This article has been accepted for publication in FEMS Microbiology Letters
Published by Oxford University Press.

FEMS Microbiology Letters, Volume 312, Issue 2, November 2010, Pages 133–141
https://doi.org/10.1111/j.1574-6968.2010.02109.x

Research Letters

The *Trypanosoma cruzi* proteins TcCox10 and TcCox15 catalyze the formation of heme A in the yeast *Saccharomyces cerevisiae*

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Editor: Albert Descoteaux

Keywords: *Trypanosoma cruzi*, heme A, cytochrome *c* oxidase, mitochondrial proteins, Chagas' disease

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Abstract

Trypanosoma cruzi, the etiologic agent for Chagas' disease, has requirements for several cofactors, one of which is heme. Because this organism is unable to synthesize heme, which serves as a prosthetic group for several heme proteins

(including the respiratory chain complexes), it therefore must be acquired from the environment. Considering this deficiency, it is an open question as to how heme A, the essential cofactor for eukaryotic CcO enzymes, is acquired by this parasite. In the present work, we provide evidence for the presence and functionality of genes coding for heme O and heme A synthases, which catalyze the synthesis of heme O and its conversion into heme A, respectively. The functions of these T. cruzi proteins were evaluated using yeast complementation assays, and the mRNA levels of their respective genes were analyzed at the different T. cruzi life stages. It was observed that the amount of mRNA coding for these proteins changes during the parasite life cycle, suggesting that this variation could reflect different respiratory requirements in the different parasite life stages.

Introduction

Trypanosomes are parasitic protists that cause significant human and animal diseases worldwide (Barrett et al., 2003). In particular, Trypanosoma cruzi, the etiological agent of Chagas' disease, is a kinetoplastid parasite with a complex life cycle. It cycles between invertebrate and vertebrate hosts, presenting several developmental stages and adapting its metabolism to changing nutrient availability [epimastigotes and metacyclic trypomastigotes in the insect vector and amastigotes and trypomastigotes in the mammalian host (Brener, 1973; Almeida-de-Faria et al., 1999)]. Trypanosoma cruzi, like other trypanosomatids, has requirements for several essential cofactors, one of which is heme. Biochemical studies have demonstrated the absence of a complete heme biosynthetic pathway (revisited in Korený 2010). This fact was corroborated by the absence of the conserved pathway in its genomic sequence (El-Sayed et al., 2005). Hence, these trypanosomatids are dependent on the uptake of this compound from their environment. After being imported, heme is transported and inserted into target proteins, which are distributed throughout different subcellular compartments. The mitochondrion is one of the most relevant heme-protein-containing organelles, and it includes the respiratory chain complexes. One characteristic of these parasites is their single and usually well-developed

mitochondrion, which presents functional and structural changes depending on the stages of its life cycle (de Souza et al., 2009). The presence of electron transport from complex II to complex IV has been demonstrated, but the contribution of complex I (NADH : ubiquinone oxidoreductase) to energy metabolism remains controversial (Opperdoes & Michels, 2008; Carranza et al., 2009).

Biochemical studies developed in T. cruzi epimastigotes showed that the main terminal oxidase is the aa3 type (Affranchino et al., 1986), the canonical cytochrome c oxidase for eukaryotic cells. Additionally, proteomic studies demonstrated the presence of subunits of complex IV (cytochrome c oxidase, CcO enzyme), other components of the respiratory chain and subunits of the FoF1 ATPase (complex V) (Parodi-Talice et al., 2007; Ferella et al., 2008).

In nonphotosynthetic eukaryotic cells, the complete heme synthetic pathway starts and finishes in the mitochondria. Trypanosoma cruzi lacks the heme biosynthetic route, and the transport and distribution of this cofactor are uncharacterized. One interesting open question is how heme A, the essential cofactor for eukaryotic CcO enzymes, is synthesized in this organism. In eukaryotic cells, heme A biosynthesis proceeds in the mitochondria. It is catalyzed by two enzymes, heme O synthase (HOS or Cox10) and heme A synthase (HAS or Cox15), which are both integral to the mitochondrial inner membrane (Barros & Tzagoloff, 2002). HOS catalyzes the synthesis of heme O by the conversion of the vinyl group on pyrrole ring A in heme B into a 17-hydroxyethylfarnesyl moiety. HAS catalyzes the oxidation of the methyl group on pyrrole ring D into an aldehyde, converting heme O into heme A.

In this work, we present the first functional characterization of enzymes involved in heme A biosynthesis from T. cruzi (herein named TcCox10 and TcCox15). Furthermore, we show that the genes encoding TcCox10 and TcCox15 are differentially transcribed during the parasite life cycle.

Materials and methods

Strains and culture media

Bacterial and yeast strains

Escherichia coli strains used for all cloning procedures were grown at 37 °C in Luria–Bertani medium supplemented with ampicillin (100 μg mL^{-1}) as necessary.

The wild-type (WT) Saccharomyces cerevisiae yeast strain used in this study was DY5113 (W303) MATa ade2-1 his3-1, 15 leu2-3, 112 trp1_, ura3-1, a generous gift from Dennis Winge (University of Utah). Strains with the ORF deletions Δcox10 and Δcox15 were generated for this work from DY5113 strains by homologous recombination with KanMX4 disruption cassettes (Wach et al., 1994): Δcox10 ::KanMX4 and Δcox15 ::KanMX4, respectively. These deletions were confirmed by PCR.

Yeast strains were transformed using lithium acetate (Gietz & Woods, 2002). The cells were grown either in a rich medium (YP, 1% yeast extract, 2% peptone) or in a synthetic complete (SC) medium lacking the appropriate nutrients for plasmid selection. Glucose 2% (Glc), galactose 2% (Gal) and/or glycerol 3%–ethanol 2% (Gly–EtOH) were used as carbon sources.

The respiratory competence of the strains was determined using growth tests on plates containing 2% glucose or 2% glycerol–3% ethanol as carbon sources, which were incubated at 30 °C for 3–5 days.

Cells and parasites

The Chinese hamster ovary cell line CHO-K_1 was routinely cultivated in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 0.15% (w/v) $NaHCO_3$ at 37 °C in a humid atmosphere containing 5% CO_2. Epimastigotes of T. cruzi, the CL strain, clone 14, were maintained in the mid-log phase by passages through liver infusion-tryptose medium supplemented with 10% FCS at 28 °C (Camargo, 1964). Intracellular forms (amastigotes) and trypomastigotes were obtained as described previously (Almeida-de-Faria et al., 1999; Silber et al., 2009). Metacyclic trypomastigotes were obtained via in vitro differentiation of epimastigote cells in the stationary phase (de Sousa, 1983) and then transferred to Grace's insect cell culture medium (pH 6.0 without FCS addition) (Gibco, Invitrogen). The purity of all the forms obtained as well as their viability were evaluated by microscopic observation.

Cloning of T. cruzi HOS and HAS coding sequences

The T. cruzi cds of HOS (Tc00.1047053509601.59/Tc00.1047053509767.59, hereafter named TcCOX10A and TcCOX10B, respectively) and HAS (Tc00.10470535112l1.70, hereafter named TcCOX15) were amplified by PCR using genomic DNA obtained from epimastigotes of the CL Brener strain. The primers listed below were designed to introduce the restriction sites BamHI or XbaI at the 5′-end and XhoI-3′ and a 3′-6xHis epitope tag.

FP.TcCOX15.XbaI: 5′-GCTCTAGAATGTTGCGATTCAGGCCGC-3′;
FP.TcCOX15.BamHI: 5′-GCGGATCCATGTTGCGATTCAGGCCGC-3′;
RP-TcCOX15-XhoI:
5′-CCGCTCGAGTTAATGGTGATGGTGATGATGACCGATAACGGTCCAAATACCA
AG-3′; FP-TcCOX10-XbaI: 5′-GCTCTAGAATGATCCGACGAGCCCTTC-3′;
FP.TcCOX10.BamHI: 5′-GCGGATCCATGATCCGACGAGCCCTTC-3′;
RP-TcCOX10-XhoI: 5′-
CCGCTCGAGTTAATGGTGATGGTGATGATGACCTGTGAGCTTCCAAGGGCGT-3′
(the restriction sites are in bold). The primers designed for TcCOX10 allowed the indistinguishable amplification of two genes: TcCOX10A and TcCOX10B. PCR

products were cloned into pGEM T-easy vectors (Promega) and sequenced to verify the amplified TcCOX10 and TcCOX15 cds. Later, TcCOX15 and TcCOX10 ORFs with a 3′-His6 epitope tag were cloned into pRS426 under the control of the MET25 promoter and the CYC1 terminator (p426.MET25) (Mumberg et al., 1994), or into pVTU101 under the ADH1 promoter and terminator sequences (Vernet et al., 1987).

In silico analysis

Sequences from the 'Tritryps' genome projects were obtained at GeneDB (http://www.genedb.org/) and TriTrypDB (http://tritrypdb.org/tritrypdb/) (Aslett et al., 2010). For the amino acid multiple sequence alignment, the CLUSTALW 2.0.12 software was used (Thompson et al., 1994). The sequences for HOS were as follows: T. cruzi CL Brener Non-Esmeraldo-like Tc00.1047053509601.59 (XP_814788.1), T. cruzi CL Brener Esmeraldo-like Tc00.1047053509767.59 (XP_817285.1), Leishmania major LmjF23.1520 (XP_001683512.1), Trypanosoma brucei Tb927.5.1310 (XP_844805.1) and the S. cerevisiae Cox10 protein (Ypl172cp, NP_015153.1). The sequences for HAS were as follows: T. cruzi CL Brener Esmeraldo-like Tc00.10470535112l1.70 (XP_817728.1), T. brucei Tb11.01.3780 (XP_829257.1), L. major LmjF28.2680 (XP_001684554.1) and the S. cerevisiae Cox15 (Yer141wp, NP_011068.1).

The transmembrane domain predictions for TcCox10 and TcCox15 were generated using the software for topology prediction TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/)

Mitochondrial purification and activity assays

Intact yeast mitochondria were isolated from yeast grown in a synthetic or a rich medium as described previously (Diekert et al., 2001). The standard Bradford assay was used to determine the total mitochondrial protein concentration (Bradford, 1976). Experimental details are included in the Supporting Information.

Mitochondrial protein membrane extraction and heme spectral analysis

Mitochondria at a protein concentration of 2–5 mg mL^{-1} were suspended in 50 mM Tris : HCl, pH 8, and were extracted with 1% sodium deoxycholate under conditions that quantitatively solubilize all the cytochromes (Tzagoloff et al., 1975). Difference spectra of the extracts reduced with sodium dithionite and oxidized with potassium ferricyanide were recorded at room temperature in a Jasco V550 spectrophotometer. The α absorption bands corresponding to cytochromes a and a_3 have maxima at about 605 nm. The corresponding maximum for cytochrome b is 560 nm and that for cytochrome c it is 550 nm.

Immunoblotting

Mitochondrial protein samples were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Proteins recognized by specific antibodies were visualized using enhanced chemiluminescence (ECL Plus) reagents (Amersham GE).

Oxygen consumption

The oxygen consumption of cells grown to the stationary phase was determined using a Clark electrode connected to a 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co.). The rate of oxygen consumption (μmolO_2 min^{-1} OD600 nm^{-1}) was calculated from the linear response as described previously (Bestwick et al., 2010). Experimental details are included in the Supporting Information.

Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Total RNA was obtained from different T. cruzi stages and CHO-K_1 cells as a control using TriZOL® reagent (Invitrogen, Lithuania). The RNA preparations were treated with RNase-free DNase I (Fermentas, Life Sciences) and checked following standard procedures (Sambrook & Russell, 2001). Each RNA extraction was carried out in triplicate. cDNAs of T. cruzi or CHO-K_1 cells (used as a control) were synthesized

through an RT reaction (Superscript III™, Invitrogen) using 5 µg of total RNA. Real-time PCR quantitative mRNA analyses were performed in a Mastercycler® ep realplex (Eppendorf, Germany) using the SYBRgreen fluorescence quantification system (Fermentas, Lithuania). The standard PCR conditions were: 95 °C (10 min), and then 40 cycles of 94 °C (1 min), 60 °C (1 min) and 72 °C (2 min), followed by the denaturation curve. The primer designs were based on nucleotide sequences of T. cruzi genes coding for TcCOX10, TcCOX15 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession numbers for TcCOX10: XM_812192.1– Tc00.1047053509767.59, and XM_809695.1– Tc00.1047053509601.59; TcCOX15: XM_812635.1– Tc00.1047053511211.70, and GAPDH: AI007393). The sequences of the primers used are listed below. The primers designed for TcCOX10 are able to recognize both cds. The data were analyzed using REALPLEX v1.5 software. The fold-change in the expression of the transcripts was obtained using the comparative method ($\Delta\Delta C_t$) (Bookout et al., 2006). The epimastigote stage was used as the reference stage for both genes.

Primers for qRT-PCR: TcCOX10-forward 5′-AGATGAAGCGAACCTGTCGT-3′, TcCOX10-reverse 5′-AACCACAAGCTCCAAACCAC-3′ (product 89 bp); TcCOX15-forward 5′-ACCACCTTCTTGTGGTGGAG-3′, TcCOX15-reverse 5′-CAATCCCAAAATGGAAATGG-3′(product 113 bp) and GAPDH-forward 5′-GTGGCAGCACCGGTAACG-3′, GAPDH-reverse 5′-CAGGTCTTTCTTTTGCGAAT-3′(product 110 bp).

The differences in the transcriptional level among the different stages were compared using Student's t-test. For this purpose, the software GRAPHPAD PRISM version 5.00 for Windows (GraphPad Software, San Diego, CA) was used. The significance level (P value) was determined with a confidence interval of 95% in a two-tail distribution. Detailed information is included in the Supporting Information.

Results and discussion

Trypanosoma cruzi is auxotrophic for heme, which is an indispensable cofactor for the biogenesis of cytochromes and other heme enzymes involved in crucial biological processes. The cytochrome c of T. *cruzi*, an important mitochondrial heme

protein, shows different properties compared with cytochrome c from other organisms. In trypanosomatids, heme is attached via only one covalent bond and none of the known cytochrome c biogenesis proteins have been identified from their genomic sequences. Several authors proposed a distinct mitochondrial pathway for cytochrome c biosynthesis in trypanosomatids (Fulop et al., 2009). Based on these data, we evaluated how heme A is synthesized by T. cruzi (and the other trypanosomatids).

Identification of cds for HOS and HAS in the T. cruzi genomic sequence

The coding sequences for putative proteins homologous to HOS and HAS have been identified in the *T. cruzi* genome. One cds, Tc00.1047053511211.70, was identified as a HAS homologue (named *TcCOX15* and TcCox15 for the corresponding protein). Two cds were associated with HOS (Tc00.1047053509601.59 and Tc00.1047053509767.59) presenting a sequence identity of 98% (named *TcCOX10A* and *TcCOX10B*, and TcCox10 A and B for the corresponding protein sequences). The predicted protein sequences [TcCox10 (A and B) and TcCox15] show about 52% and 56% homology and 37% and 41% identity to their *S. cerevisiae* orthologues, and they are also conserved in other trypanosomatids (Fig. 1). The multiple sequence alignment of HOSs includes the available trypanosomatid putative protoheme IX farnesyltransferase (HOS) and the *S. cerevisiae* Cox10 protein (Fig. 1a). The residues N196, R212, R216 and H317 (*S. cerevisiae* numbering), which are involved in the protein's function (Bestwick et al., 2010), are conserved in trypanosomatid sequences (indicated in Fig. 1a). The multiple sequence alignment of HAS proteins includes the available trypanosomatid putative HAS enzymes and the *S. cerevisiae* Cox15 protein (Fig. 1b). The alignment shows that residues involved in HAS activity based on studies from the *Bacillus subtilis* CtaA enzyme are also conserved in trypanosomatid sequences (Barros et al., 2001; Hederstedt et al., 2005). Figure 1b shows the residues H169, H245 and H393 from *S. cerevisiae* numbering, which correspond to CtaA H60, H123 and H216, respectively. Both *T. cruzi* putative proteins present eight predicted TMs, which is compatible with this type of protein (Fig. 1).

1.

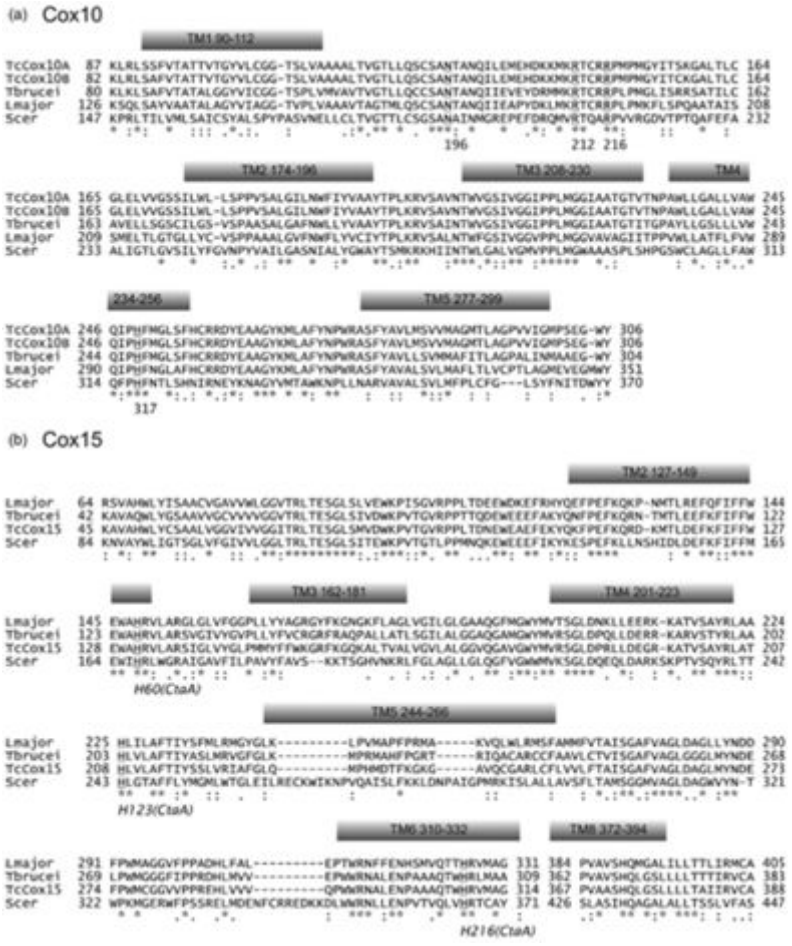

Fig. 1 Aligned sequences of Cox10 and Cox15 proteins. The multiple sequence alignments were performed using CLUSTALW 2.0.12 software (Thompson et al., 1994). The amino acid identities and similarities are marked by asterisks and dots, respectively. The transmembrane helices were predicted by TMHMM 2.0 software (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and they are shown as gray boxes above the amino acid sequences; the residue numbers included in the boxes correspond to the Trypanosoma cruzi sequence. The multiple sequence alignment for the predicted Cox10s belonging to T. cruzi [TcCox10A (Tc00.1047053509601.59) and TcCox10B (Tc00.1047053509767.59)], Trypanosoma brucei (Tb927.5.1310), Leishmania mayor (LmjF23.1520) and Saccharomyces cerevisiae are shown in (a). The conserved residues involved in the catalytic activity of HOS enzyme (Bestwick et al., 2010) are underlined and identified following the S. cerevisiae numbering. The multiple sequence alignment for the predicted Cox15s belonging to T. cruzi, TcCox15 (Tc00.1047053511211.70), T. brucei (Tb11.01.3780), L. mayor (LmjF28.2680) and S. cerevisiae are shown in (b). The conserved residues involved in the catalytic activity of HAS enzymes are underlined and identified following the numbering of the CtaA enzyme from Bacillus subtilis (Glerum et al., 1997; Hederstedt et al., 2005).

Cloning of TcCOX10 and TcCOX15 and their functional evaluation in yeast Δcox10 and Δcox15 knockout cells

The cds for *TcCOX10* and *TcCOX15* were amplified by PCR using total genomic DNA as the template and introducing a 3′-coding sequence for a 6xHis tag. As *TcCOX10* A and B cds show 98% identity, the primers designed recognize both of them equally. The amplified product for *TcCOX10* coincided with the Tc00.1047053509601.59 (*TcCOX10A*) sequence, and is named *TcCOX10* and TcCox10 hereafter for the corresponding protein. Both cds (*TcCOX10* and *TcCOX15*) were subcloned into yeast expression vectors and used to transform yeast cells lacking the corresponding genes (Δ*cox10* and Δ*cox15*). These knockout cells present a respiration-deficient phenotype due to the absence of heme A production and consequently a functionally inactive C*c*O complex (Nobrega et al., 1990; Glerum et al., 1997). This deficiency impairs the growth in a nonfermentable carbon source such as glycerol–ethanol, but they all can grow in a media containing a fermentable carbon source as glucose. Their respiratory function was restored once *TcCOX10A.6xHIS* or *TcCOX15.6xHIS* was expressed in Δ*cox10* or Δ*cox15*, respectively (Fig. 2a). Both mutants were also transformed with plasmids containing the corresponding *S. cerevisiae* genes, Δ*cox10* with *ScCOX10.6xHIS* and Δ*cox15* with *ScCOX15.6xHIS*, as positive controls. Using two different expression vectors (see Materials and methods), the same phenotype suppression was observed, demonstrating that *T. cruzi* sequences are able to complement yeast respiratory deficiencies.

2.

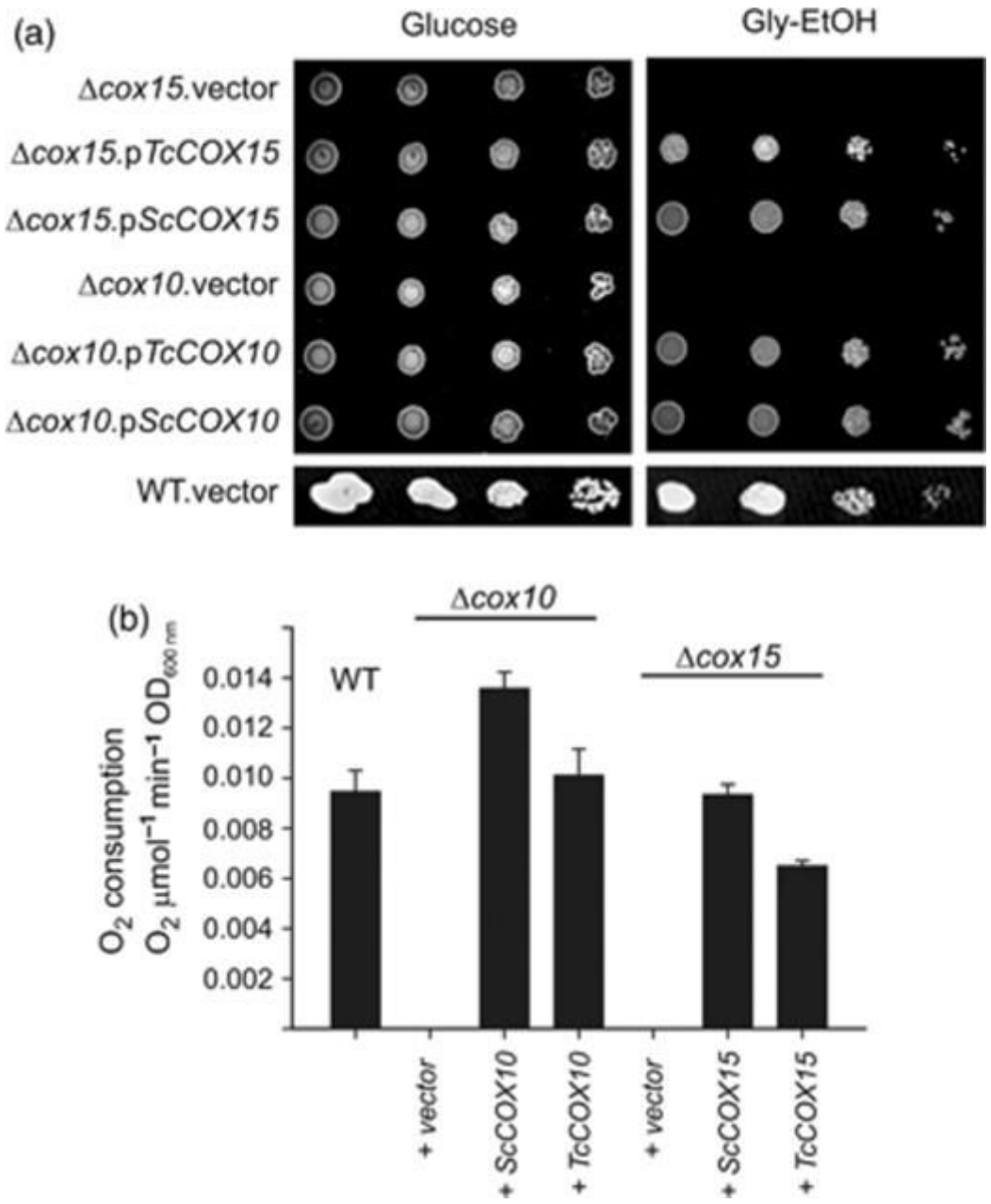

Fig. 2. Evaluation of the respiratory capacity of Δ*cox10* and Δ*cox1*, yeast knockout cells transformed with plasmids containing the *Trypanosoma cruzi* cds for *COX10* and *COX15*, respectively. (a) WT.vector: DY5113 wild type transformed with a p426.MET25 vector; Δ*cox10*.vector and Δ*cox15*.vector: knockout cells transformed with a p426.MET25 vector; Δ*cox10.*p*TcCOX10*, Δ*cox10.*p*ScCOX10*: Δ*cox10* transformed with plasmid p426.MET25 expressing either *TcCOX10.HIS* or *ScCOX10.HIS*, respectively; Δ*cox15.*p*TcCOX15*, Δ*cox15.*p*ScCOX15*: Δ*cox15* transformed with plasmid p426.MET25 expressing either *TcCOX15.HIS* or *ScCOX15.HIS*, respectively. All the plasmids encode these proteins as a C-terminal His-tag fusion protein. The transformed cells were grown in a SC selective (-URA) medium with 2% glucose overnight, serially diluted and spotted onto plates containing

glucose (SC–URA 2% glucose) or gly-EtOH (SC–URA 3% glycerol–2% ethanol) as the carbon source. The plates were incubated at 30°C for 3 days for the glucose medium and 5 days for the gly-EtOH medium. (b) The cells described in (a) were grown in SC (-URA) 2% galactose liquid medium overnight, washed and suspended in 3% glycerol. The oxygen consumption (ìmol O_2 min^{-1} $OD_{600\ nm}$−1) was measured using a Clark electrode. The data presented are the average of three independent replicates, and the error bars represent the SE of the mean.

To confirm these results, the oxygen consumption of WT, Δ*cox10*, Δ*cox15* yeast strains and their corresponding transformants was measured (Fig. 2b). As expected, the knockout cells were impaired in O_2 consumption due to their inability to produce heme A and consequently fully active C*c*O. The respiratory function was restored with the expression of the corresponding *T. cruzi COX10* and *COX15* genes, as well as with the *S. cerevisiae COX10* and *COX15* genes. Taken together, these results demonstrate that *TcCOX10* and *TcCOX15* encode HOS and HAS enzymes that are functional in the yeast model.

In order to verify the function of these proteins in heme A biosynthesis, the mitochondrial heme level was evaluated by differential absorption spectroscopy as described previously (Tzagoloff et al., 1975). The reduced minus oxidized spectra of mitochondrial cytochromes were recorded and are presented in Fig. 3a. The spectra of the knockout cells only exhibited signals corresponding to heme *b* and heme *c*, and the heme *a* signal was absent, confirming the deficiency of its biosynthesis (Nobrega et al., 1990; Glerum et al., 1997). The spectrum recorded from the mitochondria of WT cells displayed bands corresponding to heme *a*, heme *b* and heme *c*. The expression of *TcCOX10* in Δ*cox10* and *TcCOX15* in Δ*cox15* allowed the recovery of the heme *a* signal, reflecting the role in heme A synthesis of the TcCox10 and TcCox15 proteins as HOS and HAS enzymes, respectively.

3.

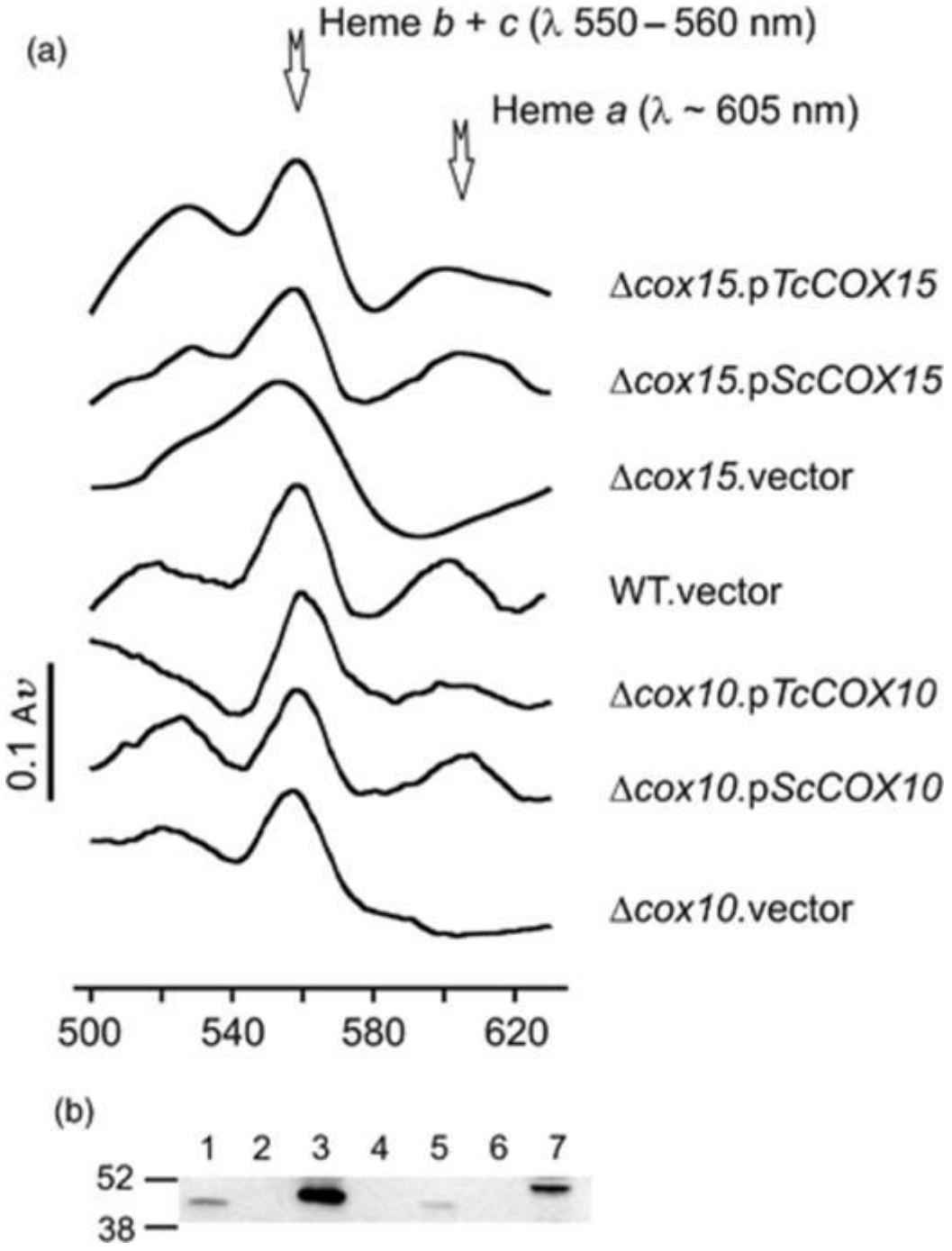

Fig. 3. Analysis of HOS and HAS activity in mitochondrial fractions. Mitochondrial fractions were obtained from the transformed yeast cells WT.vector, Δ*cox10*.vector, Δ*cox10*.p*TcCOX10*, Δ*cox10*.p*ScCOX10*, Δ*cox15*.vector, Δ*cox15*.p*TcCOX15* and Δ*cox15*.p*ScCOX15* as described previously (Diekert et al., 2001). (a) Absorption spectra of mitochondrial cytochromes. Mitochondria from the cells mentioned above were suspended in 50 mM Tris : HCl pH 8 and were extracted with sodium deoxycholate at final protein concentrations of 2–5 mg mL^{-1} as described previously (Tzagoloff et al., 1975). The extracts

were divided into two halves, one of which was oxidized with potassium ferricyanide and the other was reduced with sodium dithionite. The difference spectra were recorded using a Jasco V550 spectrophotometer at room temperature. (b) Western blot analysis of the Cox10 and Cox15 His-tagged proteins. The total proteins of the mitochondrial fractions were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and reacted with anti-His antibodies. The molecular weights of 38 and 52 kDa are marked at the left of the blot. The lanes correspond to (1) Δ*cox15*.p*TcCOX15*; (2) Δ*cox15*.vector; (3) Δ*cox15*.p*ScCOX15*; (4) WT.vector; (5) Δ*cox10*.p*TcCOX10*; (6) Δ*cox10*.vector; and (7) Δ*cox10*.p*ScCOX10*.

The protein levels of Cox10 and Cox15 were evaluated using Western blot analysis of yeast mitochondria. All these proteins (from *S. cerevisiae* and *T. cruzi*) were expressed as C-terminal his-tag fusion proteins (Fig. 3b). As expected, the proteins were detectable in the cells transformed with the plasmids expressing Tc*COX10.6xHIS*, Sc*COX10.6xHIS*, Tc*COX15.6xHIS* and/or Sc*COX15.6xHIS*, and they were not detectable in the WT, Δc*ox10* or Δ*cox15* cells transformed with control vectors. The signals detected at around 38–45 kDa were consistent with the apparent molecular weight expected for TcCox10 and TcCox15 proteins based on their primary sequences (for TcCox10 388 aa, 42 kDa and for TcCox15 396 aa, 44 kDa, both molecular weights were estimated for the preprotein without the C-terminal tag, TriTrypDB, http://tritrypdb.org/tritrypdb/).

In both cases, the band intensity of the *T. cruzi* proteins was always lower compared with the *S. cerevisiae* ones. Several factors could be involved in this observation: (1) the different mitochondrial targeting sequence [shorter in trypanosomatids (Hausler et al., 1997)] resulted in less efficient mitochondrial importation; (2) the lower stability of the *T. cruzi* proteins compared with the *S. cerevisiae* proteins in the yeast model; (3) different processing that the *T. cruzi* proteins were subjected to, which might include cleavage of the C-terminal region (which includes the his tag sequence); and (4) the yeast cells might accumulate extra amounts of ScCox10 and/or ScCox15 proteins, but might not do so for the *T. cruzi* ones, which may be more exposed to protease attack and degraded faster. The results obtained here did not differentiate between these hypotheses, but they allowed us to postulate that the amount of *T. cruzi* proteins detected was sufficient to restore the respiratory capability of yeast mutants to WT levels, recovering the biosynthesis of heme A.

Analysis of mRNA levels at different life stages of T. cruzi

Type *aa*3 cytochrome *c* oxidase was identified as the main terminal oxidase in epimastigotes, and the heme A signal was detected in epimastigotes using differential absorption spectroscopy (Stoppani et al., 1980; Affranchino et al., 1986). In addition, we showed that *TcCOX10* and *TcCOX15* sequences encode for functional HOS and HAS proteins in the yeast model. In order to find out whether the *TcCOX10* and *TcCOX15* genes are being differentially transcribed during the life cycle, their mRNA levels were quantified by qRT-PCR at different life stages. We observed that both genes were transcribed, and the data obtained showed that *TcCOX10* mRNA (Fig. 4a) varied more than *TcCOX15* mRNA (Fig. 4b) during the life cycle. However, in amastigotes, we observed that the amount of mRNA for both genes was significantly lower compared with the other stages (P <0.05 for all comparisons between amastigotes and every other stage for both genes, see Supporting Information, Appendix S1). Little is known about the metabolic changes that occur when the parasite invades host cells. A recent study showed that when the parasite differentiates into an amastigote in the host cell cytoplasm, a metabolic switch occurs (Silber et al., 2009). It is possible that the differences observed in *TcCOX10* and *TcCOX15* gene expression could be related to the metabolic adaptation afforded by the parasite, reflecting alterations in respiratory requirements in the different life stages.

4.

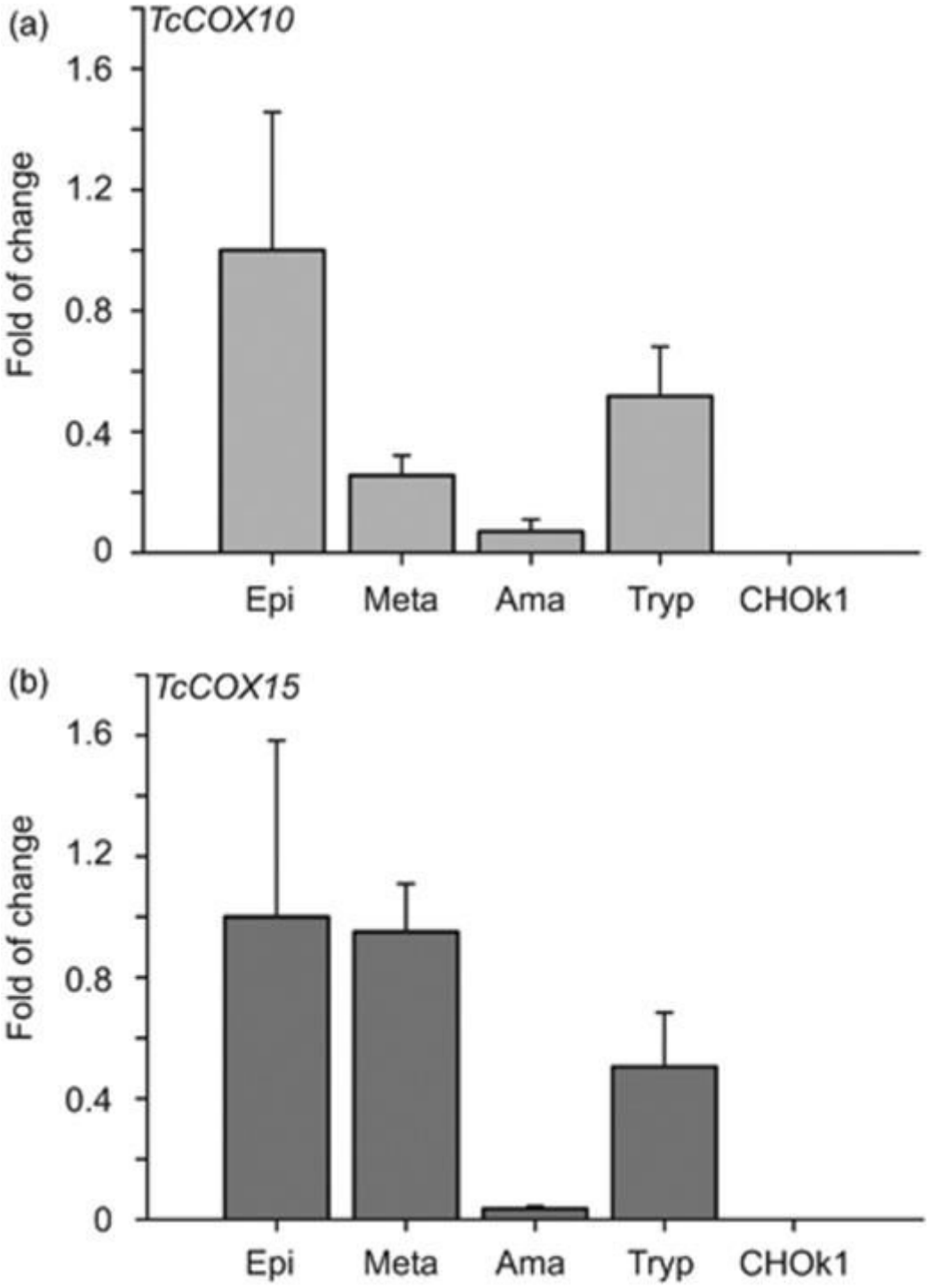

Fig. 4. Analysis of the abundance of *TcCOX10* and *TcCOX15* mRNAs during the different life stages of *Trypanosoma cruzi*. qRT-PCR was performed as described in Materials and methods for the life stages of *T. cruzi*: Epi (epimastigote), Meta (metacyclic trypomastigote), Ama (amastigote) and Tryp (blood trypomastigote). The cell line CHO-K_1 was used as a control. The fold change in mRNA is shown in (a) for *TcCOX10* and in (b) for *TcCOX15*. These data are representative of at least three biological repeats; the error bars indicate the SD.

Although mRNA quantification is not a direct measure of protein level or function, it is capable of reflecting a direct relationship. In a recent study, Wang (2009) described the relationship between *S. cerevisiae COX10* and *COX15*, proposing that these enzymes might play another unidentified role besides heme A biosynthesis.

These results confirmed the expression of the genes encoding TcCox10 and TcCox15 enzymes from *T. cruzi* at different life stages. Notwithstanding, complementary studies are necessary to discern whether Cox10 and Cox15 could have another physiological function in *T. cruzi*.

In conclusion, *T. cruzi* metabolism must adapt to different environments during its life cycle in which the parasite is under different nutritional pressures. It presents auxotrophies for various cofactors, including heme. Heme is indispensable for the biogenesis of cytochromes and other enzymes that play crucial biological roles. In this study, we demonstrated that the *T. cruzi* cds *TcCOX10* and *TcCOX15* code for HOS and HAS enzymes that are functionally active in yeast cells. Mitochondrial targeting sequences are highly conserved through evolution, and even though the sequences reported for trypanosomatids are shorter than the ones in other cells, including yeast (Hausler et al., 1997), our results showed that the *T. cruzi* sequences for Cox10 and Cox15 were recognized by the yeast mitochondrial importing machinery. These sequences were imported and properly folded to produce active enzymes in the yeast mitochondria.

The observed changes in the mRNA levels of *TcCOX10* and *TcCOX15* could be a form of regulation reflecting differences in respiratory requirements at different life stages. In order to test these hypotheses and to address how *T. cruzi* transports heme into the mitochondrion, we are working to expand our studies on this system.

Acknowledgements

We are grateful to Prof. Dennis Winge and Eric L. Hegg for the yeast plasmids and strains. This study was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). J.A.C. is a member of the carrier of scientific investigator of CONICET (Argentina). A.M.S. and B.A.S.M. are indebted to Fundacão de Amparo à Pesquisa do Estado de São Paulo (FAPESP, project #08-57596-4) and to CNPq (Project #473906/2008-2). A.M.S. is a fellow from CNPq and a member of the Instituto Nacional de Biotecnologia Estrutural e Química Medicinal em Doenças Infecciosas, INBEQMeDI (Brazil).

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Supporting Information

Additional Supporting Information may be found in the online version of this article https://doi.org/10.1111/j.1574-6968.2010.02109.x :

Appendix S1. The Trypanosoma cruzi proteins TcCox10 and TcCox15 catalyze the formation of heme A in the yeast Saccharomyces cerevisiae