed: 22873756]
- [28]. Thoden JB, Holden HM, Zhuang Z, Dunaway-Mariano D, J. Biol. Chem 2002, 277, 27468–76. [PubMed: 11997398]
- [29]. Thoden JB, Zhuang Z, Dunaway-Mariano D, Holden HM, J. Biol. Chem 2003, 278, 43709–43716. [PubMed: 12907670]
- [30]. Martin DP, Drueckhammer DG, 1992, 14–15.
- [31]. Martin DP, Bibart RT, Drueckhammer DG, 1994, 12, 4660–4668.
- [32]. Dai M, Feng Y, Tonge PJ, J. Am. Chem. Soc 2001, 123, 506–507. [PubMed: 11456558]
- [33]. Krissinel E, Henrick K, J. Mol. Biol 2007, 372, 774–797. [PubMed: 17681537]
- [34]. Zhuang Z, Gartemann K-H, Eichenlaub R, Dunaway-Mariano D, Appl. Environ. Microbiol 2003, 69, 2707–2711. [PubMed: 12732540]

Figure 1.

EsPript 3.0 generated image of the sequence alignment between PA1618, *E. coli* YdiI and *E. coli* YbdB showing the secondary structure (generated from PDB ID: 4KUQ), conserved residues (white font) and similar residues (red font). The catalytic residues Glu64 and Gln49 are conserved.

Figure 2. Overall structure of PA1618. **A)** Ribbon representation of the tetrameric PA1618 structure with subunits A, B, C, D labeled on the right. **B)** Tetrameric PA1618 bound to phenacyl-CoA (left); inset (right) shows the residues involved in binding and stabilization of the pyrophosphate moiety of the bound ligand.

Figure 3.

Active sites of ligand-bound PA1618. **A)** phenacyl-CoA Benzoyl-OdCoA and **B)** bound structure of subunit A and B on left and binding site showing the protein molecular surface displayed as a semitransparent surface covering a ribbon diagram of wt PA1618. The ligand excluded Fo-Fc electron density for the fully occupied CoA molecule is shown as a grey mesh and is contoured at the 1 σ level. **C)** Superimposition of bound benzoyl-dOCOA and phenacyl-CoA with catalytic residue side chains shown in sticks (left), active site of phenacyl-CoA ligand bound (center), and active site of benzoyl-OdCoA ligand bound (right). All H-bond interaction distances between side chains and ligand are measured in Angstroms.

Scheme 1.

Hotdog fold thioesterases can catalyze the hydrolysis of thioesters (A) by nucleophilic catalysis where the carboxylate side chain oxygen (blue) or water (red) is transferred to the organic acid (the complete transfer of the carboxylate oxygen is shown), or by (B) base catalysis where the water oxygen (red) is transferred to the organic acid. C) Truncated structural representation of benzoyl-CoA (top), benzoyl-OCOA (center) and phenacyl-CoA (bottom).

Scheme 2.

Synthesis of the acyl-OCoAs **4a-c** from pantothenic acid and O-acylethanolamine.

Table 1.

Crystallographic data collection and refinement statistics for PA1618 X-ray structures.

	PA1618 (SeMet)	PA1618 + PA-CoA	PA1618 + Benzoyl-OdCoA	Q49A variant	E64A variant
PDB code	4QD7	4QD8	4QD9	4QDB	4QDA
wavelength	0.979	1.03	0.980	1.03	0.980
resolution (Å) ^a	37.61 – 1.72 (1.81 – 1.72)	47.79 – 1.62 (1.7 – 1.62)	37.53 – 1.72 (1.82 – 1.72)	47.64 – 2.02 (2.10 – 2.02)	67.93 – 2.30 (2.38 – 2.30)
space group	C222 ₁	C222 ₁	C222 ₁	C222 ₁	C222 ₁
unit cell a, b, c (Å)	101.65 202.51 90.99	101.69 202.01 91.08	103.03 201.49 91.74	100.96 201.56 90.92	99.72 202.61 91.57
R _{merge} ^{ab} (%)	0.069 (0.76)	0.047 (0.82)	0.078 (0.72)	0.091 (0.94)	0.07 (0.55)
completeness ^a	97.94 (82.18)	99.18 (99.70)	97.85 (82.67)	98.99 (99.62)	97.57 (99.37)
I/I(σ) ^a	35.97 (15.21)	12.28 (1.54)	18.23(2.65)	8.12 (1.58)	21.77 (5.28)
redundancy ^a	6.5 (5.5)	4.4 (4.3)	12.6(10.7)	4.4 (4.4)	14.4 (14.1)
total/unique reflections	626184 / 97075	519112 / 118644	1238418 / 98312	60156 / 5992	598702 / 4073
R _{cryst} / R _{free} ^c (%)	19.73/20.94	20.51/23.82	18.87/22.37	20.32/23.27	22.12/27.67
RMSD (bond length (Å))	0.008	0.007	0.007	0.007	0.008
RMSD (bond angles (°))	1.17	1.32	1.19	1.09	1.08
Ramachandran favored (%)	97.63	98.18	98.29	97.93	97.26
Ramachandran allowed (%)	2.37	1.82	1.71	2.07	2.74
average B-factors (Å ²)	28.00	26.10	23.7	34.00	43.50
macromolecule	27.40	25.10	22.5	33.90	43.60
inhibitor	---	37.30	40.86	---	---
waters	35.20	33.10	32.5	36.20	40.30

^aValues for the highest resolution shell are given in parentheses.^b $R_{\text{merge}} = \sum |I_i - I_m| / \sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry-related reflections.^c $R_{\text{cryst}} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$, where F_{obs} and F_{calc} are observed and calculated structure factors. $R_{\text{free}} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$, where T is a test data set of 5% of the total reflections randomly chosen and set aside prior to refinement.

Table 2.

Steady state kinetic parameters with the sum of squared errors of prediction of PA1618-catalyzed hydrolysis of various acyl-CoA and aryl-CoA substrates at pH 7.5 and 25 °C. See Materials and Methods for details.

Substrate	k_{cat} (s^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1} \text{s}^{-1}$)
Acetyl-CoA	$(4.3 \pm 0.1) \times 10^{-1}$	116 ± 8	3.7×10^3
Hexanoyl-CoA	$(1.1 \pm 0.1) \times 10^{-1}$	20 ± 2	5.2×10^3
Lauroyl-CoA	$(2.8 \pm 0.1) \times 10^{-1}$	60 ± 0.2	4.7×10^3
Myristoyl-CoA	$(9.1 \pm 0.2) \times 10^{-1}$	150 ± 30	5.8×10^3
Oleoyl-CoA	$(3.9 \pm 0.1) \times 10^{-1}$	7 ± 6	1.1×10^4
Benzoyl-CoA	56 ± 1	12 ± 1	4.6×10^6
4-Hydroxybenzoyl-CoA	9.3 ± 0.2	3.6 ± 0.3	3.1×10^6
1,4-Dihydroxynaphthoyl-CoA	$(5.5 \pm 0.1) \times 10^{-1}$	2.1 ± 0.1	2.8×10^5
Coumaroyl-CoA	2.5 ± 0.1	2.7 ± 0.2	9.3×10^5

Table 3.

Steady-state inhibition constants with sum of squared errors of prediction for competitive substrate analog and product inhibitors of PA1618 hydrolysis of benzoyl-CoA at pH 7.5 and 25 °C. See Materials and Methods for details.

	PA1618 K_i (μ M)
Phenacyl-CoA	35 ± 5
Undecanyl-CoA	3.4 ± 0.3
2,4-Dihydroxyphenacyl-CoA	12 ± 1
Benzoyl-OdCoA	19 ± 3

Table 4.

Steady state kinetic parameters with the sum of squared errors of prediction of variant PA1618-catalyzed hydrolysis of benzoyl-CoA at pH 7.5 and 25 °C. See Materials and Methods for details.

PA1618	k_{cat} (s^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$)
Wild-type	56 ± 1	12 ± 1	4.6×10^6
E64A	$<1 \times 10^{-4}$	-----	-----
Q49A	$(2.2 \pm 0.3) \times 10^{-2}$	5.0 ± 0.3	4.4×10^3