J Bioenerg Biomembr (2011) 43:409–417
DOI 10.1007/s10863-011-9369-0

O_2 consumption rates along the growth curve: new insights into *Trypanosoma cruzi* mitochondrial respiratory chain

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Received: 18 February 2011 / Accepted: 9 June 2011 / Published online: 6 July 2011

Abstract Understanding the energy-transduction pathways employed by *Trypanosoma cruzi*, the etiological agent of Chagas disease, may lead to the identification of new targets for development of a more effective therapy. Herein, the contribution of different substrates for O_2 consumption rates along *T. cruzi* epimastigotes (Tulahuen 2 and Y strains) growth curve was evaluated. O_2 consumption rates were higher at the late stationary phase not due to an increase on succinate-dehydrogenase activity. Antimycin A and cyanide did not totally inhibit the mitochondrial respiratory chain (MRC). Malonate at 10 or 25 mM was not a potent inhibitor of complex II. Comparing complex II and III, the former appears to be the primary site of H_2O_2 release. An update on *T. cruzi* MRC is presented that together with our results bring important data towards the understanding of the parasite's MRC. The findings mainly at the stationary phase could be relevant for epimastigotes transformation into the metacyclic form, and in this sense deserves further attention.

Keywords *Trypanosoma cruzi* · Mitochondrial respiratory chain · Oxygen consumption · Reactive oxygen species

Introduction

It is estimated that 11 million people are infected with the parasite *Trypanosoma cruzi*, the etiologic agent of Chagas disease and that over 150 million people are at risk of contracting it (Wilkinson and Kelly 2009). Also of importance is oral transmission that in the last few years has gained relevance, especially in Brazil (Nóbrega et al. 2009), the increase in the number of cases mainly in Spain due to the intensification of immigration (Pérez de Ayala et al. 2009) and the selection of a benznidazole-resistant population of *T. cruzi* (Murta and Romanha 1998). Unfortunately, there are no immediate prospect of vaccines, and no satisfactory treatment since the two available drugs, nifurtimox and benznidazole, are toxic, may be carcinogenic, and have poor efficacy against the chronic stage of the disease (Wilkinson and Kelly 2009).

Drug discovery in trypanosomatids has proceeded through different starting points but the screening of compounds for activity against defined molecular targets, i.e., rational drug design has gained much attention in the last few years. The genome sequence of *T. cruzi* (El-Sayed et al. 2005) has made the search for target identification less complicated, but unfortunately finding a good target is not the only problem to be solved. The biological, biochemical and genetic diversity among *T. cruzi* strains represent a huge step to be overcome. Differences have been observed in the resistance to drugs (Murta et al. 1998), to oxidative stress generated by H_2O_2, cytosolic tryparedoxin peroxidase expression (Mielniczki-Pereira et al. 2007), isoenzymatic patterns (Zingales et al. 1997), cytochrome content (Engel et al. 1990) among others. It has been reported as well that strains with a higher influx of glucose through the pentose phosphate pathway are more virulent (Mancilla and Naquira 1964). Therefore, the possibility that intraspecific heterogeneity could modulate a parasite's pathogenicity, survival and adaptability (Brisse et al. 1998) has been considered.

One of the potential fields in drug research is the study of the parasite energy-yielding pathways. *T. cruzi*'s mito-

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chondrion behaves similarly to the mammalian counterpart regarding membrane potential and calcium uptake (Vercesi et al. 1991; Irigoín et al. 2008), functions that are dependent upon the mitochondrial respiratory chain (MRC). In *T. cruzi* the presence of complex I to IVof the MRC (Boveris et al. 1986; Rogerson and Gutteridge 1979) has been shown, but the importance and effective contribution of complex I in energy transduction has been a subject of debate due to: (1) the identification of only two energy coupling sites, corresponding to sites II and III in the *T. cruzi* mitochondrial fraction (Affranchino et al. 1985), (2) succinate being pointed out as the primary electron donor to the MRC (Denicola-Seoane et al. 1992), (3) succinate being produced by fumarate reductase (FR) (Tielens and Van Hellemond 2009) and (4) release of succinate to the extracellular medium probably due to the relatively inefficient respiratory chain (Denicola-Seoane et al. 1992). Besides that, bioinformatics analysis allowed the prediction of *Trypanosoma brucei* complex I to contain a minimum of 19 subunits relative to 46 subunits in the mammalian host (Opperdoes and Michels 2008). The subunits that are missing are thought to be involved in the vectorial translocation of protons across the membrane, supporting the hypothesis that in *T. brucei* complex I does not contribute to energy generation but has a major role in the regeneration of mitochondrial NAD^+ (Opperdoes and Michels 2008). This conclusion can be extended to *T. cruzi* since all mitochondrially encoded subunits and nuclear-encoded peptides identified in *T. brucei* were also detected in the parasite's genome (Opperdoes and Michels 2008). The absence of these subunits can explain the lack of inhibition of electron transport at site I by rotenone and possibly the lack of an energy coupling site since the energy conservation at site I is in some extent related to rotenone sensitivity (Affranchino et al. 1985). Despite these results, the organization of *T. cruzi*'s respiratory chain and the actual contribution of each complex to reactive oxygen species (ROS) release remain elusive (Affranchino et al. 1985; Boveris et al. 1986).

Besides being involved in energy transduction, the MRC is the main ROS-generating site, where complex I, II and III have been pointed out as the site for electron leakage and consequently ROS formation in different organisms (Kowaltowski et al. 2009). ROS serve as specific signaling molecules under both physiological and pathophysiologic conditions, and the transient generation of these species, within certain limits, is essential to maintain homeostasis (Holbrook and Ikeyama 2002). Data in the literature has been focusing on *T. cruzi*'s defense mechanisms against the ROS coming from exogenous sources (Finzi et al. 2004; Piacenza et al. 2007; Irigoín et al. 2008; among others), but far less attention has been given to the ROS generated by its own metabolism.

Taking all this into account, herein we investigated at distinct growth phases the contribution of different substrates to *T. cruzi*'s respiratory rates. Hydrogen peroxide release under MRC inhibition at distinct sites was also investigated. Additionally, to address the marked diversity that exist within this group of parasites the experiments were performed in two strains belonging to different lineages.

Material and methods

Cell cultures

T. cruzi epimastigotes (Tulahuen 2 and Y strains) were grown in LIT medium at 28 °C containing 20 mg/L hemin and 10% fetal calf serum as described (Castellani et al. 1967). After 3, 5 and 7 days of growth (*log*, early and late stationary phases, respectively), cells were harvested by centrifugation (1,000 × *g* at 4 °C), washed in phosphate buffered saline (PBS), pH 7.3 and the number of cells determined in a Neubauer chamber.

Oxygen uptake measurements

O_2 consumption was monitored in a computer-interfaced Clark-type oxygen electrode with continuous stirring, at 28 °C (Hansatech® Systems Inc., Norfolk, Eng.). Cells (10^8/mL) were incubated in a standard intracellular reaction medium (SRM) (125 mM sucrose, 65 mM KCl, 2 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10 mM HEPES pH 7.2, 1 mM EGTA and 1 mg/mL BSA) in the presence of 50 μM digitonin and the respiratory chain substrates: malate 5 mM/pyruvate 5 mM (complex I-linked substrates), malate 5 mM/pyruvate 5 mM/malonate 10 mM (complex I-linked substrates + competitive inhibitor of complex II) or succinate 5 mM (complex II-linked substrate). Respiratory control ratio (RCR) (State 3/state 4) was determined by the addition of 400 μM ADP (State 3) followed by 2 μg/mL oligomycin (State 4). Maximum oxygen consumption rates were determined in the presence of 1 μM carbonyl-cyanide-m-chlorophenylhydrazone (CCCP). Inhibitors of MRC, Antimycin A (AA, 2 μg/mL) or thenoyltrifluoroacetone (TTFA, 500 μM) were added as described in the Figures legends.

Succinate dehydrogenase (SDH) assay

Enzyme activity was determined by the reduction of phenazine methosulfate (PMS) (Singer 1974; Mirandola et al. 2008), that is reoxidized by 2,6-dichlorophelindophenol (DCIP). 10^7 cells/mL were incubated in 50 mM potassium phosphate buffer pH 7.2 in the presence of 0.05% Triton X-100, 1 mM succinate, 2 μg/mL AA, 1 mM PMS and

80 μM DCIP. Absorbance was determined at 600 nm (Singer 1974; Mirandola et al. 2008).

Mitochondrial membrane potential (ΔΨ) in intact cells

ΔΨ was estimated by flow cytometry using 3,3-dihexyloxacarbocyanine iodide dye ($DiOC_6(3)$, Molecular Probes®) (Campos et al. 2004). Cells (10^6/mL) were incubated in PBS/1 mM $MgCl_2$ in the presence of 0.3 nM $Dioc_6(3)$ at 28 °C. After 40 min, 1 μM CCCP was added to one of the tubes and a 20 min further incubation was made. Cells were then analyzed in a FACSCalibur flow cytometer (Becton Dickinson), where the F/F_{CCCP} ratio was used to normalize the results (F or F_{max}: maximum fluorescence of the probe; F_{CCCP}: fluorescence in the presence of the uncoupler) (Carranza et al. 2009).

Determination of hydrogen peroxide (H_2O_2) release

10^8cells/mL were incubated in PBS/1 mM $MgCl_2$ in the presence of 5 mM malate/5 mM pyruvate, 50 μM digitonin, 1 U/mL Horseradish peroxidase (HRP) and 25 μM Amplex Red (Molecular Probes®). Fluorescence was monitored at the excitation and emission wavelengths of 563 nm and 587 nm, respectively, on a Hitachi F4500 fluorescence spectrophotometer, with continuous stirring. A calibration curve was performed by adding known quantities of a freshly prepared H_2O_2 solution in the presence of Amplex Red and HRP (Barros et al. 2004).

Statistical analysis

Data of experiments represent averages ± standard deviations of at least six independent experiments performed in duplicate. Comparisons were conducted using Student's *t*-test in Origin 6.0 software, and data in $p<0.05$ were considered significant.

Results and discussion

The energy metabolism of *T. cruzi* strongly resembles that of *Leishmania*: all stages possess Krebs cycle activity and a mammalian-type respiratory chain linked to ATP generation (Tielens and Van Hellemond 2009; Opperdoes and Coombs 2007). However, there are still some gaps regarding maintenance of redox balance in *T. cruzi*'s MRC and the effective contribution of complex I to energy transduction.

In this sense, herein the experiments were performed using different respiratory chain substrates: malate/pyruvate (M/P) (complexes I and II); succinate (SUC) (complex II) and malate/pyruvate/malonate (M/P/Malon) (complex I), aiming to determine the contribution of each complex to O_2 consumption rates in all growth phases. Malonate, a competitive inhibitor of succinate dehydrogenase, was used at the concentration of 10 mM as described in (Carranza et al. 2009) and also because at 5 mM only a 74% inhibition was detected (Denicola-Seoane et al. 1992). Besides that, in view of the huge diversity among *T. cruzi* strains and as a comparison, we performed the experiments in two strains with differences in the resistance to oxidative stress, growth curve, doubling time (Mielniczki-Pereira et al. 2007) and phosphatase activity (Morales-Neto et al. 2009).

In relation to the oxygen consumption rates, the use of different respiratory chain substrates did not lead to significant differences in all growth phases (Fig. 1). However, these rates varied not only among strains but also at the different growth phases within the same strain. Tulahuen 2 (Fig. 1a) had an increase of approximately 28% from *log* phase (3 days) to early (5 days) stationary phase from where it remained constant. In Y strain (Fig. 1b) O_2 consumption remained constant between *log* and early stationary phases and increased 100% towards the late stationary phase (7 days). Interestingly, Tulahuen 2 had higher oxygen consumption rates than the Y strain in all

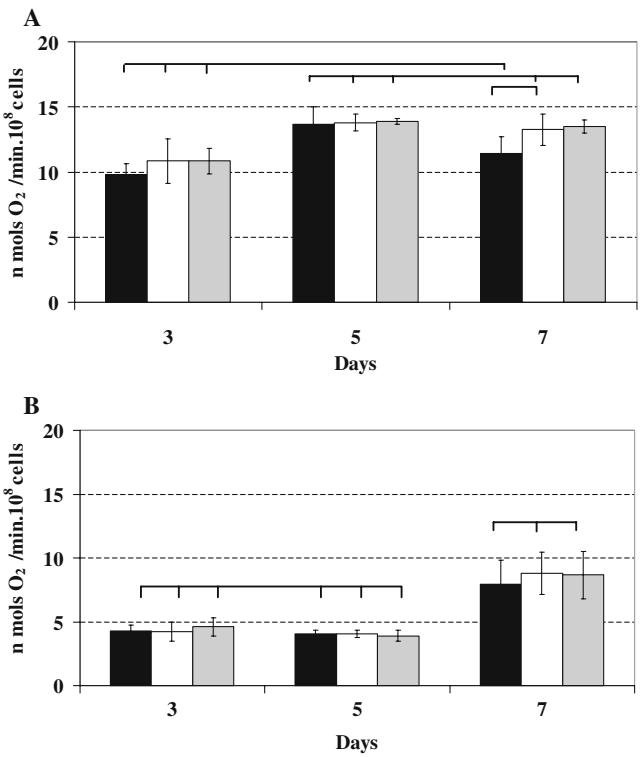

Fig. 1 Oxygen consumption of *Trypanosoma cruzi* along the growth curve. Cells (10^8/mL) at different growth phases were added to SRM in the presence of Malate 5 mM + Pyruvate 5 mM (*black bars*), Malate 5 mM + Pyruvate 5 mM + Malonate 10 mM (*white bars*) or Succinate 5 mM (*gray bars*) and oxygen consumption was determined as described under Material and Methods. **a** - Tulahuen 2 and **b** – Y. Statistical analysis: $p>0.05$ for the indicated groups

growth phases (147, 214 and 56% in *log*, early and late stationary phases, respectively) (Fig. 1a). Growth-related differences can be a consequence of the utilization of distinct metabolic substrates during growth. *T. cruzi* epimastigotes after glucose depletion from the culture medium preferentially catabolize aminoacids to CO_2 (Engel et al. 1990). In support of our results regarding the increase on the O_2 consumption rates in the late stationary phase, some *T. cruzi* strains maintain a high rate of oxygen consumption in this phase due to the utilization of amino-acids, while others decrease their rates due to their less ability to catabolize these molecules (Engel et al. 1990). Besides that, a less efficient cytochrome chain was also pointed out as a possible cause for a lower rate of oxygen utilization (Engel et al. 1990).

The ratio between the O_2 consumption rate after addition of ADP (State 3) and subsequent addition of oligomycin (State 4) is the respiratory control rate (RCR) that indicates coupling of oxidative phosphorylation with the mitochondrial respiratory chain. For both strains (Fig. 2) RCR did not vary significantly among substrates in each phase and between *log* and early stationary phase; however, a slight decrease (22 and 16% for Tulahuen 2 and Y, respectively) was detected towards the late stationary phase. In the presence of the protonophore CCCP, that permeabilizes the membrane to H^+, no significant differences were observed among substrates in both strains (data not shown). Interestingly, in the Y strain O_2 consumption rates in *log* and early stationary phase were increased to the levels of the late stationary phase and in both strains at the late stationary phase no stimulation by CCCP was detected (data not shown), suggesting that at this growth phase the mitochondrial respiratory chain was working at maximum rates, supporting the lower RCR observed (Fig. 2). As detected by flow cytometry and regardless of the differences observed among strains, $\Delta\Psi$ was similar in all growth phases for both strains studied (Fig. 3).

At this point, the similarity observed in oxygen consumption in the presence of different substrates raised the possibility that malonate although at a higher concentration than the one used to achieve 74% inhibition of complex II (Denicola-Seoane et al. 1992) could not be inhibiting it completely. Indeed, in Fig. 4 it is shown that employing SUC as substrate, malonate inhibition rates both in Tulahuen 2 (Fig. 4a) and Y (Fig. 4b) cells decreased from *log* to late stationary phases. The use of M/P in Tulahuen 2 (Fig. 4a) yielded higher inhibition rates than in the Y strain (Fig. 4b) in all growth phases, but the late stationary phase. Interestingly, although in Tulahuen 2 cells, a 41% inhibition of O_2 consumption was achieved using M/P as substrate, no significant difference was observed when compared to the ones obtained in the absence of the inhibitor (Fig. 1a). Once *T. cruzi* is able to store carbohydrates in reservosomes (Cunha-e-Silva et al. 1990), cells were starved for 3 h in PBS/1 mM $MgCl_2$ as described for *Leishmania donovani* (Santhamma and Bhaduri 1995). Under these conditions in general, for both strains inhibition of, O_2 consumption rates using M/P as a substrate were similar to cells not starved (Fig. 4). Interestingly, when SUC was used as a MRC substrate at Tulahuen 2 *log* phase a 20% decrease in inhibition related to non starved cells was observed and no difference could be detected from then on (Fig. 4a). In the

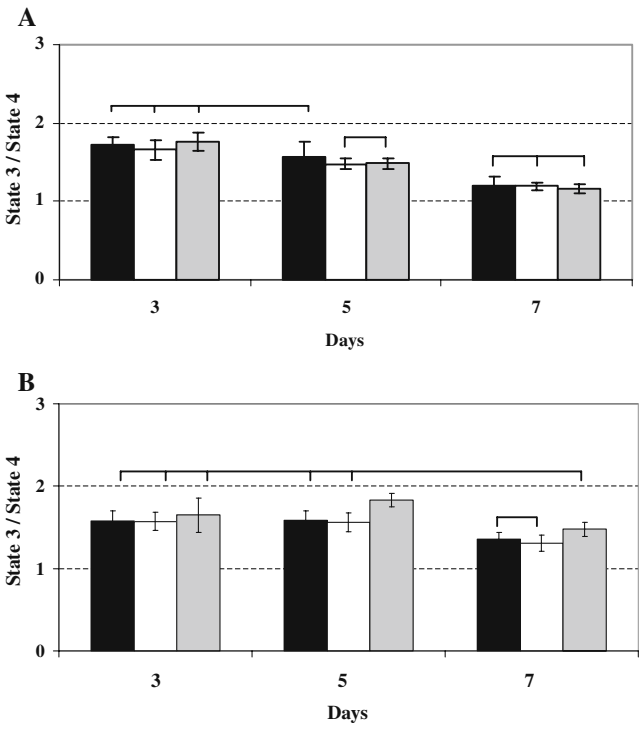

Fig. 2 *Trypanosoma cruzi* respiratory control along the growth curve. Experimental conditions were as in Fig. 1. Oxygen consumption was determined in the presence of ADP (400 μM, State 3) and Oligomycin (2 μg/mL, State 4). **a** - Tulahuen 2 and **b** – Y. Statistical analysis: $p>0.05$ for indicated groups

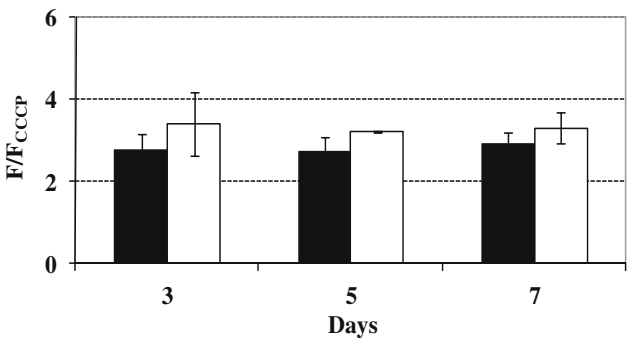

Fig. 3 *Trypanosoma cruzi* mitochondrial membrane potential along the growth curve. 10^6 cells/mL were incubated in PBS and $\Delta\Psi$ was determined as described in Materials and Methods. Tulahuen 2 (*black bars*) and Y (*white bars*). Statistical analysis: $p>0.05$ for all data

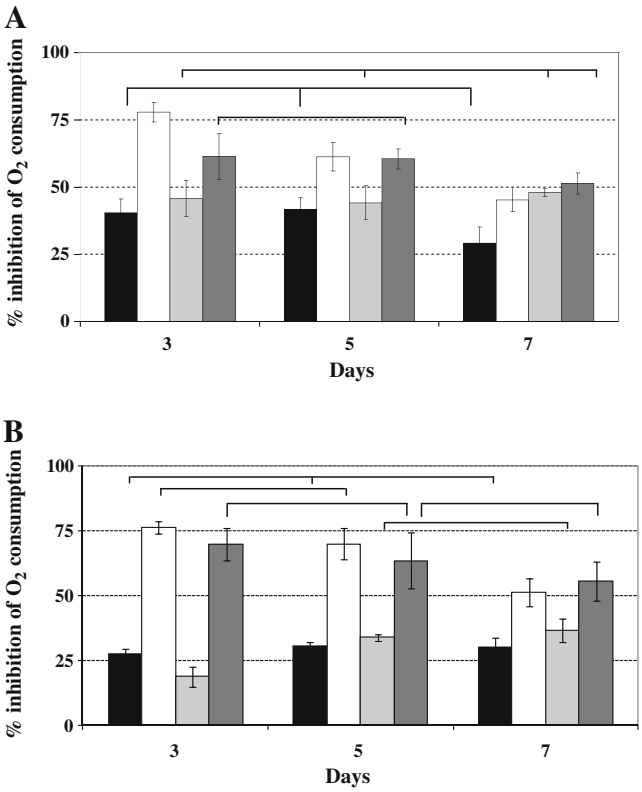

Fig. 4 Effect of malonate on *Trypanosoma cruzi* oxygen consumption along the growth curve. a- Tulahuen 2 and **b** – Y strains. 10^8 cells/mL were incubated in SRM in the presence of 5 mM malate + 5 mM pyruvate (*black bars*) or 5 mM succinate (*white bars*). After establishing the substrate-supported O_2 consumption rates, malonate (10 mM) was added and the percentage of inhibition related to the control was determined. Malonate inhibition was also evaluated in starved cells for 3 h using 5 mM malate + 5 mM pyruvate (*light gray bars*) or 5 mM succinate (*dark gray bars*) as substrates. Oxygen consumption control rates (in nmols/min.10^8 cells): Tulahuen 2 (M/P: ~8.5; 8.97 and 9.96; SUC: ~8.75; 8.07 and 9.87 for 3, 5 and 7 days, respectively) and Y (M/P: ~4.15; 5.28 and 8.44; SUC: ~3.68; 5.33 and 7.97 for 3, 5 and 7 days, respectively) and in starved cells: Tulahuen 2 (M/P: ~5.85; 5.03 and 6.66; SUC: ~5.92; 4.89 and 6.59 for 3, 5 and 7 days, respectively) and Y (M/P: ~2.74; 3.46 and 5.88; SUC: ~2.69; 3.28 and 4.79 for 3, 5 and 7 days, respectively). Statistical analysis: $p>0.05$ for the indicated groups

Y strain no difference could be detected when SUC was used as a substrate (Fig. 4b).

In view of these results we came up with the following hypotheses: malonate at 10 mM could be low in face of the intracellular succinate concentration which would reduce its competitive inhibition of complex II or this complex would have a different structure from those of mammals, as suggested by (Morales et al. 2009). Once in *L. donovani* the respiratory chain is completely inhibited with 25 mM malonate (Santhamma and Bhaduri 1995), we decided to test this concentration in *T. cruzi* only at the early stationary phase (Fig. 5). The results shown indicate that no significant differences, with the exception of M/P in Y cells, could be detected using either 10 or 25 mM malonate.

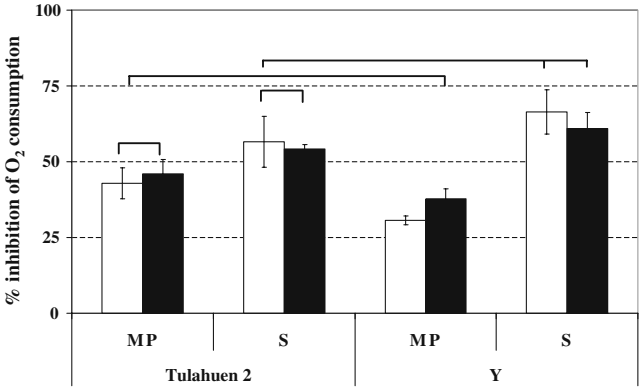

Fig. 5 Effect of malonate at different concentrations on *Trypanosoma cruzi* oxygen consumption. Experimental conditions were the same as in Fig. 4, but with the addition of malonate at 10 mM (*white bars*) or 25 mM (*black bars*) after basal Malate/Pyruvate (MP) or Succinate (S) – supported oxygen consumption. Basal oxygen consumption (values in nmols/min.10^8 cells): Tulahuen 2 (~8.97 and 8.07 for M/P- and SUC-supported oxygen consumption, respectively) and Y (~5.28 and 5.33 for M/P- and SUC-supported oxygen consumption, respectively). Statistical analysis: $p>0.05$ for indicated groups

These results corroborate with the ones that indicated that *T. cruzi* complex II is different from other organisms: it is classified as a supramolecular complex, has 12 subunits and a lower affinity for the inhibitor in comparison to the mammal enzyme (Morales et al. 2009) and even to *L. donovani* (Santhamma and Bhaduri 1995). In this sense, malonate at the concentration of 10 mM did not allow us to prove complex I's effective contribution to electron transport since complex II was not completely inhibited when M/P/Malon was used (in general around 40 and 25% inhibition in Tulahuen 2 and Y strain, respectively).

Regardless of the fact that no difference was observed in the presence of different substrates, O_2 consumption rates were higher in the late stationary phase which would suggest a higher SDH activity due to the relevance of succinate for parasite's metabolism (Tielens and Van Hellemond 2009). However, in Fig. 6, it is notable that

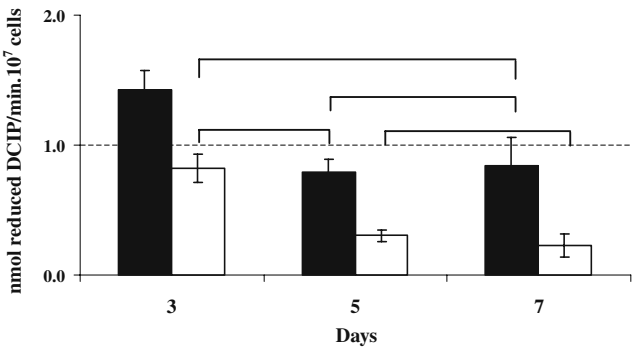

Fig. 6 Succinate dehydrogenase activity along *T. cruzi* growth curve. Experimental conditions are described in Material and Methods. Tulahuen 2 – *black bars* and Y – *white bars*. Statistical analysis: $p>0.05$ for indicated groups

the enzyme has a higher activity in the *log* phase in both strains, although in the Y strain this activity is 43% lower than the other strain. In Tulahuen 2 and Y cells this activity decreased 45% and 62%, respectively in the early stationary phase in relation to *log* phase and remained constant after that. The lower activity at the late stationary phase is in agreement with the results obtained by Cazzulo et al. (1985) showing that at this phase of growth there is not a significant consumption of succinate, and with the results from malonate inhibition that decrease toward the late stationary phase (Fig. 4).

In this sense, in the search for other O_2 consumption sources the effect of potassium cyanide (KCN) was investigated. KCN is an inhibitor of complex IV blocking O_2 reduction to H_2O resulting in ATP depletion (Lash et al. 1996). KCN (Fig. 7) did not completely inhibit O_2 consumption, in agreement with previous results not only in *T. cruzi* (Affranchino et al. 1986; Felix et al. 1978; Rogerson and Gutteridge 1979), but also in *L. donovani* (Datta and Bera 2000). These rates remained approximately around 94% in both strains, although the Y strain showed slight variations among substrates (Fig. 7b). The same percentage of inhibition for both strains was obtained even when cells were starved for 3 h. The similarity in O_2—consumption rates, in the presence of all substrates used (Fig. 1) together with these results suggest that complex I contributes to some extent to electron transport in *T. cruzi* epimastigotes.

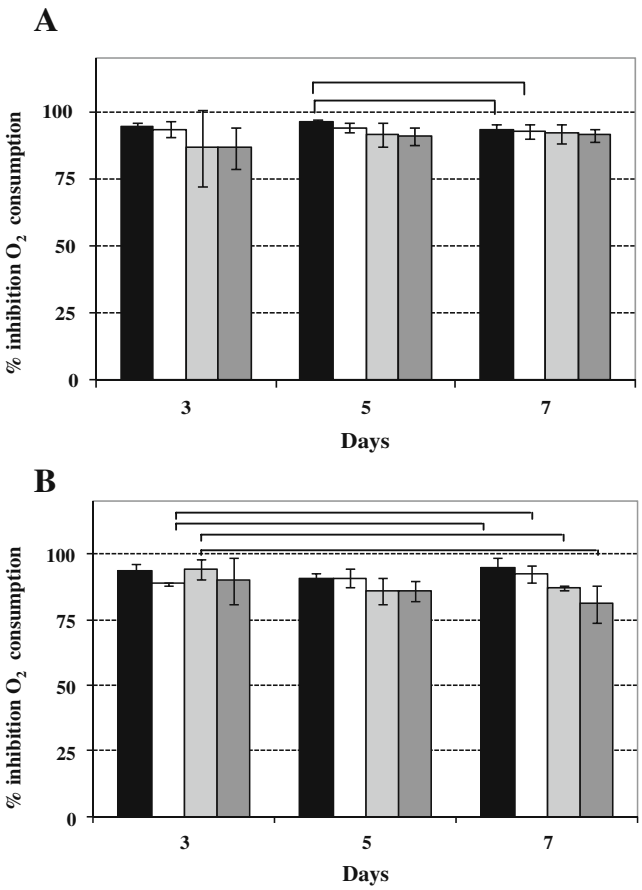

Fig. 7 Effect of cyanide on *Trypanosoma cruzi* oxygen consumption along the growth curve. Experimental conditions were the same in Fig. 4, but with the addition of KCN (2 mM) after basal oxygen consumption in Tulahuen 2 (**a**) and Y (**b**) using 5 mM malate + 5 mM pyruvate (*black bars*) or 5 mM succinate (*white bars*) as substrates. KCN was also employed to evaluate oxygen consumption inhibition in starved cells for 3 h using 5 mM malate + 5 mM pyruvate (*light gray bars*) or 5 mM succinate (*dark gray bars*) as substrates. Statistical analysis: $p<0.05$ for indicated groups

The MRC is a potential source of ROS release and in *L. donovani* it was shown that inhibition of MRC by AA, at the level of cyt_b-cyt_c, or TTFA, an inhibitor of complex II, led to $O_2^{\cdot-}$ or H_2O_2 release, respectively (Mehta and Shaha 2004). Under our experimental conditions, AA promoted approximately 90% and TTFA around 25% impairment of O_2 consumption by the MRC (Fig. 8a). No significant differences could be detected among the strains studied. Y cells led to a higher H_2O_2 release at basal levels than the Tulahuen 2 strain (Fig. 8b). When AA was used to inhibit the MRC an increase in H_2O_2 release was observed in both strains that were even higher in the presence of TTFA. Comparing complex II and III, the former one appears to be the prime area of H_2O_2 in *T. cruzi* as in *L. donovani* (Mehta and Shaha 2004).

If the higher O_2 consumption rates, especially at the late stationary phase, cannot be attributed to a higher succinate dehydrogenase activity, and AA and KCN do not inhibit by 100% the MRC, which reactions are consuming O_2 at this time point of the growth curve? The current understanding of *T. cruzi* MRC is depicted in Fig. 9. To date the presence of cytochrome *o*, aa_3, b, c_{558} (reviewed in Engel et al. 1990), and also ubiquinone 9 (Ferella et al. 2006) have been demonstrated. Due to the partial sensitivity to CN^- and AA the possibility that *T. cruzi* has a branched electron transport chain has been considered (Felix et al. 1978; Rogerson and Gutteridge 1979; Engel et al. 1990). As terminal oxidases, cytochrome aa_3 has been pointed out to be the main one and cytochrome *o* to be the other (Affranchino et al. 1986; Opperdoes 1985). Besides that, through GenBank analysis a homolog (TbAOX2) to the classic alternative oxidase (TAO), as the one present in *Trypanosoma brucei*, was detected not only in *T. cruzi* but also in *Leishmania* (Chaudhuri et al. 2006) raising new possibilities for *T.cruzi*'s MRC organization. However, the contribution of these alternatives pathways is considered to be a minor one (Tielens and Van Hellemond 1998). Nevertheless, *T. cruzi* epimastigotes depend on respiration for proliferation and normal functioning (Stoppani et al. 1980).

As discussed before, a shift in metabolism occurs after depletion of glucose from the culture medium where the catabolism of aminoacids becomes relevant (Engel et al. 1990). NAD-glutamate dehydrogenase has a considerable

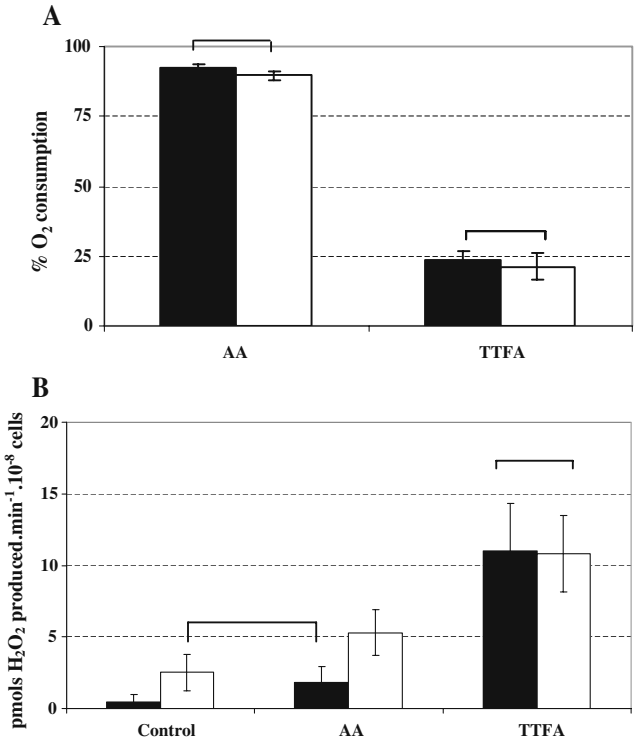

Fig. 8 Complex II inhibition increases H_2O_2 release and inhibition of complex III leads to superoxide release. Cells (10^8/mL) were incubated in PBS/1 mM $MgCl_2$ under the experimental conditions described under Material and Methods. Experiments were also performed in the presence of AA (2 μg/mL) or TTFA (500 μM). Tulahuen 2 (*black bars*) and Y (*white bars*). Statistical analysis: $p > 0.05$ for the indicated groups

increase in the activity when glucose is exhausted from the culture medium and SDH the reverse (Cazzulo et al. 1985) in agreement with Figs. 4 and 6. The former becomes important for proline metabolism (Bringaud et al. 2006) where in *T. brucei* 10 times more NADH is produced per mol of proline than per mol of glucose (Coustou et al. 2008). If this is also the case for *T. cruzi* large amounts of NADH are going to be generated from proline oxidation at this phase of growth. In analogy to what has been described for *T. brucei* and proposed to exist in *T. cruzi*, all the subunits with redox centers characterized for complex I are present (Opperdoes and Michels 2008), and as so oxidation of NADH would occur through the classical MRC, explaining the inhibition by KCN. At this time of the growth curve succinate would be secreted into the medium due to the inability of the MRC to cope with the high input of reducing equivalents (Cazzulo 1992) or converted into alanine (Tielens and Van Hellemond 1998). The ratio SDH/FR in *T. cruzi* is about 0.28 implicating that FR has an important physiological role in this parasite (Boveris et al. 1986), but there is still some speculation in the literature regarding FR contribution to Trypanosomatidae metabolism (reviewed in Tielens and Van Hellemond 1998). It appears that a minor part of succinate is generated from fumarate by FR (Tielens and Van Hellemond 1998). Besides that, it has been suggested the possible role of FR in hydrogen peroxide formation in *T. cruzi* (Denicola-Seoane et al. 1992) so the increase in O_2 consumption at the stationary phase could be a result of ROS formation. But, H_2O_2 production in *T. cruzi* is in the order of pmols (Fig. 8b) and (Carranza et al. 2009), which is much lower than O_2 consumption rates (nmols, Fig. 1). So, the contribution of H_2O_2 formation due to O_2 consumption would be unreasonable in this case.

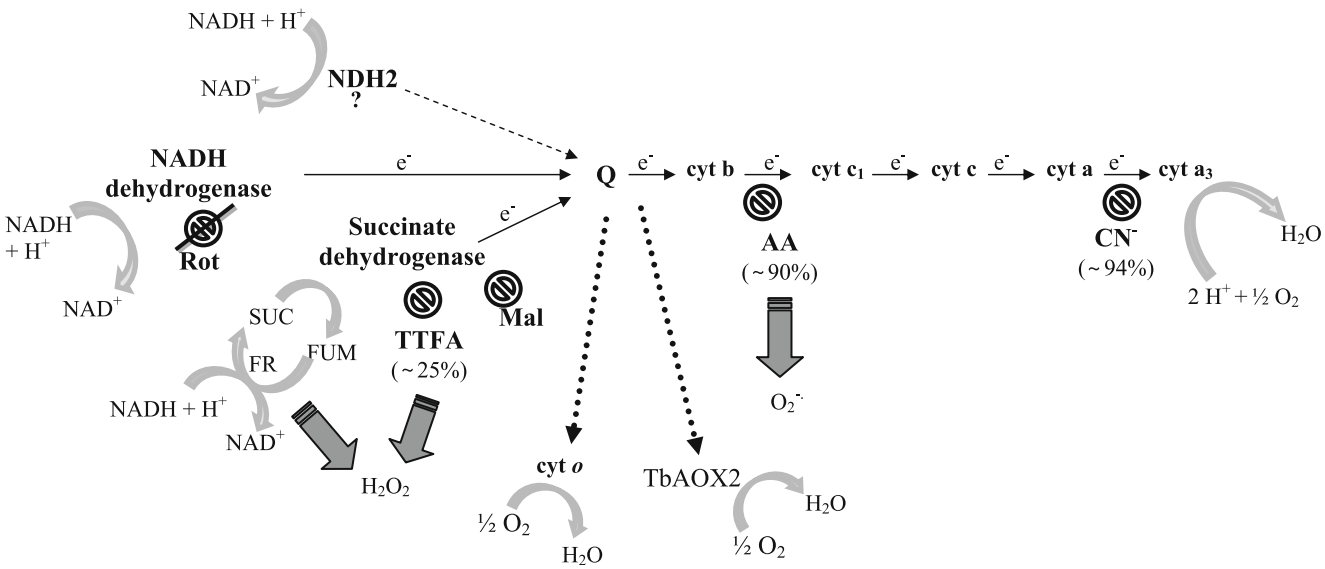

Fig. 9 The mitochondrial electron transport (MRC) system in *Trypanosoma cruzi* epimastigotes – Abbreviations: Rotenone (Rot), Succinate (SUC), Fumarate (FUM), Fumarate reductase (FR), thenoyltrifluoroacetone (TTFA), Malonate (Mal), antimycin A (AA), cyanide (CN^-), TbAOX2, ubiquinone 9 (Q) and cytochromes (cyt). Symbols: impairment (⊘) or no effect (⊘) on the MRC

In summary, tacking into account our results and what have been reported in the literature, in Fig. 9 is shown that in *T. cruzi* epimastigotes the classical components of the MRC are present and electron flow through cytochrome oxidase is the main one (Affranchino et al. 1986; Opperdoes and Michels 2008; Tielens and Van Hellemond 1998). NADH + H^+ can be oxidized by two known pathways, i.e., through the MRC or fumarate reductase (Denicola-Seoane et al. 1992) or even from another NADH-dehydrogenase as NDH2 described for *T. brucei* (Fang and Beattie 2002). Three terminal oxidases can be considered: (1) cytochrome oxidase; (2) cytochrome *o* (reviewed in Engel et al. 1990) and (3) a homolog of *Trypanosoma brucei* alternative oxidase (TbAOX2) (Chaudhuri et al. 2006). The last two appear to contribute to a less extent than cytaa_3 to O_2 consumption once CN^- promotes ~94% MRC impairment. In relation to ROS release by the MRC complexes, AA inhibits complex III by ~90% leading to superoxide anion release, while TTFA inhibiting complex II by ~25% leads to H_2O_2 release as well as FR (Denicola-Seoane et al. 1992). Further experimental evidence will be required to clear up the remaining doubts about the relative contribution of the NADH-consuming enzymes in the maintenance of *T. cruzi* mitochondrial redox balance.

Our results shed light into *T. cruzi* epimastigotes' MRC raising some important questions of unidentified O_2 consumption sources that could be not only contributing to these rates but also for ROS release. Also, since important data was obtained at the stationary phase, and could be an important event for transformation into the metacyclic form, this deserves further attention.

Acknowledgments Laura Hulshof for helping us with the initial H_2O_2 release experiments. The Brazilian agencies FAPESP and Capes for financial support and fellowship, respectively.

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