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Combining reverse genetics and nuclear magnetic resonance-based metabolomics unravels trypanosome-specific metabolic pathways

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Summary

Numerous eukaryotes have developed specific metabolic traits that are not present in extensively studied model organisms. For instance, the procyclic insect form of *Trypanosoma brucei*, a parasite responsible for sleeping sickness in its mammalian-specific bloodstream form, metabolizes glucose into excreted succinate and acetate through pathways with unique features. Succinate is primarily produced from glucose-derived phosphoenolpyruvate in peroxisome-like organelles, also known as glycosomes, by a soluble NADH-dependent fumarate reductase only described in trypanosomes so far. Acetate is produced in the mitochondrion of the parasite from acetyl-CoA by a CoA-transferase, which forms an ATP-producing cycle with succinyl-CoA synthetase. The role of this cycle in ATP production was recently demonstrated in procyclic trypanosomes and has only been proposed so far for anaerobic organisms, in addition to trypanosomatids. We review how nuclear magnetic resonance spectrometry can be used to analyze the metabolic network perturbed by deletion (knockout) or down-regulation (RNAi) of the candidate genes involved in these two particular metabolic pathways of procyclic trypanosomes. The role of succinate and acetate production in trypanosomes is discussed, as well as the connections between the succinate and acetate branches, which increase the metabolic flexibility probably required by the parasite to deal with environmental changes such as oxidative stress.

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Introduction

Our current understanding of the central metabolism has been shaped by studies performed in a small number of model organisms, so the pathways absent from this subset of model organisms are poorly represented in metabolic charts. For instance, most parasites have developed specific traits and pathways that are not present in model organisms so obtaining a comprehensive metabolic map is a major challenge faced by parasitologists. During the last decade, this area of investigation has been stimulated by completion of genome sequencing for a considerable number of human pathogenic parasites. However, the function of up to half of the sequenced parasite genes is still unknown and homology-based annotation of genomes generates numerous predictions with a low confidence of gene functions, which need to be validated or corrected by functional analyses. The development of global and targeted metabolomics approaches combined with reverse genetics tools (RNAi, gene knockout, etc.) provides a great opportunity to address this highly relevant question and to identify new metabolic steps and pathways not reported so far in model organisms.

Here we describe the contribution of nuclear magnetic resonance (NMR)-based metabolite profiling to the understanding of the central metabolism of the procyclic insect form of *Trypanosoma brucei*, a parasite responsible in its bloodstream form for sleeping sickness in humans. Early investigations using radiolabeled tracers showed that procyclic trypanosomes convert glucose into partially oxidized end-products excreted into the culture medium, i.e. mainly

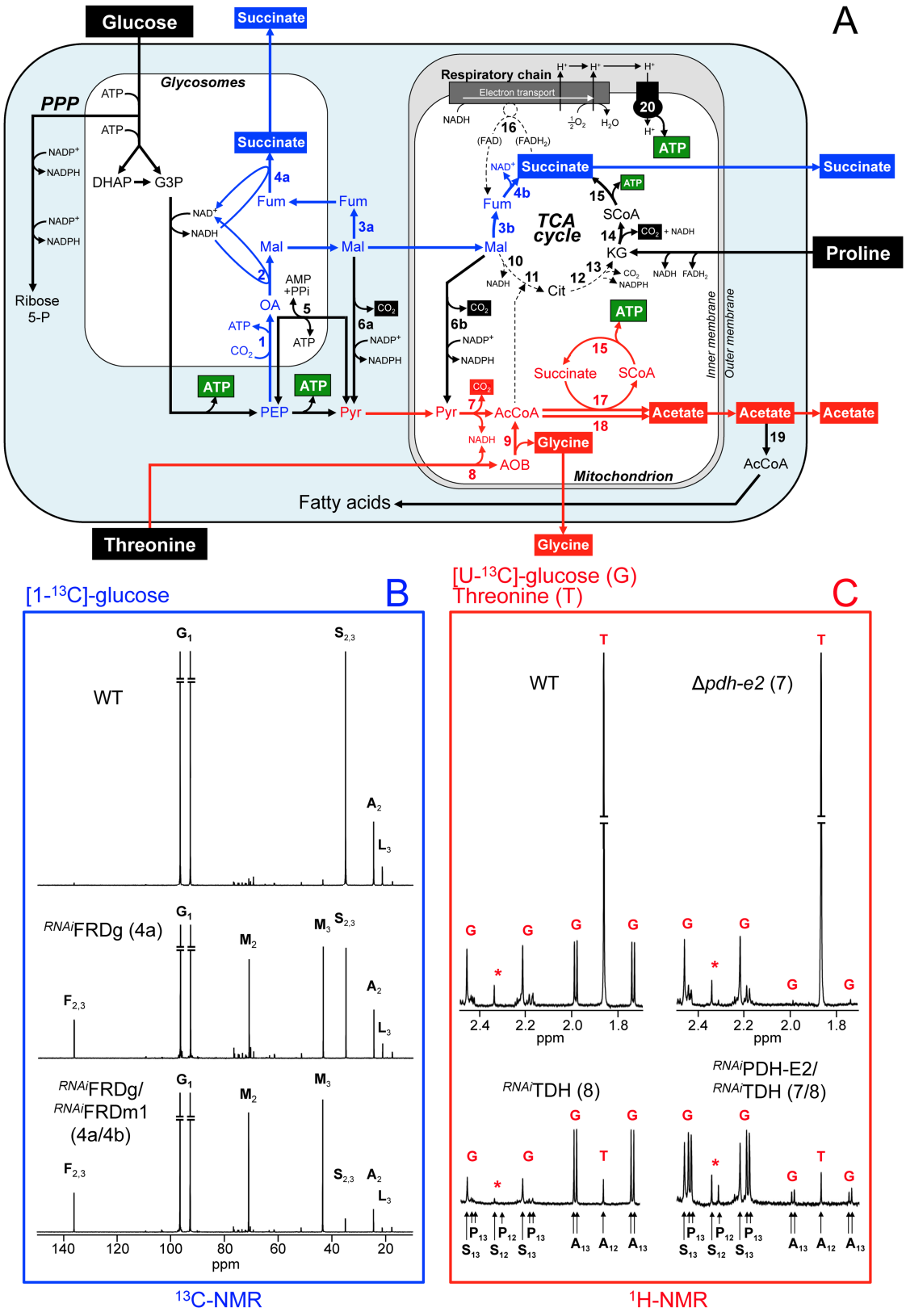
Fig. 1. Identification of two new metabolic pathway branches in trypanosomes.
A. Shows a schematic representation of the central metabolism of the procyclic trypanosomes grown in glucose-rich media. The metabolic branches leading to succinate excretion from glucose-derived PEP are in blue, while acetate production from glucose-derived pyruvate and threonine are shown in red. For the sake of clarity, the non-oxidative branch of PPP, the glycosomal contribution to PPP, the glycerol 3-phosphate/DHAP shuttle and the glycosomal glycerol production pathways are not shown. Indicated enzymes are : 1, phosphoenolpyruvate carboxykinase (PEPCK); 2, glycosomal malate dehydrogenase; 3a, cytosolic fumarase; 3b, mitochondrial fumarase; 4a, glycosomal NADH-dependent fumarate reductase (FRDg); 4b, mitochondrial NADH-dependent fumarate reductase (FRDm1); 5, pyruvate phosphate dikinase (PPDK); 6a, cytosolic malic enzyme (MEc); 6b, mitochondrial malic enzyme (MEm); 7, pyruvate dehydrogenase complex (PDH); 8, threonine 3-dehydrogenase (TDH); 9, 2-amino-3-ketobutyrate coenzyme A ligase; 10, mitochondrial malate dehydrogenase; 11, citrate synthase; 12, aconitase; 13, NADP-dependent isocitrate dehydrogenase; 14, α-ketoglutarate dehydrogenase complex; 15, succinyl-CoA synthetase; 16, succinate dehydrogenase (complex II of the respiratory chain); 17, acetate:succinate CoA-transferase (ASCT); 18, acetyl-CoA thioesterase (ACH); 19, AMP-dependent acetyl-CoA synthetase (AceCS); 20, F_o/F_1-ATP synthase (complex V or the respiratory chain). AcCoA, acetyl-CoA; AOB, amino oxobutyrate; Cit, citrate; DHAP, dihydroxyacetone phosphate; Fum, fumarate; G3P, glyceraldehyde 3-phosphate; KG, 2-ketoglutarate; Mal, malate; OA, oxaloacetate; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway; Pyr, pyruvate; SCoA, succinyl-CoA.
B. Shows the ^{13}C-NMR-based approach used to identify the succinate production pathways. The ^{13}C-NMR spectra represent metabolic end-products excreted by the wild-type procyclic cells (WT), the double RNAi mutant targeting FRDg (step 4a) and FRDm1 (step 4b) (^{RNAi}FRDg/^{RNAi}FRDm1), and the ^{RNAi}FRDg mutant incubated in PBS/$NaHCO_3$ buffer containing 11 mM [1-^{13}C]-glucose. The resonances were assigned as follows: A, acetate; F, fumarate; G, glucose; L, lactate; M, malate; S, succinate. The position of the ^{13}C-enriched in each detected molecule is indicated in indice. C2 and C3 resonances for succinate and fumarate are undistinguishable and are labeled '2,3'.
C. The ^{1}H-NMR approach was used to identify acetate-producing pathways. Metabolic end-products, acetate (A), succinate (S) and pyruvate (P), excreted by the procyclic wild-type (WT), the Δ*pdh-e2* (step 7) and the tetracycline-induced ^{RNAi}PDH-E2/ ^{RNAi}TDH cell lines (steps 7 and 8) from [U-^{13}C]-glucose (4 mM) and/or threonine (4 mM) were determined by ^{1}H-NMR. Glucose-derived and threonine-derived end-products are highlighted by red G and T characters respectively. Resonances corresponding to unenriched succinate or pyruvate, shown by red asterisks, are probably produced from an unknown internal carbon source. A part of each spectrum ranging from 1.7 ppm to 2.5 ppm is shown. The resonances were assigned as: A_{12}, acetate; A_{13}, ^{13}C-enriched acetate; P_{12}, pyruvate; P_{13}, ^{13}C-enriched pyruvate; S_{12}, succinate; S_{13}, ^{13}C-enriched succinate.

acetate and succinate, with small amounts of lactate, glycerol and/or pyruvate (Von Brand *et al*., 1955; Ryley, 1962). Carbon dioxide (CO_2) resulting from decarboxylation of malate and/or pyruvate (steps 6a, 6b and 7 in Fig. 1A) is also detected, but the acetyl-CoA molecules produced in the mitochondrion are not further metabolized through TCA cycle activity, despite the expression of all TCA cycle enzymes (Durieux *et al*., 1991; van Weelden *et al*., 2003). Indeed, procyclic trypanosomes do not use the TCA cycle as a full cycle, but use fragments of it for catabolic and anabolic purposes. For instance, when grown in glucose-rich conditions, succinate is an excreted end-product produced from both proline-derived α-ketoglutarate (steps 14–15) and glucose-derived malate (steps 3b, 4b), through oxidative and reductive branches of the cycle respectively (Fig. 1A) (van Hellemond *et al*., 2005; Coustou *et al*., 2008). Accumulation of citrate in the aconitase null mutant (step 12) demonstrated that the malate/α-ketoglutarate branch is active (steps 10–13) even at the background level, but its contribution and role are not yet understood (van Weelden *et al*., 2003). The absence of a full TCA cycle activity implies excretion of partially oxidized end-products. This is a great advantage for quantitative analysis by NMR spectrometry as quantitative analyses of CO_2 production cannot be performed using this approach. As observed for trypanosomes, most parasites have developed the capacity to ferment various carbon sources (glucose, amino acids) either aerobically or anaerobically with excretion of a number of partially oxidized end-products, which can be reliably detected and quantified by NMR spectrometry.

Nuclear magnetic resonance spectrometry approaches combined with reverse genetic tools (RNAi and gene knockout) led to the discovery and characterization of two metabolic pathways linked to aerobic fermentation in procyclic trypanosomes, i.e. succinic fermentation and mitochondrial acetate production. It is noteworthy that these two metabolic pathways also occur in the bloodstream forms of *T. brucei* (Mazet *et al*., 2013; Creek *et al*., 2015).

Succinic fermentation

In all trypanosomatids analyzed to date, glucose metabolism leads to excretion of succinate, which was originally thought to be produced from the conventional TCA cycle by succinyl-CoA synthetase (SCoAS) (step 15) (Fairlamb and Opperdoes, 1986; Cazzulo, 1992). However, as mentioned above, this hypothesis did not stand the demonstration that the TCA cycle does not work as a cycle, with glucose-derived acetyl-CoA not significantly further metabolized into CO_2 through TCA cycle activity (van Weelden *et al*., 2003). An alternative to SCoAS for succinate production is the reductive conversion of fumarate into succinate by fumarate reductase (FRD) activities. Most of the FRD characterized so far are membrane-bound proteins closely related to the TCA cycle enzyme succinate dehydrogenase (SDH). These FRD enzymes are part of a respiratory chain that transfers electrons from the quinol pool to fumarate, and are involved in ATP production by oxidative phosphorylation under anaerobic conditions (van Hellemond and Tielens, 1994). In contrast, Turrens and colleagues described a soluble NADH-



dependent FRD activity in glycosomal- and mitochondrial-enriched fractions of procyclic trypanosomes that was not associated to membrane-bound complexes (Mracek *et al*., 1991). This activity was first described in trypanosomatids. A few years before completion of the *T. brucei* genome, we identified a family composed of three different genes (*FRDg*, *FRDm1* and *FRDm2*) encoding FRD-like proteins. FRDg (step 4a) and FRDm1 (step 4b) are located in the glycosomes and the mitochondrion, respectively, and account for all the NADH-dependent FRD activity detected in the procyclic cells, as demonstrated by abolition of this activity in the ^{RNAi}FRDg/^{RNAi}FRDm1 double RNAi mutant (Coustou *et al*., 2005). This double mutant is viable with a moderately prolonged doubling time. The third FRD isoform (FRDm2) has not yet been detected at the protein level either in the procyclic or in the bloodstream forms of *T. brucei*. The respective roles of the FRD isoforms in succinate production from glucose were demonstrated by comparative ^{13}C-NMR metabolite profiling of wild-type and RNAi mutant cell lines (Besteiro *et al*., 2002; Coustou *et al*., 2005). When incubated at high density ($1–4 \times 10^8$ cells per ml) in PBS/$NaHCO_3$ containing [1-^{13}C]-glucose, the wild-type procyclic cells primarily excrete ^{13}C-enriched succinate (63.3% of the excreted ^{13}C-enriched molecules), acetate (25%) and lactate (7.4%) from glucose metabolism (Fig. 1B, top panel). RNAi-mediated downregulation of FRDg expression (^{RNAi}FRDg) induced a 2.3-fold reduction of excreted ^{13}C-succinate, the FRDg product, compensated by a 42-fold increase in excreted ^{13}C-fumarate, the FRDg substrate. Excretion of malate, the fumarate precursor produced by fumarase from oxaloacetate (step 3a), is also 20-fold increased in the ^{RNAi}FRDg cell line, which strengthens the role of FRDg in succinate production (Besteiro *et al*., 2002). The proof that FRD isoforms are responsible for all the production of succinate from glucose comes from the quasi abolition of its production in the ^{RNAi}FRDg/^{RNAi}FRDm1 mutant (Coustou *et al*., 2005) (Fig. 1B, lower panel). Similar results were also obtained with the RNAi mutant targeting both cytosolic and mitochondrial fumarase (steps 3a and 3b) (Coustou *et al*., 2006).

The role of the trypanosomatid glycosomal NADH-dependent FRD is to help maintain the glycosomal redox balance by oxidizing NADH produced from the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase back to NAD^+ (see Fig. 1A), as recently demonstrated for the procyclic trypanosomes (Ebikeme *et al*., 2010). Indeed, among the three strategies developed by trypanosomes to maintain the glycosomal NAD^+/NADH balance, i.e. (i) the glycerol 3-phosphate/dihydroxyacetone phosphate shuttle that transfers reducing equivalents to the mitochondrion (not shown in Fig. 1A), (ii) the glycosomal glycerol production pathway (not shown in Fig. 1A) and (iii) the glycosomal succinic fermentation (steps 1-4a), only the latter involving FRDg is required to sustain a high glycolytic flux in the procyclic cells (Ebikeme *et al*., 2010). The recent expression of *T. brucei* FRDg in a metabolically engineered yeast considerably improved its ethanol production yield by providing an important source of NAD^+, confirming the key role of FRDg in NADH oxidation (Salusjarvi *et al*., 2013). Interestingly, this hypothesis was proposed by Gest in 1980 (Gest, 1980), 20 and 10 years before identification of the soluble NADH-dependent FRD activity and its corresponding gene respectively (Mracek *et al*., 1991; Besteiro *et al*., 2002). Gest proposed that ancestral anaerobic organisms evolved from lactic fermentation to succinic fermentation by replacing an NADH-dependent lactate dehydrogenase by a four-step pathway composed of a CO_2-fixation enzyme [phosphoenolpyruvate carboxykinase (PEPCK) or pyruvate carboxylase], fumarase and two soluble NADH-dependent reductases (Malate dehydrogenase – MDH and FRD). This evolutionary step would offer the significant advantage of requiring 50% less pyruvate [or phosphoenolpyruvate (PEP)] to maintain the NAD^+/NADH balance, as succinic fermentation oxidizes twofold more NADH to NAD^+ than lactic fermentation. As a consequence, the remaining tri-carbon compounds can be invested in biosynthetic processes, conceived as an evolutionary step of major advantage. Characterization of the *FRDg* gene closed a long-standing debate about the existence of *bona fide* soluble NADH-dependent FRD in trypanosomes (Tielens and Van Hellemond, 1999; Turrens, 1999) and strengthened Gest's hypothesis, as glucose metabolism in the trypanosome glycosomes closely resembles his succinic fermentation model. Indeed, inclusion of the glycosomal succinate branch in the procyclic trypanosomes releases PEP molecules used to produce ATP in the cytosol through pyruvate kinase activity, which has been described as essential for the viability of the procyclic form (Morris *et al*., 2002; Coustou *et al*., 2003; Deramchia *et al*., 2014). The trypanosome FRD enzymes were the first soluble NADH-dependent FRD characterized, although the concept might be more widely applicable as soluble NADH-dependent FRD activity was reported in the parasite *Trichomonas foetus* (Müller, 2003) and the prokaryote *Hydrogenobacter thermophilus* (Miura *et al*., 2008).

In contrast, the exact role of the mitochondrial succinate branch (steps 3b and 4b) is still elusive, although it contributes to 14–44% of glucose-derived succinate production (Coustou *et al*., 2005). As demonstrated for the glycosomal succinate branch, the mitochondrial branch could be involved in the maintenance of the organellar redox balance, with FRDm1 oxidizing NADH produced by the pyruvate dehydrogenase complex (PDH, step 7), threonine dehydrogenase (TDH, step 8) and/or the proline degradation pathway (see Fig. 1A). However, no direct experimental evidence supports this hypothesis so far.

Alternatively, complex I of the respiratory chain was long considered as the main mitochondrial NADH dehydrogenase activity of procyclic trypanosomes, although it appears to be non-essential and to have a limited activity (Verner *et al.*, 2010; Duarte and Tomas, 2014), which implies that other activity(ies) oxidize mitochondrial NADH. Trypanosomes express another membrane-associated NADH dehydrogenase (NDH2), which is required to sustain the parasite growth rate and the mitochondrial membrane potential. Nevertheless, the enzyme may face the intermembrane space of the organelle and be involved in the maintenance of the cytosolic redox balance rather than the mitochondrial one (Verner *et al.*, 2013). An alternative NADH dehydrogenase activity involving the mitochondrial NADH-dependent FRD (FRDm1) and complex II of the respiratory chain (SDH, step 16) was proposed 15 years ago by Turrens (1989). In this hypothetical FRDm1/SDH cycle, electrons from NADH are transferred to fumarate by FRDm1 to produce succinate, the latter being converted back to fumarate by SDH, which transfers electrons to the quinone pool of the respiratory chain. If this cycle is functional, it is not essential for procyclic cells grown in glucose-rich media, as both the ^{RNAi}SDH and ^{RNAi}FRDm1 mutants are viable in these conditions (Coustou *et al.*, 2005; 2008). Obviously, additional experimental evidence will be required to determine the role played by each known or hypothetical NADH dehydrogenase activity in the maintenance of the mitochondrial redox balance of procyclic trypanosomes.

Mitochondrial acetate production and acetyl-CoA transfer to the cytosol

As mentioned above, glucose-derived acetyl-CoA is not significantly metabolized in the TCA cycle but is rather converted in the mitochondrion into acetate, which represents a major end-product excreted from glucose metabolism (Bringaud *et al.*, 2006). In addition, conversion of threonine into equal amounts of glycine and acetate has previously been reported (Cross *et al.*, 1975). We recently used ^{1}H-NMR spectrometry to investigate the metabolic pathways leading to acetate production and the relative contribution of both carbon sources. The advantage of ^{1}H-NMR spectrometry is the possibility to distinguish between protons linked to ^{12}C and ^{13}C carbons, while ^{13}C-NMR approaches only detect ^{13}C carbons. For instance, ^{12}C-H bonds of the acetate methyl group generate a single resonance by ^{1}H-NMR spectrometry with a chemical shift at 1.88 ppm, while ^{13}C-H bonds of the same ^{13}C-enriched molecule generate two doublets with chemical shifts at around 2.0 ppm and 1.75 ppm respectively. This approach makes it possible to distinguish end-products excreted from two different carbon sources, as long as one is uniformly ^{13}C-enriched, as illustrated in Fig. 1C. The spectra show ^{13}C-enriched and unenriched end-products excreted from metabolism of [U-^{13}C]-glucose and threonine, respectively, by wild-type procyclic trypanosomes and mutant cell lines deficient for acetate production from glucose (Δ*pdh-e2* knockout mutant – subunit E2 of PDH, step 7), threonine (^{RNAi}TDH, step 8) or both (^{RNAi}PDH-E2/^{RNAi}TDH). As expected from the metabolic map, glucose-derived and threonine-derived acetate production is abolished in both the Δ*pdh-e2* and ^{RNAi}TDH single mutants, respectively, while acetate production from both carbon sources is almost completely abolished in the double ^{RNAi}TDH/^{RNAi}PDH-E2 cell line; the residual acetate production is certainly due to incomplete RNAi-mediated downregulation of TDH and PDH-E2 expression (Fig. 1C). The lethality of the latter double mutant, while the corresponding single mutants are viable, highlights (i) the essential role of acetyl-CoA and acetate production for the procyclic trypanosomes and (ii) the primary role of threonine and glucose in acetate production, the former contributing 2.5-fold more than the latter (Millerioux *et al.*, 2013). Threonine- and glucose-derived acetyl-CoA are further converted in the mitochondrion into acetate by the same two enzymes, acetate:succinate CoA-transferase (ASCT, step 17) and acetyl-CoA thioesterase (ACH, step 18), which are synergistically essential for the parasite (Riviere *et al.*, 2004; Millerioux *et al.*, 2012). Together with the TCA cycle enzyme SCoAS (step 15), ASCT is involved in ATP production through the ASCT/SCoAS cycle, while ACH is not associated with ATP production (van Hellemond *et al.*, 1998; Millerioux *et al.*, 2012). Incidentally, ASCT is structurally related to succinyl-CoA:3-ketoacid CoA-transferases (SCOT) and was annotated as such in databases. SCOT is a well-known mammalian enzyme involved in ketone body metabolism by reversibly transferring a CoA-moiety from succinyl-CoA to acetoacetate, yielding succinate and acetoacetyl-CoA (Fukao *et al.*, 2004). This illustrates how homology-based annotations of genes may lead to mistakes that can only be fixed by functional analyses (Riviere *et al.*, 2004).

Mitochondrial production of acetate may play several roles in the procyclic trypanosomes as well as in other parasites (Bringaud *et al.*, 2010; Tielens *et al.*, 2010; Müller *et al.*, 2012). First, the strong growth phenotype observed in the Δ*asct* knockout procyclic AnTat1.1 cell line (approximately 2.3-fold increase in doubling time), while this mutant still produces acetate from glucose metabolism, strongly supports the view that the ASCT/SCoAS cycle plays a key role in ATP production (Riviere *et al.*, 2004). This was confirmed by a recent analysis performed on another procyclic strain (EATRO1125), which showed that the ASCT/SCoAS cycle and the mitochondrial F_0/F_1-ATP synthase (step 20) contribute almost equally to mitochondrial ATP production and are synergistically essential for the parasite grown in glucose-rich conditions (Millerioux

et al., 2012). It is noteworthy that the relative contribution of oxidative phosphorylation and the ASCT/SCoAS cycle is strain dependent, as exemplified by the lethal phenotype observed for the RNAi F_0/F_1-ATP synthase α-subunit in the Lister 427 29.13 strain grown in the same rich conditions (Zikova *et al.*, 2009). Second, acetate production is essential for *de novo* biosynthesis of fatty acids through a metabolic pathway branch described only in trypanosomes so far (Riviere *et al.*, 2009). In this unusual pathway, acetate is produced in the mitochondrion from acetyl-CoA by ASCT and ACH, transferred to the cytosol by a carrier-mediated process or by passive diffusion, before being converted back by the cytosolic AMP-dependent acetyl-CoA synthetase (step 19). This acetyl-CoA transfer system has been developed by the parasite to transfer acetyl-CoA produced in the mitochondrion toward the cytosol, where fatty acid biosynthesis is initiated, to replace the citrate/malate shuttle. In the latter ubiquitous shuttle, citrate produced by the mitochondrial citrate synthase (step 11) from acetyl-CoA and oxaloacetate is converted back to acetyl-CoA by the cytosolic citrate lyase. In contrast to other eukaryotes, trypanosomes do not use this shuttle for lipid synthesis as the citrate lyase enzymatic activity and gene are missing (Riviere *et al.*, 2009). Third, the lethal phenotype associated with abolition of acetate excretion in the $\Delta ach/^{RNAi}$ASCT double mutant (both corresponding single mutants are viable) did not revert in the presence of 5 mM acetate (growth of the wild-type cells is not affected by addition of up to 25 mM acetate), suggesting that another essential role could be played by mitochondrial acetate production (our unpublished data), such as participation in the generation of the mitochondrial membrane potential (Bringaud *et al.*, 2010). Indeed, acetate is nonpolar in its protonated form and can permeate membranes relatively easily by passive diffusion (Kihara and Macnab, 1981). Once across the membrane, weak acids release their protons, which leads to a net export of one proton per excreted molecule and the generation of a pH gradient. However, this would occur against the proton gradient generated by the respiratory chain, and a high concentration of acetate in the mitochondrial matrix would be required to allow its export into the cytosol by simple diffusion. Alternatively, facilitated diffusion through the inner mitochondrial membrane of the anionic form of acetate, together with a proton, could be catalyzed by a proton-linked monocarboxylate transporter family and generate a pH gradient (Halestrap and Price, 1999). Interestingly, Michels and colleagues hypothesized in the late seventies that efflux of weak acids by facilitated diffusion could also generate an electrical membrane potential if an extra proton is translocated by the carrier in addition to the proton of the undissociated solute, with the energy for this extra proton translocation provided by the transmembrane acid gradient (Michels *et al.*, 1979). This hypothesis was later experimentally proved for lactate efflux in prokaryotes (Otto *et al.*, 1980; Ten Brink and Konings, 1980). If this model is also valid for mitochondrial export of acetate, the efflux may considerably increase the mitochondrial transmembrane electrochemical proton gradient.

Proline is a possible alternative source of acetyl-CoA for the procyclic trypanosomes, even though its contribution to acetate production is ~ 50-fold lower compared with threonine (van Weelden *et al.*, 2005). In between blood meals, the midgut of the tsetse fly insect vector is glucose-free, implying that the procyclic trypanosomes rely almost exclusively on threonine present in the midgut (Balogun, 1974) for acetyl-CoA production. Alternatively, homoserine, homoserine lactones and acyl-homoserine lactones may contribute to acetyl-CoA production, as procyclic cells have maintained the last two steps of threonine biosynthesis, which can substitute for threonine in growth media (Ong *et al.*, 2014). The tsetse fly is considered to be an unlikely source for these nutrients, but the tsetse endosymbionts have the genomic ability to produce them in addition to threonine. Glycerol and acetate are also possible *in vivo* acetyl-CoA sources (van Weelden *et al.*, 2005; Millerioux *et al.*, 2013), although their presence in the midgut of the tsetse fly has not been reported so far. Theoretically, end-products of trypanosome metabolism, such as succinate or acetate, could also be reutilized by the parasite, although this hypothesis is not supported by *in vitro* data (see below).

Many other organisms produce acetate in their mitochondria or mitochondrial-like organelles, such as unicellular and multicellular parasitic (*Blastocystis* sp., *T. foetus*, *Trichomonas vaginalis*, *Fasciola hepatica*, *Ascaris lumbricoides*), commensal (*Nyctotherus ovalis*, *Piromyces* sp.) or free-living (*Chlamydomonas reinhardtii*) organisms, as well as marine invertebrates (common mussel, peanut worm, lugworm). All of them except the trypanosomatids live in anaerobic environments (Müller *et al.*, 2012). Acetate production is associated with ATP production in all these organisms, although one cannot exclude that other functions such as fatty acid biosynthesis or generation of a transmembrane proton gradient could also be attributed to its production and/or excretion.

Connections between the acetate and succinate branches provide metabolic flexibilities

Procyclic trypanosomes have developed a high level of flexibility in their central metabolism, with the ability to switch instantly from glucose-based to proline-based metabolism, and *vice versa*, without affecting their growth rate (Coustou *et al.*, 2008 and our unpublished data). Another level of flexibility resides in the metabolic flux distribution between the succinate and acetate branches,

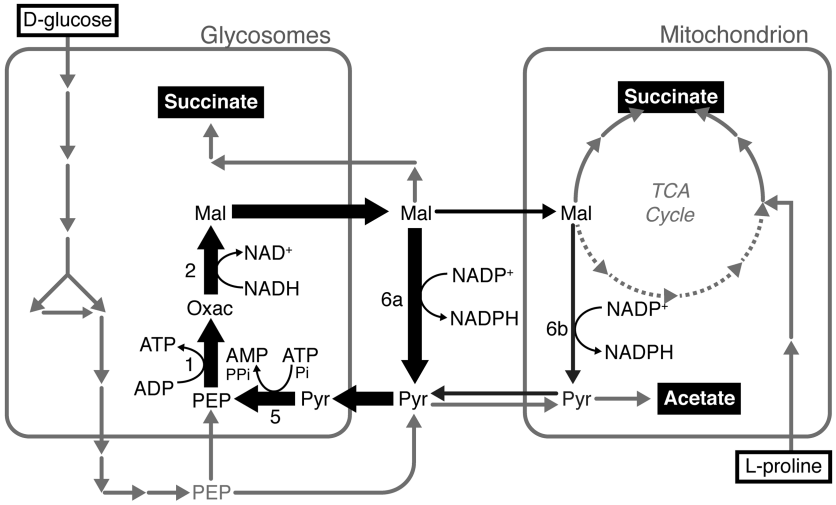


Fig. 2. Transhydrogenase-like shunt in trypanosomes. The purpose of this hypothetic shunt is to convert glycosomal NADH into cytosolic NAPDH through a cycle indicated by thick black arrows. Alternatively, NADPH could also be produced inside the mitochondrion through the additional black arrows. For enzyme numbers, see Fig. 1A.

as the percentage of succinate (and acetate) production from glucose metabolism varies between 19 and 70 depending on the analyses (Besteiro *et al.*, 2002; van Weelden *et al.*, 2003; Millerioux *et al.*, 2012). For instance, ^{1}H-NMR analyses showed that the rate of succinate excretion from glucose, ranging from 15% to 45% of the excreted end-products, depends on cell density (10^{7} to 6×10^{7} cells per ml, respectively) (our unpublished data). This explains the relatively low amounts of succinate detected by ^{1}H-NMR (2×10^{7} cells per ml, Fig. 1C) compared with ^{13}C-NMR (4×10^{8} cells per ml, Fig. 1B). The reasons for these density-dependent differences when the parasites are incubated in PBS are still unknown. However, this is not due to reutilization of excreted succinate or acetate, as the rate of excretion of these end-products is linear over time of incubation regardless of cell density (Millerioux *et al.*, 2013; M. Biran, unpubl. data). A multiobjective-criteria bioinformatics analysis of the flux distribution of the procyclic glucose metabolism showed that this high flexibility is due to flux redistribution from the succinate branch to the acetate branch through the NADP^{+}-dependent malic enzymes (steps 6a, 6b) (Ghozlane *et al.*, 2012). The procyclic trypanosomes express two malic enzyme isoforms: the cytosolic malic enzyme (MEc) isoform, which is synergistically essential with the pentose phosphate pathway for NADPH production; and the essential mitochondrial malic enzyme (Mem) isoform (Leroux *et al.*, 2011; Allmann *et al.*, 2013). One may expect that when the NADPH demand is increased, for instance in response to oxidative stress, the rate of acetate production from glucose metabolism increases because of the elevated metabolic flux through the malic enzymes. We recently proposed a new cycle (Fig. 2) that could be used temporarily when the NADPH demand is particularly high and exceeds the pentose phosphate pathway and malic enzyme capacity (Allmann *et al.*, 2013). This cycle composed of MEc (or MEm) and three glycosomal steps, i.e. pyruvate phosphate dikinase (PPDK, step 5) working in the gluconeogenic direction (Bringaud *et al.*, 1998), PEPCK (step 1) and malate dehydrogenase (step 2), may operate without any impact on the glycosomal ATP/ADP balance as ATP molecules consumed by PPDK are regenerated by PEPCK. Its utilization is certainly exceptional, as the procyclic cells growing in standard glucose-rich conditions preferentially use PPDK in the glycolytic direction to participate in the maintenance of this glycosomal ATP/ADP balance (Deramchia *et al.*, 2014). The relevance of this temporarily used hypothetical cycle is its ability to produce cytosolic NADPH sustained by NADH production in the glycosomes, which may compensate for the absence of cytosolic (and mitochondrial) transhydrogenases in trypanosomatids. A similar cycle for converting NADH into NADPH has been engineered in *Saccharomyces cerevisiae*, and is termed the transhydrogenase-like shunt owing to its function (Suga *et al.*, 2013).

Concluding remarks

Nuclear magnetic resonance-based metabolite profiling of extracellular metabolites has proved its usefulness in mapping the central metabolic network involved in the degradation of the major carbon sources used by parasites. For most of the other metabolic pathways, which do not lead to metabolite excretion, intracellular metabolite profiling or fingerprinting is required. Mass spectrometry (MS) metabolomics (LC-MS/MS, GC-MS/MS, etc.) are becoming the most popular approaches for intracellular metabolite profiling. However, the recent development of new NMR methods for measurements, as well as new methods for the analysis and interpretation of complex data sets generated by NMR spectrometry, means that

NMR spectrometry approaches are attractive alternatives to MS or that they can be used in combination with MS approaches. NMR metabolomics applications have been developed and used extensively to study complex samples such as human samples (biofluids, cells and tissues), plant organs, natural products, etc. (Larive *et al*., 2014). However, these new approaches have not been considered so far for studying parasite metabolisms, except for recent investigations on *Plasmodium* (Sengupta *et al*., 2013; Teng *et al*., 2014). The development of such NMR spectrometry approaches for parasites, including trypanosomes, would certainly complete the toolbox for studying metabolic perturbations in the numerous available mutants affecting metabolic pathways.

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