Four *Trypanosoma brucei* fatty acyl-CoA synthetases: fatty acid specificity of the recombinant proteins

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As part of our investigation of fatty acid metabolism in *Trypanosoma brucei*, we have expressed four acyl-CoA synthetase (TbACS) genes in *Escherichia coli*. The recombinant proteins, with His-tags on their C-termini, were purified to near homogeneity using nickel-chelate affinity chromatography. Although these enzymes are highly homologous, they have distinct specificities for fatty acid chain length. TbACS1 prefers saturated fatty acids in the range C_{11:0} to C_{14:0} and TbACS2 prefers shorter

fatty acids, mainly C_{10:0}. TbACS3 and 4, which have 95 % sequence identity, have similar specificities, favouring fatty acids between C_{14:0} and C_{17:0}. In addition, TbACS1, 3 and 4 function well with a variety of unsaturated fatty acids.

Key words: ACS signature motif, enzyme-coupled assay, fatty acid chain length, glycosyl phosphatidylinositol myristoylation, recombinant acyl-CoA synthetases (ACS).

INTRODUCTION

Trypanosoma brucei, the protozoan parasite that causes sleeping sickness, is a major public health problem in large regions of sub-Saharan Africa. Trypanosomes are well known for using antigenic variation to evade the immune response of their hosts. This process is mediated by a surface coat, composed of 10⁷ identical variant surface glycoprotein (VSG) molecules that protect subsurface molecules from attack by the host's immune system. The trypanosome has genes for hundreds of different VSG molecules and by switching expression from one to another it is able to sustain a chronic infection [1]. The VSG's C-terminus is linked to a glycosyl phosphatidylinositol (GPI) membrane anchor. An important feature of the trypanosome GPI is the presence of two myristates as the fatty acid component [2]. Incorporation of myristate into the GPI precursor occurs by fatty acid remodelling reactions, in which longer fatty acids in the anchor precursor are replaced by myristate [3]. The myristate donor for GPI remodelling is myristoyl-CoA.

Since the trypanosome needs massive amounts of myristate to anchor its 10⁷ VSG molecules, and since there are only low quantities of this fatty acid in the host bloodstream, this parasite has evolved special mechanisms to ensure an adequate supply of myristate [4]. Trypanosomes can efficiently import myristate from the blood [5], as well as synthesize it *de novo* using a fatty acid synthesis pathway specialized for its production [6].

Because of the fascinating mechanisms involved in the uptake and synthesis of myristate, and in its incorporation into GPIs via the fatty acid remodelling pathway, our lab has developed a strong interest in trypanosome fatty acid metabolism and of myristate metabolism in particular. One key element in fatty acid metabolism is the mechanism by which free fatty acids are activated to form fatty acyl-CoAs, a reaction catalyzed by an acyl-CoA synthetase (ACS). For example, myristate, whether it is salvaged from the host bloodstream or synthesized *de novo*, must be converted to myristoyl-CoA before it can be incorporated

into a GPI. In other cells ACSs function in membrane biogenesis, fat deposition and energy production [7], and they are likely to have at least some of these roles in *T. brucei*.

Trypanosomes, like most other organisms, have multiple ACS genes whose products presumably have different intracellular localizations, different chain-length specificities and different functions. We recently described four tandemly-linked ACS genes (*TbACS1*, 2, 3 and 4) and all four are expressed in both bloodstream and procyclic trypanosomes [8]. It is possible that there are even more TbACSs, as indicated by a search of the rapidly expanding *T. brucei* genome database. In this paper, we report the expression of the four *T. brucei* ACS genes in *Escherichia coli*, purification of the recombinant enzymes to near homogeneity, and studies of their enzymic specificity. Our long-term goal is to understand the biological functions of these enzymes, especially as they relate to the metabolism of myristate.

MATERIALS AND METHODS

Materials

All saturated fatty acids were purchased from Sigma (St. Louis, MO, U.S.A.) and unsaturated fatty acids were from Supelco (Bellafonte, PA, U.S.A.). All fatty acid stocks were dissolved in methanol and stored at –80 °C. Unsaturated fatty acids were stored under nitrogen.

PCR amplification of four *TbACS* genes

For each *TbACS* gene, the entire open reading frame, excluding the stop codon, was PCR amplified. The primers for each gene were: *TbACS1* forward, 5'-GGAATGTACGTCACCTAAGCT-TCTCTCCC; *TbACS1* reverse, 5'-CTCATACCTTGAAAAGT-TGTTCAATGACATCGCCG; *TbACS2* forward, 5'-ATGAA-

Abbreviations used: ACS, acyl-CoA synthetase; TbACS, *Trypanosoma brucei* ACS; GPI, glycosyl phosphatidylinositol; VSG, variant surface glycoprotein.

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CACGCTTCTGCGCT; *TbACS2* reverse, 5'-GCACTCCTC-CACGAAAAG; *TbACS3* forward, 5'-ATGGGGGATGTG-TGATTTCCG; *TbACS3* reverse, 5'-GCACTCCTCCACGAA-AAGTGGTGC; *TbACS4* forward, 5'-ATGGGGGATGTG-TGATTTCCG; *TbACS4* reverse, 5'-GCACTCCTCCACGAAAGTGACAC. PCR for each gene except *TbACS4* was carried out in a 50 μ l volume containing 200 ng of P1-5D7 DNA (a phage P1 clone containing genes for all four TbACSs [8]), 200 nM of each primer, 1.5 mM MgCl₂, 200 μ M of each dNTP and 2.6 units of Expand High Fidelity PCR System polymerase (Roche, Indianapolis, IN, U.S.A.). Since the PCR primers for *TbACS3* and *TbACS4* are extremely similar, the 5.3 kb *Hind*III and *Bam*HI restriction digestion product of P1-5D7 DNA was used as a template for the *TbACS4* PCR reaction. Amplification conditions were: 94 °C, 2 min, followed by 25 cycles of: denaturation at 94 °C, 30 s; primer annealing at 50 °C, 30 s; extension at 72 °C, 2.5 min; and then a final cycle of 72 °C for 7 min.

Constructs for *TbACS* expression

The PCR products were cloned into a pBAD TOPO TA cloning vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.) and then transformed into One Shot chemically competent *E. coli* (Invitrogen). Plasmids from individual colonies were prepared (Qiagen QIAprep Miniprep Kit) and the sequences were verified by sequencing both strands. Cloning in this vector results in the addition of 14 extra amino acids at the N-terminus and 28 extra amino acids at the C-terminus of each enzyme.

Expression and purification of the recombinant TbACSs

Each strain was grown to a D_{600} of 0.5 at room temperature (23–25 °C) in 0.5 litre of Luria broth supplemented with 50 μ g/ml ampicillin. The production of TbACSs was induced by the addition of arabinose (final concentration, 0.1 %). While TbACS2, 3 and 4 were induced for 2 h at room temperature (23–25 °C), TbACS1 was induced overnight at 37 °C. Cells were then harvested by centrifugation (3000 g, 4 °C, 10 min). The cell pellets were resuspended in 10 ml of buffer A [50 mM sodium phosphate, pH 7.0, 300 mM NaCl, 5 mM 2-mercaptoethanol, 5 % (v/v) glycerol, 0.1 % Triton X-100, 0.5 mM benzamidine, 40 mM imidazole], lysed with a French press and the lysates were centrifuged (10000 g, 4 °C, 20 min). Ni-NTA resin (Ni²⁺-nitrilotriacetate resin; Qiagen, 3 ml), pre-equilibrated with buffer A, was added to the resulting supernatant and the mixture was gently stirred for 1 h at 4 °C. The resin was poured into a column and washed with 10 ml of buffer A. Recombinant enzymes were eluted with 10 ml of buffer B (50 mM sodium phosphate, pH 7.0, 300 mM NaCl, 5 mM 2-mercaptoethanol, 5 % glycerol, 0.1 % Triton X-100, 0.5 mM benzamidine, 200 mM imidazole).

Assay for TbACS activity

To study the fatty acid chain-length specificity of the recombinant synthetases, an enzyme-coupled assay specifically adapted to a 96-well microplate reader was used [9]. In this assay the fatty ACS converts the fatty acid substrate to fatty acyl-CoA, which then generates hydrogen peroxide in a reaction catalyzed by acyl-CoA oxidase. Utilizing catalase, hydrogen peroxide then reacts with methanol to form formaldehyde and water. Purpald (Sigma), in the presence of formaldehyde, forms a purple dye that can be measured at 550 nm. The assay was the same as that described by Knoll et al. [9] except that the 50 μ l reaction mixture

contained 50 mM Hepes, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 mM ATP, 1 mM CoA, 1 mM dithiothreitol, 200 μ M fatty acid, 10 % (v/v) methanol, 0.1 % Triton X-100, 21000 units/ml bovine liver catalase (Sigma) and 0.1 unit/ml *Candida sp.* acyl CoA oxidase (Sigma). The reaction mixtures were incubated at 37 °C for 20 min.

RESULTS

Expression and purification of TbACSs

To determine the substrate specificity of the TbACSs, we cloned each gene into the pBAD TOPO TA vector, an expression system that adds a V5 epitope and a His-tag to the C-terminus of the recombinant protein. The AraC gene product positively regulates the pBAD promoter, which drives protein expression. After verifying the sequence of the cloned genes, we induced each plasmid to express a large amount of protein. For TbACS2, 3 and 4, induction with 0.1 % arabinose at room temperature for 2 h was sufficient. However, for TbACS1, induction under these conditions yielded very little full-length protein. Therefore, TbACS1 was induced overnight at 37 °C. Because the native TbACSs are insoluble in aqueous buffer, presumably because they are membrane-associated proteins, the recombinant enzymes were purified in the presence of 0.1 % Triton X-100. Western analysis using anti-V5 antibody showed that the majority of each protein was soluble while a relatively small amount (~20 %) was in inclusion bodies. The soluble proteins were purified using one-step nickel affinity chromatography under non-denaturing conditions. From 0.5 litre of culture, we obtained 0.2 mg of TbACS1, 1.4 mg of TbACS2, 1.6 mg of TbACS3 and 0.8 mg of TbACS4. SDS/PAGE analysis of the purified recombinant proteins is shown in Figure 1.

Fatty acid specificity of TbACSs

In the enzyme-coupled assay, the product formation was linear with respect to time and ACS concentration under our assay conditions. Since three enzymes were used in this coupled reaction, we used acyl-CoA oxidase and catalase in 10-fold excess so that they were not a limiting factor in the reaction. We also used a *Pseudomonas sp.* ACS (Sigma) that utilizes a broad range of fatty acid substrates as a positive control to show that the ACS is the limiting factor in the reaction, confirming previous results of [9].

Figure 1 SDS/PAGE analysis of purified recombinant TbACSs

The proteins were denatured at 100 °C for 5 min and 2 μ g of sample was loaded on to each lane of an SDS-polyacrylamide (8%) gel. The gel was stained with Coomassie Blue. Lane 1, TbACS1; lane 2, TbACS2; lane 3, TbACS3; lane 4, TbACS4. Size markers are shown on both sides of the gel (M).

Figure 2 Substrate specificity of purified recombinant TbACS1–4 (panels a–d respectively) using saturated fatty acids ranging from C_{4:0} to C_{24:0} and the enzyme-coupled assay

Due to the low solubility of longer fatty acids, the final concentrations of behenic acid (C_{22:0}), tricosanoic acid (C_{23:0}) and lignoceric acid (C_{24:0}) were 100 μ M, 80 μ M and 80 μ M respectively (200 μ M was used in all other cases). The enzyme used in the 50 μ l reaction was 0.2 μ g for each TbACS. Data points represent the mean for three values obtained in one experiment $\pm$ 1 S. D.

Figures 2(a)–2(d) show the saturated fatty acid specificity of the four TbACSs. Similar to ACSs from other organisms, all four purified recombinant proteins utilize a range of substrates. Analysis of saturated fatty acids indicates that TbACS1 prefers C_{11:0}–C_{14:0} with lower activity on C_{9:0}–C_{10:0} and C_{15:0}–C_{19:0} (Figure 2a). TbACS3 and TbACS4 have roughly similar specificities, with highest activity on C_{14:0}–C_{16:0} (for TbACS3) and C_{14:0}–C_{18:0} (for TbACS4), and lower activity on C_{9:0}–C_{13:0} (Figures 2c and 2d). In contrast with TbACS1, 3 and 4, all of which have a strong preference for long-chain fatty acids,

Figure 3 Substrate specificity of purified TbACS1–4 (panels a–d respectively) using selected unsaturated fatty acid substrates and the enzyme-coupled assay

The enzyme used in the 50 μ l reaction was 0.4 μ g for TbACS1, 0.4 μ g for TbACS2, 0.4 μ g for TbACS3 and 0.2 μ g for TbACS4. Data points represent the mean for three values obtained in one experiment $\pm$ 1 S. D.

TbACS2 shows the highest activity toward the medium-chain substrate decanoic acid (C_{10:0}); it also functions on fatty acids between C_{5:0} and C_{9:0} and has little activity on fatty acids longer than C_{12:0} (Figure 2b).

We also studied the specificity of the TbACSs towards unsaturated fatty acid substrates, using C_{10:0} and C_{14:0} as controls. Figures 3(a)–3(d) show that TbACS1 has a preference for oleic acid (C_{18:1}), linoleic acid (C_{18:2}), gondoic acid (C_{20:1}), arachidonic acid (C_{20:4}) and docosahexanoic acid (C_{22:6}). TbACS2 has no detectable activity on any of the unsaturated fatty acids tested. TbACS3 has a preference for palmitoleic acid (C_{16:1}), oleic acid (C_{18:1}) and linoleic acid (C_{18:2}). TbACS4 prefers oleic acid (C_{18:1}) and linoleic acid (C_{18:2}).

Table 1 Kinetic constants for TbACS1, 2, 3 and 4

K_m and k_{cat} were calculated from Lineweaver-Burke plots. Concentrations of the TbACS proteins were measured by the Bradford assay, using BSA as a standard. Molarity of the enzyme was calculated from the predicted molecular mass of the recombinant protein.

TbACS	Substrate	k_{cat} (s ⁻¹)	$10^4 \times K_m$ (M)	$10^{-3} \times k_{cat}/K_m$ (M ⁻¹ · s ⁻¹)
1	C _{14:0}	0.5	0.9	5.7
2	C _{10:0}	0.6	1.2	4.9
3	C _{14:0}	1.1	2.4	4.6
4	C _{14:0}	1.2	2.9	4.2

We measured K_m and k_{cat} for all TbACS enzymes using preferred substrates (C_{14:0} for TbACS1, 3 and 4 and C_{10:0} for TbACS2), and the results are shown in Table 1.

DISCUSSION

Because of their central role in fatty acid metabolism and especially because one or more must play a role in the metabolism of myristate, we have initiated a study of *T. brucei* fatty ACSs. Previously we described the genes for four of these enzymes [8], and now we report their enzymic specificity.

We expressed the four TbACSs in *E. coli* and purified the recombinant proteins to near homogeneity. For each enzyme the molecular mass determined by SDS/PAGE agreed with the predicted value within approx. 5%. The 68 kDa band in Figure 1 (lane 4) could be due to translation initiation from an internal methionine in TbACS4.

We found that the four TbACSs have distinct fatty acid chain-length specificities. TbACS1 mainly prefers fatty acids in the range of C_{11:0} to C_{14:0}. It is not surprising that TbACS3 and 4 have similar specificities, preferring fatty acids in the range C_{14:0} to C_{18:0}, as these enzymes have amino acid sequences that are 95% identical [8]. The most distinct specificity is expressed by TbACS2, which prefers much shorter fatty acids, in the range C_{9:0} to C_{12:0}. Since trypanosomes apparently do not have significant quantities of fatty acids shorter than myristate (C_{14:0}) [5,10,11], it is curious as to what the function of TbACS2 might be. One possibility is that the trypanosome's fatty acid synthesis system [6], which in bloodstream forms is specialized for synthesis of myristate, needs activation of intermediate species. For example, when we provided exogenous octanoate (C_{8:0}) to bloodstream trypanosomes, it was efficiently taken up and elongated to myristate (C_{14:0}) [6]. This elongation must have followed the initial conversion of the octanoate to octanoyl-CoA, possibly utilizing TbACS2.

As for the unsaturated fatty acids, linoleic acid (C_{18:2}), oleic acid (C_{18:1}) and arachidonic acid (C_{20:4}) are three of the most abundant in trypanosomes (17%, 9% and 8% respectively of the total fatty acids) [5]. All of these are efficiently activated by TbACS1 (Figure 3a), whereas TbACS3 and 4 are active on C_{18:2} and C_{18:1}, with less activity on C_{20:4} (Figures 3c and 3d). TbACS2 had virtually no activity on any of the unsaturated fatty acids tested (Figure 3b).

The ACS 'signature motif' is thought to be part of the active site involved in fatty acid binding and mutations in this sequence alter fatty acid chain-length specificity [12]. Not only is the entire amino acid sequence of TbACS1 most different from those of other TbACSs, but its ACS signature motif also differs significantly (Figure 4). Therefore, we were surprised to find that the specificity of TbACS1 is fairly similar to that of TbACS3 or TbACS4 (Figures 2 and 3). Furthermore, since TbACS2

ACS motif	D	G	W	L	H	T	G	D	I	G	X	W	X	P	X	G	X	L	K	T	I	D	R	K	K
TbACS1	G	G	W	F	R	T	G	D	V	V	E	I	G	E	D	N	A	L	R	V	I	G	R	V	K
TbACS2	D	G	W	F	H	T	G	D	V	G	S	I	G	P	N	G	T	L	R	I	I	G	R	V	K
TbACS3	D	G	W	F	H	T	G	D	V	G	S	I	D	P	N	G	T	L	R	I	I	G	R	V	K
TbACS4	D	G	W	F	H	T	G	D	V	G	S	I	G	P	N	G	T	L	R	I	I	G	R	V	K

Figure 4 Alignment of trypanosome ACS motifs for TbACS1, 2, 3 and 4, along with the consensus ACS signature motif

An asterisk marks a Lys residue in the consensus motif (see text). Identical residues are marked by black boxes.

specificity does differ dramatically from that of TbACS4, we were also surprised to find that their signature motifs are identical (Figure 4).

Both *Pseudomonas oleovorans* ACS and yeast Faa2p ACS have an aliphatic residue (Ala and Val respectively), instead of Lys in the signature motif (marked by an asterisk in Figure 4), and both have a strong preference for medium-chain fatty acids [9,13]. Furthermore, replacement of the same Lys with Ala within the *E. coli* enzyme changes the substrate preference from long-chain to medium-chain length [12]. Therefore, the amino acid at that position in the ACS signature motif seems to have a marked effect on substrate recognition. All four TbACSs have an aliphatic residue Val in that position, yet have varying activity towards medium-chain fatty acids. Our data suggests that the chain-length specificity is a complicated phenomenon and that other amino acids, outside the signature motif (since this motif is identical in TbACS2 and in TbACS4), must influence specificity.

With regard to their role in myristate metabolism, three of the four TbACSs have the potential to activate this fatty acid. We do not yet know whether one of these enzymes has the major responsibility for synthesis of myristoyl-CoA for GPI remodelling, or whether they all contribute. Further experiments, possibly involving gene silencing by RNA interference or by gene knockout, will be required to answer this question.

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