UNIVERSITY OF SÃO PAULO

SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

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

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Ribeirão Preto

2014

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Doctoral Thesis presented to the School of Pharmaceutical Sciences of Ribeirão Preto for the degree of Doctor in Sciences

Concentration area: Chemistry and Biological Physics

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Corrected version of the Doctoral Thesis presented to the Pharmaceutical Sciences Post Graduate Program in 14/03/2014. The original version is available at the School of Pharmaceutical Sciences of Ribeirão Preto/USP

Ribeirão Preto

2014

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 (*Tc*FHs) and human fumarase (*Hs*FH) to evaluate *Tc*FHs 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 *Tc*FHs belong to class I fumarases (dimeric and iron-sulfur containing enzymes) and are not homologous to *Hs*FH 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 *Tc*FHs are essential to the parasite. The kinetic characterization showed that *Tc*FHs activity is highly sensitive to oxygen whereas *Hs*FH 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 *Tc*FHm and *Tc*FHc. *Tc*FHs structural models, built by homology modeling using the *Leishmania major* fumarase crystal structure as template, were compared to the *Hs*FH crystal structure and the differences were used to design a selective ligand to the parasite fumarases. The designed ligand showed to inhibit *Tc*FHc with an IC_{50} 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.

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* (*Tc*FHs) e humana (*Hs*FH) de forma a avaliar o papel das *Tc*FHs para o parasito *Trypanosoma cruzi*, mapear o mecanismo de açãoe identificar as diferenças entre *Tc*FHs e a enzima humana de forma a serem exploradas no planejamento de inibidores seletivos às fumarases do parasito. Análise das sequências mostrou que *Tc*FHs 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 (*Tc*FHc) e uma mitocondrial (*Tc*FHm), e experimentos de nocaute gênico sugeriram que essas enzimas são essências para o parasito. A caracterização cinética das enzimas *Tc*FHc, *Tc*FHm e *Hs*FH 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 *Tc*FHs. Modelos estruturais das *Tc*FHs, 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 µM 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.

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 economical 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 motive 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 targets 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*Tryopanosoma 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 a ubiquitous enzyme responsible for the reversible conversion of fumarate into S-malate (Dakin, 1922).

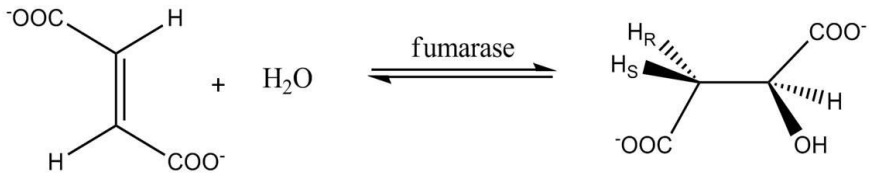

Figure 1. Fumarase reaction. Fumarase catalyze the ubiquitous reversible stereospecific hydration of fumarate (left) to S-malate (right).

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 catalityc 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 e Pines, 2011). Human fumarases (*Hs*FH) are enconded by a single gene and the dual localization is achieved by posttranslational process. *Hs*FH 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. *Tc*FHc is a 565 amino acids long peptide chain, with predicted molecular weight of 62 kDa and theoretical pI of 6.74. *Tc*FHm is a 555 amino acids long peptide chain with predicted molecular weight of 60.8 kDa and theoretical pI of 8.3. *Tc*FHc

and *Tc*FHm 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 *Tc*FHc, *Tc*FHm (Chapter 2) and *Hs*FH (Chapter 3) in a heterologous expression system and to perform thei rkinetic and structural characterization. In addition, gene knockout experiments were performed in order to validate *Tc*FHs 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 *Tc*FH enzymes (Chapter 4). This ligand was used as a chemical probe to our preliminary *in vivo* assays. The multidisciplinary work developed during in this Doctorate work, 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* metacylcic 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, metacylcic 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 anterior 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 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 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 revelance 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 (*Tc*FHc) andTcCLB.507669.10 (*Tc*FHm) 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 these enzymes constitute novel drug targets for the development of new therapies against Chagas disease.

2.2 *Conclusions*

Trypanosoma cruzi fumarases (*Tc*FHs) 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: *Tc*FHc and *Tc*FHm.

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 *Tc*FHc and *Tc*FHm 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 *Tc*FHc and *Tc*FHm 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 amastigote proliferation.

The sub-cellular localization of *T. cruzi* fumarases was perfomed and *Tc*FHc was found to be a cytosolic enzyme whereas *Tc*FHm 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 elucidade the *Tc*FHs 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 *Tc*FHs 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 a crucial 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 (*Hs*FH), a well-known citric acid cycle enzyme, therefore a key component in energy metabolism, is localized both in the mitochondria and cytosol. Both *Hs*FHs are encoded by the *fum*C gene and their subcellular localization is determined by post-translational processes (Yogev *et al.*, 2010). Different from *Tc*FH, *Hs*FH belongs to class II fumarases, a homotetrameric enzyme and independent of iron-sulfur cluster for catalysis.

Noteworthy, mutations in *Hs*FH 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, the only 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 *Hs*FH. 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 *Tc*FHs. In addition, our results introduce new perspectives to the understanding of molecular mechanisms of human diseases by characterizing different *Hs*FH 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 ***Conclusions***

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

From our biochemical and structural studies, recombinant *Hs*FH was characterized as a homotetramer and shown to perform the Michaelis-Mentem mechanism of catalysis. *Hs*FH crystals were obtained by vapor diffusion techniques and the structure was solved by X-ray diffraction experiments. *Hs*FH 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 *Hs*FH 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 *Hs*FH 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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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 *Tc*FHs 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 M*assachusetts Institute of Technology in United States*, and one of the world leaders in characterization of oxygen sensitive proteins, the first few attempts to crystallize *Tc*FHs under strictly anaerobic conditions were made. Further experiments are in progress. The extreme oxygen sensitivity raised questions whether *Tc*FHs 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 hole 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 *Tc*FHs 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 *Tc*FHs as drug targets against Chagas disease. However it is not lipophilic enough to cross membranes and reach the target enzymes.

In addition, by using the generated *T. cruzi* fumarase mutant cell lines, a very detailed phenotypic screening will be performed and with the will help to map the fumarase roles in the parasite. Special attention to the cystosolic 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.