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Invited Review

Acetate formation in the energy metabolism of parasitic helminths and protists

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ABSTRACT

Formation and excretion of acetate as a metabolic end product of energy metabolism occurs in many protist and helminth parasites, such as the parasitic helminths *Fasciola hepatica, Haemonchus contortus* and *Ascaris suum*, and the protist parasites, *Giardia lamblia*, *Entamoeba histolytica*, *Trichomonas vaginalis* as well as *Trypanosoma* and *Leishmania* spp. In all of these parasites acetate is a main end product of their energy metabolism, whereas acetate formation does not occur in their mammalian hosts. Acetate production might therefore harbour novel targets for the development of new anti-parasitic drugs. In parasites, acetate is produced from acetyl-CoA by two different reactions, both involving substrate level phosphorylation, that are catalysed by either a cytosolic acetyl-CoA synthetase (ACS) or an organellar acetate:succinate CoA-transferase (ASCT). The ACS reaction is directly coupled to ATP synthesis, whereas the ASCT reaction yields succinyl-CoA for ATP formation via succinyl-CoA synthetase (SCS). Based on recent work on the ASCTs of *F. hepatica, T. vaginalis* and *Trypanosoma brucei* we suggest the existence of three subfamilies of enzymes within the CoA-transferase family I. Enzymes of these three subfamilies catalyse the ASCT reaction in eukaryotes via the same mechanism, but the subfamilies share little sequence homology. The CoA-transferases of the three subfamilies are all present inside ATP-producing organelles of parasites, those of subfamily IA in the mitochondria of trypanosomatids, subfamily IB in the mitochondria of parasitic worms and subfamily IC in hydrogenosome-bearing parasites. Together with the recent characterisation among non-parasitic protists of yet a third route of acetate formation involving acetate kinase (ACK) and phosphotransacetylase (PTA) that was previously unknown among eukaryotes, these recent developments provide a good opportunity to have a closer look at eukaryotic acetate formation.

1. Introduction

Most parasites have a complex life cycle, which can include free-living stages as well as distinct stages inhabiting one or more host organisms. Oxygen availability is often limited during all or part of the parasite life cycle, therefore they must possess pathways of ATP synthesis that are independent of O_2 as the terminal electron acceptor. The formation of acetate from acetyl-CoA as a metabolic end product is a metabolic route that is present in many parasites, especially in those that inhabit or encounter hypoxic or anoxic habitats (Table 1) (Köhler, 1985; Tielens, 1994; Sanchez and Müller, 1996; Tielens et al., 2002). Furthermore, acetate production is also present in many non-parasitic eukaryotes, including flowering plants (Zeiher and Randall, 1990) and marine invertebrates (De Zwaan, 1991), but it is absent in mammals. Since acetate is an important end-product of energy metabolism among many parasites but not among their mammalian hosts, acetate formation is an attractive target for the development of novel anti-parasitic drugs.

Thus far, four different chemical reactions have been identified in which acetate is produced from acetyl-CoA, catalysed by either (i) a CoA-transferase, (ii) a synthetase, (iii) a hydrolase or (iv) a phosphate-acetyltransferase in combination with a kinase reaction (Fig. 1).

For all four reactions, corresponding enzymes can be found among the eukaryotes. In parasites, however, only acetyl-CoA synthetases (ADP-forming) and acetate:succinate CoA-transferases (ASCTs) have been identified to date. Therefore, this review will only briefly address the characteristics of acetate kinases and acetyl-CoA hydrolases and will focus on acetyl-CoA synthetases (ADP-forming) and ASCTs present in parasites. The similarities and differences in acetate formation between parasites will be discussed, as well as the biochemical characteristics, the sub-cellular localisations, and some remarks will be presented on the evolutionary origins of the enzymes responsible for acetate production.

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Table 1
Acetate-forming enzymes in parasitic helminths and protists. BLAST searches were performed to identify homologs of acetyl-CoA synthetase (ADP-forming) and CoA-transferases of family 1A, 1B and 1C genes in parasites, using acetyl-CoA synthetase (ADP-forming) of *Entamoeba histolytica* (AF286346) and the acetate:succinate CoA-transferases of *Trypanosoma brucei* (EAN79240), *Fasciola hepatica* (ACF06126) and *Trichomonas vaginalis* (XP_001330176), respectively. Identified homologous demonstrated significant homology with *E* values smaller than $1e^{-20}$. (—) indicates not present in a completely sequenced genome, (n.d.) not detected in available databases, (?) no literature or sequence data available.

Organism	Acetate formation from gluocse	Family I CoA-transferases			AcCoA synthetase
		Subfamily IA	Subfamily IB	Subfamily IC	(ADP-forming)
Nematodes					
Ascaris suum[a]	Major end product[e]	BI594619	BI782486	n.d.	n.d.
Haemonchus contortus[a]	Major end product[f]	n.d.	BM138769	n.d.	n.d.
Brugia malayi[b]	?[g,h]	—	XP_001900057	—	—
Onchocerca volvulus	Major end product[g]	?	?	?	?
Strongyloides stercoralis	?	?	BE580065	?	?
Trichostrongylus colubriformis	Major end product[i]	?	?	?	?
Trematodes					
Schistosoma mansoni[b]	No[j]	—	XP_002577003	—	—
Schistosoma japonicum[a]	No[j]	n.d.	AAW27410	n.d.	n.d.
Schistosoma haematobium[a]	No[j]	n.d.	n.d.	n.d.	n.d.
Fasciola hepatica[a]	Major end product[k]	n.d.	ACF06126	n.d.	n.d.
Cestodes					
Ecchinococcus multilocularis[c]	Major end product[l]	n.d.	n.d.	n.d.	n.d.
Echinococcus granulosus[a]	Major end product[l]	n.d.	n.d.	n.d.	n.d.
Taenia taeniaeformis	Major end product[m]	?	?	?	?
Taenia solium[a]	?	n.d.	EL751586	n.d.	n.d.
Moniezia expansa[a]	?	n.d.	FF677706	n.d.	n.d.
Parabasalids					
Trichomonas vaginalis[b]	Major end product[n]	—	—	XP_001330176	—
Tritrichomonas foetus[a]	Major end product[n]	n.d.	n.d.	CX154925	n.d.
Kinetoplastidae					
Trypanosoma brucei (procyclics)[b]	Major end product[o]	EAN79240	—	—	—
Trypanosoma cruzi[b]	Major end product[o]	EAN86067	—	—	—
Leishmania major [b]	Major end product[p]	CAJ06634	—	—	—
Leishmania infantum[d]	Major end product[q]	CAM70089	n.d.	n.d.	n.d.
Phytomonas sp[a]	Major end product[r]	CO723949	n.d.	n.d.	n.d.
Crithidia luciliae[d]	Major end product[o]	n.d.	n.d.	n.d.	n.d.
Archamoebae					
Entamoeba histolytica[b]	Major end product[s]	—	—	—	AF286346
Diplomonads					
Giardia lamblia[b]	Major end product[t]	—	—	—	XM_001705692
Apicomplexa					
Plasmodium falciparum[b]	No	—	—	—	XM_001348495
Toxoplasma gondii[b]	No	—	—	—	—
Theileria parva[b]	No	—	—	—	—
Babesia bovis[a]	No	n.d.	n.d.	n.d.	n.d.
Cryptosporidium parvum[b]	No	—	—	—	—
Blastocystidae					
Blastocystis hominis[a]	?	n.d.	EC648226	EC647239	EC648512
Ciliates					
Nyctotherus ovalis[a]	Major end product[u]	AJ871320	n.d.	n.d.	n.d.
Chlorophyta					
Chlamydomonas reinhardtii[b]	Major end product[v]	—	—	—	—

[a] Expressed sequence tag (EST) database.
[b] Complete genome database.
[c] DNA shotgun database.
[d] Incomplete genome database.
[e] Köhler and Bachmann (1980).
[f] Ward and Huskisson (1978).
[g] MacKenzie et al. (1989) acetate production unknown for *B. malayi*, but *B. pahangi* microfilaria are known to produce acetate as major end product (Rew and Saz, 1977).
[h] Rew and Saz (1977).
[i] Sangster and Prichard (1985).
[j] Tielens et al. (1989).
[k] van Vugt et al. (1979).
[l] McManus and Smyth (1978).
[m] von Brand et al. (1968).
[n] Steinbüchel and Müller (1986).
[o] Cazzulo (1992).
[p] Darling et al. (1989).
[q] Van Hellemond et al. (1997).
[r] Chaumont et al. (1994).
[s] Montalvo et al. (1971).
[t] Lindmark (1980).
[u] Boxma et al. (2005).
[v] Mus et al. (2007).

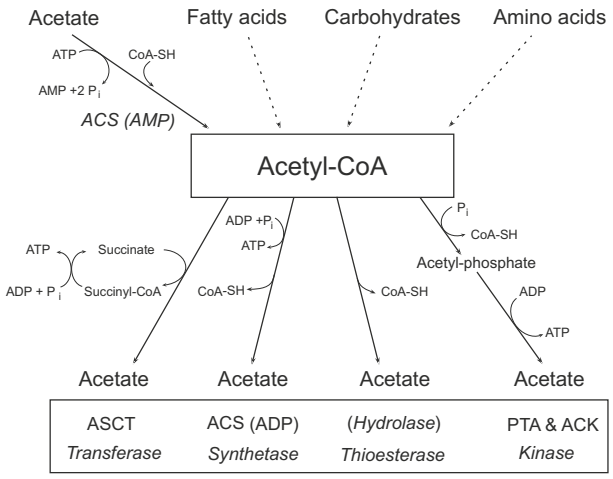


Fig. 1. Enzymatic reactions involved in the formation or utilisation of acetate. Enzymatic conversion of acetyl-CoA to acetate follows an acetate:succinate CoA-transferase reaction (ASCT), an acetyl-CoA synthetase (ADP-forming) reaction (ACS-ADP), an acetyl-CoA hydrolase reaction (ACH), or a phosphate acyltransferase reaction (PTA) followed by an acetate kinase reaction (ACK). Enzymatic conversion of acetate to acetyl-CoA is catalysed by acetyl-CoA synthetase (AMP-forming) (ACS-AMP).

2. Acetyl-CoA, the precursor for acetate production, is formed via several pathways

In core carbon and energy metabolism of eukaryotes, three main routes provide acetyl-CoA, the precursor of acetate: pyruvate breakdown, β-oxidation of fatty acids, and the breakdown of ketogenic amino acids.

In the first route, pyruvate typically stems from the degradation of carbohydrates and the gluconeogenic amino acids, alanine, glycine, cysteine, serine and tryptophane. The synthesis of acetyl-CoA from pyruvate can involve oxidative decarboxylation via the classical NAD^+-dependent pyruvate dehydrogenase complex (EC 1.2.4.1), via the ferredoxin-dependent enzyme pyruvate:ferredoxin oxidoreductase (PFO; EC 1.2.7.1) (Müller, 2003), or more rarely via an $NADP^+$-dependent variant of PFO, pyruvate:$NADP^+$ oxidoreductase (Lantsman et al., 2008). Also rare, and so far lacking in parasites, is the non-oxidative conversion of pyruvate to acetyl-CoA via pyruvate:formate lyase (PFL; EC 2.3.1.54) which is found in *Chlamydomonas* (Atteia et al., 2006), but with large strain-specific differences in formate production (Meuser et al., 2009) and to some extent in rumen fungi (see van der Giezen, 2009).

During the degradation of fatty acids via β-oxidation, in the final step of each cycle, acyl-CoA acetyltransferase (EC 2.3.1.16), more commonly called thiolase, catalyses the reaction of a β-ketoacyl-CoA with a molecule of free coenzyme A to split off the carboxyl-terminal two-carbon fragment as acetyl-CoA (Fig. 2). In the degradation of ketone bodies the same reaction is used: acetoacetate is first activated by coupling of coenzyme A donated by succinyl-CoA in a reaction catalysed by succinyl-CoA:3-ketoacid CoA-transferase, SCOT (EC 2.8.3.5) (see Section 5.2) and the resulting acetoacetyl-CoA is then split by a thiolase into two molecules of acetyl-CoA (Fig. 2).

The degradation of ketogenic amino acids, such as leucine, isoleucine and lysine directly results in the formation of acetyl-CoA (not via pyruvate). Additionally in some organisms, such as for example Trypanosomatids, threonine is degraded to acetyl-CoA (Linstead et al., 1977).

3. Acetate formation by phosphate-acetyltransferase and acetate kinase

Formation of acetate from acetyl-CoA via acetyl-phosphate occurs via two reactions catalysed by separate enzymes. In the first step, the thioester bond of acetyl-CoA undergoes phosphorolysis yielding acetyl-phosphate in a reaction catalysed by phosphate-acetyltransferase (EC 2.3.1.8; also called phosphotransacetylase, PTA) (Fig. 1). In the second step, the phosphate moiety of acetyl-phosphate is transferred to ADP in a reaction catalysed by acetate kinase (EC 2.7.2.1; ACK), yielding acetate and ATP (Anthony and Spector, 1971). This PTA–ACK pathway is a common route of ATP synthesis or acetate assimilation in prokaryotes and is found for example in *Escherichia coli* and *Salmonella typhimurium* (Wolfe, 2005). It is also found in acetogens (Ragsdale and Pierce, 2008) and methanogens (Rother and Metcalf, 2004).

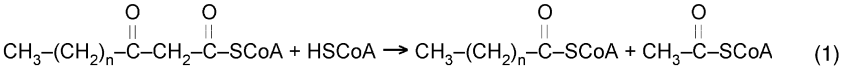
Acetate production in the protist *Entamoeba histolytica* occurs via the ACS (ADP) synthetase pathway (see below). However, in an isolated report, an acetate kinase activity was also found to be present in this protist parasite, but the corresponding enzyme was reported to use inorganic pyrophosphate (PPi) as the phosphoryl donor and not ATP, the donor in the bacterial type of ACK (Reeves and Guthrie, 1975). A gene encoding a homolog of bacterial ACK is present in *E. histolytica*, but the completely sequenced genome of this parasite appears to lack homologs for PTA (Ingram-Smith et al., 2006). Therefore, in *E. histolytica* that specific acetate kinase reaction is probably not used for the production of acetate from acetyl-CoA, but might be part of a different metabolic or signaling pathway (Wolfe, 2005). The use of ACK in a role other than the PTA–ACK pathway is suggested to occur also in fungi, which also possess ACK and lack PTA (Ingram-Smith et al., 2006). Apart from *E. histolytica*, other parasites studied seem to lack ACK and there are not yet any indications that acetate production via the PTA and ACK pathway occurs in parasites. Genes for both PTA and ACK are found, however, in the genome of the oomycete plant pathogen, *Phytophthora sojae* (Atteia et al., 2006; Ingram-Smith et al., 2006), but nothing is known about their expression or localisation in this organism.

The PTA–ACK pathway for the production of acetate, which appears to be absent in parasitic protists, is present in the free-living algal protist *Chlamydomonas reinhardtii.* In this protist, the genes for both PTA and ACK are expressed in aerobic cultures, with both proteins being localised within mitochondria (Atteia et al., 2006). *Chlamydomonas* has duplicate genes for both PTA and ACK, however, and one copy of each protein is predicted to be localised to chloroplasts (Atteia et al., 2006; Dubini et al., 2009). These copies, designated as PAT2 and ACK1, are thought to encode the chloroplast proteins and are rapidly (within 30 min) induced at the level of transcript accumulation upon transfer of *Chlamydomonas* to anaerobic heterotrophic growth conditions. Furthermore, their induction kinetics correspond closely with the accumulation of acetate as a major metabolic end product in the medium (Mus et al., 2007). In addition to acetate, *Chlamydomonas* accumulates CO_2, succinate, formate, ethanol and H_2 as major anaerobic end products (Mus et al., 2007; Dubini et al., 2009).

These are the typical end products of the anaerobic energy metabolism used by those eukaryotes that possess anaerobic mitochondria, hydrogenosomes or mitosomes (Tielens et al., 2002; Müller, 2003; van der Giezen, 2009). Hydrogenosomes are hydrogen-producing organelles evolutionarily related to mitochondria, and this evolutionary relationship has been demonstrated by the conservation of mitochondrial targeting signals, mitochondrial heat shock proteins, iron sulphur cluster biosynthesis, and the double membrane in hydrogenosomes (Benchimol and De Souza, 1983; Bui et al., 1996; Germot et al., 1996; Bradley et al., 1997; Embley et al., 1997; van der Giezen et al., 1997; Martin and Müller, 1998; Tachezy et al., 2001). Hydrogenosomes were originally characterised in trichomonads (Lindmark and Müller, 1973) but later characterised in ciliates (Yarlett et al., 1982) and other lineages (reviewed in van der Giezen, 2009). The PTA–ACK route was reported for the rumen ciliate *Dasytricha ruminantium* (Yarlett et al., 1982)

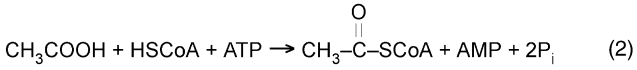
Acyl-CoA acetyltransferase, EC 2.3.1.16

Thiolase: typically involved in β-oxidation (acetyl-CoA formation)



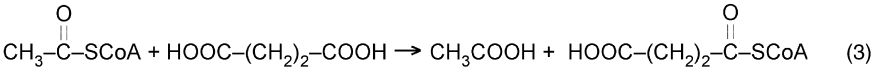
Acetyl-CoA synthetase (AMP-forming), EC 6.2.1.1

ACS (AMP): typically involved in acetate assimilation



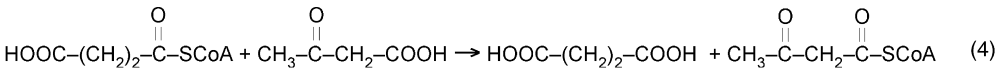
Acetate:succinate CoA-transferase, EC 2.8.3.8

ASCT: typically involved in acetate production in mitochondrion-like organelles



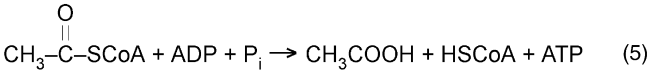
Succinyl-CoA:3-ketoacid CoA-transferase, EC 2.8.3.5

SCOT: typically involved in ketone body activation



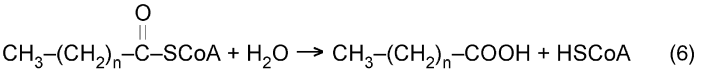
Acetyl-CoA synthetase (ADP-forming), EC 6.2.1.13

ACS (ADP): typically involved in cytosolic acetate formation



Acyl-CoA hydrolase, EC 3.1.2.1 (alternative name: short-chain acyl-CoA thioesterase),

ACH: typically involved in peroxisomal acetate formation



Phosphotransacetylase, EC 2.3.2.8, and acetate kinase, EC 2.7.2.1

PTA/ACK: typically involved in acetate production (and assimilation) in prokaryotes and *Chlamydomonas*

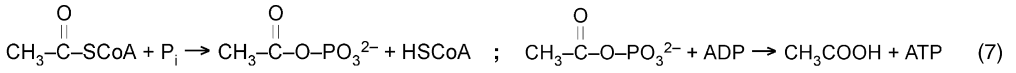


Fig. 2. Reactions catalysed by enzymes discussed in this paper that are involved in acetyl-CoA and acetate synthesis or transfer of CoA groups. For each enzyme, the EC number, the abbreviation and the typical metabolic process in which it is involved are indicated.

but that early report was not followed up. The recent molecular and biochemical characterisation of the PTA–ACK pathway in *Chlamydomonas* should renew interest in the distribution of this pathway in other eukaryotes.

4. Acetate formation by acetyl-CoA synthetase (ADP-forming)

Acetyl-CoA synthetase (ADP-forming) (EC 6.2.1.13; ACS-ADP), also called acetate thiokinase, converts acetyl-CoA into acetate, concomitantly leading to the formation of ATP from ADP (Fig. 1). The enzyme mechanism involves acetyl-phosphate and two phosphohistidine residues (Bräsen et al., 2008). This enzyme should not be confused with acetyl-CoA synthetase (AMP-forming) (EC 6.2.1.1) (Fig. 1), an unrelated enzyme present in many bacteria and eukaryotes. The physiological role of the AMP-forming acetyl-CoA synthetase is acetyl-CoA production from acetate and coenzyme A, and not the reverse, acetate formation from acetyl-CoA (Starai and Escalante-Semerena, 2004). For instance, it has recently been shown that *Trypanosoma brucei* uses ACS-AMP for

the production of acetyl-CoA in the cytosol for fatty acid synthesis (Rivière et al., 2009). Since our focus is on enzymes involved in the production of acetate, not utilisation thereof, AMP-forming acetyl-CoA synthetases will not be further addressed here.

ACS-ADP activity was first described to be involved in acetate production in the anaerobic parasitic protist *E. histolytica* (Reeves et al., 1977). Subsequently, ACS-ADP was described in another anaerobic protist, *Giardia lamblia* (Lindmark, 1980; Sanchez and Müller, 1996), in archaebacteria (Schäfer and Schönheit, 1991; Musfeldt and Schönheit, 2002), and in the rumen bacterial anaerobe *Selenomonas ruminantium* (Michel and Macy, 1990). Identification and sequence analysis of the *G. lamblia* acetyl-CoA synthetase (Sanchez et al., 1999, 2000), resulted in the identification of the homologous *E. histolytica* acetyl-CoA synthetase (Field et al., 2000), and in the identification of an acetyl-CoA synthetase gene homolog in the malaria parasite, *Plasmodium falciparum* (Sanchez et al., 2000). Furthermore, an ACS-ADP homolog has been identified in an expressed sequence tag (EST) database of *Blastocystis hominis* (Stechmann et al., 2008) (Table 1).

ACS-ADP enzymes are either heterotetrameric proteins that consist of two separate α and two separate β subunits (α_2, β_2) or homodimers that consist of two subunits representing a fusion of the respective α and β subunits $(\alpha\beta)_2$. The α and β subunits of acetyl-CoA synthetases (ADP-forming) are homologous to the α and β subunit of succinyl-CoA synthetases and together these enzymes belong to the superfamily of nucleoside diphosphate-forming (NDP-forming) acyl-CoA synthetases (Field et al., 2000; Sanchez et al., 2000; Musfeldt and Schönheit, 2002; Bräsen et al., 2008).

ACS-ADP is quite specific for ADP as the nucleotide diphosphate and most of these enzymes cannot use UDP or CDP as a substrate. The *G. lamblia* acetyl-CoA synthetase does not support CoA-SH release in the presence of UDP, CDP or GDP, and the *E. histolytica* acetyl-CoA synthetase functions poorly with guanine nucleotides (Reeves et al., 1977; Sanchez and Müller, 1996). On the other hand, the acyl-specificity of these enzymes is less strict. The *G. lamblia* ACS-ADP preferentially uses acetyl-CoA, but this enzyme can also use propionyl-CoA and succinyl-CoA as a substrate, whereas butyryl-CoA cannot be used (Sanchez and Müller, 1996). The *E. histolytica* acetyl-CoA synthetase was also reported to have a lower acyl-specificity (Reeves et al., 1977).

For both *E. histolytica* and *G. lamblia* the main metabolic end products of glucose breakdown under microaerophilic conditions are acetate, ethanol and CO_2 (Montalvo et al., 1971; Lindmark, 1980; Müller, 1988). As mentioned above, the *E. histolytica* genome contains an ACK, but lacks the PTA required for acetyl-CoA conversion to acetyl-phosphate. In the genomic databases of *G. lamblia* and *E. histolytica* no other genes could be identified that are homologous to one of the other classes of enzymes known to be involved in acetate production. Therefore, ACS-ADP is most likely essential for acetate production from glucose in both *G. lamblia* and *E. histolytica*.

ACS-ADP genes are present and highly conserved in all *Plasmodium* spp. analysed (*P. falciparum*, *Plasmodium vivax*, *Plasmodium bergei*, *Plasmodium yoelli*, *Plasmodium chabaudi chabaudi*). The energy metabolism of *Plasmodium* is currently poorly understood and biochemical analyses are hindered by the circumstance that this intracellular parasite can only be cultured while inhabiting red blood cells (Trager and Jensen, 1976; van Dooren and McFadden, 2007). It is generally thought that glucose is the main substrate for energy metabolism of *Plasmodium* species and that this glucose is oxidised via glycolysis to the main excreted end-product lactate (Homewood and Neame, 1983; Roth, 1990). Some indications have been found that at least a partial Krebs cycle and oxidative phosphorylation could be present in *Plasmodium* spp. (Suraveratum et al., 2000; Krungkrai et al., 2002) and expression profiles of metabolic enzymes of *P. falciparum* in vivo can differ considerably from the in vitro situation in which the vast majority of research is performed (Daily et al., 2007).

The presence of both an ACS-ADP and CoA-transferase-type ASCTs in an EST database of *B. hominis* (Stechmann et al., 2008) (Table 1) suggests that acetate might be an important end product of metabolism in this parasite. While the possible involvement of ACS-ADP in acetate production in *B. hominis* remains to be determined, evidence for ASCT involvement (see next section) has been presented, as Lantsman et al. (2008) measured high activities of both ASCT and the "sister activity" succinyl-CoA synthetase (SCS) needed for acetate production which, in cell fractionation studies, both clearly localise to the anaerobic mitochondria of this protist parasite (Lantsman et al., 2008).

5. Acetate formation by CoA-transferases

The transfer of a CoA group from one compound to another is catalysed by three unrelated protein families, all of which belong to the functionally defined category of CoA-transferases, which vary in substrate specificities and subunit composition (Heider, 2001). These enzymes are found in aerobic and anaerobic prokaryotes as well as in ATP-producing organelles of many eukaryotes. CoA-transferases are categorised as family I, family II or family III CoA-transferases (Heider, 2001). The CoA-transferases involved in acetate production in parasites have long been known to be succinate-dependent and are therefore called acetate:succinate CoA-transferases (ASCT, EC 2.8.3.8). The CoA-moiety of acetyl-CoA is donated to succinate yielding acetate and succinyl-CoA (Figs. 1 and 2). All ASCTs identified to date in eukaryotes are family I CoA-transferases, which are characterised by their transfer of CoA groups involving a glutamate residue in the active site of the enzyme (Heider, 2001). The general reaction mechanism of these family I CoA-transferases was elucidated by Jencks and co-workers while studying pig heart succinyl-CoA:3-oxoacid CoA-transferase (SCOT) (Solomon and Jencks, 1969; White and Jencks, 1976). They demonstrated that the CoA-moiety is not transferred directly from one substrate to the next, but is first covalently bound to a glutamate residue in the active site of the enzyme and thereafter transferred to its final acceptor. Therefore, the reaction follows a ping–pong mechanism where the first product, a free carboxylate, is released before the second substrate, the other carboxylate, enters the active site. This process is fully reversible. Most family I enzymes operate with succinyl-CoA or acetyl-CoA as possible CoA-donors and contain two dissimilar subunits in different aggregation states ($\alpha_2\beta_2$ or $\alpha_4\beta_4$) (Heider, 2001). The CoA-transferase family I contains CoA-transferases for 3-oxoacids, short-chain fatty acids and glutaconate. Family II and III CoA-transferases use different reaction mechanisms to transfer CoA groups, are not known to be involved in acetate production and will not be further discussed here (Heider, 2001).

The succinyl-CoA that is formed as a result of the ASCT reaction can subsequently be converted back into succinate by an enzyme which is also present in the Krebs cycle, SCS (EC 6.2.1.4), in a reaction in which ATP or GTP is produced concommittantly from ADP or GDP, respectively (Fig. 1). This SCS reaction is in fact very comparable to the ACS-ADP reaction described in the previous paragraph and the enzymes catalysing these two reactions are members of the same superfamily of related enzymes (Sanchez et al., 2000) (Fig. 1). Such an ASCT/SCS cycle has been documented for numerous parasites, including the parabasalids *Tritrichomonas foetus* and *Trichomonas vaginalis* (Lindmark, 1976; Steinbüchel and Müller, 1986), the trematode *Fasciola hepatica* (Barrett et al., 1978; van Vugt et al., 1979; Saz et al., 1996), and the trypanosomatids *T. brucei*, *Leishmania mexicana, Leishmania infantum* and *Phytomonas* spp. (van Hellemond et al., 1998) as well as in one

free-living species, the rumen fungus *Neocallimastix* sp. L2 (Marvin-Sikkema et al., 1993).

5.1. The CoA-transferase family I can be divided into subfamilies

Although the occurence of the ASCT reaction has long been known, it was only recently that the corresponding ASCT genes were identified in three different parasites, the protists *T. brucei* and *T. vaginalis*, and the parasitic helminth *F. hepatica* (Rivière et al., 2004; van Grinsven et al., 2008, 2009b). With the recent identification of the underlying ASCT genes, it became clear that these three ASCTs share little sequence homology among themselves (Supplementary Fig. S1). We suggest that at least three different subfamilies of the family I CoA-transferases exist and that each of the three CoA-transferases identified in parasites is in fact member of a different subfamily (Fig. 3 and Supplementary Fig. S1).

The ASCT enzymes identified in the trypanosomatid parasites *T. brucei*, *Trypanosoma cruzi* and *Leishmania major* are clear homologs of SCOT (Rivière et al., 2004). SCOTs are structurally related and all contain the conserved domain COG4670. We suggest calling this group of closely related ASCTs, subfamily A of family I CoA-transferases (subfamily IA). In general, SCOT enzymes catalyse the transfer of a CoA-moiety from succinyl-CoA to a 3-oxoacid, yielding succinate and a 3-oxoacyl-CoA (White and Jencks, 1976). This is the only subfamily of family I CoA-transferases with members present in mammals, where SCOTs have an essential function in brain and muscle mitochondria in the pathway for utilisation of ketone bodies (Fukao et al., 2004). Mammalian SCOTs transfer the CoA-moiety from succinyl-CoA to the ketoacid acetoacetate, resulting in the formation of acetoacetyl-CoA, which is subsequently cleaved by a thiolase through reaction with the thiol group of free coenzyme A to yield two molecules of acetyl-CoA in a reaction comparable to the final step in β-oxidation (Fig. 2).

The amino acid sequences of many family I CoA-transferases that resemble mammalian SCOTs are known and these are found in metazoans as well as among bacteria and fungi. In all of these SCOT-like CoA-transferases the sequence motif SENG, containing the glutamate residue of the active site, is conserved (Jacob et al., 1997; Rangarajan et al., 2005). Furthermore, these CoA-transferases are also reported to contain the conserved sequence motif GXGG, which is part of the oxyanion hole (Jacob et al., 1997; Rangarajan et al., 2005) and is present in subfamily IA (Fig. 3).

The ASCT that was identified in *F. hepatica* is clearly also a member of the family I CoA-transferases because of its substrates and reaction mechanism, which was confirmed to transfer the CoA-moiety via a ping–pong mechanism (van Grinsven et al., 2009b). However, sequence analysis demonstrates that it does not contain the SENG motif and shares no significant homology to the subfamily IA introduced above (Supplementary Fig. S1). The *F. hepatica* enzyme however is, at the sequence level, closely related to the CoA-transferase of bacteria such as *Roseburia*, and to that of *Artemia franciscana*, a brine shrimp (Fig. 3, Supplementary Fig. S1). These CoA-transferases all contain the conserved domain COG0427. Further analysis of the sequences of these related proteins shows that they all contain a conserved EXG motif, which is apparently part of the larger SENG motif of subfamily IA (Fig. 3). For *A. franciscana,* this EXG near the C-terminus was suggested to play a critical role in the catalytic formation of the thioester (Oulton et al., 2003). These CoA-transferases also possess a conserved GXGG motif (Fig. 3). We suggest naming this group of structurally related ASCTs, subfamily B of the family I CoA-transferases.

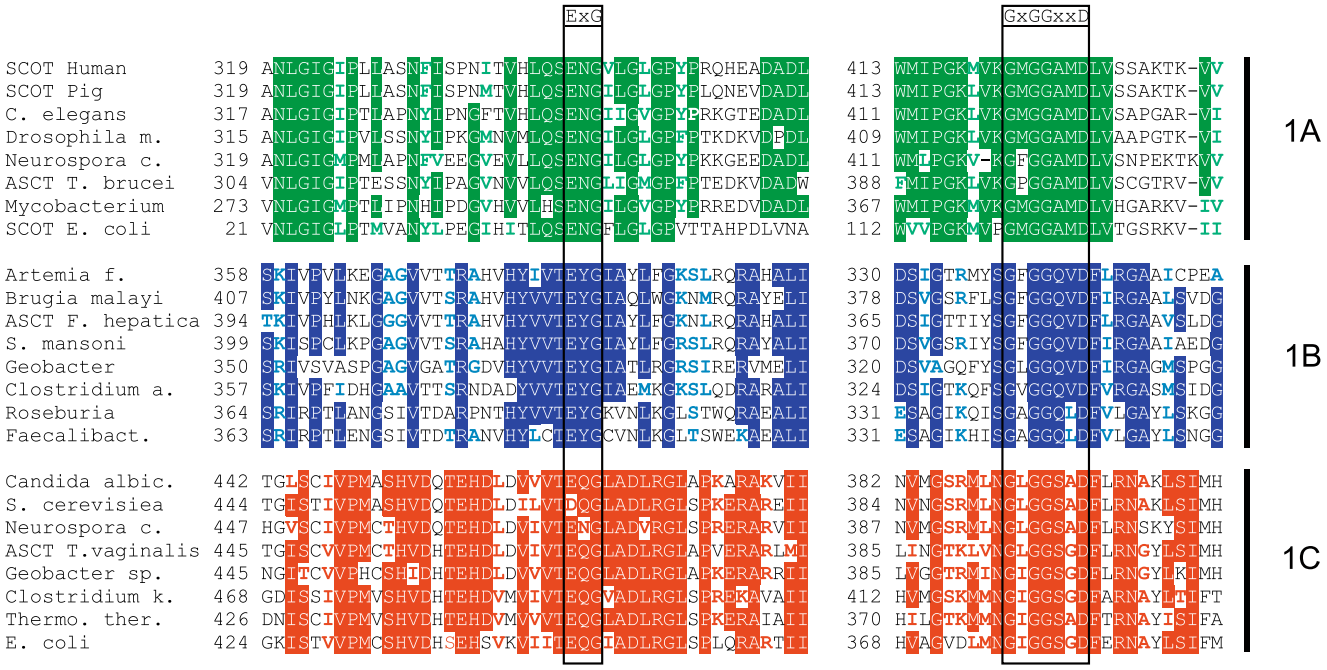


Fig. 3. Amino acid sequence alignment of the conserved EXG and GXGGXXD regions of family 1 CoA-transferases. Sequences were aligned using Clustal X (Thompson et al., 1997). Identical and conserved residues in over 75% of all listed sequences of the subfamilies A, B and C are shaded and represented by letters in green, blue and red, respectively. Identical residues in over 75% of all listed sequences are shown as consensus sequences above the alignment. Amino acid sequences of subfamily A are shown for *Homo sapiens* (humans, NP_000427), *Sus scrofa* (pig, Q29551), *Caenorhabditis elegans* (Q09450), *Drosophila melanogaster* (ACV88439), *Neurospora crassa* (XP_959228), ASCT of *Trypanosoma brucei* (XP_828352), *Mycobacterium tuberculosis* (NP_217019) and *Escherichia coli* beta subunit (CAS09915). Amino acid sequences of subfamily B are shown for the acetyl coenzyme A-transferase of *Artemia franciscana* (AAP68833), *Brugia malayi* (XP_001900057), *Fasciola hepatica* (ACF06126), *Schistosoma mansoni* (XP_002577003), *Geobacter* sp. FRC-32 (YP_002537650), *Clostridium aminobutyricum* (CAB60036), the butyryl-CoA-transferase of *Roseburia* sp. A2–183 (AAX19660) and *Faecalibacterium prausnitzii* (DQ072259). Amino acid sequences of subfamily C are shown of *Candida albicans* (P83773), *Saccharomyces cerevisiae* (P32316), *Neurospora crassa* (P15937), *Trichomonas vaginalis* (XP_001330176), *Geobacter* sp. FRC-32 (YP_002535983), *Clostridium kluyveri* (P38946), *Thermoanaerobacterium thermosaccharolyticum* (CAA93155) and *E. coli* (AAC75957). Abbreviations for characterised enzyme activities: ASCT, acetate:succinate CoA-transferase; SCOT, succinyl-coA:3-ketoacid-coenzyme A transferase.

The CoA-transferase identified in *T. vaginalis*, like all ASCTs, also belongs to the family I CoA-transferases. However, beyond shared motifs at the active site, the *T. vaginalis* ASCT shares little sequence homology to the subfamilies IA and IB defined above. On the other hand, the *T. vaginalis* enzyme clearly clusters together with CoA-transferases of many prokaryotes and fungi in what we designate here as subfamily IC (Fig. 3). Members of subfamily IC all have the same conserved domain (YgfH domain, pfam02550; TIGR03458), which is not present in the members of subfamilies IA and IB of CoA-transferases. Subfamily IC does not contain the SENG motif that characterises subfamily IA, but like subfamily IB it contains an EXG and a GXGG motif (Fig. 3). At present, subfamily IC contains no metazoan ASCTs, which might be relevant in the context of target development.

Subfamilies IA, IB and IC show little amino acid sequence homology (Supplementary Fig. S1), but comparison of the regions around the active site motif EXG by using this motif as an anchor, clearly reveal that these three subfamilies possess the same chemical moieties around this active site (Fig. 4) that is conserved within each subfamily. Whether or not these rather meagre sequence similarities reflect common ancestry of the active site or not, cannot be answered in the absence of a crystal structure, which is so far lacking for subfamilies IB and IC.

The same low sequence similarity is found in the region surrounding the oxyanion hole, which was reported to possess a GXGG motif (Jacob et al., 1997; Rangarajan et al., 2005), that seems to be a GXGGXXD motif upon closer inspection (Fig. 4). It should be noted that the position of this GXGGXXD motif of the oxyanion hole relative to the EXG motif of the active site is strikingly different in members of subfamily IA compared with the subfamilies IB and IC, i.e. on the amino-terminal side versus on the carboxy-terminal side of the active site, respectively (Fig. 3; Supplementary Fig. S1).

The CoA-transferase enzymes of subfamily IB, like the one found in *Fasciola*, and the ones of subfamily IC, found in *Trichomonas* hydrogenosomes, share on average only 22% sequence identity across subfamilies, but > 50% amino acid sequence identity within the subfamilies, respectively (Fig. 3 and Supplementary Fig. S1). Those two subfamilies IB and IC share, however, only 11% amino acid identity each with the SCOT-type enzymes (subfamily IA), the type found in *Trypanosoma*. In short, the three subfamilies of family I type CoA-transferases that we designate are only distantly related, whereas subfamilies 1B and 1C are closer to each other than each is to subfamily 1A (Fig. 3 and Supplementary Fig. S1).

The three subfamilies IA, IB and IC were defined on the basis of the three clearly distinct sequences of CoA-transferases in parasites. It can, of course, not be excluded that more subfamilies of family I CoA-transferases will be discovered when CoA-transferases from other sources are characterised.

5.2. The ASCTs of trypanosomatids

In general, SCOT enzymes catalyse the transfer of a CoA-moiety from succinyl-CoA to a 3-oxoacid, yielding succinate and a 3-oxoacyl-CoA (White and Jencks, 1976). The reaction catalysed by trypanosomal ASCT is very similar to the SCOT reaction and uses acetate as substrate instead of acetoacetate. We suggest that apparently the substrate specificity of a SCOT-like enzyme changed from 3-oxoacyl acids to acetate in a relatively recent event within the trypanosomatid lineage.

The mammalian SCOT enzymes usually exist as a homodimer with two active sites (Bateman et al., 2002). It has been shown that the polypeptide chain of pig heart SCOT (subunit composition A2) arose from a gene fusion of bacterial A and B chains. Kinetic studies showed that the CoA-moiety is transferred via a ping–pong mechanism involving a covalent thioester intermediate between CoA and a glutamate residue in the active site, as in all family I CoA-transferases (White and Jencks, 1976; Rochet and Bridger, 1994). For trypanosomatid ASCTs no kinetic data are available yet, but based on their homology to pig heart SCOT (53% identical residues, 72% conserved residues) a similar reaction mechanism seems very likely. Mammalian SCOTs are able to transfer CoA to a broad range of carboxylate substrates. In addition to the known substrates 3-ketoacids and malonate, the CoA-moiety can be transferred from succinyl-CoA to acetate, propionate and butyrate, although transfer rates to these other substrates are 10-fold lower (White and Jencks, 1976). For trypanosomatid SCOT-type ASCT no substrate specificities have yet been published.

It should be noted that at least in *T. brucei*, this ASCT is not the only enzyme involved in the production of acetate, because strains in which this enzyme was knocked out still produced acetate (Rivière et al., 2004). The identity of the alternative enzyme(s) for acetate production in *T. brucei* is still unknown, but one or more of the acyl-CoA hydrolases present in the *T. brucei* genome would be likely candidates (see Section 6).

We note that for *T. brucei*, acetate is only produced as a major end product by the procyclic trypanosomes residing in the tsetse fly and by the non-proliferating short-stumpy form in the bloodstream (van Grinsven et al., 2009a). The proliferating long, slender bloodstream form *T. brucei* parasites degrade glucose mainly to pyruvate and do not produce acetate (van Hellemond et al., 2005). Therefore ASCT in trypanosomes is probably not a suitable target for the development of anti-trypanosomal drugs. In other trypanosomatids, such as *Leishmania* spp., acetate is produced in stages in the mammalian host (Rainey and MacKenzie, 1991);

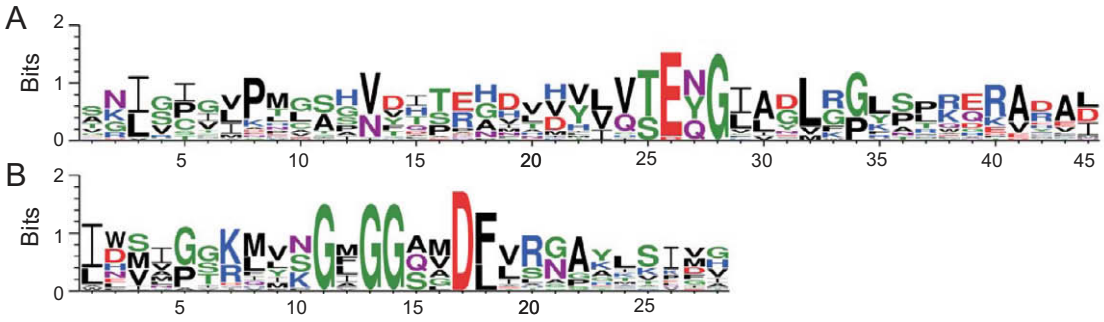


Fig. 4. Amino acid sequence logos of the conserved EXG and GXGGXXD regions of family 1 CoA-transferases. A graphical representation of the amino acid conservation of the aligned amino acid sequences shown in Fig. 3 was prepared using Weblogo version 2.8.2 (Crooks et al., 2004). A logo consists of stacks of symbols, one stack for each position in the sequence. The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino acid at that position. (A) The logo of the EXG active site and (B) the logo of the oxyanion site (GXGGXXD).

therefore ASCT could still be an interesting drug target in these parasites.

The acetate-producing ciliate *Nyctotherus ovalis,* which was once considered to be a parasite but is now known to be a commensal organism, also contains an ASCT that is homologous to this subfamily A of the family I CoA-transferases (Boxma et al., 2005). No transcripts coding for any of the other genes involved in acetate formation have been detected in *N. ovalis* to date, indicating that the reported acetate formation in *N. ovalis* might occur via this ASCT.

5.3. The ASCT of F. hepatica

The *F. hepatica* ASCT enzyme is structurally closely related to the CoA-transferases of bacteria such as *Roseburia* and to that of *A. franciscana*, a brine shrimp (Supplementary Fig. S1). These enzymes all belong to the subfamily IB of the CoA-transferases. Our analysis of the available databases indicated that the parasitic helminths *Schistosoma mansoni*, *Clonorchis sinensis*, *Paragonimus westermani*, *Ostertagia ostertagi*, *Anisakis simplex* and *Brugia malayi* also contain ASCTs that belong to subfamily IB (Table 1; and data not shown). Database searching showed that there are currently no mammalian homologs of this subfamily.

Recombinant expression studies of *T. vaginalis* and *F. hepatica* ASCTs excluded the possibility that these CoA-transferases function as heteromers, but it is yet unknown whether they function as monomers or as multimeric homomers (van Grinsven et al., 2008, 2009b).

Substrate specificities have been studied for the CoA-transferases of both *F. hepatica* and *Roseburia* sp. A2–183, and these studies have shown that both enzymes can transfer a CoA-moiety from acetyl-CoA to acetate, propionate and butyrate. Furthermore, the *F. hepatica* ASCT can transfer CoA from acetyl-CoA to succinate, whereas *Roseburia* CoA-transferase cannot use succinate as a CoA-acceptor. The latter, however, can catalyse the transfer of a CoA-moiety to pentanoate and isobutyrate, two substrates that cannot be used by the *F. hepatica* CoA-transferase (Charrier et al., 2006; van Grinsven et al., 2009b). In conclusion, subfamily IB of CoA-transferase-type ASCT displays a rather broad substrate specificity, and we expect specific differences to be found among the enzymes of different parasite lineages.

5.4. The ASCT of T. vaginalis

Like all ASCTs, the CoA-transferase identified in *T. vaginalis* also belongs to the family I CoA-transferases and clearly clusters together with CoA-transferases of many prokaryotes and fungi in what we suggested calling subfamily IC (Fig. 3). Subfamily IC currently contains no metazoan ASCTs. Members of subfamily IC all have the same conserved domain (YgfH domain, pfam02550; TIGR03458), which is not present in subfamilies IA and IB.

6. Acetate formation by acetyl-CoA thioesterases/hydrolases

A fourth way of producing acetate from acetyl-CoA occurs by simple hydrolysis of the thioester bond. Enzymes catalysing such a reaction are currently designated in the literature as acyl-CoA hydrolases and acyl-CoA thioesterases. As the reaction involves cleavage of a thioester bond it is now considered more appropriate to name these enzymes thioesterases and a revised nomenclature for the mammalian enzymes has been recommended, because over the years several unrelated acyl-CoA thioesterases have been characterised, which has led to many inconsistencies in enzyme nomenclature (Hunt et al., 2005). In contrast to the three other acetate-producing routes described so far, the thioesterase reaction releases the energy stored in the thioester bond of acetyl-CoA as heat, instead of conserving it through substrate level phosphorylation (Fig. 1). Although the physiological functions for many of the acyl-CoA thioesterases are not fully understood, they are generally thought to regulate intracellular levels of CoA esters, the corresponding free acid and coenzyme-A (Hunt et al., 2005). Acyl-CoA thioesterases are also present in peroxisomes and are involved in lipid metabolism. In humans, several of these acyl-CoA thioesterases have been characterised, including one that is active on short-chain acyl-CoAs and appears to be involved in acetate production from peroxisomal β-oxidation (Hunt and Alexson, 2008). In plant mitochondria, acetyl-CoA thioesterases are thought to produce acetate (Zeiher and Randall, 1990), which can diffuse freely to the chloroplast to serve as a substrate for fatty acid synthesis. Acetyl-CoA hydrolases seem to be present in most if not all animal and plant lineages, but there is only one description of acetyl-CoA thioesterase activity in a parasite, the nematode *Ascaris suum* (de Mata et al., 1997). The corresponding gene of this putative activity has, however, not yet been discovered in the incomplete *A. suum* database. Searching the available databases of parasites did not reveal any acetyl-CoA thioesterase gene (data not shown).

Much confusion regarding nomenclature of these enzymes takes root in reports on an enzyme from *Saccharomyces cerevisiae* that was characterised as an acetyl-CoA thioesterase (then called hydrolase) (Lee et al., 1989, 1990). Unexpectedly, however, mutants lacking this enzyme activity displayed a severe and puzzling growth defect when grown on acetate. Recently, this yeast enzyme was re-characterised and it turned out that the enzyme had only a minor hydrolase activity, and was in fact a CoA-transferase with a very highly specific activity for the transfer of the CoA-moiety from succinyl-CoA to acetate (Fleck and Brock, 2009). This yeast CoA-transferase, incorrectly annotated in the databases as a hydrolase (Accession number P32316), furthermore showed high sequence similarity to family I CoA-transferases, subfamily IC (see Section 5), explaining why many proteins that display high sequence similarity to the yeast protein are annotated in genome sequencing projects as acetyl-CoA hydrolases, although they more likely act as CoA-transferases under physiological conditions (Fleck and Brock, 2009). This effect of confusing CoA-transferase activity with hydrolase activity was strengthened by the observation of Mack and Buckel (1997) who demonstrated that replacement of the active site glutamate of *Acidaminococcus fermentans* glutaconate CoA-transferase with aspartate prevents the formation of the CoA-ester intermediate in the ping–pong reaction that defines family I CoA-transferase and results in the conversion of this CoA-transferase into a CoA-hydrolase (Mack and Buckel, 1997).

To complicate matters regarding the production of acetate even further, it is now known that acetate generation can also ensue from a side reaction of thiolase, acyl-CoA acetyltransferase, the enzyme that catalyses the final reaction of β-oxidation and that is supposed to produce acetyl-CoA (Yamashita et al., 2006) (Fig. 2).

7. Sub-cellular localisation of acetate-producing enzymes in parasites

Both *E. histolytica* and *G. lamblia* lack compartmentation of energy metabolism and their acetyl-CoA synthetases (ADP-forming) are cytosolic enzymes (Reeves et al., 1977; Sanchez and Müller, 1996). The ASCT/SCS cycle in *F. hepatica* and trypanosomatidae is localised in mitochondria (Barrett et al., 1978; van Vugt et al., 1979; van Hellemond et al., 1998). In *B. hominis*, ASCT and SCS activity are localised in the mitochondrion-like organelles of this anaerobic protistan parasite (Lantsman et al., 2008), so that would clearly appear to be the site of acetate production.

On the other hand, in trichomonads and the anaerobic fungus *Neocallimastix* the ASCT/SCS cycle is localised in hydrogenosomes (Lindmark, 1976; Steinbüchel and Müller, 1986; Marvin-Sikkema et al., 1993). Apparently, in parasites lacking compartmentation of their energy metabolism such as *G. lamblia* and *E. histolytica*, acetate production occurs in the cytosol by a single enzymatic step catalysed by acetyl-CoA synthetase (ADP-forming) (Sanchez and Müller, 1996). Acetate production in parasites that employ the ASCT/SCS cycle appears to localise to the mitochondria or hydrogenosomes (van Hellemond et al., 1998).

8. Evolutionary aspects of acetate production in parasites

Available data indicate that mitochondria were present in the eukaryote common ancestor, although their aerobic capacities are now often reduced among parasites, many of which do not use O_2 as a terminal acceptor (van der Giezen, 2009). Among eukaryotes that inhabit anaerobic and hypoxic habitats, acetate is a very common end product, hence acetate production does not correspond to a lineage-specific trait. Indeed, acetate production is distributed across all of the major eukaryotic lineages that are currently the focus of much discussion: opisthokonts (includes fungi and animals), chromalveolates (includes *Blastocystis* and ciliates), excavate taxa (includes trypanosomes and trichomonads), archaeplastida (includes *Chlamydomonas*), and amoebozoa (includes *Entamoeba*) (Mentel and Martin, 2008), an exception being the group called Rhizaria, where little biochemical data are available. This would tend to suggest that acetate production, like mitochondria, was an attribute of the eukaryote common ancestor. Moreover, with the exception of organisms possessing mitosomes, where all energy metabolism is cytosolic, acetate production is generally associated with mitochondria, including the anaerobic forms thereof, and hydrogenosomes (Tielens et al., 2002).

That we tend to be infected by anaerobically functioning parasites might seem to be a bit of a puzzle. They could surely grow more efficiently with respect to their carbon source (but probably not faster) were their mitochondria to utilise oxygen. But parasites usually don't care too much about efficiency; after all, it is the host's job to provide the continuous supply of substrates. Clearly parasites occupy a set of characteristic niches but in general their adaptive strategies differ across lineages. The limitation of acetate production to just a very few mechanisms in such highly diverse groups of organisms is striking. This puzzle of the production of acetate does not, however, have too many biochemical parts, as only a handful of enzymes is involved. There are of course cases where eukaryotic acetate production might harbour additional surprises, for example in the ascomycete *Fusarium oxysporum*, that will grow under anaerobic conditions on a variety of reduced carbon sources using elemental sulphur as the terminal electron acceptor, generating H_2S as the reduced end product in a 2:1 molar ratio relative to acetate (Abe et al., 2007).

9. Concluding remarks

In all parasites investigated to date, acetate production occurs by either a cytosolic ACS-ADP or by an organellar ASCT. Of the organellar localised ASCT enzymes, which are all members of the family I CoA-transferases, three non-homologous sub-types that are structurally unrelated have been found to date: the ASCT present in trypanosomatids, the one present in parasitic worms, such as *F. hepatica*, and the one in *T. vaginalis*. These unrelated ASCTs all catalyse the same enzymatic reaction and use the same ping-pong reaction mechanism, characterising them as family I CoA-transferases.

ASCTs are widespread throughout the eukaryotic lineages. The cytosolic acetyl-CoA synthetases, on the other hand, are present in only a very limited number of parasite species. It is perhaps a curiosity that one of the simplest biochemical aspects of eukaryote energy metabolism, acetate production, is among the last to be characterised at the molecular level.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2009.12.006.

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