The Akt C-Terminal Modulator Protein (CTMP) is an Acyl-CoA Thioesterase of the Hotdog-fold Family

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Contents: Eight pages total which include Tables SI1 and SI2 (initial velocities of Hthem4 catalyzed hydrolysis of myristoyl-CoA measured at different Akt1 concentrations), Figure S1 (molecular size determination of (Δ39)hTHEM4) and experimental protocols.

hTHEM4 Gene Cloning: (fl)hTHEM4, (Δ39)hTHEM4,(Δ99)hTHEM4

The gene encoding hTHEM4 in human (GI: 76159293) was amplified by PCR from cDNA clone MGC74423 (ATCC, GenBank accession number: BC065277) using primers 5'-CGGAGCATATGCTGAGGAGCTGCGCC-3' and 5'TTATAGGTGAGGATCCACAGCGCCTGG-3', which incorporate a NdeI site and a BamHI site. The resulting PCR fragment was digested with NdeI and BamHI restriction enzymes and ligated to a linearized pET-14b vector (Novagen) to encode the N-terminal His_6tagged full-length hTHEM4. The gene encoding the C-terminal His_6-tagged (Δ39)hTHEM4 was prepared by PCR using the full-length gene and the primers: 5'-GAGGTCATTTTCTTCATATGGAAGTCATTC-3' and 5'-CACCAGCAGCTCTCGAGTGTCAGACTTTTAGC-3'. The resulting PCR fragment was digested with NdeI and XhoI restriction enzymes and ligated to a linearized pET-23a vector. The construct that encodes the (Δ99)hTHEM4 truncation mutant was formed using primers 5'-CTTGACCCAAAGCATATGAAAGAAGAAC-3' and 5'-TTATAGGTGAGGATCCACAGCGCCTGG-3' with the hTHEM4 gene serving as the template. The PCR product was inserted into the Addgene plasmid 11540: pHM6g.TM1457 at the NdeI and BamHI cloning sites. The construct encodes His_6-MBP-(Δ99)hTHEM4 which includes the TEV protease cleave site. The gene sequences were confirmed by DNA sequencing (Health Sciences Center of the University of New Mexico)**.** The clones were used to transform BL21-codon plus (DE3) RIL competent cells for over-expression.

Preparation of (fl)hTHEM4, (Δ39)hTHEM4 and (Δ99)hTHEM4

Transformed BL21-codon plus (DE3) RIL competent cells were cultured at 20 °C with mixing at 180 RPM in LB media for 8 h (OD_{600} = 0.7), and then induced with 0.4 mM IPTG. After 12 h, the cells were harvested by centrifugation at 4°C and 6500 RPM. The 10 g of cell paste was suspended in 100 mL of ice-cold lysis buffer (50 mM NaH_2PO_4, 200 mM NaCl, 10 mM imidazole, and 0.5 mL protease inhibitor cocktail solution; pH 8.0), passed through a French press at 1200 PSIG, and then centrifuged at 48000 × g and 4 °C for 30 min. The supernatant was loaded onto a Ni-NTA agarose column (QIAGEN, 25 ml) pre-equilibrated with the lysis buffer. The column was washed with 300 mL of wash buffer (50 mM NaH_2PO_4, 200 mM NaCl, and 50 mM imidazole; pH 8.0) and then with 100 mL of elution buffer (50 mM NaH_2PO_4, 200 mM NaCl, 250 mM imidazole; pH 8.0). The fractions were analyzed by SDS-PAGE and then selectively pooled, concentrated using a Centricon (10 kDa, Pall Filtron) at 4 °C, dialyzed against 50 mM HEPES and 200 mM NaCl (pH 7.5) at 4 °C and then stored at -80°C. In the case of the His_6-MBP-(Δ99)hTHEM4 fusion protein, the His_6-MBP domain was removed using TEV protease (1 OD_{280} of TEV protease per 20 OD_{280} of protein in 50 mM NaH_2PO_4/200 mM NaCl/10 mM imidazole (pH 8), 12 h at 4°C). Ni-NTA column chromatography was carried out to remove the His_6-MBP.

Determination of the steady-state kinetic constants for hTHEM4 catalyzed acyl-CoA thioester hydrolysis.

Reactions were monitored at 25 °C by measuring the 412 nm absorbance of 5-thio-2-nitrobenzoate (ε=13.6 $mM^{-1}cm^{-1}$) formed by reaction of 5,5`-dithio-bis (2-nitrobenzoic acid) (DTNB) with the CoA liberated from the acyl-CoA substrates. Reactions were

initiated by adding hTHEM4 to 1 mL assay solutions containing substrate, DTNB (1 mM), KCl (0.2 M) and 50 mM K^+HEPES (pH 7.5). The kinetic parameters of 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 reported error was computed for the data fitting. 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.

The time course for the hTHEM4 catalyzed hydrolysis of 20 μM 4-hydroxybenzoyl-CoA (4-HBA-CoA) in 50 mM K^+HEPES/0.2 M KCl (pH 7.5; 25 ^oC) was monitored at 300 nm ($\Delta\varepsilon$ = 11.8 $mM^{-1}cm^{-1}$) to completion in order to determine if the accumulation of product reduced the reaction rate. The time course showed no evidence of product inhibition. The competitive inhibition constant K_i for CoA was determined by measuring the initial velocity of hTHEM4 catalyzed 4-HBA-CoA hydrolysis as a function of 4-HBA-CoA concentration (K_m to $10K_m$) and CoA concentration (0, 25, 50 and 75 μM). 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.

The inhibition of hTHEM4 by the 4-hydroxybenzoate, hexanoate, octanoate (25 mM stock solution prepared in 30% aq. ethanol) and lauroate (50 mM stock solution prepared in 50% aq. ethanol) was tested by measuring the initial velocity of hTHEM4 catalyzed hydrolysis of 4.2 μM lauroyl-CoA (~K_m level) in DTNB (1 mM), KCl (0.2 M) and 50 mM K^+HEPES (pH 7.5; 25 ^oC) in the absence and presence of increasing concentrations of inhibitor up to 2-5 mM. The K_i value was estimated from the concentration of inhibitor that decreased the initial velocity by half. If no inhibition was observed the K_i was assumed to be greater than the value of the highest inhibitor concentration tested.

hTHEM4 catalyzed hydrolysis of myristoylated holo-acyl carrier protein of the human cytoplasmic fatty acid synthase

The plasmids encoding ACP domain derived from human cytosolic fatty acid synthase and the human phosphopantetheinyl transferase (PPTase) were a kind gift from Dr. Stuart Smith, Children's Hospital Oakland Research Institute, Oakland, CA. The two recombinant proteins were purified as described earlier (*1,2*). The reaction mixture contained 60 μM apo-ACP, 150 μM myristoyl-CoA and 2 μM PPTase in 20 mL of 20 mM Tris/1 mM $MgCl_2$ (pH 7.0; 25 ^oC) for 3 h. The myristoyl-ACP solution was concentrated using a PALL 10K centrifuge A device and the structure was verified by ES-MS. Myristoyl-ACP calculated: 13618, observed: [M^+] 13617. The 200 μL reaction mixture initially containing 44 μM myristoyl-ACP, 10 μM (Δ39)hTHEM4, 50 mM K^+HEPES and 100 mM NaCl (pH 7.5, 37 ^oC) was incubated for 1 h and then subjected to

ES-MS analysis. The substrate peaks at [M^+] 13617, present in the spectrum of the control reaction (run in the absence of (Δ39)hTHEM4) were not observed in the spectrum of the reaction solution. Instead, a new peak at [M^+] 13405 (-210 Da = MW of myristolic acid) was present that corresponds to the deacylated holo-ACP. The steady state kinetic constants for the catalyzed hydrolysis reaction were determined by measuring the initial velocities of reaction solutions initially containing 10, 20, 40 and 80 μM myristoyl-ACP, 0.25 μM (Δ39)hTHEM4 and 0.3 mM DTNB in 50 mM K^+HEPES/0.2 M KCl (pH 7.5; 37 ^oC) and fitting the data to equation (1).

Determination of the effect of Akt1 on (fl)hTHEM4 and (Δ39)hTHEM4 activity.

The His_6-tagged full length Akt1 (3) was a kind gift from Dr. Lusong Luo of GlaxoSmithkline. Reaction solutions that initially contained (fl)hTHEM4 (0.04μM) or (Δ39)hTHEM4 (0.05 μM), myristoyl-CoA (5 μM), Akt1 (0, 0.4, 0.8, 1.5, 2.3 μM), DTNB (2 mM), KCl (0.15 M) and 50 mM K^+HEPES (pH 7.5, 25 °C) were monitored at 412 nm (ε = 13.6 mM^{-1} cm^{-1}) for CoA formation. The calculated initial velocities are reported in Tables SI1 and SI2.

References

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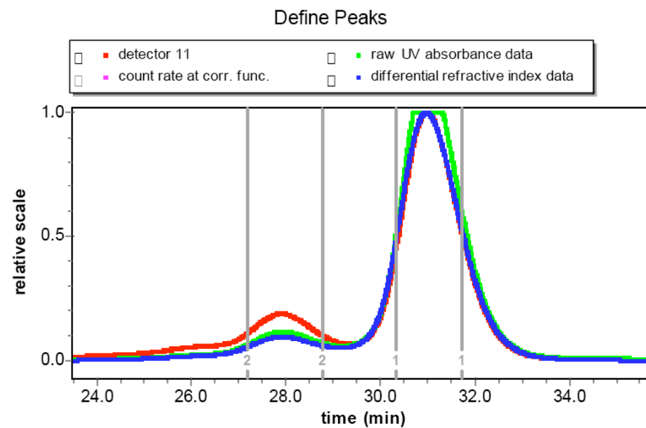
Table SI1. Hydrolysis velocities of myristoyl-CoA catalyzed by (fl)hTHEM4 at different concentrations of Akt1.

Akt, μM	Velocity (0s-50s), μM CoA/s
0	0.0135
0.4	0.0143
0.8	0.0170
1.5	0.0156
2.3	0.0155

Table SI2. Hydrolysis velocities of myristoyl-CoA catalyzed by (Δ39)hTHEM4 (0.05 μM) at different concentrations of Akt1.

Akt, μM	Velocity (0s-50s), μM CoA/s
0	0.0258
0.4	0.0291
0.8	0.0330
1.5	0.0513
3.0	0.0376

Figure SI1. The native molecular weight determination of (Δ39)hTHEM4 by SEC-LS/RI/UV analysis from ASTRA.



	Peak 1	Peak 2
Peak limits (min)	29.940-31.585	27.199-28.761
MW (kDa)	50.33	102.1
N (MW/24)	2.10	4.25
Oligomeric state	dimer	tetramer