

ATP production in isolated mitochondria of procyclic

Trypanosoma brucei

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Running title: ATP production in *T. brucei* mitochondria

Abstract

This chapter describes a luciferase-based protocol to measure ATP production in isolated mitochondria of *Trypanosoma brucei*. The assay represents an excellent method to characterize the functionality of isolated mitochondria. Comparing the ATP production induced by substrates for oxidative phosphorylation to the one induced by substrates for substrate level phosphorylation allows to draw conclusions regarding the integrity of the outer and the inner mitochondrial membranes. Furthermore, the assay provides a valuable tool to characterize RNA interference cell lines suspected to affect mitochondrial functions.

Key Words: trypanosome, *Trypanosoma brucei*, mitochondria, ATP, luciferase, oxidative phosphorylation, substrate level phosphorylation, digitonin extraction

1. Introduction

The single mitochondrion of insect stage *T. brucei* has three in part overlapping ATP production pathways (1, 2)(Fig. 1). First, as in mitochondria from other organisms ATP is produced by oxidative phosphorylation (OXPHOS) in a cyanide-sensitive electron transport chain. Second, as expected one step of substrate level phosphorylation (SUBPHOS) catalyzed by succinyl-CoA synthetase (SCoAS) occurs in the citric acid cycle. In higher eukaryotes it is GTP which is synthesized at this step, whereas the *T. brucei* enzyme directly produces ATP. Finally, mitochondrial ATP can be produced anaerobically by SUBPHOS coupled to acetate formation using the acetate:succinate CoA transferase/SCoAS cycle (ASCT cycle)(3). This pathway consists of two enzymes, the acetate:succinate CoA transferase (4) and the same SCoAS which is found in the citric acid cycle (5). Occurrence of the ASCT cycle in mitochondria is restricted; it has only been found in trypanosomatid and some parasitic helminths. Interestingly, however, the ASCT cycle is found in the hydrogenosome of trichomonads and some fungi.

While mitochondrial ATP production is of interest on its own, it also provides an excellent tool to assay the integrity and functionality of isolated mitochondria of *T. brucei* and thus has many potential applications. Assaying OXPHOS indirectly monitors the presence of the membrane potential and depending on the substrate which is used allows to test for the presence of an intact outer membrane (6). The two modes of SUBPHOS on the other hand do not depend on the membrane potential and require an intact inner membrane only. RNA interference (RNAi) is an efficient and rapid method for the generation of conditional mutants in *Trypanosoma brucei* (7, 8). Using digitonin extractions in combination with in organello ATP production assays it is possible to rapidly identify RNAi strains, which interfere with mitochondrial functions (5, 9). Furthermore, OXPHOS, in contrast to the two modes of SUBPHOS, requires some mitochondrially encoded proteins and thus indirectly depends on organellar protein synthesis. Thus, RNAi cell lines which are impaired in OXPHOS but at the same time show normal levels of mitochondrial SUBPHOS are of special interest, since they include the ones which are ablated for proteins which are required for mitochondrial translation, a process which in trypanosomes is notoriously difficult to study (10).

It is the aim of this review to provide a simple protocol to measure mitochondrial ATP production in isolated mitochondria or in digitonin-extracted *T. brucei* cells and discuss some of its applications.

2. Materials

2.1. Digitonin extraction

1. Procylic *Trypanosoma brucei* cells (see **Note 1**).
2. SDM-79 medium supplemented with 5% heat inactivated fetal bovine serum (11).
3. Wash buffer: 20 mM NaPi, pH 7.9, 20 mM Glucose, 0.15 M NaCl. Prepare as 4x stock and sterilize by filtration.
4. SoTE buffer: 20 mM Tris-HCl, pH 7.5, 0.6 M Sorbitol, 2 mM EDTA, pH 7.5. Prepare as 2x stock and sterilize by filtration.
5. Digitonin stock (cat. No. 37006; Fluka): 0.8% (w/v), dissolve in SoTE buffer. Digitonin does not dissolve well, thus heat the suspension to 95°C till it clears up then cool down to room temperature. The stock solution will now stay dissolved even at ambient temperature. Keep the stock at -20°C, if a precipitate forms after defrosting repeat the procedure described above.
6. ATP assay buffer: 20 mM Tris-HCl pH 7.4, 15 mM KH₂PO₄, 0.6 M Sorbitol, 10 mM MgSO₄, 2.5 mg/ml fatty acid free BSA (cat. No. A-6003; Sigma), sterilize by filtration.

2.2. In organello ATP production assay

1. Atractyloside stock (cat. No. A6882; Sigma): 10 mM, dissolve in DMSO. Concentration used in assay: 10 μM.
2. Antimycin stock (cat. No. A8674; Sigma): 0.2 mM, dissolve in ethanol. Concentration used in assay: 2 μM.
3. Malonate stock: 0.5 M, dissolve in water. Concentration used in assay: 7 mM.
4. Carbonyl cyanide(p-trifluoro-methoxy)-phenylhydrazone (FCCP)(cat. No. C2920; Sigma) stock: 20 mM, dissolve in ethanol. Concentration used in assay: 5 μM.
5. Substrate stocks: 0.2 M each of succinate, glycerol-3 phosphate, α-ketoglutarate and pyruvate dissolved in water. Concentration used in assay: 5 mM.
6. ADP stock: 4.5 mM, dissolve in water. Concentration used in assay: 60 μM.

2.3. Processing and luciferase assay

1. 60% perchloric acid.
2. 1 N KOH.
3. 0.5 M Tris-acetate, pH 7.75.
4. ATP Bioluminescence Assay Kit CLS II (cat No. 1699695; Roche). Prepare and store the luciferase reagent as described by the manufacturers.
5. Luminometer.

3. Methods

Principle: Mitochondrial fractions are incubated with ADP and the corresponding OXPHOS or SUBPHOS substrates. After incubation the reaction is stopped by perchloric acid and the produced ATP is quantified using a luciferase based ATP Bioluminescence kit (12).

Organellar fractions: Mitochondrial ATP production assays in *T. brucei* can be performed using mitochondria isolated by the hypotonic or the isotonic isolation protocol (see Chapter X in this volume). However, the assay is especially useful (e.g. for the phenotypic analysis of RNAi cell lines) when it is combined with digitonin extraction of whole cells (see **Subheading 3.1**), since this allows rapid analysis of multiple samples using low cell numbers only (5, 9). Low concentrations of the detergent digitonin selectively permeabilize the cell membrane but leave (at least the inner) mitochondrial membrane intact. Thus, a single centrifugation step of digitonin-extracted *T. brucei* cells will yield a pellet enriched for mitochondria.

Substrates: We routinely use four substrates: succinate and glycerol-3 phosphate which induce OXPHOS, and α -ketoglutarate and pyruvate which induce mitochondrial SUBPHOS (**Fig. 1**). Each of the substrates should be measured in the absence and the presence of the inhibitors indicated in **Table 1** in order to make sure that the correct mode of ATP production is measured. The different possible outcomes of the ATP production assays and the conclusions regarding the state of the mitochondria, which can be drawn from the results are illustrated in **Table 2**.

3.1. Digitonin extraction

1. Use 10^8 cells for each planned digitonin extraction.
2. Spin cells in 15 ml Falcon tubes at 23°C for 7 min at approx. 700 g.

3. Wash in an equal volume of wash buffer.
4. Prepare the required volume of 0.03% digitonin-containing SoTE buffer (*see Note 2*) by dilution of the 0.8% digitonin stock. Warm to ambient temperature.
5. Resuspend the pellet in 0.5 ml of SoTE buffer (prewarmed to ambient temperature) and transfer to 1.5 ml Eppendorf tube.
6. Add 0.5 ml of 0.03% digitonin-containing SoTE buffer (prewarmed to ambient temperature)(*see Note 3*).
7. Invert once and incubate 5 minutes on ice (*see Note 4*).
8. Spin in Eppendorf centrifuge at 4°C for 3 min at 5'000 g.
9. Remove supernatant.
10. Resuspend pellet in 80-120 µl of ATP assay buffer.

3.2. In organello ATP production assay

1. Decide how many reactions you want to do (*see Note 5*).
2. For each reaction resuspend 25-75 µg of isolated mitochondria or 10 µl of the resuspended digitonin pellet (*see Subheading 3.1., step 10*) in a total volume of 75 µl of ATP assay buffer.
3. Set up the required number of 75 µl reactions. Add inhibitors to control reactions (*see Note 6*), incubate on ice for 5 min.
4. Add 2 µl of the 0.2 M stocks of substrate (either succinate, glycerol-3 phosphate, α -ketoglutarate or pyruvate)(*see Note 7*).
5. Start reaction by adding 1 µl of 4.5 mM ADP.
6. Incubate for 30 minutes at 27°C.

3.3. Processing and luciferase assay

1. After incubation add 1.75 µl of 60% perchloric acid and mix immediately on vortex.
2. Incubate on ice for at least 10 min. A white precipitate will form.
3. Spin in Eppendorf centrifuge for 5 min at full speed.
4. Transfer 60 µl of the supernatant to a new tube.
5. Add 11.5 µl of 1 N KOH, the pH of the resulting mixture should be between 7 and 8.
6. Mix on vortex and incubate on ice for 3 min.

7. Spin in Eppendorf centrifuge for 5 min at full speed, keep supernatant and discard pellet (*see Note 8*).
8. Set up luciferase reaction: Use 10 μ l of supernatant (*see step 7*), 40 μ l of 0.5 M Tris-acetate, pH 7.75 and 50 μ l of luciferase reagent (*see Note 9*).
9. Measure chemiluminescence in a luminometer.
10. Analyze results by comparing ATP production in the presence and the absence of inhibitors for the different substrates. The conclusion which can be drawn from the different possible outcomes are listed in **Table 2** (*see Note 10*).

Notes

1. The procedure appears to work for any *T. brucei* cell line. We have used it for the *T. brucei* 427 (6) and 29-13 strains, as well as for many transgenic cell lines including induced RNAi strains (5, 9).
2. The digitonin concentration is the most important parameter of this experiment. Both the concentration of digitonin as well as the concentration of cells are important. The indicated final concentration of 0.015% digitonin has been optimized for cell density of 10^8 cells/ml. At this concentration we are able to detect ATP production in response to glycerol-3 phosphate, indicating that both the outer and the inner mitochondrial membranes remain intact (**Table 2**). However, if higher concentrations are used the outer membrane will be disrupted and no glycerol-3 phosphate induced ATP production can be detected. Moreover, further increasing the digitonin concentration, prior to affecting the inner membrane barrier, will remove the cytochrome c which normally, even after the disruption of the outer membrane, remains associated with the inner membrane (6). The concentration of digitonin to be used also depends on the cell line, thus some transgenic cell lines may behave differently.
3. The correct temperature is important since it influences solubilizing properties of digitonin.
4. Invert only once, more mixing will result in lower ATP production activities.
5. This depends mainly on how many substrates will be tested and how many control reactions with inhibitors are performed.
6. It is mandatory to test, as a control, at least one inhibitor which is expected to interfere with the corresponding mode of ATP production. We routinely test OXPHOS substrates in the presence

and absence of antimycin and the SUBPHOS substrates in the presence and absence of atractyloside (5). For a more complete list of inhibitors see **Table 1**. A detected ATP production is considered to be significant if it is at least 10fold reduced in the presence of the corresponding inhibitors.

7. To measure pyruvate induced ATP production 5 mM succinate has to be added as a co-substrate (5).
8. The samples are stable for at least 24 hours when kept at 4°C.
9. It is important that the chemiluminescence is measured in the linear range of the assay. Thus, using 5 µl of supernatant (*see Subsection 3.3. , step 7*) in the luciferase reaction should give half the signal which is obtained for 10 µl. If this is not the case dilute the sample accordingly.
10. When analyzing digitonin-extracted pellets, we have sometimes encountered sample to sample variations in the absolute levels of ATP production induced by a given substrate. However, it was generally possible to reproduce the relative values. Thus, in order to control for this potential variations we routinely replicate each experiment at least three times and compare the relative efficiencies of ATP production (e. g. by setting the ATP production in uninduced RNAi cell lines to 100% (5)).

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Legend**Fig. 1. Overview of mitochondrial ATP production in procyclic *T. brucei*.**

The three ATP production pathways correspond to the respiratory chain, the citric acid cycle and the ASCT cycle. The three sites of ATP production are indicated by roman numerals: I corresponds to OXPHOS; II and III correspond to SUBPHOS. The substrates which are used in the in organello ATP production assay are indicated in bold.

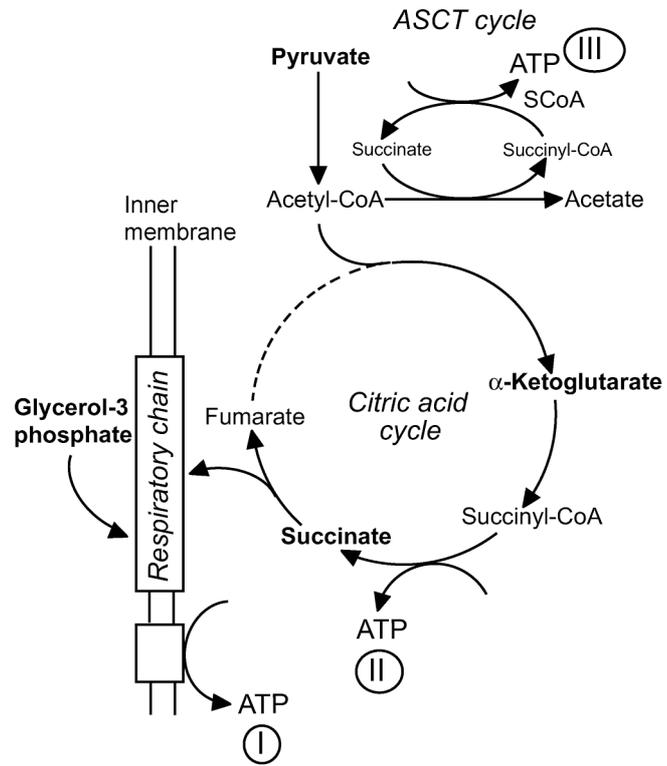


Fig. 1

Table 1. Substrates used to induce mitochondrial ATP production.

Substrate	Efficiency of ATP production ¹⁾	Mode of ATP production	Sensitivity towards inhibitors			
			Atractyloside ²⁾	Antimycin ³⁾	Malonate ⁴⁾	FCCP ⁵⁾
Succinate	100%	OXPHOS	+	+	+	+
Glycerol-3 phosphate	approx. 300%	OXPHOS	+	+	-	+
α-Ketoglutarate⁶⁾	100-200%	approx. 90% SUBPHOS (Citric acid cycle) ⁶⁾	+	-	-	-
Pyruvate⁷⁾ (co-substrate succinate)	100-200%	approx. 80% SUBPHOS (ASCT cycle) ⁷⁾	+	-	-	-

- ¹⁾ Comparison of ATP production induced by the four substrates, as measured in isolated mitochondria having an intact outer and inner membrane. ATP production induced by succinate was set to 100%. In intact mitochondria glycerol-3 phosphate is for unknown reasons by far the most efficient substrate (6).
- ²⁾ Atractyloside is a specific inhibitor of the adenine nucleotide translocater of the mitochondrial inner membrane. It prevents access of ADP to the matrix and thus serves to show that a measured ATP production is indeed mitochondrial (5, 6).
- ³⁾ Antimycin is an inhibitor of respiratory complex III and thus inhibits OXPHOS.
- ⁴⁾ Malonate is a competitive inhibitor of succinate dehydrogenase and therefore specifically inhibits OXPHOS induced by succinate.
- ⁵⁾ FCCP is a potassium ionophore and thus by disrupting the membrane potential affects OXPHOS.
- ⁶⁾ The antimycine resistant part of the α -ketoglutarate induced ATP production (ca. 90%) can be attributed to SUBPHOS. Analysis of RNAi strains has shown, that it is the SUBPHOS in the citric acid cycle which is detected (5). α -Ketoglutarate is converted into succinate in the citric acid cycle, a small fraction of which is apparently used for OXPHOS, explaining the approx. 10% of antimycine sensitive ATP production which is observed (5).
- ⁷⁾ Pyruvate on its own is not able to induce ATP production. However by adding succinate as a co-substrate efficient ATP production is observed, which to approx. 80% is due to SUBPHOS and to approx. 20% to OXPHOS. RNAi analysis has shown that the antimycine resistant part of the

pyruvate induced ATP production can be attributed to the SUBPHOS in the ASCT cycle (5). Thus in *T. brucei*, unlike in other organism, pyruvate does not enter the citric acid cycle.

Table 2. Condition of isolated mitochondria and expected outcomes of ATP production assays in wild-type *T. brucei*.

Condition of isolated mitochondria	Mitochondrial ATP production induced by:			
	Succinate ¹⁾	Glycerol-3 phosphate ²⁾	α -Ketoglutarate	Pyruvate
-intact membrane potential -OM intact -IM intact	+	+	+	+
-intact membrane potential -OM disrupted -IM intact	+	-	+	+
-no membrane Potential -OM disrupted -IM intact	-	-	+	+
-no intact mitochondria present in the tested fraction	-	-	-	-

¹⁾ Cytochrome c is a peripheral membrane protein associated with the outer face of the inner mitochondrial membrane (IM). Experiments have shown that even in the absence of the outer membrane (OM) enough of cytochrome c remains associated with the inner membrane to support OXPHOS (6).

²⁾ Glycerol-3 phosphate dehydrogenase is a soluble protein of the intermembrane space. In the absence of an intact outer mitochondrial membrane it is rapidly lost (6).