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Structure and activity of the *Pseudomonas aeruginosa* hotdog-fold thioesterases PA5202 and PA2801

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Abstract

The hotdog fold is one of the basic protein folds widely present in bacteria, archaea, and eukaryotes. Many of these proteins exhibit thioesterase activity against fatty acyl-CoAs and play important roles in lipid metabolism, cellular signaling, and degradation of xenobiotics. The genome of the opportunistic pathogen *Pseudomonas aeruginosa* contains over 20 genes encoding predicted hotdog-fold proteins, none of which have been experimentally characterized. We have found that two *P. aeruginosa* hotdog proteins display high thioesterase activity against 3-hydroxy-3-methylglutaryl-CoA and glutaryl-CoA (PA5202), and octanoyl-CoA (PA2801). Crystal structures of these proteins were solved (1.70 and 1.75 Å) and revealed a hotdog fold with a potential catalytic carboxylate residue located on the long alpha helix (Asp57 in PA5202 and Glu35 in PA2801). Alanine replacement mutagenesis of PA5202 identified four residues (Asn42, Arg43, Asp57, and Thr76), which are critical for activity and are located in the active site. A *P. aeruginosa* PA5202 deletion strain showed an increased secretion of the antimicrobial pigment pyocyanine and an increased expression of genes involved in pyocyanin biosynthesis suggesting a functional link between the PA5202 activity and pyocyanin production. Thus, the *P. aeruginosa* hotdog thioesterases PA5202 and PA2801 have similar structures, but exhibit different substrate preferences and functions.

Keywords

hotdog fold; thioesterase; crystal structure; pyocyanin; *Pseudomonas aeruginosa*

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Author Contribution: Claudio Gonzalez, Anatoli Tchigvintsev, Greg Brown and Robert Flick carried out the biochemical studies. Elena Evdokimova and Xiaohui Xu performed the crystallization experiments. Susan Lynch provided the *P. aeruginosa* PA5202 deletion strain. Jerzy Osipiuk, Marianne Cuff, Andrzej Joachimiak, and Alexei Savchenko solved and refined the crystal structures. Claudio Gonzalez and Alexander Yakunin designed the research, analyzed the data and wrote the manuscript.

Introduction

Thioesterases (thioester hydrolases, EC 3.1.2) are ubiquitous and diverse enzymes with essential roles in various processes including fatty acid, polyketide, and non-ribosomal peptide biosynthesis, acyl-CoA turnover, bioluminescence, signal transduction and removal of acyl chains from post-translationally modified proteins [1-7]. These enzymes are widespread in eukaryotes, bacteria, and archaea (4,092 sequences in the InterPro database, IPR001031).

Thioesterases are classified into two families (I and II) based on their distinct structural folds, amino acid sequence and different catalytic mechanisms. Classical α -hydrolase-fold thioesterases belong to family I [8], whereas the family II thioesterases have a “hotdog” fold, and their active sites usually contain either an Asp or Glu residue that functions as a general base [9-12]. The hotdog fold is described as an anti-parallel β -sheet (a “bun”) wrapped around a five-turn α -helix (a “sausage”) [13]. Most characterized hotdog-fold proteins possess thioesterase or thiol ester dehydratase activities [14, 15]. The hotdog fold thioesterases hydrolyze acyl-CoA thioesters to free fatty acids and CoASH, as well as the acyl thioesters of acyl-carrier proteins (acyl-ACP). A comprehensive sequence analysis of 1,357 hotdog fold proteins clustered them into 85 groups [14]. For 17 subfamilies, a conserved sequence motif was identified and some functional or structural information was found, whereas the remaining groups contained predominantly uncharacterized proteins [14].

The biochemically and structurally characterized hotdog-fold thioesterases includes: 4-hydroxybenzoyl-CoA (4HBA-CoA) thioesterases from *Pseudomonas* sp. strain CBS-3 [16, 17] and *Arthrobacter* sp. strain SU [12], phenylacetyl-CoA thioesterases PaaI from *Escherichia coli* [18] and *Thermus thermophilus* [19], Rv0098 from *Mycobacterium tuberculosis* [20], thioesterase II (TesB) from *E. coli* [9, 10, 21], and human THEM2 (hTHEM2) [22]. The characterized hotdog thioesterases have either a hexameric (trimer of dimers) or tetrameric organization (dimer of dimers) with two dimers connected through the interaction of their β -sheets (a “back-to-back” association, as in the *Arthrobacter* 4HBA-CoA thioesterase) or their α -helices (a “face-to-face” association, as in the *Pseudomonas* 4HBA-CoA thioesterase) [23-25]. The structures of the complexes of several hotdog thioesterases with their substrates or inhibitors have been determined and revealed the location of the active site residues and substrate binding sites [12, 17, 19, 22]. Two models have been proposed for the catalytic mechanism of hot dog thioesterases. Structural studies with the *Pseudomonas* 4HBA-CoA thioesterase revealed that the catalytic Asp17 is located close to the thioester carbonyl carbon and therefore might function as a nucleophile in the reaction, which proceeds via an acyl-enzyme intermediate [17]. However, the structures of the *T. thermophilus* PaaI (TtPaaI) and hTHEM2 complexed with the substrate analogs indicate that the catalysis likely proceeds via a general base-catalyzed hydrolysis with the catalytic carboxylate residue acting as a general base to activate the attacking water [19, 22]. For the TtPaaI thioesterase, an asymmetric induced fit mechanism was proposed where only two of the four active sites of the tetramer bind the substrate (“half-of-the-sites reactivity”) [19]. However, there is no evidence for the presence of this mechanism in other hotdog thioesterases.

Presently, the vast majority of hotdog proteins remain uncharacterized, and their substrate specificities remain unknown. Both prokaryotic and eukaryotic organisms produce numerous acyl-CoA thioesters (over 40 acyl-CoAs are listed on the *E. coli* database EcoCyc, <http://ecocyc.org>) which are intermediates of various biosynthetic and catabolic pathways and represent potential substrates for thioesterases. The genome of the opportunistic pathogen *Pseudomonas aeruginosa* encodes at least 23 predicted hotdog-like proteins

(Suppl. Table 1), all of which remain biochemically uncharacterized. Here, we present the results of the structural and biochemical characterization of two hotdog-fold proteins from this organism, PA5202 and PA2801, which demonstrated significant thioesterase activity *in vitro*. Their crystal structures were determined, and PA5202 was further characterized using site-directed mutagenesis and gene knock-out approaches revealing the first thioesterase with a preference to 3-hydroxy-3-methylglutaryl-CoA and glutaryl-CoA.

Experimental

Gene cloning, mutagenesis, and protein purification

Chromosomal DNA was isolated using a DNeasy miniprep kit (Qiagen), whereas plasmids and PCR products were purified using miniprep and Qiaquick purification kits (Qiagen). The genes encoding PA1835, PA2801, PA5026, PA5185, and PA5202 were PCR amplified and cloned into a modified pET15b vector (Novagen) containing an N-terminal 6His-tag followed by a tobacco etch virus protease cleavage site (ENLYFQ:G). Over-expression plasmids were transformed into the *E. coli* BL21 (DE3) Gold strain (Stratagene). Site directed mutagenesis (alanine replacement) was performed using the QuikChange™ site directed mutagenesis kit as previously described [26]. The *P. aeruginosa* mutant strain PA8379 (obtained by transposon insertion) was generously provided by the University of Washington Genome Center (UWGC). The transposon insertion site was verified using the UWGC protocol [27]. PA1835, PA2801, PA5026, PA5185, and PA5202 were overexpressed in *E. coli* and purified using metal-chelate affinity chromatography on nickel affinity resin (Qiagen) with high yield (>100 mg/liter of culture) and homogeneity (>95%) as described previously [26]. The oligomeric state of the purified proteins was determined by gel-filtration on a Superdex 200 10/300 column (GE Healthcare) equilibrated 50 mM HEPES-K buffer (pH 7.5) and 250 mM NaCl using an AKTA FPLC (GE Healthcare). Retention time of the proteins was used to estimate the relative molecular mass of the proteins via linear regression using ribonuclease A (13.7 kDa), ovalbumin (43 kDa), and aldolase (158 kDa) as standards.

Enzymatic assays

Purified hotdog-fold proteins were screened for the presence of thioesterase activity against a set of 27 commercially available (Sigma) acyl-CoA thioesters (Suppl. Table 2). The screening reactions were performed in 96-well plates at 37°C using a previously described method [28]. Thioesterase activity of PA5202 against 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and other substrates was measured spectrophotometrically in triplicates in 96-well plates at 37°C in a reaction mixture (100 µl final volume) containing 50 mM HEPES-K buffer (pH 8.0), 2 mM EDTA, 0.3 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent), 0.6 – 1.0 mM substrate, and 0.1 – 0.4 µg of protein [28]. Reactions were continuously monitored by absorbance at 412 nm over 10 minutes; the amount of thiol groups hydrolyzed was determined using the DTNB $\epsilon_{412\text{nm}} = 13.6 \text{ M}^{-1} \text{ cm}^{-1}$. For determination of K_m and V_{max} , thioesterase activity was determined over a range of substrate concentrations (between 0.05 and 1 mM for HMG-CoA or glutaryl-CoA and up to 2 mM for other substrates). Kinetic parameters were calculated by non-linear regression analysis of raw data fit to the Michaelis-Menten function using GraphPad Prism software (version 4.00 for Windows, GraphPad Software, San Diego, CA).

Growth experiments and pigments production

Growth kinetics and pigment production were evaluated using 250-ml Erlenmeyer flasks containing 25 ml of LB medium and incubated at 37°C with aeration (250 rpm). Cell growth was monitored by measuring optical density at 600 nm ($\text{OD}_{600\text{nm}}$). Cultures were inoculated with cells from overnightgrown LB cultures (the starting $\text{OD}_{600} = 0.1$). Pyocyanin was

quantified spectrophotometrically by absorbance at 700 nm ($\epsilon = 4,310 \text{ M}^{-1} \text{ cm}^{-1}$) and purified as previously described [29, 30]. The ability of cells to grow in a variety of carbon sources was evaluated using MOPS-buffered minimal medium [31] supplemented with selected carbon sources (0.2% final concentration). The experimental cultures were inoculated with cells from an overnight LB-grown culture (initial $\text{OD}_{600}=0.05$) and grown in 250-ml Erlenmeyer flasks containing 25 ml of culture at 37 °C with aeration (220 rpm).

Quantitative Real Time PCR (qRT-PCR)

The expression of genes encoding selected enzymes related to ketone metabolism was quantified by qRT-PCR using protocols and procedures previously described [26]. Briefly, culture samples were collected from exponentially growing cells ($\text{OD}_{600} \sim 0.6\text{--}0.7$), mRNA extraction was carried out using a Ribopure® extraction kit (Ambion), and cDNA synthesis was performed with a Superscript first-strand synthesis kit (Invitrogen). The qRT-PCR experiments were carried out on an Applied Biosystems 7300 apparatus (Applied Biosystems) using SYBR Green qPCR SuperMix UDG (Invitrogen). All samples were normalized to *recA* expression as a control. The relative expression values (Rex) were estimated by determination of mRNA abundance compared to the wild type strain under the same culture conditions as previously described [30].

Protein crystallization

Crystals of selenomethionine (SeMet)-substituted PA2801 were grown at 22 °C using the hanging drop vapor diffusion method. 2 μl of protein sample (21.6 mg/ml) was mixed with an equal volume of reservoir solution as previously described [32]. Crystals appeared after several days in the presence of 0.2 M calcium chloride (pH 5.1) and 28% PEG-3350. The crystals were transferred to a reservoir solution containing 16% glycerol as a cryoprotectant and then mounted on the beam. Crystals of SeMet-substituted PA5202 were grown using crystallization solution containing 0.1 M Mg-acetate, 0.1 M Tris-HCl (pH 8.5), 20% PEG-3350, and 2% MPD. 25% ethylene glycol was used as a cryoprotection agent.

Structure determination

Diffraction data were collected using the SBC-Collect program at the 19-BM and 19-ID beamlines of the Structural Biology Center at the Advanced Photon Source [33]. Data were integrated and scaled using the HKL2000 software package [34]. The structures of PA5202 and PA2801 were determined by MAD (multi-wavelength anomalous diffraction) or SAD (single wavelength anomalous diffraction) phasing, respectively, using the HKL2000 suite incorporating the following programs: SHELXC, SHELXD, SHELXE, MLPHARE, and SOLVE/RESOLVE [34, 35]. The initial protein models were further processed with ARP/wARP software [36], with final models built using the program COOT [37] and refined with the REFMAC5 program of the CCP4 suite [38, 39]. Full-length PA5202 was used as one TLS (Translation/Libration/Screw motion) group, whereas for PA2801, five TLS groups were determined by the TLSMD server [40]. The quality of the structures was checked using the validation tools of COOT [37] and Molprobity [41]. All residues with the exception of Asp27 in chain B of PA5202 were within acceptable regions of the Ramachandran plot. A summary of data collection and refinement statistics is shown in Table 1.

Protein Data Bank Accession Codes

Coordinate and structure factors have been deposited to PDB with accession codes 1ZKI (PA5202) and 3QY3 (PA2801).

Results and Discussion

Thioesterase screen of five purified *P. aeruginosa* hotdog-fold proteins

As the first step for the biochemical characterization of *P. aeruginosa* hotdog-fold proteins, we selected the five proteins for which the crystal structures were solved by the Midwest Center for Structural Genomics: PA1835 (PDB code 1YOC), PA2801 (2ALI), PA5026 (1SH8), PA5185 (2AV9), and PA5202 (1ZKI). To reveal the biochemical activity and *in vitro* substrates of these proteins, they were purified and screened for thioesterase activity against a set of 27 various CoA-thioesters (Suppl. Table 2). In these screens, PA5202 was the most active enzyme (specific activity over 2 μ moles/min per mg protein) and showed thioesterase activity against glutaryl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), and succinyl-CoA, as well as toward several other substrates (Fig. 1A). PA2801 exhibited high thioesterase activity against octanoyl-CoA and glutaryl-CoA and significant activity toward lauroyl-CoA, hexanoyl-CoA, and hydroxybutyryl-CoA (Fig. 1B). PA5185 showed significant thioesterase activity only against propionyl-CoA (Fig. 1C), whereas no hydrolytic activity against the available thioester substrates was found in PA1835 and PA5026 (data not shown).

Based on amino acid sequence, PA5202, PA2801 and PA5185 share a low overall sequence similarity (14–31 % sequence identity) and belong to two different hotdog subfamilies (Fig. 2). PA5202 is a member of the PaaI subfamily (subfamily-11; predicted thioesterases), whereas PA2801 and PA5185 belong to the YbaW subfamily (subfamily-16, unknown proteins) [14, 25]. However, it has been mentioned that the consensus sequence motifs are not well conserved in hotdog-like proteins [25]. Sequences of PA5202, PA2801, and PA5185 show the presence of a potential catalytic carboxylate (Asp57 in PA5202, Glu35 in PA2801, and Asp41 in PA5185) located on the longest α -helix (4 in PA5202 and 2 in PA2801) (Fig. 2). Recent bioinformatic and experimental studies of hotdog-fold proteins disclosed several quaternary associations including dimers, tetramers with helix/helix interactions (“face-to-face”), tetramers with β -sheet/ β -sheet interactions (“back-to-back”), and three types of hexamers [14, 22, 25]. Analysis of the oligomeric state of the *P. aeruginosa* proteins using gel-filtration revealed a tetrameric organization of active hotdog-fold proteins (PA2801, PA5185, and PA5202), whereas the proteins without thioesterase activity (PA1835 and PA5026) were found to exist as dimers in solution (Suppl. Table 3). PA1835 and PA5026 belong to the hotdog subfamily-13 (YiiD-like), which includes the *E. coli* YiiD protein with the acetyltransferase and hotdog domains fused together, but no enzymatic activity has been reported for its hotdog domain [25]. Thus, the hotdog-fold proteins PA2801, PA5185, and PA5202 from *P. aeruginosa* are thioesterases with different *in vitro* substrate preferences. Of the three proteins, PA5202 showed the highest specific activity under the screening conditions, whereas PA2801 exhibited the highest level of substrate promiscuity. Although the three proteins showed different preferred *in vitro* substrates, they shared at least two secondary substrates: hydroxybutyryl-CoA and acetyl-CoA (Fig. 1).

To our knowledge, PA5202 represents the first hotdog-fold thioesterase with a preference for the polar aliphatic acyl-CoAs: glutaryl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Glutaryl-CoA has been shown to be an intermediate of the degradation of benzoate, pyridines, tryptophan, or lysine [42, 43]. HMG-CoA has been shown to be a key intermediate of the mevalonate pathway, which produces precursors of various isoprenoids (sterols, cholesterol, dolichols, triterpenes, ubiquinone) and prenyl groups for the C-prenylation of proteins or various aromatic natural products including phenazines [44]. Presently, it is unclear if the mevalonate pathway is present in *P. aeruginosa* [45]. HMG-CoA is also an intermediate of the synthesis and degradation of ketone bodies (acetoacetate, β -hydroxybutyrate, acetone) and several amino acids (L-valine, L-isoleucine, and L-leucine)

[46]. Fifty years ago, the presence of thioesterase activity against HMG-CoA was demonstrated in extracts from *E coli*, *Neurospora crassa*, *Tetrahymena pyriformis* and mammalian tissues [47]. However, a HMG-CoA-specific thioesterase has never been purified and characterized.

To determine if the hydrolytic activity of PA5202 against HMG-CoA or glutaryl-CoA is physiologically pertinent, its thioesterase activity was further characterized. This enzyme showed maximal activity at alkaline pH (8.5 to 9.5) and was stimulated by low concentrations of Mg^{2+} , Mn^{2+} , and Ca^{2+} (0.1 mM; 80%, 60%, and 45% stimulation, respectively), whereas low concentrations (0.01 mM) of Cu^{2+} and Zn^{2+} were inhibitory (20% of residual activity). In addition, the thiol inhibitors N-ethylmaleimide and iodoacetate (1 mM) completely inhibited the activity of PA5202. Under optimal reaction conditions, PA5202 exhibited classical Michaelis-Menten kinetics with hyperbolic saturation curves for all positive substrates (Suppl Fig. 1). The steady-state kinetic constants measured for the acyl-CoA and aryl-CoA thioesters as substrates are summarized in Table 2. The protein demonstrated high catalytic activity toward glutaryl-CoA ($k_{cat} = 26.8 \text{ s}^{-1}$) and HMG-CoA ($k_{cat} = 3.9 \text{ s}^{-1}$), but substrate affinity was higher to HMG-CoA ($K_m = 0.1 \text{ mM}$). Substrate affinity of PA5202 was higher to stearoyl-CoA ($K_m = 5 \text{ }\mu\text{M}$) or palmitoyl-CoA ($K_m = 23 \text{ }\mu\text{M}$), but the catalytic activity was much lower than that with HMG-CoA (Table 2). Therefore, PA5202 exhibits a high catalytic efficiency with glutaryl-CoA and HMG-CoA *in vitro* ($k_{cat}/K_m = 0.3 - 0.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). Both thioesters are known to be metabolized by HMG-CoA reductase (EC 1.1.1.34; K_m range 3 - 60 μM), HMG-CoA hydrolase (EC 4.2.1.18; K_m range 7 - 10 μM), HMG-CoA lyase (EC 4.1.3.4; K_m range 0.02 - 3.1 mM) and glutaryl-CoA dehydrogenase (EC 1.3.99.7; K_m range 10 - 50 μM) (BRENDA database, <http://www.brenda.uni-koeln.de/>). No information is presently available on the intracellular concentration of acyl-CoA thioesters in *Pseudomonas*, but in *E coli* it has been shown to vary in the range of 4 - 600 μM depending on growth conditions [48-50]. Thus, both HMG-CoA and glutaryl-CoA thioesterase activities of PA5202 are likely to be physiologically significant.

Crystal structures of PA5202 and PA2801

PA5202 and PA2801 were crystallized using a hanging drop method, and their crystal structures were determined at 1.7 Å and 1.75 Å resolution, respectively, using MAD (for PA5202) or SAD (for PA2801) methods (Table 1). The crystal structure of PA5185 was reported previously [51]. Both PA5202 and PA2801 protomers have a classical hotdoglike fold with an anti-parallel β -sheet (a “bun”) wrapped around a long α -helix (a “sausage”) (Fig. 3A, 3B). In the PA5202 structure, three additional, short α -helices (α -1, α -2, and α -3) cover the long α -helix α -4, whereas in PA2801 two short α -helices (α -1 and α -3) are positioned near two opposite ends of the β -sheet, close to the ends of the long α -2 helix (Fig. 3A, 3B). The order of the secondary structure elements in the PA5202 protomer (β -sheet, α -1, α -2, α -3, α -4) is the same as in the *Arthrobacter* 4HB-CoA thioesterase (1Q4U) and human hTHEM2 thioesterase (3F5O), but is different from that in PA2801 (α -1, α -3, β -sheet, α -2).

Although PA5202 and PA2801 belong to different hotdog-fold subfamilies, they dimerize in a similar way through the formation of a large common β -sheet with additional interactions between the ends of two long α -helices, as well as between the protein loops (Fig. 3C, 3D). The structures also suggest a tetrameric organization of both proteins that was confirmed by gel-filtration studies. In these experiments, PA5202 had a molecular weight of ~ 54.0 kDa (predicted monomer size 13.8 kDa), whereas PA2801 had a molecular weight of 87.6 kDa (predicted monomer size 17.7 kDa). Presently, two tetrameric hotdog protein clades are known with the “back-to-back” (sheet-to-sheet) or “face-to-face” (helix-to-helix) association of dimers [14, 22, 25]. As shown in Fig. 3 (3E, 3F), the structure of the PA5202 tetramer revealed a “back-to-back” dimer association, whereas PA2801 has a “face-to-face”

oligomerization structure. Like PA2801, the structure of PA5185 (also from the YbaW subfamily) showed a hotdog-like tetramer with the “face-to-face” association of two dimers [51].

A Dali search identified several structural homologs of PA5202 in PDB: *TtPaaI* (1WN3; Z-score 20.7, r.m.s.d. 1.1 Å) and the uncharacterized protein Q7W9W5_BORPA from *Bordetella parapertussis* (3DKZ; Z-score 21.0, r.m.s.d. 1.2 Å). For PA2801, the structurally similar proteins were the *E. coli* YbgC (1S5U; Z-score 18.6, r.m.s.d. 1.7 Å) and PA5185, another biochemically uncharacterized hotdog-like protein from *P. aeruginosa* (2AV9; Z-score 18.1, r.m.s.d. 1.5 Å). The last protein shares 31% sequence identity with PA2801 and also belongs to the YbaW hotdog subfamily (Fig. 2).

Site-directed mutagenesis of PA5202 and potential active site

We used site-directed mutagenesis (alanine replacement) to assess the residues of PA5202 important for its enzymatic activity. 17 conserved or semi-conserved residues located in the large cavity with the long α -helix at the bottom were mutated to Ala, and the mutant proteins were purified and tested for thioesterase activity against HMG-CoA (Fig. 4). The Y14A mutant protein was expressed in an insoluble form, whereas the other proteins were found to be soluble. Enzymatic assays revealed that four proteins (N42A, R43A, D57A, and T76A) showed negligible activity, five proteins (R41A, H48A, S54A, Y83A, and R104A) exhibited a greatly reduced activity (25% and lower), whereas other proteins retained at least 50% (R86A and R103A) or wild-type level (S12A, F53A, S65A, S105A, and K117A) activity (Fig. 4). Except for S54A, the active mutant proteins demonstrated a reduced substrate affinity to HMG-CoA with the strongest effect in R103A (Table 2).

Our attempts to obtain a crystal structure of PA5202 in complex with HMG-CoA or other substrate have been unsuccessful so far. Therefore, we compared the PA5202 apostructure with the structure of *TtPaaI* (1WN3) complexed with a substrate analog (hexanoyl-CoA) to assign the potential roles for the PA5202 residues. The ligand binding pocket and active site of *TtPaaI* are formed by the residues from three subunits of the protein tetramer [19, 22], and its structure can be quite well superimposed with the PA5202 structure (r.m.s.d. 1.11 Å for the C atoms of 115 aligned residues) (Suppl. Fig. 2). We propose that these proteins have similar binding sites for the CoA part of their substrates, but use different residues for the coordination of the substrate acyl part (hexanoyl in *TtPaaI* and HMG in PA5202). Since *TtPaaI* and PA5202 have the same (“back-to-back”) dimer-dimer association and share significant sequence and structure similarity, it is likely that PA5202 has a similar active site organization too. For both *TtPaaI* and hTHEM2, a general base catalytic mechanism has been proposed which involves activation of a catalytic water molecule by a conserved carboxylic residue located on the long α -helix (Asp65 in hTHEM2 and Asp48 in *TtPaaI*) [19, 22]. The homologous carboxylate residue Asp57 is also critical for the activity of PA5202 (Fig. 4) suggesting that it likely functions as a general base in this enzyme. In addition, this residue makes a hydrogen bond to the side chain of the semi-conserved Thr76 (2.7 Å), which is also required for PA5202 activity (Fig. 4, 5A). This interaction is expected to maintain the Asp57 side chain in an unprotonated state [52–54], important for the coordination of a catalytic water molecule. There is a similarly positioned Ser residue in the active site of hTHEM2 (Ser83, 3.6 Å from Asp65), which has been proposed to cooperate with Asp65 in the coordination of the catalytic water [22]. This might also be true for the PA5202 Thr76, since Ala replacement of this residue has a dramatic effect on PA5202 thioesterase activity (Fig. 4). The *E. coli* thioesterase II (from the TesB-like family) has Thr228 at a similar position, whereas in the crystal structure of the 4HBA-CoA thioesterase from *Arthrobacter* sp. strain SU (the 4HBT-II subfamily) the catalytic Glu73 is hydrogen bonded to the side chain of Asn96 [10, 12]. However, structures of *TtPaaI* (1WN3) and several other hotdog thioesterases including PA2801 (3QY3) and *E. coli* PaaI (2FS2) have

revealed no presence of a hydroxyl or polar side chain near the catalytic carboxylate suggesting some differences in their catalytic mechanisms.

In the PA5202 active site there are three hydrophilic amino acids located near the catalytic Asp57 (Asn42, His48, and Ser54) whose replacement to Ala produced mutant proteins with a greatly reduced activity, suggesting that these residues are also important for PA5202 activity (Fig. 4, 5A). The homologous mutant proteins N50A and H56A of hTHEM2 and N33A of *TpaaI* showed low thioesterase activity too [19, 22]. In these two proteins and PA5202, the His residue is part of the highly conserved HGG motif (His56-Gly57-Gly58 in hTHEM2) present in many hotdog thioesterases (Fig. 2). For hTHEM2 and *TpaaI*, it has been proposed that the side chain of Asn50 (Asn33 in *TpaaI*) and the backbone amide of Gly57 (Gly40 in *TpaaI*) bind and polarize the carbonyl oxygen of the substrate thioester, stabilizing the negatively charged tetrahedral intermediate of the thioesterase reaction and facilitating hydrolysis of the thioester bond (an oxyanion hole) [19, 22]. In PA5202, the side chain of Asn42 and the main chain amide of Gly49 probably play the same role and function as an oxyanion hole. The role of the His48 and Ser54 side chains in PA5202 activity is presently unclear.

Similar to *TpaaI*, the phosphopantetheine part of the acyl-CoA substrate likely interacts with the side chain of the highly conserved Tyr83 in the PA5202 active site, hence, the very low activity of the Y83A protein (Fig. 4). The CoA adenosine 3',5'-diphosphate moiety appears to be coordinated by the side chains of Arg85, Arg103, Arg104, Ser105, and Lys117 with the phosphate-binding residues (Arg85, Arg103, Arg104, Lys117) located on the loops connecting the last three α -strands. Accordingly, the PA5202 R85A, R103A, and R104A mutant proteins showed reduced activity and substrate affinity, whereas S105A and K117A exhibited wild-type level activity and reduced substrate affinity (Fig. 4, Table 2). Similar results were obtained with hexanoyl-CoA as substrate except for the wild-type level activity in the R43A protein (Suppl. Fig. 3), which was almost inactive against HMG-CoA (Fig. 4) suggesting that this residue might be involved in the coordination of the HMG carboxyl.

The structure of PA2801 also revealed the presence of a potential catalytic carboxylate (Glu35) located on the long α -helix in the active site, and the side chain of Tyr65 seems to be appropriately positioned to interact with the phosphopantetheine part of the substrate (Fig. 5B). In addition, there are three positively charged residues (Arg83, Lys111, and Arg121) which can coordinate the substrate adenosine 3',5'-diphosphate moiety (Fig. 5B). However, in contrast to PA5202, the PA2801 active site contains two aromatic side chains (Trp16 and Tyr22) and the positively charged Arg38 near the catalytic Glu35, which might determine a different substrate preference of PA2801 (Fig. 5B). Overall, the PA2801 tetramer organization ("face-to-face" dimer-dimer association) and active site residues are similar to that of the *Pseudomonas* 4HBA-CoA thioesterase (1BVQ), whose active site is formed by the residues from two protomers [16, 17, 19]. This appears to be true for PA2801 too. Thus, PA5202 and PA2801 have the same structural fold, but evolved a different substrate preference, and future structural studies will reveal the molecular details of the thioester bond hydrolysis in their active sites.

Growth and gene expression experiments with the PA5202 deletion strain

To provide insight into the physiological function of the PA5202 thioesterase in *P. aeruginosa*, we performed growth experiments with the *P. aeruginosa* PA5202 deletion strain using 26 different carbon sources (Suppl. Table 4). This strain showed wild-type growth rates on rich (LB) medium with a doubling time of 37-40 min. However, the PA5202 deletion strain showed a delayed growth (a 1-2 h lag phase) on minimal medium with TCA cycle intermediates as carbon sources (data not shown). The growth delay was even more prominent (3 – 6 hours) when glutarate, propionate, or acetate was used as carbon sources

(Fig. 6A). One of the possible reasons for the observed growth delay of the PA5202 deletion strain might be the toxic effect of the temporal accumulation of the physiological substrate of PA5202 (HMG-CoA or glutaryl-CoA). This is supported by a recent observation that intracellular accumulation of HMG-CoA inhibits the growth of metabolically engineered *E. coli* cells expressing the heterologous HMG-CoA synthase [50]. In *E. coli*, this toxic effect was eliminated by co-expression of the HMG-CoA reductase. It has been suggested that high intracellular levels of HMG-CoA might inhibit enzymes in the early steps of the *E. coli* type II fatty acid biosynthetic pathway [50].

In addition, the PA5202 deletion strain showed a greatly increased production of the extracellular pigment pyocyanin in the stationary growth phase on rich medium (LB) (Fig. 6B, Suppl. Fig. 3). Pyocyanin (1-hydroxy-5-methyl-phenazine) is a blue antibiotic pigment and a known quorum sensing-dependent virulence factor produced by *P. aeruginosa*, which kills mammalian and bacterial cells through the generation of reactive oxygen species [55-57]. Although pyocyanin represents one of the *P. aeruginosa* virulence factors and has broad biological importance, the biochemistry of the biosynthesis of the tricyclic phenazine ring system is not well understood [29, 58, 59]. After five hours of growth, the culture of the PA5202 deletion strain developed a characteristic greenish color, and spectrophotometric analysis of culture supernatants demonstrated a greatly increased (six fold) production of pyocyanin (Fig. 6B). The observed increase in pyocyanin production by the PA5202 deletion strain can be attributed to the increase in the intracellular level of the PA5202 *in vivo* substrate (HMG-CoA or glutaryl-CoA), which might have a direct or indirect effect on the pyocyanin synthesis. It is known that in *Pseudomonas*, HMG-CoA and glutaryl-CoA are intermediates of the degradation pathways of several amino acids (tryptophan, lysine, leucine), which eventually produce acetyl-CoA [46, 60]. These reactions are not directly connected to the known reactions of pyocyanin biosynthesis (which do not involve any acyl-CoA intermediate), suggesting that the increase in the pyocyanin production is not likely due to the direct effect of the PA5202 gene deletion, but probably mediated by presently unknown metabolic reactions. Recently, a redox homeostasis maintenance mechanism based on the pyocyanin production has been proposed for *P. aeruginosa* stationary phase cultures suggesting that pyocyanin may influence the intracellular redox state by decreasing carbon flux through central metabolic pathways [29]. In addition, although hotdog thioesterases are mainly known to use acyl-CoAs as substrates, several enzymes can also hydrolyze acyl-ACPs [14, 15, 25, 61]. Therefore, we can not rule out that the increase in the pyocyanin production in the PA5202 deletion strain reflects a balancing of the *P. aeruginosa* intracellular redox state or acyl-ACP pool.

We also determined the effect of the PA5292 gene deletion on the expression of three groups of *P. aeruginosa* genes involved in ketone-body metabolism (PA0266, PA0447, PA1999, PA2003, PA2011, and PA5015), pyocyanin biosynthesis (PA4209, PA4210, PA4211, and PA4214), and pyocyanin-induced shock response (PA2274, PA4205, and PA4206) (Table 3). After three hours of growth, the total RNA was extracted from *P. aeruginosa* cells (one hour before the visible production of pyocyanin) and the selected mRNAs were quantified using real-time PCR as described in Materials and Methods. As shown in Table 3, the deletion of PA5202 had a small effect on the expression of the group-1 genes suggesting that the enzymes of ketone-body metabolism have a limited role in the utilization of the PA5202 substrates (HMG-CoA or glutaryl-CoA). In contrast, expression of the genes involved in pyocyanin biosynthesis or pyocyanin-induced stress response was greatly increased in the PA5202 deletion strain compared to the wild type cells (Table 3). In *P. aeruginosa*, the synthesis of pyocyanin is known to be induced by the quorum-sensing quinolone signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS), whose precursors are derived from chorismate or through the degradation of tryptophan and include anthranilate-CoA, -ketodecanoyl-CoA, and -ketodecanoyl-ACP, as well as

glutaryl-CoA as intermediates [62–64]. Potentially, the *in vivo* activity of PA5202 might reduce the intracellular level of PQS thereby suppressing the induction of the pyocyanin synthesis.

Thus, biochemical and structural characterization of two hotdog proteins from *P. aeruginosa* identified two new thioesterases with similar structures, but different substrate preferences and active site residues. PA2801 is most active toward octanoyl-CoA, whereas PA5202 is the first thioesterase with a preference to HMG-CoA and glutaryl-CoA. Growth experiments with the PA5202 deletion strain revealed an increased production of pyocyanin and increased expression of genes involved in pyocyanin biosynthesis suggesting a functional link between the PA5202 activity and pyocyanin synthesis *in vivo*. Future biochemical and structural studies of the *P. aeruginosa* hotdog proteins will identify novel thioesterases and provide further insight into their activity and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ACP	acyl-carrier protein
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
4HBA-CoA	4-hydroxybenzoyl-CoA
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
MAD	multi-wavelength anomalous diffraction
MPD	2-methyl-2,4-pentanediol
PQS	2-heptyl-3-hydroxy-4-quinolone

SAD	single-wavelength anomalous diffraction
TLS	translation-libration-screw motion

Fig. 1. Substrate profiles of PA5202 (**A**), PA2801 (**B**), and PA5185 (**C**): thioesterase activity against 27 different CoA thioesters (only substrates with detectable activity are shown). Each bar represents an average of the results from at least two independent determinations, with S.D. indicated by error bars (in all figures).

Fig. 2. Structure-based sequence alignment of PA5202 (**A**) and PA2801 (**B**) with other hot-dog thioesterases with solved structures. (**A**), the PaaI subfamily proteins; (**B**), the YbaW subfamily proteins. The secondary structure elements of PA5202 and PA2801 are shown above and those of *Tt*PaaI and EC TesC below the alignment. Residues conserved in all aligned proteins are highlighted in gray, and the predicted catalytic carboxylate residues are boxed. PA5202 residues mutated to Ala in this work are marked with black triangles above the alignment and numbered. The compared proteins are: (**A**), PA5201 (Q9HTY7), *E. coli* PaaI (P76084), *T. thermophilus* PaaI (TTHA0965; Q5SJP3); (**B**), PA2801 (Q9I042), PA5185 (Q9HU04), and *E. coli* TesC (P77712).

Fig. 3.

Overall structure of the *P. aeruginosa* hot-dog proteins PA5202 and PA2801. (A), PA5202 monomer; (B), PA2801 monomer; (C), PA5202 dimer; (D), PA2801 dimer; (E), PA5202 tetramer; (F), PA2801 tetramer. In (A) and (B), the helices and strands are labelled and shown in magenta and green, respectively. Protein subunits are shown in different colors in (C), (D), (E), and (F). Note that in the PA5202 tetramer (E) two dimers interact by their β -sheets (a “back-to-back” orientation), whereas in the PA2801 tetramer (F) the dimers are connected by their α -helices (a “face-to-face” orientation).

Fig. 4. Alanine replacement mutagenesis of PA5202: thioesterase activity of the purified mutant proteins against HMG-CoA. Reaction mixtures contained 0.6 mM HMG-CoA and 0.2 μ g of purified protein.

Fig. 5.

Close-up stereo view of the active sites of PA5202 (**A**) and PA2801 (**B**). The side chains of the active site residues are shown as *sticks* along with the protein ribbon. The protein monomers are colored in different colors: pale cyan, gray, pale green, and blue-white. Note that the residues from three monomers (pale cyan, gray, and pale green) contribute to one active site in PA5202 (**A**), whereas the PA2801 active site is formed by the residues from two monomers (**B**). In (**A**), R103* and R104* denote two Arg residues which are part of another active site, but are located close to the first active site shown on the picture, suggesting that the second active site might be affected by the substrate binding to the first active site.

Fig. 6. Growth and pyocyanin production by the wild-type and PA5202 deletion strains of *P. aeruginosa*. (A), growth on minimal media with the addition of different carbon sources (0.2%): propionate (■), acetate (●), or glutarate (▲). Experiments were carried out in triplicates using the inoculum grown on LB at 37°C (16-18 h). The flasks were inoculated (OD₆₀₀ 0.05) with the *P. aeruginosa* wild type (dark symbols) or PA5202 (open symbols) strains, and cellular growth was monitored by measuring the culture optical density (OD₆₀₀). (B), growth (○) and production of pyocyanin (□) by the wild type (●) and PA5202 deletion (○) strains on rich media (LB) at 37 °C. Pyocyanin concentration was estimated by absorbance measurements of culture supernatants at 700 nm.

Table 1
Data collection and refinement statistics for the crystal structures of PA5202 and PA2801

	PA5202			PA2801
Data collection				
Space group	P3 ₁ 21			I222
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)	57.95,57.95,113.50			53.816 58.907 85.509
α , β , γ (°)				
	Inflection	Peak	Remote	Peak (SAD)
Wavelength	0.97945	0.97929	0.96411	0.9792
Resolution (Å)	50-1.8(1.84-1.8)	50-1.7(1.74-1.7)	50-1.95(2-1.95)	40-1.75 (1.80-1.75)
<i>R</i> _{sym} or <i>R</i> _{merge}	6.1(44.0)	6.1(37.9)	6.6 (43.7)	6.3 (54.9)
<i>I</i> / <i>I</i>	9.7(2.0)	9.8(1.7)	9.3(3.4)	47.7 (1.90)
Completeness (%)	96.5(79.1)	92.3(63.5)	99.8(99.1)	98.0 (79.7)
Redundancy	5.7(2.9)	5.3(2.0)	6.2(5.1)	8.7 (3.6)
Refinement				
Resolution (Å)	2.9-1.7			40-1.75
Number of reflections	22000			12434
<i>R</i> _{work} / <i>R</i> _{free}	16.0/19.4			16.53/20.76
Number of atoms				
Protein	1964			1066
Solvent	256			104
<i>B</i> -factors (Å ²)	20.05			32.33
R.m.s deviations				
Bond lengths (Å)	0.011			0.019
Bond angles (°)	1.225			1.681
Ramachandran plot (%)				
Favored regions	99.2			97.8
Allowed regions	99.6			100
Outliers	0.40			0
PDB code	1ZKI			3QY3

Values in parentheses are for the highest-resolution shell.

Table 2
Kinetic parameters of the wild type and mutant PA5202 with various substrates

Protein	Variable substrate	$K_m (mM)$	$K_{cat} (s^{-1})$	$k_{cat}/K_m (M^{-1}s^{-1})$
WT	Acetyl-CoA	0.5 ± 0.1	0.20 ± 0.03	0.4×10^3
WT	Succinyl-CoA	0.10 ± 0.02	0.80 ± 0.03	0.7×10^4
WT	<i>n</i> -Hexanoyl-CoA	0.20 ± 0.02	1.20 ± 0.03	0.6×10^4
WT	<i>n</i> -Octanoyl-CoA	0.40 ± 0.06	2.4 ± 0.2	0.7×10^4
WT	Palmitoyl-CoA	0.02 ± 0.01	0.30 ± 0.03	1.3×10^4
WT	Stearoyl-CoA	0.005 ± 0.001	0.040 ± 0.003	0.8×10^4
WT	Methylmalonyl-CoA	0.40 ± 0.04	0.30 ± 0.01	0.8×10^3
WT	-Hydroxybutyryl-CoA	0.50 ± 0.05	2.0 ± 0.1	0.4×10^4
WT	Phenylacetyl-CoA	1.10 ± 0.01	0.10 ± 0.01	0.1×10^3
WT	Glutaryl-CoA	0.7 ± 0.1	26.8 ± 0.9	0.4×10^5
WT	HMG-CoA	0.10 ± 0.01	3.9 ± 0.1	0.3×10^5
S12A	HMG-CoA	0.30 ± 0.03	3.0 ± 0.2	0.1×10^5
R41A	HMG-CoA	0.40 ± 0.06	1.3 ± 0.1	0.4×10^4
H48A	HMG-CoA	0.20 ± 0.02	1.1 ± 0.1	0.5×10^4
F53A	HMG-CoA	0.20 ± 0.04	3.3 ± 0.2	0.1×10^5
S54A	HMG-CoA	0.10 ± 0.01	0.5 ± 0.1	0.5×10^4
S65A	HMG-CoA	0.50 ± 0.04	3.9 ± 0.1	0.7×10^4
R85A	HMG-CoA	0.50 ± 0.05	2.1 ± 0.1	0.4×10^4
R103A	HMG-CoA	1.0 ± 0.1	1.8 ± 0.1	0.2×10^4
R104A	HMG-CoA	0.40 ± 0.03	1.2 ± 0.1	0.3×10^4
S105A	HMG-CoA	0.50 ± 0.04	2.70 ± 0.01	0.5×10^4
K117A	HMG-CoA	0.30 ± 0.03	3.30 ± 0.01	1.1×10^4
R43A	<i>n</i> -Hexanoyl-CoA	0.60 ± 0.05	1.10 ± 0.03	0.2×10^4
S65A	<i>n</i> -Hexanoyl-CoA	0.55 ± 0.02	1.70 ± 0.03	0.3×10^4

Table 3
Relative expression of selected genes involved in ketone body metabolism determined by qRT-PCR in the PA5202 mutant background

Group	Protein ¹	Enzyme encoded or gene name	Relative expression ²		
			PA5202 (LB)	wild type (LB- -KBA)	PA5202 (LB- -KBA)
I	PA0266	4-aminobutyrate aminotransferase	1.6 ± 0.1	0.60 ± 0.01	0.80 ± 0.01
	PA0447	Glutaryl-CoA dehydrogenase	0.60 ± 0.01	0.30 ± 0.01	0.20 ± 0.02
	PA1999	3-ketoacid-CoA transferase	1.0 ± 0.1	1.0 ± 0.1	0.70 ± 0.01
	PA2003	3-Hydroxybutyrate dehydrogenase	1.4 ± 0.1	0.30 ± 0.02	0.50 ± 0.01
	PA2011	HMG-CoA Lyase	1.3 ± 0.1	1.00 ± 0.01	1.00 ± 0.01
	PA5015	Pyruvate dehydrogenase	1.2 ± 0.1	8.1 ± 1.1	2.00 ± 0.02
II	PA4209	<i>phzM</i>	9.2 ± 1.1	0.10 ± 0.01	3.2 ± 0.3
	PA4210	<i>phzA</i>	19.2 ± 1.5	0.2 ± 0.1	14.9 ± 1.1
	PA4211	<i>phzB</i>	17.8 ± 2.4	0.1 ± 0.0	9.7 ± 0.9
	PA4214	<i>phzE</i>	18.3 ± 2.0	0.2 ± 0.1	11.0 ± 1.3
III	PA2274	Putative monooxygenase	69.9 ± 12.2	0.6 ± 0.1	20.2 ± 1.4
	PA4205	<i>mexG</i>	108.9 ± 10.5	0.4 ± 0.0	21.2 ± 2.4
	PA4206	<i>mexH</i>	89.3 ± 10.7	0.5 ± 0.1	26.7 ± 1.8

¹The gene names used correspond to the nomenclature utilized in the *Pseudomonas* genome database V2.

²According to the equation described (35), the relative expression (R_{ex}) of each gene in the wild type background equals 1. The over-expressed genes will show the R_{ex} values >1, whereas the down-regulated genes will have R_{ex} <1.