Comparative Modelling, Characterization And Molecular Dynamics Study Of Trypanothione Reductase (Ldtr) From Leishmania Donovani

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ABSTRACT

Visceral Leishmaniasis (VL) is the most lethal form of Leishmaniasis caused by Leishmania donovani. This disease is the second largest parasitic killer in the world. Trypanothione reductase (TR) is an enzyme commonly present in all members of Trypanosomatidae, including Leishmania. This enzyme, analogous to Glutathione Reductase (GR) of mammals, is crucial for the management of oxidative stress of the parasite; as it recycles Trypanothione. The three-dimensional structure of TR from *L. donovani* (LdTR) has not been determined till date. In this study, the three-dimensional structure of LdTR was built by homology modelling and refined using molecular dynamics program. Various properties of the structural hierarchy of LdTR was also attempted to study along with the recognition and characterization of catalytic domains present in the enzyme. The main tools and servers used for the research were- MODELLER 9.11, VEGA ZZ 3.01, and MESSA etc. The results involved creation of an energy-minimized, refined comparative 3-D model of LdTR. The study also predicted LdTR to be a mitochondrial protein that uses FAD as an electron donor and is involved directly involved in homo-dimerization as well as indirectly involved in reduction of reactive oxygen species. The structure was submitted in PMDB (Protein Model Database).

Index Terms- Energy Minimization; Homology Modelling ; Modeller 9.11; Trypanothione reductase; Visceral Leishmaniasis; Vega ZZ 3.01.

INTRODUCTION

Leishmaniasis is a disease of protozoan origin, caused by the members of *Leishmania* genus. The insect vector for this parasite is sand fly of *Phlebotomus* and *Lutzomyia* genus [1]. Amidst the various forms of this disease, Visceral Leishmaniasis (VL) is the most acute. Also known as Kala-azar, it is caused by *Leishmania donovani* and is second largest parasitic-killer in the world after malaria [2]. The main characteristic of VL is that it is a systemic infection where the parasite invades important organs like spleen, liver etc gradually destroying immune cells like macrophages and making the host immune-suppressed. Eventually, the host succumbs to any opportunistic infection like pneumonia, tuberculosis, dysentery and AIDS. *L. donovani* is commonly found in tropical and sub-tropical areas of East Africa and Indian sub-continent where such pathogens are omnipresent [3].

The lifecycle of *Leishmania* revolves around two forms- the non-motile amastigotes and the motile promastigotes. Amastigotes are usually present a host's body but when ingested by a sand fly, they develop into promastigotes. Once inside a mammalian host, the promastigotes invade macrophages, return to the amastigotes form and increase their number asexually, lysing the macrophages by pressure during the process [4].

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VL is usually diagnosed by visualising amastigotes in splenic and bone marrow aspirates or serological testing using K39 dipstick [5]. VL is usually treated using pentavalent antimonials like sodium stibogluconate or Urea stibamine. But, traditional chemotherapeutics is being limited by rapidly emerging drug resistance [6,7]. Currently used drugs for treating resistant infections are Amphotericin B and Miltefosine Impavido [8,9,10]. As of 2012, no preventive vaccine has been found against VL.

Trypanothione reductase (TR) is an enzyme that plays a crucial role in redox damage management in *Leishmania*. Trypanothione dithiol ($T[SH]_2$) functions as an antioxidant which reduces Reactive oxygen species (ROS) while getting converted to Trypanothione disulfide ($T[S]_2$). The recycling or regeneration of ($T[SH]_2$) from ($T[S]_2$) is carried out by TR [11,12]. TR is unique in *Leishmania* and other trypanosomatids but absent in mammalian hosts, which makes it a potential drug target for development of antileishmanial drug [13]. Its mammalian counterpart is Glutathione reductase. However, Trypanothione reductase from *L. donovani* (LdTR) has no record of a three dimensional (3D) structure in PDB. It is a well-acknowledged fact that the tertiary structure of an enzyme determines its function. 3D structures can be determined most accurately by X-ray and NMR but it is also possible to design a hypothetical structure through *in silico* tools using structural data from its closest homologues.

In this study, we studied the primary and secondary structure of LdTR, and predicted the tertiary structure of LdTR. Post sequence retrieval from NCBI Protein Database, ProtParam was used for primary

analysis of molecular weight, pI, GRAVY etc [14, 15]. The secondary structure was predicted and analysed using MESSA, RAMPAGE and FoldIndex respectively [16, 17, 18]. Blastp, PSI-Blast and PROPSEARCH were employed to select a template [19, 20, 21]. The 3D structure modelling was done by MODELLER and further, energy minimization and side chain refinement was performed using VEGA ZZ 3.01 [22, 23]. The 3D model was analysed using various tools like PredictProtein, ProSA and ModFOLD4 [24, 25, 26]. The predicted 3D structure was submitted in the PMDB (Protein Model DataBase) [27].

METHODOLOGY

1. PRIMARY STRUCTURE RETRIEVAL:

The amino acid sequence of Trypanothione reductase was obtained from NCBI Protein database (Accession No. CBZ31498.1). The sequence was analyzed using the ProtParam online tool.

2. SECONDARY STRUCTURE PREDICTION:

The secondary structure as well as ontology and associated biological activity was predicted using MESSA server. Ramachandran plot for the query sequence was built using RAMPAGE. FoldIndex was used to assess the folding capacity and disordered fragments of the enzyme.

3. TEMPLATE SELECTION AND TERTIARY STRUCTURE PREDICTION:

Homology modelling of the three-dimensional structure of an enzyme requires a template which usually, is the X-ray crystallography or NMR-determined 3D structure of a close homologue. Therefore, PSI-Blast, PROPSEARCH and BlastP tools were used to search for the closest structural homologue for LdTR. After template selection, the comparative 3D models were built using MODELLER.

4. MODEL REFINEMENT AND ENERGY MINIMIZATION:

Vega ZZ molecular modelling suite 3.01 was employed to refine the 3D model of LdTR. Firstly, the correct side chains as well as hydrogens were added and atom type along with residues with unusual geometry were fixed. The energy minimization steps were performed using the in-built molecular AMMP molecular dynamics, simulation and modelling program [28]. After correction of bond types, charges were assigned using AMBER force field and Gasteiger charges. In the AMMP window, the conjugate gradients algorithm was selected and default parameters were set, i.e., Minimum steps-3000, Toler value- 1.0000 and Steepest steps-0. After the program finished running, the total energy of force field was calculated.

5. QUALITY ANALYSIS OF PREDICTED 3D MODELS:

The overall model quality analysis was done using MODFold4 and ProSA servers.

6. CHARACTERIZATION OF THE 3D MODEL:

The tools used for post-model analysis were InterProSurf, PredictProtein, SignalP 4.0, SFLD (Structure function linkage database) and Q-Site Finder. [30, 31, 32, 33] InterProSurf server calculated Accessible surface area of LdTR. PredictProtein was used for subcellular localization and detection of transmembrane regions. SignalP predicted whether the protein is not a signalling peptide or not. Q-SiteFinder predicted the presence of ligand binding pockets. The SFLD database was accessed using Hidden Markov Model (HMM) to link amino acid sequence and 3D structure of LdTR to its functional domains.

RESULTS

1. PRIMARY STRUCTURE ANALYSIS:

The amino acid sequence of Trypanothione reductase was found to be 491 amino acids. There were no corresponding experimental 3D structures available in PDB database for LdTR. With whole genome of the host organism being sequenced in 2011 and complete structural analysis on its way, it is crucial to predict an almost-accurate 3D model of the enzyme using the bioinformatics tools available at present.

Analysis using ProtParam online tool showed following results: Molecular weight of 52993.3, Theoretical pI of 5.94, Instability index of 31.63, aliphatic index of 81.20 and Grand Average of Hydropathicity (GRAVY) score of -0.125.

2. SECONDARY STRUCTURE ANALYSIS:

The MESSA server predicted that the enzyme consisted of equal number of α-helices and β-sheets with a few loops. No consensus sequences for coiled coils were detected. Gene ontology of the protein predicted sub-cellular localization to be mitochondria and molecular functions of the protein to be homo-dimerization and catalytic activity but it could not predict any biological process associated with the enzyme. The MESSA server also predicted that the query enzyme is closely related to glutathione disulfide reductase from *Crithidia fasciculata* which is a flavoprotein and whose activity is dependent on cystine at the active site.

The RAMPAGE plot exhibited presence of almost all amino acid residues (98.6%) in favoured regions, with an exception of a few **[Fig.-1]**.

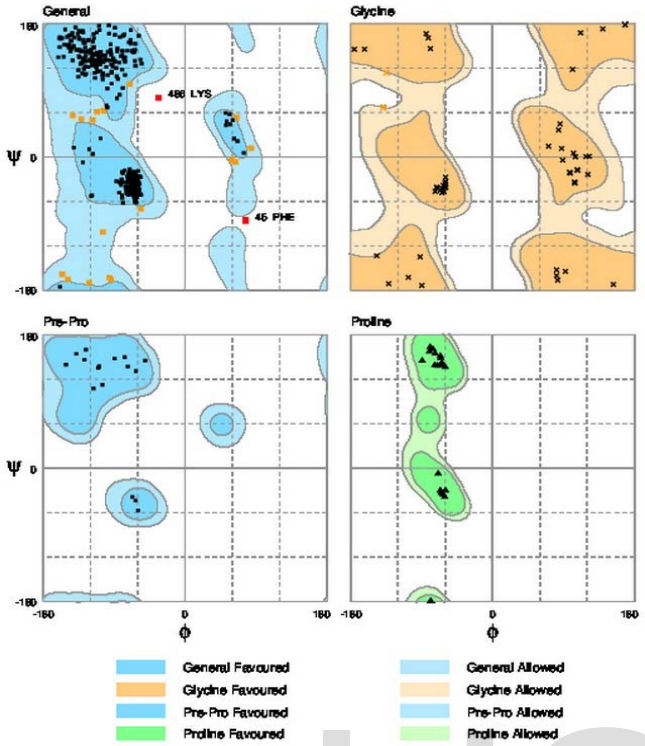

Figure 1. Rampage plot for LdTR.

FoldIndex calculated the enzyme's unfoldability index to be 0.187, which is a very low score **[Fig. 2].**

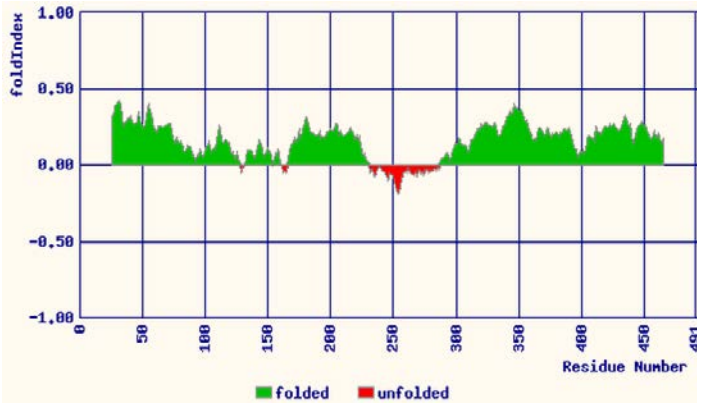

Figure 2. FoldIndex graph showing folding index and predicted disordered fragments.

3. TERTIARY STRUCTURE PREDICTION:

All the three tools, namely, PSI-Blast, PROPSEARCH and BlastP exhibited Trypanothione disulfide reductase of *Leishmania infantum* as the closest homolog of our query enzyme; hence its corresponding PDB structure was selected as the template (PDB id-2JK6). Ten 3D structures were built using MODELLER and the best was selected on the basis of the least DOPE (Discrete Optimized Potential Energy) score.

4. HOMOLOGY MODEL REFINEMENT AND ENERGY MINIMIZATION:

The best 3D model selected in the previous step was refined using Vega ZZ 3.01. Seven residues (Pro 42, 43, 59, 90, 255, 336 and Leu 491) were discovered with unusual geometry within the enzyme; they were fixed. In the next step, we attempted to make the structure more energetically favourable. The value of total energy was minimized from the value of -6627.161 KJ/Mol to -26063.002 KJ/Mol. The structure was finally saved in .pdb format **[Fig. 3(A) & 3(B)].**

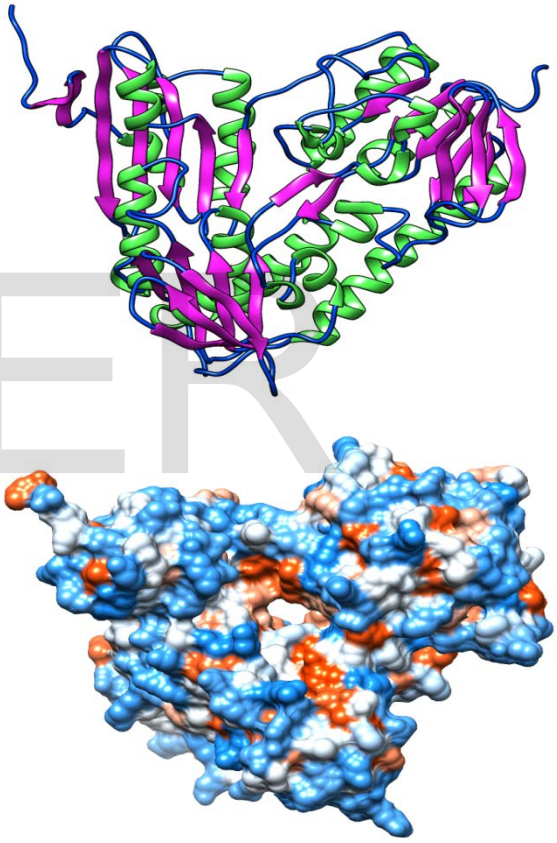

Figure 3. The refined 3D model; A- Image generated using secondary structure depiction tool (α-helix in green, β-sheet in purple & coils in blue); B- Hydrophobicity surface of the protein. (Both images were generated using Chimera 1.7 [29].)

5. QUALITY ANALYSIS OF PREDICTED 3D MODEL:

ModFOLD4 server provided a p-value of 4.264×10^{-5} and Global quality score of 0.9581 for our 3D model (the threshold p-value <0.001 suggests that there is less than a 1/1000 chance that the model is incorrect). Again, ProSA

depicted a Z-score of -11.76 which lies well within the X-ray crystallography range of scores typically found for native proteins of similar size **[Fig. 4(A)]**. Similarly, local model quality was found to be in negative values in both, energy per 40 and 10 residues of the structure, which is a good indication of the model being correct **[Fig. 4(B)].**

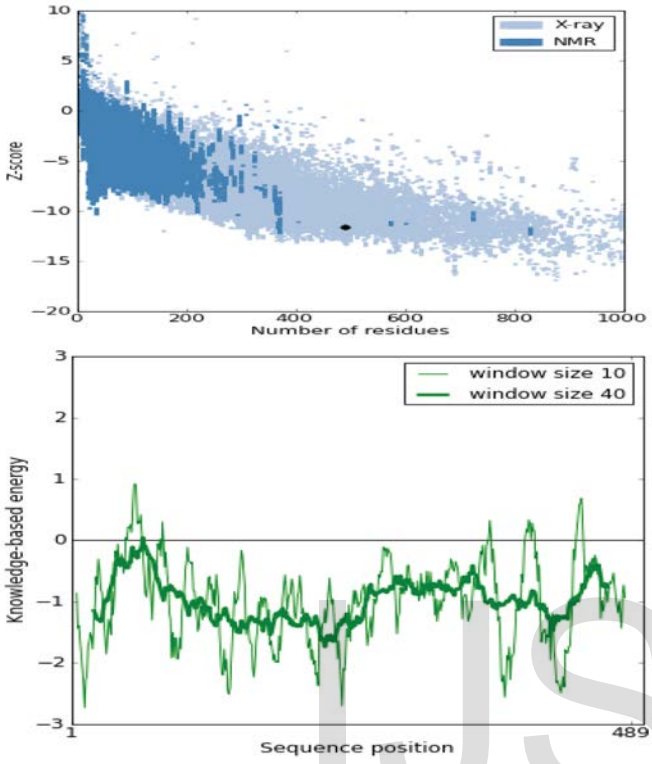

Figure 4(A)- ProSA Z-score graph, the black dot denotes the Z-score; 4(B)- ProSA Local model quality, graph peaks lie mostly below 0.

6. CHARACTERIZATION OF THE 3D MODEL:

After assurance of good quality of the designed model, it is pertinent to infer the properties of the protein. InterProSurf server calculated a Probe radius of 1.400, Polar area/energy of 7988.84, Apolar area/energy of 13690.68, Total area/energy of 21679.52, Number of surface atoms of 2022 and Number of buried atoms of 1694 for the 3D model. Its subcellular localization was predicted to be mitochondria using PredictProtein. It also predicted the presence of a helical transmembrane region from amino acid 191-208 with a length of 18 residues. SignalP predicted that with a mean value of 0.212 (< cut-off value 0.450), the protein is not a signalling peptide. Q-SiteFinder predicted the presence of 62 ligand binding pockets in the protein. The SFLD database predicted that our query has the highest similarity with Glutathione Reductase (GR) family. On alignment of the family with the query sequence, it was observed that G133, G135 and G138 residues were involved in FAD-binding; C185 and C190 in disulfide bond formation to stabilize the tertiary structure as well as electron shuttling between FAD and substrate; K193 in electrostatic interactions; G358, G360, A363 in NADP binding; E365 in formation of H-bonds with NADP; and H732 and E737 in acid/base catalysis of the enzyme **[Table-1].**

TABLE-1 CONSERVED RESIDUES OF QUERY SEQUENCE IN RELATION TO OTHER MEMBERS OF GR FAMILY AND THEIR FUNCTIONS

POSITION	TYPE	FUNCTION	EVIDENCE CODE
133	Gly (G)	Assists in FAD Binding	Inferred from crystal structure (ICS)
135	Gly (G)	Assists in FAD Binding	ICS
138	Gly (G), Ala (A)	Assists in FAD Binding	ICS
185	Cys (C)	Forms disulfide bonds with Cys70 and shuttles electrons between FAD and Substrate	Inferred from catalytic residues (ICR)
190	Cys (C)	Forms disulfide bonds with Cys65 and shuttles electrons between FAD and Substrate	ICR
193	Lysine (K)	Electrostatic interaction with N5 of FAD	ICR
358	Gly (G)	Assists in NADP Binding	ICS
360	Gly (G)	Assists in NADP Binding	ICS
363	Ala (A)	Assists in NADP Binding	ICS
365	Glu (E)	Hydrogen bonds with	ICS

		carboxy amide of nicotinamide ring of NADP	
732	His (H)	Acid/Base catalyst	ICR
737	Glu (E)	Orients H698 for acid/base catalysis	ICR

DISCUSSION

From the primary and secondary structure analysis data, it can be inferred that the Trypanothione reductase (LdTR) enzyme is of mitochondrial origin, consisting of more hydrophobic residues than hydrophilic (high aliphatic index and negative GRAVY score); the overall structure presenting α-helix (30.96%), β-sheets (22.40%), turns (8.76%) and random coils (37.88%) and it is well-folded in its tertiary form (extremely low unfoldability index). It also can be presumed that the enzyme is involved in homo-dimerization and uses FAD as a cofactor. After creation of a full-fledged, stereochemically as well energetically corrected theoretical model of LdTR in three-dimension, we put efforts to characterize the enzyme's tertiary structure. We learnt that LdTR consists of two transmembrane domains and its binding sites mainly constitute of negatively-charged, hydrophobic residues. This information fits in perfectly with the fact that the cognate substrates of LdTR are positively-charged polyamines. The predicted model clearly suggests presence of 62 pockets as well presence of two disulfide linkages (Cys65-Cys190 and Cys70-Cys185) in its catalytic site. It also infers that there are two FAD binding domains, two NADP binding domains and two Trypanothione binding site in the stable, dimer of the enzyme. This fact, however, requires further experimental proof. In conclusion, Trypanothione Reductase from *Leishmania donovani* can be defined as a mitochondrial protein that uses FAD/NADP as an electron donor and is directly involved in homo-dimerization and thiol metabolism as well as indirectly involved in reduction of reactive oxygen species.

Trypanothione reductase (LdTR) enzyme is crucial for the survival of *Leishmania donovani* inside any host cell, because it helps the organism to suppress oxidative stress that might be fatal if not dealt with. Thus, it can be considered to be a potential drug target for elimination of VL. Though LdTR is analogous to the mammalian Glutathione reductase (GR), both are mutually exclusive in recognizing their respective substrates. Hence, the mammalian GR surpasses the risk of being falsely recognised as LdTR by the targeted drugs. The facts cited above signify the fact that LdTR can, undoubtedly, be regarded as a drug target for treating VL caused by *Leishmania donovani* [34]. In order to carry out drug–related studies on this target, a three-dimensional structure of the enzyme must be present. So, the absence of a three-dimensional structure of LdTR motivated us to carry out this study. The findings of this study does satisfy our aim to understand the Trypanothione reductase enzyme at least partially, if not completely.

Future prospects of this study would involve determining the 3D structure of LdTR using experimental methodologies like X-ray crystallography or NMR. Elucidation of other structure-related prospects like changes in tertiary structure after binding to a substrate is also crucial. *In vitro* as well as *in vivo* validation would certainly establish LdTR as a drug target. Once we are past that phase, designing of inhibitors against this drug target could be undertaken to treat Visceral Leishmaniasis that is rampant in the Indian sub-continent. Studies on LdTR gene should also be encouraged to determine its importance in survival of the protozoa.

CONFLICT OF INTEREST

The authors have declared that they harbour no conflict of interest in any matter.

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