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Characterisation of the fumarate hydratase repertoire in *Trypanosoma cruzi*

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ABSTRACT

Nifurtimox and benznidazole represent the only treatments options available targeting Chagas disease, the most important parasitic infection in the Americas. However, use of these is problematic as they are toxic and ineffective against the more severe stages of the disease. In this work, we used a multidisciplinary approach to characterise the fumarases from *Trypanosoma cruzi*, the causative agent of Chagas Disease. We showed this trypanosome expresses cytosolic and mitochondrial fumarases that *via* an iron-sulfur cluster mediate the reversible conversion of fumarate to S-malate. Based on sequence, biochemical properties and co-factor binding, both *T. cruzi* proteins share characteristics with class I fumarases, enzymes found in bacteria and some other protozoa but absent from humans, that possess class II isoforms instead. Gene disruption suggested that although the cytosolic or mitochondrial fumarase activities are individually dispensable their combined activity is essential for parasite viability. Finally, based on the mechanistic differences with the human (host) fumarase, we designed and validated a selective inhibitor targeting the parasite enzyme. This study showed that *T. cruzi* fumarases should be exploited as targets for the development of new chemotherapeutic interventions against Chagas disease.

1. Introduction

Throughout Latin America approximately 8 million people suffer from Chagas disease, a neglected tropical infection caused by the flagellated protozoan parasite *Trypanosoma cruzi*. This disease is characterised by a series of life-threatening mega syndromes that promote damage to the heart, intestines and central nervous system, severely affecting the quality of life of infected patients [1].

The normal route of transmission of this zoonotic infection is *via* the hematophagous behaviour of insects belonging to the Reduviidae family, in which part of *T. cruzi*'s life cycle occurs, to humans, where the cycle is completed [2]. However, as a result of alternative modes of transmission such as through blood transfusions, organ transplantation, congenital and ingestion of contaminated food [3], and population migration, Chagas disease has started to emerge as a public health problem at non-endemic sites such as the USA and Europe [4].

Benznidazole and nifurtimox currently represent the only treatment options available against Chagas disease but their use is controversial. They are highly toxic and not effective in curing the infection during the lethal disease staging while some strains are refractory to treatment. In addition, the drug regimens are prolonged with a course of treatment requiring multiple daily doses taken over a 1 to 4 month period. Often, the recommended schedules are not completed, usually because of their side effects, resulting in considerable scope for the development of resistance. There is a considerable need for novel therapeutic strategies targeting Chagas disease and exploring the biochemistry of *T. cruzi* is seen as an important strategy to identify and validate new drug targets for the development of innovative treatments [5].

Fumarases (EC 4.2.1.2), also known as fumarate hydratases, are ubiquitous enzymes that catalyse the reversible conversion of fumarate to L-malate [6]. Based on sequence, structure, biochemical properties and co-factors, they can be divided into three distinct types [7]. Class I fumarases primarily expressed by

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bacteria and some protozoa, are homodimeric iron–sulfur (4Fe-4S) containing enzymes of approximately 120 kDa in size, and are readily inactivated by superoxide anions, heat or radiation. In contrast, class II fumarases are homotetrameric, iron independent proteins of approximately 200 kDa in size that are expressed by bacteria and numerous eukaryotes including higher plants, fungi and mammals [8]. Higher eukaryotic cells express one class II enzyme that is dual localized by reverse translocation after undergoing proteolytic processing to generate different echoforms [9–11]. One of these forms is localized to the mitochondrion where it participates in the Kreb's cycle while the second, located in the cytosol, plays a role in amino acid and fumarate metabolism. Intriguingly, the cytosolic variant is postulated to participate in the cellular response to DNA double strand breaks with this enzyme undergoing transport into the nucleus [12]. A third fumarase class has been identified from prokaryotes. This enzyme is composed of a heterodimer with an alpha and a beta subunit resembling the N-terminal and C-terminal parts of class I fumarase, respectively [13,14].

Analysis of the trypanosomal databases has shown that trypanosomes have the potential to express two class I fumarases, enzymes that are completely distinct from the class II counterparts found in humans. This, coupled with the observation that the total fumarase activity in an insect-stage *Trypanosoma brucei* cell is essential for parasite viability [15], prompted us to evaluate whether these enzymes constitute anti-chagasic drug targets. Here, we show that the total fumarase activity is important, if not essential, for *T. cruzi* viability and that both *T. cruzi* enzymes catalyse the reversible conversion of fumarate to L-malate with this activity being readily inhibited by the sulfur-containing malate derivative, thiomalate. Our results suggest that fumarase could be exploited as a potential drug target in *T. cruzi* and thus selective inhibition of fumarase may constitute a new strategy against Chagas disease.

2. Materials and methods

2.1. Cell culturing

T. cruzi epimastigote Sylvio X.10.6 were grown at 28 °C in RPMI 1640 medium supplemented with 5 g L^{-1} trypticase, 20 mM HEPES pH 7.5, 10% (v/v) heat-inactivated calf fetal serum, 0.22 g L^{-1} sodium pyruvate, 0.34 g L^{-1} sodium glutamate, 2500 U L^{-1} penicillin, 0.25 g L^{-1} streptomycin and 20 mg L^{-1} hemin. DNA was introduced into *T. cruzi* epimastigotes using the Human T-cell Nucleofector® kit and an Amaxa® Nucleofector™ (Lonza AG) set to program X-001. Transformed parasites were grown in the presence of 5 or 10 μg mL^{-1} puromycin, 5 or 10 μg mL^{-1} blasticidin, 5 μg mL^{-1} hygromycin and/or 100 μg mL^{-1} G418.

T. cruzi amastigote parasites were grown in African green monkey kidney epithelial (Vero) cells at 37 °C in a 5% (v/v) CO_2 atmosphere in RPMI 1640 medium. Uninfected Vero cells were maintained in this medium and sub-cultured following trypsin treatment. To produce *T. cruzi* metacyclic cells, epimastigote cultures were allowed to grow to late stationary phase resulting in differentiation of the parasite. These were used to infect Vero cells. Following overnight incubation at 37 °C in a 5% (v/v) CO_2 atmosphere, non-internalized parasites were removed by washing in liver infusion tryptose medium containing non-inactivated fetal calf serum. Bloodstream form metacyclics emerged 10 days after the initial infection and used to infect new Vero cells at a ratio of 10 parasites per mammalian cell. After 2 days, the medium was aspirated and the cells examined microscopically following Giemsa staining. The percentage of infected Vero cells and the number of amastigotes per infected cell were determined by analyzing 100 host cells randomly distributed across several fields of view. The mean number of parasites found in all host cells (mean abundance) was calculated using the quantitative software (QPweb 1.0) with 95% confidence interval by the bias-corrected and accelerated (BCa) bootstrap method with 2000 bootstrap replications.

2.2. Protein purification

The vectors used to express various fumarases in *E. coli* were generated as follows: full length Tc*fhc* was amplified from *T. cruzi* genomic DNA (gDNA) with the primers gaattcATGAGTCTGTGCGAAAACT and gcggccgcATCAAAGGAGTTTGGAAAAAAAG (lower case correspond to restriction sites incorporated into the primers to facilitate cloning). The amplicon was digested with *Eco*RI and *Not*I then cloned into the corresponding sites of the pET-28a vector (Novagen). The fumarase gene was subcloned into pET-28a-SUMO [16] using *Eco*RI and *Xho*I such that a DNA sequence gene coding for a hexahistidine-tagged SUMO was inserted in-frame at the 5′ end of the Tc*fhc*-derived DNA fragment. For TcFHm heterologous expression, the full length Tc*fhm* gene was amplified from *T. cruzi* gDNA with the primers ggatccATGCTGCGCCGTTCTGC and gcggccgcagcaTTGGACTCATTTGAGCTG, the resultant fragment digested with *Bam*HI and *Not*I, and cloned into the corresponding sites in pET-28a-SUMO. The human fumarase gene (Hs*fh*) was subcloned from the pET-28a-*HsFH* construct [17] into pET-28a-SUMO using *Bam*HI and *Xho*I restriction sites.

Overnight cultures of *E. coli* BL21 (DE3) harboring a pET-28a-SUMO vector containing the fumarase genes were diluted 1:100 in Lysogeny Broth containing 30 μg mL^{-1} kanamycin and grown at 37 °C with aeration until the culture reached the logarithmic phase of growth ($OD_{600\,nm} \sim 0.5$–0.6). *E. coli* cultures were transferred to 18 °C for 30 min and protein expression induced by addition of β-D-thiogalactopyranoside (IPTG) (TcFHc: 5 μM; TcFHm: 50 μM; HsFH: 500 μM). Cultures were incubated at 16 °C for a further 24 hours before harvesting the cell. For expression of the *T. cruzi* enzymes, 200 mg L^{-1} ferrous sulfate heptahydrate, 200 mg L^{-1} ferric citrate and 2 mM cysteine were added to the growth medium. All subsequent purification steps were performed under anaerobic conditions. The cells were resuspended in buffer A (50 mM NaH_2PO_4, pH 8.5, 300 mM NaCl, 10 mM imidazole) containing 1 mM phenylmethylsulfonyl fluoride then disrupted by sonication using 10× 30 s pulses on ice. The clarified lysate was passed through a Ni-NTA resin (Qiagen) equilibrated with buffer A. The resin was washed with the same buffer containing 25 mM imidazole and then re-equilibrated with buffer A. The histidine tagged protease ULP1 (500 μg) was added to the resin, the column incubated overnight at 8 °C. Tag-free enzymes were then eluted off the column with buffer A. Enzymes were concentrated and dialyzed against the storage buffer (50 mM Tris pH 8.5, and 150 mM NaCl) using an ultra-filter unit with 30 kDa cutoff (Amicon-Millipore).

Size exclusion chromatography of TcFHc was carried out using an isocratic run (50 mM Tris pH 8.5, 600 mM NaCl, 10 mM imidazole and 1 mM DTT) in a Superdex 200 10/300 (GE Life Sciences). The oligomeric state was estimated using molecular weight markers according to the column manufacturer's instructions.

2.3. Enzyme kinetics

TcFHc and TcFHm activities were monitored under anaerobic conditions by following the change in absorbance at 250 nm due to consumption or formation of fumarate ($\varepsilon_{250\,nm} = 1.45\ mM^{-1}\ cm^{-1}$ [18]). A reaction mixture containing 50 mM Tris pH 8.5, 150 mM NaCl, fumarate (500, 250, 125, 62.5 and 31.25 μM) or malate (8, 4, 2, 1, and 0.5 mM) was incubated at 25 °C. The background rate of fumarate consumption/formation was determined and the reaction initiated by addition of fumarase (10 μg mL^{-1}). The protein amount used was corrected based on the iron–sulfur content measured spectrophotometrically ($\varepsilon_{410\,nm} = 15{,}000\ M^{-1}\ cm^{-1}$