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Thioesterase superfamily member 2 (Them2)/acyl-CoA thioesterase 13 (Acot13): a homotetrameric hotdog fold thioesterase with selectivity for long-chain fatty acyl-CoAs

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Abstract

Them2 (thioesterase superfamily member 2) is a 140-aminoacid protein of unknown biological function that comprises a single hotdog fold thioesterase domain. On the basis of its putative association with mitochondria, accentuated expression in oxidative tissues and interaction with StarD2 (also known as phosphatidylcholine-transfer protein, PC-TP), a regulator of fatty acid metabolism, we explored whether Them2 functions as a physiologically relevant fatty acyl-CoA thioesterase. In solution, Them2 formed a stable homotetramer, which denatured in a single transition at 59.3 °C. Them2 exhibited thioesterase activity for medium- and long-chain acyl-CoAs, with K_m values that decreased exponentially as a function of increasing acyl chain length. Steady-state kinetic parameters for Them2 were characteristic of long-chain mammalian acyl-CoA thioesterases, with minimal values of K_m and maximal values of k_{cat}/K_m observed for myristoyl-CoA and palmitoyl-CoA. For these acyl-CoAs, substrate inhibition was observed when concentrations approached their critical micellar concentrations. The acyl-CoA thioesterase activity of Them2 was optimized at physiological temperature, ionic strength and pH. For both myristoyl-CoA and palmitoyl-CoA, the addition of StarD2 increased the k_{cat} of Them2. Enzymatic activity was decreased by the addition of phosphatidic acid/phosphatidylcholine small unilamellar vesicles. Them2 expression, which was most pronounced in mouse heart, was associated with mitochondria and was induced by activation of PPAR α (peroxisome-proliferator-activated receptor α). We conclude that, under biological conditions, Them2 probably functions as a homotetrameric long-chain acyl-CoA thioesterase. Accordingly, Them2 has been designated as the 13th member of the mammalian acyl-CoA thioesterase family, Acot13.

Keywords

fatty acid; fatty acyl-CoA; homotetramer; hotdog fold thioesterase; phospholipid; StarD2

AUTHOR CONTRIBUTION

Jie Wei and David Cohen designed the research study, Jie Wei and Hye Won Kang performed the experiments, Jie Wei, Hye Won Kang and David Cohen analysed the data, and Jie Wei and David Cohen wrote the paper.

INTRODUCTION

Acyl-CoA thioesterases (Acots) catalyse the hydrolysis of the thioester bonds of a variety of CoA esters, most notably fatty acyl-CoAs. As both their substrates and products may play fundamental roles in metabolism, signalling, gene transcription and enzyme regulation [1], Acots appear to be critical for cellular homeostasis. The Acot gene family in mice encodes 12 proteins, with diverse tissue expression and intracellular distribution [2].

On the basis of structural and functional data, Acot proteins have so far been assigned to the α/β -fold hydrolase [3,4] and the hotdog fold thioesterase/dehydratase [5,6] superfamilies. The hotdog fold superfamily is characterized by a conserved fold that consists of an antiparallel β -sheet 'bun' that wraps around an α -helical 'sausage' [5,7]. A consistent feature of the hotdog fold domain is multimerization to create an active enzyme. On the basis of structure predictions [5], five of the 12 Acot genes in the mouse (Acots 7, 8, 9, 11 and 12) are members of the hotdog fold superfamily. This has been verified for Acot7 [6], which contains two hotdog fold domains that assemble to form a quaternary structure comprising a trimer of hotdog fold dimers. A distinguishing characteristic of Acots 11 and 12 is the presence of two hotdog fold thioesterase domains combined with a C-terminal START (steroidogenic acute regulatory transfer-related) domain [5,8]. As START domain proteins, Acot11 [also known as Them1 (thioesterase superfamily member 1), BFIT (brown-fat-inducible thioesterase) and THEA (thioesterase adipose associated)] and Acot12 [also known as CACH (cytosolic acetyl-CoA hydrolase)] have been designated StarD14 and StarD15 respectively [9,10]. Together, they comprise the thioesterase START group, which has been classified as the fifth of six subfamilies of START domain proteins [9,10].

StarD2 [also known as PC-TP (phosphatidylcholine-transfer protein)], is called a START-domain minimal protein because the entire amino acid sequence comprises the START domain [11,12]. Recently, a yeast two-hybrid screen of a mouse liver cDNA library identified Them2 (thioesterase superfamily member 2) as a StarD2-interacting protein [13]. Them2 is an 140-amino-acid protein that comprises a single hotdog fold thioesterase domain. Whereas a preliminary crystal structure suggests dimerization of mouse Them2 (PDB code 2CY9), the crystal structure of human THEM2 revealed a tetramer [14]. An initial substrate survey suggested that THEM2 targets a CoA thioester of a functionalized polar aromatic compound (e.g. phenylacetyl-CoA) [14], but discounted fatty acyl-CoAs as potential physiological substrates for THEM2. A role for THEM2 in cellular proliferation has been demonstrated and the protein localized in part to microtubules in certain cell lines [15], but the relationship of these findings to the thioesterase activity of THEM2 remains undefined.

The present study systematically explored the potential function and regulation of Them2 as an acyl-CoA thioesterase. This was motivated by several observations: (i) Them2 is putatively associated with mitochondria [16] and mRNA expression is accentuated in oxidative tissues [13], suggesting a role in fatty acid metabolism, (ii) Them2 interacts physically with StarD2, which plays a key role in fatty acid metabolism [17], and (iii) this interaction appears to favour a complex consisting of Them2 and StarD2 in a 2:1 Them2/StarD2 stoichiometric ratio. This is the same ratio that is observed for the thioesterase and START domains contained within Them1 [9,10,13]. Them1 functions as an acyl-CoA thioesterase with substrate specificity for medium-to long-chain fatty acyl-CoAs and is postulated to play a key role in the metabolism of lipids by brown fat [18].

The findings of the present study indicate that Them2 forms a homotetramer with substrate preference for the hydrolysis of long-chain fatty acyl-CoAs and has steady-state enzyme kinetics similar to other mammalian acyl-CoA thioesterases. Them2 activity was increased

by addition of StarD2, but not influenced by microtubules or tubulin dimers. Consistent with a role in fatty acyl-CoA metabolism, expression of Them2 was highest in heart, associated with mitochondria and was increased by activation of PPAR α (peroxisome-proliferator-activated receptor α). Them2 activity was regulated by the concentration and composition of model membranes, which represents a newly appreciated, physiologically relevant mechanism for the control of acyl-CoA thioesterase activity. On the basis of these data, mouse *Them2* and human *THEM2* have been added to the Acot and ACOT families as *Acot13* and *ACOT13* respectively, by the HUGO Gene Nomenclature Committee and the Mouse Genomic Nomenclature Committee.

EXPERIMENTAL

Expression and purification of recombinant proteins

Recombinant mouse Them2 was expressed and purified as described in [13], with modifications. Briefly, pET19b (Novagen) containing the open reading frame of Them2 was transformed into *Escherichia coli* BL21 (DE3) cells, and protein expression was induced by the addition of 1 mM IPTG (isopropyl β -D-thiogalactoside) to LB (Luria-Bertani) broth followed by 4 h of shaking (250 rev./min) at 37°C. The pET19b-Them2 expression vector introduced an in-frame N-terminal His₁₀ tag followed by an enterokinase recognition site that facilitated its removal following affinity purification. Bacteria were harvested by centrifugation and the pellet resuspended in BugBuster™ containing Benzonase (Novagen) plus protease inhibitors, and subjected to rotary shaking for 30 min at room temperature (23 °C). After ultracentrifugation using a Beckman L8-80M ultracentrifuge with a SW41Ti rotor at 24149 rev./min for 30 min at 4°C, the supernatant containing Them2 was applied to a HisTrap column (GE Healthcare). Bound His₁₀-tagged Them2 was eluted using a stepped gradient of 5 ml of 150 mM imidazole followed by 5 ml of 500 mM imidazole. Fractions containing Them2 were pooled and then dialysed against 20 mM sodium-phosphate buffer (pH 7.4). Them2 protein yielded a single band by SDS/PAGE (15% gel) followed by Coomassie Brilliant Blue staining. Recombinant StarD2 was purified as described previously [13]. As we had determined, in preliminary experiments, that the presence of the His₁₀-tag did not appreciably influence Them2 enzymatic activity, the tag was not removed for the current studies. Concentrations of Them2 were determined according to its molar absorption coefficient at 280 nm, which was calculated based on the primary sequence (<http://www.expasy.org>), or by using the Bradford method [19] using a Bio-Rad reagent.

Multimerization of Them2

Multimerization of Them2 was assessed by FPLC. Purified Them2 (100 μ l of 0.1 mM protein) was applied to a Superdex 75 10/300 GL column (GE Healthcare) that was pre-equilibrated with buffer consisting of 20 mM Tris/HCl, 100 mM NaCl and 10% glycerol (pH 7.5). Them2 was eluted with the same buffer at a flow rate of 0.5 ml/min at 4°C, with detection by absorbance at 280 nm. The column was calibrated using a Gel Filtration Calibration Kit LMW (GE Healthcare). Protein standards (2 mg/ml in 100 μ l of buffer containing 20 mM Tris/HCl, 100 mM NaCl and 10% glycerol, pH 7.5) were loaded on to the Superdex 75 10/300 GL column and eluted in the same buffer. Elution volumes of the protein standards were used to create a calibration curve for determining the molecular mass of Them2.

Thermal denaturation of Them2

Heat-induced denaturation of Them2 was characterized using CD spectroscopy using a JASCO model 810 spectropolarimeter fitted with a 0.1 cm cuvette. CD of Them2 in 10 mM sodium-phosphate (pH 7.4) was measured at wavelengths ranging from 190 to 260 nm in 0.5 nm increments at a rate of 10 nm/min. Two scans were averaged for each spectrum, with

baseline correction by buffer subtraction. For thermal denaturation, molar ellipticity at 208 nm (Θ_{208}) was measured as a function of temperature ranging from 10 to 90°C. The data were fitted using a two-state model (folded and unfolded protein) as described in [20]: $[\Theta] = (1 - f_D)\Theta_N + f_D\Theta_D$, where Θ_N and Θ_D represent the pre-transition (N) and post-transition (D) ellipticity values respectively, and f_D is the fraction of denatured protein. The equilibrium constant (K) was determined at each temperature using the fitted value of f_D : $K = 2f_D^2C_t/(1 - f_D)$, where C_t is the concentration of Them2. The midpoint of protein unfolding transition (T_m) was determined for the midpoint of the transition curve (i.e. $f_D = 0.5$) using the relationship $\Delta G = -RT\ln K$, where ΔG is the Gibbs–Helmholtz free energy of protein unfolding, and the relationship $\Delta G^\circ(T) = \Delta H(T_m)(1 - T/T_m) + \Delta C_p[(T - T_m) - T\ln(T/T_m)] - RT_m\ln C_t$, where ΔC_p is the heat capacity of unfolding at constant pressure, and $\Delta H(T_m)$ is the apparent enthalpy of unfolding at T_m .

Thioesterase activity assay

The acyl-CoA thioesterase activity of purified Them2 was measured using DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] (Molecular Probes) [21]. Hydrolysis of acyl-CoA molecules leads to a rapid reaction between DTNB and CoA to form 5-thio-2-nitrobenzoate, which absorbs light at 412 nm with a molar absorption coefficient of $13.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [22]. Reactions were carried out in 200 μl volumes within the wells of 96-well Nunc plates (Fisher Scientific). Pure acyl-CoAs of various hydrocarbon chain length and saturation were obtained from Avanti Polar Lipids. Acetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA, β -hydroxybutyryl-CoA, malonyl-CoA, 2-butenoyl-CoA and phenylacetyl-CoA were purchased from Sigma–Aldrich. Substrates were prepared as 10 mM stock solutions in water. Appropriate volumes were mixed with reaction buffer to achieve final concentrations in 50 mM KCl, 10 mM Hepes (pH 7.5) and 0.3 mM DTNB. Them2 was then added to initiate acyl-CoA hydrolysis. Plates were immediately loaded into a temperature-controlled SpectraMax M5 microplate reader (Molecular Devices), mixed for 5 s and absorbance readings at 412 nm (A_{412}) were taken at 1 min intervals for up to 60 min.

Kinetic characterization of Them2 acyl-CoA thioesterase activity

Steady-state kinetic parameters of Them2 activity were determined from rates of CoA liberated as functions of time following mixing of the enzyme (E) Them2 with the substrate (S) acyl-CoA. Initial rates (V_0) were determined using SoftMax Pro software (Molecular Devices) as the maximal slope of A_{412} against time curves. Values of V_0 were determined by iterative linear regressions, each of which utilized five successive time points. Upon varying [S] to create saturation curves, values of V_0 were fitted to the Michaelis–Menten equation $V_0 = V_{\max}[S]/([S] + K_m)$ using Prism 4 (GraphPad Software) to yield $V_{\max}$, the maximum velocity and K_m , the Michaelis–Menten constant. When substrate inhibition was observed, the Michaelis–Menten equation was modified to: $V_0 = V_{\max}[S]/\{K_m + [S](1 + [S]/K_i)\}$, where K_i is the inhibition constant. Values of k_{cat} were calculated as $V_{\max}/[E]$.

The pH-dependence of Them2 activity was characterized using a universal tribuffer system containing 50 mM sodium-acetate, 50 mM 2-(*N*-morpholino) ethanesulfonate and 100 mM Tris/HCl [23]. The influence of ionic composition on Them2 was explored by varying the concentrations of NaCl and KCl in 10 mM Hepes (pH 7.5). The influence of temperature was determined by measuring steady-state kinetic parameters at 25, 37 and 50°C. The effects of recombinant StarD2 on Them2 activity were determined by addition to Them2 in the same buffer at 37°C. The influence of membrane concentration and charge on Them2 activity was explored using small unilamellar vesicles. Egg-yolk phosphatidylcholine and egg phosphatidic acid were obtained from Avanti Polar Lipids. Phospholipids dissolved in chloroform were combined in various phosphatidic acid/phosphatidylcholine molar ratios, dried under a stream of nitrogen, resuspended in 10 mM Hepes plus 50 mM KCl (pH 7.5)

and sonicated (using a Special Ultrasonic Cleaner, Laboratory Supply Corp.) as described previously [24]. The influence of microtubules and heterodimeric tubulin subunits on V_0 of Them2-mediated acyl-CoA hydrolysis was determined at 37°C. To prepare microtubules, purified bovine brain tubulin (Cytoskeleton) was dissolved in 80 mM sodium-Pipes (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 5% glycerol and 20 μM Taxol (Cytoskeleton) to achieve final concentrations of 5 mg/ml in 50 μl within the wells of 96-well Nunc plates. Polymerization was initiated by incubation at 37°C with shaking and monitored by time-dependent increases in A_{340} in the SpectraMax M5 Microplate Reader until values stabilized. For experiments using heterodimeric tubulin subunits, tubulin was dissolved (5 mg/ml) in 50 μl sodium-Pipes (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA plus 30 μM nocodazole (Sigma), which prevented polymerization. The absence of polymerization at 37°C was confirmed by measuring the A_{340} . After 1 h of incubation with shaking at 37°C, 10 μl , containing microtubules or tubulin dimers, was added to a preheated reaction mixture (37°C) that included palmitoyl-CoA and DTNB. Acyl-CoA hydrolysis was initiated by adding Them2 to achieve a final volume of 200 μl , which contained 10 μM palmitoyl-CoA, 50 mM KCl, 10 mM Hepes (pH 7.5) and 0.3 mM DTNB.

Critical micellar concentrations of acyl-CoAs

Values of CMC (critical micellar concentration) for acyl-CoAs were determined by a fluorescence technique [25]. Briefly, the fluorescent probe TNS [6-(p-toluidino)-2-naphthalene sulfonate] was obtained from Sigma–Aldrich. Acyl-CoAs were dissolved in buffer containing 50 mM KCl, 10 mM Hepes (pH 7.5) and 15 μM TNS in wells of a 96-well Nunc plate. Relative fluorescence at 37°C was measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm using the SpectraMax M5 Microplate Reader. CMC values were determined from plots of fluorescence intensity as function of acyl-CoA concentration using linear regression analysis as described in [25].

Membrane binding of Them2

Binding of Them2 to small unilamellar vesicles was measured as described previously [24], with minor modifications. Purified Them2 was mixed with small unilamellar vesicles to a final volume of 200 μl . The final protein concentration (40 μM) was held constant, whereas the phospholipid concentration or composition was varied. Mixtures were incubated for 10 min at room temperature to allow binding of proteins to small unilamellar vesicles, and then unbound proteins were collected by ultrafiltration through Microcon YM100 100 kDa molecular mass cut-off filters (Millipore) by centrifugation at 3000 g for 5 min. Unbound proteins remaining within the filter were eluted by centrifugation at 3000 g for 2 min with the addition of 300 μl of 50 mM KCl and 10 mM Hepes (pH 7.5), and were combined with initial ultrafiltrates. Ultrafiltrates containing unbound proteins were subjected to SDS/PAGE (15% gel) followed by Coomassie Brilliant Blue staining. The influence of membrane binding on Them2 structure was characterized by CD at 25 and 37°C [24]. Final concentrations of Them2 and phospholipid were 86 μM and 1.0 mM respectively in 10 mM sodium phosphate (pH 7.4).

Tissue and organelle expression of Them2 and regulation by PPAR α

Eight week-old male FVB/NJ mice, as well as *Ppara*^{-/-} and wild-type 129S3/SvImJ mice were obtained from The Jackson Laboratory. Mice were housed in a standard 12 h light/12 h dark cycle facility and had free access to standard rodent diet 5001 (LabDiets) and water. Mice were killed by cardiac puncture following intraperitoneal injection of anaesthesia containing 90 mg of ketamine/kg of body weight (Webster Veterinary) and 10 mg of xylazine/kg of body weight (Webster Veterinary). Liver, kidney, heart and brown fat tissues were immediately harvested and snap-frozen in liquid nitrogen. Tissues were homogenized in RIPA buffer (1% Tergitol NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl,

50 mM Tris/HCl and 2 mM EDTA) using a PRO200 homogenizer (Midsco) at maximal speed. After incubation with shaking at 4°C for 40 min, homogenates were centrifuged at 14000 g for 10 min to remove tissue debris. Standard procedures were used to purify liver nuclear proteins [26], cytosol [27] and endoplasmic reticulum [28]. Purified mitochondria were prepared by Percoll gradient centrifugation [29]. The peptide EKVTLVSAAPEKLIC designed to match a sequence of amino acids 26–40 of mouse Them2 was synthesized, covalently attached to keyhole-limpet haemocyanin and used as an antigen to produce rabbit anti-mouse Them2 polyclonal serum (Covance Research Products). A polyclonal antibody against StarD2 was as described previously [13]. A monoclonal antibody against mouse β -actin was purchased from Sigma–Aldrich. Proteins were fractionated by SDS/PAGE (15% gel) and subjected to Western blot analysis, with detection by enhanced chemiluminescence (Western Lightning chemiluminescence reagent, PerkinElmer).

To assess regulation by PPAR α , a fenofibrate-supplemented chow (0.2%, w/w) diet was prepared (Bioserve) by pelleting fenofibrate (Sigma–Aldrich) with standard rodent diet 5001 (Purina). Mice were fed on chow or the fenofibrate-supplemented diet for 7 days and then killed as described above. Livers were harvested immediately and snap-frozen in liquid nitrogen for subsequent analyses. Total RNA samples were prepared from the liver using TRizol® (Invitrogen). cDNA was synthesized and used to quantify *Them2* mRNA expression by quantitative PCR [13]. Protocols for animal use were approved by the institutional committee of Harvard Medical School.

RESULTS

Oligomerization and thermal stability of Them2

We first explored multimerization of Them2 (Figure 1). The FPLC elution profile of Them2 (Figure 1A) yielded one major peak corresponding to a molecular mass of 60 kDa, with a very small peak eluting at slightly less than 20 kDa. Considering the 15 kDa molecular mass of Them2, this finding is consistent with the formation of a homotetramer in aqueous solution. We next examined the thermal stability of Them2 using CD spectroscopy. Figure 1(B) demonstrates the influence of temperature on the fraction of denatured Them2, which was calculated based on changes in Θ_{208} . As shown in the inset of Figure 1(B), the shape of the far-ultraviolet CD spectrum of Them2 reflects contributions from both α -helical and β -sheet structural elements. The sharp thermally induced transition of Them2 was well represented ($R^2 > 0.99$) by the two-state model with $T_m = 59.3$ °C, at which 50% of Them2 was dissociated and denatured. The absence of additional temperature-dependent transitions suggests that denaturation of Them2 subunits occurred in concert with dissociation of Them2 tetramers.

Acyl-CoA thioesterase activity of Them2

The acyl-CoA thioesterase activity of Them2 was monitored according to time-dependent increases in A_{412} values that reflected the formation of 5-thio-2-nitrobenzoate (Figure 2). Figure 2(A) shows the influence of various Them2 concentrations at a constant concentration of myristoyl-CoA (100 μ M). Although time-dependent increases in A_{412} values following mixing of Them2 and myristoyl-CoA were nearly linear, there was the suggestion of a sigmoidal shape that is consistent with a modest induction lag. Absorbance values levelled off during the 50 min time course for Them2 concentrations exceeding 3.3 μ M. The rate of increase in A_{412} varied in proportion to Them2 concentration, such that at very high concentrations of Them2 (>13.2 μ M), substantial proportions of myristoyl-CoA were hydrolysed before the first spectrophotometric reading that could be obtained. The concentration of Them2 did not influence maximum A_{412} values, which reflected the constant total concentration of myristoyl-CoA hydrolysed in the reaction. Figure 2(B)

demonstrates the activity of Them2 at a fixed concentration as functions of various myristoyl-CoA concentrations. In accordance with increases in substrate concentrations, maximal A_{412} values increased under these conditions.

Influence of acyl-CoA molecular species on thioesterase activity of Them2

We next explored the capacity of Them2 to hydrolyse various acyl-CoA molecular species (Figure 3). Figure 3(A) shows saturation curves for saturated acyl-CoAs with hydrocarbon chain lengths that varied from 6 to 14. Values of K_m and V_{max} , k_{cat} and k_{cat}/K_m are summarized in Table 1. Consistent with substrate inhibition, values of V_0 for the longer chain acyl-CoAs (i.e. palmitoyl and oleoyl) increased up to concentrations of 40 μ M and then declined (Figure 3B). On the basis of this observation, K_m and V_{max} values were determined using the modified form of the Michaelis–Menten equation, which incorporates substrate inhibition and are listed together with k_{cat} and k_{cat}/K_m values in Table 1. This analysis yielded K_i values of 56.1 and 31.2 for palmitoyl-CoA and oleoyl-CoA respectively. Saturation curves and kinetic parameters for additional Them2 substrates are shown in Figure 3(C) and are listed in Table 1 respectively. These demonstrate that compounds with a variety of structures exhibited approx. 10-fold higher values of K_m and 10-fold lower values of k_{cat}/K_m when compared with medium and long-chain fatty acyl-CoA molecules. As long-chain acyl-CoA micelles form in the concentration ranges that were studied [30–32], we tested whether the decline in V_0 values occurred at acyl-CoA concentrations in Figure 3(B) that coincided with the CMC values. Figure 3(D) shows results of CMC measurements under the same conditions (i.e. temperature and ionic strength) as were used in Figures 3(A) and 3(B). CMC values were 75 μ M for palmitoyl-CoA and 67 μ M for oleoyl-CoA. Results are not shown for the shorter-chain acyl-CoA molecular species, which showed no evidence of micelle formation at concentrations up to 200 μ M.

Effect of temperature, pH and ionic strength on Them2 activity

We next explored the influence of temperature on the myristoyl-CoA activity of Them2. Considering that the T_m for Them2 unfolding was observed to be 59.3 °C, with the transition beginning at temperatures in excess of 50°C (Figure 1B), we characterized the kinetics of Them2 activity at temperatures ranging from 25°C to 50°C (Figure 4). Figure 4(A) demonstrates the influence of increasing temperature on saturation curves using myristoyl-CoA as a substrate. Each curve was fitted ($R^2 > 0.97$) to the Michaelis–Menten equation to determine the steady-state kinetic parameters listed in Table 1. As shown in Figure 4(B), there was a substantial effect of temperature on both K_m and V_{max} , which increased 3-fold and 2-fold respectively from 25 to 50°C (Figure 4B). By contrast, there was only a modest 30% decrease in the value of k_{cat}/K_m over the same range of temperatures.

Figure 5 shows the effects of ionic strength and pH on the myristoyl-CoA thioesterase activity of Them2. For both NaCl and KCl (Figure 5A), there were modest increases (15–20%) in V_0 from 0–80 mM, which levelled off and then declined at concentrations exceeding 160 mM. For concentrations exceeding 320 mM, modest inhibition (up to 10%) of Them2 activity was observed compared with the absence of NaCl or KCl. As shown in Figure 5(B), there were marked (5-fold) increases in V_0 as pH was increased from 4.5–7.2, which then levelled off. Optimal ranges of ionic strengths and pH for the myristoyl-CoA activity of Them2 are noted in Table 1.

Influence of StarD2 and microtubules on the long-chain acyl-CoA thioesterase activity of Them2

We demonstrated previously that the addition of StarD2 increased Them2 rates of myristoyl acyl-CoA hydrolysis [13]. To confirm and extend these findings, we explored the influence of StarD2 on Them2 steady-state kinetics for myristoyl-CoA and palmitoyl-CoA at two

StarD2/Them2 molar ratios of 0.5 and 1.0. As listed in Table 1 for myristoyl-CoA, addition of StarD2 resulted in a decreased K_m , but increased V_{max} , such that k_{cat}/K_m values were increased 2-fold. For palmitoyl-CoA, both K_m and V_{max} of Them2 were increased by StarD2, so that k_{cat}/K_m remained essentially unchanged.

A report that Them2 co-localizes to microtubules in a U2OS osteogenic sarcoma cell line [15] suggests that these structures or their protein component (i.e. heterodimeric subunits of α - and β -tubulin) could influence the activity of Them2. However, under the conditions of our *in vitro* experiment, values of V_0 (nmol/min per mg) for palmitoyl-CoA thioesterase activity (mean $\pm$ S.D.) of Them2 ($V_0 = 21.4 \pm 2.8$) were not influenced by the presence of microtubules ($V_0 = 24.6 \pm 4.0$) and were somewhat reduced by dimeric tubulin subunits ($V_0 = 14.3 \pm 1.4$).

Small unilamellar vesicles inhibit the myristoyl-CoA activity of Them2

In cells, Them2 would be expected to interact with fatty acyl-CoAs in the context of membrane bilayers. We therefore examined the influence of membrane phospholipid composition and concentration on the hydrolysis of myristoyl-CoA by Them2 (Figure 6). As shown in Figure 6(A), the addition of pure phosphatidylcholine small unilamellar vesicles (i.e. phosphatidic acid/phosphatidylcholine molar ratio = 0) led to progressive inhibition of Them2 activity as the phospholipid concentration was increased, which levelled off near 75%. Table 1 lists the steady-state kinetic parameters for Them2 myristoyl-CoA thioesterase activity in the presence of 4 mM phosphatidylcholine small unilamellar vesicles. These data indicate that the addition of small unilamellar vesicles was associated with increased K_m and decreased V_{max} , with a corresponding decrease in k_{cat}/K_m . At a phosphatidic acid/phosphatidylcholine molar ratio of 0.1, there was also progressive inhibition in Them2 activity, reaching 95% by 10 mM phospholipid. When the phosphatidic acid/phosphatidylcholine molar ratio was increased to 0.2 or 0.3, there was potent inhibition (70%) at only 1 mM phospholipid concentration, which increased to 95% with increasing addition of small unilamellar vesicles. By contrast, small unilamellar vesicles with phosphatidic acid/phosphatidylcholine molar ratios of 0.4 were less effective at inhibiting Them2 activity, levelling off near 60% at phospholipid concentrations exceeding 6 mM. Figure 6(B) shows the same data as the percentage of V_0 for Them2-mediated hydrolysis of myristoyl-CoA in the absence of vesicles. In this format, it is apparent that Them2 activity was maximally suppressed by small unilamellar vesicles with phosphatidic acid/phosphatidylcholine molar ratios of 0.2 and 0.3.

In order to assess interactions between Them2 and model membranes, we measured binding to small unilamellar vesicles as functions of phospholipid concentration and composition (Figure 6C). As shown in the upper panel, there was a steady increase in Them2 binding as a function of phospholipid concentration for small unilamellar vesicles composed of pure phosphatidylcholine. The lower panel reveals that increasing phosphatidic acid contents of small unilamellar vesicles (1.0 mM phospholipid) resulted in a gradual increase in Them2 binding for phosphatidic acid/phosphatidylcholine molar ratios of 0–0.2. Between the molar ratios of 0.2 and 0.3, there was a sharp increase in binding, with little additional binding occurring at 0.4. Similar results were also observed at a higher phospholipid concentration of 4.0 mM (results not shown). Finally, we used CD in order to examine the influence of membrane binding on Them2 structure (Figure 6D). Compared with small unilamellar vesicles composed of pure phosphatidylcholine, a phosphatidic acid/phosphatidylcholine molar ratio of 0.1 was associated with more negative values of both Θ_{208} and Θ_{222} , consistent with increased α -helical content. With further increases in the phosphatidic acid/phosphatidylcholine molar ratio, Θ_{222} levelled off, whereas Θ_{208} returned to similar values as observed for pure phosphatidylcholine. The associated increase in the $\Theta_{222}/\Theta_{208}$ ratio was indicative of a conformation change, but there was no evidence that reduced acyl-CoA

thioesterase activity was attributable to denaturation of Them2 due to membrane-binding. Similar results were observed when these experiments were repeated at 25°C (results not shown).

Them2 is expressed at high levels in heart, is primarily associated with mitochondria and is regulated by PPAR α

Of the four oxidative tissues surveyed, Figure 7(A) shows that Them2 protein is most highly expressed in heart, followed by kidney, brown adipose tissue and liver. Although included as a loading control, we note that variations in expression of β -actin may themselves reflect differences in tissue expression. Figure 7(B) demonstrates that Them2 in the liver is primarily associated with mitochondria, but a significant fraction is present in the cytosol. This analysis also shows that StarD2 is concentrated in the cytosol, but is also associated with mitochondria and endoplasmic reticulum. As shown in Figure 7 (C), fenofibrate feeding increased the levels of *Them2* mRNA by 2.6- and 1.4-fold in FVB/JN and 129S3/SvImJ mice respectively. Up-regulation of *Them2* mRNA expression was not observed in 129S3/SvImJ *Ppara*^{-/-} mice, which supports PPAR α activation by fenofibrate as the mechanism. To garner further evidence for PPAR α -mediated regulation of Them2, we analysed a 2 kb region of the 5'-UTR (untranslated region) from mouse *Them2* (<http://www.ensembl.org>) for potential PPRES (PPAR α -response-elements) using the Transcription Element Search System (<http://www.cbil.upenn.edu/cgi-bin/tess>). This analysis identified three putative PPRES, which were located at nucleotides -1376, -1183 and -443.

DISCUSSION

The identification of Them2 as a StarD2-interacting protein with accentuated expression in oxidative tissues [13] suggested that its physiological substrates might be fatty acyl-CoAs. The results of the present study support the assertion that Them2 functions biologically as a long-chain acyl-CoA thioesterase and have led to its new designation as Acot13.

Whereas a preliminary report of the mouse Them2 crystal structure suggests that the protein self-assembles as a homodimer, the molecular mass of mouse Them2 in solution, observed by FPLC, is indicative of a homotetramer. This is in agreement with dynamic light scattering measurements, as well as the crystal structure determined for human THEM2 [31]. Our thermal denaturation data extend these structural data by demonstrating that both dissociation of the homotetramer and the denaturation of secondary-structural elements occur coincidentally as a single sharp transition at a temperature (59.3 °C) that is well above the pathophysiological range.

The steady-state kinetic constants of Them2, which were optimized under physiological conditions of temperature, ionic strength and pH, are consistent with a long-chain acyl-CoA thioesterase. In the present study, Them2 was localized to mitochondria and cytosol. Because the concentrations of long-chain acyl-CoAs are in the range 0.2–3.1 mM within mitochondria and 30–90 μ M in cytosol [1], the K_m values of Them2 for long-chain acyl-CoAs are sufficiently low to be physiologically relevant. This was not the case for the other metabolic substrates tested for which Them2 was either inactive (i.e. acetyl-CoA, 2-butenoyl-CoA) or for which the K_m values were an order of magnitude higher than their respective intracellular concentration ranges (i.e. 3-hydroxy-3-methylglutaryl-CoA, malonyl-CoA and β -hydroxybutyryl-CoA) [33–35].

We observed substrate inhibition for palmitoyl-CoA and oleoyl-CoA, but not for acyl-CoAs with shorter chain lengths. Acyl-CoAs are amphipathic molecules that form micelles, depending upon concentration, acyl chain length and saturation, temperature and ionic

strength [30–32]. For acyl-CoAs with saturated fatty acyl chains, CMC values decrease exponentially as a function of increasing chain length [31]. When measured at room temperature under similar conditions as used in the present study, the CMC values of myristoyl-CoA and lauroyl-CoA were 210 and 1130 μM respectively [31]. Considering that the CMCs of acyl-CoAs increase with temperature [30], their values would be expected to easily exceed the highest concentrations that were used to generate saturation curves for Them2 (100 μM). Indeed, we did not detect micelle formation of these fatty acyl-CoAs under conditions used to characterize Them2. By contrast, CMC values at room temperature for palmitoyl-CoA (30–80 μM) and oleoyl-CoA (32–33 μM) [25,30–32] fall within the range of substrate concentrations that we studied. Although these published values were measured under similar conditions of ionic strength and pH as in the present study, we characterized Them2 at 37°C. Consistent with the expected effect of increased temperature, at 37°C the CMC values of palmitoyl-CoA (75 μM) and oleoyl-CoA (67 μM) fell at or above the upper range of reported values at room temperature. Appreciating that the micelles form over a range of concentrations that include the empirically determined CMC value, as opposed to a single discrete concentration, the inhibition of Them2 activity at palmitoyl-CoA and oleoyl-CoA concentrations exceeding 40 μM was consistent with a detergent effect of these acyl-CoAs on Them2. Values of K_i determined for substrate inhibition for palmitoyl-CoA (56.1 μM) and oleoyl-CoA (31.2 μM) were also in reasonable agreement with the concentrations of acyl-CoAs that inhibited Them2. Overall, these findings support the assertion that micelle formation was the mechanism leading to substrate inhibition, as has been postulated for other acyl-CoA thioesterases [36–39].

A comparison of the steady-state kinetic parameters obtained in the present study with those of other Acot/ACOT proteins supports the assertion that Them2 functions biologically as a long-chain acyl-CoA thioesterase. As shown in Figure 8, the K_m for acyl-CoA thioesterase activity of Them2 was represented well ($R^2 > 0.99$) by an exponential function of saturated acyl chain length. When taken together with reported K_m values for short-chain fatty acyl-CoAs that exceed 100 μM [14], this relationship suggests that short- and medium-chain fatty acyl-CoAs are unlikely to be physiological substrates of Them2. Also plotted in Figure 8 are available K_m values for mammalian cytosolic [4,40], peroxisomal [39] and mitochondrial long chain acyl-CoA thioesterases [40,41], which follow a similar pattern as observed for Them2. By contrast, a non-specific bacterial hotdog fold thioesterase exhibits high affinity for all acyl chain lengths [42]. These observations suggest that the hydrophobicity of acyl-CoA molecular species, at least in part, plays a role in the access of the acyl-CoA to the enzyme active site of Them2 and the other mammalian acyl-CoA thioesterases. This possibility is strengthened further by the positive correlation between CMC values of acyl-CoAs [31] and the K_m for Them2 (inset of Figure 8).

It is important to note that our substrate survey for Them2 was performed at 37°C, whereas the literature values for other acyl-CoA thioesterases were measured at room temperature. Because the K_m of Them2 increases as a function of temperature, our results are not directly comparable. Indeed, K_m values for Them2 would probably be even more similar to the other long-chain acyl-CoA thioesterases plotted in Figure 8 if corrected for the effects of temperature. Another experimental difference is that values of K_m for the other acyl-CoA thioesterases were obtained following the addition of BSA to the media. BSA was included because substrate inhibition was generally observed when long-chain acyl-CoA concentrations exceeded ~ 10 μM . This was presumably due to the lower CMC values of long-chain acyl-CoAs at room temperature compared with at 37°C. As BSA may influence presentation of the substrate to the enzyme, its addition may have contributed, in part, to the lower K_m values observed for these enzymes.

Other steady-state parameters also support the assertion that Them2 may function as a long-chain acyl-CoA thioesterase. Consistent with the substrate specificities of other long-chain acyl thioesterases [1,6,39,40], values of k_{cat}/K_m for Them2 were maximized for myristoyl-CoA and palmitoyl-CoA (Figure 8). We note that values of V_{max} (nmol/min per mg) for Them2 do fall below other reported values [4,39,40], which vary (mean $\pm$ S.D.) from 524 ± 222 for ACOT2 [39] to 3750 ± 871 for Acot1 [39]. However, a direct comparison of V_{max} requires expression of this parameter in molar terms (i.e. k_{cat}). As Them2 forms a tetramer in solution, this necessitated the upward adjustment of k_{cat} values in Table 1 by 4-fold. With few exceptions [6,39], multimerization of other acyl-CoA thioesterases has not been defined, nor are sufficient data provided so that values of k_{cat} can be computed. In addition, literature values for V_{max} were derived using protein concentrations that were measured using the Bradford dye-based assay. As noted in Table 1, there was a discrepancy between this method and concentrations measured using the molar absorbance coefficient. This was most likely attributable to the amino acid sequence of Them2, which exhibits low contents of both aromatic amino acids, as well as dye-binding basic residues. If protein concentrations measured by the Bradford method were used for our calculation of V_{max} , this would have further increased values of k_{cat} 3-fold. As was the case for K_m , it is also possible that the addition of BSA to the medium may have influenced values obtained for V_{max} . Finally, it is important to consider that a comparison of the capacity for Acots to hydrolyse long-chain fatty acyl-CoAs under biological conditions depends not only on k_{cat} , but also on the relative cellular expression levels, which are not represented by these *in vitro* data.

In cells, it would be expected that access of Them2 to long-chain acyl-CoA molecules occurs in the context of membrane bilayers. Driven by hydrophobic interactions, palmitoyl-CoA has been shown to partition into model membranes [43], which sharply reduces monomeric concentrations in solution [44]. As myristoyl-CoA does not form micelles that might perturb membrane structure, we chose this molecular species to examine the effects of small unilamellar vesicles on Them2 activity. The observed dependence on both phospholipid concentration and net negative charge suggests that interactions between Them2 and membrane bilayers may modulate access of the enzyme to acyl-CoA molecules. This could occur by a mechanism in which a non-specific association of the protein with the membrane bilayer reduces its enzymatic activity [45]. In support of this possibility, we observed increased binding of Them2 to small unilamellar vesicles as functions of both concentration and phosphatidic acid content. It is noteworthy, however, that the sharpest decrease in Them2 activity occurred between phosphatidic acid/phosphatidylcholine molar ratios of 0.1 and 0.2, whereas the sharpest increase in binding occurred between molar ratios of 0.2 and 0.3. This suggests that the magnitude of membrane-binding does not entirely account for the observed changes in enzyme activity. CD measurements demonstrated that small unilamellar vesicles induced conformational changes in Them2, which also varied as functions of phosphatidic acid content and presumably contributed to the observed decreases in enzymatic activity. It is also possible that reduced Them2 activity may have been in part due to reduced free concentrations of myristoyl-CoA molecules in solution. The reduction in Them2 inhibition at the highest phosphatidic acid/phosphatidylcholine molar ratio of 0.4 is not readily explained by protein-membrane interactions based on charge alone, as both the extent of binding and the CD spectrum were similar to those observed for a molar ratio of 0.3. In this regard, Them2 activity might also have been in part governed by effects of phosphatidic acid on the curvature of small unilamellar vesicles [46], which may have influenced access of Them2 to its substrate. These issues notwithstanding, it is noteworthy that cellular membranes are composed of approx. 10–20% anionic phospholipids [47]. This creates the potential for the biologically relevant modulation of Them2 acyl-CoA thioesterase activity based on the effects of membrane charge. Whereas we attempted to substantiate these findings by examining the influence of purified mitochondria, the intrinsic acyl-CoA thioesterase activity of mitochondria (presumably due to the presence of native

Them2 and other mitochondrial acyl-CoA thioesterases [1]) did not allow us to detect inhibition of recombinant Them2 (results not shown).

The expression pattern and regulation of Them2 further supports the notion that this protein functions biologically as a fatty acyl-CoA thioesterase. Whereas Cheng et al. [15] reported that *Them2* mRNA levels measured by real time-PCR were highest in kidney, Northern blot analysis revealed that highest mRNA expression was in heart, followed by kidney, muscle and liver [13]. The latter distribution is supported by the protein expression pattern of Them2. Moreover, both heart and brown fat tissues are highly oxidative and preferentially consume fatty acids. Their high expression level of Them2 suggests that the enzyme is probably involved in endogenous metabolic activity and not simply detoxification of exogenous substances, such as phenylacetyl-CoA, for which the kinetic constants of Them2 (Table 1) and THEM2 [14] are suboptimal.

The subcellular localization of Them2 to mitochondria was first proposed based on a proteomic analysis of mitochondrial-associated proteins [16]. Previous confocal microscopy findings using both fluorescence-tagged proteins and immunofluorescence suggested that Them2 was bound to microtubules in U2OS cells [15] and was principally cytoplasmic/cytosolic, with a small fraction entering the nucleus of HEK (human embryonic kidney) 293T cells [13]. We did not observe an effect of microtubules on Them2 activity, and the mechanistic implications of this interaction remain to be defined. The subcellular fractionation approach in the present study revealed that Them2 is primarily associated with mitochondria, with an appreciable fraction also detected in the cytosol. This is consistent with a reversible interaction with StarD2, which is concentrated in the cytosol, but also detected in purified mitochondria, as shown in the present study and by others [48]. The association of both proteins with mitochondria further suggests that Them2 plays a role in the metabolism of fatty acids. The same is true of transcriptional regulation by PPAR α , which is a characteristic feature of fatty acyl-CoA thioesterases [1]. As activation of PPAR α also up-regulates both StarD2 [12,49] (which in turn increases the k_{cat} of Them2) and cellular fatty acyl-CoA concentrations [1], this may provide an integrated mechanism for the regulation of fatty acid oxidation in the cell.

While the present paper was under review, Cao et. al. [50] reported a structure–function analysis of human THEM2. On the basis of the structural characteristics of the catalytic site and high values of $k_{\text{cat}}/K_{\text{m}}$, these authors conclude that medium-to long-chain acyl-CoAs represent the biological substrates for THEM2. A long hydrophobic acyl-binding site is in keeping with our observation in Figure 8 of an exponential relationship between K_{m} and acyl chain length. Cao et al. [50] also demonstrate that, for certain hydroxylated phenylacetyl-CoA molecules only, an advantageous orientation of the thioester occurs within the catalytic site of the enzyme. This probably explains the unusually high $k_{\text{cat}}/K_{\text{m}}$ for these substrates [14]. This structure–function analysis further indicates that acyl proteins are unlikely to be physiological substrates of THEM2.

Abbreviations

Acot/ACOT	acyl-CoA thioesterase
CMC	critical micellar concentration
DTNB	5,5'-dithiobis(nitrobenzoic acid)
PPAR	peroxisome-proliferator-activated receptor
PPRE	PPAR-response element

START	steroidogenic acute regulatory transfer-related
Them	thioesterase superfamily member
TNS	6-(p-toluidino)-2-naphthalene sulfonate.

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Figure 1. Oligomerization and thermal stability of Them2

(A) FPLC elution profile of Them2, showing that the protein oligomerizes in solution to form a homotetramer. * denotes protein peaks. Arrows indicate elution volumes of molecular mass (kDa) standards: 1) 75, 2) 43, 3) 29, 4) 13.7 and 5) 6.5. (B) As described in the text, the fraction of denatured Them2 (f_D) was determined from Θ_{208} , and these data were fitted (solid line) to a two-state model (folded tetramer and unfolded monomer) with T_m (at $f_D = 0.5$) of 59.3°C. The inset shows the circular dichroic spectrum of Them2 at 37°C.

Figure 2. Acyl-CoA thioesterase activity of Them2

(A) The time-dependent Them2-mediated hydrolysis of 100 μM myristoyl-CoA was monitored at A_{412} for various Them2 concentrations: 0 μM (●), 1.7 μM (○), 3.3 μM (■), 6.6 μM (□), 13.2 μM (▲), 19.8 μM (△), 26.4 μM (◆) and 33.0 μM (◇). (B) The time-dependent hydrolysis by 5.0 μM Them2 of varied myristoyl-CoA concentrations: 0 μM (●), 10 μM (○), 20 μM (■), 40 μM (□), 60 μM (▲), 80 μM (△), 100 μM (◆) and 120 μM (◇). Reactions were carried out at 37°C in mixtures that included 10 mM Hepes (pH 7.5), 50 mM KCl and 0.3 mM DTNB. Each data point represents the average of two A_{412} determinations.

Figure 3. Acyl-CoA substrate specificity of Them2

(A) Saturation curves of initial velocity (V_0) for acyl-CoAs with ≤ 14 -carbon acyl chains: hexanoyl-CoA (●), decanoyl-CoA (■), lauroyl-CoA (▲) and myristoyl-CoA (◆). Solid lines indicate fitting of the data to the Michaelis–Menten equation. (B) Saturation curves of V_0 for acyl-CoAs with ≥ 16 -carbon acyl chains: palmitoyl-CoA (○) and oleoyl-CoA (□). Values of V_0 were fitted to a modified Michaelis–Menten equation, which takes substrate inhibition into account. (C) Saturation curves of V_0 for additional substrates: phenylacetyl-CoA (Δ), β -hydroxybutyryl-CoA (◇), malonyl-CoA (▽) and 3-hydroxy-3-methylglutaryl-CoA (X). For (A)–(C) reactions were carried out at 37°C in 10 mM Hepes (pH 7.5), 0.3 mM DTNB and 50 mM KCl. (D) CMCs of oleoyl-CoA (□) and palmitoyl-CoA (○) were determined at 37°C by fluorescence spectroscopy (excitation wavelength, 360 nm; emission wavelength, 460 nm) as described in the text using 15 μ M TNS in 10 mM Hepes (pH 7.5) and 50 mM KCl. Intersections of regression lines were used to determine CMC values of 75 μ M for palmitoyl-CoA and 67 μ M for oleoyl-CoA.

Figure 4. Temperature dependence of Them2 myristoyl-CoA thioesterase activity
(A) Saturation curves of V_0 for myristoyl-CoA were constructed for measurements at 25°C (●), 37°C (■) and 50°C (▲). Solid lines indicate fitting of the data to the Michaelis–Menten equation. **(B)** The influence of temperature on values of the kinetic parameters K_m (◇), V_{max} (◆) and k_{cat}/K_m (○) relative to 25°C for Them2 myristoyl-CoA thioesterase activity. Reactions were carried out in mixtures that included 10 mM Hepes (pH 7.5), 50 mM KCl and 0.3 mM DTNB.

Figure 5. Effects of ionic strength and pH on Them2 myristoyl-CoA thioesterase activity
(A) Them2 activity was determined in the presence of 100 μ M myristoyl-CoA for a wide range of NaCl (●) and KCl (○) concentrations. Values of V_0 are plotted as the fold change compared with the absence of either salt. **(B)** Thioesterase activity of Them2 was determined over a range of pH values as described in the text. Reactions were carried out at 37°C in mixtures that included 10 mM Hepes (pH 7.5), 50 mM KCl and 0.3 mM DTNB.

Figure 6. Phospholipid concentration and composition of small unilamellar vesicles regulate Them2 myristoyl-CoA thioesterase activity

Values of V_0 for 10 μ M Them 2 were determined using 100 μ M myristoyl-CoA in the presence of phosphatidic acid/phosphatidylcholine small unilamellar vesicles. **(A)** Percentage inhibition of V_0 when compared with the absence of small unilamellar vesicles for phosphatidic acid/phosphatidylcholine molar ratios of 0 (●), 0.1 (○), 0.2 (■), 0.3 (△) and 0.4 (▲). **(B)** The same data are plotted as a percentage of V_0 measured in the absence of small unilamellar vesicles (●) for total phospholipid concentrations of 1 mM (○), 2 mM (■), 4 mM (□), 6 mM (▲), 8 mM (△), 10 mM (◆) and 12 mM (◇). Reactions were carried out at 37°C in mixtures that included 10 mM Hepes (pH 7.5), 50 mM KCl and 0.3 mM DTNB. **(C)** Membrane binding of Them2 was determined according to the free fraction of protein (described in the text) as a function of increasing phospholipid (pure phosphatidylcholine) or phosphatidic acid/phosphatidylcholine molar ratios (1.0 mM phospholipid). These data are representative of two experiments. **(D)** Influence of small unilamellar vesicles on CD of Them2 at 37°C. Molar ellipticity $[\Theta]$ of 86 μ M Them2 was measured in the presence of small unilamellar vesicles (1.0 mM phospholipid) with increasing phosphatidic acid/phosphatidylcholine molar ratios: 0 (●), 0.1 (○), 0.2 (■), 0.3 (△) and 0.4 (▲).

Figure 7. Tissue distribution, subcellular localization and PPAR α -mediated regulation of Them2
(A) Them2 tissue distribution was ascertained by Western blot analysis of tissue homogenates harvested from two FVB/NJ mice (40 μ g of protein/lane). The blot was also probed with an anti- β -actin antibody. **(B)** Subcellular localization of Them2 and StarD2 was determined by Western blot analysis of proteins (40 μ g/lane) from liver homogenate (L), nuclei (N), cytosol (C), mitochondria (M) and endoplasmic reticulum (E) following purification from the liver of an FVB/NJ mouse. **(C)** Regulation of *Them2* mRNA by PPAR α was ascertained using FVB/NJ mice ($n = 8$ /group), as well as wild-type ($n = 4$ /group) and *Ppara*^{-/-} 129S3/ SvImJ ($n = 6$ /group). Mice were fed on chow (closed bars) or chow supplemented with 0.2% fenofibrate (open bars) for 7 days. *Them2* mRNA was

quantified by real-time PCR using cyclophilin as an invariant reference gene. The expression in chow-fed wild-type mice was set at 100%. Results are means $\pm$ S.E.M. * $P < 0.05$, using a two-tailed Student's t test.

Figure 8. Influence of fatty acyl-CoA chain length on steady-state kinetic parameters of acyl-CoA thioesterases

Values of K_m (solid lines) and k_{cat}/K_m (broken line) plotted as functions of chain length for saturated acyl-CoAs. Data for Them2 (●) are from Table 1, and the curved solid line represents the fit ($R^2 > 0.99$) of the data to an exponential function: $K_m = 675e^{-0.27(\text{acyl chain length})}$. Also plotted are K_m values for cytosolic Acot1 (◆) [4] and ACOT1 (▲) [40], peroxisomal Acot8 (◇) [39], mitochondrial ACOT2 (△) [40] and *Haemophilus influenzae* YciA (○) [42]. The inset plots the relationship between K_m determined from Figure 3 and CMC values of palmitoyl-CoA (□), myristoyl-CoA (◆) and lauroyl-CoA (■) from [31]. Values of K_m for Them2 were measured at 37°C in the absence of BSA, whereas for the other enzymes, K_m values were determined at room temperature with BSA added to the medium.

Table 1
Them2-catalysed acyl-CoA hydrolysis: steady-state kinetic constants and characteristics

Reactions were carried out at 37°C in 50 mM KCl, pH 7.5, unless otherwise specified. As determined for Them2 using myristoyl-CoA as the substrate, the optimal pH was ≥ 7.2 and the optimal range for ionic strength was 80–160 mM for both NaCl and KCl. Values of K_m and V_{max} were determined by fitting the experimental data to the Michaelis–Menten equation as described in the text. For the determination of kinetic constants, each data point (V_0) in the saturation curve represents the average of duplicate determinations. The kinetic constants reported for an experiment represent non-linear regression fits to these data. Protein concentrations were determined using molar absorbance values of Them2. Them2 concentrations measured using the Bradford method were 3-fold lower. Therefore, if used for calculating steady-kinetic constants, protein concentrations measured by the Bradford method would increase V_{max} , k_{cat} and k_{cat}/K_m 3-fold. Values of k_{cat} were calculated assuming that Them2 was tetrameric in solution. We observed slight differences in activity among Them2 preparations, which may have been due to the storage time of the protein and times required for mixing of reagents. In order to estimate these effects, the kinetic constants were determined on three separate occasions for myristoyl-CoA at 37°C. This revealed a variability (1 S.D.) of 8% for K_m and 13% for V_{max} .

Substrate	K_m (μ M)	V_{max} (nmol/min per mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Fatty acyl-CoAs				
Acetyl-CoA	Inactive			
2-Butenoyl-CoA	Inactive			
Hexanoyl-CoA (C _{6:0})	138.2	29.8	3.0×10^{-2}	2.2×10^2
Decanoyl-CoA (C _{10:0})	47.2	54.3	5.5×10^{-2}	1.2×10^3
Lauroyl-CoA (C _{12:0})	27.5	37.7	3.8×10^{-2}	1.4×10^3
Myristoyl-CoA (C _{14:0})	15.5	46.9	4.7×10^{-2}	3.1×10^3
25°C	7.3	30.4	3.1×10^{-2}	4.4×10^3
50°C	21.4	61.4	6.2×10^{-2}	2.9×10^3
Phosphatidylcholine (4 mM)*	22.8	32.2	3.3×10^{-2}	1.4×10^3
StarD2/Them2,0.5	10.6	68.1	6.9×10^{-2}	6.5×10^3
StarD2/Them2,1.0	13.6	84.6	8.6×10^{-2}	6.3×10^3
Palmitoyl-CoA (C _{16:0})	10.0	38.1	3.8×10^{-2}	3.8×10^3
StarD2/Them2,0.5	18.0	56.8	5.7×10^{-2}	3.2×10^3
StarD2/Them2,1.0	15.9	65.2	6.6×10^{-3}	4.2×10^3
Oleoyl-CoA (C _{18:1,n-9,cis})	27.4	60.4	6.1×10^{-2}	2.2×10^3
Other acyl-CoAs				
β -Hydroxybutyryl-CoA	162.8	45.4	4.6×10^{-2}	2.8×10^2
3-Hydroxy-3-methylglutaryl-CoA	305.7	6.9	7.0×10^{-3}	2.3×10^1
Malonyl-CoA	142.3	14.0	1.4×10^{-2}	1.0×10^2
Phenylacetyl-CoA	191.6	119.8	1.2×10^{-1}	6.3×10^2

* Determined in the presence of small unilamellar vesicles composed of pure phosphatidylcholine at 4 mM total phospholipid concentration.