Correlation of Structure and Function in the Human Hotdog-fold Enzyme hTHEM4.[†]

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Sixteen pages containing experimental protocol and Table SI1, Table SI2 and Figures SI1-5.

Experimental

Preparation of Enzymes.

His_6-tagged recombinant full-length hTHEM4 and the N-terminal truncated (Δ1-39)hTMEM4 were prepared from the *E. coli* clones as reported in reference (*1*). His-tagged full-length Akt1 was expressed and purified from baculovirus and activated with purified PDK1 to phosphorylate Thr^{308} and purified MK2 to phosphorylate Ser^{473} according to published procedure (*2*). Akt1 used in the pull-down experiments was prepared using the *E. coli* clone and His_6-MBP TEV protease cleavable tag according to the protocols described in reference (*3*).The cDNA clone of the gene *AKT1* (BC000479) encoding full length Akt1 was purchased from Thermo Scientific (MHS1011-58771). The gene was inserted into the Addgene plasmid 11540: pHM6g.TM1457 (to produce the His_6-MBP TEV protease cleavable construct) cleaved using NdeI and BamHI.

Site-directed Mutagenesis

Site-directed mutagenesis was carried out using a PCR-based strategy with the (Δ1-39)hTHEM4/ pET-23a (+) plasmid as template, commercial primers and dNTP (Invitrogen), P*fu Turbo* DNA polymerase, and the Techgene thermal cycler manufactured by TECHNE (Princeton, NJ). The PCR products were treated with DpnI to remove the wild type plasmid before transformation into competent *Escherichia coli* cells (BL21 Star™ (DE3) One Shot). The sequence of the mutated gene was confirmed by DNA sequencing carried out by the DNA Sequencing Facility of the Health Sciences Center in the University of New Mexico, the plasmid was prepared using a QIAprep Spin Miniprep Kit (Qiagen). The hTHEM4 mutants were purified to homogeneity (monitored by SDS-PAGE) by the same procedure used to purify the wild-type enzyme.

hHTEM4 Native Molecular Weight Determination

The association states of full-length and (Δ1-39) hTHEM4 were determined at the Biophysics Resource of Keck Facility (Yale University) using HPLC (High Performance Liquid Chromotography) SEC (Size Exclusive Chromotography) (LS) Light Scattering (RI) Refractive Index analysis to.

Crystal structure determination

Crystals of hTHEM4 were obtained by vapor diffusion in hanging drops at room temperature. The reservoir solution contained 11 % w/v PEG 3350 and 0.1 M ammonium phosphate monobasic in the case of hTHEM4. The protein sample, kept in 0.2 M NaCl and 50 mM HEPES, pH 7.5, was mixed with the enzyme inhibitor undecan-2-one-CoA to a final protein concentration of 8 mg/mL and inhibitor concentration of 4 mM. Equal volumes of the protein solution and reservoir solution were equilibrated with the reservoir solution. Needle-like crystals of hHTEM4 appeared within three days (approximate dimensions 0.8 x 0.02 x 0.02 mm^3). The crystals were transferred to a mother liquor solution supplemented with 20% glycerol and flashed-cooled in propane cooled with liquid nitrogen.

X-ray diffraction data were collected at the GM/CA-CAT 23-ID beamline at the Advanced Photon Source, Argonne National Laboratory. The beamline was equipped with a MAR 300 CCD detector. Data were processed with the program CrystalClear (Rigaku). The lattice parameters and the space group indicated that the crystals contained 49% solvent and two molecules in the asymmetric unit.

PSI-BLAST (*4*) showed that the closest hTHEM4 sequence homolog in the PDB is a protein from *Rhodococcus* sp. with a hotdog fold (E score of 10^{-7}, PDB entry code 2OV9, determined by the Midwest Center for Structural Genomics), although the protein function has not been

defined. This structure was used as the search model to determine the hTHEM4 structure by the Molecular Replacement method. The PSI-BLAST sequence alignment spanned the hTHEM4 residues 142 to 231 corresponding to most of the *Rhodococcus* thioesterase hotdog core fold (residues 121 to 208) with 33 % amino acid identity. The N-terminal region of hTHEM4 (residues 39 – 120) has no identifiable homolog with known structure.

Molecular Replacement was performed with the PHASER program (*5,6*) and the dimeric *Rhodococcus* sp. thioesterase search model was truncated to include the entire hotdog core fold (residues 101-209). A single solution was readily obtained, with the dimer occupying the crystal asymmetric unit. The initial model was energy minimized with Refmac (*7*) followed by a non-crystallographic averaging and solvent-flattening using Resolve (*8*).

Building of the missing N-terminal polypeptide chain was accomplished gradually by repeated cycles of model building with Coot (*9*) and phase improvement with Resolve (*8*). The electron density map also showed a bound undecan-2-one-CoA inhibitor in each active site and these were modeled once the building of the polypeptide chain was completed. Final refinements cycles and positioning of water molecules were performed with the Phenix program (*10*). The structure refinements summary is provided in Table SI1.

hTHEM4 Thioesterase Activity Assay

Reaction solutions were monitored at 412 nm. The reactions were initiated by adding hTHEM4 to solutions initially containing substrate (at varying concentration: 1-10 fold K_m), DTNB (2 mM), KCl (0.2 M) and 50 mM K^+HEPES (pH 7.5, 25 ^oC). The kinetic parameters V_{max} and K_m were determined from initial velocity data, measured as a function of substrate concentration, by

using equation (1) and KinetAsyst (IntelliKinetics, PA),

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

where [A] is the substrate concentration, V is the initial velocity, V_{max} is the maximum velocity and K_m is the Michaelis constant. The k_{cat} was calculated from the ratio of V_{max} and the total enzyme concentration. The enzyme concentration was determined using the Bradford method.

hTHEM4 Inhibition

Competitive inhibition constants for undecan-2-one-CoA, hexanoate, octanoate and lauroate were determined by measuring the initial velocity of hTHEM4 catalyzed substrate hydrolysis as a function of substrate concentration (K_m to $10K_m$) and inhibitor concentration (0, $1K_i$ and $2K_i$). The initial velocity data were fitted to equation (2) using KinetAsyst (IntelliKinetics, PA),

$$V = V_{max}[A] / ([A] + K_m (1 + [I]/K_i)) \qquad (2)$$

where [A] is the substrate concentration, V is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, K_i is the competitive inhibition constant and [I] is the inhibitor concentration. Because the DTNB-based kinetic assay that is used to measure the initial velocities of hTHEM4 catalyzed acyl-CoA thioester hydrolysis is not compatible with experiments using exogenous CoASH as a product inhibitor, an alternate assay method was employed. Accordingly, 4-hydroxybenzoyl-CoA (4-HBA-CoA) ($k_{cat} = 2.9 \pm 0.2 \times 10^{-1}$ s^{-1} and $K_m = 29 \pm 4$ μM) was used as substrate because its hydrolysis can be monitored by measuring the decrease in absorbance at 300 nm ($\varepsilon = 11.8$ $mM^{-1} \bullet cm^{-1}$).

Akt1 Kinase Activity Assay

The catalytic activity of activated, full-length Akt1 (*1*) was measured using GSK3a peptide (RPRAATF) and ATP as substrates in the presence or absence of hTHEM4. Reaction buffer containing 25 mM MOPS, 0.5 mM CHAPS, 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT and 0.1 mg/mL BSA (pH 7.5) was used to prepare Akt1, GSK3a, ATP and hTHEM4 solutions. To a non-binding surface plate, 10 μL of the GSK3a (10 μM) and ^{33}P-ATP (50 μM) mix were added along with 10 μL of hTHEM4 (0-6 μM) solution. The reaction was initiated by the addition of 20 μL of Akt1 (1 nM) and terminated at 0, 5, 15, 30, 60, 120, 180 and 240 min by adding 40 μL of 1% H_3PO_4 to the plate. After 10 min, 60 μL of the quenched reaction solution was transferred to a Millipore MAPH filter plate. After 30 min the filter plate was washed 5-times with 100 μL of 1% H_3PO_4 and then the filter plate was dried at 50°C for 30 min. Next, 50 μL of Microscint20 cocktail was added and the plate was read in TopCount using a ^{33}P protocol.

Co-Immunoprecipitation of recombinant full-length Akt1 and hTHEM4

Anti-Akt1 antibody (Cell Signaling #9272) covalently immobilized agarose beads were prepared following the manufacturer's instructions (Thermo Scientific #26148:Pierce Direct IP Kit). Briefly, 20 μg of the Akt1 antibody were incubated with 40 μL of the agarose bead slurry in the presence of $NaCNBH_3$ at room temperature for at least 2 h. The uncoupled antibody was removed by extensive washing with wash buffer and the uncoupled reactive sites in agarose beads were quenched by incubation with the primary amine and $NaCNBH_3$. Purified recombinant full-length Akt1(150 μg) (*3*) and full-length hTHEM4 (35 μg) were incubated with the anti-Akt1antibody immobilized agarose beads in 600 μL of IP lysis buffer at 4°C for 12 h. As a control, the same amount of hTHEM4 was incubated with the anti-Akt1antibody immobilized agarose beads in the absence of Akt1. Unbound or weakly bound protein was removed by using

washing steps prior to eluting the specifically bound protein from the beads with the elution buffer (10 mM Tris with 100 mM glycine pH 7.4). Protein samples were separated by SDS-PAGE before being transferred to a nitrocellulose membrane, which was then incubated for 12 h with the anti-Akt1 (Cell Signaling) or anti-THEM4 (Abnova, H00117145-B01) antibody at 4°C. After extensive washing, the membrane was treated with anti-mouse HRP and anti-rabbit HRP antibody (Invitrogen). Chemiluminescence was carried out using enhanced chemical luminescent reagent (PerkinElmer). The image was obtained by exposing the membrane to Kodak Biomax light film (Sigma-Aldrich).

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Table SI1. Summary of X-ray data collection and structure refinement.

Data collection	
Space group	$P2_12_12_1$
Cell dimension (Å)	a = 56.6, b = 58.8, c = 135.4
Wavelength (Å)	0.9202
Resolution (Å)	2.3
No. of observed reflections	85,318
Completeness (%)[a]	99.8 (99.9)
No. of unique reflections	20,650
R_{merge}[b]	0.086 (0.331)
<I/s(I)>	9.5 (3.4)
Redundancy	4.1 (4.2)
Refinement	
No. of reflections used	20,649
No. of protein atoms	2,903
No. of UNC atoms	120
No. of water atoms	213
R_{cryst}[c]	0.198 (0.264)
R_{free}[d]	0.255 (0.330)
RMSd from ideal geometry	
Bond length (Å)	0.006
Bond angle (°)	1.3
Average B factor (Å^2)	
Protein	34
UNC	38
Water	40
Ramachandran plot (%)[e]	91.4, 8.6, 0.0, 0.0

[a]The values in parentheses are for the highest resolution shell, 2.38-2.30 Å.

[b]R_{merge} = å$_{hkl}$[(å$_j$ | I_j – <I> |) / å$_j$| I_j|].

[c]R_{cryst}=å$_{hkl}$| |F_o| – |F_c| | / å$_{hkl}$|F_o|, where F_o and F_c are the observed and calculated structure factors, respectively.

[d]R_{free} is computed from 1,001 randomly selected reflections omitted from the refinement.

[e]Ramachandran plot categories are most favored, allowed, generously allowed, and disallowed (*11, 12*).

Table SI2. hHTEM4 native molecular weight determination by SEC-LS/RI/UV.

RUN	PEAK Elution at UV trace (mL)	MW (kDa) Calculated average for the major peak (Mw)	MW (kDa) Calculated range of Mw observed	Sequence Predicated MW for monomer (kDa)	Oligomeric association
Full length hTHEM4	15.33	61	60-66	29	dimer

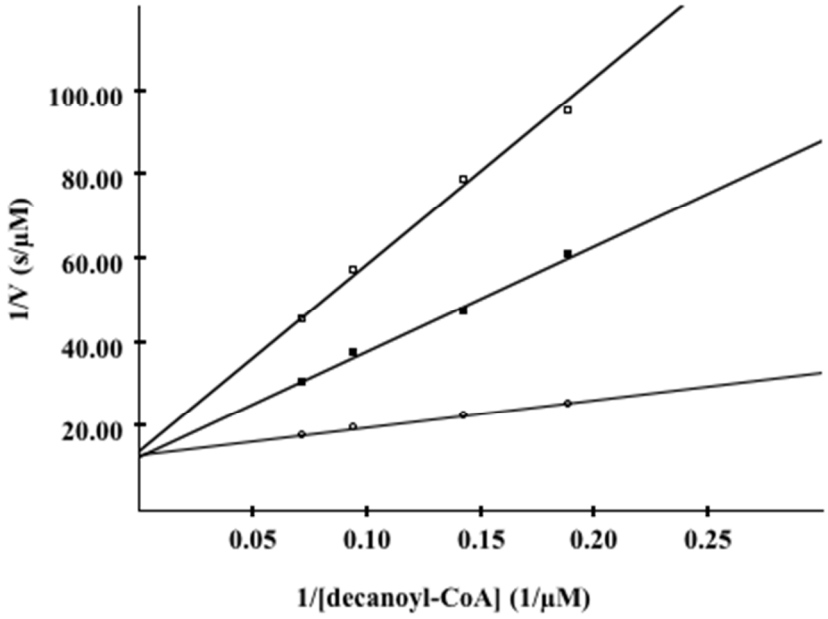


Figure SI1. Double reciprocal plot of the initial reaction velocity (V) vs the concentration of the decanoyl-CoA substrate. Reaction solutions initially contained 5 to 15 µM decanoyl-CoA, 0.02 µM His_6-hTHEM4, 2 mM DTNB (pH 7.5, 25^{o}C) and 0.0 (O), 2.5 (■) and 5.0 µM (□) undecan-2-one-CoA.

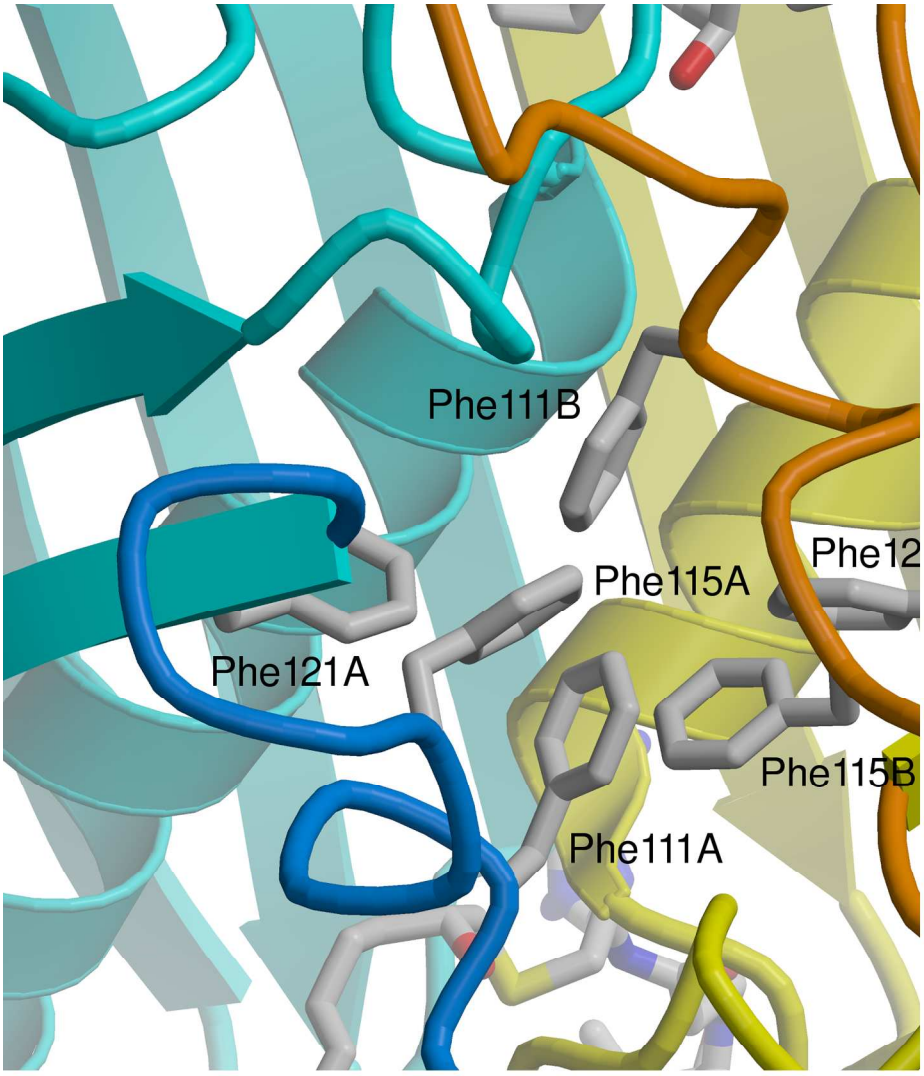


Figure SI2. The cluster of Phe residues at the hTHEM4 dimer interface.

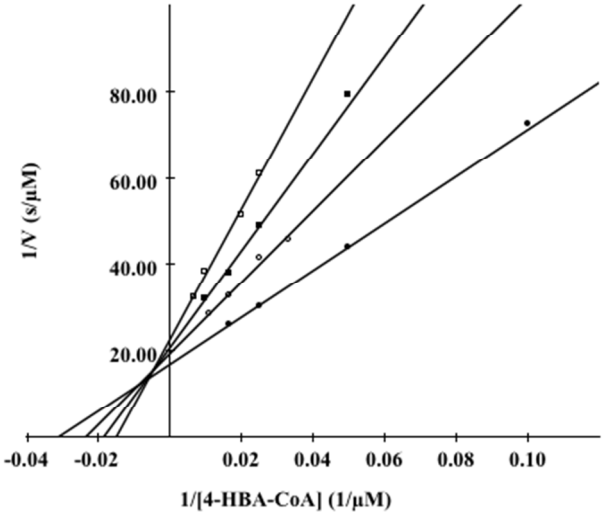


Figure SI3. Double reciprocal plot of the initial reaction velocity (V) vs the concentration of the 4-hydroxybenzoyl-CoA substrate. Reaction solutions initially contained 10 to 150 μM 4-HBA-CoA, 0.3 μM hTHEM4, 50 mM HEPES (pH 7.5, 25°C) and 0 (●), 50 (O), 100 (■) and 150 (□) μM CoA.

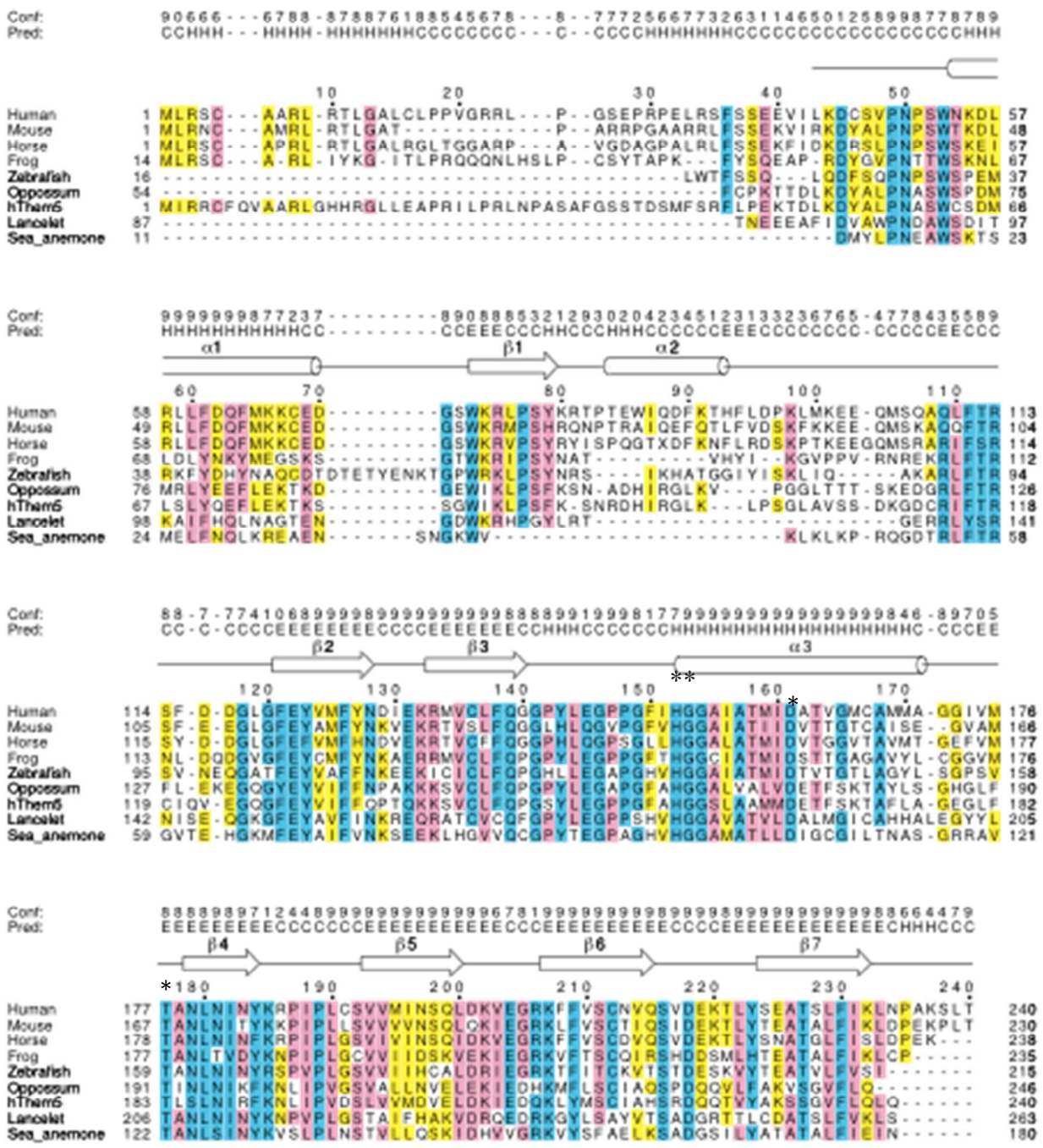
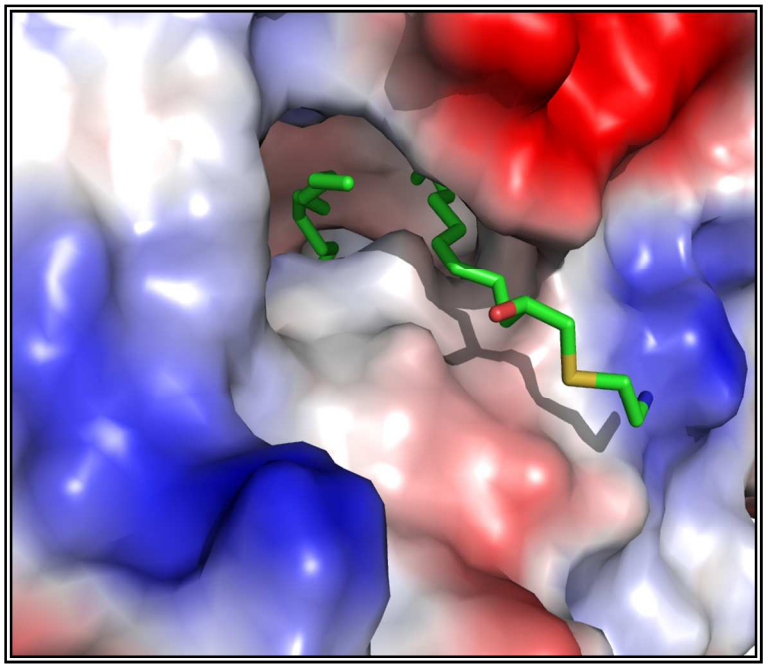


Figure SI4. Alignment of eukaryotic THEM4 sequences plus the human THEM5 sequence. Catalytic residues Asp161, Thr177, His152 and Gly153 are indicated by *.



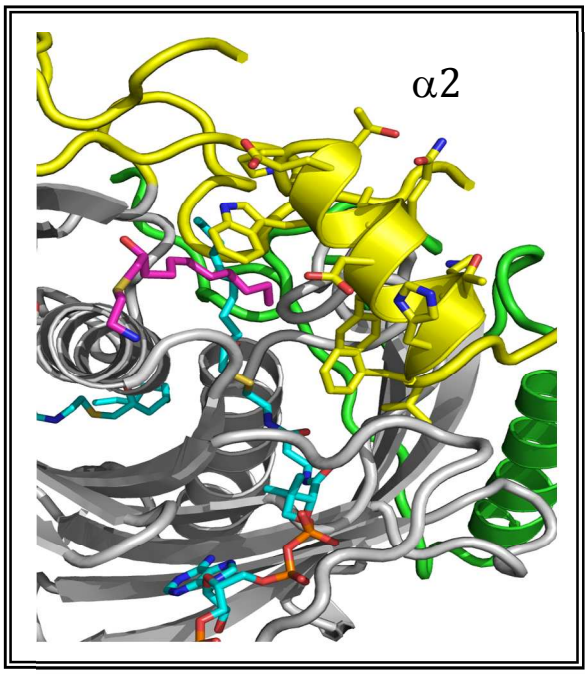


Figure SI5. (Left) The undecan-2-one-CoA C_9-(C=O)-C-S-C-C-N fragment shown in stick in relation to the terminal region of the undecan-2-one-CoA ligand (also shown in stick) bound in the substrate-binding site. Undecan-2-one-CoA carbon atoms are colored green, oxygen atoms red, nitrogen atoms blue and sulfur atoms yellow. hTHEM4 surface carbon atoms are colored white, nitrogen atoms blue and oxygen red. (**Right**) Hotdog-fold domains are colored gray, N-terminal domain of subunit A green, N-terminal domain of subunit B yellow. The undecan-2-one-CoA C_9-(C=O)-C-S-C-C-N fragment is shown in stick with magenta carbon atoms, red oxygen atoms and blue nitrogen atoms. The undecan-2-one-CoA ligand bound in the substrate-binding site is shown in stick with cyan carbon atoms, red oxygen atoms and blue nitrogen atoms. The amino acid side chains of α2 of subunit B are shown in stick with carbon atoms colored yellow, oxygen atoms red and nitrogen atoms blue.