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Downregulation of the nuclear-encoded subunits of the complexes III and IV disrupts their respective complexes but not complex I in procyclic *Trypanosoma brucei*

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Summary

The function, stability and mutual interactions of selected nuclear-encoded subunits of respiratory complexes III and IV were studied in the *Trypanosoma brucei* procyclics using RNA interference (RNAi). The growth rates and oxygen consumption of clonal cell lines of knock-downs for apocytochrome c_1 (apoc_1) and the Rieske Fe-S protein (Rieske) of complex III, and cytochrome *c* oxidase subunit 6 (cox6) of complex IV were markedly decreased after RNAi induction. Western analysis of mitochondrial lysates using specific antibodies confirmed complete elimination of the targeted proteins 4–6 days after induction. The Rieske protein was reduced in the apoc_1 knock-down and vice versa, indicating a mutual interdependence of these components of complex III. However, another subunit of complex IV remained at the wild-type level in the cox6 knock-down. As revealed by two-dimensional blue native/SDS-PAGE electrophoresis, silencing of a single subunit resulted in the disruption of the respective complex, while the other complex remained unaffected. Membrane potential was reproducibly decreased in the knock-downs and the activities of complex III and/or IV, but not complex I, were drastically reduced, as measured by activity assays and histochemical staining. Using specific inhibitors, we have shown that in procyclics with depleted subunits of the respiratory complexes the flow of electrons was partially re-directed to the alternative oxidase. The apparent absence in *T. brucei* procyclics of a supercomplex composed of complexes I and III may represent an ancestral state of the respiratory chain.

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Introduction

Trypanosomes and related parasitic flagellates belong to the most unusual eukaryotes. Most transcripts of their mitochondrial DNA undergo extensive editing of the uridine insertion/deletion type (Stuart *et al*., 2005). With the exception of one ribosomal protein, all identified mitochondrial protein-coding genes of *Trypanosoma brucei* encode subunits of respiratory complexes (Alfonzo *et al*., 1997). During life cycle that involves a vertebrate host and a tse-tse fly vector, the mitochondrion of *T. brucei* undergoes dramatic changes. The bloodstream stage of vertebrate hosts possesses a functionally downregulated mitochondrion that lacks cyanide-sensitive oxygen uptake (Bienen *et al*., 1993) and sustains its membrane potential by ATPase (F_0F_1-ATP synthase) (Nolan and Voorheis, 1992) which also allows Ca^{2+} transport. The mitochondrion contains trypanosome alternative oxidase (TAO), an enzyme related to alternative oxidases of plants and fungi (Clarkson *et al*., 1989; Chaudhuri *et al*., 1998) that is in the bloodform linked via the ubiquinone/ubiquinol pool with glycerol-3-phosphate dehydrogenase (Clarkson *et al*., 1989; Hannaert *et al*., 2003).

The procyclic stage of the insect host is characterized by the presence of a metabolically fully active mitochondrion, equipped with branched electron-transport chain that uses oxygen as final electron acceptor (Njogu *et al*., 1980). The inner membrane of the procyclics contains both TAO and a classical cytochrome-mediated respiratory chain, which is composed of four complexes, ubiquinone/ubiquinol pool and cytochrome *c* (Opperdoes, 1985). As the procyclic stage possesses cytochrome *c* oxidase, the metabolic function of the repressed TAO may rest in protecting the mitochondrion from reactive oxygen production (Fang and Beattie, 2003), one of the functions earlier attributed to TAO-like oxidase in plant mitochondria (Maxwell *et al*., 1999).

Complex I (NADH dehydrogenase) is the only respiratory complex the presence of which in procyclic *T. brucei* remains to be unambiguously proven. The effect of rela-

tively high concentrations of rotenone, an inhibitor of complex I, on procyclics *in vivo* was interpreted either as a proof of specific inhibition of this complex (Beattie and Howton, 1996; Tielens and Van Hellemond, 1998; Fang *et al*., 2001) or as a non-specific blockade of other activities (Hernandez and Turrens, 1998; Christmas and Turrens, 2000). Although a functional complex I remains to be purified and characterized, biochemical evidence provided recently (Gonzáles-Halphen and Maslov, 2004) and the presence of putative homologues of mitochondrial and nuclear-encoded subunits in the trypanosomatid genomes support its existence in procyclics.

Complex II (succinate dehydrogenase) is the only electron-transport complex exclusively encoded by the nuclear genome (Lemire and Oyedotun, 2002). In the model trypanosomatids *Crithidia fasciculata* and *Leishmania tarentolae*, complex III (cytochrome *c* reductase) is composed of 10 and 11 subunits, respectively, thus achieving a level of complexity standard for this complex (Priest and Hajduk, 1992; Horváth *et al*., 2000a). Mitochondrial-encoded apocytochrome *b*, and nuclear-encoded apocytochrome c_1 (apoc_1) and Rieske Fe-S protein (Rieske) are similar to homologues in other eukaryotes. The specific activity of complex III of *L. tarentolae* is well in the range of its activities in other eukaryotes (Horváth *et al*., 2000a). Complex IV (cytochrome *c* oxidase) contains 10–11 nuclear-encoded and three mitochondrial-encoded subunits in the above-mentioned trypanosomatids (Speijer *et al*., 1996a; Horváth *et al*., 2000b). Only one nuclear-encoded subunit (subunit 8) has a similarity with a protein outside the Kinetoplastida, namely with mammalian subunit IV and *Neurospora crassa* subunit V (Speijer *et al*., 1996b). The largest imported subunit (trCOIV) has no homology with known genes (Maslov *et al*., 2002). Despite the apparently unique protein composition, trypanosomatid complexes III and IV feature standard spectral and enzymatic characteristics.

With the exception of complex I, kinetoplastid respiratory complexes have been to some extent characterized in respect to their structure and enzymatic activities. However, very little is known about the stability and turnover of these complexes, their mutual interactions or the function of individual subunits. In bacteria, yeast, plants and mammals, the imported subunits have a role in regulation, stabilization, insulation or assembly (Scheffler, 1999). Knockouts for most subunits fail to assemble their respective complex, and usually also influence the activity and/or stability of the other complexes (Acin-Perez *et al*., 2004; Schägger *et al*., 2004; Stroh *et al*., 2004; Dudkina *et al*., 2005).

Here we present an analysis of *T. brucei* phenotypes following the downregulation of nuclear-encoded subunits of complex III (apoc_1 and Rieske) and a subunit of complex IV (cytochrome *c* oxidase subunit 6; cox6) by RNA interference (RNAi). We show that all three subunits are not only essential for a proper function of their respective complexes, but are also indispensable for their assembly/stability. In addition, in the absence of complex III or IV, complex I remains fully active with electron flow being partially redirected to TAO.

Results

RNA interference causes selective depletion of target proteins

RNA interference cell lines have been prepared to knock-down nuclear-encoded subunits of respiratory complexes, in particular apoc_1 and Rieske of the cytochrome *c* reductase (complex III), and subunit 6 of the cytochrome *c* oxidase (complex IV). Upon induction with tetracycline (tet+) which triggered degradation of the target mRNA, proliferation of all three representative clonal cell lines was markedly inhibited. Growth slowed down after 4 days, whereas the growth of tet– cells remained unaffected, as compared with the parental cells (Fig. 1). In all knock-downs, RNAi induction resulted in a massive transcription of double-stranded RNA (dsRNA) (Fig. 1; insets). The Rieske, apoc_1 and cox6 mRNAs were virtually completely degraded 24 h after the addition of tet, and remained absent throughout the experiment (Figs 1 and 3).

Depletion of target proteins was verified by Western analysis using specific antibodies (Fig. 2). All proteins interfered against were very stable and remained present for several generations after the depletion of respective transcripts (data not shown). It took about 6 days of RNAi induction for the Rieske, apoc_1 and cox6 proteins to become almost undetectable in their respective knock-downs (Fig. 2).

Cox6 is a bona fide subunit of complex IV

Because of the high degree of similarity of the *T. brucei* Rieske and apoc_1 genes and those of other eukaryotes (Priest and Hajduk, 1992; 1995; data not shown), both proteins are likely components of complex III. However, despite extensive analysis of its stage-specific regulation (Matthews and Gull, 1998; Timms *et al*., 2002), cox6 was never rigorously proven to be a *bona fide* subunit of complex IV in *T. brucei*. Therefore, we raised polyclonal antibodies against the purified cox6 polypeptide (Fig. 4A), which in the *T. brucei* lysates specifically recognizes a single band of 20 kDa (Fig. 2). No polypeptide of expected size was recognized in *Phytomonas serpens* (Fig. 2), which is in agreement with the postulated absence of complexes III and IV in this flagellate (Nawathean and Maslov, 2000). Immunoreactivity in two-dimensional (2D) blue native/Tricine-SDS-PAGE gels of the anti-cox6 antis-

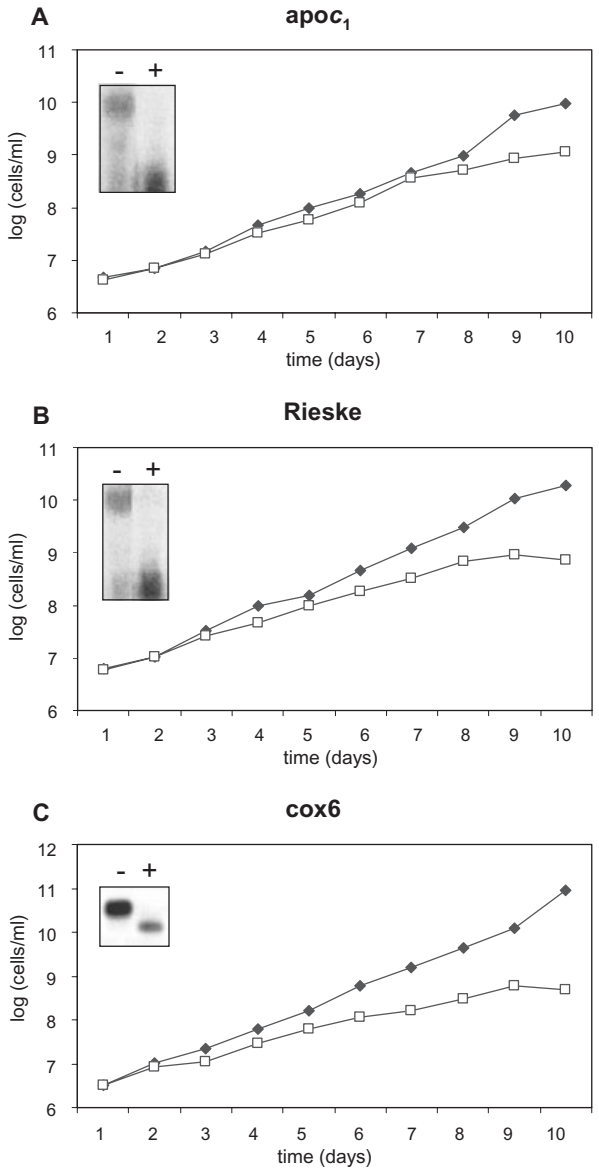

Fig. 1. Effect of apoc_1, Rieske and cox6 RNAi on cell growth. Growth inhibition following induction of apoc_1 dsRNA (A), Rieske protein dsRNA (B) and cox6 dsRNA (C). Cell numbers were measured using Beckman Z2 Coulter®. The numbers of tet– (diamonds) and tet+ cells (squares) are indicated. The *y*-axis is labelled by a log scale and represents the product of measured cell densities and the total dilution. Insets show Northern analysis of mRNA levels from non-induced (–) cells and those expressing dsRNA 48 h after induction (+). The cox6, Rieske and apoc_1 mRNAs were 0.75 kb, 2.35 kb and 2.0 kb long respectively.

era with a protein that is a component of cytochrome *c* oxidase confirmed the identity of this subunit (Fig. 4B).

Complex III subunits are mutually dependent

To test whether the elimination of one subunit influenced stability of the other subunits, Northern blots of the non-induced and induced cells were screened with probes against all three downregulated subunits. Only targeted mRNA was eliminated in each knock-down, while the level of mRNAs for the other analysed subunits remained unaffected (Fig. 3). However, anticipated ablation of the apoc_1 protein in respective RNAi cells was accompanied by the loss of the Rieske protein. A similar situation was observed in Rieske-silenced cells, in which a decrease of 'the other' complex III subunit occurred (Fig. 2). As only the targeted mRNA is eliminated, the decrease of the non-targeted component must be caused by destabilization of the protein. Interestingly, when the cox6 knock-down was assayed for the presence of another subunit of complex IV, namely subunit trCOIV (Maslov *et al*., 2002), we did not see the same phenomenon as with complex III subunits, i.e. the mutual dependence of individual subunits. In the absence of the cox6 protein, trCOIV remained at the wild-type level. Both on the RNA and on the protein levels, cox6 is unaffected in knock-downs for complex III subunits and vice versa (Figs 2 and 3).

Mitochondria were purified following a protocol previously described for *L. tarentolae* (Horváth *et al*., 2000b), which has been adjusted for the lower number of the *T. brucei* procyclics. In protein complexes solubilized from pelleted mitochondria with mild detergents and separated by 2D blue native/Tricine-SDS-PAGE electrophoresis, respiratory complexes III and IV were identified on the basis of previous work (Speijer *et al*., 1996a,b; Horváth *et al*., 2000b; Maslov *et al*., 2002). Using this gel system, we have further analysed the composition of respiratory complexes in the knock-downs. While mitochondria of tet– cells contained both complex III and complex IV, interference against the apoc_1 and Rieske mRNA caused the disappearance of complex III at day 4, leaving complex IV intact (Fig. 5A and B). Similarly, the elimination of cox6 protein was sufficient to cause the disruption of complex IV. In the cox6 RNAi knock-downs, complex III retained its wild-type composition as expected (Fig. 5C).

Along with the respiratory subunits, we have also followed the dynamics of the alternative oxidase. While the levels of TAO varied in individual experiments to a larger extent than the levels of other analysed proteins, a clear pattern emerged. TAO was increased even before RNAi induction and, when compared with the parental strain, the amount of protein reproducibly more than doubled in all induced knock-downs (Fig. 2). This increase was accompanied by the appearance of a high-molecular-weight band (Fig. 2), which is thought to be unproperly solubilized dimeric form (Chaudhuri *et al*., 2005).

Cytochrome c *oxidase and reductase activities are altered*

The disappearance of either complex III or complex IV in the Rieske, apoc_1 and cox6 knock-downs visualized in 2D

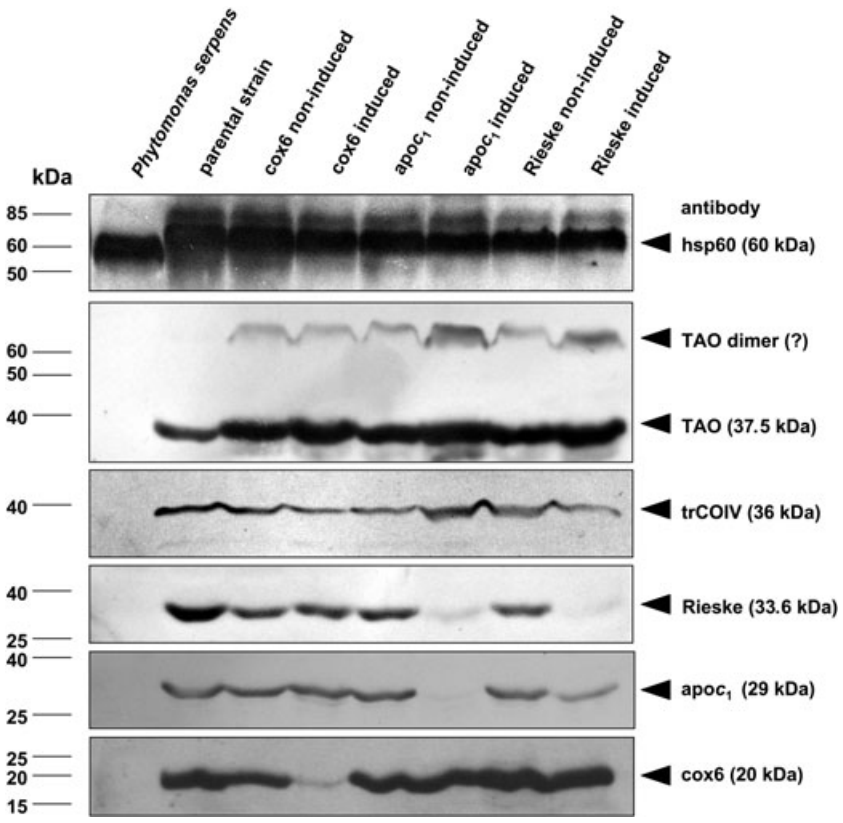

Fig. 2. Effects of apoc$_1$, Rieske and cox6 RNAi on protein levels. Protein levels of hsp60, TAO, trCOIV, Rieske, apoc$_1$ and cox6 (listed according to the declining molecular weight) were analysed by Western blot analysis in extracts from *P. serpens*, *T. brucei* parental strain and non-induced and induced cell lines. Each lane was loaded with protein from ~10^7 cells and blots were immunostained using various specific polyclonal antisera as described in *Experimental procedures*. α-hsp60 was used as a loading control.

gels (Fig. 5) was confirmed by the measurement of their specific activities. We have followed the cytochrome *c* reductase and oxidase activities of purified mitochondrial lysates incubated in the reductase/oxidase buffer with the change of absorbance being measured at 550 nm during 60/600 s intervals (see *Experimental procedures*). The activities in different mitochondrial lysates of the *T. brucei* parental cells fluctuate between 400–1100 mU mg^{-1} and 0.8–3.9 mU mg^{-1} for cytochrome *c* reductase and oxidase respectively. The levels obtained for apoc$_1$ and Rieske RNAi cells 4 days after induction showed a drastic decrease of cytochrome *c* reductase activity (Table 1). The same activity, however, was either not affected or slightly increased in the induced cox6 knock-downs when compared with the tet– cells, which clearly showed that the absence of even an essential subunit of complex IV had no influence on complex III (Table 1). Eventually, the reductase activity decreased in these cells (data not shown), which can be attributed to the secondary effects of cytochrome *c* oxidase deficiency.

As shown in Table 1, cytochrome *c* oxidase activity follows a more complex pattern in the analysed cell lines. As expected, in the cox6 RNAi cells, the oxidase activity became undetectable already after 4 days of induction, and remained so for a prolonged period of time (data not shown). Unexpectedly, in the apoc$_1$ and Rieske RNAi cells, in most experiments the oxidase activity dropped in the non-induced cells, whereas in the induced cells it always rebounced close to the wild-type level.

In an attempt to confirm this result by a different approach, we have introduced to the field of trypanosome research an assay used previously in human cells (Jung *et al.*, 2000). Direct histochemical detection of the cytochrome *c* oxidase activity in the gel after blue native electrophoresis fully confirmed the activity measurements in solution. There was no detectable signal in the induced cox6 kinetoplast lysates, and varying decrease of this activity was observed in the non-induced apoc$_1$ and Rieske RNAi cells (Fig. 6). In perfect agreement with the measurements in solution, upon RNAi induction of both cell lines, the oxidase activity was restored to a level comparable with the parental strain (Fig. 6).

Complex I is not impaired in the knock-downs

In bacteria and human cultured cells mutations in the complex III subunit caused the decrease of activity and/or stability of complex I. To investigate possible association between these complexes in *T. brucei*, we have measured the activity of complex I in all three knock-downs. In different mitochondrial lysates of the parental strain the activities ranged from 180 to 420 μU mg^{-1}. The NADH dehydrogenase activity obtained for apoc$_1$, Rieske and cox6 RNAi cells 6 days after induction showed no

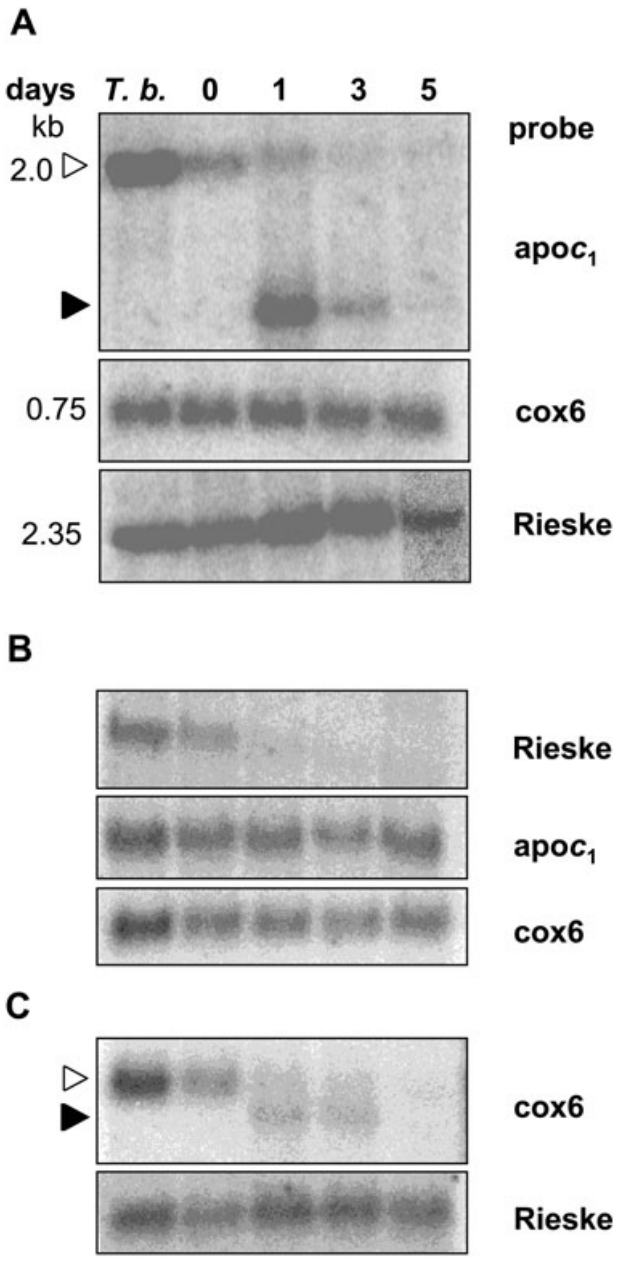

Fig. 3. Effects of apoc_1, Rieske and cox6 RNAi on mRNA levels.
A. Parental strain (*T. b.*), apoc_1, cox6 and Rieske mRNA levels were analysed by Northern blot analysis in extracts from the *T. brucei* parental strain, non-induced cells and cells isolated 1, 3 and 5 days after apoc_1 RNAi induction. The positions of the targeted mRNA and the dsRNA synthesized following induction are indicated with open and closed arrowhead respectively.
B. Rieske, apoc_1 and cox6 mRNA levels were analysed by Northern blot analysis in extracts from the *T. brucei* parental strain, non-induced cells and in extracts isolated 1, 3 and 5 days after Rieske RNAi induction.
C. Cox6 and Rieske mRNA levels were analysed by Northern blot analysis in extracts from the *T. brucei* parental strain, non-induced cells and in extracts isolated 1, 3 and 5 days after cox6 RNAi induction.

decrease (Table 1). Results obtained by spectrophotometry have been compared with histochemical detection of NADH dehydrogenase activity in the blue native gel. We were able to detect equally strong specific signal in the non-induced and induced cells (Fig. 6).

Dependence on terminal oxidases shifts and membrane potential decreases

As the *T. brucei* procyclics rely partially on oxidative phosphorylation for their ATP production (Coustou *et al*., 2003; Besteiro *et al*., 2005), we wondered about the effect incurred on oxygen uptake in these knock-downs. Oxygen consumption was monitored in both non-induced cells and cells after 6 days of induction. The application of selective inhibitors of two modes of oxygen consumption enabled us to distinguish between the electron flow through the terminal oxidases, namely complex IV and TAO. Parental cells showed an oxygen uptake rate of $(3.57 \pm 1.48) \times 10^{-10}$ μmol cell^{-1} min^{-1}.

After the addition of KCN, which selectively inhibits the activity of complex IV, oxygen uptake decreased in the non-induced cells by about half (44–55% in different knock-downs). Subsequent inhibition of TAO by the addition of SHAM left the non-induced cells with only a residual consumption of oxygen that ranged from 14% to 18% of the wild-type consumption (Fig. 7A). In contrast, the addition of KCN to the tet+ cells showed less dramatic decrease of oxygen uptake. In all three interfered cell lines, KCN decreased oxygen consumption by about 13–18% only, further validating the anticipated increased dependence of the Rieske-, apoc_1- and cox6-depleted cells on the KCN-insensitive TAO (Fig. 7A). Additional support for a shift in electron flow from respiratory chain complexes to TAO in the procyclics came from experiments in which cells were treated with specific inhibitors in a switched order. An early addition of SHAM indeed caused significantly stronger decrease of oxygen consumption in the induced cells compared with the non-induced (Fig. 7B). Subsequent challenge of the induced cultures with KCN had a less severe effect than the same treatment incurred on the non-induced cells (Fig. 7B). In all induced cell lines, the percentage of SHAM-sensitive oxygen consumption markedly increased (Fig. 7C).

As mitochondrial membrane potential depends on the proper function of respiratory complexes and is indispensable for the import of mitochondrial proteins, we expected its impairment in the interfered cells. Previously, rhodamine 123 was evaluated as a membrane potential-specific dye suitable for the measurements in *T. brucei* procyclics (Divo *et al*., 1993). The initial observation by light microscopy of decreased uptake of mitotracker red (Molecular Probes) by the tet+ cells (data not shown) was followed by the measurement of the import into the

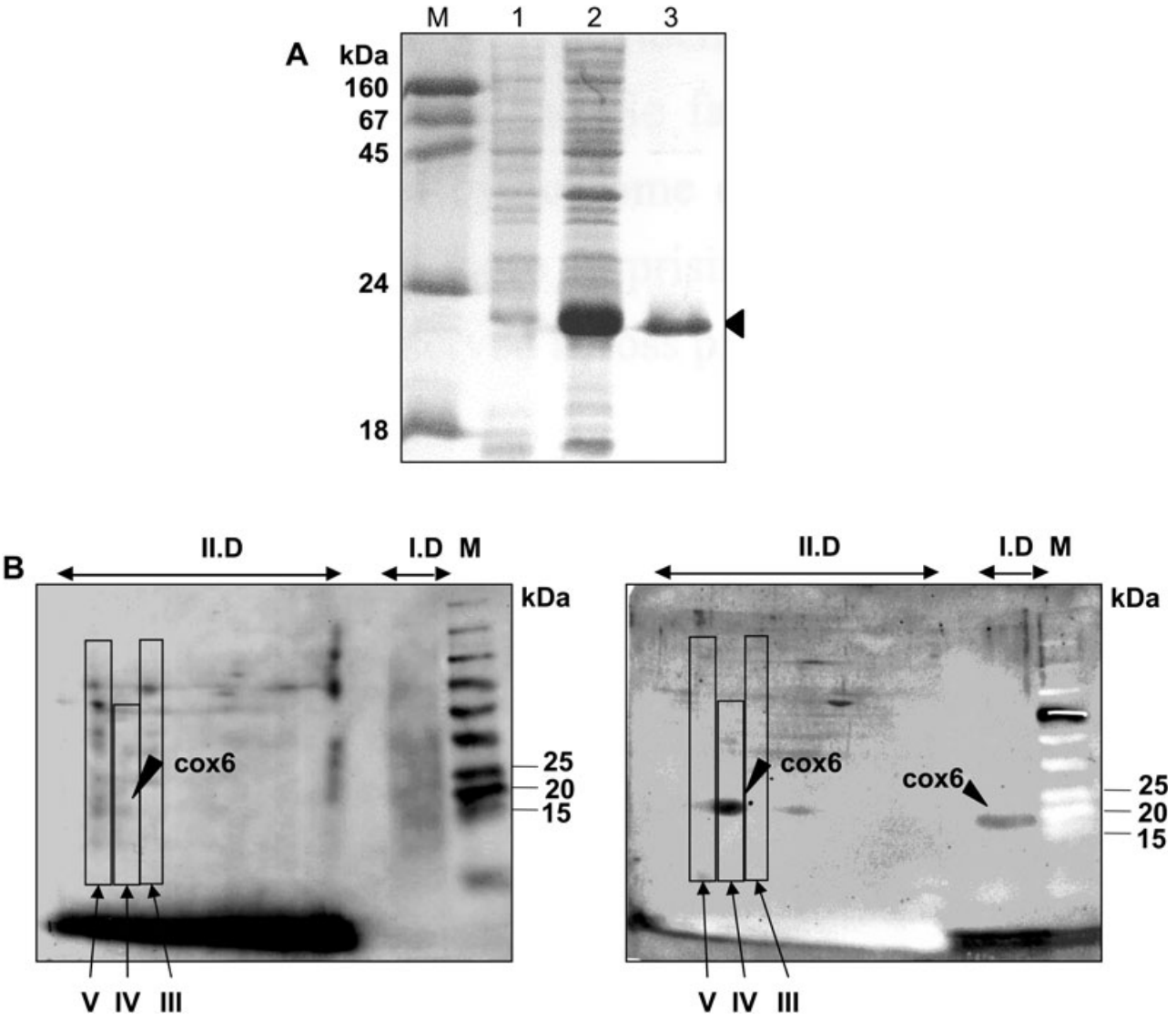

Fig. 4. Expression of cox6 protein in *E. coli* and its association with complex IV.
A. Expression of cox6 protein in *E. coli*. The polypeptides in the non-induced bacterial lysate (lane 1), the induced lysate (lane 2) and purified recombinant protein (lane 3) are shown. Lane M represents the marker (ICN); band sizes are given on the left.
B. Preferential association of cox6 with complex IV. Respiratory complexes of the parental strain were resolved in one-dimensional (I.D) and two-dimensional (II.D) 6% blue native (3% stacking)/10% Tricine SDS-PAGE gels (left). Parallel gels were blotted and probed with the anti-cox6 antiserum (right). Localization of the cox6 protein within complex IV is indicated in first and second dimension by arrowheads. Positions of the respiratory complexes are indicated under the panels. Lane M represents the marker (ICN) for first dimension; band sizes are given on the right.

organelle of tetramethylrhodamine ethyl ester (TMRE), a compound related to rhodamine 123. Indeed, RNAi directed against any of the studied subunits of the respiratory complexes caused a decrease of membrane potential, quantified by the uptake of TMRE using flow cytometry (Fig. 8). A shift of the peak and a wider distribution of obtained values for the measured cells testify to the impairment of membrane potential maintenance.

Discussion

We have used the RNAi technology to gain insight into the function of the respiratory complexes in procyclic *T. brucei*. Thus far, several studies have shown that this stage generates ATP via standard oxidative phosphorylation, with complex IV transfering electrons to oxygen (Tielens and Van Hellemond, 1998). This source of ATP, however, does not seem to be essential (Bochud-Allemann and Schneider, 2002; Hannaert *et al*., 2003; Van Weelden *et al*., 2003), especially in the presence of glucose (Besteiro *et al*., 2005; Lamour *et al*., 2005). In line with this view, the elimination of key subunits of respiratory complexes reported here proved not to be lethal in the glucose-containing SDM-79 medium, although the growth

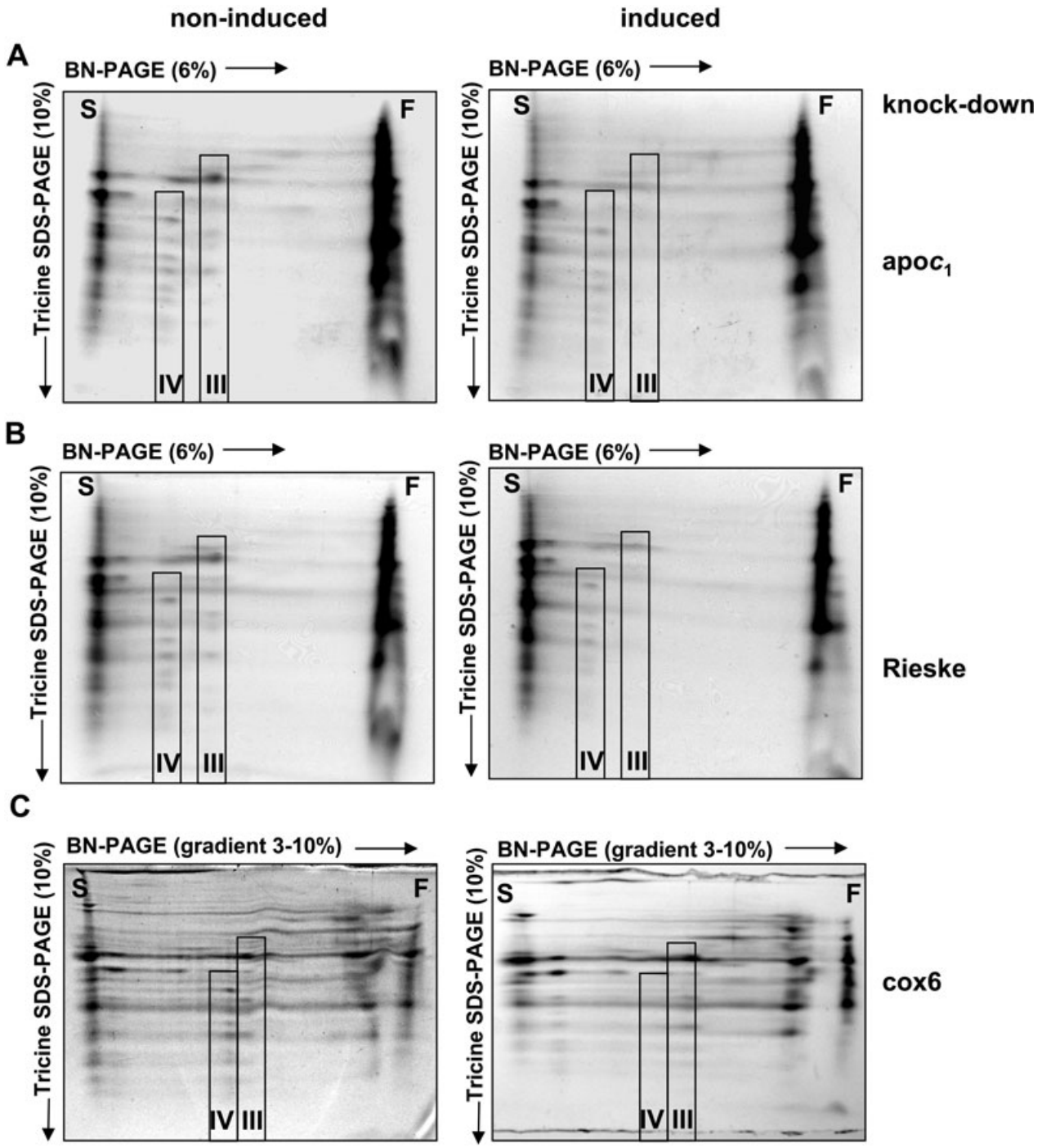

Fig. 5. Two-dimensional (2D) gel analysis of mitochondrial membrane proteins of apoc_1, Rieske and cox6 RNAi cells. The first dimension was performed in 6% (apoc_1 and Rieske) or 3–10% gradient (cox6) blue native (BN) gels. The second dimension was performed in 10% Tricine SDS-PAGE gels and the gel was stained with Coomassie blue. Positions of the respiratory complexes III and IV are indicated in the gel. The start (S) and the migration front (F) on the first dimension are indicated in the gel. Mitochondrial lysates from the non-induced apoc_1 (A), Rieske (B) and cox6 (C) knock-downs (left) and cells 4 days after RNAi induction (right) are shown.

rate of the knock-downs was markedly reduced and eventually the cells ceased dividing. Western analysis of cells depleted for one of these subunits with a panel of antibodies against other subunits revealed that the absence of one complex III subunit leads to the degradation of the interacting subunit. The apoc_1 knock-down is in fact a double knock-down for apoc_1 and Rieske, and in parallel to the downregulation of Rieske, apoc_1 is ablated. As the

Table 1. Functional assays for complex I, complex III and complex IV activities.

RNAi strain	Induction	NADH dehydrogenase activity (μU mg^{-1})	Cytochrome *c* reductase activity (mU mg^{-1})	Cytochrome *c* oxidase activity (mU mg^{-1})
Parental		217 ± 34	928 ± 169	2.85 ± 1.55
apoc_1	Non-induced	312 ± 33	572 ± 190	0.45 ± 0.25
	Induced	344 ± 80	147 ± 17	1 ± 0.29
Rieske	Non-induced	285 ± 42	596 ± 228	0.3 ± 0.092
	Induced	290 ± 85	112 ± 29	1.1 ± 0.37
cox6	non-induced	285 ± 45	959 ± 211	0.57 ± 0.14
	Induced	312 ± 45	736 ± 205	0

All activities were measured in mitochondrial lysates prepared from at least three independent RNAi induction experiments as described in *Experimental procedures*. One unit (U) of activity catalyses the reduction/oxidation of 1 μmol of cytochrome *c* per min (complexes III and IV) and oxidation of 1 nmol of NADH per min (complex I). Specific activity is calculated as U per mg of mitochondrial proteins. The calculations have been performed according to Horváth *et al.* (2000a). Medium values and SD are shown.

transcripts of the non-interfered genes are present, the other subunit must be degraded in the absence of the targeted protein. Therefore, apoc_1, Rieske and cox6 seem to be essential for the assembly of their respective complexes in procyclics. Furthermore, subunits that cannot be incorporated into the *de novo* assembled complex III are quickly degraded. We can only speculate about the peculiar stability of the trCOIV protein in the cox6 knock-downs. This subunit may be stored in a manner different from that of apoc_1 and Rieske. Alternatively, as a component of complex IV without a structural function, disassembly of the complex may not be a signal for the degradation of trCOIV.

Given the importance of apoc_1 and Rieske for the function of complex III in other eukaryotes (Scheffler, 1999; Schneider *et al*., 2004), their essentiality for the assembly of the complex is not surprising. Cox6, shown here for the first time to be a *bona fide* subunit of *T. brucei* complex IV, is also critical for the existence of this complex. However, it is only a small nuclear-encoded subunit that has no similarity to the cytochrome *c* oxidase subunits of other eukaryotes. With the available data, however, we cannot rule out the less likely explanation that the import into the mitochondrion of components of a non-functional complex is blocked.

In the absence of complex III, complex IV is not only properly assembled, but remains functional and the same applies vice versa. This result indicates a lack of cross-talk between both complexes which are functionally interconnected and indispensable for the cytochrome-mediated electron transfer to oxygen. The lack of dependence between the interfered complexes and complex I is even more surprising. In bacteria, mouse and human cells, mutations in the subunits of complex III exert a profound influence on the stability and/or activity of complex I (Acin-Perez *et al*., 2004; Schägger *et al*., 2004; Stroh *et al*., 2004). As this is not the case in procyclic *T. brucei*, we propose that the apparent absence of a supercomplex in this model protist represents an ancestral state. We have for the first time directly measured the activity of complex I without the interfering activity of alternative NADH dehydrogenase. Our data thus provide additional compelling evidence for the existence of complex I in studied trypanosomatids. Its rather small size (~650 kDa) seems to correlate with the paucity of identifiable homologues of the nuclear-encoded subunits of complex I in the completed *T. brucei* genome (our unpublished results) and may reflect its possible ancestral state.

Unlike the bloodstream stage that respires solely via the SHAM-sensitive TAO, the insect stage contains both a cyanide-sensitive cytochrome-bearing electron-transport chain and TAO (Opperdoes, 1985; Schneider, 2001). Moreover, as dyskinetoplastic mutants cannot be obtained for procyclics (Schnaufer *et al*., 2002), the respiratory chain seems to be essential for this stage (Bochud-Allemann and Schneider, 2002). Further support for such an assumption came from experiments in which prolonged incubation with KCN and SHAM killed the procyclics (Coustou *et al*., 2003; Van Weelden *et al*., 2003). As TAO is 100 times less abundant in this stage than in the bloodform (Chaudhuri *et al*., 1998), and the stage-specific upregulation of Rieske and apoc_1 is just the opposite (Priest and Hajduk, 1994), procyclics were generally thought to rely on complex IV as their terminal oxidase (Schneider, 2001; Hannaert *et al*., 2003).

So far, the flexibility of procyclics in regard to the way electrons flow in their mitochondrion was tested only using selective inhibitors of individual complexes, which may have side-effects on the cell. The data presented here show that these cells can survive with a non-functional cytochrome pathway by switching to TAO. The decreasing amount of the newly assembled complexes III and IV, as a consequence of subunits eliminated via RNAi, enables such a gradual switch. In trypanosomatids, it has to be a particularly gradual process, as experiments with *in organello* translation revealed that the mitochondrion contains an ample supply of nuclear-encoded subunits (Horváth *et al*., 2002). Recently, engineered overexpression of TAO in procyclics triggered a phenotype similar to that described in this study, i.e. the rate of respiration and the

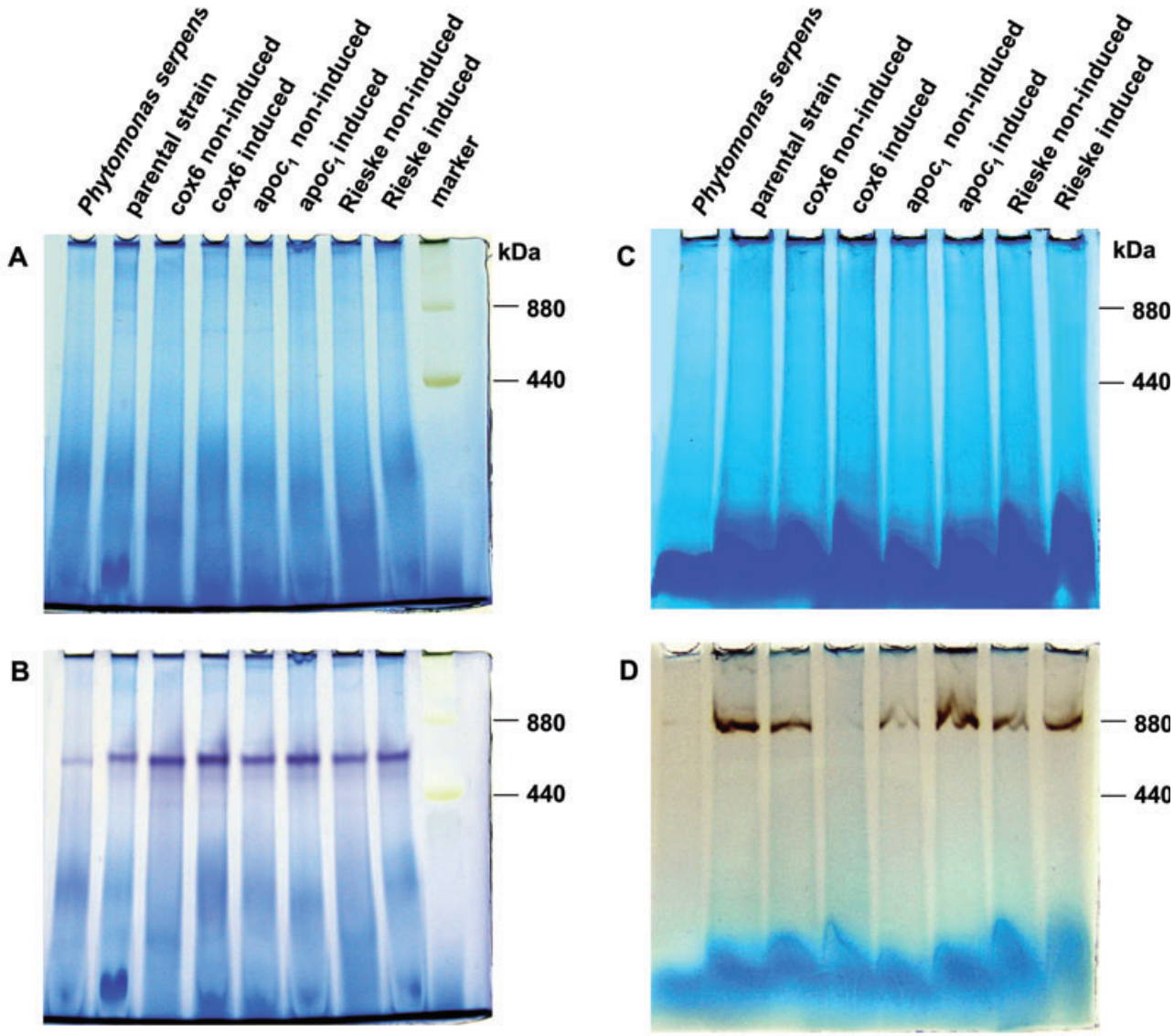

Fig. 6. Histochemical staining of complexes I and IV.
A and C. Blue native gels photographed immediately after the run.
B. Same gel as in (A) after histochemical staining for the NADH dehydrogenase activity.
D. Same gel as in (C) after histochemical staining for cytochrome *c* oxidase activity.
Electrophoresis was performed in 4–15% blue native gradient gel. Mitochondrial lysates from *P. serpens* and *T. brucei* parental strain, non-induced cells and cells after 6 days of induction are shown. The position of molecular weight markers is indicated (Ferritin, Sigma).

SHAM-sensitive respiratory pathway capacity was increased at the expense of the cytochrome-mediated pathway (Walker *et al*., 2005).

As a computer model predicted considerable overcapacity of TAO in *T. brucei* (Helfert *et al*., 2001), we propose that already at its wild-type level, TAO has a capacity to handle an increasing number of electrons redirected from the downregulated cytochrome-mediated electron-transport chain. Eventually the higher performance and hence turnover of TAO enforces its increased import. Due to its sensitivity to SHAM (Fernández-Becerra *et al*., 1997), *P. serpens* is expected to contain TAO. We therefore explain the lack of signal obtained with the anti-TAO antisera in the *Phytomonas* lysate as a reflection of the evolutionary distance between the homologues rather than the absence of TAO in these flagellates.

Functional analysis provides convincing evidence that complexes III and IV are not essential for T. brucei procyclics. Hence, it is presumably complex I and/or ATPase that are indispensable, as the failure to edit and subsequently translate mRNAs of mitochondrial-encoded respiratory subunits is imminently lethal for the procyclics

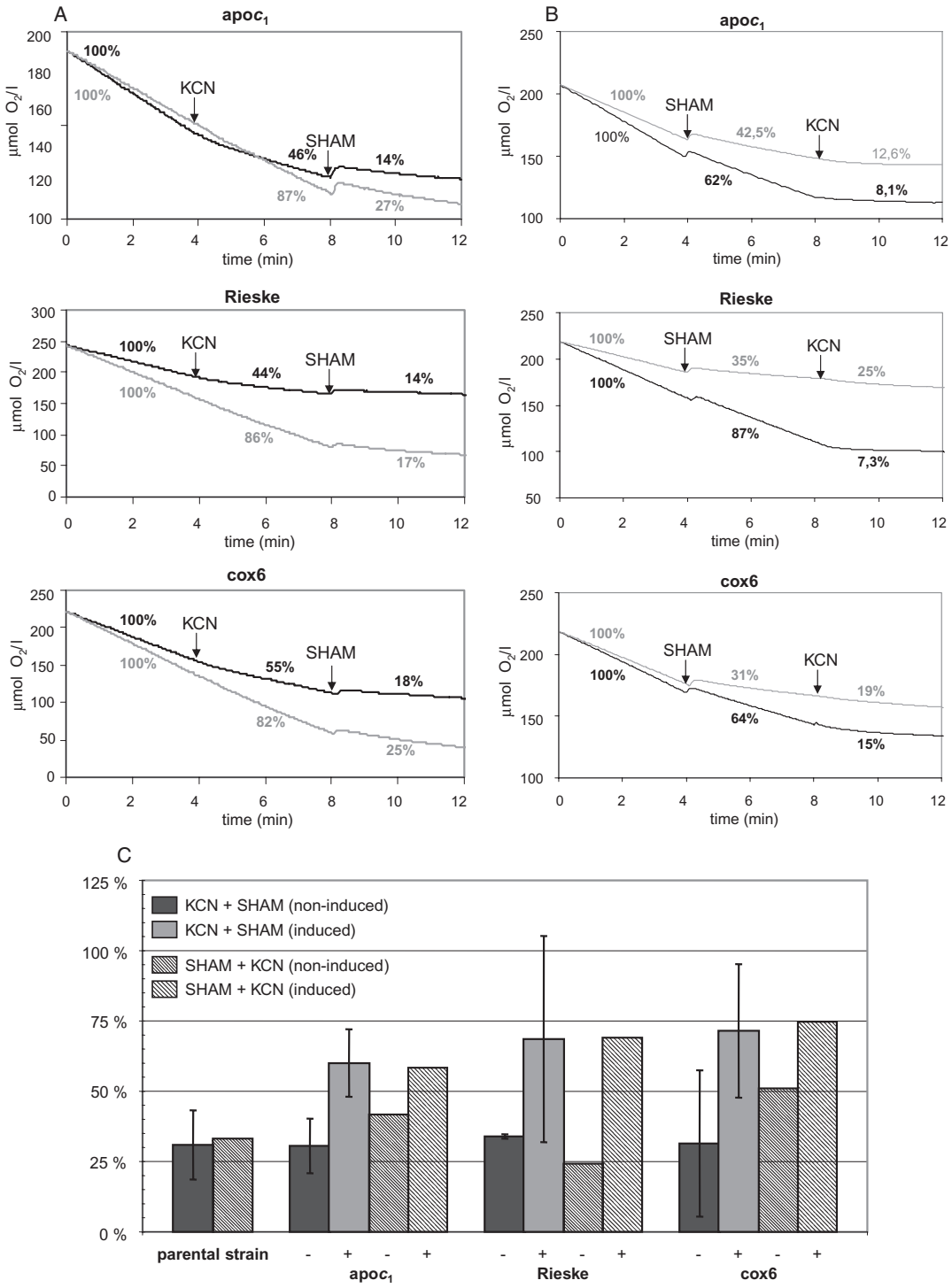

Fig. 7. Effect of apoc_1, Rieske and cox6 RNAi on respiration.
A and B. Non-induced cells (black line) and apoc_1, Rieske and cox6 cells 6 days after RNAi induction (grey line) were incubated in SDM-79 medium at 27°C and oxygen consumption was monitored with an oxygen electrode. KCN was added at 0.1 mM concentration before (A) or after (B) the addition of SHAM (at 0.03 mM). The tracings of a representative experiment show the decrease of oxygen concentration as it is consumed by the procyclics. Numbers next to the lines indicate percentage of (residual) oxygen consumption with respiration of non-induced and induced cells in the absence of both drugs set at 100%.
C. Percentage of SHAM-sensitive respiration following subsequent additions of KCN and SHAM (solid bars) and SHAM and KCN in switched order (hatched bars) to the parental strain, non-induced (–) and induced (+) knock-down cell lines (apoc_1, Rieske and cox6). In each sample, oxygen consumption in the presence of both drugs (see A and B) was subtracted from oxygen consumption of the same untreated cells and the difference was taken as a reference value for percentage calculations. The mean and the SD values of four experiments are shown.

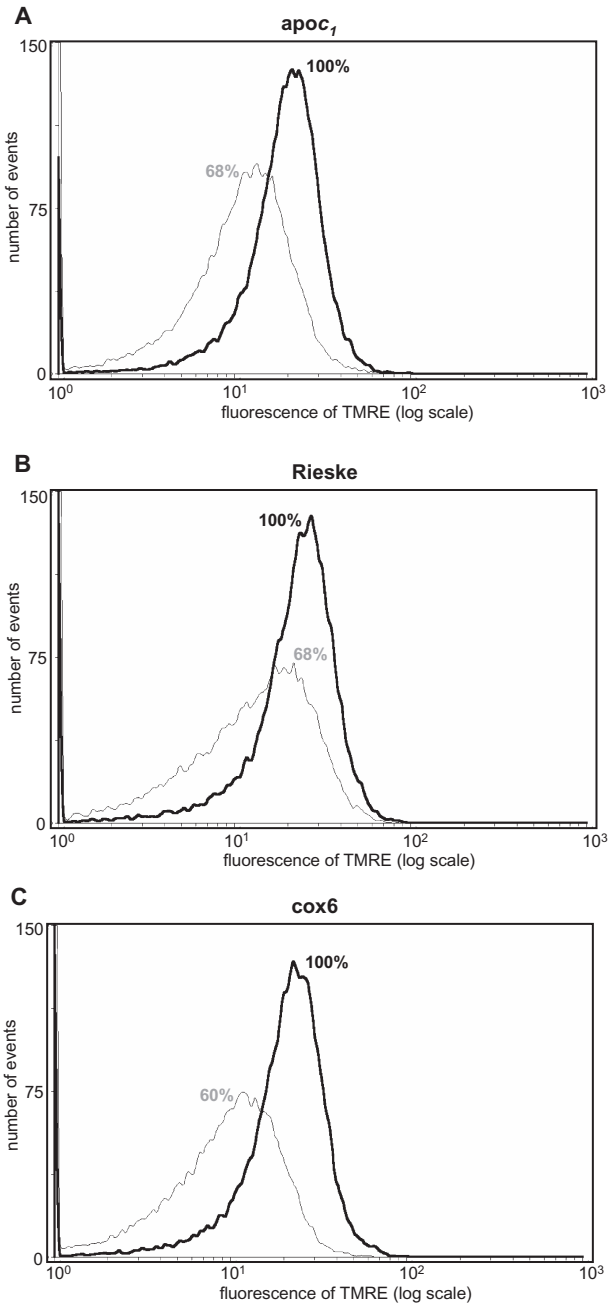

Fig. 8. Effect of apoc_1, Rieske and cox6 RNAi on mitochondrial membrane potential. Mitochondrial membrane potential was measured in non-induced cells (thick black line) and apoc_1, Rieske and cox6 cells 6 days of RNAi induction (thin grey line) that were, after incubation with 0.4% TMRE, analysed using flow cytometry. The fluorescence distribution was plotted as frequency histogram. (A) apoc_1, (B) Rieske and (C) cox6 knock-downs.

(Aphasizhev et al., 2002; O'Hearn et al., 2003; Wang et al., 2003; Neboháčová et al., 2004).

In the apoc1, Rieske and cox6 knock-downs, complex I and residual complexes III and IV may be able to sustain mitochondrial membrane potential at a level sufficient for protein import. Alternatively, one can envisage that in these modified procyclics, ATPase generates the transmembrane proton gradient from ATP hydrolysis, a function normally confined to the bloodstream stages (Nolan and Voorheis, 1992). Although oxidative phosphorylation may not be a major source of ATP for the insect forms (Coustou et al., 2003; Besteiro et al., 2005), its absence has dramatic effects. Cells with collapsed complex III or IV grow poorly, and have decreased membrane potential and low oxygen consumption. Interestingly, the elimination of terminal uridylyl transferase in procyclics also caused a decrease in oxygen uptake similar to that occurring in our cell lines, and resulted in the disappearance of respiratory complexes III and IV (Neboháčová et al., 2004). While novel for protozoans, upregulation of TAO as a consequence of a non-functional cytochrome pathway is not unprecedented, as it has previously been described in plants and fungi (Dufour et al., 2000; Funes et al., 2004). The ability to survive in the presence of potent inhibitors of cytochrome complexes, such as cyanides, nitric oxide and sulphide (McDonald and Vanlerberghe, 2004), may be a novel adaptation of T. brucei and represents another facet of an enormous flexibility of these ancient parasites.

Experimental procedures

Construction of vectors, transfection, cloning, RNAi induction and growth

A 496-nucleotide (nt)-long insert spanning 13 nt of the 5′ untranslated region, the entire cox6 gene and 11 nt of the 3′ untranslated region, was polymerase chain reaction (PCR)-amplified using oligonucleotides cox6-F1 (5′-CTCGAGC GAGAAAGTAATC**ATG**CC-3′) and cox6-R1 (5′-AAGCTTC ACTAA**CTA**CTCATCATATAC-3′) (added XhoI and HindIII restriction sites are underlined; start and stop codons are in bold). The amplified fragment was cloned into pCR2.1 TOPO^R (Invitrogene) and subcloned into pZJM (Wang *et al*., 2000), resulting in the construct for the cox6 knock-down. The 5′ part of the Rieske gene spanning nt 11–457 (the A of the AUG initiation codon being no. 1) was PCR-amplified using oligonucleotides Rieske-F (5′-ACATATGCCGCAGGTCCTGCATA TCAGC-3′) and Rieske-R (5′-CAAGCTTCACGGCATACCG CACCATAAG-3′) (added HindIII and NdeI restriction sites are underlined), cloned into pGEM-T^R (Promega) and subcloned via the same restriction sites into pZJM, creating the Rieske RNAi construct. Finally, the 5′ half of the apoc_1 gene spanning nt 3–444 was obtained by PCR amplification with oligonucleotides apoc_1-F (5′-ACTCGAGGCAGGTAAGAAAGCT CACCCC-3′) and apoc_1-R (5′-ATCTAGACCATCAACTCAC CGTTGCCCC-3′) (added XbaI and XhoI restriction sites are underlined). The amplicon was cloned into pGEM-T^R, and subcloned via the same restriction sites into pZJM, resulting in the apoc_1 RNAi construct.

Cultivation, electroporation and cloning of the procyclic *T. brucei* parental (29-13) strain were performed as described elsewhere (Vondrušková *et al*., 2005). Synthesis of dsRNA was induced by the addition of 1 μg ml^{-1} tet. Growth curves

of parental cells and clonal cell lines were obtained using the Beckman Z2 Coulter® over a period of 11 days after the induction of RNAi.

Northern blot analysis

Total RNA was isolated using Trizol (Sigma) according to the manufacturer. Ten micrograms of RNA per lane were loaded on a 1% formaldehyde agarose gel and transferred to a membrane overnight by capillary transfer. After UV cross-linking, the membranes were pre-hybridized in NaPi solution (0.5 M Na_2HPO_4 and NaH_2PO_4, pH 7.2; 7% SDS; 1 mM EDTA) at 55°C. Hybridization with probes labelled by random priming (HexaLabel™ kit, MBI Fermentas) with [α^{32}-P]-dATP (ICN) was carried out overnight in the same solution at 55°C. A wash in 2× SSC + 0.1% SDS at room temperature for 15 min was followed by a wash in 0.2× SSC + 0.1% SDS for 20 min at 55°C. The radioactive signal was quantified using the phosphoimager Storm 860 (MD).

Expression of cox6, preparation of antibodies and Western blot analysis

The entire coding region of cox6 gene (477 nt) was PCR-amplified using oligonucleotides cox6-F2 (5′-CACCTC GAGCCCTTTGTCGATCACAAT-3′) and cox6-R2 (5′-CCCAAGCTTCACTAACTACTCATCATATAC-3′) (added XhoI and HindIII restriction sites are underlined), cloned into pRSET-A (Invitrogen), equipped with an N-terminal His-tag and transfected into *Escherichia coli* BL21(DE3)pLys cells (Novagen). Overexpression, purification and antibody preparation followed a protocol described elsewhere (Maslov *et al*., 2002). Cell lysates of the *T. brucei* parental strain, non-induced and induced clonal cell lines and *P. serpens* were loaded on a 13% SDS-PAGE gel (5×10^6 cells per lane), blotted and probed with the anti-cox6 antiserum (1:500). The monoclonal mouse antibody against the *L. tarentolae* Rieske protein was used at 1:500 dilution (provided by L. Simpson). The polyclonal rabbit antisera against the *T. brucei* apoc_1 (provided by S.L. Hajduk), heat-shock protein 60 (hsp60) (provided by P.A.M. Michels), TAO (provided by G.C. Hill), cox6 and *L. tarentolae* trCOIV were used at 1:500, 1:20 000, 1:100, 1:500 and 1:500 dilutions respectively. Secondary anti-rabbit and anti-mouse immunoglobulin G antibodies (1:2000) (Sevapharma) coupled to horseradish peroxidase were visualized using the ECL kit (Amersham Biosciences)

Isolation of mitochondria

The protocol for isolation of kinetoplast-mitochondrial vesicles by hypotonic lysis (Horváth *et al*., 2000b) has been adjusted for the low amount of cells as follows. A pellet of 2–3×10^8 cells was resuspended in 1.5 ml of NET buffer (0.15 M NaCl; 0.1 M EDTA; 10 mM Tris-HCl, pH 8.0), spun in microcentrifuge (12 000 r.p.m. per 10 min, 4°C) and resuspended in 1.5 ml of DTE buffer (1 mM Tris-HCl, pH 7.9; 1 mM EDTA) and the lysate was pushed through a 25G needle. The sheared lysate was supplemented with 180 µl of 60% sucrose and spun as described above. The resulting pellet was resuspended in 500 µl of STM buffer (20 mM Tris-HCl, pH 7.9; 2 mM $MgCl_2$; 0.25 mM EDTA) and DNase solution [10 mM Tris-HCl, pH 7.4; 10 mM $CaCl_2$; 50% (w/v) glycerol; 2 mg ml^{-1} DNase I] was added to a final concentration of 10 µg ml^{-1}. After incubation for 30 min at 4°C, the reaction was stopped by the addition of 500 µl of STE buffer (20 mM Tris-HCl, pH 7.9; 2 mM EDTA; 0.25 mM sucrose). Pelleted mitochondrial vesicles were washed twice in the same buffer, and stored at −70°C.

Two-dimensional gel electrophoresis

Analysis of respiratory complexes of purified mitochondria was performed by 2D blue native/Tricine-SDS-PAGE gels. Mitochondria isolated from 2–3×10^8 cells were resuspended in 20 µl of ACA solution (0.75 M aminocaproic acid; 50 mM Bis-Tris), and the addition of 5 µl of 10% dodecylmaltoside was followed by a 1 h incubation at 4°C. The lysate was spun in a microcentrifuge (12 000 r.p.m. per 10 min, 4°C), and 1.5 µl of CB solution [0.5 M aminocaproic acid; 5% (w/v) Coomassie Brilliand Blue G-250] was added to the supernatant. The mixture was incubated for 10 min at 4°C and loaded on a 3–10% gradient or 6% blue native gel followed by resolution in a 10% Tricine-SDS-PAGE gel. Transfer of resolved proteins by semi-dry blotting and staining was performed as described previously (Maslov *et al*., 2002).

NADH dehydrogenase, cytochrome c *reductase and oxidase activity assays*

NADH dehydrogenase (complex I) activity was measured in a 1 ml cuvette containing NDH buffer (50 mM potassium phosphate buffer, pH 7.5; 1 mM EDTA; 0.2 mM KCN), 2 µl of mitochodrial lysate and 5 µl of 20 mM NADH. After addition of 10 µl of mixture of 2 mM coenzyme Q_2 and 5 mM ferricyanide the change in absorbance at 340 nm was measured every 10 s for 3 min.

Cytochrome *c* reductase (complex III) activity was measured in a 1 ml cuvette containing QCR buffer [40 mM sodium phosphate buffer, pH 7.4; 0.5 mM EDTA; 20 mM sodium malonate; 50 µM horse heart cytochrome *c* (Sigma); 0.005% dodecylmaltoside]. After the parallel addition of 2 µl of mitochondrial lysate and 2 µl of 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol [decylubiquinone (Sigma) was, before use, reduced as described; Trumpower and Edwards, 1979], the solution was quickly mixed with a pipette, and the change in absorbance at 550 nm was measured every 5 s over a period of 60 s. KCN was added to a final concentration of 200 µM as an inhibitor of interfering oxidase activity.

Cytochrome *c* oxidase (complex IV) activity was measured in a 1 ml cuvette containing COX buffer (40 mM sodium phosphate buffer, pH 7.4; 0.5 mM EDTA; 20 µM horse heart cytochrome *c*; 30 µM sodium ascorbate; 0.005% dodecyl maltoside) to which 20 µl of mitochondrial lysate was added. The change in absorbance at 550 nm was measured every 20 s for 10 min. Antimycin A (Sigma) at final concentration 300 ng ml^{-1} was used to selectively inhibit interfering reductase activity.

Protein concentrations were determined by Bradford. For complex I, a unit of activity was defined as an amount of

enzyme that catalyses the oxidation of 1 nmol of NADH per minute, assuming an extinction coefficient of 6.2 mM^{-1} cm^{-1} (Gonzáles-Halphen and Maslov, 2004). For complexes III and IV, a unit of activity was defined as the amount of enzyme that catalyses the reduction or oxidation of 1 μmol of cytochrome *c* per minute, assuming an extinction coefficient of 21.1 mM^{-1} cm^{-1} (Horváth *et al*., 2000b).

Histochemical staining of complexes I and IV

For the staining of complex I, 60 μg of mitochondrial lysate prepared as described above was loaded per lane and analysed on a 4–15% gradient blue native PAGE gel. Immediately after the run, the gel was stained in the reaction buffer (0.1 M Tris-HCl, pH 7.4; 0.14 mM NADH; 1 mg ml^{-1} nitrotetrazolium blue chloride) by slow agitation overnight. Complex IV was resolved under the same conditions and after the run, the gel was stained in the reaction buffer (50 mM phosphate buffer, pH 7.4; 1 mg ml^{-1} 3,3′diaminobenzidine; 24 U ml^{-1} catalase; 1 mg ml^{-1} cytochrome *c*; 75 mg ml^{-1} sucrose) by slow agitation for 3 h. After staining, the enzymatic activities of complexes I and IV appear as a specific colourful precipitate (violet for complex I, brown for complex IV). The gels were subsequently fixed in a mixture of 30% methanol and 10% acetic acid (Jung *et al*., 2000).

Measurement of oxygen consumption and membrane potential

Logarithmically growing cells were harvested, washed and resuspended in 1 ml of SDM-79 medium at a concentration of 3×10^7 cells ml^{-1}. Oxygen consumption at 27°C was determined with a Clark-type polarographic electrode (1302 Microcathode Oxygen Electrode, model 782; Strathkelvin Instruments). Cyanide (KCN) and salicylhydroxamic acid (SHAM) were added in 4 min intervals to final concentrations of 0.1 mM and 0.03 mM respectively.

Tetramethylrhodamine ethyl ester (Molecular Probes) uptake was used as a measure of the mitochondrial membrane potential. Cells (1×10^6) were incubated in 1 ml of SDM-79 medium with 0.4% TMRE for 20 min at 27°C. After staining, the cells were pelleted by centrifugation at 2500 r.p.m. per 5 min, resuspended in dye-free medium and immediately used for flow cytometry analysis, performed on an Epics XL flow cytometer (Coulter). Twenty thousands events were measured in each experiment. The data were analysed using WinMDI software.

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References

Acin-Perez, R., Bayona-Bafaluy, M.P., Fernandez-Silva, P., Moreno-Loshuertos, R., Perez-Martos, A., Bruno, C., *et al.* (2004) Respiratory complex III is required to maintain complex I in mammalian mitochondria. *Mol Cell* **13:** 805–815.

Alfonzo, J.D., Thiemann, O., and Simpson, L. (1997) The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. *Nucleic Acids Res* **25:** 3751–3759.

Aphasizhev, R., Sbicego, S., Peris, M., Jang, S.-H., Aphasizheva, I., Simpson, A.M., *et al.* (2002) Trypanosome mitochondrial 3′ terminal uridylyl transferase (TUTase): the key enzyme in U-insertion/deletion RNA editing. *Cell* **108:** 637–648.

Beattie, D.S., and Howton, M.M. (1996) The presence of rotenone-sensitive NADH dehydrogenase in the long slender bloodstream and the procyclic forms of *Trypanosoma brucei brucei*. *Eur J Biochem* **241:** 888–894.

Besteiro, S., Barrett, M.P., Riviere, L., and Bringaud, F. (2005) Energy generation in insect stages of *Trypanosoma brucei*: metabolism in flux. *Trends Parasitol* **21:** 185–191.

Bienen, E.J., Maturi, R.K., Pollakis, G., and Clarkson, A.B. (1993) Non-cytochrome-mediated mitochondrial ATP production in bloodstream form *Trypanosoma brucei brucei*. *Eur J Biochem* **216:** 75–80.

Bochud-Allemann, N., and Schneider, A. (2002) Mitochondrial substrate level phosphorylation is essential for growth of procyclic *Trypanosoma brucei*. *J Biol Chem* **277:** 32849–32854.

Chaudhuri, M., Ajayi, W., and Hill, G.C. (1998) Biochemical and molecular properties of the *Trypanosoma brucei* alternative oxidase. *Mol Biochem Parasitol* **95:** 53–68.

Chaudhuri, M., Ott, R.D., Saha, L., Williams, S., and Hill, G.C. (2005) The trypanosome alternative oxidase exists as a monomer in *Trypanosoma brucei* mitochondria. *Parasitol Res* **96:** 178–183.

Christmas, P.B., and Turrens, J.F. (2000) Separation of NADH-fumarate reductase and succinate dehydrogenase activities in *Trypanosoma brucei*. *Mol Biochem Parasitol* **183:** 225–228.

Clarkson, A.B., Bienen, E.J., Pollakis, G., and Grady, R.W. (1989) Respiration of bloodstream forms of the parasite *Trypanosoma brucei brucei* is dependent on a plant-like alternative oxidase. *J Biol Chem* **264:** 17770–17776.

Coustou, V., Besteiro, S., Biran, M., Diolez, P., Bouchaud, V., Voisin, P., *et al.* (2003) ATP generation in the *Trypanosoma brucei* procyclic form – cytosolic substrate level phosphorylation is essential, but not oxidative phosphorylation. *J Biol Chem* **278:** 49625–49635.

Divo, A.A., Patton, C.L., and Sartorelli, A.C. (1993) Evaluation of rhodamine-123 as a probe for monitoring mitochondrial function in *Trypanosoma brucei* spp. *J Eukaryot Microbiol* **40:** 329–335.

Dudkina, N.V., Eubel, H., Keegstra, W., Boekema, E.J., and Braun, H.P. (2005) Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. *Proc Natl Acad Sci USA* **102:** 3225–3229.

Dufour, E., Boulay, J., Rincheval, V., and Sainsard-Chanet, A. (2000) A causal link between respiration and senescence in *Podospora anserina*. *Proc Natl Acad Sci USA* **97:** 4138–4143.

Fang, J., and Beattie, D.S. (2003) Alternative oxidase present in procyclic *Trypanosoma brucei* may act to lower the mitochondrial production of superoxide. *Arch Biochem Biophys* **414:** 294–302.

Fang, J., Wang, Y., and Beattie, D.S. (2001) Isolation and characterization of complex I, rotenone-sensitive NADH: ubiquinone oxidoreductase, from the procyclic forms of *Trypanosoma brucei*. *Eur J Biochem* **268:** 3075–3082.

Fernández-Becerra, C., Sánchez-Moreno, M., Osuna, A., and Opperdoes, F.R. (1997) Comparative aspects of energy metabolism in plant trypanosomatids. *J Eukaryot Microbiol* **44:** 523–529.

Funes, S., Nargang, F.E., Neupert, W., and Herrmann, J.M. (2004) The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. *Mol Biol Cell* **15:** 1853–1861.

Gonzáles-Halphen, D., and Maslov, D.A. (2004) NADH-ubiquinone oxidoreductase activity in the kinetoplasts of the plant trypanosornatid *Phytomonas serpens*. *Parasitol Res* **92:** 341–346.

Hannaert, V., Bringaud, F., Opperdoes, F.R., and Michels, P.A. (2003) Evolution of energy metabolism and its compartmentation in Kinetoplastida. *Kinetopl Biol Dis* **2:** 11–42.

Helfert, S., Estévez, A.M., Bakker, B., Michels, P.A.M., and Clayton, C. (2001) Roles of triosephosphate isomerase and aerobic metabolism in *Trypanosoma brucei*. *Biochem J* **357:** 117–125.

Hernandez, F.R., and Turrens, J.F. (1998) Rotenone at high concentrations inhibits NADH-fumarate reductase and the mitochondrial respiratory chain of *Trypanosoma brucei* and *T. cruzi*. *Mol Biochem Parasitol* **93:** 135–137.

Horváth, A., Berry, E.A., and Maslov, D.A. (2000a) Translation of the edited mRNA for cytochrome *b* in trypanosome mitochondria. *Science* **187:** 1639–1640.

Horváth, A., Berry, E.A., Huang, L.S., and Maslov, D.A. (2000b) *Leishmania tarentolae*: a parallel isolation of cytochrome *bc*(1) and cytochrome *c* oxidase. *Exp Parasitol* **96:** 160–167.

Horváth, A., Neboháčová, M., Lukeš, J., and Maslov, D.A. (2002) Unusual polypeptide synthesis in the kinetoplast-mitochondria from *Leishmania tarentolae* – identification of individual *de novo* translation products. *J Biol Chem* **277:** 7222–7230.

Jung, C., Higgins, C.M.J., and Xu, Z. (2000) Measuring the quantity and activity of mitochondrial electron transport chain complexes in tissues of central nervous system using blue native polyacrylamide gel electrophoresis. *Anal Biochem* **286:** 214–223.

Lamour, N., Riviere, L., Coustou, V., Coombs, G.H., Barrett, M.P., and Bringaud, F. (2005) Proline metabolism in procyclic *Trypanosoma brucei* is down-regulated in the presence of glucose. *J Biol Chem* **280:** 11902–11910.

Lemire, B.D., and Oyedotun, K.S. (2002) The *Saccharomyces cerevisiae* mitochondrial succinate: ubiquinone oxidoreductase. *Biochim Biophys Acta* **1553:** 102–116.

McDonald, A.E., and Vanlerberghe, G.C. (2004) Branched mitochondrial electron transport in the animalia: presence of alternative oxidase in several animal phyla. *IUBMB Life* **56:** 333–341.

Maslov, D.A., Zíková, A., Kyselová, I., and Lukeš, J. (2002) A putative novel nuclear-encoded subunit of the cytochrome *c* oxidase complex in trypanosomatids. *Mol Biochem Parasitol* **125:** 113–125.

Matthews, K.R., and Gull, K. (1998) Identification of stage-regulated and differentiation-enriched transcripts during transformation of the African trypanosome from its bloodstream to procyclic form. *Mol Biochem Parasitol* **95:** 81–95.

Maxwell, D.P., Wang, Y., and McIntosch, L. (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc Natl Acad Sci USA* **96:** 8271–8276.

Nawathean, P., and Maslov, D.A. (2000) The absence of genes for cytochrome *c* oxidase and reductase subunits in maxicircle kinetoplast DNA of the respiration-deficient plant trypanosomatid *Phytomonas serpens*. *Curr Genet* **38:** 95–103.

Neboháčová, M., Maslov, D.A., Falick, A.M., and Simpson, L. (2004) The effect of RNA interference down-regulation of RNA editing 3′-terminal uridylyl transferase (TUTase) 1 on mitochondrial *de novo* protein synthesis and stability of respiratory complexes in *Trypanosoma brucei*. *J Biol Chem* **279:** 7819–7825.

Njogu, R.M., Whittaker, C.J., and Hill, G.C. (1980) Evidence for a branched electron transport chain in *Trypanosoma brucei*. *Mol Biochem Parasitol* **1:** 13–29.

Nolan, D.P., and Voorheis, H.P. (1992) The mitochondrion in blood-stream forms of *Trypanosoma brucei* is energized by the electrogenic pumping of protons catalyzed by the F_1F_0-ATPase. *Eur J Biochem* **209:** 207–216.

O'Hearn, S.F., Huang, C.E., Hemann, M., Zhelonkina, A., and Sollner-Webb, B. (2003) *Trypanosoma brucei* RNA editing complex: band II is structurally critical and maintains band V ligase, which is nonessential. *Mol Cell Biol* **23:** 7909–7919.

Opperdoes, F.R. (1985) Biochemical peculiarities of trypanosomes, African and South-American. *Br Med Bull* **41:** 130–136.

Priest, J.W., and Hajduk, S.L. (1992) Cytochrome-*c* reductase purified from *Crithidia fasciculata* contains an atypical cytochrome c_1. *J Biol Chem* **267:** 20188–20195.

Priest, J.W., and Hajduk, S.L. (1994) Developmental regulation of *Trypanosoma brucei* cytochrome *c* reductase during blood-stream to procyclic differentiation. *Mol Biochem Parasitol* **65:** 291–304.

Priest, J.W., and Hajduk, S.L. (1995) The trypanosomatid Rieske iron-sulfur proteins have a cleaved presequence that may direct mitochondrial import. *Biochim Biophys Acta* **1269:** 201–204.

Schägger, H., de Cool, R., Bauer, M.F., Hofmann, S., Godinot, C., and Brandt, U. (2004) Significance of respirasomes for the assembly/stability of human respiratory chain complex I. *J Biol Chem* **35:** 36349–36353.

Scheffler, I.E. (1999) Mitochondrial electron transport and oxidative phosphorylation. In *Mitochondria*. Scheffler, I.E. (ed.). New York: Wiley-Liss, pp. 141–246.

Schneider, A. (2001) Unique aspects of mitochondrial biogenesis in trypanosomatids. *Int J Parasitol* **31:** 1403–1415.

Schnaufer, A., Domingo, G.J., and Stuart, K. (2002) Natural and induced dyskinetoplastic trypanosomatids: how to live without mitochondrial DNA. *Int J Parasitol* **32:** 1071–1084.

Schneider, D., Berry, S., Volkmer, T., Seidler, A., and Rogner, M. (2004) PetC1 is the major Rieske iron-sulfur protein in the cytochrome *b*(6)*f* complex of *Synechocystis* sp PCC 6803. *J Biol Chem* **279:** 39383–39388.

Speijer, D., Breek, C.K.D., Muijsers, A.O., Hartog, A.F., Berden, J.A., Albracht, S.P.J., *et al.* (1996a) Purification and characterization of cytochrome c oxidase from the insect trypanosomatid *Crithidia fasciculata*. *Mol Biochem Parasitol* **85:** 171–186.

Speijer, D., Breek, C.K.D., Muijsers, A.O., Groenevelt, P.X., Dekker, H., De Haan, A., and Benne, R. (1996b) The sequence of a small subunit of cytochrome *c* oxidase from *Crithidia fasciculata* which is homologous to mammalian subunit IV. *FEBS Lett* **381:** 123–126.

Stroh, A., Anderka, O., Pfeiffer, K., Yagi, T., Finel, M., Ludwig, B., and Schägger, H. (2004) Assembly of respiratory complex I, III, and IV into NADH oxidase supercomplex stabilizes complex I in *Paracoccus denitrificans*. *J Biol Chem* **279:** 5000–5007.

Stuart, K.D., Schnaufer, A., Ernst, N.L., and Panigrahi, A.K. (2005) Complex management: RNA editing in trypanosomes. *Trends Biochem Sci* **30:** 97–105.

Tielens, A.G.M., and Van Hellemond, J.J. (1998) Differences in energy metabolism between trypanosomatidae. *Parasitol Today* **14:** 265–272.

Timms, M.W., van Deursen, F.J., Hendriks, E.F., and Matthews, K.R. (2002) Mitochondrial development during life cycle differentiation of African trypanosomes: evidence for a kinetoplast-dependent differentiation control point. *Mol Biol Cell* **13:** 3747–3759.

Trumpower, B.L., and Edwards, C.A. (1979) Purification of a reconstitutively active iron-sulfur protein (oxidation factor) from succinate cytochrome *c* reductase complex of bovine heart mitochondria. *J Biol Chem* **254:** 8697–8706.

Van Weelden, S.W.H., Fast, B., Vogt, A., Van Der Meer, P., Saas, J., Van Hellemond, J.J., *et al.* (2003) Procyclic *Trypanosoma brucei* do not use Krebs cycle activity for energy generation. *J Biol Chem* **278:** 12854–12863.

Vondrušková, E., van den Burg, J., Zíková, A., Ernst, N.L., Stuart, K., Benne, R., and Lukeš, J. (2005) RNA interference analyses suggest a transcript-specific regulatory role for MRP1 and MRP2 in RNA editing and other RNA processing in *Trypanosoma brucei*. *J Biol Chem* **280:** 2429–2438.

Walker, R., Jr, Saha, L., Hill, G.C., and Chaudhuri, M. (2005) The effect of over-expression of the alternative oxidase in the procyclic forms of *Trypanosoma brucei*. *Mol Biochem Parasitol* **139:** 153–162.

Wang, Z., Morris, J., Drew, M., and Englund, P.T. (2000) Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J Biol Chem* **275:** 40174–40179.

Wang, B.B., Ernst, N.L., Palazzo, S.S., Panigrahi, A.K., Salavati, R., and Stuart, K. (2003) TbMP44 is essential for RNA editing and structural integrity of the editosome in *Trypanosoma brucei*. *Eukaryot Cell* **2:** 578–587.