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ScienceDirect
Biochemical and Biophysical Research Communications 349 (2006) 172–177
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Crystal structure of human thioesterase superfamily member 2 ☆,☆☆

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Received 2 August 2006
Available online 14 August 2006

Abstract

Hotdog-fold has been identified in more than 1000 proteins, yet many of which in eukaryotes are less studied. No structural or functional studies of human thioesterase superfamily member 2 (hTHEM2) have been reported before. Since hTHEM2 exhibits about 20% sequence identity to *Escherichia coli* PaaI protein, it was proposed to be a thioesterase with a hotdog-fold. Here, we report the crystallographic structure of recombinant hTHEM2, determined by the single-wavelength anomalous dispersion method at 2.3 Å resolution. This structure demonstrates that hTHEM2 indeed contains a hotdog-fold and forms a back-to-back tetramer as other hotdog proteins. Based on structural and sequence conservation, the thioesterase active site in hTHEM2 is predicted. The structure and substrate specificity are most similar to those of the bacterial phenylacetyl-CoA hydrolase. Asp65, located on the central α-helix of subunit B, was shown by site-directed mutagenesis to be essential to catalysis.

Keywords: Hotdog-fold; Thioesterase; Crystal structure; hTHEM2; PaaI; Active site; Substrate screening

Human thioesterase superfamily member 2 (hTHEM2, NCBI Protein 8923812; Expasy accession Q9NPJ3) displays about 20% sequence identity to *Escherichia coli* PaaI, a component of the phenylacetic acid degradation pathway that functions to recycle CoA [1]. In addition, hTHEM2 exhibits some sequence similarity to *Arthrobacter* 4-hydroxybenzoyl-CoA thioesterase (4HBT) that catalyzes the final step of 4-chlorobenzoate degradation pathway [2]. The phenylacetic acid degradation pathway in *E. coli* contains 14 proteins encoded by a gene cluster *paaX* [3]. However in human, genomic sequence data suggest that 7 *PaaX* genes were lost during evolution, indicating that the phenylacetic acid degradation pathway does not exist in human. Therefore, the biological functions of human thioesterase superfamily members remain unclear although they are essential in human metabolism and associated to genetic diseases [4–6]. In order to understand the structure and function of hTHEM2, we cloned its gene, overexpressed in *E. coli.*, and purified the hTHEM2 protein. The crystal structure of hTHEM2 was determined by the single-wavelength anomalous dispersion method at 2.3 Å resolution.

Materials and methods

Preparation of recombinant wild-type, selenoMet, and D65A hTHEM2. The cDNA encoding hTHEM2 was amplified from a human brain cDNA library (Clontech) by PCR and ligated into pET22b(+) (Novagen). The purified recombinant plasmid (named WT-hTHEM2/pET22b(+)) was

☆ *Abbreviations:* hTHEM2, human thioesterase superfamily member 2; 4HBT, 4-hydroxybenzoyl-CoA thioesterase; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid).

☆☆ The atomic coordinates and structure factors of the hTHEM2 structure have been deposited in the Protein Data Bank with Accession code 2F0X.

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.08.025

confirmed by DNA sequencing and then used to transform competent *E. coli* BL21(DE3) cells (Stratagene). Cells were grown in 1 l of LB media with shaking at 37 °C for 2 h, induced with 1 mM IPTG for 4 h, and then harvested by centrifugation at 4 °C. The 5 g cell pellet was suspended in lysis buffer (4 °C, pH 7.5; 20 mM Tris–HCl, 200 mM NaCl, and 0.2 mM PMSF), sonicated at 4 °C for 40 min using a microtip equipped ultrasonic crasher JY92-II (SCIENTZ BIOTHECHNOLOGY) and then centrifuged at 4 °C (15,000 rpm, 30 min). The supernatant was loaded onto a Chelating Sepharose™ Fast Flow affinity column (Amersham Bioscience) and chromatographed with elution buffer (200 mM imidazole, 20 mM Tris–HCl, pH 7.5, 200 mM NaCl) at 4 °C. The thioesterase-containing fractions were pooled, dialyzed against 20 mM Tris–HCl/50 mM NaCl (pH 7.5), and concentrated to 10 mg/ml for storage at −80 °C (yield: 30 mg/l cell culture). Protein purity was verified by SDS–PAGE analysis. The subunit mass was determined by ESI-MS and by SDS–PAGE analysis with protein molecular size standards. The native mass was determined by dynamic light scattering analysis. To prepare the Se-Met thioesterase used for MAD phasing, the hTHEM2 encoding gene was overexpressed in the methionine auxotroph B834 (DE3) *E. coli* strain, and the protein was purified as described above with the exception that, pH 8.5, Tris–HCl buffer was used. Mutagenesis was carried out using a PCR-based strategy based with the WT-hTHEM2/pET-22b(+) plasmid as template, commercial primers (Invitrogen), the PCR kit supplied by Stratagene, and the Techgene thermal cycler manufactured by TECHNE (Princeton, NJ). The PCR products were used to transform competent *E. coli* BL21(DE3) cells and the plasmid was prepared using a QIAprep Spin Miniprep Kit (Qiagen). The sequence of the mutated gene was confirmed by DNA sequencing. The D65A hTHEM2, purified as described above for the wild-type enzyme in a yield of 4 mg/g wet cells, was shown to be homogenous by SDS–PAGE analysis.

Crystallization, data collection, and structure determination. Se-Met hTHEM2 crystals were prepared by the hanging drop vapor diffusion method. One microliter of protein (15 mg/ml) was mixed with 1 μl reservoir solution (15% polyethylene glycol 2000, 150 mM ammonium sulfate, 50 mM HAc-NaAc (pH 4.8) and 10 mM dithiothreitol) to set a hanging drop. Crystals grew to their full length at 4 °C after a 2 week period. MAD data were collected at 100 K with cryo-protectant containing additional 20% glycerol on beamline 3WIA at Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences. All data were processed with DENZO and SCALEPACK [7].

By direct method, 39 Se positions (of a total 48 Se atoms) were identified using the peak data set by SHELXD [8]. Se coordinates were refined and phases were calculated by SOLVE [9]. RESOLVE [10] was used for density modification and non-crystallographic 8-fold symmetry averaging. The electronic density map was clearly defined and the initial model was traced manually in O [11]. Subsequently, refinement was conducted in CNS [12] against the remote data set within resolution of 50–2.3 Å. The quality of final model was checked by PROCHECK [13]. Data collection and structure refinement statistics were listed in Table 1.

Enzymatic assays. The thioesterase activity was monitored 412 nm ($\Delta\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$) by coupling the reaction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) with the CoA liberated from the acyl-CoA substrate. The 0.2 ml reaction solutions consisted of enzyme (0.08 μM for 3-hydroxyphenylacetyl-CoA, 4-hydroxyphenylacetyl-CoA, 3,4-dihydroxyphenylacetyl-CoA, 3,5-dihydroxyphenylacetyl-CoA, and β-hydroxybutyryl-CoA, 0.8 μM for 3 hydroxybenzoyl-CoA, and 8 μM for all others), DTNB (1 mM), KCl (0.2 M), and various concentrations of substrate ($0.5–5 \times K_m$) in 10 mM K^+ Hepes (pH 7.5, 25 °C) contained in a quartz cuvette of 1 cm light path-length. The initial velocity data, measured as a function of substrate concentration, were analyzed using Eq. (1) and the computer program KinetAsyst (IntelliKinetics, PA).

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

V, initial velocity; V_{max}, maximum velocity; [S], substrate concentration; and K_m, Michaelis constant. The k_{cat} was calculated from V_{max}/[E] where [E] is the total enzyme concentration determined using the Bradford method [14]. The activity of the D65A mutant was measured with 0.2 mM 3-dihydroxyphenylacetyl-CoA.

Table 1
Data collection, phasing, and refinement statistics

Data collection	Peak	Remote
Wavelength (Å)	0.9789	0.95
Resolution range (Å)	50–2.5 (2.56–2.5)	50–2.3 (2.35–2.30)
No. of total reflections	318,771	408,282
No. of unique reflections	41,105(2730)	52,710(3479)
I/σ	20.2(5.8)	21.2(5.3)
Completeness (%)	100	100
R_{merge}	0.113(0.362)	0.095(0.347)
Space group	C2	
Unit cell dimensions (Å)	$131.94 \times 122.08 \times 90.98$	$\beta = 123.9°$
SAD phasing (20–2.5 Å)		
Mean FOM[a]	0.32	
Mean FOM[a] after DM	0.62	
Structure refinement		
R_{work}[b]/R_{free}[c]		0.217/0.243
Protein atoms		7906
Water atoms		490
Sulfate molecules		20
rms bond lengths (Å)		0.006
rms bond angles (°)		1.3
Average B-factor (Å^2)		32
B-factor from Wilson plot (Å^2)		28
Ramachandran plot (%)		
Most favorable		91.7
Additional allowed		8.3

Peak and remote data for selenomethionine (Se-Met) were collected from a single crystal. Refinement was performed against the remote data. Numbers in parentheses represent the value for the highest resolution shell.

[a] Mean FOM (figure of merit) $= <|\sum P(\alpha)e^{i\alpha}/\sum P(\alpha)|$, where α is the phase and $P(\alpha)$ is the phase probability distribution.

[b] $R_{work} = \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated structure factors.

[c] $R_{free} = \sum_T||F_{obs}| - |F_{calc}||/\sum_T|F_{obs}|$, where T is a test data set of 10% of the total reflections randomly chosen and set aside prior to refinement.

Results and discussion

Protein purification and characterization

hthem2 Gene was cloned and overexpressed in *E. coli*. The hTHEM2 protein was purified to homogeneity by affinity chromatography. Thirty milligrams of protein could be purified from 1 l cell culture. This protein was very stable in K^+ Hepes (pH 7.5) buffer. SDS–PAGE electrophoresis showed only one band at about 16 kDa, which is in agreement with the mass (15,895) determined by ESI mass spectroscopy. The theoretical mass of 16,026 Da indicates loss of the N-terminal Met (−131) during posttranslational modification. Interestingly, dynamic light scattering analysis of hTHEM2 showed a native molecular weight of 64 kDa, suggesting its tetrameric form in solution.

Three-dimensional structure

There are eight hTHEM2 molecules per asymmetric unit. Ribbon representation of hTHEM2 monomer is

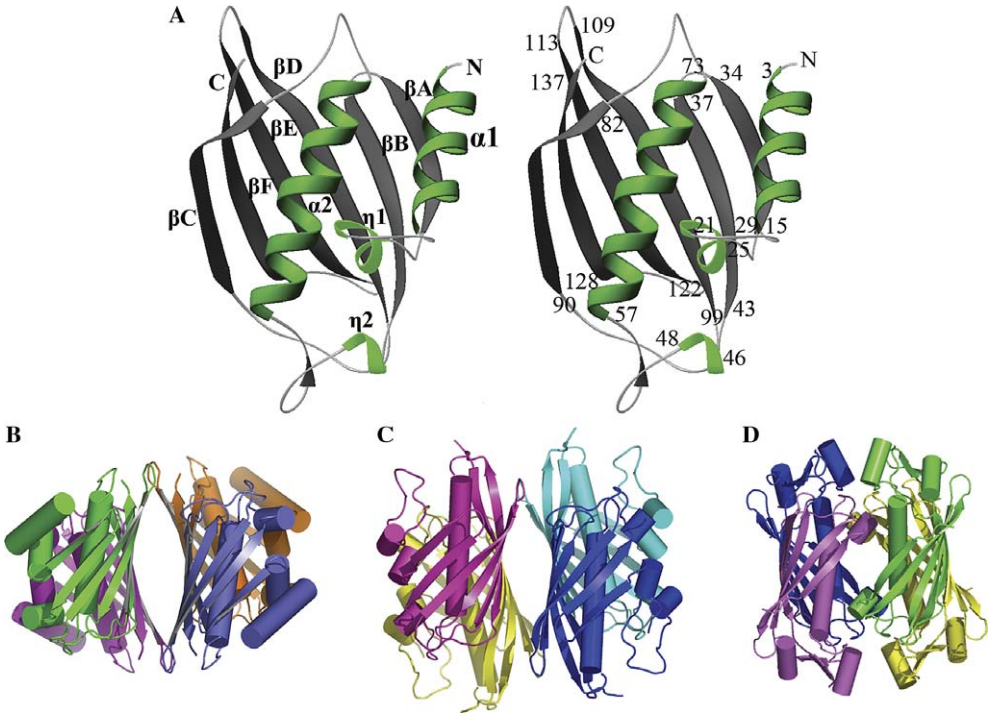

Fig. 1. Overall structure of hTHEM2 and tetrameric forms of some thioesterases (hTHEM2 and 4HBT from *Arthrobacter* sp. and *Pseudomonas* sp.). (A) Stereodiagram of hTHEM2 with secondary structural elements indicated. (B) The tetrameric form of hTHEM2. (C) The tetrameric form of 4HBT from *Arthrobacter* sp. and (D) from *Pseudomonas* sp. The four subunits are in four different colors, respectively. The α-helices were shown in cylindrical model. (A) was generated using the program Ribbons [22], (B–D) were produced by PyMOL [23] .

shown in Fig. 1A. For each monomer, the architecture is clearly a hotdog-fold [15]. Six β-strands wrap up a long α-helix and an additional α-helix is flanking along the β1-strand. A DALI search [16] with hTHEM2 monomer serving as query, identified nine structures with *Z* scores greater than 10. Among them, four are bacterial thioesterases with known substrates [1,17–20], while the other five (PDB ID code: 2cy9, 1o0i, 1yoc, 1nng, 2ess) possess unclear functions.

hTHEM2 dimerize by a stable anti-parallel β-sheet formed at residues Ser83–Tyr90 on βC-strand from each monomer. Since each monomer contains six β-strands, an extended 12-stranded anti-parallel β-sheet is included in the hTHEM2 dimer. The dimer is further stabilized by hydrogen bonds between the two central α-helices. Consistent with its molecular weight in solution, hTHEM2 forms a tetramer in crystal packing, with two dimers stacked back-to-back (Fig. 1B). This particular quaternary structure is also observed in the *Arthrobacter* sp. Strain SU 4-hydroxybenzoyl-CoA thioesterase (4HBT) [19] (Fig. 1C) and other related phenylacetyl-CoA thioesterases (PaaIs) [1,20]. In contrast to hTHEM2, the tetramer of 4HBT from *Pseudomonas* sp. adopts another style, in which the two dimers stack face-to-face to form the native tetramer [17] (Fig. 1D). The residues involved in the tetramerization of hTHEM2 are Asn87, Asp85, Thr135, Met91, and Phe115. In the PaaI from *Thermus thermophilus* HB8, Arg67 stabilizes the tetramer [20]. In hTHEM2, the corresponding residue Asn87 might play a similar role.

Putative active site

By superimposing the structures of hTHEM2 and 4HBT/inhibitor complex (PDB entry 1Q4T) [19], the active site of hTHEM2 was proposed (Fig. 2A). The active site of hTHEM2 is located at subunit interface and it is formed by residues from three subunits, including Glu22, Asp65, Asn66, Thr69, Met70, Pro80, Val82, Ser83, His134, and Lys136 from the first subunit, Asn50, His56, Gly57, Tyr90, Met91, Ser92, and Pro93 from the second and Lys108, Gly110, Lys11, Thr112, and Leu113 from the third. Of these residues, Asn50, His56, Gly57, Asp65 are most conserved and may play important roles in catalytsis (Fig. 2C).

Substrate specificity

A focussed substrate screen was applied to define chemical function. The substrate activities of various aromatic and aliphatic acyl-CoA thioesters were tested with the purified thioesterase at 25 °C and pH 7.5 (see Table 2). Among the compounds screened, 3-hydroxyphenylacetyl-CoA (k_{cat}/K_m 3.5×10^4 $M^{-1}s^{-1}$) and 3,4-dihydroxyphenylacetyl-CoA (k_{cat}/K_m 1.8×10^4 M^{-1} s^{-1}) were found to be the most active substrates. The benzoyl-CoA and aliphatic acyl-CoA thioesters, including the fatty acyl-CoA thioesters showed limited substrate activity. If the phenyl group is not hydroxylated (such as is the case with phenylacetyl-CoA) the catalytic activity is decreased 100-fold. Thus, we conclude that the hTHEM2 is targeting a CoA thioester that is functionalized with a polar aromatic substituent.

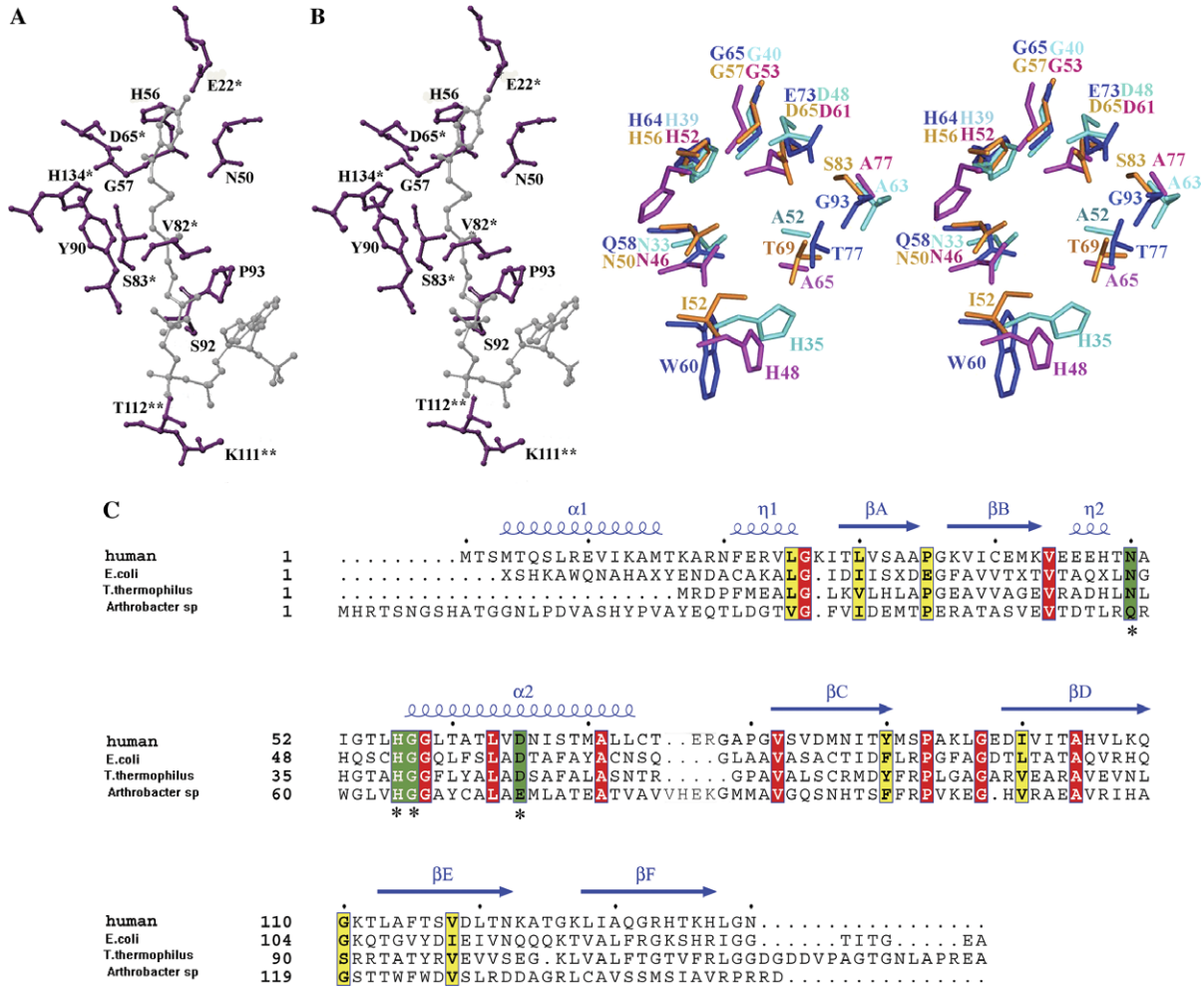

Fig. 2. The putative active site identified by substrate analog modeling and sequence alignment of hTHEM2 with other three hotdog proteins. (A) Modeling of 4-hydroxyphenacyl-CoA ligand (gray), the substrate analog in *Arthrobacter* 4HBT structure (PDB entry: 1Q4T), into the hTHEM2 structure by superimposing the two thioesterases. The hTHEM2 residues (purple) that are located within 4 Å of the modeled substrate analog are indicated in the figure. Non-labeled residues are located in the first hTHEM2 subunit, while (*) and (**) residues are in the second and third subunits, respectively (see text). (B) Stereo pictures of the active sites residues D65, N50, H56, G57, I52, T69, and S83 of hTHEM2 thioesterase (orange) overlaid with D61, N46, H52, G53, H48, A65, and A77 of *E. coli* PaaI (magenta), D48, N33, H39, G40, H35, A52, and A63 of *T. thermophilus* PaaI type 2 (cyan) and E73, Q58, H64, G65, W60, T77, and G93 of *Arthrobacter* 4HBT (blue). (C) Structure-based sequence alignment of hTHEM2 with the other three hotdog proteins. Protein sequences were from *Homo sapiens* (human), *E. coli* (1psu), *Thermus thermophilus* (1j1y), and *Arthrobacter* sp. (1q4s). Sequence accession numbers of the Swiss-Prot database are Q9NPJ3, P76084, Q5SJP3, and Q7BUB8, respectively. The secondary structure of hTHEM2, which is mainly defined by the analysis of the structure using DSSP program [24], is indicated above the alignment. The identical residues were labeled in red and similar residues in yellow. The putative catalytic site residues Asn50, His56, Gly57, and Asp65 was labeled in green and indicated with a star. This sequence alignment was produced by ESPript [25]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Substrate recognition and catalysis

The catalytic sites of the *Arthrobacter* 4-hydroxybenzoyl-CoA thioesterase and the PaaIs from *E. coli* and *T. thermophilus* are overlaid with that of the hTHEM2 in Fig. 2B. The key residue of the hotdog-fold thioesterase active site is the catalytic carboxylate residue (Asp or Glu), which functions in nucleophilic catalysis or in general base catalysis [19]. In hTHEM2, the Asp65 on the central α-helix is analogous to the catalytic Glu73 of the *Arthrobacter* 4-hydroxybenzoyl-CoA thioesterase, and the catalytic Asp61 and Asp48 of *E. coli* and *T. thermophilus* PaaIs, respectively. To determine whether the hTHEM2 Asp65 is essential to hTHEM2, it was replaced with Ala by site-directed mutagenesis and then the thioesterase activity of the purified D65A mutant towards 3-hydroxyphenylacetyl-CoA was measured. The absence of detectable activity confirms that Asp65 is the essential catalytic residue (Table 2). The highly conserved residue His56, the counterpart of the residue from *Arthrobacter* 4-hydroxybenzoyl-CoA thioesterase, may play a critical role in the orientation of the orientation of the carboxylate of Asp65. Gly57, which is located at the N-terminus of the central α-helix, is positioned to interact with the substrate thioester C=O, thus orienting activating it for nucleophilic attack. The locations of these three residues Asp65, His56, and Gly57 as well as the amide residue (Asn50 in hTHEM2) that is located opposite to the carboxylate are highly conserved in the superfamily, so the catalytic strategy might be conserved.

Table 2
Steady-state kinetic constants for wild-type for hTHEM2 catalyzed hydrolysis of acyl-CoAs at 25 °C, pH 7.5

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (μM) [K_i (μM)]	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Wild-type	3-Hydroxyphenylacetyl-CoA[a]	1.4 ± 0.1	$(4.1 \pm 0.4) \times 10^1$	3.5×10^4
	3,4-Dihydroxyphenylacetyl-CoA[a]	$(1.9 \pm 0.1) \times 10^{-1}$	$(1.0 \pm 0.1) \times 10^1$	1.8×10^4
	3,5-Dihydroxyphenylacetyl-CoA[a]	$(1.9 \pm 0.1) \times 10^{-1}$	$(2.5 \pm 0.3) \times 10^1$	7.9×10^3
	4-Hydroxyphenylacetyl-CoA[a]	$(1.4 \pm 0.1) \times 10^{-1}$	$(4.9 \pm 0.4) \times 10^1$	2.9×10^3
	Phenylacetyl-CoA[b]	$(8.4 \pm 0.6) \times 10^{-2}$	$(2.4 \pm 0.3) \times 10^2$	3.5×10^2
	4-Hydroxybenzoyl-CoA[a]	No activity	—	—
	3-Hydroxybenzoyl-CoA[a]	$(1.1 \pm 0.1) \times 10^{-2}$	$(3.2 \pm 0.2) \times 10^1$ [1.7 ± 0.2]	3.5×10^2
	4-Hydroxyphenacyl-CoA[a]	No activity	—	—
	4-Hydroxybenzyl-CoA[a]	No activity	—	—
	4-Chlorobenzoyl-CoA[a]	$(3.2 \pm 0.2) \times 10^{-3}$	$(1.3 \pm 0.2) \times 10^2$	2.5×10^1
	n-Butyryl-CoA[b]	$(4.7 \pm 0.5) \times 10^{-2}$	$(6.0 \pm 0.1) \times 10^2$	7.8×10^1
	β-Hydroxybutyryl-CoA[b]	$(1.8 \pm 0.1) \times 10^{-1}$	$(1.1 \pm 0.2) \times 10^2$	1.6×10^3
	n-Octanoyl-CoA[b]	$(4.7 \pm 0.1) \times 10^{-3}$	$(2.6 \pm 0.3) \times 10^1$	1.8×10^2
	n-Palmitoyl-CoA[b]	$(4.4 \pm 0.1) \times 10^{-3}$	$(1.6 \pm 0.1) \times 10^1$	2.8×10^2
	Glutaryl-CoA[b]	$(5.0 \pm 0.2) \times 10^{-2}$	$(6.7 \pm 0.5) \times 10^2$	7.5×10^2
	Malonyl-CoA[b]	$(6.6 \pm 0.6) \times 10^{-2}$	$(2.8 \pm 0.8) \times 10^2$	2.4×10^2
	Crotonyl-CoA[b]	$(7.6 \pm 0.2) \times 10^{-3}$	$(1.8 \pm 0.1) \times 10^2$	4.7×10^2
	DL-3-Hydroxy-3-methylglutaryl-CoA[b]	$(7.9 \pm 0.2) \times 10^{-2}$	$(2.9 \pm 0.2) \times 10^3$	2.7×10^1
D65A	3-Hydroxyphenylacetyl-CoA[a]	$<1.0 \times 10^{-5}$	—	—

[a] Compounds were synthesized as reported in [18,21].
[b] Compounds were purchased from Sigma.

In the case of the *E. coli* PaaI, 3,4-dihydroxyphenylacetyl-CoA, and the corresponding monohydroxyphenylacetyl-CoAs are the most active substrates at $k_{cat}/K_m = 2\text{–}6 \times 10^6\ M^{-1}\,s^{-1}$ (phenylacetyl-CoA is 2 orders of magnitude less reactive; 4-hydroxybenzoyl-CoA is not a substrate) [1]. In the present study, we identified these same substrates as being the most active with hTHEM2. However, hTHEM2 enzymatic activity is only about one percent toward above compounds compared to *E. coli* PaaI and its exact physiological substrate needs to be further investigated.

Acknowledgments

This work is supported by NIH Grant GM28688 (to D.D.M) and by the National Foundation of Talent Youth (Grant No. 30225015), the 973 programs (No. 2004CB720000), National Natural Science Foundation of China (Grant Nos. 10490913 and 30500100) and Chinese Academy of Sciences (KSCX2-SW-224). We thank Dr. Peng Liu and Yuhui Dong in the Institute of High Energy Physics, and Mr. Yi Han in the Institute of Biophysics for diffraction data collection.

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