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

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Natacha Bochud-Allemann and Andre Schneider‡
From the Department of Biology/Zoology, University of Fribourg, Chemin du Musée 10, CH-1700 Fribourg, Switzerland

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 trypanosomes 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 bc1 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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‡ To whom correspondence should be addressed. Tel.: 41-26-3008877; Fax: 41-26-3009741; E-mail: andre.schneider@unifr.ch.

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/SCoS cycle; RNAi, RNA interference.
Mitochondrial Metabolism in T. brucei

EXPERIMENTAL PROCEDURES

Cells—Procylic 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 × 10⁷ cells/ml. Procylic 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 × 10⁷ cells/ml.

Isolation of Mitochondria—Mitochondria of T. brucei 427 were 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 (10⁸ cell equivalents each) was re-suspended in 750 μl of the buffer used for the in organello ATP production assays.

ATP Production Assays—ATP production assays were done as described (15). For each sample, 50–100 μg of mitochondrially purified mitochondrial fractions or 10⁷ 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: atracyloside (33.3 μM/ml), malonate (6.7 μM), antymycin (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_brucel) were 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 XhoI and HindIII sites, respectively. The resulting fragments were cloned into the corresponding restriction sites of the pZJIM vector, which contains opposing T7 RNA polymerase pro-
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**A**

|                   | Succinate | α-Ketoglutarate | Pyruvate / Succinate |
|-------------------|-----------|-----------------|----------------------|
| ATP production (%)|           |                 |                      |
| None              | 100       | 100             | 100                  |
| Atract.           | 95        | 95              | 95                   |
| Mal.              | 95        | 95              | 95                   |
| Antim.            | 95        | 95              | 95                   |

**B**

|                   | Succinate | α-Ketoglutarate | Pyruvate / Succinate |
|-------------------|-----------|-----------------|----------------------|
| ATP production (%)|           |                 |                      |
| None              | 100       | 100             | 100                  |
| Atract.           | 95        | 95              | 95                   |
| Mal.              | 95        | 95              | 95                   |
| Antim.            | 95        | 95              | 95                   |

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 antimycin (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 antimycin-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. 2B), 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. 3A). 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. 3B). 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. 3C 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. 4D 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...
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 pleomycine 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. 4A). 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. 3C).

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. 4B). 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. 3A and 4B). 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. 3C) or by removing KDH and PDH simultaneously (Fig. 4A), the cells stop growing.

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. 3A). 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 diskinotoplastic mutants (24) and the observed growth arrest of a cytochrome c1 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 glycero-3-phosphate. Indeed, an intermembrane space localized glycero-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 glycero-3-phosphate is able to induce ATP production in isolated organelles. Interestingly, there is even more ATP synthesized in response to glycero-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).

| RNAi-ablated activities | Oxidative phosphorylation | Substrate level phosphorylation | Growth |
|------------------------|---------------------------|-------------------------------|-------|
|                        | Citric acid cycle | ASCT cycle               |       |
| SDH                    | –              | +                             | +     |
| KDH                    | +              | –                             | +     |
| SCoAS                  | +              | –                             | +     |
| PDH                    | +              | –                             | +     |
| KDH/PDH                | +              | +                             | +     |
| SDH/PDH                | –              | +                             | +     |

* Plus or minus symbol indicates the presence or absence of the respective ATP production pathway.

+ ++, growth rate identical to uninduced cells; ++, slightly slower growth than uninduced cells; –, no growth.
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 knock-down 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. Procyclic *T. brucei* is adapted to life in the digestive tract of the tsetse fly. In particular, succinyl-CoA is essential for growth of procyclic *T. brucei*, is the only enzymatic activity specific for the ASCT cycle, the acetate:succinate CoA transferase, whose gene has not been identified yet, would be required.

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