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Short communication

Complex I (NADH:ubiquinone oxidoreductase) is active in but non-essential for procyclic *Trypanosoma brucei*

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ABSTRACT

The requirement of complex I (NADH:ubiquionone oxidoreductase) for respiration in *Trypanosoma brucei* is controversial. Recent identification of homologues of its subunits in mitochondrial proteome resolved a question of its presence or absence. However, with one exception, no data have been available concerning the function(s) of complex I or its subunits. Here we present a functional RNAi study of three (NUBM, NUKM, NUEM) putative subunits of this complex. Although no changes were detected in growth, mitochondrial membrane potential or reactive oxygen species production in cell lines depleted for target transcript, the NUBM and NUKM RNAi knock-downs showed decreased specific NADH:ubiquinone oxidoreductase activity. Moreover, glycerol gradients of all cell lines revealed the presence of two distinct peaks of NADH dehydrogenase activity, with shifted sensitivity to inhibitors of complex I upon RNAi induction. Thus complex I is not only present in the procyclic stage of *T. brucei* 29-13 strain, but it does participate in electron transport chain.

Trypanosoma brucei is a causative agent of African sleeping sickness, a disease rampant in sub-Saharan Africa, where it is estimated to infect between 50 and 70,000 people each year. The mitochondrion of the procyclic stage transmitted by tsetse flies (*Glossina* spp.) differs significantly from that of the bloodstream stage, which dwells in mammalian hosts. The latter stage is known to have a metabolically suppressed and morphologically reduced mitochondrion lacking cytochromes, while the procyclic stage contains a reticulated and metabolically active organelle (for review see [1]) with respiratory complexes III, IV and V proven to be essential [2–4].

The very presence of complex I (NADH:ubiquinone oxidoreductase) in *T. brucei*, which in some eukaryotes forms the largest protein complex in the respiratory chain known so far, is a controversial issue. The prototypic complex I removes electrons from NADH and transfers them to ubiquinone (coenzyme Q), at the same time contributing to the proton gradient by moving four protons across the membrane (for review see [5]). Its subunits are also involved in fatty acid synthesis, regulation of apoptosis and organellar protein synthesis, while others may function as redox shields and/or scaffolds (for review see [6]).

Despite this multitude of its functions, several reports suggested complex I is absent from trypanosomes [7,8] and from other kinetoplastids [9], although data claiming this complex being active in African trypanosomes were also published [10,11]. It was the indirect nature of these experiments that exposed them to criticism, however. Biochemical assays used to identify complex I activity were performed with too high concentrations of inhibitors, potentially influencing other activities [12]. Furthermore, alternative dehydrogenase (NDH2) was discovered in *T. brucei* [13]. This single-protein enzyme catalyzes the same biochemical reaction as complex I (NADH:ubiquinone oxidoreduction) and, as such, it might have masked the activity of genuine complex I in the above-mentioned studies [12]. Apart from that, not all subunits crucial for complex I activity were found in an *in silico* search [14].

However, several lines of evidence emerged recently that shift the debate once again in favor of the existence of a (sub)complex I in these flagellates. Active complex I was detected in *Phytomonas serpens* [15] and between 12 and 19 putative subunits were found *in silico* in *T. brucei* [14,16]. Moreover, a key mitochondrial-encoded subunit considered to be missing [14] was identified in the kinetoplast DNA maxicircle [17]. Due to the high divergence of *T. brucei*, numerous putative subunits of complex I may still be annotated as hypothetical proteins [18,19]. Recently, a 24-subunit oxidoreductase complex has been pulled down, containing homologues of complex I [20], which were subsequently confirmed by a high-throughput study [18]. In spite of our considerable knowledge

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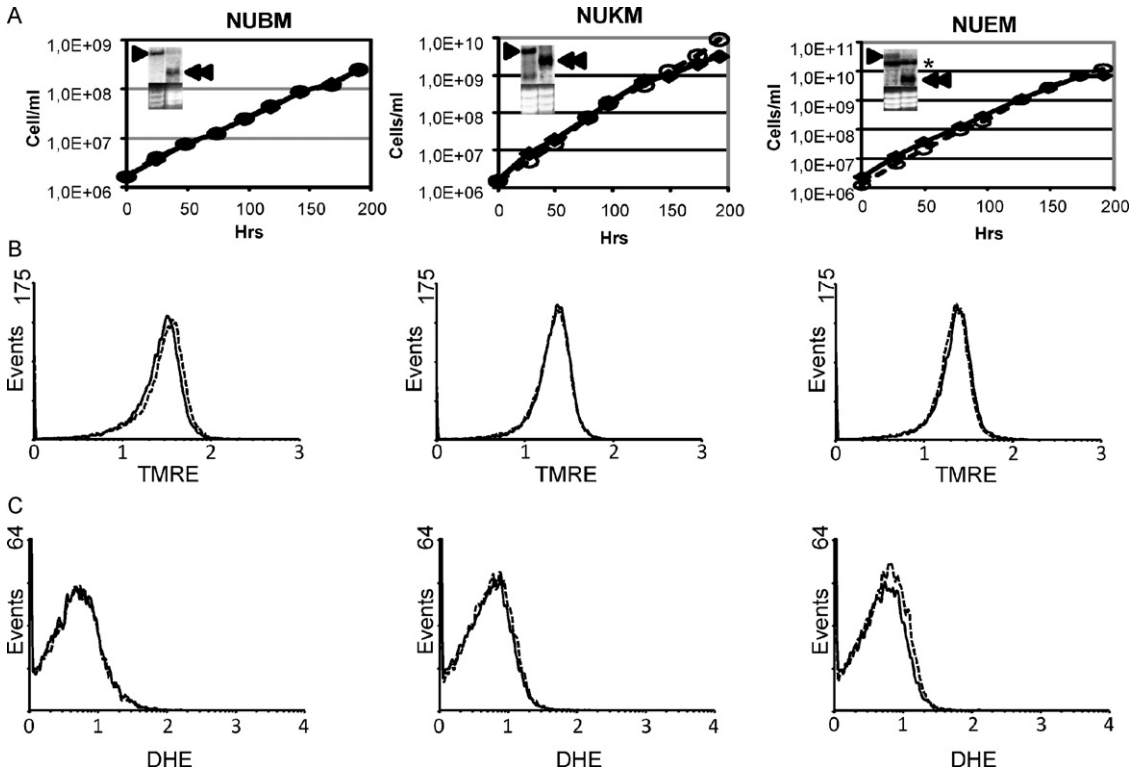

Fig. 1. Phenotypes of *T. brucei* procyclics interfered against the NUKM, NUEM and NUBM subunits of complex I. (A) Growth curves of RNAi cell lines in SDM-79. Full and dashed lines correspond to the non-induced (tet−) and RNAi-induced (tet+) cells, respectively. *x* – hours post-induction; *y* – cell concentration/ml. Insets show effect of RNAi (left lane – non-induced cells; right lane – RNAi-induced cells). The position of the targeted mRNA and the double stranded RNA are indicated with a single and double arrowhead, respectively. As a control, the gel was stained with ethidium bromide to visualize rRNA bands. The NUEM cell line contains a persistently cross-reacting non-specific band between the NUEM mRNA and double stranded RNA (asterisk). (B) Mitochondrial membrane potential and (C) ROS production were measured in the non-induced cells (full lines) and cells 6 days of RNAi-induction (dashed lines). *x* – log scale of fluorescence; *y* – number of events.

about the composition of putative complex I in *T. brucei*, virtually nothing is known about its function and activity. In fact, it was only shown that one of its subunits, acyl carrier protein, participates in fatty acid synthesis in the procyclic stage [21]. In order to shed light on this enigmatic complex, we decided to analyze the function of three of its highly conserved subunits.

Selected subunits are non-essential for growth in culture. Guided by the L-shaped crystal structure of prokaryotic complex I [5] and using the GeneDB database (http://www.genedb.org), the following three conserved subunits with homologues present in virtually all known complexes I were selected (for review see [14]): i) NUKM (Tb11.47.0017), the last subunit in the chain of Fe–S clusters in the hydrophilic arm and a homologue of the PSST subunit with a molecular mass of 23 kDa; ii) NUBM (Tb927.5.450), a homologue of a 51 kDa subunit with a predicted mass 55.3 kDa, is the electron entry point and contains both an FMN coenzyme and Fe–S cluster; iii) NUEM (Tb09.244.2620), a 42.2 kDa part of the membrane subdomain that is a homologue of the 39 kDa subunit containing NADP(H) binding domain. Next, we have PCR-amplified fragments of the target genes for their down-regulation via RNA interference (RNAi). Primers K-FW (5′-T**GGATCC**ATAGTTTGCAATGCTTCGTC-3′) and K-RV (5′-A**AAGCTT**GTATTGCTCTTTCACAGCCT-3′) amplified 503 bp-long fragment of NUKM, while primers 40-FW (5′-C**GGATCC**CCACCATCATTCGGGGCACGG-3′) and 40-RV (5′-C**AAGCTT**CGTGGGATTCCAAAGTCCTCC-3′) were used to obtain 486 bp-long piece of NUEM. Finally, primers 50-FW (5′-C**TCTAGA**GTGGGTTTTCTTTCCGC-3′) and 50-RV (5′-TC**TCTAGA**CGCACGATCTTTCCG-3′) were used to amplify whole 1481 bp-long NUBM gene (the BamHI and HindIII restriction sites are in bold for NUKM and NUEM; XbaI sites were added to NUBM primers). The PCR products were cloned via pCR2.1-TOPO® (Invitrogen) into the p2T7-177 RNAi vector. The NUBM fragment was cut-out from the plasmid using XbaI and SalI, resulting in two pieces (800 and 691 bp), of which the smaller one was cloned into p2T7-177 via XbaI and XhoI sites and used for RNAi. Several attempts to raise polyclonal antibodies against overexpressed NUBM and NUKM, as well as against synthetic oligopeptides derived from them, were unsuccessful (data not shown).

Upon electroporation and selection, clonal cell lines of the *T. brucei* 29-13 procyclics obtained as described elsewhere [21] were tested for the efficiency of RNAi by Northern analysis using the plasmid inserts as probes (hybridization at 55 °C overnight followed by washes with 2 × SSC + 0.1% SDS at RT and 0.2 × SSC + 0.1% SDS at 55 °C). Growth of the cells, diluted 10× upon reaching concentration of 1×10^7 cells/ml, was measured for 8 days. Despite efficient ablation of the targeted transcripts, none of the induced cell lines showed a growth phenotype (Fig. 1A insets). To check whether the lack of phenotype is due to diminished oxidative phosphorylation as was shown for respiratory complex II [4], we have cultured the NUKM and NUEM cells in SDM-80 supplemented with standard amount of glucose and in the same medium lacking glucose [4]. Since the growth remained unaffected (Fig. 2), following experiments were performed under standard cultivation conditions. Lack of growth effect of the *T. brucei* procyclics depleted for the nuclear-encoded subunits of complex I is in accordance with the data obtained from *Trypanosoma cruzi*, the viability of which was unaltered by the deletion of the kinetoplast DNA region carrying the mitochondrial-encoded subunits of the same complex [22]. These results resonate with other data presented in this paper that complex I only participates in NAD^+ regeneration.

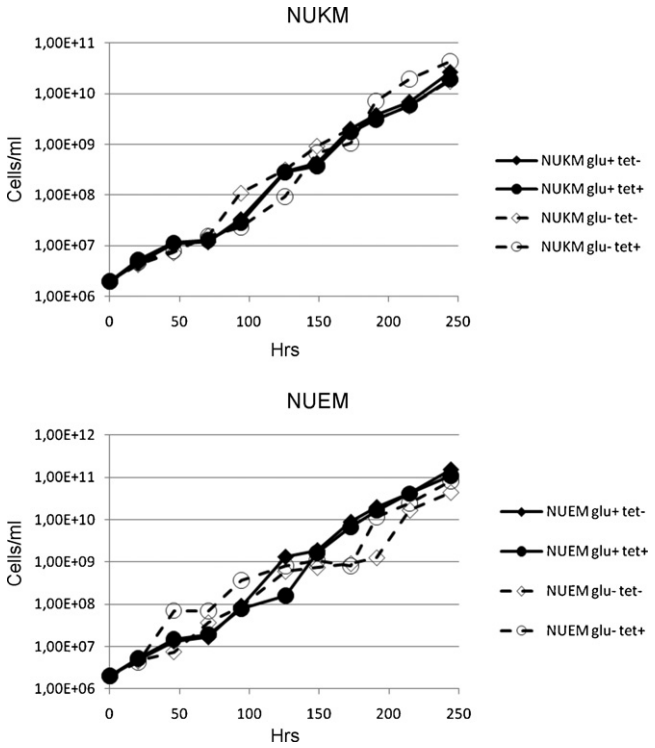

Fig. 2. Effect of RNAi on the NUKM and NUEM cells grown in SDM-80 medium either supplemented with or lacking glucose. Full and dashed lines correspond to the cells grown in glucose-containing (glu+) and glucose-lacking SDM-80 (glu−), respectively. Diamonds and circles represent non-induced (tet−) and RNAi-induced (tet+) cells, respectively. Full and empty symbols correspond to presence and absence, respectively, of glucose in the medium. *x* – hours post-induction; *y* – cell concentration/ml.

Specific NADH:ubiquinone oxidoreductase activity is decreased. Next, we have measured the specific NADH dehydrogenase activity in the parental cell line and subsequently in the RNAi-induced cells. For biochemical assays, mitochondria from 5×10^8 cells were isolated and lysed as described elsewhere [2]. The protein content was established by Bradford and 5 μl of the cleared lysate was added to the NDH solution (100 mM KPi, pH 7.5; 1 mM EDTA, pH 8.5; 0.2 mM KCN; 20 μM coenzyme Q2). The reaction was started by the addition of NADH to a final concentration 100 μM and monitored for 5 min at 340 nm. Piericidin A and rotenone, specific inhibitors of complex I from higher eukaryotes, and diphenylen iodonium (DPI), an inhibitor of the flavin enzymes, were added to final concentrations of 5, 10 and 100 μM, respectively. Results of five to ten independent measurements of the parental strain in the presence or absence of the drugs are summarized in Table 1. Contrary to other reports [10,23], rotenone had only a very weak effect (~12% inhibition) on the NADH dehydrogenase activity in the parental cells, while piericidine A had no effect at all. This observation is surprising when compared with the data obtained for the related trypanosomatid, *P. serpens*, where complex I is sensitive to both inhibitors with almost the same specific activity [15]. Since we did not test higher concentrations of piericidine A than the ones used successfully for *P. serpens*, we cannot exclude the possibility that in *T. brucei* the NADH dehydrogenase activity is piericidine A-sensitive, yet in a different concentration range. Nevertheless, RNAi cell lines did not show any significant differences in their sensitivity to rotenone (data not shown).

Table 1
NADH:ubiquinone oxidoreductase activities of parental and RNAi cell lines. Average activity and standard deviations of several independent inductions [$n = 5$–10] is shown. 1U corresponds to oxidation of 1 nmol of NADH per min. Residual activity represents activity upon inhibition by 5, 10 and 100 μM Piericidine A, rotenone and DPI respectively. Activity of the RNA-induced cells is shown in % with the non-induced cells representing 100%.

Parental strain activity (U/mg)	38 ± 09
	Residualactivityuponinhibition(%)
Piericidine A	105 ± 17
Rotenone	88 ± 08
DPI	20 ± 12
	Residualactivitiesofinducedcelllines(%)
NUKM	77 ± 7
NUEM	102 ± 3
NUBM	77 ± 11

In cells ablated for the NUBM and NUKM proteins, a 20% decrease in specific NADH dehydrogenase activity occurred, confirming participation of these complex I subunits in this activity. Although the down-regulation of the NUEM subunit had no effect on the NADH dehydrogenase activity measured in crude mitochondrial lysate (Table 1), it caused a significant decrease of rotenone sensitivity of the glycerol gradient-purified NADH:Q2 oxidoreductase activity (see below and Supplementary Tables 1 and 2). Next, we followed the NADH dehydrogenase activity with two other artificial acceptors, ferricyanide and dichlorophenolindophenol, which are capable of receiving electrons with energy lower than those accepted by ubiquinone. That way we tested the possibility that the studied proteins participate in a pathway not connected with respiratory chain, utilizing electron acceptors with redox potential other than coenzyme Q2, such as NADH:ferredoxin oxidoreductase. Overall, the measured activities were an order of magnitude higher than that of coenzyme Q2 (data not shown), a result expected due to the participation of more enzymes in NADH dehydrogenation. Nevertheless, none of the RNAi knock-downs showed a reproducible decrease of this activity (data not shown). This lack of effect of RNAi strongly suggests that the down-regulated activity represents a small fraction of the total NADH dehydrogenase(s).

The sensitivity to DPI of both 29-13 parentals and RNAi cells suggests that a flavin-containing enzyme may be responsible for NADH dehydrogenation upon the use of coenzyme Q2 as an acceptor. Type-II NADH dehydrogenase (NDH2) [13] is an imminent candidate for this function, and its characterization is currently under way in our laboratory. Although the activity of complex I in the procyclic stage of *T. brucei* is similar to that measured for *P. serpens* [15], both activities are more than 100× lower than the NADH dehydrogenase activity in the yeast *Yarrowia lipolytica* [24]. However, it cannot be excluded that such a low activity is just caused by lower enzymatic affinity towards the artificial acceptor coenzyme Q2 used in the activity experiments. At present, we can only speculate that the rather limited decrease of the NADH dehydrogenase activity in the RNAi-interfered cells might be caused by either the genuinely low activity of complex I or by a possible up-regulation of NDH2 as a result of decreased activity of this complex.

Characterization of the RNAi cells using glycerol gradients showed two NADH:Q2 activity peaks. The mitochondrial lysate of procyclic *T. brucei* contains at least two NADH:Q2 oxidoreductase activities. To partially purify them, mitochondria from 5×10^8 cells were lysed using dodecylmaltoside (final concentration 2%) in a mitochondrial lysis buffer (10 mM Tris, pH 7.2; 100 mM KCl; 10 mM $MgCl_2$; 1 mM DTT) including Mini-protease inhibitor cocktail tablets (Roche) in a total volume of 1 ml for 1 h on ice. Cleared lysate was loaded onto a 10–30% glycerol gradient containing the same buffer and spun at 38,000 rpm in SW40Ti rotor (Beckman) for 12 h at 4 °C. 500 μl fractions were collected from the top and, immediately, in each fraction the NADH:Q2 oxidoreductase activity was measured using Tecan Infinite® M200 reader. 20 μl of the fraction and 180 μl of the buffer containing 100 mM KPi, pH 7.5 and 140 μM NADH, and 20 μM Q2 were loaded into each well and change of absorbance at 340 nm was monitored for 5–7 min using 60 cycles. For inhibitions, the same buffer was supplemented with rotenone

Table 2
Distribution of NADH:Q2 oxidoreductase activity and rotenone- and DPI-sensitivity in glycerol gradient fractions of the NUKM knock-downs. For activity lines, + denotes 5–10 U activity, ++ 10–15 U and +++ >15 U. U was calculated as (ΔOD (340 nm) per min per mg of protein) × 100. For inhibitory lines, + denotes inhibition 1–30%, ++ 30–70%, +++ >70% as compared with the activity without inhibitor. tet− represents the non-induced cells; tet+ represents the RNA-induced cells.

Fractionnr.	1–10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
NUKM															
Activity (tet−)		+	+	+	+	+	+		+	+	+++				
Activity (tet+)				+	+	++	+	++		+		++		++	
Rotenone (tet−)				+		+++	++		++	+++	++				
Rotenone (tet+)					+					++		++			
DPI (tet−)		+++					+				+				
DPI (tet+)				+++	+++	++	+++	++		++		+++		+++	

or DPI in the same concentrations as described above. Next, protein content in each fraction was established using Bradford assay. Moreover, the NADH:nitrotetrazolium blue (NTB) oxidoreductase activity was checked using 1 mg of NTB per ml of reaction buffer as an acceptor instead of Q2.

Protein distribution showed a peak in the top third of the gradient (Supplementary Table 1). Interestingly, the NADH:NTB oxidoreductase activity, known in *P. serpens* to represent the rotenone-sensitive complex I [15], does not correspond to the NADH:Q2 oxidoreductase activity in *T. brucei*. It localizes to the upper third of the gradient, an area corresponding to one of the peaks of dihydrolipoyl dehydrogenase [20], whose activity can be visualized under the same conditions as complex I [25]. Separation on a native 2–15% BN-PAGE gel and staining for the NTB activity revealed in these fractions a high molecular weight complex [2], previously considered to be a homologue of the *P. serpens* complex I [15].

Results from a representative NADH:Q2 oxidoreductase experiment are shown in Table 2, with all data available in Supplementary Tables 1 and 2. In the non-induced cells the dehydrogenase activity constitutes two peaks in the lower two thirds of the gradient. Glycerol gradients from all three RNAi-induced knock-downs shared approximately the same protein and activity profiles and revealed a parallel decrease and increase of rotenone- and DPI-sensitive NADH dehydrogenase activity, respectively (Table 2). Thus, it appears that silencing of the complex I activity leads to an up-regulation of flavin-containing dehydrogenase(s), with NDH2 being the likely candidate.

An antibody previously shown to recognize on a dot-blot a native epitope of an oxidoreductase [20], which was further characterized to bind to an epitope of α + λ subcomplex of complex I (A. Zíková, pers. commun.) positively reacted with all fractions containing the NADH:Q2 oxidoreductase activity (data not shown). This observation strongly indicates that complex I or its parts are present in both activity peaks. We cannot conclude whether this represents two or even more forms of the incomplete complex I or a described stand-alone oxidoreductase complex [20]. Slight increase of the DPI-sensitive activity in all three knock-downs suggests a co-localization of complex I with alternative NADH:Q2 oxidoreductase(s) (Table 2 and Supplementary Table 2). Its distribution in a region far from the main protein peak could signify an interaction between complex I and its alternative enzymes in *T. brucei*.

Membrane potential and ROS production are not affected. In bacteria as well as in eukaryotes, complex I participates in the generation of membrane potential. To test whether this is also the case in trypanosomes, the non-induced and RNAi-induced cells were incubated for 20 min in SDM-79 medium to which tetramethyl rhodamide ethyl ester perchlorate (TMRE), a cationic dye sequestered to cellular compartments according to their negative charge, was added to a final concentration 250 nM. Subsequently, 1 ml of the treated cells was diluted by 4 ml of an isotonic solution suitable for flow cytometry and immediately analyzed by Beckman Coulter Epics-XL flow cytometer as described previously [2] (Fig. 1B). We did not notice any change in membrane potential, which corresponds with the lack of growth phenotype. This suggests that complex I is probably not involved in proton transfer in mitochondrial respiratory chain of procyclic *T. brucei*. This result agrees with the prediction of Opperdoes and Michels that although individual subunits may be present, the complex itself is likely not participating in proton translocation [14]. However, we cannot exclude the highly unlikely possibility that changes in the activity or abundance of complexes downstream from complex I would compensate for the loss of the first proton pump.

Next, the studied RNAi knock-downs were incubated with 5 μg/ml of dihydroethidium, using the same protocol as described for TMRE. This dye enters cell freely and stains DNA upon reaction with reactive oxygen species (ROS), which can be quantified by flow cytometry. This is a logical experiment, as complex I is well known to be a site of ROS production [24]. While no change was observed in cells with down-regulated NUBM and NUKM, a slight but reproducible increase in ROS production occurred upon the ablation of the NUEM subunit (Fig. 1C). However, since neither growth nor membrane potential, nor NADH dehydrogenase activity of this cell line are affected, this increase might rather be caused by changes in other metabolic processes than that of complex I. No detectable effect of ablation of the tested complex I subunits on ROS production agrees with the low decrease in the NADH:ubiquinone oxidoreductase activity. However, if the finding that the overall low superoxide formation rate represents only 0.2% of the NADH oxidation rate in isolated membranes of *Y. lipolytica* [24] is applied to our results in *T. brucei*, any change in ROS caused by complex I would be below the detection threshold of used methods.

Concluding remarks

Taken together, our data show that complex I does not play any major role in energy metabolism of the *T. brucei* procyclics, a situation similar to that observed in *T. cruzi* [22]. It is worth mentioning here, however, that both parasites contain in their nuclear genomes Ind1, a chaperone responsible for the incorporation of Fe–S clusters into complex I [26]. This suggests that this complex is indeed indispensable at least in some part of the life cycle of these flagellates. Due to several sources of electrons feeding the respiratory chain, *T. brucei* is probably able to sustain sufficient mitochondrial membrane potential via complexes III and IV, the ablation of which leads to a dramatic shift in membrane potential and subsequent cell death [2]. The NADH dehydrogenase activity of complex I can be bypassed via NDH2, with enough protons being translocated downstream of ubiquinone by the two above-mentioned complexes. This situation dramatically differs from that in *P. serpens*, a parasite of plants lacking complexes III and IV [27], and thus relying on complex I to generate the proton gradient used for ATP synthesis and protein import. Furthermore, our data are in agreement with the non-essentiality of complex II under glucose-rich conditions [4]. These findings suggest that other enzymes, such as NDH2

and glycerol-3-phosphate dehydrogenase, may be major sources of electrons for the respiratory chain. We speculate that with glucose as an energy source, the mitochondrion uses the respiratory chain in a way reminiscent of the bloodstream stage: as an electron sink for glycerol-3-phosphate dehydrogenase, the enzyme connecting glycolysis and the mitochondrion [7]. Essentiality of complexes III and IV for the procyclic stage [2] can be explained as a result of the overall low activity of complex I mirrored in its lack of participation on mitochondrial membrane potential by proton translocation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2010.11.003.

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