UNIVERSITY OF SÃO PAULO
SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

**Structural and functional characterization of *Trypanosoma cruzi*
fumarate hydratase isoforms**

Ricardo Augusto Pereira de Pádua

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Structural and functional characterization of *Trypanosoma cruzi*
fumarate hydratase isoforms

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Physics

Candidate: Ricardo Augusto Pereira de Pádua

Supervisor: Prof. Dr. Maria Cristina Nonato Costa

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Inscription

Aos meus pais, Benedito e Vera, à minha irmã Lígia e à minha
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Epigraph

"I do not think there is any thrill that can go through the human heart like that felt by the inventor as he sees some creation of the brain unfolding to success..."

- Nikola Tesla

RESUMO

PEREIRA DE PÁDUA, R. A. **Caracterização estrutural e funcional das isoformas da enzima fumarato hidratase de *Trypanosoma cruzi***. 119 f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2014.

Trypanosoma cruzi é um protozoário flagelado que ao infectar seres humanos causa a doença de Chagas, uma doença tropical negligenciada que afeta milhões de pessoas no mundo todo. As fumarato hidratases (FH), ou fumarases, são enzimas que catalisam a reação estéreo-específica reversível de hidratação do fumarato em S-malato, e foram recentemente consideradas essenciais para a viabilidade do parasito *Trypanosoma brucei*, sugerindo seu potencial como alvo macromolecular para o desenvolvimento de novos fármacos tripanocidas. O presente trabalho visou à caracterização funcional, bioquímica, biofísica e estrutural das fumarases de *T. cruzi* (TcFHs) e humana (HsFH) de forma a avaliar o papel das TcFHs para o parasito *Trypanosoma cruzi*, mapear o mecanismo de ação e identificar as diferenças entre TcFHs e a enzima humana de forma a serem exploradas no planejamento de inibidores seletivos às fumarases do parasito. Análise das sequências mostrou que TcFHs pertencem à classe I das fumarases (enzimas diméricas dependentes de ferro) e não são homólogas à HsFH que pertence a classe II (tetraméricas independentes de ferro). Estudos de localização celular confirmaram a existência de duas fumarases em *T. cruzi*, uma citosólica (TcFHC) e uma mitocondrial (TcFHM), e experimentos de nocaute gênico sugeriram que essas enzimas são essenciais para o parasito. A caracterização cinética das enzimas TcFHC, TcFHM e HsFH mostrou que as fumarases de *T. cruzi* são sensíveis ao oxigênio enquanto a enzima humana se mantém ativa em condições aeróbicas. Estudos de ressonância eletrônica paramagnética mostraram a presença de um cluster de ferro-enxofre, sensível a oxidação por oxigênio, envolvido no mecanismo enzimático das enzimas TcFHs. Modelos estruturais das TcFHs, construídos por homologia à estrutura cristalográfica da fumarase de *Leishmania major*, foram comparados à estrutura cristalográfica obtida para a fumarase humana e as diferenças entre as duas estruturas foram utilizadas no planejamento de ligantes seletivos às fumarases do parasito. O ligante planejado inibiu a fumarase citosólica de *T. cruzi* na faixa de 1 μ M e não apresentou efeito na atividade da enzima humana. Testes *in vivo* demonstraram o efeito tripanocida do inibidor provavelmente por interferir na produção de ATP pela mitocôndria do *T. cruzi*. Os resultados obtidos com o desenvolvimento desse projeto apresentam uma proposta inovadora no desenvolvimento de novas terapias contra a doença de Chagas, o uso da enzima fumarase como alvo macromolecular, assim como apresenta um inibidor potente e seletivo para a enzima do parasito a ser utilizado como protótipo no desenvolvimento de fármacos contra *Trypanosoma cruzi*. A síntese de moléculas análogas ao inibidor de forma a melhorar suas propriedades farmacológicas encontra-se em andamento.

Palavras-chave: Fumarase, Fumarato hidratase, *T. cruzi*, doença de Chagas, inibidor seletivo, estrutura cristalográfica.

ABSTRACT

PEREIRA DE PÁDUA, R. A. **Structural and functional characterization of *Trypanosoma cruzi* fumarate hydratase isoforms.** 119 f. Thesis (Doctorate). School of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2014.

Trypanosoma cruzi is a flagellate protozoan parasite that infects humans and causes Chagas disease, a tropical neglected disease that affects millions of people worldwide. Fumarate hydratases (FH), or fumarases, are enzymes responsible for the reversible stereo-specific hydration of fumarate into S-malate, and were recently considered to be essential to *Trypanosoma brucei* viability, suggesting, therefore, a potential role for FHs as macromolecular targets to the drug development against trypanosomatids. The present work focused on the functional, biochemical, biophysical and structural characterization of *T. cruzi* fumarases (TcFHs) and human fumarase (HsFH) to evaluate TcFHs role for *T. cruzi*, map the reaction mechanism and identify and exploit differences between the parasite and host enzymes in order to design selective inhibitors to the parasite enzyme. Sequence analysis revealed that TcFHs belong to class I fumarases (dimeric and iron-sulfur containing enzymes) and are not homologous to HsFH which belongs to class II fumarases (tetrameric iron independent enzymes). Cellular sub-localization studies confirmed the presence of a cytosolic and a mitochondrial fumarases in *T. cruzi* and gene knockout experiments suggested TcFHs are essential to the parasite. The kinetic characterization showed that TcFHs activity is highly sensitive to oxygen whereas HsFH activity remained stable in aerobic conditions. Electron paramagnetic experiments further revealed the presence of an iron-sulfur cluster highly sensitive to oxidation and involved in the catalytic mechanism in both TcFHm and TcFHc. TcFHs structural models, built by homology modeling using the *Leishmania major* fumarase crystal structure as template, were compared to the HsFH crystal structure and the differences were used to design a selective ligand to the parasite fumarases. The designed ligand showed to inhibit TcFHc with an IC₅₀ of 1 µM and showed no effect on the human fumarase activity. *In vivo* assays using *T. cruzi* epimastigotes demonstrated the trypanocidal effect of the designed inhibitor probably caused by stalling ATP production. The results obtained with the development of this project represent an innovative proposal on the development of new therapies against Chagas disease, the use of fumarase enzyme as a macromolecular target, as well as present a potent and selective inhibitor to the parasite enzyme to be further used as a prototype in the development of drugs against Chagas disease. The synthesis of inhibitor analogues with optimized pharmacological properties are currently in progress.

Keywords: fumarase, fumarate hydratase, Chagas disease, selective inhibitors, crystal structure.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BLA	blasticidin resistance gene
bp	base pair
BSA	bovine serum albumine
CD	circular dichroism
DAPI	diamidino 2 phenylindole
ddH ₂ O	double distilled water
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
E1cb	elimination unimolecular conjugate base
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidiumbromide
EPR	Electron Paramagnetic Resonance
FCS	fetal calf serum
FH	fumaratehydratase (fumarase)
gDNA	genomic DNA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HYG	hygromycin resistance gene
IPTG	isopropyl β -D-1-thiogalactopyranoside
K _{cat}	enzyme turnover
kDa	kiloDalton
kDNA	kinetoplastic DNA
K _m	Michaelis constant
KO	knockout
LB	lysogenic broth
mRNA	messenger RNA
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NADH	reduced NAD
NEO	neomycin resistance gene
NZCYM	NZ amine, NaCl, yeast extract, casamino acids, magnesium sulfate
O.D. _{600nm}	optic density at 600 nm
PAC	puromycin resistance gene
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
PTS1	type 1 peroxisome targeting signal
PTS2	type 2 peroxisome targeting signal
QMUL	Queen Mary University of London
rDNA	ribosomic DNA
RNA	ribonucleicacid
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	TRIS acetate EDTA buffer
TcFHc	<i>Trypanosoma cruzi</i> cytosolic fumaratehydratase
TcFHm	<i>Trypanosoma cruzi</i> mitochondrial fumarate hydratase

TEMED	N,N,N',N'-tetramethylenediamine
TRIS	2-amino-2-hydroxymethyl-propane-1,3-diol
Tub	tubulin
ULP1	ubiquitin like protein 1
USP	University of São Paulo
UTR	untranslated region
UV	ultraviolet
v/v	volume/volume
$V_{\max}$	maximum velocity
w/v	weight/volume
WHO	World Health Organization

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Chapter 1. Initial Considerations

1.1 *Initial considerations*

Neglected Tropical Diseases (NTDs) consist of a large group of infirmities caused by a variety of pathogens such as viruses, bacteria, protozoa and helminths. Affecting more than 1 billion people, especially in poor and marginalized areas, they represent a serious burden to the public health (Liese *et al.*, 2010).

The reality is cruel when it comes to NTDs. More than sad statistics, behind large numbers there are real people struggling over the lack of efficient and safe treatment to control parasitic diseases. They live in pain, experience prejudice, all of this immersed in a severe limiting socio-economic condition (Conteh *et al.*, 2010). They have lost the right and the perspective to live with dignity and with consequences that extend beyond the personal loss in life quality and which also affect the family and the State.

Unfortunately, the lack of interest and limitations of both public and private health sectors on the development of new diagnostic methods and new therapies to fight neglected diseases put on the shoulders of the Scientific Academy the great responsibility of not only identifying and characterizing new drug targets, but also to innovatively develop new and efficient strategies to control and treat these parasitic diseases. In order to move forward in the complex and multidisciplinary drug discovery pipeline it is of need to restructure the currently *modus operandi* of the academic research, where additionally to the quest for high quality science, now it is also necessary to put different expertise and free thinking upon a common topic, a formerly intrinsic part of the everyday university researcher, now molded economical and bureaucratic criteria that must be taken into account during project development and execution.

Our laboratory (Laboratório de Cristalografia de Proteínas de Ribeirão Preto, LCP-RP) is a few hundred meters far from a regional hospital that daily treats many Chagas disease patients (Kamiji and De Oliveira, 2005), one of the seventeen NTDs listed as priority by the World Health Organization in 2013. This disease is caused by the protozoan parasite *Trypanosoma cruzi* and despite infecting humanity for more than nine thousand years (Aufderheide *et al.*, 2004) it was only described in 1909 by the Brazilian physician Carlos Chagas (Chagas, 1909). However, even after more than a century, no definitive cure or vaccine were obtained for Chagas disease, and the current chemotherapy based on a long term intake of two highly toxic drugs: nifurtimox and benznidazole, is not effective enough and cure is not achieved for more than 80% of treated patients (Cancado, 1999). There is undoubtedly an urgent need to develop new drugs and tools for the treatment of *Chagas disease*.

The proximity to this reality combined with the great interest in innovative research is a driving force for the prospection of this new chemotherapy treatment. Nifurtimox and benznidazole

were empirically discovered and only recently their molecular mechanism started to be evaluated (Hall *et al.*, 2011; Hall and Wilkinson, 2012). In a different approach, our work focuses primarily on the identification and characterization of a new drug target in *T. cruzi* and further exploits the knowledge for the design of drug prototypes. The first *T. cruzi* drug target to be studied in our lab was the enzyme dihydroorotate dehydrogenase (DHODH) that provides orotate for the essential *de novo* pyrimidine biosynthesis pathway (Annoura *et al.*, 2005; Pinheiro *et al.*, 2008; Pinheiro *et al.*, 2013). DHODH uses fumarate as a co-substrate which is in turn provided by the cytosolic fumarase (FH) enzyme. In addition to its relevance for nucleotide biosynthesis, RNA interference experiments showed fumarase activity is essential for the viability of procyclic *Trypanosoma brucei* (Coustou *et al.*, 2006). Then, we decided to investigate the importance of fumarase activity for *T. cruzi* in order to evaluate their potential as drug targets against Chagas disease.

Fumarase is an ubiquitous enzyme responsible for the reversible conversion of fumarate into S-malate (Dakin, 1922).

Figure 1. Fumarase reaction. Fumarase catalyze the ubiquitous reversible stereospecific hydration of fumarate (left) to S-malate (right).

Traditionally, fumarases are divided in two distinct classes (Woods, Schwartzbach e Guest, 1988): class I fumarases are homodimeric, contains an iron-sulfur cluster (4Fe-4S) in the catalytic center and are considered thermolabile. Class II fumarases are homotetrameric, iron independent and thermostable enzymes. Recently, a third class has been described and consists of an α -subunit that resembles the N-terminal portion of class I fumarases and a β -subunit homologous to the C-terminal region of class I fumarases (Shimoyama *et al.*, 2007).

Eukaryotic organisms contain in general two fumarases: a cytosolic (FHC) and a mitochondrial isoform (FHM) (Yogev, Naamati and Pines, 2011). Human fumarases (HsFH) are encoded by a single gene and the dual localization is achieved by posttranslational process. HsFH is a 467 amino acids long peptide chain, with predicted molecular weight of 50.2 kDa and theoretical pI of 6.91 and belong to class II fumarases. Differently, preliminary analysis revealed that *T. cruzi* contains a cytosolic and a mitochondrial class I fumarase genes and are encoded by genes TcCLB.507257.60 and TcCLB.507669.10 named *TcFHC* and *TcFHM*, respectively. *TcFHC* is a 565 amino acids long peptide chain, with predicted molecular weight of 62 kDa and theoretical pI of 6.74. *TcFHM* is a 555 amino acids long peptide chain with predicted molecular weight of 60.8 kDa and theoretical pI of 8.3. *TcFHC*

and *TcFHm* share about 59.7% of sequence identity. Parasite and human enzymes share about 15% sequence identity.

The present work consisted of functional and structural characterization of *Trypanosoma cruzi* and human fumarases. We combined molecular biology, biochemical, biophysical, molecular modeling and X-ray crystallography techniques to develop a successful protocol to overproduce *TcFHc*, *TcFHm* (Chapter 2) and *HsFH* (Chapter 3) in a heterologous expression system and to perform their kinetic and structural characterization. In addition, gene knockout experiments were performed in order to validate *TcFHs* as drug targets (Chapter 2). Structural and kinetic distinct properties found between human and parasite enzymes were used to design a potent and selective ligand against *TcFH* enzymes (Chapter 4). This ligand was used as a chemical probe to our preliminary *in vivo* assays. The multidisciplinary work developed during this Doctorate, besides the development of scientific knowledge, had also the aim to contribute, even modestly, for the search of new opportunities for the relief of those suffering from Chagas disease.

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Chapter 2. *Typanosoma cruzi* fumarases

2.1 Introduction

Trypanosoma cruzi is a flagellated protozoan parasite that infects humans and is responsible for the Chagas disease, or American Trypanosomiasis, which affects about 8 million people mainly in Latin America (WHO Fact Sheet, 340, 2013). Chagas disease, named in honor of the Brazilian physician Carlos Chagas who first described its pathology and pathogenesis (Chagas, 1909; Coura e Dias, 2009), is characterized by potentially life-threatening damage to the heart, intestines and central nervous system, significantly affecting the quality of life of infected patients (Punukollu *et al.*, 2007; Diego Maya *et al.*, 2010).

T. cruzi life cycle has two distinct phases: one in the vertebrate host and the other in insects. Different forms of parasite transmission between hosts have already been reported including blood transfusion, organ transplantation, congenitally and by the ingestion of contaminated food (Pereira *et al.*, 2009; Alarcon De Noya *et al.*, 2012; Kotton, 2012; Howard *et al.*, 2013). However, the classic route of transmission is through the contact with the feces of insect vector from Reduviidae family.

The transition between hosts demands *T. cruzi* to adapt to different environmental conditions such as oxidative stress and nutrient starvation, causing profound changes in cell metabolism and morphology. In this sense, different developmental stages were assigned to each part of *T. cruzi* life cycle.

During the insect blood meal, *T. cruzi* metacyclic trypomastigotes, non-dividing infective cells, are excreted with the feces nearby the wound left by the insect bite, where they infect the host exposed tissues. Once inside the host, metacyclic trypomastigotes actively invade cells where they become amastigotes which are dividing and non-infective forms of *T. cruzi*. After replicating, the amastigotes differentiate into trypomastigotes which burst the cells and are free to invade new cells or to be taken up by reduviidae insects. At the medium part of the insect intestinal tract *T. cruzi* is found as epimastigotes, dividing and non-infective cells, and at the posterior intestine they become metacyclic trypomastigotes completing the life cycle.

After *T. cruzi* infection, the disease progresses in three different phases: the acute, the indetermined or latent, and the chronic phases. The acute phase is not necessarily symptomatic but in some cases presents flu like symptoms which disappear spontaneously and therefore is often ignored (Marin-Neto e Rassi, 2009). If not treated, the disease evolves to the latent phase characterized by absence of symptoms, positive serology and mild inflammation and may last 10 to 30 years. The symptoms of Chagas disease at the chronic phase includes, mega colon, mega heart, cardiac arrests, arrhythmia and arterial and venous thromboembolisms (Diego Maya *et al.*, 2010).

Despite being known for more than a century, affecting millions of people and causing deadly symptoms, only two therapies are available and consist of the long term use (for the chronic phase) of the drugs benznidazole and nifurtimox, both highly toxic and only effective at the acute phase of Chagas disease.

Additionally, both benznidazole and nifurtimox are nitro-heterocyclic pro-drugs which rely on the activation by the trypanosomatid type I nitroreductase enzyme to exert the anti-parasitic effect (Hall, Bot e Wilkinson, 2011; Hall e Wilkinson, 2012). Recent studies showed that a single mutation in this enzyme is enough to confer resistance to the parasite (Maria Mejia *et al.*, 2012) and since the same activation mechanism is shared by both benznidazole and nifurtimox, cross resistant strains is a major concern since no alternative treatment is available (Wilkinson *et al.*, 2008). It is also problematic the fact that benznidazole and nifurtimox chemotherapy for the acute phase of Chagas disease does not guarantee complete elimination of the parasites. Therefore, even when circulating parasites are not detected, immunosuppression events can reactivate the infection (Pinazo *et al.*, 2010). In light of these facts, there is an urgent need for the development of new therapies to fight Chagas disease.

The modern process of rational drug discovery requires a multidisciplinary approach that involves target identification and validation, lead drugs identification strategies and effective screening methodologies (Burley e Park, 2005). In this process, the knowledge of basic *biology and biochemistry* of the target *organisms, at genomic and proteomic levels, constitutes a key step in the* identification of potential biomarkers and new macromolecular drug targets. Thus, exploring the biochemistry of *T. cruzi* can bring an important contribution to the development of effective therapies for Chagas disease.

A recent study using *Trypanosoma brucei* as a model organism, the causative agent of African trypanosomiasis, demonstrated that the enzyme fumarate hydratase, also called fumarase, is essential for the viability of the procyclic (insect) stage of this parasite (Coustou *et al.*, 2006). Those results shed light on the relevance of fumarase activity on trypanosomatids and prompted us to explore the potential of fumarase as drug target against American trypanosomiasis. In order to verify that hypothesis this chapter describes our first approaches to unravel the function of fumarases in *T. cruzi*. Our studies involved genetic, biochemical, biophysical and structural characterization of the *T. cruzi* proteins encoded by the genes TcCLB.507257.60 (*TcFHC*) and TcCLB.507669.10 (*TcFHM*) with putative fumarase activity (Hertz-Fowler *et al.*, 2004).

The genes were cloned and expressed in *Escherichia coli* and the proteins purified to homogeneity. The biochemical and biophysical characterization under inert and oxygen containing atmospheres showed that the product of the TcCLB.507257.60 and TcCLB.507669.10 genes indeed have fumarase activity, following a Michaelis-Menten mechanism and depend on an iron-sulfur

cluster as prosthetic group for their activity. Reverse genetic studies in *T. cruzi* suggested that fumarases are essential enzymes to *T. cruzi* supporting the idea that these enzymes constitute novel drug targets for the development of new therapies against Chagas disease.

2.2 Objectives

To characterize the structure and function of *Trypanosoma cruzi* fumarases, *TcFHc* and *TcFHm*, in order to validate them as drug targets. To accomplish this goal the following steps were pursued:

- Cloning of *TcFHc* and *TcFHm* genes in expression vectors.
- Production of pure *TcFHc* and *TcFHm* enzymes by means of recombinant expression in *E. coli* and purification by immobilized metal affinity chromatography (IMAC)
- Biochemical characterization
 - Quaternary structure
 - Enzyme kinetics
- Biophysical characterization
 - Circular dichroism
 - Electron paramagnetic resonance
- Structural characterization
 - Protein crystallization
 - X-ray diffraction and structure determination
 - Homology modeling
- Functional characterization
 - Reverse genetics in *T. cruzi* for both *TcFHc* and *TcFHm* genes
 - Phenotypic analysis of fumarase deficient *T. cruzi* cell lines
 - Sub-cellular localization of *TcFHc* and *TcFHm* in *T. cruzi*

2.3 Methodology

2.3.1. *In silico* analysis

Prediction of signal peptides was performed by using the programs Mitoprot (Claros e Vincens, 1996) to specifically predict mitochondrial import signal and TargetP (Emanuelsson *et al.*, 2007) for prediction of protein sub-cellular localization and of cleavage sites in signal peptides.

2.3.1. Recombinant *T. cruzi* fumarases production

2.3.1.1. Vectors construction

The propagation of the vectors described in this session was carried out using *E. coli* DH5 α strain in Lysogenic Broth (LB) medium containing 30 μ g/mL kanamycin for vectors of pET series and 100 μ g/mL ampicillin for pTZ cloning vector. Primers and PCR conditions used to generate our constructs are summarized in Table 1 and Table 2.

Table 1. Primers used for *T. cruzi* fumarase enzymes recombinant production

Primers	Sequence	Restriction enzyme
TcFHc1-F	gacgac gaattc ATGAGTCTGTGCGAAAAC	<i>EcoRI</i>
TcFHc1-R	gacgacgac gcggccgc ATCAAAGGAGTTTGAAAAAAG	<i>NotI</i>
TcFHm1-F	gacgac ggatcc ATGCTGCGCCGTTCTGC	<i>BamHI</i>
TcFHm1-R	gacgacgac gcggccgcagc aTTGACTCATTTGAGCTG	<i>NotI</i>
FHc-Cterm-F	ggg gaattc AACGTGGTGAATGTCGATCTG	<i>EcoRI</i>
FHc-Nterm-F	ggg catatg gaattc TCTGATCCGCACACGACGAG	<i>NdeI EcoRI</i>
FHc-Nterm-R	ggg ctcgag tca CAGGTATTGTGCGGGGTCCCG	<i>XhoI</i>
Δ mtTcFHm	ggg ggatcc TTGACGAAGGACTACGTTTCT	<i>BamHI</i>
ExtpET28	AAAGCCGGCGAACGTGGG	-

Table 2. PCR conditions for *T. cruzi* fumarase enzymes recombinant production

Fragment	Primer 1	Primer 2	Annealing temperature (°C)	[Mg ²⁺](mM)	Size (bp)
TcFHc*	TcFHc1-F	TcFHc1-R	49	2	1698
TcFHm*	TcFHm1-F	TcFHm1-R	59.6	2	1668
TcFHc-Cterm	FHc-Cterm-F	ExtpET28	50	1-5	868
TcFHc-Nterm	FHc-Nterm-F	FHc-Nterm-R	50	1-4	1005
Δ mtTcFHm	Δ mtTcFHm	ExtpET28	50	4	1880

* The PCR was carried out without the addition of DMSO

2.3.1.1.1. pET-28-SUMO-TcFHc

Genomic DNA (gDNA) of *T. cruzi* trypomastigote cells (Y strain) was extracted using the Illustra Tissue and Cells Genomic Prep Mini Spin Kit (GEHealthcare Life Sciences) and used as template to amplify the *TcFHc* gene by PCR Table 1 and Table 2.

After amplification the gene was cloned into pET-28a (MerckMillipore) using *EcoRI* and *NotI* restriction sites. The *TcFHc* gene was subcloned into a modified version of the pET-28a expression vector called pET-28-SUMO using *EcoRI* and *XhoI* restriction enzymes. The generated vector was called pET-28-SUMO-TcFHc.

The pET-28-SUMO vector contains the SUMO protein, between the *NheI* and *BamHI* restriction sites what enables the removal of both poly-histidine and SUMO tags by selective cleavage using the ubiquitin like protein 1 (ULP1). The pET-28-ULP1 vector, used to express and purify the ULP1 protease was kindly provided by Dr. André Ambrosio from LNBIO-Campinas and it was used for removal of SUMO fusion tags when applicable.

2.3.1.1.2. pET-28-SUMO-TcFHm

The full length *TcFHm* gene was amplified from *T. cruzi* (Y Strain) gDNA using primers and conditions described (Table 1 and Table 2). The amplicon was digested with *BamHI* and *NotI* restriction enzymes and ligated into pET-28-SUMO, previously digested with the same enzymes, generating the pET-28-SUMO-TcFHm vector.

2.3.1.1.3. pET-28-SUMO-ΔmtTcFHm

The first 35 residues at the N-terminal end of the *TcFHm* enzyme was predicted as mitochondrial target signal. Considering that mitochondrial import normally requires this signal to be cleaved off in the mature protein and, therefore, it is not important for enzyme activity, a truncated version of *TcFHm* lacking the signal peptide (Δmt*TcFHm*) was also cloned. The Δmt*TcFHm* was amplified by PCR (Table 1 and Table 2) using the pET-28-SUMO-TcFHm vector as template. The amplicon was cloned into pTZ vector using the InsTAclone PCR Cloning Kit (Thermo Scientific) and further subcloned into pET-28-SUMO using *BamHI* and *XhoI* restriction enzymes. The generated vector was called pET-28-SUMO-ΔmtTcFHm.

2.3.1.1.4. pET-28-TcFHc-Nterm and pET-28-SUMO-TcFHc-Nterm

The N-terminal portion (residues from 23 to 372) of the *TcFHc* enzyme alone was produced in an attempt to maximize the chances of crystallization. The TcFHc-Nterm fragment was amplified by PCR (Table 1 and Table 2) using pET-28-SUMO-TcFHc as template and was cloned into pET-28-SUMO and pET-28a expression vectors using *EcoRI* and *XhoI* restriction enzymes to generate the pET-28-TcFHc-Nterm and pET-28-SUMO-TcFHc-Nterm constructs.

2.3.1.1.5. pET-28-TcFHc-Cterm and pET-28-SUMO-TcFHc-Cterm

The C-terminal fragment of TcFHc (residues from 383 to 565) was also cloned into both pET-28a and pET-28-SUMO expression vectors. The TcFHc-Cterm fragment was amplified by PCR (Table 1 and Table 2) and ligated into the host vectors between *EcoRI* and *XhoI* restriction sites generating the pET-28-TcFHc-Cterm and pET-28-SUMO-TcFHc-Cterm constructs.

2.3.1.2. Expression and purification

Chemically competent *E. coli* BL21 DE3 cells were transformed by heat-shock with the following *T. cruzi* fumarase expressing vectors: pET-28-SUMO-TcFHc, pET-28-SUMO-TcFHm, pET-28-SUMO-TcFHc-Cterm, pET-28-SUMO-TcFHc-Nterm. A single colony of each cell line was inoculated in 10 mL of LB containing 30 µg/mL kanamycin and grown overnight at 37 °C under 180 rpm agitation. The 10 mL starter culture was diluted in 1 L of fresh media containing 30 µg/mL kanamycin and grown until optic density at 600 nm (O.D._{600nm}) reached 0.5. At this point 200 mg FeSO₄·7H₂O, 200 mg ferric citrate, 242 mg cysteine and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the following concentrations: TcFHc – 5 µM, TcFHm – 50 µM, TcFHc-Cterm – 1000 µM, TcFHc-Nterm – 5 µM. Protein expression was carried out overnight at 18 °C under 180 rpm agitation. Cells were harvested by centrifugation at 8,000 g and the pellets stored at -20 °C until used.

A pellet corresponding to 1 L of cell culture was resuspended in 20 mL of buffer A (50 mM NaH₂PO₄, pH 8.5, 600 mM NaCl and 10 mM imidazole) added of and 1 mM phenylmethanesulfonylfluoride (PMSF). Cells were disrupted by sonication using 15 pulses of 30 s with 30 s intervals on ice. The soluble fraction was obtained by centrifugation of the cell lysate for 30 min at 16,000 g and 4 °C and poured into 2 mL of Ni-NTA resin pre equilibrated with buffer A. The resin was washed with 30 mL of buffer A followed by a second washing step of 30 mL of buffer B (50 mM NaH₂PO₄, pH 8.5, 600 mM NaCl and 25 mM imidazole). After re-equilibration of the column with buffer A, 500 µg ULP1 was added and the mixture incubated for 3 hours under slow agitation in a cold room. Tag-free protein was eluted with 30 mL of buffer A, dialyzed and concentrated in buffer C (50 mM Tris pH 8.5, 150 mM NaCl) using a centrifugal ultra filter unit of appropriate cutoff (Amicon – Millipore). All purification steps were monitored by SDS-PAGE.

Full length TcFHc and TcFHm were also expressed and purified under strict anaerobic conditions. This work was performed by Elizabeth C. Witternborn by applying the same purification protocol in an anaerobic chamber (MBraun), as part of a scientific collaboration between our laboratory and Catherine L. Drennan Laboratory at Massachusetts Institute of Technology (MIT).

2.3.2. Biophysical and biochemical characterization

2.3.2.1. Size-exclusion chromatography

Pure *TcFHc* obtained from the metal affinity purification was loaded in a Superdex 200 10/300 column connected to an Äkta Purifier (GE Lifesciences) and subject to a chromatographic run with buffer 50 mM Tris pH 8.5, 600 mM NaCl, 10 mM imidazol and 1 mM DTT using 0.5 mL/min flow rate. The column was calibrated under the same experimental conditions by the technician Iara A. Cardoso following the column manufacturer's instructions. From the relation between mass and elution volume the oligomeric state of *TcFHc* could be determined using the Equation 1.

Equation 1

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

V_e is the elution volume, V_0 is the column void determined by blue dextran and V_t is the total column volume. The K_{av} of each molecular marker was plotted in function of the logarithm of its molecular mass. Linear regression analysis provided an equation to predict the relationship between molecular mass and K_{av} which was used to retrieve *TcFHc* molecular mass from its V_e .

2.3.2.2. Circular Dichroism (CD)

CD experiments were carried out for *TcFHc* using a Jasco J-810 spectropolarimeter equipped with a temperature controller. The measurements were performed using a 0.2 mg/mL *TcFHc* sample in buffer 1.5 mM Tris pH 8.5 and 1.5 mM NaCl in a quartz rectangular cuvette of 0.1 cm optical path. The far UV spectra (195-250 nm) were scanned with 100 mDegrees sensitivity, 50 nm/minute scan speed, 2 nm band width and 2 seconds response time. The final spectrum is the average of 16 scans. The values of the CD spectra at a given wavelength were converted to a calculated mean value for the molar ellipticity per residue $[\theta]_{\lambda} = (\theta_{\lambda} M_0)/(10 l c)$ where θ_{λ} is the observed ellipticity in mDegrees, M_0 is the mean mass per residue ($M_0 = 115$), c is the protein concentration (mg/mL) and l is the optical path (cm). The *TcFHc* stability was analyzed through a thermal denaturation experiment in which the CD spectrum (195-250 nm) was measured over a range of temperatures (10 – 90 °C).

2.3.2.3. Electron paramagnetic resonance (EPR)

EPR measurements were performed to assess the presence of an iron-sulfur cluster in *TcFHc*. A BrukerElexsys E580 spectrometer operating at X band (9.5 GHz) equipped with a rectangular cavity was utilized. The optimal temperature for the experiments, around that of liquid helium (4-10 K), was reached using an Oxford ITC 503 cryostat. The parameters were optimized to avoid signal saturation and distortion with the final values: 10 mW microwave potency, 0.2 mT magnetic field modulation

and 100 kHz magnetic field modulation frequency. Samples contained 87 μM of protein in a total volume of 50 μL .

2.3.2.4. Optimum pH determination

The *TcFHc* activity was monitored by spectrophotometric measurements of fumarate consumption/formation at 250 nm ($\epsilon=1.45 \text{ mM}^{-1}.\text{cm}^{-1}$ (Bock e Alberty, 1953)) at 25°C. The reaction assay mixture consisted of 50 mM of buffer (KCl/NaOH pH 12, NaHCO_3 /NaOH pH 11, glycine/NaOH pH 10, Tris pH 9, 8 and 7, sodium cacodylate pH 6 and sodium acetate pHs 5 and 4), 150 mM NaCl and 1 mM malate/fumarate in 1 mL. Experiments were carried out using a Hitachi U-2800 spectrophotometer in aerobic atmosphere.

2.3.2.5. Kinetics assay

The kinetics parameters, V_{max} and K_m were determined for both substrates, malate and fumarate, by varying the concentration of fumarate (500, 250, 125, 62.5 and 31.25 μM) and malate (8, 4, 2, 1, and 0.5 mM). For reactions performed under nitrogen atmosphere, 10 $\mu\text{g/mL}$ of *TcFHc* or *TcFHm* was used. The reactions in the presence of oxygen were carried out with 40 and 80 $\mu\text{g/mL}$ of *TcFHc* in presence of fumarate and malate, respectively. At 25 °C, assays were performed for both substrates, in a 1mL reaction mixture containing 50 mM Tris pH 8.5, 150 mM NaCl.

2.3.3. Structural characterization

2.3.3.1. Crystallization assays

Full length *TcFhc* and *TcFhm*, and the C-terminal domain *TcFhc*-Cterm were subjected to crystallization assays using both hanging and sitting drop vapor diffusion techniques utilizing the sparse matrix protocol (Jancarik e Kim, 1991) implemented in commercial kits such as Crystal Screen, PEG/Ion, MembFac, PEGRx, Index Screen (Hampton Research), Nextal MbClass (Qiagen) and the homemade kit Morpheus (Gorrec, 2009) at 294 K. In order to optimize crystal growth the additives glycerol, malate, fumarate, thiomalate, DTT, β -mercatpoethanol (BME) were also tested. Different combination of chromatographic techniques such as affinity and molecular exclusion were used to prepare the protein samples.

2.3.3.2. Homology model

The *TcFhc* and *TcFhm* homology models were built using the coordinates of *Leishmania major* fumarase crystal structure, recently solved in our laboratory. The program Modeller 9.11 (Salic e Blundell, 1993) was used to generate molecular models based on the variable target function method with conjugated gradients. The first refinement step was performed by implementing simulated annealing followed by molecular dynamics with additional refinement cycles carried out using the REFMAC structure idealization tool (Murshudov *et al.*, 1999) using MolProbity as indicator of model quality (Davis *et al.*, 2007). Electrostatic potential surfaces were calculated by solving the Poisson-Boltzman equation using the APBS plugin (Baker *et al.*, 2001) in PyMOL (The PyMOL Molecular Graphics System, version 1.3 Schrödinger, LLC).

2.3.4. Functional characterization

2.3.4.1. Cell culture

2.3.4.1.1. *T. cruzi* epimastigotes

Epimastigotes of *T. cruzi* strain Sylvio X.10.6 were grown in static culture at 28°C in RPMI 1640 media supplemented with 5 g/L trypticase, 20 mM HEPES pH 7.5, 10% inactivated calf fetal serum, 0.22 g/L sodium pyruvate, 0.34 g/L sodium glutamate, 2500 U/L penicillin, 0.25 g/L streptomycin and 20 mg/L hemin. Alternatively, epimastigotes were cultured in liver infusion tryptose (LIT) medium (Camargo, 1964).

2.3.4.1.2. *T. cruzi* metacyclics

T. cruzi metacyclics were obtained by letting an epimastigote culture age for up to 30 days without passage. In order to assess the metacyclogenesis for the different cell lines, cell aliquots were taken at different time points, fixed on a glass slide and stained with DAPI. The kinetoplast DNA position relative to the nuclear position was observed in a fluorescence microscope (excitation wavelength 358 nm and emission at 461 nm) and metacyclogenesis was determined in terms of metacyclics per 100 cell counted.

2.3.4.1.3. Mammalian cells

Renal cells of African green monkey (Vero cells) were cultivated in RPMI 1640 at 37 °C in a 5% CO₂ atmosphere. Cells were subcultured by trypsinization when 90% confluency was reached. Briefly, 90% confluent cells cultured in a 25 cm² bottle had the medium discarded and 2 mL of a 0.05% Trypsin-0.02% EDTA was added and incubated for 15 min. After all cells were detached, 4 mL of RPMI medium was added and used to inoculate other culture bottles.

2.3.4.1.4. *T. cruzi* amastigotes

For the infection experiments 2mL of a *T. cruzi* culture containing a mixture of epimastigotes and metacyclics was added to an 80 % confluent Vero cell culture in a 25 cm² flask. After 16 hours, the non adhered parasites were washed out with LIT medium containing non-inactivated fetal calf serum to lyse remaining epimastigote cells. After 10 days metacyclics emerged from infected Vero cells and were used to infect new Vero cells (10:1 ratio) in 24 well plates containing 13 mm glass slides at the bottom of the wells. Cells were kept at 37 °C in a 5% CO₂ incubator. After 2 days the medium was aspirated and cells were stained with Giemsa. The percentage of infected Vero cells and the number of amastigotes per infected cell were determined by analyzing 100 host cells distributed in randomly chosen microscopic fields. The mean number of parasites found in all host cells (mean abundance) was calculated using the quantitative parasitology software (QPweb 1.0) with 95% confidence interval by the bias-corrected and accelerated (BCa) bootstrap method with 2000 bootstrap replications.

2.3.5. Knockout cell lines

2.3.5.1. Knockout vectors

T. cruzi knockout cell lines for the genes *TcFhc* and *TcFhm* were constructed using the strain Sylvio X10/6. The DNA fragments used to transfect *T. cruzi* epimastigotes were generated using the pKO^{BLA}-TcNTR and pKO^{PAC}-TcNTR vectors as templates (Wilkinson *et al.*, 2008). The pKO vectors are composed of the 5' and 3' extremities of the gene to be knocked out flanking a resistance cassette

containing the resistance gene and the intergenic region of tubulin which guarantees correct mRNA processing. The resistance genes used were: blasticidin (BLA), puromycin (PAC), geneticin/G418 (NEO) and hygromycin (HYG). The primers and amplicons generated are listed in Table 3 and Table 4, respectively. PCR reactions were carried out using Vent DNA polymerase (New England Biolabs) and supplied with 10% dimethyl sulfoxide (DMSO). The propagation of the vectors described in this session was carried out using *E. coli* XL1 Blue strain in NZCYM medium containing 100 µg/ml ampicillin. All constructs were checked by sequencing.

Table 3. Primer sequences used to generate the knockout vectors.

Primers	Sequence	Restriction enzyme
TcFHc1-KO1	aaa gagctccgcgg ATGAGTCTGTGCGAAAAC TGC	SacI SacII
TcFHc1-KO2	aaa tctaga TCCCGTCCAACAGAGTTCGCC	<i>XbaI</i>
TcFHc-KO5	aaa gggccc ATCGTGATGGATATCGCCTGT	<i>ApaI</i>
TcFHc-KO6	aaa ggtacc CATTCCCTTTGGAGTCTTCGC	<i>KpnI</i>
TcFHm1-KO1	ggg gagctccgcgg TTTCTTTGACGCACACATCAC	SacI SacII
TcFHm1-KO2	aaa tctaga AAATCTTGTAGACGCCACGAC	<i>XbaI</i>
TcFHm1-KO3	aaa gggccc CAAGACACGACTAAGTTTGAG	<i>ApaI</i>
TcFHm1-KO4	aaa ggtacc TCATTGAGCTGTTGGAAGAA	<i>KpnI</i>
HYG-1	ggg aagctt ATGAAAAAGCCTGAACTCACC	<i>HindIII</i>
HYG-2	ggg atcgat CTATTCCTTTGCCCTCGGACG	<i>Clal</i>
NEO-1	ggg aagctt ATGGGATCGGCCATTGAACAA	<i>HindII</i>
NEO-2	ggg atcgat TCAGAAGAACTCGTCAAGAAG	<i>Clal</i>

Table 4. PCR conditions for the generated knockout fragments

Fragment	primer 1	primer 2	Annealing temperature (°C)	[Mg ²⁺](mM)	Size (pb)
5' TcFHc KO1-KO2	TcFHc1-KO1	TcFHc1-KO2	50	1-5	450
3' TcFHc KO5-KO6	TcFHc1-KO5	TcFHc1-KO6	54	2-3	471
5' TcFHm KO1-KO2	TcFHm1-KO1	TcFHm1-KO2	50	2-5	490
3' TcFHm KO3-KO4	TcFHm1-KO3	TcFHm1-KO4	50	2-5	484
HYG	HYG-1	HYG-2	50	2-5	1150
NEO	NEO-1	NEO-2	50	2-5	803

2.3.5.1.1. Vector pKO^{BLA}-TcFHc

The 450 bp 5' region of *TcFHc* gene was amplified with primers TcFHc1-KO1 and TcFHc1-KO2 using *T. cruzi* Sylvio X 10/6 genomic DNA. The TcFHc KO1-KO2 fragment was cloned into pKO^{BLA}-*TcNTR* between *SacII* and *XbaI* restriction sites generating the pKO^{BLA}-5'*TcFHc*-3'*TcNTR*. The 471 bp 3' extremity of *TcFHc* gene, TcFHc KO5-KO6, was initially cloned in pGEM T-easy vector (Promega) and

then subcloned into $\text{pKO}^{\text{BLA}}\text{-5'TcFHc-3'TcNTR}$ between *Apal* and *KpnI* to generate $\text{pKO}^{\text{BLA}}\text{-TcFHc}$ vector (Figure 2).

Figure 2. Map of the $\text{pKO}^{\text{BLA}}\text{-TcFHc}$ vector: The 5' and 3' extremities of the *TcFHc* gene (blue) flanking the blasticidin resistance cassette (BLA) (green). The tubulin intergenic regions (black) guarantee correct mRNA processing. The numbers correspond to base pair length of each fragment.

2.3.5.1.2. Vector $\text{pKO}^{\text{PAC}}\text{-TcFHc}$

The 5' fragment *TcFHc* KO1-KO2 was cloned into $\text{pKO}^{\text{PAC}}\text{-TcNTR}$ using *SacI* and *XbaI* restriction sites followed by cloning the fragment 3' *TcFHc* KO5-KO6 between *Apal* and *KpnI*. The resultant vector was called $\text{pKO}^{\text{PAC}}\text{-TcFHc}$ (Figure 3).

Figure 3. Map of the $\text{pKO}^{\text{PAC}}\text{-TcFHc}$ vector: The 5' and 3' extremities of the *TcFHc* gene (blue) flanking the puromycin resistance cassette (PAC) (yellow).

2.3.5.1.3. $\text{pKO}^{\text{BLA}}\text{-TcFHm}$

Similarly to $\text{pKO}^{\text{BLA}}\text{-TcFHc}$ vector, the 5' and 3' extremities of *TcFHm* were amplified and cloned flanking the blasticidin resistance cassette (BLA) in $\text{pKO}^{\text{BLA}}\text{-TcNTR}$ vector. The 5' *TcFHm* KO1-KO2 fragment was cloned between *SacII* and *XbaI* at the 5' prime position whereas the 3' *TcFHm* KO3-KO4 fragment was cloned at the 3' flank using *Apal* and *KpnI* restriction enzymes generating the $\text{pKO}^{\text{BLA}}\text{-TcFHm}$ vector (Figure 4).

Figure 4. Map of the pKO^{BLA}-TcFHm vector: The 5' and 3' extremities of the TcFHm gene (orange) flanking the blasticidin resistance cassette (BLA) (green).

2.3.5.1.4. pKO^{PAC}-TcFHm

The pKO^{PAC}-TcFHm vector was built by replacing the blasticidin resistance cassette in the pKO^{BLA}-TcFHm vector by the puromycin resistance cassette from pKO^{PAC}-TcNTR vector using *XbaI* and *ApaI* restriction sites (Figure 5).

Figure 5. Map of the pKO^{PAC}-TcFHm vector: The 5' and 3' extremities of the TcFHm gene (orange) flanking the puromycin resistance cassette (PAC) (yellow).

2.3.5.1.5. pKO^{NEO}-TcFHc

The geneticin (G418) resistance cassette (NEO) was amplified from the vector pTEX-TcNTR-9e10 (Maria Mejia *et al.*, 2012) with primers NEO-1 and NEO-2. After amplification the NEO gene was digested with the enzymes *Clal* and *HindIII* and inserted into pKO^{PAC}-TcFHc replacing the puromycin resistance cassette and generating the pKO^{NEO}-TcFHc (Figure 6).

Figure 6. Map of the pKO^{NEO}-TcFHc vector: The 5' and 3' extremities of the *TcFHc* gene (blue) flanking the geneticin (G418) resistance cassette (NEO) (red).

2.3.5.1.6. pKO^{HYG}-TcFHc

At first, the hygromycin resistance cassette (HYG) was amplified with the primers HYG-1 and HYG-2 using the pBABE-HYG vector as a template (Morgenstern e Land, 1990). The amplified fragment was digested with the enzymes *HindIII* and *ClaI* and used to replace the puromycin resistance cassette from the pKO^{PAC}-TcFHc generating the pKO^{HYG}-TcFHc vector (Figure 7).

Figure 7. Map of the pKO^{HYG}-TcFHc vector: The 5' and 3' extremities of the *TcFHc* gene (blue) flanking the hygromycin resistance cassette (HYG) (purple).

2.3.5.1.7. pKO^{NEO}-TcFHm

The NEO cassette amplified and previously used to build the pKO^{NEO}-TcFHc vector was used to replace the PAC gene in the pKO^{PAC}-TcFHm between *HindIII* and *ClaI* restriction sites. The resulting vector was named pKO^{NEO}-TcFHm (Figure 8).

Figure 8. Map of the pKO^{NEO}-TcFHm vector: The 5' and 3' extremities of the *TcFHm* gene (orange) flanking the geneticin (G418) resistance cassette (NEO) (red).

2.3.5.1.8. pKO^{HYG}-TcFHm

The HYG cassette amplified and previously used to build the pKO^{HYG}-*TcFHc* vector was subcloned into the pKO^{PAC}-*TcFHm* vector between the *HindIII* and *ClaI* restriction sites replacing the PAC cassette. The generated vector was called pKO^{HYG}-*TcFHm* (Figure 9).

Figure 9. Map of the pKO^{HYG}-TcFHm vector: The 5' and 3' extremities of the *TcFHm* gene (orange) flanking the hygromycin resistance cassette (HYG) (purple).

2.3.5.2. Transfection and cell line selection

Initially, the pKO^{PAC}-*TcFHc* and pKO^{PAC}-*TcFHm* vectors were digested with *SacI* and *KpnI* restriction enzymes and the pKO^{BLA}-*TcFHc* and pKO^{BLA}-*TcFHm* were digested with *SacII* and *KpnI* restriction enzymes. The released linear fragments containing the resistance cassette were gel purified and used to transfect *T. cruzi* Sylvio X 10/6 epimastigotes by electroporation as described earlier (Xu *et al.*, 2009). After 24 hours following the transfection, the cells were transferred to a 75 cm² culture flask with 40 mL and the appropriate drug was added (puromycin 10 µg/mL, blasticidin

10 µg/mL). The cells were seeded in 24 well plates (2 mL per well) and plates were incubated at 28°C for 3-6 weeks in order to select resistant cells.

When the majority of wells were positive for transfectants, the cells were subjected to limited dilution in order to obtain clonal cell population. To confirm the gene knockouts, the obtained cell line obtained was analyzed by Southern blot.

The cell lines generated after the first round of transfections had a single copy of *TcFhc* or *TcFhm* replaced by either PAC or BLA resistance cassette (genotypes: *TcFhc*^{+/-BLA}, *TcFhc*^{+/-PAC}, *TcFhm*^{+/-BLA}, *TcFhm*^{+/-PAC}) (Figure 10).

Figure 10. Representation of the first round of knockout for *TcFhc* and *TcFhm* genes: a) Replacement of one copy of the gene *TcFhc* by the linear fragment containing blasticidin resistance cassette obtained from the vector pKO^{BLA}-*TcFhc*. b) Replacement of one copy of the gene *TcFhm* by the linear fragment containing blasticidin resistance cassette obtained from the vector pKO^{BLA}-*TcFhm*. c) Disruption of one copy of the gene *TcFhc* by the linear fragment containing puromycin

resistance cassette obtained from the vector $\text{pKO}^{\text{PAC}}\text{-TcFhc}$. d) Disruption of one copy of the gene *TcFHm* by the linear fragment containing puromycin resistance cassette obtained from the vector $\text{pKO}^{\text{PAC}}\text{-TcFHm}$.

The $\text{TcFhc}^{+/-\text{BLA}}$ and $\text{TcFHm}^{+/-\text{BLA}}$ cell lines were used in a second round of transfections, in order to replace the remaining copy of each gene by the PAC cassette released from the $\text{pKO}^{\text{PAC}}\text{-TcFhc}$ and $\text{pKO}^{\text{PAC}}\text{-TcFHm}$ vectors, respectively (Figure 11). The selection of the new cell lines generated was performed using 5 $\mu\text{g/mL}$ puromycin and 5 $\mu\text{g/mL}$ blasticidin.

Figure 11: Graphical representation of the second round of *TcFhc* and *TcFHm* gene knockouts:a) The $\text{TcFhc}^{+/-\text{BLA}}$ cell line was transfected with the linear fragment containing puromycin resistance gene released from the $\text{pKO}^{\text{PAC}}\text{-TcFhc}$. b) The $\text{TcFHm}^{+/-\text{BLA}}$ cell line was transfected with the linear fragment containing puromycin resistance gene released from the $\text{pKO}^{\text{PAC}}\text{-TcFHm}$.

The next step in order to obtain a *T. cruzi* cell line lacking all fumarase genes consisted of knocking out the mitochondrial fumarase gene copies in a null cytosolic fumarase background. For the third round of knockouts, the $\text{TcFhc}^{-\text{PAC}/-\text{BLA}}$ cells were transfected with the linear fragment containing the NEO cassette obtained from the vector $\text{pKO}^{\text{NEO}}\text{-TcFHm}$ after digestion with *SacI* and *KpnI* restriction enzymes. Parasite selection was performed with 5 $\mu\text{g/mL}$ puromycin, 5 $\mu\text{g/mL}$ blasticidin and 100 $\mu\text{g/mL}$ geneticin (G418) (Figure 12).

Figure 12. Representation of the third round of *TcFH* genes knockout: The linear fragment containing the NEO cassette was obtained from the digestion of the pKO^{NEO}-*TcFHm* vector and transfected into *TcFHc*^{-PAC/-BLA} cells.

Finally, the remaining *TcFHm* gene copy was targeted by transfecting the *TcFHc*^{-PAC/-BLA}-*TcFHm*^{+/-NEO} cells with the linear DNA fragment containing the hygromycin cassette produced after digestion of the pKO^{HYG}-*TcFHm* vector with *SacI* and *KpnI* restriction enzymes. Selection of *TcFHc*^{-PAC/-BLA}-*TcFHm*^{-HYG/-NEO} cells was carried out using 5 µg/mL puromycin, 5 µg/mL blasticidin, 100 µg/mL geneticin (G418) and 5 µg/mL hygromycin (Figure 13).

Figure 13. Representation of the fourth and last round of *TcFH* genes knockout: *TcFHc*^{-PAC/-BLA}-*TcFHm*^{+/-NEO} cells were transfected with the hygromycin resistant cassette (HYG) obtained after digestion of the pKO^{HYG}-*TcFHm* with *SacI* and *KpnI* restriction enzymes in order to delete all fumarase genes from *T. cruzi* genetic repertoire.

2.3.5.3. Southern Blot

In order to verify the integration of the knockout fragments into the *T. cruzi* genomic DNA (gDNA) used to disrupt the fumarase genes, the Southern blot technique was used (Southern, 1975). The disruption of the wild type gene sequences by the insertion of resistance cassettes increased the length of the fragment released by specific restriction enzymes allowing the visualization of knockout fragments in the *T. cruzi* genomic DNA by DNA labeled probes.

Single stranded DNA probes labeled with digoxigenin-conjugated dUTP were synthesized by PCR using the DIG System (Roche) following the manufacturer's instructions. The primer pair 5' *TcFHc* KO1 and 5' *TcFHc* KO2 was used to synthesize a *TcFHc* specific probe whereas 5' *TcFHm* KO1 and 5' *TcFHm* KO2 primers were used to generate the *TcFHm* probe.

gDNA was extracted from each *T. cruzi* cell line to be analyzed using the phenol-chloroform method (Sambrook, 1983). The gDNA digestion with *NcoI* restriction enzyme was performed to cleave a DNA segment of TcChr36-P chromosome where the *TcFHc* gene is located (Figure 14).

Figure 14. Fragments released upon *NcoI* digestion at *TcFHc* locus. The digestion with *NcoI* at the *TcFHc* gene locus in the TcChr36-P chromosome released a 2155 bp fragment for the wild type cell line, a 2687 bp fragment for the integrated BLA resistance cassette and a 2687 bp fragment for the integrated PAC resistance cassette. All fragments were detected by the DIG conjugated single stranded DNA 5'*TcFHc* KO1-KO2 probe.

Similarly, the enzyme *XhoI* was used to retrieve a DNA fragment of chromosome TcChr26-S where the *TcFHm* gene is found (Figure 15).

Figure 15. Fragments released upon *XhoI* digestion at *TcFHm* locus. The digestion with *XhoI* at the *TcFHm* gene locus in the TcChr26-S chromosome released a 4262 bp fragment for the wild type cell line, a 4571 bp fragment for the integrated BLA resistance cassette, a 4771 bp fragment for the integrated PAC resistance cassette, a 4966 bp fragment for the integrated NEO resistance cassette and a 5194 bp fragment for the integrated HYG resistance cassette. All fragments were detected by the DIG conjugated single stranded DNA 5'TcFHm KO1-KO2 probe.

2.3.5.4. *T. cruzi* fumarases sub-cellular localization

T. cruzi epimastigotes were transfected with the pTEX-9e10 episomal vector to overexpress the fumarases in fusion with the C-terminal c-myc epitope tag (Tibbetts, Klein e Engman, 1995). C-myc corresponds to the EQKLISEEDL peptide and is recognized by the 9e10 antibody enabling the immunodetection of c-myc tagged proteins. The vectors were named pTEX-*TcFhc*-9e10 and pTEX-*TcFHm*-9e10, for the cytosolic and mitochondrial fumarase isoforms respectively. In addition, both N-terminal c-myc tagged version (pTEX-TagN-9e10-*TcFhc*) and untagged (pTEX-*TcFhc*) constructs were built for the *TcFhc* gene, *T. cruzi* cells transfected with pTEX vectors and containing the geneticin resistance gene were selected with 200 µg/mL G418 after the transfections and cultured at concentration of 100 µg/mL G418 to maintain the selective pressure.

2.3.5.5. Construction of pTEX episomal vectors

The propagation of the vectors described in this session was carried out using *E. coli* XL1 Blue strain in NZCYM medium containing 100 µg/ml ampicillin.

2.3.5.5.1. pTEX-TcFHc-9e10

TcFHc gene was initially amplified by PCR with TcFHc1-OX1 and TcFHc1-OX2 primers (Table 5 and Table 6) using *T. cruzi* Sylvio X10/6 gDNA as template and further digested with *EcoRI* and *HpaI* restriction enzymes. The pTEX-*TcNTR*-9e10 vector (Maria Mejia *et al.*, 2012) was cut with *EcoRI* and *EcoRV* restriction enzymes and the *TcNTR* gene replaced by the *TcFHc* gene, generating the pTEX-*TcFHc*-9e10 construct. Joining the blunt end *EcoRV* and *HpaI* cleaved sites resulted in the GATGTT sequence and further loss of the restriction site. The pTEX-*TcFHc*-9e10 map is shown in Figure 16.

Table 5. Primers used in the construction of pTEX-*TcFHs* vector series

Primers	Sequence	Restriction enzyme
TcFHc1-OX1	ggg gaattc ATGAGTCTGTGCGAAAAGTGC	<i>EcoRI</i>
TcFHc1-OX2	ggg gttaac AAGGAGTTTGGAAAAAAGTC	<i>HpaI</i>
TcFHc-OX3	gggagatctctcgag TCAAAGGAGTTTGGAAAAAAG	<i>BglII</i> <i>SacI</i>
TcFHc-OX4	ggg tctaga AGTCTGTGCGAAAAGTCAAT	<i>XbaI</i>
TcFHm1-OX1	ggg gaattc ATGCTGCGCCGTTCTGCGGCA	<i>EcoRI</i>
TcFHm1-OX2	ggg gatata TTTGAGCTGTTGGAAGAAGTC	<i>EcoRV</i>
EcoRI-cmyc	ggg gaattc ATGGAACAGAAAGTATCTCT	

Table 6. PCR conditions used to build pTEX-*TcFHs* vector series

Fragment	Primer 1	Primer 2	Annealing temperature (°C)	[Mg ²⁺](mM)	Size (bp)
TcFHc OX1-OX2	TcFHc1-OX1	TcFHc1-OX2	50	5	1695
TcFHm OX1-OX2	TcFHm1-OX1	TcFHm1-OX2	50	2-5	1665
TcFHc-TagN OX4-OX3	TcFHc-OX4	TcFHc-OX3	50	3	1698
c-myc-TcFHc	EcoRI-cmyc	TcFHc-OX3	50	3	1698
TcFHc-NoTag	TcFHc1-OX1	TcFHc1-OX3	50	3	1698

Figure 16. Map of the pTEX-TcFhc-9E10 vector: The *TcFhc* gene (blue) was amplified from *T. cruzi* gDNA and cloned between the *EcoRI* and *EcoRV* restriction sites replacing the *TcNTR* gene from the pTEX-*TcNTR*-9E10 vector. In this construction a c-terminal c-myc epitope (gray) was fused to the *TcFhc* gene enabling the immunodetection with the 9E10 antibody. The selection of transfected cells was enabled by the NEO gene (red) and was carried out with G418.

2.3.5.5.2. pTEX-TcFHm-9E10

For the construction of pTEX-*TcFHm*-9E10 vector, the *TcNTR* gene in the vector pTEX-*TcNTR*-9E10 was replaced by the fragment TcFHm OX1-OX2 amplified from the *T. cruzi* Sylvio X 10/6 gDNA (Table 5 and Table 6) and digested with *EcoRI* and *EcoRV* restriction enzymes (Figure 17).

Figure 17. Map of the pTEX-TcFHm-9E10 vector: The *TcFHm* gene (orange) was amplified from *T. cruzi* gDNA and cloned between the *EcoRI* and *EcoRV* restriction sites replacing the *TcNTR* gene from the pTEX-*TcNTR*-9E10 vector. In this construction a c-terminal c-myc epitope (gray) was fused to the *TcFHm* gene enabling the immunodetection with the 9E10 antibody. The selection of transfected cells was enabled by the NEO gene (red) and was carried out in presence of G418.

2.3.5.5.3. pTEX-TagN-9E10-TcFhc

An N-terminal c-myc tagged version of pTEX-*TcFhc* was built anticipating the possible presence of a cryptic type 1 peroxisome targeting signal (PTS1) placed at the C-terminus end of the

TcFHc enzyme (-SKLL) (Opperdoes e Szikora, 2006) which could lead to the mislocalization of the C-terminal tagged *TcFHc*.

As a first step, the fragment *TcFHc*-TagN OX4-OX3 (Table 5 and Table 6) was amplified from *T. cruzi* Sylvio X 10/6 gDNA, digested with *Bgl*II and *Xba*I restriction enzymes and cloned into pLEW-tagN (Wilkinson *et al.*, 2005), previously digested with *Bam*HI and *Xba*I. The resulting vector, called pLEW-tagN-*TcFHc* positioned the c-myc tag to the 5' of *TcFHc* gene.

TcNTR gene was then cut out of pTEX-*TcNTR*-9E10 with *Eco*RI and *Xho*I restriction enzymes and replaced with the c-myc-*TcFHc* fragment amplified from pLEW-tagN-*TcFHc* (Table 5 and Table 6) between *Eco*RI and *Xho*I restriction enzymes. The final construct was named pTEX-TagN-9e10-*TcFHc* (Figure 18).

Figure 18. Map of the pTEX-TagN-9e10-*TcFHc* vector: The c-myc tag (gray) was placed at the 5' end of *TcFHc* gene (blue) in order to avoid a possible mislocalization of the *TcFHc* enzyme tagged at the C-terminus in the pTEX-*TcFHc*-9e10 vector. The *TcFHc* gene was first cloned into pLEW-tagN to position the c-myc sequence at the 5' end of the gene and the whole sequence (tag+gene) was amplified again and used to replace the *TcNTR*-cmyc sequence in pTEX-*TcNTR*-9e10 vector to generate the pTEX-9e10-NTAg-*TcFHc*.

2.3.5.5.4. pTEX-*TcFHc*

A rescue vector was constructed for the case when the fumarase genes depletion would trigger a deleterious phenotype. Thus, *TcNTR* was removed from pTEX-*TcNTR*-9E10 vector using *Eco*RI and *Xho*I restriction enzymes and replaced with *TcFHc*-NoTag fragment (Table 5 and Table 6) previously digested with *Eco*RI and *Xho*I enzymes (Figure 19).

Figure 19. Map of the pTEX-*TcFHc* vector: The pTEX-*TcFHc* was conceived as a rescue vector in the case of fumarase gene copies would be essential. The vector was built cutting out both *TcNTR* gene and c-myc tag from the pTEX-*TcNTR*-9e10 and replacing by the *TcFHc* gene between *EcoRI* and *XhoI* restriction sites.

2.3.5.6. Western Blot

The western blot or protein blotting technique was used to detect the c-myc tagged *TcFHc* and *TcFHm* in *T. cruzi* cells transfected with pTEX-*TcFHc*-9e10 and pTEX-*TcFHm*-9e10 episomal vectors respectively (Renart, Reiser e Stark, 1979; Towbin, Staehelin e Gordon, 1979; Burnette, 1981).

1×10^8 parasites in logarithmic phase of growth were harvested by centrifugation (10 minutes at 1,500 g) and the pellet washed with sterile 1x PBS and spun down. The cells were resuspended in 100 μ L of Laemmli buffer (10^7 parasites/10 μ L), sonicated and incubated at 95 °C for 5 minutes. 5 μ L sample were loaded in each lane of a sodium dodecyl sulfate (SDS) containing polyacrylamide gel and subjected to an electrophoretic run (SDS-PAGE) (Laemmli, 1970).

The separated proteins were then electrophoretically transferred to a nitrocellulose membrane using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) according to the manufacturer's protocol.

Detection of c-myc tagged proteins were carried out using the Amershan ECL Western blotting detection reagents and analysis system (GE healthcare), using monoclonal mouse anti-c-Myc Antibody (9e10) (Santa Cruz Biotechnology) as primary antibody (1:2500 dilution) and goat anti-mouse conjugated with horseradish peroxidase (1:2500 dilution).

2.3.5.7. Sub-cellular localization by digitonin permeabilization

The distribution of the *TcFHm* enzyme within *T. cruzi* cellular compartments was assessed by gradually permeabilizing the parasite's membranes by increasing digitonin concentrations. The

protocol designed was adapted from two different studies previously reported (Ferella *et al.*, 2008; Feliciano *et al.*, 2012).

4×10^9 cells of the *T. cruzi* cell line harboring the pTEX-*TcFHm*-9e10 vector in logarithmic phase of growth were harvested by centrifugation for 10 minutes, 1000 g at room temperature (RT). The cells were washed twice with buffer D (20 mM Tris-HCl pH 7.2, 225 mM sucrose, 20 mM KCl, 10 mM KH_2PO_4 , 1 mM EDTA and 5 mM MgCl) and spun down. The pellet was resuspended in 3 mL of buffer D containing protease inhibitor cocktail (Roche) and cells were kept on ice. 50 μL aliquot of the cell suspension was combined with an equal volume of Triton X-100 0.2% and 300 mM NaCl and incubated for 20 minutes at RT. This sample was used to determine the protein concentration of the cell suspension using the Pierce BCA Protein Assay Kit (Thermo).

The cell suspension was diluted in buffer D to 3 mg/mL total protein concentration. 100 μL of cells (300 μg total protein) were combined with 200 μL of different digitonin stock solutions in buffer D to reach the following final digitonin concentrations: 0, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 500, 750 and 1000 $\mu\text{g}/\text{mL}$. The samples were incubated for 5 minutes at 28 °C, centrifuged at 14,000 g for 2 minutes and the supernatant carefully aspirated. A total lysis control sample was obtained by incubating cells with 0.1 % Triton X-100 for 20 min at 4 °C.

Western blot analyses of the supernatants were carried out as described before. Three different primary antibodies were used as organelle markers: rabbit anti-enolase as cytosolic marker (Lai, Bontempi and Lukes, 2012), rabbit anti-aldolase as glycosomal marker (Galland *et al.*, 2007) and rabbit anti-ASCT (acetyl:succinate CoA transferase) as mitochondrial marker (Riviere *et al.*, 2004).

2.3.5.8. *T. cruzi* luciferase expressors

Fluorescent or bioluminescent parasites are of great use to assess infection spread and parasitemia in host cells or animal models and hence constitute a powerful tool to evaluate efficacy of new drugs or, in this particular case, investigate phenotypic responses mutant parasites.

In this context, *T. cruzi* fumarase wild type and knockout cell lines (*TcFHC*^{-PAC/-BLA} and *TcFHm*^{-PAC/-BLA}) were transfected with the linear DNA released after digesting the pTRIX2-RE9hum (Bot *et al.*, 2010) vector with *SacI* and *Ascl* (Figure 20). The integration of this linear DNA into the ribosomal array enables the parasite cells to constitutively express the luciferase gene which catalyses the handy bioluminescent reaction observed in fireflies when the substrate luciferin is present.

Figure 20. Map of pTRIX2-RE9h vector: The DNA fragment released upon digestion of the vector with *SacI* and *AscI* contains the luciferase gene (green), the geneticin resistance gene (red) flanked by the GAPDH UTRs and a rDNA sequence which enable the integration into the *T. cruzi* gDNA.

The positively transfected cells were selected using 100 µg/mL G418 maintaining the additional puromycin and blasticidin selective pressure for the knockout cell lines.

After cloning by limited dilution five clones of each cell line (WT-TRIX, *TcFhc*^{-PAC/-BLA}-TRIX and *TcFhm*^{-PAC/-BLA}-TRIX) were assayed to check the luciferase activity using the Steady Glo Luciferase Assay System (Promega) in a luminometer (Wallac). Briefly, 10⁷ parasites of each clone were centrifuged and resuspended in 100 µL of assay lysis buffer and incubated by 5 minutes at RT. 10 µL of the cell lysate were transferred to a luminescence compatible 96 well microplate where 50 µL of luciferase assay reagent (luciferin) was added before measurements were started.

2.4 Results and Discussion

2.4.1. Sequence analysis

In silico prediction of signal peptides was performed for *Trypanosoma cruzi* fumarases TcFHc and TcFHm, as well as for the orthologues in *Trypanosoma brucei* TbFHc and TbFHm and *Leishmania major* LmFH-1 and LmFH-2.

The output of Mitoprot showed a high probability ($\geq 90\%$) of TcFHm and TbFHm to be found inside the mitochondrion and a low probability of TcFHc (14%) and TbFHc (3%) to be addressed to this organelle, the same result was obtained using TargetP. TbFHm is indeed a mitochondrial protein as shown by sub-cellular localization experiments in procyclic *T. brucei* (Coustou *et al.*, 2006). Neither Mitoprot or TargetP could correctly predict the mitochondrial import of LmFH-1 as detected by confocal microscopy and digitonin fractionation experiments (Feliciano *et al.*, 2012). These predictions suggested that TcFHc is cytosolic, or at least non-mitochondrial, and TcFHm is a mitochondrial enzyme.

In addition, both TcFHs protein sequences seem to contain peroxisomal targeting sequences (PTS) indicating these proteins could be also localized to glycosomes (peroxisomes derived organelles specialized in glucose metabolism highly present in kinetoplastids). The TcFHc C-terminus end (-SKLL) resembles the classic type 1 PTS (PTS1), -SKL, which is recognized by proteins (PEX proteins) of the machinery responsible for the import of proteins into the glycosome lumen (Parsons, 2004). However, the effect of the extra leucine residue on the recognition by PEX proteins is unknown. A less conserved type of PTS (PTS2) was identified at the N-terminus of TcFHm (-KYVPLIPHV-), TbFHm (-KVFPLLPHL-) and LmFH-1 (-KVSHKETKY-) enzymes (Opperdoes e Szikora, 2006). Nevertheless, the experimental data available so far for both *L. major* and *T. brucei* fumarases could not detect a glycosomal fumarase (Coustou *et al.*, 2006; Feliciano *et al.*, 2012). These features can be visualized in the sequence alignment between *T. cruzi*, *T. brucei*, *L. major* and *E. coli* fumarase homologues (Figure 21).

The *E. coli* fumarase A (EcFumA) shares many conserved residues with the trypanosomatid fumarases. Three cysteines in particular are conserved in all class I fumarases (highlighted in yellow - Figure 21) and they are responsible for the binding of an iron-sulfur cluster.

Figure 21. Sequence alignment of class I fumarases. The *TcFhc*, *TbFhc*, *LmFH-2*, *EcFumA*, *TcFhm*, *TbFhm* and *LmFH-1* enzyme sequences were aligned using Multalin (Corpet, 1988) and the alignment was further edited using Jalview 2.8 (Waterhouse *et al.*, 2009). Black boxes indicate the mitochondrial signal peptides predicted by TargetP; regions highlighted in purple corresponds to the PTS2; the sequence highlighted in green shows the non-canonical PTS1 sequence for *TcFhc* and *TbFhc*; the conserved cysteines highlighted in yellow are responsible for binding of an iron sulfur cluster present in all class I fumarases.

In addition, sequence identity between the trypanosomatid fumarases predicted to be in the same cell compartment (e.g., *TcFHm* and *LmFH-1* – 75.3%) is higher than when comparing the mitochondrial and cytosolic isoforms within the same organism (e.g., *TcFHm* and *TcFHc* – 59.7%). These data suggest that a gene duplication event occurred in a common ancestor of *Leishmania* and *Trypanosoma*. In fact, this event possibly occurred in a very early stage of the tree of life since this gene duplication is also present in *E. coli* which possess the 90% identical fumarases *fumA* and *fumB*. Interestingly, all trypanosomatid fumarases, regardless of their cell localization, share about 59% identity with *E. coli* *fumA*. Thus, it is tempting to suggest that both fumarase genes were acquired together with the symbiotic organism that gave rise to the mitochondrion, and were further transferred to the nuclear genome in different chromosomes evolving separately.

2.4.2. Protein production

Both fumarases isoforms, *TcFHc* and *TcFHm*, were successfully cloned, expressed and purified by nickel affinity chromatography (Figure 22). The pET-SUMO vector showed better yield (10 mg/L of *E. coli* culture) and were chosen instead pET-28a derivatives.

Figure 22. SDS-PAGE analysis of nickel affinity chromatography of *TcFHc* and *TcFHm* enzymes: Both full length genes of *TcFHc* and *TcFHm* enzymes were cloned into pET-28-SUMO vector and used to transform *E. coli* BL21 DE3 cells to overexpress the target enzymes fused with a N-terminal 6xHis-SUMO tag. This tag enabled the *TcFHc* and *TcFHm* purification by metal affinity chromatography and was removed by ULP1 digestion. M stands for molecular marker; A) 1- Ni-NTA bound *TcFHc* during cleavage by ULP1; 2 and 3 show pure *TcFHc* (62kDa) fractions eluted from Ni-NTA; B) 4- *TcFHm* during cleavage by ULP1; 5- *TcFHm* after cleavage; 6 and 7 – *TcFHm* (60.8 kDa) elution from the column; 8 – column wash with 500 mM imidazole and elution of the 6xHis-SUMO tag (15.1 kDa).

Pure *TcFHc* and *TcFHm* fractions presented a brownish color suggestive of iron-sulfur containing enzymes. Addition of Fe^{3+} and Fe^{2+} salts and cysteine to the culture medium showed to be

crucial to increase cluster incorporation with consequent enhancement in protein stability and activity. *TcFHm* showed higher tendency to precipitate than *TcFHc*, limiting its characterization.

The N-terminal and C-terminal domains of *TcFHc* were cloned separately, expressed and purified as described for *TcFHc* and *TcFHm* full length enzymes. *TcFHc* C-terminal domain yielded about 10 mg of pure protein per liter of *E. coli* culture (Figure 23).

Figure 23. SDS-PAGE analysis of nickel affinity chromatography of the C-terminal domain of *TcFHc* enzyme. M stands for the molecular marker; 1 – Insoluble fraction after cell lysis by sonication; 2 – soluble fraction containing C-terminal domain of *TcFHc* enzyme fused to the 6xHis-SUMO tag (~35 kDa); 3 – wash step carried out with buffer B (containing 25 mM imidazole); 4-7 – elution of the tag free C-terminal domain of *TcFHc* (19.8 kDa); 8 and 9 – resin wash with buffer A (containing 500 mM imidazole) with elution elution of ULP1 (~20 kDa) and the 6xHis-SUMO tag (15.1 kDa).

On the contrary, the majority of *TcFHc*N-terminal domain remained in the insoluble fraction after cell lysis. Despite the fact that small amounts of soluble protein could be detected, cleavage by ULP1 and consequent removal of 6xHis-SUMO showed not to be efficient (Figure 24). *TcFHc*N-terminal domain showed to be unstable and not suitable for pursuing further experiments.

Figure 24. SDS-PAGE analysis of nickel affinity purification of *TcFhc* N-terminal domain: M stands for molecular marker; 1 - insoluble fraction containing the most part of the expressed target protein (50 kDa); 2 – soluble fraction; 3 and 4 elution after cleavage with buffer A, the processed protein appears as a thin band (35 kDa) and the tagged protein as a strong band (50 kDa).

In addition to full length *TcFhc*, *TcFHm* and the isolated N- and C-terminal domains of *TcFhc*, we have also tested an expression system for the truncated version of *TcFHm*, Δ mt*TcFHm*, where the predicted mitochondrial signal peptide had been removed. However, despite several attempts, expression of the target protein could not be detected. A suitable expression system for the truncated version of *TcFHm* is still under development.

2.4.3. Biochemical and biophysical analysis

2.4.3.1. Size-exclusion chromatography

Size-exclusion chromatography was performed to determine the native quaternary structure of recombinant full length *TcFHc*. In addition, the effect of DTT on preventing aggregation by non-specific disulfide bond formation was also investigated. Size exclusion chromatography for *TcFHc*, previously purified by nickel affinity chromatography, was carried out in presence and absence of DTT. The chromatographic profile of pure enzyme showed two main populations for *TcFHc*. Addition of 1 mM DTT increased protein homogeneity suggesting that improper disulfide bond may occur for *TcFHc* (Figure 25). Different size markers were used to build a calibration curve and an approximated molecular weight of 147 kDa was found for *TcFHc* peak. This is close to the expected size of a dimer (142 kDa) reported as the classic quaternary structure of class 1 fumarases.

Figure 25. Elution profile of *TcFHc* enzyme on a Superdex 300 column. In red is shown the chromatographic profile of *TcFHc* purified without DTT and in blue the *TcFHc* sample prepared with 1mM DTT. DTT clearly reduces *TcFHc* aggregation probably by avoiding the formation of unspecific disulfide bonds.

2.4.3.2. Circular Dichroism

Circular dichroism was used to evaluate *TcFHc* secondary structure and thermo stability. The far-UV CD spectra of *TcFHc* measured as a function of the temperature are shown in (Figure 26 A). The spectrum at 20 °C contains the usual CD bands attributed to transitions occurring in α -helix elements (centred at 190 and at 208 nm). However, the third typical absorption of an α -helix at 222 nm is poorly resolved at all temperatures. This is probably due to an admixture of the 222 nm α -helix

transition with the 215 nm β -sheet transition, thus suggesting the presence of α -helix and β -sheet elements in the protein structure. Deconvolution of CD spectra to estimate the secondary structure content of *TcFhc* using k2d (Andrade *et al.*, 1993; Merelo *et al.*, 1994) at 10 °C indicated a composition of 40% α -helix, 16% β -strand and 44 % random structure.

The estimated secondary structure content for *TcFhc* was compared with that of class II fumarases of known crystal structures (PDBIDS: 3QBP, 3OCE, 3NO9, 3GTD, 3E04, 1YFE, 1VVK, 1YFM and 3RRP) which show a conserved secondary structure distribution of approximately 57% α -helix and 7% β -strand. In addition to the sequence similarity with other members of class I fumarase and the oxygen sensitivity, our findings thus suggest that *TcFhc* has important structural differences to class II fumarases.

Interestingly, *TcFhc* differed slightly from the CD spectra of *Leishmania major* homologues despite being highly similar proteins (Feliciano *et al.*, 2012). If these differences translate into actual structural or dynamic changes between leishmania and trypanosome fumarases remain to be determined.

TcFhc thermal denaturation was evaluated by measuring the optical activity at 222 nm (θ_{222}) as a function of temperature. Values of θ_{222} are plotted in (Figure 26B) and show that *TcFhc* follows a sigmoid-like (two-state) thermal transition from its native to an unfolded state with a temperature transition of 50 °C.

Figure 26. Circular dichroism spectra of *TcFhc*: A) *TcFhc* CD spectra shows the α helical minimum in 190 nm and maximum at 208 nm and a poorly resolved minimum at 222 nm probably due to contamination of β -strand minimum at 215 nm indicating that *TcFhc* is $\alpha\beta$ protein. B) *TcFhc* thermal denaturation was monitored at 222 nm and showed a sigmoid thermal transition from the native to the unfolded state.

2.4.3.3. Electron paramagnetic resonance

The EPR spectra of both *TcFHc* and *TcFHm* contain resonances centered on $g \sim 2.019$ which are typical of $S=1/2$ spin system in an axial local symmetry as shown in Figure 27. This type of spectra was assigned to $[3\text{Fe-4S}]^+$ clusters (Flint, Emptage e Guest, 1992). This result differs from previously published data which reported that $[4\text{Fe-4S}]^{2+}$ clusters are present in the structures of other fumarate hydratases (Flint, Emptage e Guest, 1992; Vankuijk *et al.*, 1996; Shimoyama *et al.*, 2007; Van Vugt-Lussenburg *et al.*, 2009). $[4\text{Fe-4S}]$ clusters are described to be crucial to class I fumarases catalytic mechanism where they behave as a Lewis acid that activates the substrates (Flint, Emptage e Guest, 1992).

Figure 27. EPR spectra of *TcFHc* and *TcFHm*: Both enzymes EPR spectra contain a resonances centered in $g \sim 2.019$ which is considered a fingerprint of a $[3\text{Fe-4S}]^+$ cluster.

It is known that $[3\text{Fe-4S}]$ clusters can be reversibly produced from $[4\text{Fe-4S}]$ clusters by oxidation and consequent loss of one iron atom when the preparation of iron-sulfur proteins is carried out under aerobic conditions (Telser, Lee e Hoffman, 2000; Brown *et al.*, 2002; Ossa *et al.*, 2011). Although the purification was prepared under nitrogen atmosphere, the conditions were not strictly anaerobic resulting in the conversion of $[4\text{Fe-4S}]$ to $[3\text{Fe-4S}]$. To check for that possibility, we quantified the EPR signal of *TcFHc* by comparing the double-integrated area of the EPR spectrum of *TcFHc* and a standard sample (1mM CuCl_2 with 10 mM EDTA) with EPR resonances in the same magnetic field. The quantification showed that the doubly-integrated area of the *TcFHc* spectrum accounted for less than 10% of the expected cluster concentration (based on the protein concentration spectrophotometrically determined) suggesting that either the iron-sulfur incorporation was low during overexpression in bacteria or total cluster destruction by oxygen

occurred during sample preparation. The second hypothesis is more likely to be the explanation since a *TcFHc* sample prepared under strictly anaerobic environment showed 70% cluster content measured spectrophotometrically at 410 nm and using $\epsilon=15000 \text{ M}^{-1}\text{cm}^{-1}$ (Nakamaru-Ogiso *et al.*, 2002). Further oxidation beyond the [3Fe-4S] cluster and cluster destruction was also reported for *E. coli* fumarase A (Flint, Emptage e Guest, 1992). In addition, experiments performed under anaerobic environment showed that *TcFHm* had only 9% of cluster incorporation. This finding may explain *TcFHm* instability and tendency to precipitate.

Finally, EPR measurements of *TcFHs* incubated with substrates, malate and fumarate, showed changes in resonance evidencing a direct interaction between the cluster and catalysis (Figure 28). Therefore, remaining substrates carried from the *E. coli* and subsequent purification steps could in turn contribute to underestimate the cluster content in these enzymes.

Figure 28. EPR spectra of *TcFHc* with malate and fumarate. EPR spectra were obtained for *TcFHc* (blue) incubated with excess of both sodium malate (red) and sodium fumarate (green). A reduction in the intensity indicates that the iron sulfur cluster interactis with both carboxylic acids tested.

2.4.3.4. Optimum pH and kinetic

TcFHc showed its highest catalytic activity between pHs 8 and 9 and negligible activity above pH 10 and below pH 6 (Figure 29). Similar values were reported for *Euglena gracilis* class I fumarase (Shibata, Gardiner e Schwartzbach, 1985). The pH 8.5 was chosen to perform full kinetic characterization of both *TcFHc* and *TcFHm* enzymes.

Figure 29. Fumarate formation/consumption measured at 250 nm versus pH. Optimum pH determination *TcFhc* catalytic activity: A) Malate dehydration to fumarate was monitored by fumarate formation at 250 nm at different pHs. Highest peaks were found between pH 8 and 9; B) Similarly, fumarate hydration to malate reached its max velocity between pH 8 and 9.

Kinetic parameters for *TcFhc* and *TcFhm* were obtained for both formation and consumption of fumarate in anaerobiosis (Table 7). The protein amount used was corrected based on the iron-sulfur content measured spectrophotometrically at 410 nm wavelength.

Table 7. Kinetics parameters calculated for *TcFhc* and *TcFhm* enzymes.

Enzyme	Substrate	Kcat (s ⁻¹)	Km (mM)	Kcat/Km (M ⁻¹ s ⁻¹)
<i>TcFhc</i>	Malate	2.6 (±0.25) × 10 ⁴	3.8 ± 0.7	7.0 × 10 ⁶
	Fumarate	4.0 (±0.6) × 10 ⁴	1.5 ± 0.3	2.7 × 10 ⁷
<i>TcFhm</i>	Malate	1.00 (±0.05) × 10 ⁴	2.4 ± 0.3	4.3 × 10 ⁶
	Fumarate	1.4 (±0.2) × 10 ⁴	0.7 ± 0.2	1.9 × 10 ⁷
<i>EcFumA</i>	Malate	7 × 10 ²	0.7 ± 0.1	1.0 × 10 ⁶
	Fumarate	1.9 × 10 ³	0.46 ± 0.08	4.2 × 10 ⁶
<i>EcFumB</i>	Malate	4.8 × 10 ²	0.30 ± 0.07	1.6 × 10 ⁶
	Fumarate	1.3 × 10 ³	0.32 ± 0.04	4.5 × 10 ⁶

TcFhc and *TcFhm* enzymes presented similar kinetic properties. Both enzymes showed lower Km for fumarate than for malate which in addition to a faster turnover (Kcat) for the formation of malate resulted in a catalytic efficiency (Kcat/Km) for the hydration direction five times higher than for the dehydration. Unless some unknown regulation occurs within the *T. cruzi* cells the hydration direction from fumarate to malate catalyzed by both *TcFHs* should be the most common one. It remains elusive the utilization of a classical oxidative direction of the TCA cycle in *T. cruzi*, however procyclic *T. brucei* uses the dehydration direction to provide fumarate to fumarate reductase (Van

Weelden *et al.*, 2003). In spite of the intrinsic preference for the malate formation, the reverse reaction could easily occur in a broader metabolic context where malate concentration is higher.

In addition, *TcFHs* showed two fold higher *K_{cat}* for both substrates when comparing to *E. coli* class I fumarases (*EcFumA* and *EcFumB*) and one fold higher *K_m* resulting higher catalytic efficiencies for the trypanosomal enzymes (Van Vugt-Lussenburg *et al.*, 2013).

However, when purified in aerobic atmosphere, *TcFHs* exhibited low activity. Thus, *TcFHs* are highly susceptible to oxygen which is a common feature among iron-sulfur lyases such as *E. coli* *fumA* (Flint, Emptage e Guest, 1992) and aconitase (Robbins e Stout, 1985) as a consequence of [4Fe-4S] cluster oxidation to [3Fe-4S] cluster.

2.4.3.5. Crystallization

Several attempts in order to crystallize both full length *TcFHc* and *TcFHm* enzymes failed for reasons not completely understood. Hypotheses such as protein flexibility, heterogeneity, instability, were evaluated with the use of different additives and the cloning of predicted stable domains.

Based on the crystal structure of the cytosolic *Leishmania major* fumarase it was possible to predict two domains, N-terminal and C-terminal, for *TcFHc*. As described earlier, the predicted N-terminal domain showed to be unstable and not adequate for crystallization experiments. Differently, crystals of the C-terminal domain of *TcFHc* were readily obtained by mixing 2 µL of protein at 6.6 mg/mL with 2 µL of crystallization solution with overnight incubation at 293 K. Two different conditions from the Crystal Screen kit (Hampton Research) were identified as promising crystallization conditions: 100 mM Tris-HCl pH 8.5, 200 mM sodium acetate trihydrate and 30 % (w/v) polyethylene glycol (PEG) 4000 (condition 22 - Crystal Screen 1) and 100 mM Tris pH 8.5, 200 mM magnesium chloride hexahydrate and 3.4 M 1,6-hexanediol (condition 39 - Crystal Screen 2). All crystals grew as hollow cylinders and diffracted poorly (7 Å) (Figure 30). Hollow crystals arise when crystal growth is faster than protein diffusion. Under these circumstances the protein molecules get trapped in the crystal wall before they can reach its interior leaving a solvent channel. In order to decrease crystal growth rates lower temperature (277 K), use of additives such as glycerol and addition of paraffin/silicon oil to the reservoir were tested but no improvement on diffraction could be observed. Interestingly, addition of paraffin to the reservoir solution had no effect in the crystallization rate which means vapor diffusion plays little, if any, role in the *TcFHc* C-terminal domain crystallization in such conditions.

Figure 30. Straw like crystals of *TcFHc* C-terminal domain. Crystals were obtained by sitting drop vapor diffusion mixing 2 μ L of purified protein at 6.6 mg/mL with 2 μ L of reservoir solution consisted of 100 mM Tris-HCl pH 8.5, 200 mM sodium acetate trihydrate and 30% PEG 4K.

As an additional attempt, *TcFHc* C-terminal domain crystals were crushed and used as crystal seeds for the crystallization of the full length *TcFHc* resulting in too tiny crystals not suitable for X-ray diffraction experiments even for microfocus beamline (I24 - Diamond, UK) and optimization is in progress.

2.4.3.6. Homology models

TcFHc and *TcFHm* homology models were built using the *LmFH-2* crystal structure recently solved by Patricia R. Feliciano as part of her Doctorate studies in our lab. This is the first and only structure available for class 1 fumarases. *LmFH-2* shared approximately 73.9 % of sequence identity against *TcFHc* and 59.8 % against *TcFHm*.

TcFHc and *TcFHm* models present a homodimeric structure where each monomer presents a α/β fold. Analyses of secondary structure for both protein models suggested that each *TcFHc* monomer is composed of 25.6% β -strands, 27.8% of α -helices, 3.5% of 3_{10} helices 43.1% of disordered regions (loops and turns) and of *TcFHm* is composed of 22.0% β -strands, 28.3% of α -helices, 1.1% of 3_{10} helices and 48.5 % of disordered regions (loops and turns) (Figure 31).

The comparison between the *TcFHc* and *TcFHm* homology models suggested *TcFHs* are structurally similar, as expected from the high sequence similarity (75.6%) which in turn is explained by the shared origin of both *TcFHc* and *TcFHm* genes (Figure 31 and Figure 21). Nevertheless, significant differences mainly at the N-terminal region could be observed.

As mentioned earlier, we predicted the presence of an N-terminal 35 residue long mitochondrial signal peptide, using TargetP, which is expected to be removed during mitochondrial import. Based on this prediction *TcFHm* enzyme was truncated accordingly but overexpression in *E. coli* could not be detected. Since the deletion of the first 35 residues of *TcFHm* implied loss of secondary structure (Figure 31) it may have stalled protein folding. However, structural analysis

together with *in silico* predictions suggested a 15 residues long mitochondrial signal peptide instead and new attempts to produce a new truncated version of *TcFHm* are in progress.

In addition, considering both fumarases shared a common ancestral, we speculate that *TcFHc* gained an insertion of 12 residues at the N-terminal extremity over the course of evolution. Therefore, *TcFHc* is 27 residues longer than the mature *TcFHm* (Figure 33), but the reason behind this N-terminal extension in *TcFHc* as well as if this feature has an impact on protein function remains to be understood.

Figure 31. *TcFHc* and *TcFHm* homology models: *TcFHs* are dimeric proteins and each monomer formed by two domains the N-terminal (green) and the C-terminal domain (blue). The N-terminal domain holds all cysteines responsible for iron-sulfur cluster binding. The cluster is located at the catalytic tunnel formed at the N-terminal and C-terminal domains interface. *TcFHc* contains a β -hairpin which is missing in *TcFHm* (yellow). The regions highlighted in red in *TcFHc* were deleted for the pET28-SUMO-*TcFHc*-Cterm and pET28-SUMO-*TcFHc*-Nterm vectors construction. The region in red in *TcFHm* corresponds to the signal peptide predicted by TargetP and was removed to generate the pET-28-SUMO- Δ mt*TcFHm* vector.

Each *TcFH* chain can be described as two main domains, the N- and C-terminal domains. The N-terminal domain (residues 1 – 383, *TcFHc* numbering) accommodates all three cysteines (C 131, C 250 and C 344) involved in the iron-sulfur cluster binding and is responsible for all intermolecular interactions between the monomers. The C-terminal domain (residues 384 – 565) is important for substrate interaction. The interface between the C-terminal and N-terminal domains forms the catalytic tunnel with the iron-sulfur cluster positioned at the back. 10 residues are involved in substrate interaction: 5 of chain A C-terminal domain (R 419, T 465, T 466, R 469 and K 489) 4 of chain A N-terminal domain (Q 132, D 133, R 170, G 214) and 1 of chain B N-terminal domain (H 332) (Figure 32).

Figure 32. Catalytic site of kinetoplastid fumarases. Homology models of *TcFHc* (green) and *TcFHm* (blue) and crystal structure of *LmFH2* (magenta). All residues involved in substrate interaction are conserved throughout the enzymes. The catalytic site is composed by 10 residues and 3 cysteines responsible for iron-sulfur cluster binding. Two malate molecules are shown one chelating the labile iron of the 4Fe-4S cluster and the other at the entrance of the catalytic site.

N and the C-terminal domains are only connected by a long coil which could lead to movement between domains and further impairment of crystallization. Hence, *TcFHc* domains were separately produced and possible flexible regions such as the first 41 residues and the domains linker were removed (Figure 31 and Figure 33). The poor solubility found for the N-terminal domain could indicate that the n-terminal residues removed are somehow important for protein structure. In fact, some organisms express these domains as two independent proteins and the resulting fumarase enzyme is assembled as a heterodimer (Van Vugt-Lussenburg *et al.*, 2009).

Figure 33. Sequence alignment and secondary structure description of *TcFhc* and *TcFhm* enzymes. N-terminal domain residues which interact with the substrate are highlighted in green and C-terminal residues in blue. The yellow cysteines are responsible for cluster binding. *TcFhc* region 50-70 corresponds to a β -hairpin that is structurally missing in *TcFhm* due to the presence of two prolines in the mitochondrial isoform.

2.4.4. Reverse genetic studies in *T. cruzi*

2.4.4.1. *TcFHc* double knockout

Both *TcFHc* gene copies located at *T. cruzi* chromosome TcChr36-P were disrupted by homologous recombination with two linear DNA fragments containing BLA and PAC resistance cassettes. Proper integration of the knockout fragments was analyzed by Southern blot (Figure 34).

Figure 34. Southern blot analysis of the *T. cruzi* *TcFHc* knockout cell lines: The first lane corresponds to *T. cruzi* Sylvio X10/6 gDNA containing both copies of the wild type (WT) *TcFHc* gene. Second lane shows the same region of the genome where one copy of *TcFHc* was disrupted with the blasticidin resistance cassette. Similarly the third lane corresponds to the disruption of one *TcFHc* copy by the puromycin resistance cassette. The fourth lane shows the double *TcFHc* mutant in a polyclonal population. The last lane represents a clonal population of *TcFHc* double knockout *T. cruzi*. Gene drawings at scale are represented on the left. At the bottom, a simple representation of the chromosomal arrangement for each cell line.

2.4.4.2. *TcFHm* double knockout

Similarly, both copies of the *TcFHm* gene were successfully disrupted by BLA and PAC resistance cassette (Figure 35).

Figure 35. Southern blot analysis of the *T. cruzi* *TcFHm* knockout cell lines: The first lane corresponds to *T. cruzi* Sylvio X10/6 gDNA containing both copies of the wild type (WT) *TcFHm* gene. Second lane shows the same region of the genome where one copy of *TcFHm* was disrupted with BLA resistance cassette. Similarly the third lane corresponds to the disruption of one *TcFHm* copy by PAC resistance cassette. The fourth lane shows the double *TcFHm* mutant in a polyclonal population. The last lane represents a clonal population of *TcFHm* double knockout *T. cruzi*. Gene drawings at scale are represented on the left. At the bottom, a simple representation of the chromosomal arrangement for each cell line.

During construction of fumarase knockout cell lines several parasites presented trisomic chromosomes where both resistance cassette were integrated and a third chromosome containing the wild type fumarase gene was kept (results not shown). Although those cell lines had no use for downstream characterization, trisomy raised important questions about the importance of fumarase genes to *T. cruzi* cells.

2.4.4.3. *TcFHc* double *TcFHm* single knockout

T. cruzi cell line possessing only one copy of fumarase gene (*TcFHm*) was obtained by transfecting the *TcFHm* knockout fragment containing the NEO resistance cassette in the clone 1 *TcFHc*^{-PAC/-BLA} cell line generating a *TcFHc*^{-PAC/-BLA}/*TcFHm*^{+/-NEO} genotype (Figure 36).

Figure 36. Southern blot analysis of the *T. cruzi* *TcFHc* double *TcFHm* single knockout cell line. gDNA of WT (first lane), *TcFHc*^{-PAC/BLA} (second lane) and *TcFHc*^{-PAC/BLA} *TcFHm*^{+/-NEO} (third lane) *T. cruzi* cell lines were analyzed by southern and revealed the disruption of a *TcFHm* copy in a double *TcFHc* knockout background.

In fact, only one clonal population was generated in this experiment and was further used in the transfection of the last knockout fragment: the HYG resistance cassette flanked by *TcFHm* gene extremities. Despite many attempts, no viable cells lacking all four fumarase gene copies were obtained. Incubation of transfected cells with various concentrations of malate and fumarate substrates, previously reported to rescue the fumarase knockdown phenotype in *T. brucei* (Coustou *et al.*, 2006), was tested with no success. Therefore, the reverse genetics studies suggested an essential role for fumarase enzymes in *T. cruzi* viability.

2.4.5. Phenotypic analysis

2.4.5.1. Growth curves

Curves of logarithm of cell density versus time were plotted for *T. cruzi* WT (blue), *TcFhc*^{-PAC/-BLA} (green) and *TcFhm*^{-PAC/-BLA} (red) cell lines. Growth was monitored by counting cells seeded at 1×10^5 parasites every 48 hours for 20 days (Figure 37).

Figure 37. *T. cruzi* cell lines growth curves. WT (blue), *TcFhc*^{-PAC/-BLA} (green) and *TcFhm*^{-PAC/-BLA} (red) cell lines growth was monitored by counting cell every 48 hours for 20 days and cell density (cells/mL) was plotted against elapsed time.

Figure 37 shows small effect of fumarases gene knockout in *T. cruzi* growth. A pronounced lag phase was observed for the *T. cruzi* cell line lacking the mitochondrial fumarase. Nevertheless, normal growth is readily restored in log phase indicating metabolic adaptation. Interestingly, the transition to stationary phase also differs from wild type *T. cruzi*. The data suggested *TcFhm* deficiency in *T. cruzi* somehow disrupts metabolic adaptation between growth phases.

The presence of carrier proteins that shuttle malate and fumarate substrates between compartments explain a crosstalk between the mitochondrion and cytosol in *T. cruzi*. Hence, despite the absence of the fumarase enzyme in each compartment of knockout cell lines, the phenotype is compensated by the exchange of substrates between these compartments. This further explains why it was feasible to disrupt 3 out of 4 fumarase gene copies but not all of them. In our hypothesis, the

remaining gene is able to compensate the lack of fumarase activity in the whole cell by means of substrate/product transport across membranes by specific carriers.

2.4.5.2. Metacyclogenesis

T. cruzi epimastigotes undergo metacyclogenesis when stressed by starvation. Cultures of *T. cruzi* WT, *TcFHc*^{-PAC/-BLA} and *TcFHm*^{-PAC/-BLA} cell lines of 6, 16 and 18 days old were verified for the presence of metacyclis by visualizing nuclear morphology (Figure 38).

Figure 38. Evaluation of *T. cruzi* cell lines metacyclogenesis: kinetoplast DNA (kDNA) is denser than nuclear DNA and corresponds to the brighter spheres. The fainter structures correspond to the nuclei shown as a sphere in epimastigotes and as a cylinder in metacyclis. The table to the right contains the percentage of metacyclis cells for each cell line at different time points.

T. cruzi epimastigotes from the tested knockout cell lines differentiated into metacyclis suggesting that either fumarases are not involved in this process or it requires low fumarase activity. This result is important once it makes it feasible to analyze the role of fumarase in infection and amastigotogenesis, the most relevant processes from the clinical perspective.

2.4.5.3. Mammalian cell infection

Metacyclis of the *T. cruzi* WT, *TcFHc*^{-PAC/-BLA}, *TcFHm*^{-PAC/-BLA} and *TcFHc*^{-PAC/-BLA}/*TcFHm*^{+/-NEO} cell lines were used to infect Vero cells in glass slides. The slides were stained with Giemsa, the number of infected cells and the number of amastigotes per infected cell determined (Figure 39).

Figure 39. Infection of mammalian cells by different *T. cruzi* cell lines: the *T. cruzi* cell lines WT, $TcFhc^{PAC/-BLA}$ (dKO-FHc), $TcFhm^{PAC/-BLA}$ (dKO-FHm) and $TcFhc^{PAC/-BLA}/TcFhm^{+/-NEO}$ (dKO-FHc/sKO-FHm) were used to infect mammalian (Vero) cells. The number of infected cells and the number of amastigotes formed by mammalian cell were counted and the mean abundance of parasites was calculated using the quantitative parasitology software QPweb 1.0. The figure to the left shows a mammalian cell infected with the wild type *T. cruzi* Sylvio X10/6.

The results showed that the cytosolic fumarase depletion had little effect in infection although decreased the number of amastigotes per infected cell. A very similar phenotype was observed for the $TcFhc^{PAC/-BLA}/TcFhm^{+/-NEO}$ supporting the hypothesis that the deletion of a single gene copy has little effect on cell metabolism. Distinct from the $TcFhc^{PAC/-BLA}$ and $TcFhc^{PAC/-BLA}/TcFhm^{+/-NEO}$ cell lines, a pronounced decrease in both percentage of infected cells and number of amastigotes per infected cell was observed for the *TcFhm* double knockout cell line ($TcFhm^{PAC/-BLA}$). The results suggested an important role for the mitochondrial isoform in *T. cruzi* infection and amastigote proliferation.

2.4.5.4. Sub-cellular localization

The distribution of both *TcFhc* and *TcFhm* enzymes throughout the *T. cruzi* cell compartments were analyzed. The data obtained is in good agreement with both *in silico* prediction and experimental reports for the *T. brucei* and *L. major* homologues (Coustou *et al.*, 2006; Feliciano *et al.*, 2012).

Digitonin permeabilization of *T. cruzi* cells expressing the c-myc tagged *TcFhm* together with western blot analysis clearly demonstrated a mitochondrial localization for *TcFhm* isoform. Only soluble fractions obtained at high concentrations of digitonin, required for mitochondrial permeabilization, were found positive for c-myc tagged *TcFhm* as well as for the protein recognized by the anti-*TbASCT* antibody, a known mitochondrial enzyme (Figure 40).

Figure 40. Digitonin permeabilization of *T. cruzi* cells expressing c-myc tagged TcFHm: TcFHm was released concomitantly with ASCT at high digitonin concentration. These results are in agreement with our *in silico* analysis which predicted the mitochondrial cell compartmentalization for TcFHm.

Confocal microscopy analysis of *T. cruzi* CL Brener expressing TcFHc and TcFHm tagged with green fluorescent protein (GFP) were carried out by Dr. Shane Wilkinson and Mr. Ali Kia. *T. cruzi* expressing TcFHc-GFP showed fluorescence throughout the whole cell indicating a cytosolic localization for TcFHc (Figure 41).

Figure 41. Confocal microscopy of *T. cruzi* CL Brener expressing TcFHc-GFP. Parasites expressing TcFHc-GFP (green) were co-stained with DAPI (DNA; blue). A pattern throughout the whole cell was observed typical of cytosolic proteins.

Parasites expressing TcFHm-GFP were stained with the mitochondrial marker Mitotracker (Life Technologies) and analyzed by confocal microscopy. Merged images confirmed the mitochondrial localization for TcFHm (Figure 42).

Figure 42. Confocal microscopy of *T. cruzi* CL Brener expressing TcFHm-GFP:Parasites expressing TcFHm-GFP (green) were co-stained with Mitotracker (red). The pattern of co-localization (yellow) is shown in the merged image.

2.4.5.5. Luciferase expressors

T. cruzi WT, *TcFhc*^{-PAC/-BLA} and *TcFHm*^{-PAC/-BLA} cell lines were modified to express the luciferase enzyme, which will be used in the future for compound screening in animal models. Since integration to the parasite ribosomal array is not controlled, expression levels of luciferase may differ among clones and screening for the best expressors was carried out (Figure 43).

Figure 43. *T. cruzi* cell lines expressing luciferase. The blue bars indicate luminescence intensity by each of five clones of WT (WT 1-5), *TcFhc*^{-PAC/-BLA} (C 1-5) and *TcFHm*^{-PAC/-BLA} (M 1-5) cell lines in comparison with the *T. cruzi* WT (wt CONTROL) which lacks the luciferase enzyme.

Five clones of each *T. cruzi* cell line were tested for luminescence after previous incubation with the substrate luciferin. The best expressors of each cell line, WT1, C3 and M1 were selected and will be further used in both *in vivo* and *in vitro* characterization of fumarase knockout cell lines.

2.5 Conclusions

Trypanosoma cruzi fumarases (TcFHs) are products of a gene duplication event which occurred early in the tree of life and further evolved separately giving rise to two very similar enzymes: TcFHc and TcFHm.

Both enzymes were successfully cloned, heterologously expressed in *E. coli*, purified to homogeneity and characterized by biophysical and biochemical approaches.

The results shown in this chapter depict TcFHc and TcFHm as dimeric enzymes which contain in each monomer a 4Fe-4S cluster as prosthetic group in the catalytic center directly involved with the reversible hydration of fumarate to malate. These features are classic of class I fumarases and therefore a fumarase function could be assigned to the putative fumarase genes of *T. cruzi*.

The structural characterization by homology molecular modeling showed differences between TcFHc and TcFHm evolutionarily arisen after gene duplication. Nevertheless, the residues within the catalytic center are conserved among all kinetoplastid fumarases which is important from the drug design perspective.

In order to verify the importance of TcFHc and TcFHm genes to this parasite, genetic reverse studies by gene knockout were performed and suggested fumarases are essential to *T. cruzi* epimastigotes. Infection of mammalian cells with fumarase mutant *T. cruzi* cell lines suggested TcFHm may play an important role in host cell invasion and/or amastigote proliferation.

The sub-cellular localization of *T. cruzi* fumarases was performed and TcFHc was found to be a cytosolic enzyme whereas TcFHm was encountered in the mitochondrion.

In addition, fumarase mutant *T. cruzi* cell lines were genetically engineered to express the firefly luciferase, constituting an important tool to further elucidate the TcFHs roles in various cellular processes.

Altogether, these results point to the fumarase enzymes as new macromolecular targets in the long lasting fight against Chagas disease.

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Chapter 3. Human fumarase

3.1 *Introduction*

The potential of TcFHs as promising targets for drug development against Chagas disease depends on their central position in metabolism of *T. cruzi* and their characteristic differences compared with the human host enzyme.

As seen in the previous chapter, our studies support an important role for fumarase activity in *T. cruzi*, mainly by providing substrates for important metabolic pathways. Thus, it became of high importance to understand and compare the mechanism of action of human and *T. cruzi* enzymes and further identify differences to be exploited during the development of potent and selective inhibitors against the parasitic enzyme.

Human fumarase (HsFH), a well-known citric acid cycle enzyme, therefore a key component in energy metabolism, is localized both in the mitochondria and cytosol. Both HsFHs are encoded by the *fumC* gene and their subcellular localization is determined by post-translational processes (Yogev *et al.*, 2010). Different from TcFH, HsFH belongs to class II fumarases, a homotetrameric enzyme and independent of iron-sulfur cluster for catalysis.

Noteworthy, mutations in HsFH have been implicated in a variety of human diseases, including progressive encephalopathy, fumaric aciduria, hereditary leiomyomatosis and renal cell carcinoma (Whelan *et al.*, 1983; Kerrigan *et al.*, 2000; Yang *et al.*, 2013). Despite its remarkable importance, there is only one structure of HsFH deposited in PDB (PDB ID: 3E04) from the Structure Genomic Consortium with no publications associated and only a structural study presently available regarding the clinical relevance of human fumarase was performed on the *Escherichia coli* FumC homologue, which shares 60% identity with the human enzyme (Estevez *et al.*, 2002).

In the present work, we have performed a structural and biochemical characterization of HsFH. Our findings will contribute to the mapping of distinct mechanisms adopted by class I and class II fumarases, as well as it will provide the structural basis for the development of selective ligands against TcFHs. In addition, our results introduce new perspectives to the understanding of molecular mechanisms of human diseases by characterizing different HsFH mutations in deficient fumarase activity and their relationship to distinct genetic disorders. The knowledge achieved and the experimental tools developed in this project represent an important step towards this goal.

3.2 **Objectives**

In order to develop a cross-validation model for the design of class I fumarase selective inhibitors, human fumarase has been characterized by biochemical and structural approaches.

In order to achieve our objectives the following steps have been pursued:

- Cloning of the human fumarase in pET-28a expression vector
- *HsFH* expression in *E. coli*
- Purification by metal affinity chromatography
- Development of an enzymatic assay and determination of enzyme kinetic parameters
- Determination of quaternary structure assembly by size exclusion chromatography
- Crystal structure determination of *HsFH*

3.3 Methodology

3.3.1. Protein production

The human fumarase (*HsFH*) gene (GenBank: AAA52483.1) was codon optimized for expression in *Escherichia coli*, synthesized and cloned between the 5' *Bam*HI and 3' *Hind*III restriction sites in a pUC57 vector by GeneScript (USA). The *HsFH* gene was subcloned into pET-28a vector (MerckMillipore) using *Bam*HI and *Hind*III restriction enzymes. The vector generated was called pET-28a-*HsFH* and was used to transform chemocompetent *E. coli* BL21 DE3 cells by heat shock. A single colony was used to inoculate 10 mL of LB broth containing 30 μ g/mL kanamycin and incubated overnight at 37°C and 180 rpm. The starter culture was inoculated in 1 L of LB broth and let grow for 2.5 hours at 37°C when IPTG was added to 500 μ M. The temperature was changed to 18°C and *HsFH* expression was carried out overnight. Cells were harvested by centrifugation at 8,000 g and pellets were kept at -20 °C until used.

A pellet corresponding to 167 mL of cell suspension was resuspended in 20 mL of buffer A (50 mM NaH₂PO₄ pH 8.5, 300 mM NaCl) containing 1 mM PMSF. The cells were lysed by sonication using 10 pulses of 30 seconds with an interval of 30 seconds resting in ice. The soluble fraction containing *HsFH* was obtained after centrifuging the cell lysate at 16,000 g for 30 min. The supernatant was poured into 2 mL Ni-NTA resin (Qiagen) packed in a poly-prep column previously equilibrated with buffer A. The resin was washed with 15 mL of buffer A containing 50 mM imidazole and *HsFH* was eluted with 10 mL of buffer A containing 500 mM imidazole. Pure *HsFH* fractions were combined and dialyzed against buffer B (50 mM Tris pH 8.5 and 150 mM NaCl) and concentrated using a 30 kDa cutoff centrifugal filter unit (Millipore). *HsFH* was quantified using the theoretical extinction coefficient $\epsilon_{280\text{nm}} = 24410\text{M}^{-1}\text{cm}^{-1}$ obtained from ProtParam server (Wilkins *et al.*, 1999).

3.3.2. Kinetics

All studies were performed with the oligo-histidine tagged enzyme. Initially, *HsFH* optimum pH was determined by monitoring the consumption or formation of fumarate at 250 nm using $\epsilon_{250\text{nm}} = 1450\text{M}^{-1}\text{cm}^{-1}$ under a wide range of pH (3, 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10, 11). Catalytic constants (K_m , V_{max} and K_{cat}) were determined by monitoring initial velocities while varying both substrate concentrations in buffer B: fumarate (62.5, 125, 250 and 500 μ M) and malate (0.25, 0.5, 1, 2 and 4 mM). All measurements were performed using 2.5 μ g/mL (46.5

nM) *HsFH*. Michaelis-Menten hyperbola (Equation 2) was fit by nonlinear regression implemented in Origin Lab software

Equation 2

$$V = V_{max} \frac{[S]}{K_m + [S]}$$

where V is reaction rate and $[S]$ is substrate concentration.

3.3.3. Gel Filtration

500 μ L sample of pure *HsFH* at 8 mg/mL (4 mg) was injected in a Superdex 30/200 GL (GE Lifesciences) column pre-equilibrated with buffer 50 mM Tris pH 8.5 600 mM NaCl. The same buffer was used to perform an isocratic run at a 0.5 mL/min flow rate. *HsFH* oligomeric state was determined from the calibration curve (plot of K_{av} versus the logarithm of molecular weight) once its K_{av} value is calculated from its measured elution volume (Equation 3)

Equation 3

$$K_{av} = \frac{V_e - V_0}{V_c - V_0}$$

where V_c is the column void volume, V_e is the elution volume, and V_g is the geometric column volume.

3.3.4. Crystallization

Pure *HsFH* obtained from metal affinity chromatography was concentrated to 4 mg/mL in buffer Band used for crystallization screening. The sitting drop vapor diffusion technique was used with Crystal Screen1-2 and PEG/Ion 1-2 commercial kits (Hampton Research). The plates were kept at 293K and drops were frequently checked for crystals. Crystals were grown overnight in 100 mM sodium malonate pH 5.0 with 12% (w/v) polyethylene glycol 3350 (PEG 3350) in a 4 μ L drop containing 2 μ L of protein and 2 μ L of crystallization solution and 500 μ L in the reservoir. The Additive Screen (Hampton Research) was used together with the crystallization condition described above in order to reduce nucleation and promote crystal growth.

3.3.5. Data collection, processing and phasing

A single *HsFH* crystal was harvested and transferred to a drop of cryoprotectant solution consisting of 100 mM sodium malonate pH 5.0, 14% (w/v) PEG 3350, and 30% glycerol and was further dehydrated by exposure to air for 2 hours. The dehydrated crystal was then mounted in a cryo-loop for X-ray diffraction experiments and data collection was carried out at MX2-LNLS beamline (Campinas, Brazil) using 1.459 Å X-ray wavelength. The crystal was positioned 121.6 mm far from the 3x3 MarMosaic 225 CCD detector and was kept at 100 K by using the Oxford Nitrogen Cryojet XL system. The full dataset comprised 102 images collected by using 1.0 degree oscillation with an exposure time of 60 s. Images were indexed in iMosflm (Leslie and Powell, 2007) and scaled in SCALA (Evans, 2006). Initial phases were obtained by molecular replacement with the program Phaser in Phenix (Adams *et al.*, 2010) utilizing the PDB 3E04 structure as the template.

3.3.6. Structure refinement

The *HsFH* structure refinement was performed by adjusting side chain rotamers using the software Coot (Emsley and Cowtan, 2004) interspersed with torsion angle simulated annealing and positional and individual B-factor refinement using the phenix.refine program (Adams *et al.*, 2010). Iterative manual building and refinement of the model were performed until satisfactory R-factor and R-free were obtained. Model geometry was evaluated using Molprobit (Davis *et al.*, 2007).

3.4 Results and Discussion

3.4.1. HsFH production

The HsFH was successfully subcloned into pET-28a vector from the synthetic construct and was overexpressed using *E. coli* BL21 DE3. Soluble HsFH was purified to homogeneity by nickel affinity chromatography with a yield of 110 mg of pure protein per liter of *E. coli* culture (Figure 44).

Figure 44. SDS-PAGE analysis of HsFH nickel affinity purification. M corresponds to the molecular markers (labeled in kDa to the left); Lane 1, soluble fraction obtained after cell lysate centrifugation; Lane 2, resin washing step with buffer A containing 50 mM imidazole; Lane 3, pure HsFH (53.7 kDa) eluted after passing buffer A containing 500 mM imidazole.

3.4.2. HsFH kinetics

In activity assays, the recombinant HsFH catalytic performance was monitored by the conversion of malate to fumarate and vice-versa (Table 8). The catalytic rate versus substrate concentration ($V/[S]$) plot for HsFH showed classic Michaelis-Menten saturation curves over the range of substrate concentration tested. The catalytic efficiency (K_{cat}/K_m) shown for fumarate is about 2.3 times higher than for malate which is in agreement with the classic fumarase role in converting fumarate into malate as part of the TCA cycle.

Table 8. Kinetic parameters for several members of class II fumarases.

Enzyme	Substrate	Kcat (s ⁻¹)	Km (μM)	Kcat/Km (s ⁻¹ M ⁻¹)
<i>HsFH</i>	Fumarate	26 ± 1	404 ± 32	6.54E+04
	Malate	56 ± 4	1989 ± 258	2.83E+04
<i>EcFH</i>	Fumarate	1149	207	5.56E+06
	Malate	595.2	857	6.95E+05

In comparison to the *E. coli* fumC, *HsFH* catalytic efficiency is 85 and 25 times lower for the hydration and the dehydration reaction directions respectively (Table 8).

We initially speculate whether the presence of an N-terminal tag in *HsFH* could interfere with protein activity. Worth mentioning, in the *E. coli* FumC construct its affinity tag is located in the C-terminus. On the other hand, class II fumarases were reported to have a much faster rate in presence of higher substrate concentrations (5-fold the Km) than used for *HsFH* characterization (Keruchenko *et al.*, 1992; Weaver and Banaszak, 1996; Beeckmans and Van Driessche, 1998). Unfortunately, the range of substrate concentration used for *E. coli* FumC was not stated in the cited article, compromising a more accurate analysis. In addition, some class II fumarases were also found to be activated by divalent cations such Mg²⁺ (Jiang *et al.*, 2010) and the effect of activators in *HsFH* remains to be determined.

Differently from *TcFHs*, *HsFH* activity remained constant for days in aerobic conditions which was expected for oxygen insensitive class II fumarases.

3.4.3. Size exclusion chromatography

In order to determine the quaternary structure of the recombinant protein, a gel filtration chromatography was performed using a calibrated column with different molecular markers. Pure recombinant *HsFH* was subject to size exclusion column and active enzyme eluted as a major peak at a retention volume of 11.45 mL corresponding to a molecular mass of 212 kDa (Figure 45). In addition, during gel filtration a second peak and a small shoulder were observed, suggesting the presence of small aggregates.

Considering the theoretical molecular weight of *HsFH* monomer, including the histidine tag, as 53.7 kDa (Wilkins *et al.*, 1999), the results suggest that the native enzyme exists as a homotetramer. Our findings are in agreement with the tetrameric structure observed for members of class II FHs (Woods *et al.*, 1988). In addition, the use of a gel filtration chromatography step during protein purification has allowed the isolation of high homogenous protein sample.

Figure 45. Size exclusion chromatogram of *HsFH* enzyme: The profile obtained showed a major peak indicating a homogenous population of *HsFH* purified by nickel metal affinity. Using a calibration curve built with molecular markers *HsFH* showed an estimated molecular weight of 212 kDa which corresponds to a tetrameric state (*HsFH* monomer: 53 kDa).

3.4.4. *HsFH* crystallization

Several crystallization conditions from the commercial kits promoted *HsFH* crystallization. However, the majority of crystals obtained presented hollow morphology which could lead to poor diffraction. Crystals obtained in the condition containing 100 mM sodium malonate pH 5.0 and 12% (w/v) PEG 3350 presented a different morphology (Figure 46-A) which was selected to follow optimization. Some of the additives tested improved crystal growth and reduced nucleation and the best additive consisted of 4% (v/v) Pentaerythritolethoxylate (3/4 EO/OH) (Figure 46-B).

Serendipitously, one contaminated condition was replaced by Milli-Q water and the crystallization drop consisting of 4 μ L of 2 mg/mL *HsFH*, 25 mM Tris pH 8.5 and 75 mM NaCl promoted crystal growth after 2 weeks (Figure 46-C). Albeit protein crystallization at salting-in region is common among crystallization technique such as microdialysis, to our knowledge this is the first time it was achieved by using vapor diffusion. Unfortunately, these crystals showed to be fragile when manipulated during the X-ray experiments.

Figure 46. HsFH crystals: A) crystal obtained using 100 mM sodium malonate pH 5.0 and 12% (w/v) PEG 3350; B) HsFH crystal using the same condition in A) with the addition of 4% (v/v) Pentaerythritolethoxylate (3/4 EO/OH); C) HsFH crystals obtained using ultrapure water as crystallization condition.

3.4.5. X-ray diffraction, data processing and structure determination

Crystal dehydration proved to be important for improvement in crystal order and diffraction resolution and was performed by exposing the drop to air for 2 hours. Longer dehydration induced the formation of precipitates and a thin skin covering the drop, both undesirable for crystal handling and X-ray diffraction experiments.

Unit-cell parameters, merging statistics and the lack of systematic absences indicated that the enzyme crystals belonged to space group C222 (Table 9). HsFH has a calculated molecular weight of approximately 53.7 kDa and the calculated Matthews coefficient is $2.43 \text{ \AA}^3 \text{Da}^{-1}$ assuming the presence of one molecule in the asymmetric unit, based on a partial specific volume of $0.74 \text{ cm}^3 \text{g}^{-1}$, the calculated solvent content was approximately 49.3%. These values lie within the range commonly observed for protein crystals (Matthews, 1968; Kantardjieff and Rupp, 2003).

Table 9. Data collection and processing: Outer shell values are shown in parentheses.

Diffraction source	MX2, LNLS
Wavelength (Å)	1.459
Temperature (K)	100
Detector	3 × 3 MAR Mosaic 225 CCD
Crystal-to-detector distance (mm)	121.6
Rotation range per image (°)	1
Total rotation range (°)	102
Exposure time per image (s)	60
Space group	C222
Unit-cell parameters (Å, °)	$a = 124.42, b = 147.15, c = 57.01, \alpha = \beta = \gamma = 90$
Mosaicity (°)	1.29
Resolution range (Å)	31.67-2.10 (2.175-2.100)
Total No. of reflections	105423 (15169)
No. of unique reflections	27725 (4192)

Completeness (%)	89.9 (94.4)
Multiplicity	3.8 (3.6)
$\langle I/\sigma(I) \rangle$	3.6 (2.1)
$R_{r.i.m.}$	0.210 (0.495)
$R_{p.i.m.}$	0.108 (0.247)
R_{merge}	0.179 (0.423)
Overall B factor from Wilson plot ($\text{\AA}^2$)	14.8

Data phasing was performed by molecular replacement, and initial refinement was performed by several rounds of adjustment of side-chain rotamers using Coot (Emsley and Cowtan, 2004) interspersed with torsion-angle simulated-annealing and positional and individual B-factor refinement using phenix.refine (Adams *et al.*, 2010). Refinement reached an R factor of 18% and an Rfree of 23%.

3.4.6. Structural characterization

The coordinates of *HsFH* have been previously deposited in the Protein Data Bank (PDB: 3E04) by the Structural Genomics Consortium. However neither data about the structural model or biochemical characterization were found available in the literature.

From our structural analysis, *HsFH* structure solved in our laboratory is almost identical to the previously deposited human fumarase crystal structure with 0.6 Å RMSD, but provided additional information regarding the position of the catalytic loop which is missing in 3E04. The *HsFH* structure is also very similar to the *fumC* structure with 0.8 Å RMSD (PDB: 1FUO) (Weaver *et al.*, 1995).

The tetramer is composed of four identical monomers that can be described as composed of three domains named D1, D2 and D3 (Figure 47). D1 (residues 49-188) is located at the N-terminus and is formed by one β -hairpin and five α -helices (Figure 47 – red). D2 (residues 189-439) contains 6 α -helices 5 of them responsible for the intermolecular interactions that hold the tetramer (Figure 47 – green). D3 (residues 440 – 510) is the smallest domain and presents 2 helix-turn-helix motives and one additional helix that connects D3 to D2 (Figure 46 – blue).

Figure 47. HsFH domains: D1 is represented in red and accommodates all residues of the B-site (yellow) and some residues of A site (magenta). The region in green corresponds to D2 where the majority of intermolecular interactions responsible for the tetramer formation take place. The majority of A-site residues are located at D2. D3 is represented in blue.

Class II fumarases possess two different substrate binding sites (Figure 48) (Weaver *et al.*, 1995; Weaver and Banaszak, 1996). The catalytic site is formed by the juxtaposition of three out of the four chains from the tetramer and it is called the A site. 12 Å distant of the A site, towards the enzyme surface, is located a less conserved region among class II fumarases entitled the B site whose residue composition is concealed within each chain. It is believed that the B site participates in the catalysis helping in the exchange of substrates with the solvent.

The A site is formed by the juxtaposition of three monomers where only D1 and D2 domains participate. The B site is formed exclusively by D1 residues (H 176 - N 182) (Figure 48).

Figure 48. Stereo view of HsFH sites: The catalytic site (A-site) is composed by the D2 residues K 371/A, N 373/A, E 378/A, E 362/A from chain A (light green), the D2 residue H 235/C (chain C – dark green) and D1 residues S 186/D, S 187/D and N 188/D (chain D - red). The B-site (yellow) is located 12 Å towards the surface from the A-site and is formed by the residues from H 176/D to N 182/D of the D1 domain.

Although a high level of structural similarity is observed among class II fumarases, striking differences can be observed when comparing class I and class II fumarases (Figure 49). It is interesting to point out that, although class II fumarases can be found widespread in nature, class I fumarases are found preferentially in unicellular organisms. To date, there are few genomes from multicellular species where class I fumarase homologues are present and most of them are model organisms for studying the origin of multicellularity. Both fumarase classes constitute an example of molecular adaptive convergence since no clear homology can be found between both classes and they still possess one common function.

Finally, the important role of fumarase activity for *T. cruzi* metabolism together with structural dissimilarities found between human and parasite enzymes provide the perfect scenario for TcFHs to be exploited as drug targets against Chagas disease (Figure 49).

Figure 49. *HsFH* X *TcFHc* structural comparison. A) *HsFH*: tetrameric iron independent enzyme. B) *TcFHc*: dimeric iron-sulfur containing fumarase.

3.5 **Conclusions**

The human fumarase enzyme (*HsFH*) was successfully cloned, expressed and purified to homogeneity by nickel affinity chromatography.

From our biochemical and structural studies, recombinant *HsFH* was characterized as a homotetramer and shown to perform the Michaelis-Menten mechanism of catalysis. *HsFH* crystals were obtained by vapor diffusion techniques and the structure was solved by X-ray diffraction experiments. *HsFH* purification, crystallization and structure determination have been recently published (Pereira de Pádua and Nonato, 2014).

The development of a purification protocol and an enzymatic assay for *HsFH* allowed the obtainment of pure enzyme in a scale suitable for cross-validation inhibitory studies. In addition, the 3D models of both human and *Trypanosoma cruzi* fumarases will be exploited to design selective inhibitors for class I fumarases.

Finally, the structural and kinetic analysis of native and mutant forms of *HsFH* will be performed in order to evaluate the role of specific residue substitutions for fumarase structure and activity and their relationship to distinct genetic diseases. Work is currently in progress on these topics.

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Chapter 4. Ligand design

4.1 **Introduction**

Treatment of chronic phase of Chagas disease is restricted to the long term use of toxic drugs with limited efficacy in the chronic phase of the disease. Alternative treatments have been investigated for more than a century, but no effective cure for the distinct phases of the disease has been found so far. In 2002 the World Health Organization prioritized 6 important biological processes in *T. cruzi* to be targeted: the ergosterol biosynthesis, the protein prenylation machinery, proteases (especially cruzapain), glycolytic enzymes, the trypanothione biosynthesis and the enzyme trans-sialidase. After 14 years only a few new drugs emerged, and even the most promising case of triazoles (ergosterol biosynthesis inhibitors), which are currently in clinical trials, is already too expensive to be considered an effective strategy to widespread use in endemic countries (Pinazo *et al.*, 2010; Urbina, 2010; Barrett and Croft, 2012). Even more aggravating, triazoles efficacy was found to vary widely among different *T. cruzi* strains (Diniz *et al.*, 2013).

Considering the lack of an effective treatment for Chagas disease and the increasing number of people worldwide infected with *T. cruzi* there is an urgent need for prospection of alternative routes to pharmacological intervention. In this chapter we describe our preliminary efforts towards this goal.

In Chapter 2 the main structural and biochemical features of class I fumarases were described, and the importance of those enzymes for the survival of *T. cruzi* were evaluated. Chapter 3 comprised the structural and biochemical characterization of class II human fumarase. Together, the previous chapters described our work in producing and characterizing both parasite and host target enzymes and in providing important tools for validating TcFH as a molecular target for drug development. Our next step, found in this chapter, consisted of combining the knowledge and the experimental tools for the design of selective inhibitors to the *T. cruzi* enzymes.

Although class I and class II fumarases share neither sequence nor structural similarity, both exerts the same enzymatic role by catalyzing the reversible hydration of fumarate into malate. In detail, all fumarases employ the E1cb elimination mechanism (Figure 50): first a base (B_{FH}) abstracts the acidic hydrogen from β -carbon of malate forming a carbanion. After rearrangement, the electron pair moves to the adjacent atom with concomitant elimination of the hydroxyl group and formation of the double bond. Finally, the hydroxyl group abstracts a

proton from an acid (HA_FH) generating the water molecule (Mcmurry, 2005; Grossman, 2008).

Figure 50. General reaction mechanism for fumarases. Fumarases employ the E1cb elimination to reversibly convert malate into fumarate. A fumarase residue acts as base (B_FH) and abstracts the acid hydrogen of β -carbon generating a carbanion. In a rearrangement step, the lone pair moves to the adjacent carbon expelling the hydroxyl group which, in turn, abstracts a proton from a nearby acid residue.

Despite several peculiarities, the most striking difference between class I and class II fumarases relies on the presence of iron-sulfur cluster that acts as a Lewis acid in class I fumarases whereas class II fumarases do not rely on prosthetic groups for catalysis. As observed in the crystal structure of *LmFH-2*, one iron of the cluster was predicted to coordinate with the hydroxyl groups of the substrates (Figure 51) similar to the mechanism described for the aconitase, also a cluster containing lyase (Flint *et al.*, 1992).

Figure 51. Model for iron-sulfur cluster-substrate complex found in class I fumarases. Class I fumarases use a [4Fe-4S] cluster as a Lewis acid to activate the hydroxyl group of malate to elimination. The figure was extracted from (Flint *et al.*, 1992).

By exploiting these unique bioinorganic properties of iron-sulfur cluster-substrate complex it was possible to design the first selective inhibitor of class I fumarase. Its IC_{50} was determined *in vitro* for the *TcFHc* enzyme and the compound was also tested *in vivo* against *T. cruzi* epimastigotes cells. Our findings, not only proved the principle that class I selective inhibition can be achieved, but also gave rise to a new strategy, both in terms of molecular target and chemical probe, for the development of new drugs to treat Chagas disease.

4.2 **Objectives**

The aim of the work described in this chapter was to search and exploit structural and mechanistic differences between the *TcFH* and *HsFH* enzymes in the development of inhibitors against the parasite enzyme, and to perform preliminary assays in order to evaluate in vitro and in vivo inhibitor potency and selectivity. The following steps were taken to achieve our goals:

- Design of a ligand candidate.
- Spectrophotometric measurements to evaluate inhibition potency against both *TcFHc* and *HsFH*.
- Evaluation of ligand trypanocidal activity on *T. cruzi* epimastigotes.
- Evaluation of ligand effects on mitochondrial membrane potential in *T. cruzi* cells.

4.3 Methodology

4.3.1. Ligand design

In the design of selective inhibitors for class I fumarases the presence of iron-sulfur cluster, a unique feature within this class, was exploited.

According to the model proposed by Flint, after confirmed by our structural studies, the malate interacts with the labile iron of the iron-sulfur cluster through two coordination bonds: one with the hydroxyl oxygen and the other with the closest carboxyl oxygen.

By analyzing both chemical and stereo properties of this interaction, it seemed feasible to predict that the replacement of the hydroxyl group from malate by a thiol group, thiomalate, would trigger the formation of a strong thiolate-metal bond with consequent loss of enzymatic activity (Figure 52). In theory, class II fumarases would not be inhibited since they lack metals in the catalytic center.

Figure 52. Ligand design for selective inhibition of class I fumarases. Replacing the hydroxyl group of malate (left) by a thiol group of thiomalate (right) would form a thiolate-metal bond, therefore impairing fumarase reaction to proceed. Theoretically, class II fumarases would not be inhibited since they lack metals in the catalytic center.

4.3.2. In vitro inhibition assays

4.3.2.1. *TcFHc inhibition assay*

Thiomalate was bought from Sigma and tested against *TcFHc* purified under aerobic conditions (see Chapter 2 for details). Different thiomalate concentrations (300, 105, 36.75, 12.86, 4.5, 1.58, 0.55, 0.19, 0.068, 0.024, 0.008 and 0 μ M) were tested. The reaction media consisted of thiomalate mixed in buffer 50 mM Tris pH 8.5, 150 mM NaCl in presence of either 500 μ M sodium fumarate or 2 mM sodium malate and incubated with 40 μ g/mL of *TcFHc*. The formation or consumption of fumarate was monitored at 250 nm. Absorbance values over time were plotted and

the relationship between them was calculated by linear regression. The slopes were used to calculate the percentage of inhibition compared to the control reaction (0 μ M thiomalate). The percentage of inhibition versus thiomalate concentration was plotted, non-linear regression was used to fit the Boltzmann function and the IC₅₀ calculated (Equation 4).

Equation 4

$$y = A2 + (A1 - A2) / (1 + \frac{\exp(x_0 - x)}{dx})$$

where A1 is y high asymptote, A2 is y low asymptote, x_0 is the inflexion (IC₅₀) and dx is the width.

TcFHc inhibition by different thiol containing compounds, 1 mM of cysteine, β -mercaptoethanol (BME) and DTT, was also evaluated.

4.3.2.2. HsFH inhibition assay

The *HsFH* enzyme was purified as described in Chapter 3. Activity measurements were carried out using 2.5 μ g/mL of *HsFH* in buffer 50 mM Tris pH 8.5, 150 mM NaCl in presence of 500 μ M sodium fumarate and 1 mM thiomalate.

4.3.3. *In vivo inhibition assay*

4.3.3.1. T. cruzi epimastigote growth inhibition assay

3x10⁶ WT and *TcFHm*^{-PAC/-BLA} (dKO-FHm) *T. cruzi* epimastigotes in log phase of growth were seeded in 96 well plates containing sodium malate, sodium thiomalate (10mM-10nM range with 1:10 serial dilution) and benznidazole (100 μ g/mL) and incubated at 28 °C for 48 hours. The cell viability indicator resazurin was added to the wells and the plate further incubated for 5 hours. The level of resazurin reduction/oxidation was quantitated by dual wavelength absorbance at 490-595 nm (Rolon *et al.*, 2006).

4.3.3.2. Mitochondrial membrane potential measurements

Mitochondrial membrane potential of wild type *T. cruzi* epimastigotes was measured by monitoring the fluctuations in the fluorescence of safranin O using 495 nm wavelength excitation and emission in 586 nm. Safranin O is a cationic fluorescent dye which migrates electrophoretically in the mitochondria matrix towards the negative charge formed in the inner membrane (Lemasters *et al.*, 1987). 2 x 10⁸ epimastigotes in logarithmic phase of growth were incubated in the reaction medium (125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH pH 7.2, 1 mM MgCl₂, 2 mM KH₂PO₄, 5 mM potassium succinate) added of 10 μ M safranin O and 2.5 or 5 mM thiomalate in 2 mL total volume. Additions of digitonin (30.45 μ M), ADP (200 mM) and the uncoupler FCCP (10 μ M) were indicated in the spectra. The decrease in membrane potential, observed by the cation electrogenic influx, is

linearly correlated to the increase in the fluorescence intensity of the dye when it exits the mitochondrion (Akerman and Wikstrom, 1976; Emaus *et al.*, 1986).

4.4 Results and Discussions

4.4.1. Fumarase inhibition by thiomalate

Thiomalate was evaluated as inhibitor of *TcFhc* and *HsFH*, model enzymes of class I and class II fumarases respectively. Considering both enzymes could potentially catalyze thiomalate into malate and hydrogen sulfide (H_2S instead of H_2O), incubation of *TcFhc* and *HsFH* with sodium thiomalate alone was monitored for the formation of fumarate. Neither fumarate could be detected at 250 nm nor could H_2S bubbles be visualized in the buffer after the incubation period.

TcFhc showed to be inhibited by thiomalate for both reaction directions at low micromolar IC_{50} values: $3.0 \pm 0.5 \mu\text{M}$ and $5.9 \pm 0.3 \mu\text{M}$ of thiomalate for hydration and dehydration reactions (Figure 53).

Figure 53.Thiomalate IC_{50} determination for *TcFhc*. Both hydration (left) and dehydration (right) reactions catalyzed by *TcFhc* were inhibited at low micromolar levels by thiomalate (TM).

The differences in IC_{50} values found for malate and fumarate are probably due to the use of different substrate concentration and at this moment cannot be associated to distinct mechanisms of inhibition. Kinetic assays, including the determination of K_i values against both substrates, as well as a detailed structural analysis of the ligand-enzyme complex will be further used to unravel the mechanism of inhibition

As predicted, in our cross-validation assays no inhibition of *HsFH* could be detected, even in presence of thiomalate twice as more concentrated than the natural substrate (Figure 54).

Figure 54. *HsFH* versus thiomalate. *HsFH* incubated with 500 μ M sodium fumarate (Fum) is shown in blue and the comparison with the same reaction in the presence of 1 mM sodium thiomalate (red – Fum+TM) showed no loss of activity.

Moreover, the slightly difference in the slope (Figure 54) suggest that activation of *HsFH* is taking place. Considering thiomalate blocked *TcFHc* catalytic site by forming a thiolate-metal bond with the iron-sulfur cluster, it became important to evaluate if different thiol containing compounds could also interfere likewise with *TcFHc* activity. Activity assays in presence of cysteine, BME or DTT were performed for both fumarate and malate substrates and the results indicated that minor, if not negligible, *TcFHc* inhibition occurred for these thiol compounds (Figure 55). The results indicated that not only the thiol group is important for inhibition but also the other atoms in thiomalate are crucial to adequate access and binding to the catalytic cleft.

Figure 55. *TcFH* activity measurement based on fumarate consumption/production monitored at 250 nm. Effect of various thiol compounds on *TcFHc* activity. Various thiols were tested against *TcFHc* at 1 mM concentration cysteine (red), BME (blue) and DTT (green). Thiomalate (36 μ M) is shown in magenta as a matter of comparison. A relatively small effect on both formation (left) and consumption (right) of fumarate by the different thiols was detected taking into account the concentrations used. BME reproducibly interfered in the hydration reaction but no loss in activity was detected.

Altogether, the studies showed that thiomalate is a selective inhibitor for class I fumarases and a low micromolar inhibitor for *TcFHs*. Besides the possibility of exploiting thiomalate and its analogues as prototypes for drug development to treat Chagas Disease, they consist of a powerful tool to perform *in vitro* and *in vivo* assays in order to validate *TcFHs* as a drug target.

4.4.2. Thiomalate effect in *T. cruzi* epimastigotes

WT and dKO-FHm (null *TcFHm* mutant) *T. cruzi* epimastigotes were challenged with a wide range of thiomalate (TM) and malate (M) concentrations and the effect on cell viability evaluated. The results, depicted in Figure 56, revealed important information on thiomalate effect on parasite cells. It was clear that thiomalate showed poor trypanocidal activity. Thiomalate killed less than 2% of *T. cruzi* cells whereas our control experiment performed in presence of 100 µg/mL benznidazole (BZ) showed 26% of trypanocidal activity. However, the difference between 10 mM thiomalate with respect to 10 mM malate indicated that the single replacement of the hydroxyl to a thiol group indeed generated a trypanocidal compound.

Moreover, increasing the malate concentration from 1 to 10 mM slightly increased dKO-FHm parasites viability without altering the survival of WT parasites. The same increment in thiomalate concentration caused a decrease in both WT and dKO-FHm parasites viability, with a pronounced trypanocidal activity found for the mitochondrial fumarase deficient cells (Figure 56).

From the data, it is tempting to speculate whether both sodium malate and sodium thiomalate are not lipophilic enough to cross the parasite membrane at low concentrations. At higher concentrations small quantities could be passively diffused into the *T. cruzi* cytosol and malate could be partially rescued the dKO-*TcFHm* phenotype (with the aid of mitochondrial shuttles in a similar fashioned as predicted for *TcFHm*^{-PAC/-BLA} in Chapter 3). Instead, thiomalate inhibited the cytosolic fumarase causing a depleting effect on both WT and dKO-*TcFHm* parasites but a worse effect on dKO-*TcFHm* mutant cell line due to the impossibility of exchange malate/fumarate across the mitochondrion. This model is in agreement with the lack of success to rescue the *TcFHc*^{-PAC/-BLA}/*TcFHm*^{-NEO/-HYG} knockout cell lines by using external sources of the dicarboxylic acids. 10 mM concentration used in our assays was probably was not enough to induce any effect, however experiments at higher concentrations may induce osmotic shock in parasite cells.

Figure 56. Percentage of trypanocidal activity versus thiomalate (TM) and malate (M) concentrations. *T. cruzi* WT and *TcFHm*^{-PAC/-BLA} sensitivity to benznidazole (BZ) and thiomalate: sodium thiomalate (TM) is a poor trypanocidal agent due to its low lipophilicity. 10 mM concentration of malate (M) and thiomalate may enable passive diffusion of compounds. Inside the cell TM aggravates the mitochondrial mutant phenotype and M partially rescue it.

In addition, mitochondrial membrane potentials were measured using safranin O as a fluorescent indicator (Figure 57). In this experiment, the mitochondrion must be first accessed by means of digitonin permeabilization. Under correct experimental conditions, parasite cell membrane becomes permeable to the reaction medium substrates and safranin O, the mitochondrion membranes are kept intact and a potential can be observed by reduction in fluorescence. The membrane potential is generated when substrates enter into mitochondrion generating reducing equivalents to the respiratory chain which in turn pumps protons to the intermembrane space. Addition of ADP triggers the synthesis of ATP by the F_0F_1 ATP synthase which uses the proton gradient causing the increase in fluorescence. The compound FCCP is an ionophore that allows protons to flow inside the mitochondrion before they are used by ATP synthase. 2.5 mM thiomalate significantly diminished the membrane potential and 5 mM impaired the generation of proton motive force. The fact that high thiomalate concentrations were necessary to affect the mitochondrion matrix even after digitonin addition suggested that thiomalate cannot use or inhibit malate shuttles. Therefore, thiomalate inhibited the energy production by oxidative phosphorylation in *T. cruzi*. Considering thiomalate inhibits class I fumarases it is tempting to assume that the deleterious phenotype caused by the *TcFHC*^{-BLA/-PAC}/*TcFHm*^{-NEO/-HYG} genotype in *T. cruzi* is due to lack of ATP production.

Figure 57. Thiomalate effect on WT *T. cruzi* membrane potential. Digitonin was added to allow influx of substrates into the cytosol of *T. cruzi*. Consumption of substrates produced a proton gradient between the mitochondrial matrix and the intermembrane space with reduction in fluorescence (RFU: relative fluorescence units). Addition of ADP triggered ATP production, reduction of the proton motive force and increase of fluorescence. FCCP allowed equilibrium to be established and hence increase in fluorescence. Thiomalate (TM) at 2.5 mM caused a decrease in membrane potential and at 5 mM the proton motive force generation was impaired.

4.5 **Conclusions**

In the present chapter the reaction mechanisms of the *T. cruzi* class I and the human class II fumarases were described and the most important difference between them, the iron-sulfur cluster, was used to design a selective inhibitor against the parasite enzymes.

The ligand design strategy was inspired in the very nature of class I fumarases. Initially, the ligand skeleton was borrowed from the substrate malate and exploring the idea of the stable thiol-iron bond present in the iron-sulfur cluster, the ligand thiomalate was coined.

Fortunately, thiomalate is a cheap commercially available compound which was, therefore, tested against the target *T. cruzi* fumarase enzyme. Thiomalate showed to inhibit TcFHc at low micromolar level, and further cross-validated against the human enzyme, proving the original concept of being class I selective. Other thiol containing compounds failed to reproduce thiomalate inhibition in *T. cruzi* fumarases suggesting that inhibition of TcFHc requires more interactions than metal-thiolate.

The exciting results on selective inhibition against TcFHc were not reproduced when thiomalate was tested in *T. cruzi* epimastigotes cells. However, given its dicarboxylic nature it is reasonable to speculate that thiomalate was not lipophilic enough to penetrate both cell and mitochondrion membranes and reach the enzyme targets. In order to test our hypothesis, synthesis of thiomalate analogues which display better lipophilic properties are currently in progress.

Finally, the effect of thiomalate on mitochondrial membrane potential, when no proton gradient could be detected, provided strong evidences on the essential role of *T. cruzi* fumarase in ATP synthesis by oxidative phosphorylation. In addition, this provided a feasible explanation for our difficulties in obtaining the null fumarase mutant.

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Chapter 5. Final remarks

5.1 *Final Remarks*

The work here described and entitled “Structural and functional characterization of *Trypanosoma cruzi* fumarate hydratase isoforms” was carried out between January 2009 and January 2014.

Initially, our work focused on the structural characterization of *T. cruzi* fumarases by using X-ray crystallography techniques. Our first big challenge involved the development of a reproducible protocol for protein expression and purification for further pursuing the crystallization and X-ray diffraction experiments. Without previous experience and lack of adequate infrastructure, it took us sometime to figure out what level of experimental difficulties we were facing and what was necessary to overcome them. It is now well known that purification of iron-sulfur cluster proteins is often troublesome and characterized by low levels of iron-sulfur cluster incorporation and high levels of oxygen sensitivity.

After using a combination of EPR, circular dichroism, size exclusion chromatography, activity assays, and a careful monitoring of the behavior of protein samples under different oxygen environments, it is feasible to consider that crystallization of TcFHs failed due to sample heterogeneity induced by oxidation of the cluster and consequent generation of different protein populations. Only recently, as part of a collaboration with Catherine Drennan’s lab from *Massachusetts Institute of Technology in United States*, and one of the world leaders in characterization of oxygen sensitive proteins, the first few attempts to crystallize TcFHs under strictly anaerobic conditions were made. Further experiments are in progress. The extreme oxygen sensitivity raised questions whether TcFHs could be involved in oxygen, or more likely, reactive oxygen or nitrogen species (ROS or NOS) cell signaling. *T. cruzi* trypomastigotes for instance, either invade human cells where they are bombarded with ROS and NOS, especially in macrophages, or they can be picked up by triatomine bugs together with the host blood. In the insect gut, the release of iron from hemoglobin triggers Fenton’s reaction which forms hydroxyl radicals. Thus, considering fumarases are even more sensitive to radicals than to oxygen and that these enzymes are essential to the parasite, the fumarase cluster destruction could somehow signal *T. cruzi* cell to raise its anti-oxidative stress defense mechanism. In fact, aconitase, also a 4Fe-4S cluster containing lyase, binds RNA when in its apo form and signals the cell to increase iron mobilization from cellular storage and uptake. Aconitase is then what is called a moonlight protein, since it holds a crucial role at the initial steps of TCA cycle inside the mitochondria and at the same time its cytosolic isoform regulates the intracellular iron concentration. Interestingly, a highly positive charge region was identified in TcFHs that could therefore be associated with nucleic acid binding.

The discovery of the first class I fumarase selective inhibitor described in this work was of great importance to validate *TcFHs* as drug targets against Chagas disease. However, thiomalate is not lipophilic enough to cross membranes and reach the target enzymes. The same problem was faced by researchers in Germany when they suggested fumarate as a chemotherapy to treat psoriasis. They overcame the problem by simply converting the carboxylic acids in esters (Mrowietz *et al.*, 1998). Nowadays, dimethyl fumarate is a licensed oral therapy for psoriasis in Germany (trade name Fumaderm). In addition, dimethyl fumarate was approved by the FDA in 2013 as a drug to treat multiple sclerosis (trade name Tecfidera).

In collaboration with Prof. Dr. Flávio Emery, dimethyl thiomalate (DMT) was synthesized (Kato *et al.*, 2009) and is already being tested in *T. cruzi* epimastigotes. It is expected that the more lipophilic DMT would cross the cell membrane and once inside the cytosol the *T. cruzi* esterases would demethylate the DMT generating the thiomalate (TM) *in situ*. To check this mechanism *in vitro*, the putative *T. cruzi* esterase will be produced. Since human cells also possess esterases in the cytosol, the premature conversion of DMT in TM may limit the efficacy to kill *T. cruzi* amastigotes, where the compound needs to cross three different membranes. To address this matter, a different strategy exploiting the benznidazole activation mechanism by the unique trypanosomatid nitroreductase was considered and the proposition consisted of a central thiomalate molecule flanked by two 4-nitrobenzyl moieties. Other modifications like the inclusion of a second thiol group to increase binding are also being planned.

In addition, by using the generated *T. cruzi* fumarase mutant cell lines, a very detailed phenotypic screening will be performed and will help to map the fumarase roles in the parasite. Special attention to the cytosolic fumarase function will be necessary since it possesses a less obvious role. Recently, the cytosolic human fumarase was found to migrate to the cell nucleus upon DNA damage by hydroxyurea or ionizing radiation. If such behavior is shared by class I fumarases in lower eukaryotes remains to be studied.

5.2 *Bibliography*

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