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Research paper

Interaction of frataxin, an iron binding protein, with IscU of Fe–S clusters biogenesis pathway and its upregulation in AmpB resistant *Leishmania donovani*

Amir Zaidi [a], Krishn Pratap Singh [a], Shadab Anwar [a], Shashi S. Suman [a], Asif Equbal [a], Kuljit Singh [a], Manas R. Dikhit [b], Sanjeeva Bimal [c], Krishna Pandey [d], Pradeep Das [e], Vahab Ali [a, *]

[a] Laboratory of Molecular Biochemistry and Cell Biology, Department of Biochemistry, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Agamkuan, Patna 800007, India
[b] Biomedical Informatic Centre, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Agamkuan, Patna, India
[c] Department of Immunology, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Agamkuan, Patna, India
[d] Department of Clinical Medicine, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Agamkuan, Patna, India
[e] Department of Molecular Biology, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Agamkuan, Patna, India

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ABSTRACT

Leishmania donovani is a unicellular protozoon parasite that causes visceral leishmaniasis (VL), which is a fatal disease if left untreated. Certain Fe–S proteins of the TCA cycle and respiratory chain have been found in the *Leishmania* parasite but the precise mechanisms for their biogenesis and the maturation of Fe–S clusters remains unknown. Fe–S clusters are ubiquitous cofactors of proteins that perform critical cellular functions. The clusters are biosynthesized by the mitochondrial Iron–Sulphur Cluster (ISC) machinery with core protein components that include the catalytic cysteine desulphurase IscS, the scaffold proteins IscU and IscA, and frataxin as an iron carrier/donor. However, no information regarding frataxin, its regulation, or its role in drug resistance is available for the *Leishmania* parasite. In this study, we characterized Ld-frataxin to investigate its role in the ISC machinery of *L. donovani*. We expressed and purified the recombinant Ld-frataxin protein and observed its interaction with Ld-IscU by co-purification and pull-down assay. Furthermore, we observed that the cysteine desulphurase activity of the purified Ld-IscS protein was stimulated in the presence of Ld-frataxin and Ld-IscU, particularly in the presence of iron; neither Ld-frataxin nor Ld-IscU alone had significant effects on Ld-IscS activity. Interestingly, RT-PCR and western blotting showed that Ld-frataxin is upregulated in AmpB-resistant isolates compared to sensitive strains, which may support higher Fe–S protein activity in AmpB-resistant *L. donovani*. Additionally, Ld-frataxin was localized in the mitochondria, as revealed by digitonin fractionation and indirect immunofluorescence. Thus, our results suggest the role of Ld-frataxin as an iron binding/carrier protein for Fe–S cluster biogenesis that physically interacts with other core components of the ISC machinery within the mitochondria.

1. Introduction

Leishmania is a unicellular kinetoplastid protozoan that belongs to the Excavata, arguably the most ancient domain, and is responsible for serious diseases in humans and animals. The organism alternates between two forms: the motile extracellular promastigote form in the gut of *Phlebotomine* insects (sandfly) and the non-motile intracellular amastigote form adapted to macrophages of mammalian hosts. During this protozoan's life cycle, *Leishmania* must cope with hostile environmental factors, such as oxidative stress caused by the reactive oxygen species (ROS) generated inside macrophages. Thus, the iron level must be delicately regulated during the protozoan's life cycle, because apart from being an integral precursor for Fe–S clusters, the metal is also

* Corresponding author. Tel.: +91 9304836301 (mobile), +91 0612 2631565 (office); fax: +91 0612 2634379.
E-mail address: vahab_ali@yahoo.com (V. Ali).

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an amplifier of ROS. Fe–S clusters are essential cofactors of proteins that perform important functions in central cellular processes, such as electron transfer, catalysis, DNA repair, tRNA thiolation, ribosome biogenesis, and iron regulation [1–3]. These clusters are found in all living organisms and are most commonly assembled as [2Fe–2S], [3Fe–4S], and [4Fe–4S], with the Fe^{2+} ions being coordinated by a cysteine thiol group from the sulphide-linked proteins. Three major Fe–S cluster assembly pathways have been identified to date: the ISC (Iron–Sulphur Cluster) system, SUF (Sulphur Utilization Factor) system, and NIF (Nitrogen Fixation) system [4]. The SUF and NIF systems are active under specific conditions or present in some organisms, whereas ISC system performs general house-keeping functions in Fe–S cluster assembly in bacteria and have highly conserved orthologs in eukaryotes [5]. The majority of protozoan parasites have retained ISC system in either mitochondria or mitochondria-like organelles, such as mitosomes, hydrogenosomes, and mitochondria-related organelles (MROs) [6,7]. However, *Plasmodium* spp. and *Blastocystis hominis* possess the SUF system (or some of its components) in addition to the canonical ISC system, which is functional under oxidative stress and iron-deficient conditions [6–8]. The NIF system is present in nitrogen-fixing bacteria, cyanobacteria, and microaerophilic bacteria but absent in eukaryotes and protozoan parasites, except *Entamoeba histolytica* [7,9] and free-living amoeba (*Mastigamoeba balamuthi*) [10].

The ISC system involves about 30 protein components [3,11] and among them, 10 proteins have been conserved from bacteria to humans [12,13]. In eukaryotes, the biogenesis of Fe–S clusters occurs in the mitochondrial matrix space. The core components of this process include a cysteine desulphurase complex, Nfs1–Isd11, which provides sulphur to the scaffold protein Isu1 [14,15], Yfh1 (yeast frataxin homologue), an iron donor in yeast and mammals [16–19], and the proteins that assist in the transfer of Fe–S clusters to target proteins. Frataxin is a nuclear-encoded mitochondrial metallochaperone protein that is most likely directly involved in Fe–S cluster assembly and iron homeostasis and is conserved from bacteria to humans [20]. Frataxin depletion is associated with specific defects in the *de novo* Fe–S cluster formation on Isu of yeast [21].

Frataxin is a compact and globular protein with a well-characterized 3Dstructure consisting of two α-helices and five to seven aligned anti-parallel β-sheets that form an α/β sandwich. In prokaryotes, the bacterial frataxin orthologue (CyaY) interacts in an iron-dependent manner with IscU in the presence of IscS [1,22]. In contrast, in yeast, Yfh1 interacts with the Isu/Nfs1–Isd11 complex and an interaction between Isu and Nfs1–Isd11 was essential for the formation of the tripartite complex with Yfh1 [23]. Similarly, human frataxin was also shown to interact with the core Isu/Nfs1/Isd11 complex rather than with the individual components for Fe–S cluster assembly [18].

The major functions of frataxin include iron storage and haeme metabolism, Fe–S cluster biogenesis, oxidative phosphorylation, and protection against oxidative stress [24–28]. In humans, the loss of frataxin expression or function results in Friedreich's ataxia (FRDA), an autosomal recessive neuro- and cardio-degenerative disorder characterized by mitochondrial iron accumulation, sensitivity to oxidants, depletion of mitochondrial DNA, impaired respiration, and decreased activity of Fe–S proteins. These phenotypic changes and other putative functions of frataxin have been extensively studied in yeast, mouse and human cells [25,29,30] and, lately, also in *Arabidopsis thaliana* [31,32] and *Trypanosoma brucei* [33,34]. Frataxin of *T. brucei* plays a protective role in oxidative stress [34] but not in iron storage and haeme metabolism. Recently, the Fe–S cluster biogenesis machinery in the procyclic and blood stream stages of trypanosomes and the role of their components in iron acquisition and tRNA thiolation was reviewed [35]. However, the precise role of frataxin is presently unknown and difficult to predict, as it is a multifunctional protein.

Leishmania is able to use iron from several sources, such as transferrin, lactoferrin or hemin. To survive within host macrophage parasitophorous vacuoles (PV), *Leishmania* must acquire iron. These protozoans take iron directly from the host endocytosed transferrin, where the amastigotes expressing *Leishmania* ferric reductase (LFR1) on the membrane convert the Fe^{3+}-transferrin complex into soluble ferrous iron. The element is then transported by *Leishmania* iron transporter 1 (LIT1), a protein that is upregulated in iron poor environments [36]. Iron plays a crucial role in regulating redox balance in all trypanosomatids due to its essential role in the activity of superoxide dismutases (SODs), metalloenzymes that depend on iron for activity. For this reason, modulation in iron availability directly impacts cellular function [37]. Recent studies have shown the role of iron in amastigote differentiation through a mechanism that involves the production of ROS [38].

Frataxin as an iron donor/chaperone may have an important role in the parasite's life cycle, thus its characterization is imperative for understanding the mechanisms of iron homeostasis and parasite biology. We did not find the ferritin homologue, an iron storage protein in bacteria and higher eukaryotes, with a *Leishmania* genome database search. Yeasts also lack ferritin, but possess frataxin to supply iron for the biogenesis of Fe–S clusters and the storage of iron within the mitochondria [24,27]. Such functions are still unexplored in *Leishmania* parasites. Therefore, how the parasites store/transport iron within the mitochondria is a subject of investigation and Ld-frataxin is one of the potential candidates, given its orthologs' similar roles in other eukaryotes [27,39–41]. Here, we identified a frataxin-like protein of *Leishmania* (Ld-frataxin), purified the recombinant Ld-frataxin (rLd-frataxin), analyzed its sub-cellular localization and showed its interaction with Ld-IscU, the mitochondrial scaffold protein. We also show that Ld-frataxin stimulates the cysteine desulphurase activity of Ld-IscS in the presence of Ld-IscU. Further, we observed 1.5–2.0-fold upregulation of Ld-frataxin expression in AmpB-resistant *Leishmania donovani* isolates. This work is the first description and identification of a frataxin-like protein of *Leishmania* species.

2. Material and methods

2.1. Chemicals and reagents

All chemicals of analytical grade were purchased and used from Sigma–Aldrich, Amresco (USA), and USB (USA) unless otherwise stated. Ni^{2+}-NTA agarose matrix and Gel extraction kit were purchased from Qiagen. Plasmids and restriction enzymes were purchased from Novagen and Fermentas. Culture medium M199 and RPMI-1640 were used from Hyclone and Sigma.

2.2. Animal ethics statement

Balb/c female mice 8–10 weeks old were used for raising antibodies after prior approval of Animal Ethical Committee, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Indian Council of Medical Research (ICMR). The RMRIMS, ICMR follows "The Guide for the Care and Use of Laboratory Animals", 8th edition by the Institute for Laboratory Animal Research.

2.3. Parasite and culture conditions

L. donovani strains, Ag83 (MHOM/IN/1983/Ag83), Dd8 (MHOM/80/IN/Dd8) promastigotes used in experiments (designated, S1 &