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Mitochondrial Substrate Level Phosphorylation Is Essential for Growth of Procyclic *Trypanosoma brucei**

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Oxidative phosphorylation and substrate level phosphorylation catalyzed by succinyl-CoA synthetase found in the citric acid and the acetate:succinate CoA transferase/succinyl-CoA synthetase cycle contribute to mitochondrial ATP synthesis in procyclic *Trypanosoma brucei*. The latter pathway is specific for trypanosome but also found in hydrogenosomes. *In organello* ATP production was studied in wild-type and in RNA interference cell lines ablated for key enzymes of each of the three pathways. The following results were obtained: 1) ATP production in the acetate:succinate CoA transferase/succinyl-CoA synthetase cycle was directly demonstrated. 2) Succinate dehydrogenase appears to be the only entry point for electrons of mitochondrial substrates into the respiratory chain; however, its activity could be ablated without causing a growth phenotype. 3) Growth of procyclic *T. brucei* was not affected by the absence of either a functional citric acid or the acetate: succinate CoA transferase/succinyl-CoA synthetase cycle. However, interruption of both pathways in the same cell line resulted in a growth arrest. In summary, these results show that oxygen-independent substrate level phosphorylation either linked to the citric acid cycle or tied into acetate production is essential for growth of procyclic *T. brucei*, a situation that may reflect an adaptation to the partially hypoxic conditions in the insect host.

Trypanosoma brucei is a unicellular parasite responsible for human sleeping sickness and nagana in cattle. It cycles between the bloodstream of a mammalian host and the digestive tract of the tsetse fly. During transmission, *T. brucei* differentiates into different life cycle stages characterized by distinct morphologies, surface proteins, and energy metabolisms.

The energy metabolism of the long slender bloodstream forms in the vertebrate host is based on glycolysis, which is localized in a specialized organelle, called the glycosome. Long slender bloodstream forms have a mitochondrion whose volume is much reduced when compared with the one in the other life cycle stages. Furthermore, since the cytochromes and many citric acid cycle enzymes are missing, it is not capable of performing oxidative phosphorylation (1, 2).

The procyclic form in the midgut of the fly, on the other hand, is characterized by a large mitochondrion. It has a complete citric acid cycle and a fully functional respiratory chain (3). Energy production in procyclic cells is mainly based on the mitochondrion. There are three partially overlapping pathways in which ATP can be produced (indicated as *types I–III* in Fig. 1, as discussed below).

In type I, as in mitochondria from other organisms, ATP is produced by oxidative phosphorylation in a cyanide-sensitive electron transport chain. The main mitochondrial respiratory substrate appears to be succinate (4). Succinate is oxidized by succinate dehydrogenase (SDH), [1] which transfers the electrons to the cytochrome bc_1 complex via the lipid soluble electron carrier ubiquinone. The presence of a functional NADH: ubiquinone oxidoreductase has also been reported (5, 6); however, to what extent this complex is involved in ATP production is a matter of debate (7).

In type II, as expected, one step of substrate-level phosphorylation catalyzed by succinyl-CoA synthetase (SCoAS) occurs in the citric acid cycle. In higher eukaryotes, it is GTP that is synthesized at this step, whereas the *T. brucei* enzyme directly produces ATP.

In type III, finally, mitochondrial ATP can be produced anaerobically by substrate level phosphorylation coupled to acetate formation using the as yet poorly characterized acetate: succinate CoA transferase/SCoAS cycle (ASCT cycle) (8). This pathway consists of two enzymes, the acetate:succinate CoA transferase, which is responsible for transferring the CoA from acetyl-CoA to succinate, and a SCoAS activity, which conserves the energy in the thioester bond of succinyl-CoA. Occurrence of the ASCT cycle in mitochondria is very restricted; it has only been found in trypanosomatid and some parasitic helminths (8).

Interestingly, however, the ASCT cycle is also found in the hydrogenosome of trichomonads and some fungi. Hydrogenosomes are double membrane-bounded organelles involved in pyruvate degradation. They do not perform oxidative phosphorylation and do not have their own genome (9). Nevertheless, morphological and molecular data suggest that mitochondria and hydrogenosomes are related. *T. brucei* belongs to the earliest branching eukaryotes that have mitochondria (10). The fact that they share the ASCT cycle with hydrogenosomes therefore supports a common evolutionary origin for both organelles.

In this study, we have established a system that allows the separate analysis of the three mitochondrial ATP production pathways in procyclic *T. brucei* by measuring ATP production in an *in organello* system in response to different substrates and a panel of specific inhibitors. Furthermore, RNA interference (RNAi)-induced ablation of key enzymes of each pathway was used to determine their relative importance for the survival of cultured procyclic *T. brucei* cells.

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[1] The abbreviations used are: SDH, succinate dehydrogenase; KDH, α−ketoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; SCoAS, succinyl-CoA synthetase; ASCT cycle, acetate:succinate CoA transferase/SCoAS cycle; RNAi, RNA interference.

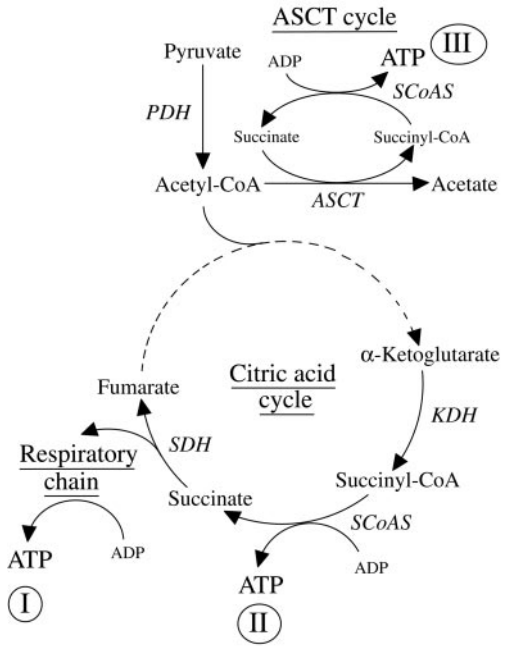

FIG. 1. **Outline of the mitochondrial energy metabolism in procyclic *T. brucei*.** The three pathways responsible for mitochondrial ATP production corresponding to the ASCT cycle, the citric acid cycle, and the respiratory chain are indicated. The three sites of ATP production are indicated by *roman numerals*: *I*, oxidative phosphorylation; *II* and *III*, substrate level phosphorylation in the citric acid cycle and the ASCT cycle, respectively. Key substrates, intermediates and end products are indicated. Enzymes are shown in *italics*.

EXPERIMENTAL PROCEDURES

Cells—Procyclic *T. brucei*, stock 427, was grown at 27 °C in SDM-79 medium supplemented with 5% fetal calf serum. Cells were harvested at 3.5–4.5 × 10^7 cells/ml. Procyclic *T. brucei*, strain 29-13, on which the RNAi knock-down cell lines are based, was grown in SDM-79 (11) supplemented with 15% fetal calf serum, 50 μg/ml hygromycine, and 15 μg/ml G-418, and it was harvested at a density of 0.5–2 × 10^7 cells/ml.

Isolation of Mitochondria—Mitochondria of *T. brucei* 427 used for the *in organello* ATP production assays shown in Fig. 2 were isolated under isotonic conditions (12), resulting in a mitochondrial preparation having intact outer and inner membranes (13). The final fraction of mitochondria was resuspended at 30–50 mg of protein/ml in SoTE (0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA) containing 20 mg/ml fatty acid-free bovine serum albumin and frozen in aliquots. Mitochondria used for *in organello* ATP production assays from the RNAi knock-down cell lines were prepared by digitonin extraction as described (14). The resulting mitochondrial pellet (10^8 cell equivalents each) was resuspended in 750 μl of the buffer used for the *in organello* ATP production assays.

ATP Production Assays—ATP production assays were done as described (13). For each sample, 50–100 μg of isotonically purified mitochondrial fractions or 10^7 cell equivalents of the digitonin-extracted samples were used. To induce ATP production, 5 mM of the indicated substrates (succinate, α-ketoglutarate, or pyruvate/succinate) and 67 μM ADP was added. After incubation at 25 °C for 30 min, the reaction was processed, and the ATP concentration was determined as described. Inhibitors were preincubated with mitochondria for 10 min on ice and used at the following final concentrations: atractyloside (33.3 μg/ml), malonate (6.7 mM), antimycin (2.7 μM). ATP production was then induced by the addition of substrate and ADP.

RNA Interference—The *T. brucei* databases (obtained from The Sanger Institute web site at www.sanger.ac.uk/Projects/T_brucei) was analyzed with the BLAST software using default parameter settings to search for the genes for SDH, α-ketoglutarate dehydrogenase (KDH), SCoAS, and pyruvate dehydrogenase (PDH) by homology to the corresponding genes in other organisms (Table I). Fragments corresponding to the 5′-part of the coding region of the flavoprotein subunit of the SDH and the E1 subunit of the KDH gene were amplified using 5′- and 3′-primers having flanking *Xho*I and *Hind*III sites, respectively. The resulting fragments were cloned into the corresponding restriction sites of the pZJM vector, which contains opposing T7 RNA polymerase promoters (15). The RNAi constructs for the β subunit of SCoAS and the E1 α-subunit of PDH were based on pLew100. pLew100 was modified by replacing the *Hin*dIII/*Bam*HI fragment with a fragment corresponding to 690 nucleotides of the trypanosomal spliced leader sequence. The inserted fragment carries adjacent *Hin*dIII/*Xba*I sites at its 5′-end and adjacent *Xho*I/*Bam*H sites at its 3′-end. Essentially the vector represents a version of the previously described stem loop construct carrying convenient cloning sites (15). Fragments corresponding to the 5′-part of the coding region of the SCoAS and PDH genes were amplified using 5′-primers carrying flanking *Bam*HI/*Hind*III sites and 3′-primers carrying *Xba*I/*Xho*I sites. The PCR fragments from the two genes were then digested with *Hind*III/*Xba*I and inserted into the corresponding sites of the modified stem loop vector. The resulting plasmids, carrying one gene fragment each, as well as the original PCR fragments, were then digested by *Bam*HI/*Xho*I, allowing insertion of the same gene fragments in the opposite direction.

TABLE I
Oligonucleotides used for the construction of the RNAi-plasmids

Enzyme	Subunit	GenBank™ accession numbers[a]	Oligonucleotides[b]
SDH	Flavoprotein	AQ659505	gaggtatcaacgctgctc
		AL496599.1	gctttcggagcataccgc
KDH	E1	AQ657187	gagaaactgtgggtgtgg
		AQ656461	cttgcccagcaaaagcag
SCoAS	β	AQ659061	cttcccacgaaggctgcg
		AQ655451	agcattttcagcggcgcg
PDH	E1 α	AQ953618	gcttaagtgtgtcagccg
			tcgagggcatagcgccac

[a] Sequencing of the *T. brucei* genome was performed by the following sequencing centers: Sanger Centre and The Institute of Genomic Research (TIGR).

[b] 5′- and 3′-primers used for the amplification of the inserts in the corresponding RNAi constructs are shown.

All RNAi plasmids were linearized with *Not*I and transfected into the procyclic *T. brucei* strain 29-13, which expresses T7 RNA polymerase and the tetracycline repressor. Selection with phleomycine, cloning, and induction with tetracycline were done as described (16).

For the double RNAi cell lines described in Fig. 4, in the RNAi constructs for SDH and KDH, we have replaced the pleomycine resistance gene with the puromycine resistance gene. The plasmids were linearized as before and used to transfect the previously characterized single knock-down for PDH (Fig. 3*D*).

RESULTS

In Organello ATP Production Induced by Different Substrates—Using a commercially available luciferase kit, we have established a method to measure the ATP production in isolated mitochondria of procyclic *T. brucei*. It was shown previously that isolated mitochondria are depleted of nucleotides as well as of endogenous substrates (13). Addition of ADP is therefore required for *in organello* ATP production, irrespective of which substrate is being used. Treatment of mitochondria with atractyloside, a specific inhibitor of the ADP/ATP translocator, blocks import of ADP and therefore provides a simple way to test whether an observed ATP production is indeed mitochondrial. The use of malonate, a competitive inhibitor of SDH, and antimycine, which inhibits the cytochrome bc_1 complex, permits us to determine which fraction of an observed ATP production is due to oxidative phosphorylation (13).

Fig. 2*A* shows the nature of the ATP production in isolated mitochondria induced by succinate, α−ketoglutarate, and pyruvate, the three substrates that have been used throughout this study. As shown previously, succinate induces an ATP production that is completely abolished in the presence of malonate or antimycine and therefore is entirely due to oxidative phosphorylation (type I) (13). Addition of α−ketoglutarate, on the other hand, leads to an ATP production essentially due to substrate level phosphorylation occurring in the citric acid cycle and catalyzed by SCoAS (type II). The low but statistically significant amount of ATP synthesis that is inhibited by malonate and antimycine (~20%) is due to the produced suc-

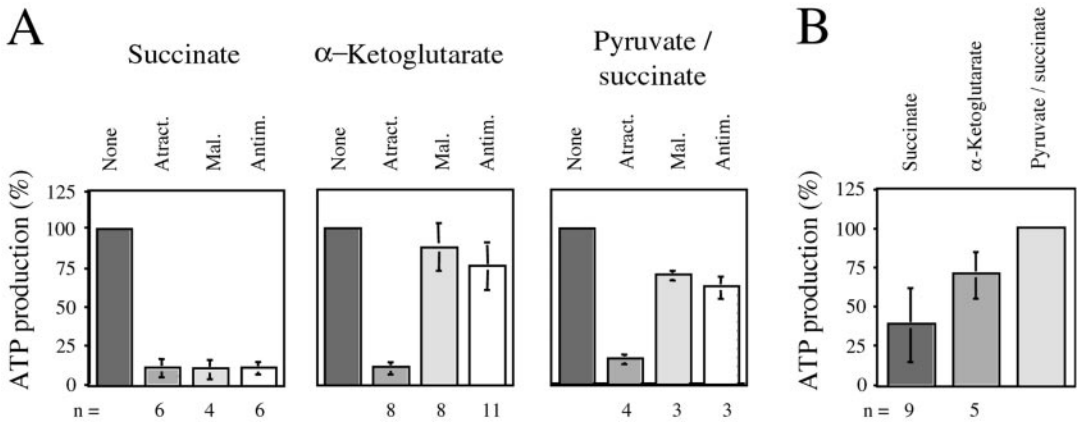

FIG. 2. **ATP production in isolated mitochondria of wild-type *T. brucei*.** *A*, nature of ATP production in isolated mitochondria induced by the indicated substrates. The samples without any additions (*None*) were set to 100%. The three other values corresponding to the assays done in the presence of atractyloside (*Atract.*), malonate (*Mal.*), and antimycine (*Antim.*) are means expressed as percentages from this sample. *B*, comparison of the ATP production induced by the indicated substrates. The pyruvate/succinate combination was set to 100%, and the means of the assays done in the presence of succinate and α-ketoglutarate are shown. The number of experiments (*n*) used to calculate the mean and standard deviation is indicated at the *bottom* of each *bar*.

cinate that is fed into the respiratory chain. No ATP production was measured in the presence of pyruvate alone. This was surprising since pyruvate is expected to be metabolized to acetyl-CoA, which can be used by the citric acid as well as by the ASCT cycle. A possible explanation for these results is that co-substrates were missing. Indeed, when the pyruvate-containing reaction was supplemented with succinate, ATP production could be induced. Approximately 68% of the observed production was malonate- and antimycine-insensitive and therefore caused by substrate level phosphorylation most likely linked to the ASCT cycle (type III). The remaining ~32% are due to succinate alone giving its electrons to the respiratory chain. A small amount of ATP production (~20% of the pyruvate succinate combination) was also observed when pyruvate was combined with either malate or fumarate, indicating that pyruvate can also enter the citric acid cycle (not shown).

Comparing the absolute values of ATP production (Fig. 2*B*), it is clear that more ATP is synthesized in response to α−ketoglutarate and pyruvate/succinate, which induce mainly substrate level phosphorylation (type II and III), than to succinate alone, which induces oxidative phosphorylation only (type I). However, the amounts of ATP produced by substrate level phosphorylation cannot directly be compared with the amounts produced by oxidative phosphorylation, for the following reason. During the mitochondrial isolation procedure, fragmentation of the large procyclic mitochondria cannot be avoided. The resulting mitochondrial vesicles reseal, but the membranes may still be more leaky to protons than *in vivo*, resulting in an underestimation of the ATP that can be produced by oxidative phosphorylation within the intact cell.

In Organello, ATP Production in Single RNAi Knock-down Cell Lines—RNAi is a recently discovered process in which the presence of a double-stranded RNA in a cell causes specific degradation of the corresponding mRNA in a variety of organisms. RNAi was shown to be a powerful method for the inhibition of gene expression in *T. brucei* (15, 17, 18). Therefore, to dissect the different ATP production pathways and to determine their relative importance for procyclic *T. brucei*, we established tetracycline-inducible (19) RNAi cell lines that have reduced amounts of SDH, KDH, SCoAS, and PDH. Subsequently, all cell lines were analyzed for a growth phenotype in SDM 79 medium (11) and for the presence of the three mitochondrial ATP production pathways described in the Introduction. For all experiments, two sets of controls were performed: 1) All biochemical assays were done in parallel with mitochondria isolated from cells uninduced and induced for the expression of double-stranded RNA. It is expected that in all experiments, mitochondria from uninduced cells should behave like the ones from wild-type *T. brucei* shown in Fig. 2. This was indeed the case since the results of the *in organello* ATP assays performed with cells grown in the absence of tetracycline were essentially identical for all cell lines and substrates tested (Fig. 3, *left sides* of the *bar diagrams*). 2) For each of the four cell lines, the ATP production pathways expected not to be affected by the RNAi knock-down effect were used as positive controls (Fig. 3, *CONTROL*) to show the integrity of the mitochondria.

SDH links the citric acid cycle to the respiratory chain. A RNAi knock-down of the flavoprotein subunit of SDH results in an essentially complete loss of oxidative phosphorylation in response to succinate (Fig. 3*A*). Substrate level phosphorylation induced by either α-ketoglutarate or pyruvate/succinate, however, was not affected. Interestingly, despite the severe biochemical phenotype, no effect on growth was observed.

The KDH complex converts α-ketoglutarate into succinyl-CoA. RNAi-induced ablation of the E1 subunit of KDH selectively affects the substrate level phosphorylation occurring in the citric acid cycle (Fig. 3*B*). As expected, oxidative phosphorylation and ATP production in the ASCT cycle were not affected. Similarly to SDH knock-down cell lines, growth of procyclic *T. brucei* in culture is not affected by the at least 90% reduction of KDH activity in the RNAi knock-down cell line.

Fig. 3*C* shows the effect of RNAi-induced ablation of the β-subunit of SCoAS on *in organello* ATP synthesis. As expected, oxidative phosphorylation is not affected. ATP production induced by α−ketoglutarate, however, is essentially eliminated. Furthermore, the malonate-resistant substrate level phosphorylation induced by pyruvate/succinate is also abolished in organelles from the induced cell line. These results show that the same SCoAS activity is used for substrate level phosphorylation in the citric acid and in the ASCT cycle. Interestingly, there appears to be a compensatory change in induced cells since the succinate present in the pyruvate/succinate mixture is ~1.7-fold more efficiently used in oxidative phosphorylation than in the uninduced cells. SCoAS activity is essential for procyclic *T. brucei,* as evidenced by the observed growth arrest of induced cells after 3–5 days.

Fig. 4*D* shows *in organello* ATP production assays from cell lines ablated for the E1 α-subunit of PDH. As expected, ATP production induced by succinate and α-ketoglutarate is normal. Substrate level phosphorylation in the ASCT cycle, however, is

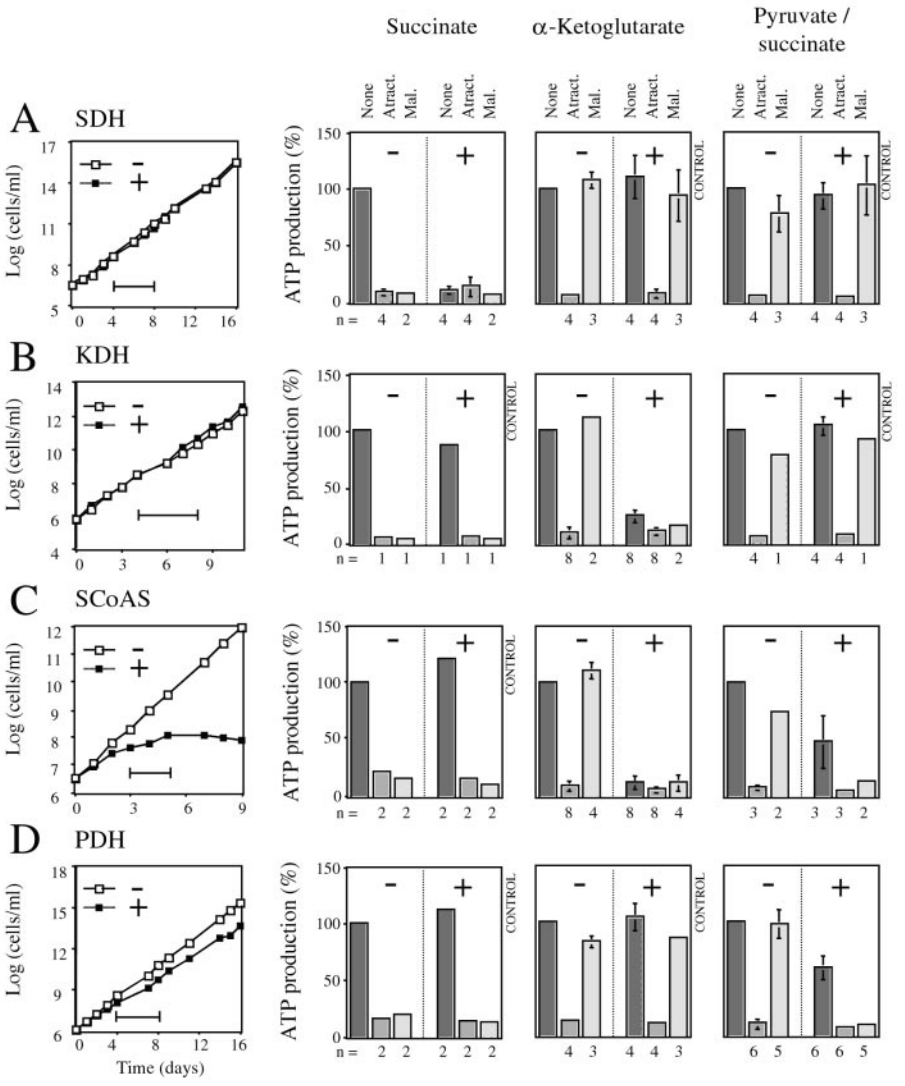

FIG. 3. **Characterization of growth and mitochondrial ATP production of single RNAi knock-down cell lines.** Cell lines ablated for SDH (*A*), KDH (*B*), SCoAS (*C*), and PDH (*D*) activities. Growth curves of the four RNAi cell lines uninduced (grown without tetracycline, −) and induced for the expression of double-stranded RNA (grown in the presence of tetracycline, +) are shown on the *left*. The three bar diagrams on the *right* show the nature of ATP production in digitonin-extracted mitochondria in response to the substrates indicated at the *top*. Each panel shows the results for mitochondria isolated from cells uninduced (−) and induced for RNAi (+). The induction time for the experiments performed with induced cells is indicated by a *bracket* in the *growth curve*. ATP production in mitochondria isolated from uninduced cells and tested without any additions (*None*) was set to 100%. All other values in each panel are means expressed as percentages of this sample. For each panel, the means for assays done without further additions (*None*) or in the presence of atractyloside (*Atract.*) and malonate (*Mal.*) are shown for mitochondria from uninduced and induced cells, respectively. For each cell line, the panels for the substrates not expected to give a change in ATP production are marked as *CONTROL*. The number of independent experiments (*n*) used to calculate the mean and the indicated standard deviation is shown at the *bottom* of each *bar*. In the case of $n = 2$, only the mean is shown.

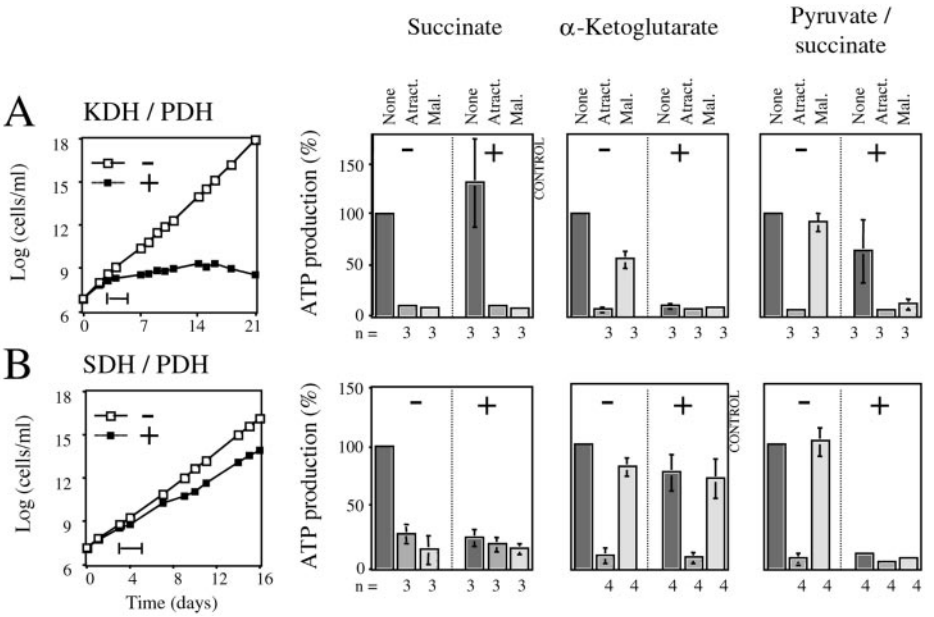

FIG. 4. **Characterization of growth and mitochondrial ATP production of double RNAi knock-down cell lines.** Cell lines ablated for KDH/SDH activities (*A*) and for SDH/PDH activities (*B*). *Growth curves* of the two RNAi cell lines uninduced (grown without tetracycline, −) and induced for the expression of double-stranded RNA (grown in the presence of tetracycline, +) are shown on the *left*. The three bar diagrams on the *right* show the nature of ATP production in digitonin-extracted mitochondria in response to the substrates indicated at the *top*. Panels are arranged identically to Fig. 3.

completely abolished. Furthermore, similar to the SCoAS knock-down cells, there appears to be a compensatory elevation of oxidative phosphorylation; the added succinate that does not contribute to oxidative phosphorylation in mitochondria from uninduced cells results in a net ATP production reaching ~50% of that observed in organelles from induced cells. Despite the fact that RNAi-induced ablation appears to completely abolish PDH activity, only a marginal growth phenotype is observed.

This might, however, not only be due to the lack of ATP synthesis in the ASCT cycle but also to the reduced levels of acetyl-CoA, which is also expected to interrupt the citric acid cycle at the level of citrate synthase.

In organello ATP Production in Double RNAi Knock-down Cell Lines—Except for SCoAS, no strong growth phenotypes were observed in the RNAi knock-down cell lines for any of the tested enzymatic activities. This allowed us to produce RNAi cell lines in which two enzymatic activities are ablated simultaneously. In a practical sense, this was done by replacing the phleomycine resistance marker in the RNAi plasmids with a puromycine resistance gene. A previously characterized, clonal RNAi knock-down cell line for a single gene can then be transfected with a second plasmid carrying another gene.

In a first such experiment, we created a cell line ablated for both KDH and for PDH activities. As expected, both type II and III ATP productions are eliminated in mitochondria from induced cells (Fig. 4*A*). Furthermore, despite the fact that no or only a marginal growth phenotype is observed in the corresponding single RNAi knock-down cell lines (Fig. 3, *B* and *D*), a complete growth arrest is observed if both activities are ablated simultaneously. In fact, the cell line exhibits the same biochemical and growth phenotypes as were observed in the single RNAi-SCoAS knock-down cell line (Fig. 3*C*).

In a second double knock-down cell line, both SDH and PDH activities were ablated. *In organello* ATP production assays show that oxidative phosphorylation induced by succinate as well as substrate level phosphorylation in the ASCT cycle are abolished. The substrate level phosphorylation in the citric acid cycle, however, is not affected (Fig. 4*B*). Furthermore, the cell line shows no stronger growth phenotype than the corresponding single RNAi cell lines (Fig. 3, *A* and *D*). It therefore appears that the substrate level phosphorylation in the citric acid cycle alone is sufficient to support growth of procyclic *T. brucei*, albeit at a somewhat slower rate than in wild-type cells. In summary, these results suggest that substrate level phosphorylation linked to either the citric acid cycle or the ASCT cycle is essential for growth of procyclic *T. brucei*.

DISCUSSION

We have used *in organello* ATP production in response to different substrates to study mitochondrial energy metabolism in procyclic *T. brucei*. Energy metabolism of trypanosome mitochondria has been investigated before, mainly by monitoring oxygen consumption in intact cells or isolated organelles (5, 20). In our study, we have directly measured ATP production, which has the advantage that it is not influenced by oxygen reduction linked to the alternative oxidase (21). *In organello* ATP production assays were combined with RNAi-induced ablation of specific enzyme activities, which allowed us to selectively disrupt the distinct ATP production pathways.

Growth of the RNAi knock-down cell lines was monitored in standard SDM-79 medium containing proline as well as glucose and pyruvate as carbon sources (11). Proline is expected to metabolize into α-ketoglutarate, fueling substrate level phosphorylation in the citric acid cycle as well as oxidative phosphorylation. Glucose and pyruvate, on the other hand, are used for substrate level phosphorylation in the ASCT cycle but can also be fed into the citric acid cycle. The SDM-79 medium therefore provides all required substrates for the three mitochondrial ATP production pathways. A summary of the phenotypes observed in the RNAi cell lines is shown in Table II. Succinate-induced oxidative phosphorylation can be abolished without significantly inhibiting growth (Figs. 3*A* and 4*B*). Substrate level phosphorylation either linked to the citric acid cycle or to the ASCT cycle, however, is essential since if both pathways are interrupted in the same cell line, either by ablating the SCoAS activity (Fig. 3*C*) or by removing KDH and PDH simultaneously (Fig. 4*A*), the cells stop growing.

TABLE II
Phenotypes of RNAi cell lines

RNAi-ablated activities	ATP production pathways[a]			Growth[b]
	Oxidative phosphorylation	Substrate level phosphorylation		
		Citric acid cycle	ASCT cycle	
SDH	–	+	+	+++
KDH	+	–	+	+++
SCoAS	+	–	–	–
PDH	+	+	–	++
KDH/PDH	+	–	–	–
SDH/PDH	–	+	–	++

[a] Plus or minus symbol indicates the presence or absence of the respective ATP production pathway.
[b] +++, growth rate identical to uninduced cells; ++, slightly slower growth than uninduced cells; –, no growth.

All *in organello* oxidative phosphorylation detected, irrespectively of which substrate is used, is entirely inhibited by malonate (Fig. 2) or absent in the SDH RNAi knock-down cell line (Fig. 3*A*). This is also true for the ~20% of oxidative phosphorylation induced by the NADH-generating substrate α-ketoglutarate (Fig. 2) and the one induced by glutamate (not shown), suggesting that no functional NADH:ubiquinone oxidoreductase linked to the respiratory chain exists in procyclic *T. brucei*. If this is indeed the case, the only entry point of mitochondrial reducing equivalents into the classic respiratory chain is via SDH. Intramitochondrial NAD may be regenerated in this scenario by NADH dehydrogenases, which are not linked to the respiratory chain (22) or by fumarate reductase (23). There is overwhelming evidence, such as the inability to obtain dyskinetoplastic mutants (24) and the observed growth arrest of a cytochrome c_1 knock-down cell line (not shown), that the respiratory chain is essential in procyclic *T. brucei*. It is therefore surprising that the lack of SDH activity does not result in a growth defect. There are at least two possible explanations for these results: (*a*) The essential function of the respiratory chain may be to use electrons from the cytosolic substrate glycerol-3-phosphate. Indeed, an intermembrane space localized glycerol-3-phosphate dehydrogenase activity has been shown in procyclic *T. brucei* (13). Unlike in bloodstream cells, this enzyme is at least in part linked to the classic respiratory chain since glycerol-3-phosphate is able to induce ATP production in isolated organelles. Interestingly, there is even more ATP synthesized in response to glycerol-3-phosphate than to succinate (13). Furthermore, succinate is known to accumulate during growth of procyclic *T. brucei* (25), suggesting that only a fraction of it is used for oxidative phosphorylation. (*b*) It may not be the ATP production that is the essential function of the respiratory chain but the depletion of cytosolic oxygen, which according to the Ox Tox model was the primary function of ancestral mitochondria (26).

Neither ablation of SDH nor of KDH activity results in a growth phenotype, suggesting that a complete citric acid cycle is not essential for procyclic *T. brucei*. SCoAS, on the other hand, is required for growth. Unlike in most other organisms, the trypanosomal SCoAS is not only linked to the citric acid but also linked to the ASCT cycle (8). Ablation of SCoAS therefore interrupts two pathways, which may explain why it is required for growth. The essential function of SCoAS is in agreement with a previous study showing that an inhibitor of SCoAS phosphorylation was able to arrest growth of procyclic *T. brucei* (27).

Detection of substrate level phosphorylation with pyruvate/succinate, which depends on PDH and SCoAS but not on KDH activity, for the first time directly demonstrates ATP production in the ASCT cycle. Removal of PDH activity interrupts the

ASCT cycle in the *in organello* system as well as the citrate synthase branch of the citric acid cycle, which, however, only marginally contributes to ATP production (not shown). The slight growth phenotype observed in the PDH RNAi knockdown cell line therefore suggests that the ASCT cycle is dispensable for procyclic *T. brucei*, provided that substrate level phosphorylation in the citric acid cycle is still operational. However, *in vivo*, not all acetyl-CoA might be produced by PDH alone. To definitively decide whether the ASCT pathway is essential, a knock-out cell line for the only enzymatic activity specific for the ASCT cycle, the acetate:succinate CoA transferase, whose gene has not been identified yet, would be required.

The procyclic stage of *T. brucei* depends on a functional respiratory chain (see above), and the same is true for *Saccharomyces cerevisiae*, which is grown on non-fermentable carbon sources. Interestingly, the relative importance of the different citric acid cycle enzymes appears to be very different in the two organisms. Whereas in *T. brucei* SDH, KDH, and PDH appear to be dispensable, they are required for growth of yeast on non-fermentable carbon sources. On the other hand, SCoAS, which is essential for growth of procyclic *T. brucei*, is the only citric acid cycle enzyme of yeast that can be disrupted without abolishing growth on non-fermentable carbon sources (28, 29).

In summary, our results suggest that substrate level phosphorylation is essential for the growth of procyclic *T. brucei*. However, it does not seem to matter in which pathway, the citric acid or the ASCT cycle, the ATP is being synthesized. Neither the ATP produced in the citric acid cycle, assuming that succinate accumulates as an end product, nor the ATP produced in the ASCT cycle depends on oxygen. Procyclic *T. brucei* is adapted to life in the digestive tract of the tsetse fly. The fact that at least one pathway encompassing substrate level phosphorylation is essential for survival may therefore reflect an adaptation to the partly hypoxic conditions in the fly. Furthermore, our study also shows that in *T. brucei*, RNAi-induced ablation of defined enzymatic activities is a powerful tool to untangle the complex interrelations of different pathways in a metabolic network.

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