

Structural analysis of the *Babesia microti* thioredoxin reductase: a potential drug target for babesiosis treatment.

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Abstract

Babesia microti, the causative agent of human babesiosis which was endemic in United States and parts of Europe with sporadic cases in other regions is expanding its geographical range. Being a common transfusion threat, *Babesia* has become a cause of concern in researchers and epidemiologists. Human babesiosis is now recognized as an emerging disease and gains more attention now than ever before. Thioredoxin reductase, a promising drug target in apicomplexan parasite has been validated in several species. This study focused on *in-silico* analysis of physicochemical properties, functional and structural aspects of thioredoxin reductase of *B. microti*. Comparative modeling approach was adopted for developing the three dimensional structural model of Thioredoxin reductase of *Babesia microti*. The model developed was found to be of reasonably good stereo-chemical quality by structure validation servers. This study will provide valuable insights about the function and structure of the enzyme thioredoxin reductase of *B. microti* and aid in developing effective chemotherapeutic agents for control and treatment of *Babesia*.

Keywords: *Babesia microti*, Babesiosis, Thioredoxin reductase.

Accepted on April 28, 2018

Introduction

Babesiosis in humans and animals is caused by apicomplexan zoonotic hematotropic parasites of the genus *Babesia* transmitted by ticks. *Babesia* is quite ubiquitous in nature with a wide range. *Babesia* is one of the most common blood parasites of mammals. Babesiosis is one of the most common infections of animals and causes profound economic, medical and veterinary impact globally. After years of being recognized as major pathogen in cattle causing huge economic losses, babesiosis has recently gained recognition as an important pathogen of human [1]. First case of babesiosis was reported in Croatia in 1957 [2]. Babesiosis caused by the *B. microti* in human in United States was first documented in 1969 [3].

Out of more than 100 known species of *Babesia*, only a fraction cause human babesiosis. Among these, genetically distinct *Babesia microti* is the predominant species in US and major culprit responsible for causing human babesiosis. The spectrum of Babesiosis ranges from asymptomatic to severe malaria-like symptoms including chills, sweats, headache, arthralgia, myalgia, anorexia, cough and occasionally causing deaths in humans. *Babesia* disease cycle progresses in two hosts involving black legged deer ticks of belonging to *Ixodes* genus as definite host and a vertebrate intermediate host primarily. *Peromyscus leucopus* while humans are accidental

hosts or dead-end hosts who acquire the infection with the bite of infected ticks. The disease is tightening its grip in many states of United States as well as other parts of the world including Europe and a few Asian countries. Co-morbidity of babesiosis with Lyme disease and possibility of under-reporting in asymptomatic patients often compounds the situation. The fact that Babesiosis can be transmitted congenitally as well as through blood transfusion and is difficult to diagnose makes the situation worse [4,5]. *Babesia* can even survive for prolonged period in blood storage conditions. Babesiosis has emerged as a major transfusion transmitted threat of the recent times [5]. *Babesia* remains understudied unlike another apicomplexan parasite *Plasmodium* and *Toxoplasma*. Owing to increased number of incidences over past few years, human babesiosis is now classified as emerging disease [6]. CDC has included babesiosis as nationally notifiable diseases in 2011. Immunocompromised individuals like those suffering with HIV infection, cancer, hemoglobinopathy, organ transplantation are at high risk of contacting babesiosis and demonstrate more severe symptoms and persistent infection. Other factors that make one vulnerable to contacting babesiosis are age, drug regime of immunosuppressive drugs or recent splenectomy. The disease shows a seasonal trend coinciding with the seasonal activity of ticks. Like any other protozoan parasites, *Babesia* inhabits an oxygen rich environment inside

blood cells of mammalian host and faces a challenge of shielding its system against oxidative stress which may wreak havoc by damaging its membrane lipids, nucleic acids, and proteins. To counteract Reactive Oxygen Species (ROS), *Babesia* employs anti-oxidant systems including Thioredoxin-thioredoxin reductase (Trx/TrxR) and Glutathione-Glutathione Reductase (GSH/GR). Thioredoxin system comprises of Thioredoxin reductase (TrxR), various thioredoxins and Thioredoxin-dependent peroxidases (TPx). Thioredoxin reductase has been biochemically characterized in *Babesia* [7]. Thioredoxin reductase belongs to a family of dimeric flavoenzymes. Thioredoxin reductase is an essential enzyme required for counteracting the oxidative stress and hence, in survival of these parasites. It is considered an attractive drug target as it plays key role in many cellular processes and its inhibition can affect many vulnerable stages in apicomplexan parasites. Not much is known about the functional and structural aspects of thioredoxin reductase of *Babesia*. Comparative modeling or homology modeling is an alternate method that aid in deriving structural insight about protein in absence of experimentally derived structure. Homology modeling has been used successfully for providing structural information of key enzymes in various instances in important human pathogens in past [8-17]. The enzyme of *Babesia* has pronounced and significant differences with the human counterpart. Hence, we undertook the exercise of developing a 3-dimensional model of Thioredoxin reductase enzyme of *Babesia microti* for obtaining insights about its structure and function.

Materials and Methods

Sequence retrieval and physicochemical characterization

Uniprot database (<http://www.uniprot.org/>) was searched using keyword “*Babesia microti*” and thioredoxin reductase”. The query yielded 7 entries. Primary sequence of the enzyme thioredoxin reductase of *Babesia microti* (Accession: A0A1R4AAX8) was collected in Fasta format from the database. Physicochemical properties of the enzymes were determined using ExPASy-ProtParam and Protscale tool [18]. Charge and Pepstats of Emboss was used to determine position-wise distribution of charge mole percentage of various classes of amino acids in Thioredoxin reductase respectively.

Propensity of crystallization

CRYSTALP2 web server available at [19] and Xtalpred [20] were used to predict the propensity of enzyme to crystallize.

Functional characterization of thioredoxin reductase of *Babesia microti*

CYS REC server [21] that identifies the positions of cysteines, total number of cysteines and computes the most probable SS bond pattern of pairs in the protein sequence was used to analyse SS bonds in primary sequence.

Motifs were predicted using default parameters in MEME suite (Multiple Em for Motif elicitation) [22].

Consurf server was employed for identification of biologically important protein residues in the protein sequence [23,24]. As three-dimensional structure of the protein is not available in Protein Data Bank (PDB) (<http://www.rcsb.org/pdb>), this study aimed at development of 3D model of thioredoxin reductase of *Babesia microti* and conducting in-depth sequence and structural analysis.

Secondary structure prediction from target sequence

Secondary structure of a protein denotes the arrangement of residues in alpha helix, beta sheets, extended strands or turns. Secondary structure of the selected protein was predicted employing NPS server [25] using various methods viz. DPM [26], DSC [27], GOR4 [28], HNNC [29], PHD [30], Predator [31], SOPMA [32], SIMPA96 [33] and secondary consensus [34] keeping default parameters for 4 state predictions keeping output width=70.

Prediction of protein disorder

Often post translational modifications, attachment of signal peptides, evolutionary path of a protein molecule are dictated by the disordered regions present in a protein molecule. Thus, identification of such important disordered regions is of immense importance to understand the structure and function of a protein. Disordered regions of the thioredoxin reductase were identified using DisEMBL [35], GLOBPLOT [36], RONN [37] and Protein Disorder Prediction System (PRDOS) [38] server.

Generation of three dimensional models

Comparative modeling approach was adopted to derive a 3D model of thioredoxin reductase of *Babesia microti*. The spatial restraint based Modeller 9.19 version was used for this purpose. The complete modeling exercise included target based template searching and selection, target-template alignment, model development employing spatial restraint, model evaluation, loop optimization and final structural quality evaluation. For further assurance and better template consideration, we have also attempted parallel template selection through Swiss model [39].

Selection of template

For template selection, the first approach model generation method of Swiss model [39] was used. On the basis of obtained result, high-resolution X-ray crystallography structure of thioredoxin glutathione reductase at a resolution of 2.30 Å (PDB ID: 2x99, A chain) [40] having 48.22% identity with the query sequence was selected as template for homology modeling exercise. In parallel, template searches were performed through Modeller 9.19 version [41] via profile development and searching. Reasonable number of hits (20; 1aogA, 1ojt, 3grs, 1trb, 1dxlA, 1ebdA, 1nhp, 1fecA, 1gesA, 1h6vA, 1jehA, 3ladA, 1lpfA, 1lvI, 1mo9A, 1onfA, 1vdc,

1xdia, 1qlra, 1xhcA) was obtained through this process and all the obtained templates were compared on the basis of their sequence length coverage (sequence identity) and crystal structure resolution.

Target-template alignment

Out of the selected total number of templates (20 from Modeller analysis and 2x99A from SwissModel), further reduction in number of templates was done following the sequence identity and structural resolution based criterion. The following templates were selected: 1mo9A, 1xhcA, 1nhpA, 1xdia, 1lvla, 1h6vA, 1qlra. A template-template comparison was done to reach such conclusion. Target-template alignment was done using the Modeller based scripts (Modeller version 9.19). The template (2x99A) selected through the Swiss Model tool was also included in the final template list.

Model development and evaluation

The target sequence was carefully aligned with the generated multi-template alignment. Total 10 models were generated based upon the multi-template-target alignment. All the models were subjected to PDF, DOPE and GA341 evaluation. The best model was selected after carefully analyzing the obtained values, especially the DOPE scores. GA341 output suggested that the structures were similar to the original crystal structures in quality.

Loop optimization and further structural evaluation

The best model was selected and visually inspected using VMD [42] and Chimera [43]. The observation suggested that the model contained multiple sheets and helices along with several loops. Considering the generation of multiple loops, multi-template based approach was adopted so as to generate a structure with comparatively shorter loops. Loop optimization was mandatory considering the presence of multiple loops in the structure. Based on the visual inspection and other external systematic evaluation (RAMPAGE [44], ERRAT [45] etc.), regions with loops having comparatively poor energy were subjected to refinement. Total 10 structures with loop refinement were generated and further evaluated. Obtained results pertaining to the template selection through structure development and evaluation are described in the result section.

Structure validation

Followed by the structure validation through DOPE score, GA341 and modeler objective function, all the structures were further subjected to validation through RAMPAGE, PROSA (Protein Structure Analysis) and ANOLEA (Atomic Non-Local Environment Assessment). Analysis detail is provided in the result section.

Secondary structural analysis from final protein structure

To understand the three-dimensional structure with details of secondary, tertiary and quaternary features, further structural

analysis with PDBSUM and PROMOTIF [46] was performed. Detailed insight on all the sheets, helices, loops, hairpins, disulphide linkages along with specific motifs and orientation was obtained for the generated structure.

Active site prediction

Identification of active site is important to understand the plausible pockets present in a protein which can be considered for safe binding sites for potential inhibitors which may aid in hindering the structural and functional mechanism of an important protein molecule belonging to a pathogen. With such objective, potential active site mapping was done using CASTp tool [47] to retrieve information regarding the microenvironment of the pockets of the target protein.

Ligand selection and docking analysis

Antibiotic resistance has become a cause of concern globally. The drug pipeline should be replenished from time to time with novel natural or synthetic molecules to provide remedy to the patients from various infections. Similar situation demands for novel active molecules for *Babesia* too. Recent report by Harbut et al. [48] suggested that Auranofin is having excellent potential to exhibit bactericidal role affecting the thiol-redox homeostasis. Interestingly, it was found in the same study that Auranofin can be used for other gram positive bacteria too such as *Bacillus subtilis*, *Enterococcus faecalis*, *Enterococcus faecium* and *Staphylococcus aureus*. Auranofin functions through creating oxidative stress for the bacteria through diminishing their reducing capacity. Thus, this potential molecule may become a common purpose inhibitor for several gram positive pathogens.

Following the vital information, similar compounds from the PubChem databases were utilized which showed maximum structural similarity with the auranofin molecule. Total 33 such molecules were selected including different conformations that were used for the docking purpose against the developed structure. Structures having 3D information were only considered. SwissDock server [49] was utilized for this purpose.

Protein dynamics simulation for fluctuations

Fluctuation of protein provides insight in relation to the stability and flexibility of a protein. Simulated molecular motion analysis provides ample information in this regard. Different types of molecular motion analysis were performed for the target protein to understand the flexibility and plausible direction of the motion in the target protein. CABSflex [50] was used for this purpose.

Results and Discussion

The goal of this computational analysis was to retrieve in-depth molecular information with relation to the target protein considered for this study. Analyzing the protein in a comparative manner through integrated sequential, structural and functional analyses yielded novel insights for *Babesia*

microti thioredoxin reductase protein. The following result section describes such information in detail.

Sequence retrieval and physicochemical characterization

Initial investigation of the protein sequence suggested some important information that is summarized in the Table 1. The length of the protein was found to be 553 amino acids having overall molecular weight of 60329.49. The higher molecular weight suggests that out of various types of thioredoxin reductase, this protein might have similarity with glutathione reductase, trypanothine reductase and such other enzymes [51].

The enzyme was predicted to be non-crystallizable with 0.463 confidence by CRYSTALP and least crystallizable both in EP crystallization (Class: 3) and RF crystallization classes (Class: 11). SPpred (Soluble Protein prediction) results showed a score indicating that thioredoxin reductase of *Babesia microti* is a soluble protein.

Theoretical pI of the protein was estimated to be 7.56, suggesting the point where the net charge of the protein could be nil. The theoretical pI value helps in different experimental set up pertaining to the protein for isolation and purification. The extinction coefficient value is another important parameter in relation to experimental calculations providing the absorption value of the protein. The obtained instability index value (Table 1) 31.12 represents the theoretical stability of the protein even after being a quite large molecule. Similarly, the aliphatic index value of 93.58 (Table 1) suggests the major relative volume occupancy of the aliphatic side chains for this *Babesia* thioredoxin reductase. Grand Average of Hydropathicity (GRAVY) value of -0.016 demonstrates the hydrophilic nature of the protein.

Table 1. Physicochemical properties of thioredoxin reductase of *B. microti* determined using protparam.

S. no.	Property	Value
1.	Number of amino acids	553
2.	Molecular weight	60329.49
3.	Theoretical pI	7.56
4.	Total number of negatively charged residues	56
5.	Total number of positively charged residues	57
6.	Formula	C ₂₆₈₇ H ₄₂₇₆ N ₇₂₂ O ₇₉₈ S ₂₇
7.	Total number of atoms	8510
8.	Ext. coefficient	43945
9.	Ext. coefficient*	43320
10.	Instability index	31.12
11.	Aliphatic index	93.58
12.	Grand average of hydropathicity (GRAVY)	-0.016

13.	Estimated half-life (mammalian reticulocytes, <i>in vitro</i>)	>30
14.	Estimated half-life (yeast, <i>in vivo</i>)	>20
15.	Estimated half-life (<i>Escherichia coli</i> , <i>in vivo</i>)	>10

Amino acid composition

Prediction results showed that leucine (Table 2) was the most abundant amino acid followed by glycine and valine. Secondary structure of a protein is representation of repetitive geometrical conformations formed as a result of intermolecular and intramolecular hydrogen bonding. Most of the servers predicted that random coils were predominant structures in the protein followed by alpha helices and extended strands.

Eleven cysteines at position 17, 105, 110, 200, 251, 278, 306, 419, 471, 547 and 552 were predicted using CYSRec. Out of these, 7 are not S-S bounded, 1 is probably S-S bounded. Only 2 cysteines (536) showed high score and are probably S-S bounded. Most probable pattern is 105-110.

Table 2. Amino acid composition of thioredoxin reductase of *B. microti* predicted using protscale.

S. no.	Amino acid	Number	Percentage
1	Ala	37	6.70%
2	Arg	21	3.80%
3	Asn	29	5.20%
4	Asp	34	6.10%
5	Cys	11	2.00%
6	Gln	12	2.20%
7	Glu	22	4.00%
8	Gly	48	8.70%
9	His	13	2.40%
10	Ile	38	6.90%
11	Leu	51	9.20%
12	Lys	36	6.50%
13	Met	16	2.90%
14	Phe	22	4.00%
15	Pro	23	4.20%
16	Ser	39	7.10%
17	Thr	34	6.10%
18	Trp	3	0.50%
19	Tyr	18	3.30%
20	Val	46	8.30%

Proportion of different classes of amino acids in thioredoxin reductase of *B. microti* is represented in Table 3 suggesting dominance of non-polar amino acids followed by small and

polar amino acids. Periodicity of polar and non-polar amino acids along with the existence of disordered regions determines the secondary structure of a protein molecule, especially its tendency of developing α -helices and β -sheets [52].

Table 3. Mole percentage of different classes of amino acids present in thioredoxin reductase of *B. microti* using emboss.

S. no.	Property	Residues	Number	Mole (%)
1	Tiny	(A+C+G+S+T)	169	30.561
2	Small	(A+B+C+D+G+N+P+S+T+V)	301	54.43
3	Aliphatic	(A+I+L+V)	172	31.103
4	Aromatic	(F+H+W+Y)	56	10.127
5	Non-polar	(A+C+F+G+I+L+M+P+V+W+Y)	313	56.6
6	Polar	(D+E+H+K+N+Q+R+S+T+Z)	240	43.4
7	Charged	(B+D+E+H+K+R+Z)	126	22.785
8	Basic	(H+K+R)	70	12.658
9	Acidic	(B+D+E+Z)	56	10.127

Improbability of expression in inclusion bodies for the considered protein was found to be 0.792 suggesting the probable tendency of the protein with reference to its presence in the inclusion bodies and thus hinting the possible way of purifying the same without losing the enzymatic activity. The other important physicochemical properties along with their respective value ranges are provided in Table 4.




Table 4. Minimum and maximum values of physicochemical properties of thioredoxin reductase of *B. microti* predicted using protscale.

S. no.	Property	Minimum value	Maximum value
1.	Bulkiness	0.197	0.828
2.	Polarity	0.001	0.556
3.	Refractivity	0.108	0.515
4.	Recognition factor	0.065	0.632
5.	Hydrophobicity (Kyte and Doolittle)	0.246	0.819
6.	Transmembrane tendency	0.329	0.839
7.	Buried Residues	0.218	0.846
8.	Accessible residues	0.323	0.701
9.	Ratio hetero end/side	0.030	0.442
10.	Average area buried	0.115	0.542
11.	Average flexibility	0.329	0.852
12.	Relative mutability	0.218	0.767

Sequential motif analysis

Sequential and structural motifs are majorly conserved in nature and serve as determinant for important structural component or vital supporting functional elements. The protein sequence was subjected to motif analysis and 3 motifs were found to be conserved in the protein sequences which are represented in Table 5.

Table 5. Motifs discovered in thioredoxin reductase of *B. microti* using MEME.

Logo	p-value	Start	E-value	Site count	Width
	1.27E-15	98	1.90E+01	2	13
	1.28E-14	540			
	2.30E-23	451	1.60E+01	2	21
	1.18E-22	399			
	1.82E-08	211	2.50E+01	3	6
	1.50E-07	375			
	2.17E-07	14			

Secondary structure analyses from target sequence

A multi-server based analysis was performed to gain insight into the secondary structure of the *B. microti* thioredoxin reductase protein. Table 6 depicts the obtained result from various tools along with the percentage of secondary structural elements predicted.

Table 6. Secondary structure of thioredoxin reductase of *Babesia microti* predicted using NPS server.

Secondary structure	DSC	HNHC	MLRC	PHD	Predator	Sec. cons.
Alpha helix	25.50%	30.56%	29.66%	22.24%	20.43%	25.50%
310 helix	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pi helix	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Beta bridge	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Extended strand	16.64%	23.69%	22.06%	24.41%	20.25%	20.25%
Beta turn	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bend region	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Random coil	57.87%	45.75%	48.28%	53.35%	59.31%	50.27%

The obtained results hinted towards dominance of coiled structural component followed by helices and extended strands. No further hint was obtained for the other delicate structural components such as 310 helix, Pi helix, beta turns, bend regions or hinge regions. Thus, it is necessary to have structure based secondary structure analysis for minute information missing from the sequence based analysis. As thioredoxin reductase are of different types [51] with difference in

structure, mechanism of action, variation in coding genes, therefore, each thioredoxin structure should be analysed with individual attention.

Table 7. Disordered regions by definition using different servers.

S. no	Server	Definition by type	Region
1	Pondr	Disordered regions	172-177, 187-188, 213-218, 336-357, 367-367, 377-382
2	DisEMBL	Disordered by loops/coils definition	4-71, 62-70, 86-112, 133-144, 180-195, 208-240, 289-322, 327-380, 394-423, 456-481, 488-495, 509-553
		Disordered by hot-loops definition	93-103, 134-145, 167-196, 208-217, 460-469, 536-553
		Disordered by Remark-465 definition	538-553
3	GLOBPLOT	Disordered by Russell/Linding definition	61-71, 92-106, 210-219, 230-237, 463-468, 536-553
		Potential globular domains (GlobDoms) by Russell/Linding definition	1-91, 107-551

Transmembrane region prediction

As the transmembrane helix regions scoring above 500 only are considered significant, only 2 regions (2-21, 239-266, inside to outside helices) and 1 (96-114, Outside to inside helices) predicted were considered (Figure 1).

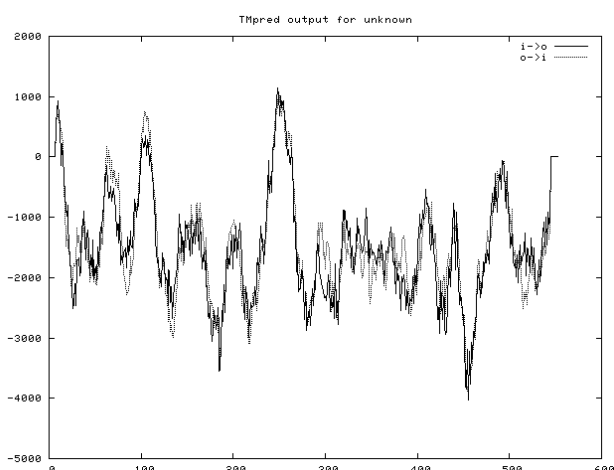


Figure 1. Transmembrane region prediction using TMPRED.

Disordered regions in the target protein

Identification of disordered regions in a protein molecule is of vital importance to understand the structural, functional and evolutionary aspect of a protein. It is imperative to understand the disordered regions of this protein considering its structural and functional diversity. The predicted disordered regions are provided in Table 7 and Figure 2.

Sequence-structure conservation analysis

Consurf server [23,24] was used for finding the evolutionary significance of amino acids in thioredoxin reductase protein sequence of *B. microti* with the default options of BLAST E-value threshold: 0.001, maximum number of homologs: 50, iteration=1. Multiple sequence alignment was built using MAFT and UNIREF90 was used for collection of homologues

using HMMER homology search algorithm. Out of 1366 HMMER hits, 1242 were found to be unique and the calculation was performed on the 50 sequences closest to the query. The conservation scores versus residue number and the unrooted phylogenetic tree constructed using the tree building facility of CLUSTAL-W employing the multiple sequence alignment obtained from MUSCLE are shown in Figure 3.

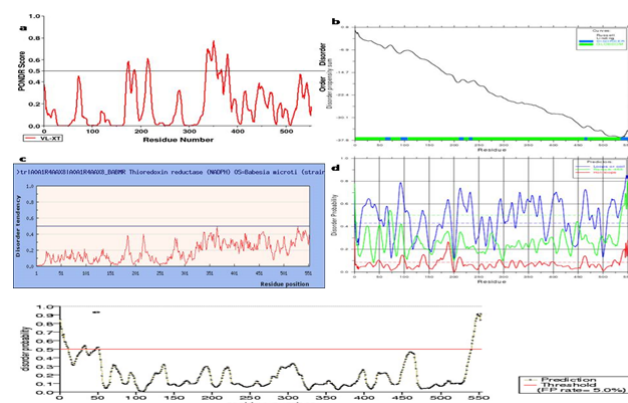


Figure 2. Protein disorder of thioredoxin reductase of *Babesia microti* predicted using (a) PONDR, (b) GLOBPLOT (c) IUPRED (d) DisEMBL and (e) PRDOS.

Structure generation through homology modeling

As mentioned in the methodology section, homology modeling for the target sequence was attempted using Modeller 9.19 version. Following the protocol discussed, profile was built and relevant structures were searched through Modbase. Rigorous experiment was conducted to develop a model from a single template where PDB ID 2x99 chain "A" (best template found through SwissModel server) and 1mo9 chain "A" (best template found in Modbase template search) were used as templates. But due to template-target length difference, single template based approach was not adequate to provide expected result. Therefore, a multi-template based approach was adopted. Figure 4A shows the potential important templates obtained during template search along with their respective

sequence identity, structural resolution and sequence coverage. Altogether, twenty one (21) templates were obtained and the best identity and templates with good structural resolution containing (1mo9A, 1xhcA, 1nhpA, 1xdia, 1lvla, 1h6vA, 1qlrA and 2x99A) were considered for multi-template based modeling (Figure 4B). Generated target-template alignment is shown in Figure 5.

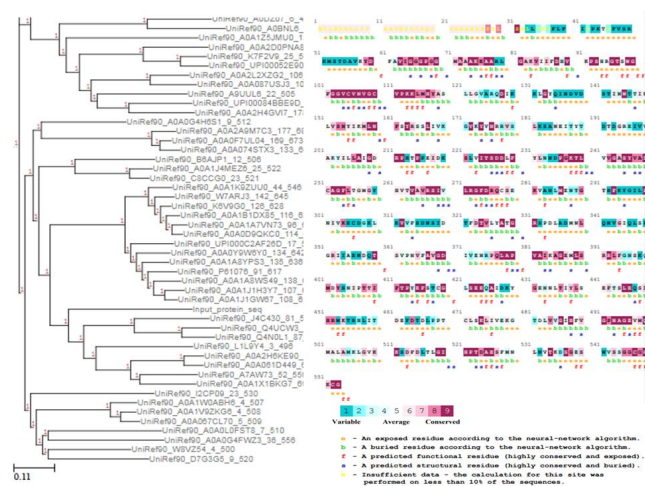


Figure 3. Consurf results showing conserved amino acid with scores.

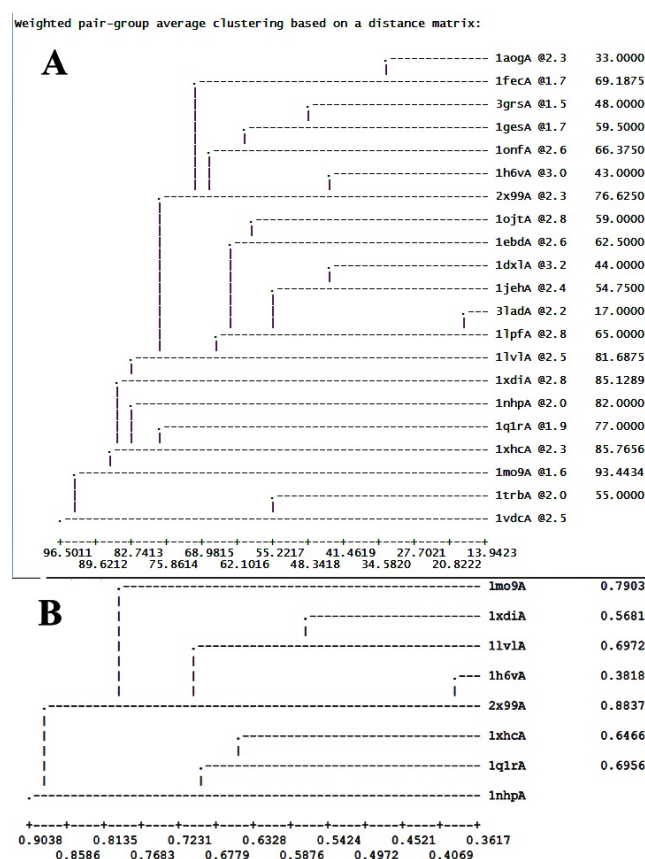


Figure 4. Template selection for three dimensional structural development through modeller. (A) The obtained initial hits as templates. (B) Comparative identity of the selected templates for multi-template based modelling.



Figure 5. Generated target sequence and multiple template alignment.

Once the target-template alignment was generated, ten protein structures were developed for the target protein using the multi-template and target sequence alignment. All the proteins were further subjected to evaluation through molpdf, DOPE and GA341 scoring. The obtained results are displayed in Table 8 along with their respective model numbers. The 4th model was found to be comparatively good based on the above mentioned evaluation scoring and was considered for the further analysis. The obtained model is represented in new cartoon format and surface view in Figure 6. The developed structure showed the alpha helices and beta sheets almost similar to other thioredoxin reductase proteins along with some long loops.

Table 8. Molpdf, DOPE score and GA341 score of best ten three dimensional structural models developed with multiple templates.

Model number	molpdf	DOPE	GA341
Model 4	27163.44336	-55457.5	1
Model 1	26907.65625	-55397.7	1
Model 10	26742.1875	-55374.7	1
Model 7	26880.80469	-55139.8	1
Model 8	26782.80469	-54850.4	1
Model 6	27027.62305	-54804.3	1
Model 5	27123.99609	-54774.9	1
Model 2	27446.76563	-54381.3	1
Model 3	27149.9707	-54333.4	1
Model 9	27038.10547	-54202.5	1

Loop optimization of the best structure

The existence of multiple loops suggested the further need for loop optimization. Therefore, all loop regions with poor energy profile and structural orientation were identified using energy profiling and structural evaluation and visualization and the structure was subjected to loop optimization.

Table 9. molpdf and DOPE score of best ten loops optimized three dimensional structural models developed with multiple templates.

Structure model number	molpdf	DOPE
Model 8	1184.14331	-10252.12012
Model 2	1651.75635	-9835.49707
Model 1	903.81464	-9727.25488
Model 4	2020.79944	-9114.76074
Model 9	5959.34863	-8566.29785
Model 6	3826.18115	-8330.34473
Model 5	4257.93311	-8059.69629
Model 3	5646.4541	-8047.19873
Model 7	4218.79346	-7961.8457

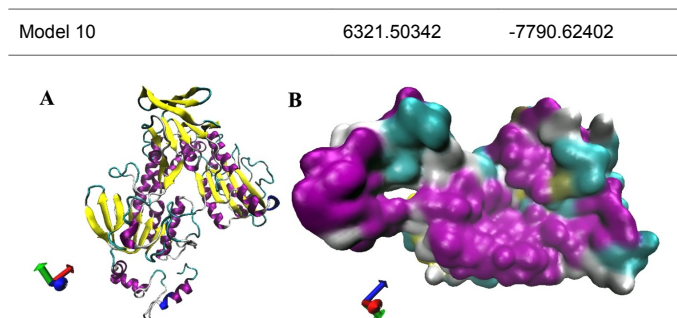


Figure 6. Three dimensional structure of thioredoxin reductase of *B. microti* visualized using VMD. (A) Representation of the α helices (purple), β sheets (yellow) and loops (cyan) of the final protein generated after loop optimization through multiple-template based modeling approach. (B) Surface view of the protein where purple colored surface refers to the α -helix region and cyan loops.

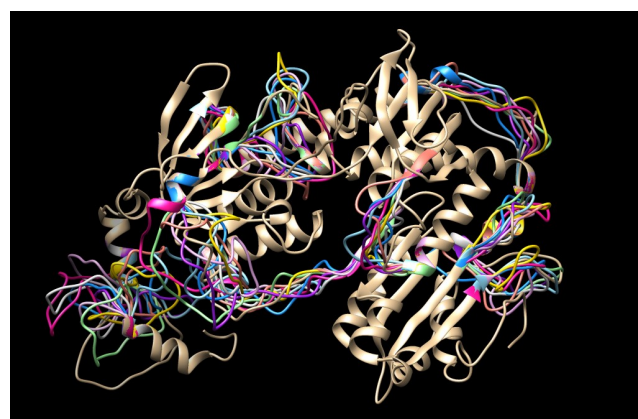


Figure 7. Three dimensional structure of thioredoxin reductase of *B. microti* with the loops optimized for 10 structures visualized using chimera.

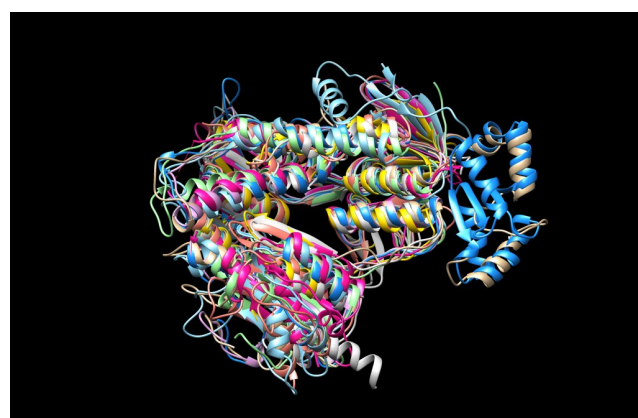


Figure 8. Superimposed target-template structures. The target is represented in light grey and all other templates are depicted in various other colors superimposed with the target protein molecule.

The evaluation scores for the 10 models developed during loop optimization considering the previous multi-template based best structure as starting structure are provided in Table 9. The model 8 was found to be the best loop optimized model and was considered for all relevant analysis. Figure 7 represents the structures with loops after optimization.

Structure validation

The final structure was identified after the loop modeling and subjected to superimposition with the considered multiple templates. The obtained RMSD was within the allowed region for most of the templates. The superimposition of the final modeled structure with multiple templates is shown in Figure 8. Respective RMSD value ranges and distance observed between the target and the templates are represented in Figure 9. The RMSD values ranged from 0.506 Å to 2.457 Å.

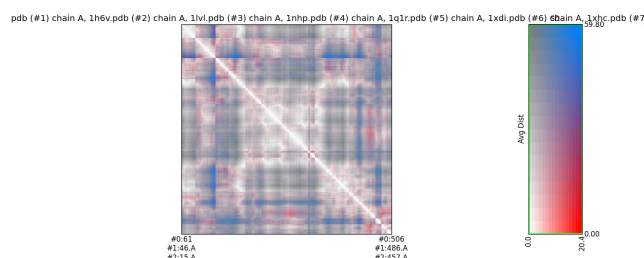


Figure 9. Target-templates superimposition distance matrix with distance values and standard deviations. This is a 2D matrix representation of the target and the template proteins where ranges of deviations are represented in angstrom.

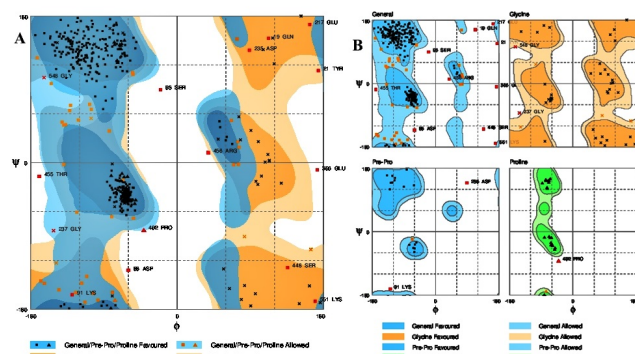


Figure 10. Stereochemical quality check using RAMPAGE.

The final structure was further evaluated for stereo-chemical structural quality through various standard tools such as RAMPAGE [44], PROSA [53] and ANOLEA.

RAMPAGE analysis

Stereochemical quality of model was checked using RAMPAGE server (Figure 10). Ramachandran plot obtained using RAMPAGE revealed that 90.2% of residues were within the most favored regions. Residues falling in additionally allowed regions and outlier residues in outlier region were 7.1 % and 2.7% respectively.

Validation through PROSA

PROSA (Protein Structure Analysis) was used to evaluate the quality of 3D models of protein structures. Z-score is a measure of overall model quality and denotes the deviation of the total energy of the structure compared to energy distribution derived from random conformations. The PROSA

score was -7.71 for the modeled protein, which indicates its correctness.

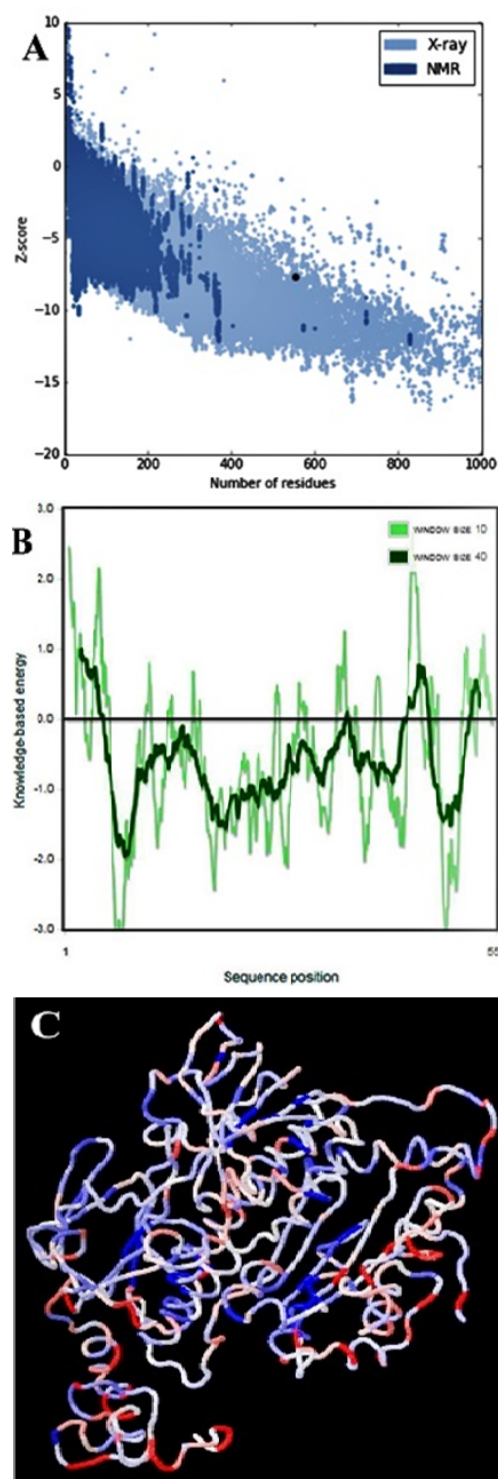


Figure 11. PROSA analysis of the modeled structure for structural evaluation (A) The black dot shows the position of the modeled structure with comparison to the existing X-ray crystallography generated and NMR based structures. (B) Comparative estimation of the energy profile with relation to the knowledge-based energy profiles. (C) Depiction of the structure based on the energy values where "blue" represents lowest energy and "red" represents highest energy in the protein.

PROSA profile for the target protein model was found better than all other template structures (PDB ID: 1mo9, chain "A"-11.77; PDB ID: 2x99, chain "A"-11.87; PDB ID: 1xhc, chain "A"-9.98; PDB ID: 1nhp, chain "A"-12.77; PDB ID: 1xdi, chain "A"-11.07; PDB ID: 1lv1, chain "A"-11.0; PDB ID: 1h6v, chain "A"-10.93 and PDB ID: 1qlr chain "A"-11.43). Z-score computed by PROSA for thioredoxin reductase of *B. microti* was found to be better than the Z-score of all the templates (Figure 11A). Negative values in PROSA plot with comparison to the knowledge based energy values indicated the stable regions of the protein and authentic model development (Figure 11B).

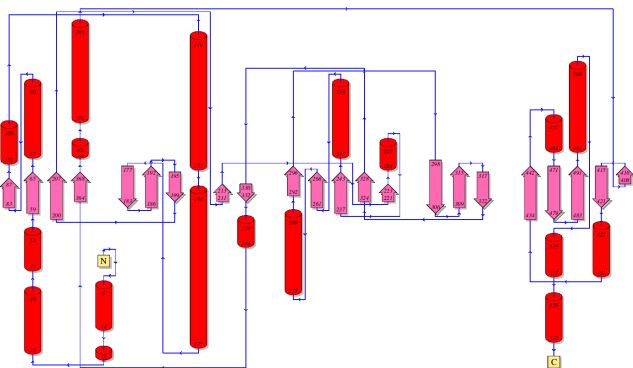


Figure 12. Complex protein topology displaying the direction of multiple helices and parallel and anti-parallel beta sheets generated for the target protein.

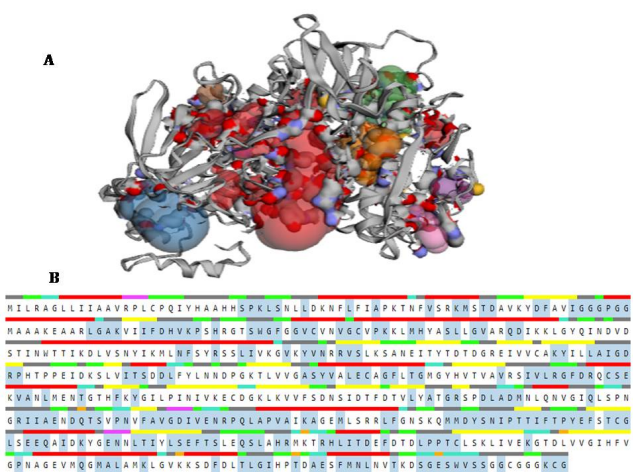


Figure 13. Depiction of top 10 pockets in target protein molecule.

Analysis by ANOLEA

Analysis of the modeled protein structure with ANOLEA (Atomic Non-Local Environment Assessment) [53] also provided expected outcome. The analysis was conducted with a window size of 9 amino acids. The results suggested that out of 553 amino acids, only 114 (20.61%) showed comparatively higher energy. The Figure 11C also provided the similar information with a structural view. Computed non-local normalized energy Z-score of 2.80 was considerable for this large protein. The amino acids with high energy values were residue number 42; 52-56; 99-101; 132-135; 137-139; 151;

212; 228-233; 256; 310-318; 332-337; 351-354; 440-460; 494-498; 500-501; 507-539; 541-549.

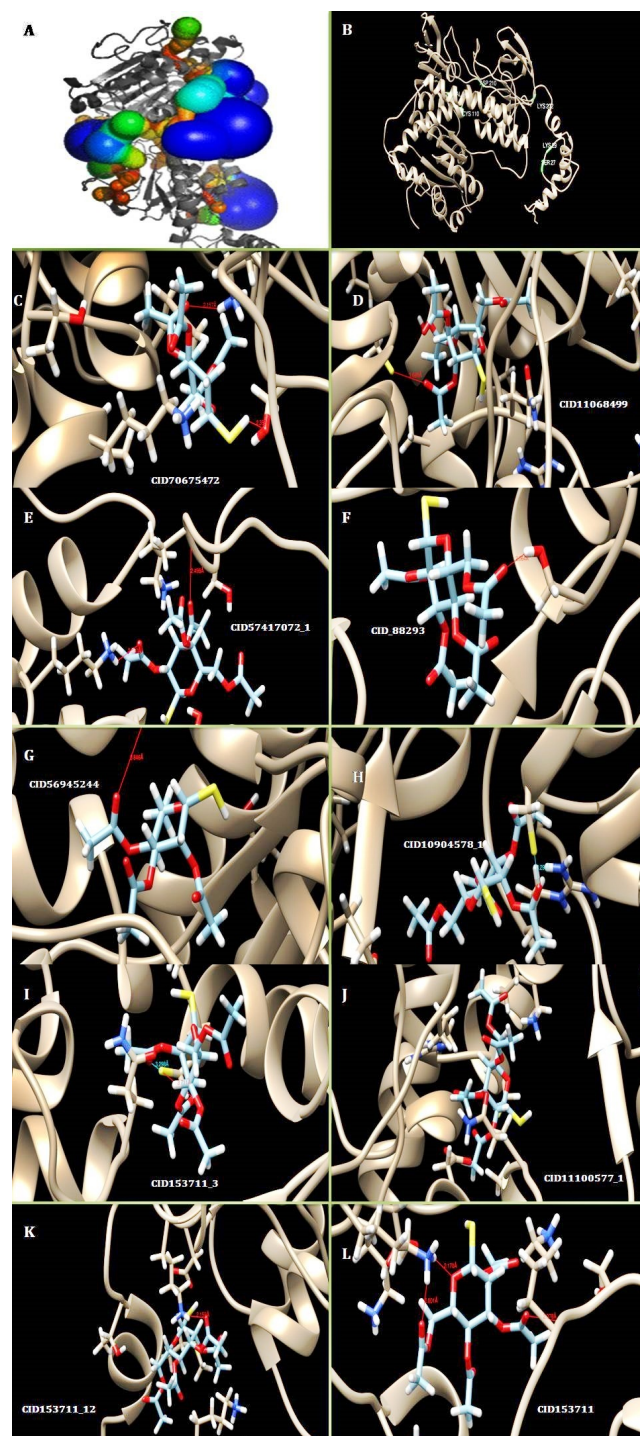


Figure 14. (A): Representation of the identified pores; (B): major residues participating in the interaction with ligand molecules and (C-L): docking modes of the top 10 molecules along with respective hydrogen bond interactions. Information related to detailed interactions is provided in Table 19.

Structural topological analysis

Structural topological analysis is important for this protein considering its complex structural and functional diversity. The

outcome of the PROMOTIF topological analysis is provided in Figure 12.

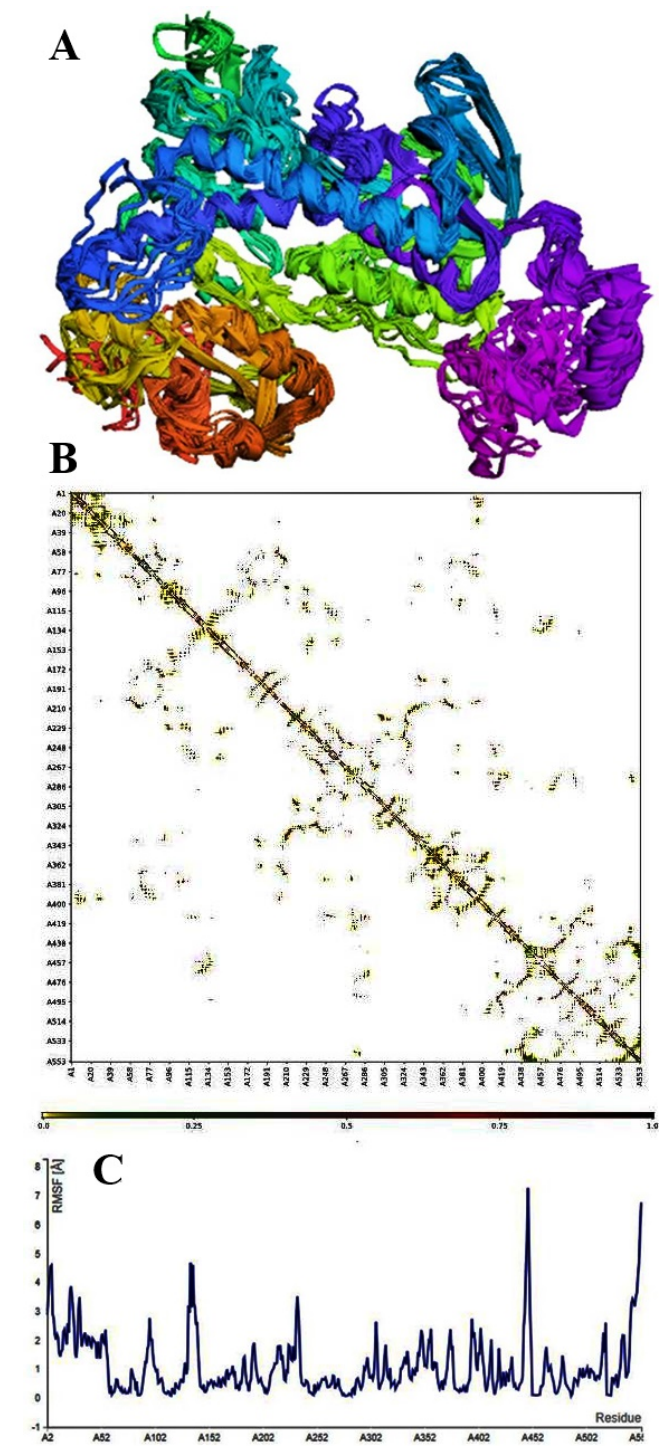


Figure 15. Flexibility analysis of the target protein. (A) Cartoon representation of top 10 models with fluctuations depicted in different color code. (B) Contact map generated for all 10 proteins. (C) Representation of obtained residue wise RMSF values for the structural models depicting the fluctuation for each residue in the protein structure.

Table 11. Distribution of the amino acid residues in the beta-alpha-beta units.

The figure depicts the alpha helices, parallel and anti-parallel beta sheets and loop regions in detail along with the residue numbers. It is evident that the overall structure contains 22 β -sheet regions, 19 helices and several loop regions.

Secondary structural analysis from structure

Detail secondary structural analysis was performed for the final protein structure developed. The post loop optimized best structure was utilized for this purpose and PROMOTIF was considered for the analysis.

As expected, the alpha helix portion was found to be maximum in the structure with 179 amino acids out of 553 being part of it which was 32.4% followed by the strand portion (108 amino acids (19.5%)). 3-10 helix structures were found to be comprising of 5 amino acids (0.9%) and other important structural component were constituted by 261 (47.2%) amino acids. The overall structure contains several structural components such as beta sheets, beta-alpha-beta structural unit, beta-hairpins, beta-bulges, strands, helices, helix-helix interactions, beta turns, gamma turns and disulphide linkage. The detail description is provided in the following section.

Beta sheets: Total 6 beta sheets were found in the *Babesia* thioredoxin reductase structure. The number of strands varied from 2 to 5 as shown in Table 10. No barrel like structures were found for these predicted sheets. Presence of parallel and anti-parallel sheets were observed for this structure as represented with their respective topology in Table 10.

Table 10. Beta sheets present in the modeled structure along with number of strands, type and topology.

Sheet	No. of strands	Type	Barrel	Topology
A	4	Parallel	No	-1X 2X 1X
B	3	Antiparallel	No	1 1
C	2	Antiparallel	No	1
D	5	Parallel	No	-2X -1X -1X 3X
E	3	Antiparallel	No	1 1
F	5	Antiparallel	No	-1 -3 1 1

Beta-alpha-beta units: Beta-alpha-beta units are important structural components of thioredoxin reductase structure. Table 11 represents the strands along with the start, end residues and residues distributed in the helix and loop regions. This topology suggests the presence of alpha helical region sandwiched by beta sheets and most of the time such structural arrangement serves as a conserved topology in a protein structure.

Strand 1			Strand 2			No. of helices	No. of residues	
Start	End	Length	Start	End	Length	No. of residues in loop	No. of residues in helices	
Tyr 59	Ile 64	6	Val 84	Phe 87	4	1	19	14
Lys 238	Val 242	5	His 261	Val 266	6	1	18	14
His 261	Val 266	6	His 292	Tyr 295	4	1	25	15

Beta-hairpins units

The beta-hairpin structural components suggest the presence of the anti-parallel beta sheets separated by a short loop region. Obtained structural regions are listed in Table 12 along with their respective hairpin class. Many hairpin classes were observed as provided in Table 12. Beta hairpin structural elements often indicate the folding pattern of a protein molecule and aid in unraveling the protein folding.

Table 12. Beta-hairpin regions in the modeled structure along with their classes.

Strand 1			Strand 2			Hairpin class
Start	End	Length	Start	End	Length	
Arg178	Lys182	5	Ile187	Asp191	5	2:4
Ile187	Asp191	5	Glu196	Val199	4	3:5 IG
Pro299	Cys306	8	Lys309	Phe314	6	2:2 II
Lys309	Phe314	6	Ser318	Phe322	5	2:4
Thr408	Ile410	3	Phe416	Gly420	5	5:5
Leu435	Phe442	8	Cys471	Glu478	8	28:28
Cys471	Glu478	8	Val484	Val490	7	4:6

Beta-bulges: Beta bulges are important structural regions which are responsible for slight structural deviation in the beta sheets through introducing additional H-bonds in a beta sheet. They are categorized based on the length of disruption in the beta strand. Apart from simple distortion in the native structures, a beta bulge may have far reaching functional impact as well. Often, beta bulged regions may indicate an important structural region which may have been mutated yet maintained structural conservation considering the importance of the structural conservation in that particular region. The obtained beta bulges along with their respective types and impacted residue as res X are presented in Table 13.

Table 13. Beta-bulges in the modeled structure along with classes.

Bulge type	Res X	Res 1	Res 2
Parallel bent		Lys202	Asp60
Antiparallel classic	His213	Thr329	Gly330
Antiparallel G1	Cys306	Gly308	Lys309

Antiparallel classic	Lys309	Glu305	Cys306
Antiparallel wide	Lys311	Asp320	Thr321
Antiparallel classic	Val313	Ile300	Asn301
Antiparallel classic	Ile476	Val485	Gly486
Antiparallel G1	Glu478	Asp482	Leu483

Strands: The combination of helices and strands in thioredoxin reductase structure makes it unique and complex for its important function. The strand regions are presented in Table 14 along with their respective length.

Helices: Apart from strand regions, multiple helix regions also develop the backbone of the large thioredoxin protein. Obtained helices are presented in Table 15 along with their respective length.

Table 14. Components of the strand for the generated protein model.

Start	End	Sheet	No. of residues
Tyr59	Ile64	A	6
Val84	Phe87	A	4
Arg178	Lys182	B	5
Ile187	Asp191	B	5
Glu196	Val199	B	4
Ala201	Leu206	A	6
Arg211	Pro212	C	2
Leu222	Val223	D	2
Lys238	Val242	D	5
His261	Val266	D	6
His292	Tyr295	D	4
Pro299	Cys306	E	8
Lys309	Phe314	E	6
Ser318	Phe322	E	5
Thr324	Tyr327	D	4
Arg331	Ser332	C	2
Val365	Ala367	A	3
Thr408	Ile410	F	3
Phe416	Gly420	F	5

Leu435	Phe442	F	8
Cys471	Glu478	F	8
Val484	Val490	F	7

Table 15. Helix regions found in the modeled structure of thioredoxin reductase of *Babesia microti*.

Start	End	Type	No. of residues
Leu7	Val13	H	7
Arg14	Leu16	G	3
Lys36	Thr45	H	10
Val48	Ser53	H	6
Pro68	Arg79	H	12
Gly103	Val108	H	6
Cys110	Leu132	H	23
Trp145	Lys170	H	26
Thr225	Asp228	H	4
Tyr246	Gly257	H	12
Arg276	Asn288	H	13
Leu340	Val343	H	4
Gly369	Ile371	G	3
Ala379	Phe394	H	16
Glu423	Tyr430	H	8
Glu432	Asn434	G	3
Leu458	Glu462	H	5
Ala494	Leu507	H	14
Lys511	Leu516	H	6
Ser527	Val533	H	7

Helix-helix interactions: Helix-helix interaction is crucial in understanding the folding pattern of a protein structure especially in membrane proteins. Detail information about the obtained helix-helix interaction is provided in Table 16.

Table 16. Depiction of helix-helix interaction of the modeled protein.

Helices	Helix types	Interaction type	No. interacting residues	
			Helix 1	Helix 2
3	4	H H C N	2	1
5	6	H H n n	1	2
5	8	H H C C	4	3
5	14	H H I I	7	8
6	7	H H c n	4	3
6	8	H H C I	2	4

6	9	H H c c	1	2
6	14	H H N I	1	1
7	8	H H I I	4	5
7	9	H H n c	1	1
7	10	H H I I	4	4
7	14	H H n n	1	1
7	17	H H C C	2	1
8	9	H H I C	2	1
8	10	H H n c	1	1
9	10	H H N I	2	3
10	11	H H C C	3	2
11	15	H H n n	2	2
18	19	H H I N	1	1
18	20	H H N N	2	1
19	20	H H I I	3	5

Beta turns: Information on beta turns provides important hint about the reverse turn in a large protein molecule. Table 17 represents the observed β -turns found in the modeled protein. Abundance of proline and glycine is common in β -turns. The obtained result suggests that turn types VIII and IV are dominant for this protein. In some of cases, H-bonds were also found. The type IV turn suggests they are not perfectly defined turns whereas type VIII suggest that these turns are having -60 degree Phi (i+1), -30 degree Psi (i+1), and -120 degree Phi and Psi angle for i+2 residues for developing the dihedral angle.

Table 17. Beta turns observed in the modeled protein.

Turn	Sequence	Turn type	H-bond
Met1-Arg4	MILR	IV	
Arg4-Leu7	RAGL	IV	Yes
Val57-Asp60	VKYD	VIII	
Lys91-His94	KPSH	IV	
His94-Thr97	HRGT	IV	
Arg95-Ser98	RGTS	IV	
Lys182-Asn185	KSAN	IV	
Ser183-Glu186	SANE	IV	
Ala184-Ile187	ANEI	IV	
Asp191-Gly194	DTDG	I	
Asp219-Leu222	DKSL	IV	
Asp228-Tyr231	DLFY	IV	
Leu229-Leu232	LFYL	IV	
Phe230-Asn233	FYLN	IV	
Leu232-Asp235	LNND	IV	

Pro236-Thr239	PGKT	IV	
Leu271-Phe274	LRGF	IV	
Lys294-Ile297	KYGI	IV	
Lys304-Asp307	KECD	IV	
Cys306-Lys309	CDGK	II	Yes
Phe314-Asn317	FSDN	IV	
Phe322-Val325	FDTV	VIII	
Leu335-Met338	LADM	IV	Yes
Leu347-Asn350	LSPN	I	Yes
Thr360-Pro363	TSVP	VIII	
Val362-Val365	VPNV	I	Yes
Val372-Arg375	VENR	II	Yes
Met401-Ser404	MDYS	IV	
Thr412-Glu415	TPYE	VIII	
Ser444-Gln447	SLEQ	IV	
Arg452-Thr455	RMKT	IV	
Met453-Arg456	MKTR	IV	Yes
Thr455-Leu458	TRHL	IV	
Asp464-Leu467	DTDL	VIII	
Glu478-Thr481	EKGT	IV	
Lys479-Asp482	KGTD	II'	
Gly491-Ala494	GPNA	IV	Yes
Ile520-Thr523	IHPT	VIb	
Thr534-Ser537	TKDS	I	
Asp536-Glu539	DSGE	IV	Yes
Glu539-Val542	ESWV	IV	
Ser540-Ser543	SWVS	I	
Gly545-Gly548	GGCG	IV	
Gly548-Lys551	GGGK	IV	

Gamma turns: Followed by beta turns, gamma turns are the predominant tight turns present in the protein structures. Table 18 represents the observed gamma turns in the modeled structure. Abundance of inverse gamma turns is common as found in this case also. Rare classic gamma turns are also found in this structure (Table 18). The gamma turns may have impact on structural folding of the protein and may help in developing the required quaternary structural configuration.

Table 18. Observed gamma turns in the structure.

Start	End	Sequence	Turn type
Met338	Leu340	MNL	INVERSE
Pro376	Leu378	PQL	INVERSE

Ile410	Thr412	IFT	INVERSE
Met453	Thr455	MKT	INVERSE
Lys479	Thr481	KGT	CLASSIC
Val533	Lys535	VTK	INVERSE

Disulphide linkage: Existence of disulphide linkage is important for proteins involved in redox associated reaction. The analysis suggested that the modeled structure is having disulphide linkage between the cysteine 105 and cysteine 110 residue number.

Active site identification

Castp server was used to predict possible binding sites of modeled structure. Out of all the sites predicted, best 10 sites were selected as shown in Figure 13. Binding sites having highest surface area and volume were selected as the most probable active sites for the modeled protein (Table 19).

Table 19. The top 10 pockets with area and volume information.

Pocket ID	Area (SA)	Volume (SA)
1	1437.150	1334.336
2	517.250	693.072
3	266.266	132.424
4	137.962	81.917
5	201.313	77.787
6	127.716	77.102
7	135.291	48.060
8	88.826	42.928
9	65.263	33.284
10	82.267	29.309
11	88.254	25.771

Molecular docking analysis

As mentioned in the method section, molecular docking analysis was carried out employing SwissDock docking facility. All the molecules were minimized employing CHARMM force field. The PSF, PAR files and the docking clusters were generated using the protocol provided. The parameters used are passive flexibility distance=0.0, wanted confs=5000, nbfact seval=5000, nbseeds=250, sdsteps=100, abnr steps=250, clustering radius=2.0 Å and maximum cluster size=8.

The target protein and the selected ligands were prepared through molecular mechanical calculations before docking exercises as per the tool protocol. The Supplementary Table 1 contains the detailed docking results of all the molecules considered. The docking analysis revealed the interactions between the ligand molecules and the target protein

considered. The ligands are mostly auranofin derivatives as mentioned in the method section. The obtained ten best molecules along with their specific interactions are represented in Table 20. The energy values of the ligand-protein binding affinity were found between -16.51 and -20.88 kcal/mol. Some of the residues in the target proteins showed extensive affinity towards forming hydrogen bonds with the ligand molecules which are presented in “H-H bond receptor atom” column of Table 20. Interestingly, LYS 29, SER 245, CYS 110, LYS 210, SER 27 and LYS 202 showed more affinity towards the ligand molecules than other residues in the target protein. The poses of the docking are presented in Figure 14.

Target protein flexibility analysis

Molecular motion analysis provided information related to the most flexible as well as the rigid part of the target protein considered for this study. Estimated output of the flexibility of

the modeled targeted protein computed through CABSflex server is provided in Figure 15. The outcome shows the deviation of the structural components of native protein as depicted in the Figure 15A. Fluctuation pattern is depicted through different color codes where maximum fluctuations are depicted towards the “C” and “N” terminal of the protein and medium range of RMSF was observed for the rest of the structural part. The contact map of ten different modes of the protein shows residue-residue fluctuations for all the amino acid residues. The RMSF fluctuation plot (Figure 15B) of all the modes shows that RMSF (root mean square fluctuation) value ranged approximately between 0 Å and 8 Å. The analysis suggests that the movement of the protein is higher around amino acid residue number 100, 245, 450 (Figure 15C) (region represented in green and dark brown in Figure 15A). The fluctuations obtained around the C-terminal and the N-terminal of the protein may be ignored considering them as free ends.

Table 20. Docking result of the top 10 molecules with specific interactions and hydrogen bonds. The “Compound ID” column contains the conformations after the ID represented with (“_”) followed by the number.

Compound ID	Cluster	Cluster rank	Energy (kcal/mol)	H-bond number	First bond receptor atom	H-H bond atom	First bond ligand atom	H-H bond length (Å)	Second bond receptor atom	H-bond atom	Second bond ligand atom	H-bond length (Å)	Third bond receptor atom	H-H bond atom	Third bond ligand atom	H-H bond length (Å)		
CID7067547_2	7	1	-20.88	2	LYS 29	HZ3	1.5	LIG 1	O5	2.157	SER OG	27	1.52	LIG 1	H9	2.353		
CID1106849_9	0	2	-20.02	1	CYS 110	SG	1.3	LIG 1	O8	3.689								
CID5741707_2_1	2	0	-18.61	2	LYS 29		1.15	LIG 1	O7	2.498	LYS HZ1	202	1.15	LIG 1	O8	2.36		
CID88293	0	0	-18.39	1	SER HG1	245	1.1	LIG 1	O9	2.064								
CID5694524_4	1	5	-18.37	1	CYS 110	SG	1.14	LIG 1	O6	3.846								
CID1090457_8_1	4	0	-17.54	1	CYS 110	SG	1.30	LIG 1	O8	3.294								
CID153711_3	2	5	-17.44	2	CYS 110	SG	1.22	LIG 1	O8	3.298	CYS SG	110	1.22	LIG 1	O7	3.711		
CID1110057_7_1	1	0	-17.40															
CID153711_12	8	0	-17.00	1	LYS 202	HZ3	1.50	LIG 1	O7	2.159								
CID153711	14	0	-16.51	3	LYS 29		1.92	LIG 1	O7	2.378	LYS HZ1	202	1.92	LIG 1	O9	2.001		
													LYS HZ3	202	1.92	LIG 1	O1	2.17

Conclusion

Lack of vaccine for combating human Babesiosis also implores us to search for novel drug targets and development of new, safe and effective chemotherapeutic agents. The infection is caused by the intra-erythrocytic parasitic genus *Babesia*. The transmission cycle of this parasite is maintained in both vertebrate and non-vertebrate hosts and the parasite infects a wide range of vertebrates. Across various geographical regions, human Babesiosis is caused by different parasitic species such as *B. microti*, *WAI piroplasm*, *B. divergens* etc.

Thus, it is cumbersome to develop a single potent and effective vaccine for effective prevention or control of human babesiosis. Several research efforts have been made to develop and crosscheck the possibility of a recombinant vaccine against different species of *Babesia*, such as *Babesia bovis*, *B. microti* etc. None of these efforts have been successful in providing complete solution in this regard. The complex process of host cell invasion of this parasite should be further explored to target several molecular events and multiple stages for vaccine design and development.

An effective inhibitor which may act against almost all the species would be a great support to the existing treatment as we cannot ignore the lethal consequences of the infection in several cases. Keeping in view the possibility of relapse and the emergence of atovaquone resistant strains after treatment, there is a dire need for exploring new drug targets and drugs.

In the current study, we developed a reasonably good high quality three dimensional structure of thioredoxin reductase of *B. microti* using homology modeling approach. The good quality of refined model was obtained using the state-of-the-art computational methodology and the outcome of the analysis was confirmed by using standard structure validation tools. As no experimentally determined structure of the considered enzyme is available, we believe that this model will aid in initiating drug discovery exercises and studies based on structural and functional insights obtained in this study. The docking analysis with derivatives of inhibitor auranofin which has already shown huge potential against an array of gram positive bacteria may pave a way towards procuring a series of excellent and effective medication against the pathogen.

Acknowledgement

Neelima Arora thanks University grants Commission for Postdoctoral fellowship.

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