ELSEVIER

Molecular and Biochemical Parasitology 111 (2000) 87–94

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com.

ATP production in isolated mitochondria of procyclic *Trypanosoma brucei*

Natacha Allemann, André Schneider *

Department of Biology, Institute of Zoology, University of Fribourg, Pérolles, CH-1700 Fribourg, Switzerland

Received 2 May 2000; received in revised form 29 June 2000; accepted 5 July 2000

Abstract

Membrane potential-dependent ATP production was measured in mitochondrial fractions of procyclic *Trypanosoma brucei* using a luciferase based assay. Mitochondria isolated under hypotonic conditions were able to produce ATP using succinate as substrate. The same was observed with mitochondria isolated under isotonic conditions, however, in this case a 6–7-fold higher amount of ATP was produced with glycerol-3-phosphate as substrate. Disruption of the outer membrane of isotonically prepared mitochondria lead to a selective loss of the glycerol-3 phospate induced ATP production, indicating that glycerol-3-phosphate dehydrogenase is a soluble enzyme of the intermembrane space. Isolation of mitochondria under hypotonic conditions, therefore, results in disruption of the outer membrane, whereas in the organelles isolated under isotonic conditions both the membranes remain intact.

Keywords: Kinetoplasts; Organelles; Respiratory chain; Succinate; Glycerol-3-phosphate dehydrogenase

1. Introduction

Mitochondria of *Trypanosoma brucei* and other kinetoplastids are unique in many respects. Their genome is organized into an intercalated network of maxi-and mincircles. Many mitochondrial genes represent cryptogenes whose primary transcripts have to be modified by RNA editing in order to become functional mRNAs [1]. Furthermore, mitochondrial translation is complicated by the fact that all the required tRNAs are encoded and produced in the nucleus and therefore, need to be imported into mitochondria [2,3]. The function of RNA editing, as well as tRNA import is to synthesize the small number of mitochondrial encoded proteins which, except for one ribosomal protein, are all hydrophobic components of the respiratory chain. The ultimate function of the mentioned processes is, therefore, to produce ATP. Though, bloodstream forms produce ATP only by glycolysis, energy metabolism of procyclic trypanosomes is based on the mitochondria [4]. ATP is produced by oxidative phosphorylation in a cyanide sensitive electron transport chain. Un-

Abbreviations: FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone.

* Corresponding author. Tel.: +41-26-3008877; fax: +41-26-3009741.

E-mail address: andre.schneider@unifr.ch (A. Schneider).

0166-6851/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0166-6851(00)00303-0

like in mammalian mitochondria it has been suggested that the main respiratory substrate is succinate which transfers the electrons to the respiratory chain at the level of the succinate dehydrogenase (complex II) [5,6]. In this scenario no NADH would be produced inside mitochondria but the Krebs cycle would be used to produce succinate. In a more recent study, however, it was shown that a rotenone sensitive NADH dehydrogenase (complex I) activity exists in procyclic *T. brucei* and that subunits of complex I can be detected on immunoblots [7]. In these studies digitonin permeabilized cells or crude mitochondrial fractions from hypotonically lysed cells were used to measure parameters such as NADH oxidation or oxygen consumption.

The aim of our study was to use more defined mitochondrial fractions in order to investigate respiration in procyclic *T. brucei*. Firstly we determined the purity and intactness of mitochondria obtained by the two most common isolation procedures [8–11]. Instead of measuring substrate or oxygen consumption as was done in previous publications [5,7], we decided to directly measure the final product of mitochondrial energy metabolism: ATP synthesis.

2. Material and methods

2.1. Cells

Procyclic wild-type *T. brucei*, stock 427, were grown in SDM-79 medium supplemented with 5% fetal bovine serum (FBS). Cells were harvested at late log phase corresponding to 2.5×10^7–4.0×10^7 cells ml^{-1}.

2.2. Purification of mitochondria

Isolation of mitochondria under hypotonic conditions was done as described [8,12]. Cells were resuspended in DTE (1 mM Tris–HCl pH 8.0; 1 mM EDTA) at 1.2×10^9 cells ml^{-1} and lysed immediately by passage through a hypodermic needle (No. 26). After DNase I digestion the lysate was loaded on 20–35% (w/v) Percoll gradients. The final fraction of mitochondria was resuspended at high protein concentration (30–50 mg ml^{-1}) in STE (20 mM Tris–HCl pH 8.0, 0.25 M sucrose, 2 mM EDTA) containing 20 mg ml^{-1} fatty acid free bovine serum albumine (BSA) and frozen in aliquots in liquid N_2. The samples were kept at -70°C.

Isolation of mitochondria under isotonic condition was done as described [10]. Cells were lysed by intracytoplasmic cavitation with nitrogen gas in SoTE (0.6 M sorbitol, 20 mM Tris–HCl, pH 7.9 and 2 mM EDTA) at a concentration of 2×10^9 cells ml^{-1}. Two subsequent low speed spins (300 g) were performed to remove the remaining intact cells. After a DNase I digestion, the lysate was applied to 18–28% (w/v) Nycodenz gradients. The final fraction of mitochondria was resuspended at high protein concentration (30–50 mg ml^{-1}) in SoTE (20 mM Tris–HCl pH 7.5, 0.6 M sorbitol, 1 mM EDTA, 4 mM DTT) containing 20 mg ml^{-1} fatty acid free BSA and frozen as above.

2.3. Determination of purification factor and yield

Protein concentration of total cellular extract, the fractions applied to the gradient and the final isolated organelles were determined for both the hypotonic and the isotonic isolation procedure using the BCA assay (Pierce). Sample from each fraction corresponding to 10 and 20 μg of proteins, respectively, were used to isolate RNA as described. The resulting RNA was quantitatively subjected to Northern analysis, using standard procedures. The blot was then probed with the radioactively kinased oligonucleotide (5'AGGAGAGTAGGACTTGCCCT3') specifically recognizing the mitochondrial encoded 12S rRNA. The signals were quantified using a phosphorimager and purification factors and yields were calculated (Table 1).

2.4. ATP production assay

Standard ATP production assay was performed in 75 μl of assay buffer (20 mM Tris–HCl pH 7.4, 15 mM KH_2PO_4, 0.6 M sorbitol, 10 mM $MgSO_4$, 2.5 mg ml^{-1} fatty acid free BSA) using 50–100 μg of purified mitochondrial fractions. To induce

ATP production 5 mM of the indicated substrates and 67 μM of ADP was added. After incubation at 25°C the reaction was denatured by the addition of 1.75 μl of 60% perchloric acid, left on ice for 10 min and centrifuged for 5 min at 15 300 × *g*. A volume of 60 μl of the supernantant was collected and neutralized with 11.5 μl of 1 M KOH. Next, the reaction was incubated on ice for 3 min and centrifuged as before [13]. Finally, the ATP concentration was measured using ATP Bioluminescence Assay Kit CLS II (Roche, Switzerland) according to the instructions supplied by the manufacturer using 5–10 μl of the resulting supernatant.

The following substances expected to interfere with mitochondrial ATP production were tested (the solvent of the stock solution and the minimal concentrations giving maximal inhibition which were tested are indicated in parentheses): malonate (H_2O, 5 mM), antimycin (ethanol, 0.2 μM), KCN (H_2O, 1 mM), FCCP (ethanol, 1 μM), atractyloside (DMSO, 5 μg ml^{-1}) and oligomycin (ethanol, 25 μg ml^{-1}). Furthermore rotenone (DMSO) which did not inhibit ATP production was tested at 67 μM. In all the experiments with mitochondria in assay buffer were incubated for 10 min on ice in the presence of the corresponding inhibitor. ATP production was then induced by the addition of substrate and ADP.

Table 1
Comparison of mitochondrial preparations[a]

	Enrichment (fold)	Yield (%)
MIT_{hypo}		
Before gradient	0.9/1.3 ($n = 2$)	17/24 ($n = 2$)
After gradient	4.8/5.5 ($n = 2$)	9/10 ($n = 2$)
MIT_{iso}		
Before gradient	0.8–1.4 ($n = 6$)	23–34 ($n = 4$)
After gradient	1.2–3.1 ($n = 8$)	5/8 ($n = 2$)

[a] Enrichment and yield of mitochondria isolated under hypotonic (MIT_{hypo}), or isotonic conditions (MIT_{iso}) calculated as described in Section 2. Values are given for the DNase digested fractions before and after the Percoll gradient for hypotonically isolated mitochondria and before and after the Nycodenz gradient for isotonically isolated mitochondria. For hypotonically isolated organelles values from two independent experiments are given, for isotonically isolated mitochondria the range of values for the indicated number (n) of independent experiments are shown.

2.5. Mitoplasting

Conversion of isotonically isolated mitochondria to mitoplasts (mitochondria with a disrupted outer membrane) was performed as follows. A frozen aliquot of mitochondria was diluted 10-fold with H_2O incubated for 10 min on ice before isotonic conditions were restored by the addition of one third of the total volume of 2.4 M sorbitol. As a control a mock treatment was performed. The organelles were diluted 10-fold with 1 × SoTE and after 10 min on ice were supplemented with a further aliquot of 1 × SoTE corresponding to one third of the total volume. Both the samples were centrifuged at 0°C for 3 min at 5200 × *g*. The supernatant was removed and used to determine FAD-linked glycerol-3-phosphate dehydrogenase activity [7]. The resulting mitoplasts, or in the case of the mock-treated sample the mitochondria-containing pellets, were resuspended in assay buffer and used to measure ATP production or FAD-linked glycerol-3-phosphate dehydrogenase activity.

3. Results

3.1. Hypotonic and isotonic preparations

There are two common but conceptually different methods to isolate mitochondria from *T. brucei*. In the original protocols, lysis of the cells is done under hyptonic conditions by passage through a hypodermic needle [8,9]. After a DNase digestion the organellar vesicles are then isolated on Percoll gradients. New methods have employed cell lysis under isotonic conditions by nitrogen cavitation [10,11]. In one protocol the organelles are further purified after a DNase digestion using equilibrium centrifugation in Nycodenz gradients [10]. As is seen in Table 1 the hypotonic procedure yielded mitochondria which were enriched approximately 5-fold when compared with whole cells. The enrichment of the mitochondrial fraction obtained by the isotonic method, however, was only approximately 2-fold.

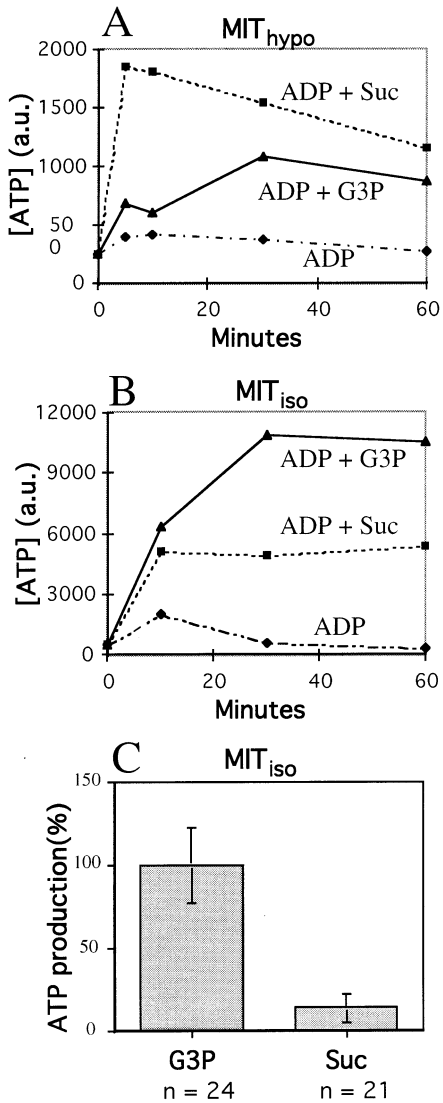


Fig. 1. ATP production in isolated mitochondria of *T. brucei*. (A) Time course of ATP production of mitochondria isolated under hypotonic conditions (MIT_{hypo}) in the presence of ADP alone, or ADP and glycerol-3-phosphate (G3P) or succinate (Suc). A.u., arbitrary units. (B) same as (A) but mitochondria isolated under isotonic condition (MIT_{iso}) were used. (C) Comparison of relative ATP production by glycerol-3-phosphate or succinate after 30 min of incubation in a large number (*n* is indicated below the bar) of independent experiments performed with different, isotonically isolated mitochondrial preparations. The mean value of ATP production in the presence of glycerol-3-phosphate was set to 100%. ATP production induced by ADP alone was substracted in each case. Standard deviation (S.D.) is indicated.

The yield in both cases was approximately 5–10%.

3.2. ATP production in isolated mitochondria

It was our aim to compare respiratory functions of the mitochondrial fractions prepared by either method. To do so ATP production was measured using a commercially available luciferase based kit. No significant level of ATP could be measured if isolated mitochondria were incubated without further supplements, indicating that the preparations are depleted of ATP. Furthermore, addition of ADP alone was not sufficient for mitochondrial ATP production indicating that endogenous substrates are used up (Fig. 1A and B). ATP production could be induced only if the reaction was supplemented with ADP in combination with a suitable respiratory substrate. Using succinate as substrate, sustained ATP production was observed for both preparations. Interestingly, when glycerol-3-phosphate was used as a substrate, efficient ATP production was observed in isotonically prepared mitochondria whereas only residual activity was obtained in the hypotonic preparation (Fig. 1A and B). In the isotonically prepared organelles glycerol-3-phosphate was by far the superior substrate yielding on average a 7-fold higher ATP production than succinate (Fig. 1 C). None of the other substrates tested (pyruvate, glucose, proline) lead to any measurable ATP production. This was also true for NADH and malate, which are expected to produce NADH inside mitochondria.

3.3. Nature of ATP production

In further experiments the nature of the measured ATP production was characterized. Should the measured ATP production indeed be due to the respiratory chain, it is expected to require a membrane potential and to be sensitive to inhibitors of the respiratory chain. Furthermore, ATP should also be produced inside the organelle by the mitochondrial ATP synthetase. Fig. 2 shows that the ATP production by succinate and glycerol-3-phosphate in both types of mitochondria requires a proton gradient across the mito-

chondrial inner membrane since it is abolished in the presence of the protonophore FCCP. The one exception is the membrane potential independent residual ATP production observed in hypotonically purified mitochondria in the presence of glycerol-3-phosphate. This production can, therefore, not be attributed to the respiratory chain. Addition of rotenone, an inhibitor of complex I (NADH- dehydrogenase) did not significantly impair ATP synthesis in either organellar preparation. This is expected as succinate and glycerol-3-phosphate, both feed their electrons directly to ubiquinon and would therefore bypass complex I. Malonate, an inhibitor of complex II (succinate dehydrogenase) showed no effect on ATP production by glycerol-3 phosphate but as expected abolishes ATP synthesis by succinate. Furthermore, mitochondrial ATP production in all cases was inhibited by antimycin which interferes with complex III (bc1-complex) and cyanide which poisons complex IV (cytochrome c oxidase) confirming that ATP is formed by the respiratory chain. In order for ATP to be synthesized inside mitochondria ADP needs to be transported across the mitochondrial inner membrane by the ADP/ATP translocator. Atractyloside blocks this carrier and prevents ADP from entering mitochondria, therefore as ADP concentration is limiting in isolated mitochondria no ATP production should be observed in the presence of atractyloside. Fig. 2 shows that this is indeed the case. However, inhibition by atractyloside is less pronounced in mitochondria isolated under hypotonic mitochondria which may indicate that some membrane damage might have occurred during hypotonic cell lysis. Finally, it was tested whether oligomycin a inhibitor of mitochondrial ATP synthetase interferes with ATP production. It has

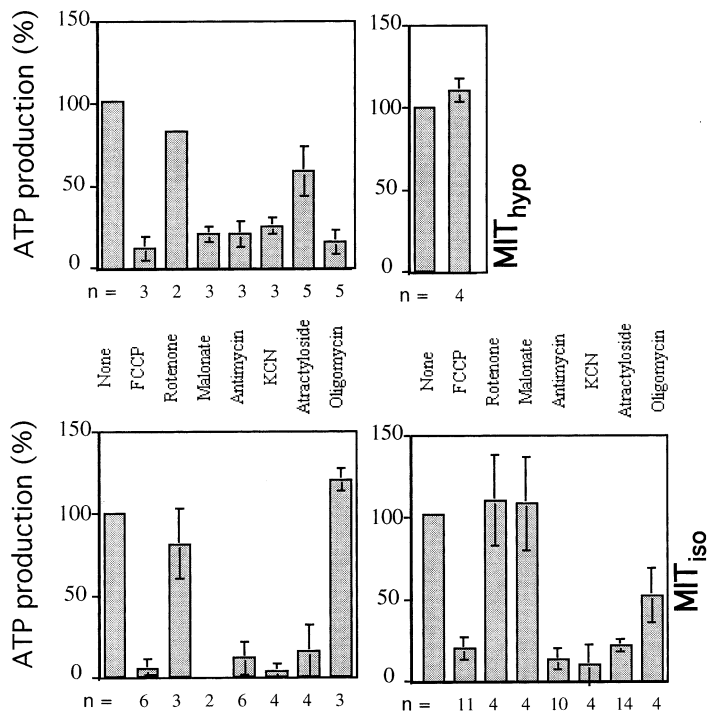
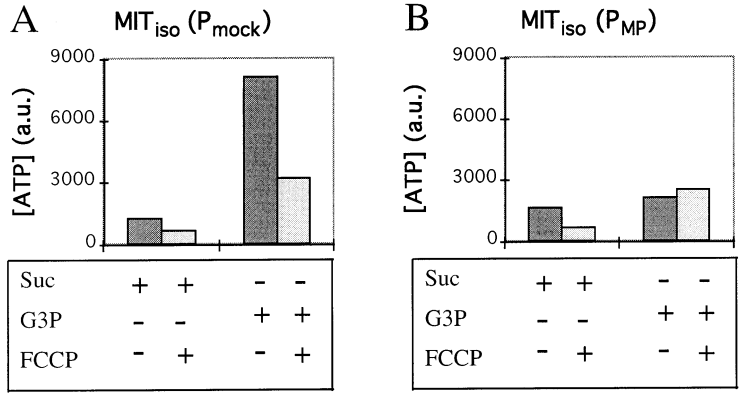


Fig. 2. Nature of ATP production in isolated mitochondria of *T. brucei*. ATP production after 30 min incubation induced by succinate (suc, left panels) or glycerol-3-phosphate (G3P, right panels) measured in hypotonically isolated mitochondria (MIT_{hypo}, top panels) or isotonically isolated mitochondria (MIT_{iso}, bottom panels). ATP production was measured in the presence of ADP and the corresponding substrate without or with the indicated additions. S.D. for the indicated number (*n*) of independent experiments is shown. For concentration of inhibitors see Section 2.



C

			MIT iso		MIT iso	
	MIT_{hypo}	MIT_{iso}	P_{MP}	SN_{IMS}	P_{mock}	SN_{mock}
FAD-linked G3P dehydrogenase activity (a.u.)	78	180	39	15	128	4

Fig. 3. Comparison of ATP production in mitochondria and mitoplasts. (A) ATP production in the presence of ADP after 30 min of incubation in isotonically isolated mitochondria (MIT_{iso}) subjected to a mock mitoplasting procedure (P_{mock}) measured as indicated in the presence of succinate (Suc), glycerol-3-phosphate (G3P) and the protonophore FCCP. (B) same as (A) but ATP production was measured in mitoplasts (P_{MP}) derived by hypotonic treatment of isotonically isolated mitochondria. (C) FAD-linked G3P dehydrogenase activity [7] in 400 μg of MIT_{hypo} and isotonically MIT_{iso} or in P_{MP} and intermembrane space fraction (SN_{IMS}) derived from 400 μg of isotonically isolated mitochondria or the corresponding mock-treated fractions (P_{mock}; SN_{mock}). For mitoplasting procedure see Section 2.

been shown that isolated ATP synthase activity of *T. brucei* can be inhibited by oligomycin to only 50% [14]. In agreement with this, we observe a partial inhibition of ATP synthesis in hypotonically and isotonically prepared mitochondria if glycerol-3-phosphate is used as substrate. However, if succinate is used in the latter preparation no inhibition is observed. Though at present we have no explanation for this observation it should be kept in mind that succinate induced ATP production is at least 7-fold less efficient than if glycerol-3-phosphate is used substrate.

3.4. Glycerol-3-phosphate dehydrogenase is localized in the intermembrane space

Hypotonically purified mitochondria are unable to utilize glycerol-3-phosphate as substrate. This could be explained if the hypotonic isolation procedure only yields mitoplasts, mitochondria with a disrupted outer membrane, and if mitochondrial glycerol-3-phosphate dehydrogenase is a soluble enzyme of the intermembrane space. In order to test this prediction we compared glycerol-3-phosphate induced ATP synthesis in isotonically isolated mitochondria and mitoplasts. Mitoplasts were prepared by exposing mitochondria to hypotonic conditions for 10 min and after addition of sorbitol to equalize osmolarity mitoplasts were recovered by centrifugation. As a mock control, mitochondria were treated identically but isotonic conditions were maintained throughout the experiment. As shown in Fig. 3A and B, mitochondria as expected, were able to produce ATP with both succinate and glycerol-3-phosphate as substrates and mitoplasts behaved identical in regard of succinate but lost the ability to utilize glycerol-3-phophate. These results were confirmed by the fact that 3.3-fold more FAD-linked glycerol-3-phosphate dehydrogenase activity was detected in isotonically prepared mitochondria than mitoplasts. Mitochondria derived from isotonically prepared organelles showed, after correction for the different purification factors, even 6-fold more

FAD-linked glycerol-3-phosphate dehydrogenase activity than organelles isolated under hypotonic conditions. Furthermore, the enzyme activity was only detected in the intermembrane space fraction but not in the corresponding isotonic supernatant of the mock control. The fact that only very little activity is recovered might be due to the low protein concentration of the intermembrane space fraction.

4. Discussion

Our results demonstrate differences between the hypotonically and isotonically isolated mitochondria. If purity is of major importance, the hypotonic preparation [8] should be chosen, as it is at least twice as pure than organelles purified by the isotonic procedure [10]. However, if an intact energy metabolism is important the isotonic isolation is far superior. Both the preparations were able to produce ATP with succinate, implying entry of the electrons into the respiratory chain at complex II (succinate dehydrogenase) which associated with the matrix side of the inner membrane. Glycerol-3-phosphate on the other hand was only able to induce ATP formation in mitochondria purified by the isotonic preparation. Furthermore, ATP production in isotonic mitochondria was 7-fold more efficient with glycerol-3-phosphate than with succinate (Fig. 1 C). This might be due to the fact that succinate needs to be imported into the matrix, possibly a rate-limiting step, before complex II can transfer its electrons to the respiratory chain. Glycerol-3-phosphate, however, does not need to be imported but transfers its electrons directly from the outside to ubiquinon. In agreement with that, glycerol-3-phosphate induced ATP synthesis was shown to be sensitive to antimycin and cyanide, inhibitors of complex III or IV, but not to malonate or rotenone, inhibitors of succinate- or NADH-dehydrogenase. Furthemore, it was possible to show that glycerol-3-phosphate dehydrogenase is released into the supernatant upon mitoplasting of isotonically isolated mitochondria and is, therefore, a soluble enzyme of the intermembrane space (Fig. 3). The enzyme is more loosely associated with the mitochondrial inner membrane than cytochrome c an essential component of the respiratory chain, which remains associated with the inner membrane even in mitoplasts.

Analysis of glycerol-3-phosphate as respiratory substrate has been complicated by the fact that the two glycerol-3-phosphate dehydrogenases exist in *T. brucei*; one linked to FAD in mitochondria and a second one linked to NAD in glycosomes [4]. Glycerol-3-phosphate oxidation has previously been observed using crude mitochondrial preparations prepared by hypotonic cell lysis [7]. Surprisingly, however, in that case glycerol-3-phosphate oxidation was sensitive to rotenone. The suggested explanation was that glycosomal glycerol-3-phosphate dehydrogenase contaminating the mitochondrial fraction produced NADH which was then oxidized by the rotenone sensitive NADH dehydrogenase in mitochondria. Our results show that analysis of the energetics of mitochondria should be done with mitochondria isolated under isotonic conditions. If hypotonic conditions or digitonin are used the outer membrane gets destroyed and at least one important component of the energy metabolism, the mitochondrial glycerol-3-phosphate dehydrogenase, is lost. In *T. brucei* mitochondrial glycerol-3-phosphate dehydrogenase has so far mainly been studied in bloodstream forms where the enzyme is directly linked to the alternative oxidase [15]. Our results suggest a previously unrecognized important role of mitochondrial glycerol-3-phosphate dehydrogenase in cyanide sensitive respiration of procyclic *T. brucei*.

No evidence of a functional rotenone sensitive NADH dehydrogenase was found. Neither NADH producing substrate malate or pyruvate nor NADH itself was able to induce ATP synthesis. Furthermore, all ATP synthesis detected in mitochondria was resistant to rotenone. Nevertheless, we did not show in the above-given experiment whether NADH was actually produced inside mitochondria so the presence of rotenone sensitive NADH dehydrogenase in *T. brucei* needs further study.

In summary our work emphasizes the need to use intact mitochondria for bioenergetics studies and provides the means to manipulate intramito-

chondrial ATP concentrations using various combinations of mitochondrial substrates and inhibitors.

Acknowledgements

We thank Elke Horn for excellent technical assistance, members of our group for helpful discussion and Timothy Tan for editing the manuscript. This study was supported by grants 31-056825.99 and 4037-55154 from the Swiss National Foundation and by a fellowship of the 'Professor Max Cloëtta'-Foundation (to A. Schneider).

References

[1] Estevez AM, Simpson L. Uridine insertion/deletion RNA editing in trypanosome mitochondria — a review. Gene 1999;240:247–60.
[2] Simpson AM, Suyama Y, Dewes H, Campbell DA, Simpson L. Kinetoplastid mitochondria contain functional tRNAsb which are encoded in nuclear DNA and also contain small minicircle and maxicircle transcripts of unknown function. Nucl Acids Res 1989;17:5427–45.
[3] Hancock K, Hajduk SL. The mitochondrial tRNAs of *Trypanosoma brucei* are nuclear encoded. J Biol Chem 1990;265:19208–15.
[4] Clayton CE, Michels P. Metabolic compartmentation in African typanosomes. Parasitol Today 1996;12:465–71.
[5] Turrens JF. The role of succinate in the respiratory chain of *Trypanosoma brucei* procyclic trypomastigotes. Biochem J 1989;259:363–8.
[6] Turrens JF, 1991. Mitochondrial metabolism of African trypanosomes, Biochem. Protozool. 145-53.
[7] Beattie DS, Howton MM. The presence of rotenone-sensitive NADH dehydrogenase in the long slender bloodstream and the procyclic forms of *Trypanosoma brucei brucei*. Eur J Biochem 1996;241:888–94.
[8] Harris ME, Moore DR, Hajduk SL. Addition of uridines to edited RNAs in trypanosome mitochondria occurs independently of transcription. J Biol Chem 1990;265:11368–76.
[9] Braly P, Simpson L, Kretzer F. Isolation of kinetoplast-mitochondrial complexes from *Leishmania tarentolae*. J Protozool 1974;21:782–90.
[10] Hauser R, Pypaert M, Häusler T, Horn EK, Schneider A. In vitro import of proteins into mitochondria of *Trypanosoma brucei* and *Leishmania tarentolae*. J Cell Sci 1996;109:517–23.
[11] Priest JW, Hajduk SL. In vitro import of the rieske iron-sulfur protein by trypanosome mitochondria. J Biol Chem 1996;271:20060–9.
[12] Hauser R, Schneider A. tRNAs are imported into mitochondria of *Trypanosoma brucei* independent of their genomic context and of their genetic origin. EMBO J 1995;14:4212–20.
[13] Glick BS, Wachter C, Reid GA, Schatz G. Import of cytochrome b2 to the mitochondrial intermembrane space: the tightly folded heme-binding domain makes import dependent upon matrix ATP. Prot Sci 1993;2:1901–17.
[14] Williams N. The mitochondrial ATP sythetase of *Trypanosoma brucei*: structure and regulation. J Bioenerg Biomembr 1994;26:173–8.
[15] Clarkson AB, Bienen EJ, Pollakis G, Grady RW. Respiration of bloodstream forms of the parasite *Trypanosoma brucei* brucei is dependent on a plant-like alternative oxidase. J Biol Chem 1989;264:17770–6.