Interface analysis of DnaK-GrpE-DnaJ chaperon system of *Mycobacterium tuberculosis*

Surface analysis by using Bioinformatics

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Abstract: **The biological functions of proteins are governed by their proper folding and assembly. Protein chaperones are essential in all domains of life to prevent and resolve protein misfolding during translation and proteotoxic stress. *Mycobacterium tuberculosis* capsular heat shock protein DanK is a molecular chaperone. This chaperone interacts with protein substrates in an ATP-dependent manner to prevent aggregation and promote protein folding. This activity is stimulated by DnaJ and GrpE. ATP-dependent Mycobacterium DnaK (Hsp70) chaperone's function is controlled in part by the nucleotide exchange factor (NEF) co-chaperone GrpE. Recently crystal structure of *Mycobacterium tuberculosis* DnaK has been deciphered but there is no three-dimensional structure of the DnaK-DnaJ and DnaK-GrpE complex reported to date. In this piece of work, we modeled the DnaJ and GrpE protein and docked with the crystal structures with DnaK.**

Index Terms: **DnaK, GrpE, DnaJ, GrpE, Chaperone, Hsp70**

I. INTRODUCTION

Chaperones have an essential role in the regulation of protein conformation. The functions of molecular chaperones in cells include the facilitation of the folding of proteins and assistance in the correct assembly or disassembly of oligomeric protein complexes. One major cellular system to prevent aggregation and premature folding of proteins in bacteria is constituted by molecular chaperones of the conserved Hsp70 family protein DnaK (Wang *et al*., 1993). The best-studied Hsp70 chaperone of bacteria is *E. coli*. ATP-dependent *Mycobacterium tuberculosis* DnaK (PDB ID4RTF/Rv0350) (Hsp70) chaperones whose function is controlled in part by the nucleotide exchange factor co-chaperone GrpE (Rv0351) and DnaJ (Rv0352) (Hsp40 family) (Liberek *et al*., 1991; McCarty *et al.*, 1995). Specifically, ATP binding to DnaK facilitates substrate release, and substrate binding promotes ATP hydrolysis. In the cell, substrates enter the DnaK/DnaJ/GrpE chaperone cycle mainly by binding DnaJ or ATP-DnaK. Stimulation of ATP hydrolysis by the substrate and DnaJ locks the substrate in the DnaK peptide-binding pocket. DnaK ATPase activity was stimulated by substrates (nine-fold) and DnaJ (13-fold) (McCarty *et al*., 1995). Stimulation of the γ-phosphate cleavage reaction by DnaJ is much more efficient (complete conversion of bound ATP to ADP within five seconds) than that by substrates (McCarty *et al*., 1995). Subsequently, the nucleotide exchange factor GrpE facilitates the release of the bound ADP. The binding ATP to the empty nucleotide-binding pocket of DnaK releases the bound substrate, resetting the chaperone cycle. DnaK contains the N-terminal nucleotide-binding domain (NBD) and C-terminal substrate-binding domains (SBD) (Qi *et al*., 2013). The C-terminal substrate-binding domain (SBD) consists of a substrate-binding pocket covered with a helical subdomain that is responsible for client protein recognition (Zhu *et al*., 1996; Chou *et al*., 2003).The initiation of a tuberculosis infection involves the adherence and phagocytosis of *Mycobacterium tuberculosis* bacilli by host cells. It is generally thought that the primary host niche of *M. tuberculosis* is the alveolar macrophage. After a sudden temperature rise, organisms from *Escherichia coli* to *Homo sapiens*, induce the synthesis of a group of so-called heat shock proteins), some of which are highly conserved among species (Craig, 1985). Intracellular ATP levels appear to fluctuate during the Heat shock response, and this fluctuation may be necessary for the induction of thermo tolerance (Bloom *et al*., 1986). In addition to its effectors' function in the heat shock response, DnaK also regulates this response by destabilizing the alternative sigma factor, σ32, preventing aberrant induction of the heat shock response during non-stress conditions and turning off the response after heat shock (Straus *et al*., 1990). It is reported that the high-level expression of chaperone proteins under stress conditions is necessary for preventing irreversible loss of protein function(Lindquist, 1988). *Escherichia coli* DnaK (PDB ID 4JNF) and GrpE (PDB ID 1DKG). Heat shock proteins interact both in vivo and in vitro processes. Heat shock proteins (Hsp) appear to be constituents of the cellular machinery of protein folding, degradation, and repair (Skowyra *et al*., 1990; Martin *et al*., 1991; reviewed in Morimoto *et al*., 1990; Rothman, 1989). Client proteins bind to the apical domains of chaperone protein (Fenton *et al*., 1994), predominantly via hydrophobic interactions. *Escherichia coli* DnaJ and GrpE (PDB ID 1DKG) heat shock proteins jointly stimulate ATPase activity of DnaK (Liberek *et al*, 1991). There is a common mechanism of substrate binding and release for the function of DnaK, DnaJ, and GrpE in protein folding with the help of ATP hydrolysis (Szabo *et al*., 1994).DnaJ activates the intrinsic ATPase activity of DnaK but in the presence of a saturating concentration of client peptide, DnaJ does not influence on ATPase activity of DnaK (Jordan *et al*., 1995). Substrate protein and DnaJ stimulate γ-phosphate cleavage by binding to a preformed complex of DnaK with ATP (McCarty *et al*., 1995). GrpE increases the nucleotide (ADP) exchange rates drastically for the DnaK chaperone system (Packschies *et al*., 1997). Thus, in performing these ATPase-related functions, DnaJ and GrpE are also deeply involved in regulating substrate binding by DnaK. To this end, their respective activities are finely balanced to achieve optimal chaperone activity. The DnaK substrate -binding motif of *E. coli* comprises about five hydrophobic residues flanked on either side by positively charged side chains. Substrate specificity of the DnaK chaperone is determined by screening cellulose-bound peptide libraries chaperone proteins in general recognize exposed hydrophobic surfaces of stress denatured protein (Bukau *et al*., 1998). However, the average total protein concentration in E. coli is 5–8 mM, that is, much larger than the in vivo DnaK

concentration (30 μM) (Mogk *et al*., 1999). Thus, it is clear that it is not possible for every protein in the cell to be associated with one DnaK molecule at all times DnaJ action required the binding of protein substrates to the central hydrophobic pocket of the substrate-binding cavity of DnaK (Laufen *et al*., 1999). In *T. thermophilus*, although the thermophilic DnaK system displays no stimulation of the DnaK-ATPase activity by DnaJ, nucleotide exchange is still efficiently stimulated by GrpE (Groemping *et al.*, 2001). A ternary complex of GrpE, DnaK, and a peptide substrate could be observed only when the peptide binding to DnaK precedes GrpE binding (Brehmer *et al*., 2004). The N-terminal half of GrpE coils (residues 34-68) interacts with DnaK interdomain linker, regulates the nucleotide exchange activity of the co-chaperone, and is required to stabilize DnaK-substrate complexes in the ADP-bound conformation (Moro *et al*., 2007). Some authors suggest a thermal sensor role, to regulate DnaK folding activity in heat-shock conditions (18,19), while others indicate a role in modulating dynamics of the chaperone substrate-binding domain through interaction with DnaK (SBD) (Moro *et al*., 200).

Crystal Structure of DnaK Protein complexed with Nucleotide Exchange Factor GrpE in Chaperone System of *Geobacillus kaustophilus* is already determined (Papers & Doi, 2008). In *E. coli* ATP binding allosterically opens the peptide-binding site of DnaK(PDB ID:4JNF) (Qi *et al*., 2013). The DnaK's substrate selection mechanism depends upon polypeptides' kinetic and thermodynamic stabilities (Sekhar et al., 2012). The long N-terminal GrpE (PDB ID 4ANI) of *Geobacillus kaustophilus* α-helices stabilize the linker between SBD and NBD of *G. kaustophilus*'s DnaK (PDB ID: 2V7Y) in the complex. Furthermore, interactions between the DnaK SBD and the N-terminal disordered region of GrpE may accelerate substrate release from the DnaK of *G. kaustophilus* (Intoi *et al*., 2012). In *Mycobacterium tuberculosis*, DnaK (PDB ID 4RTF/Rv0350) regulates the heat shock response through its interaction with the HspR C-terminal tail, which becomes insoluble upon heat shock, thereby relieving the repression of chaperone genes (Fay & Glickman, 2014). Mycobacterium surface protein DnaK is essential for cell growth. Host Host-inflictedeotoxic stress is likely a significant in vivo stress for *M. tuberculosis* during infection, yet the function of the mycobacterium chaperone network in native and stress induced proteo-stasis is incompletely understood. Mycobacterium cells restart cell growth after proteotoxic stress by isolating persistent DnaK (PDB ID4RTF/Rv0350) containing protein aggregates away from daughter cells. The host cell binding activity of *M. tuberculosis* is enhanced by the DnaK chaperone protein (Hickey *et al*., 2009).

Furthermore, the proteins that interact with DnaK indicate that most client proteins that require DnaK for proper folding and/or stability are largely non-essential, suggesting that loss of function of these proteins in the absence of DnaK does not impact viability. However, in the absence of both DnaK and transcription factors, the E. coli cell suffers proteo stasis collapse characterized by global insolubility of nascent proteins (Calloni *et al*., 2012). The kinetic competition between protein folding and chaperone binding has not been carefully analyzed, especially in the context of proteins that can fold independently even in the absence of molecular chaperones (Sekhar *et al*., 2012). In unstressed cells, DnaK of *E. coli* localizes to multiple, dynamic foci, but relocalizes to focal protein aggregates during stationary phase or upon expression of aggregating peptides (Fay & Glickman, 2014).

The three-dimensional structures of Mycobacterium heat shock co-chaperone protein DnaJ and GrpE are unavailable. During heat stress, these two proteins form a transient complex with DnaK and hence their interaction is very important for substrate protein proper folding. The active site residues are also essential for ideal drug targeting to avoid the pathogenesis of *Mycobacterium tuberculosis*. Heat shock protein GrpE in *Mycobacterium tuberculosis* Participates actively in the response to hyper osmotic and heat shock contains N-terminal disordered region (alpha-helix c-terminal β sheet).

In *M. tuberculosis* DnaK is the dominant chaperone responsible for folding of native peptides in the absence of exogenous stress such as heat shock and DnaK is required for solubility of large multimodular lipid synthases (Fay *et al.*, 2014). The function of DnaK in mycobacterium, a genus that includes multiple human pathogens, and find that DnaK is required for cell growth (Fay *et al.*, 2014). ATP bound state (T state) of DnaK lowers substrate affinity than ADP bound state(R state) of DnaK. So ATP binding to DnaK (NBD) induces substrate release from the DnaK (SBD) (Palleros *et al*, 1993) Schmid *et al.*, 1994). Substrate binding in the DnaK (SBD) stimulates ATP hydrolysis in DnaK (NBD). GrpE accelerates exchange of ADP for ATP in DnaK 5000 fold (Theyssen *et al.*, 1996). The GrpE N-terminal disordered region may accelerate the release of substrate bound to DnaK (Brehmer *et al*., 2004; Moro *et al*., 2007). The bacterial Hsp70 chaperone system possesses an intrinsic substrate selection mechanism that relies on the folding kinetics of the client protein (Sekhar *et al*., 2012). Without this, extracellular Mycobacterium DnaK Polarizes Macrophages to the M2-Like Phenotype (Devaraj *et al*., 2011).

The complex structure of DnaK and GrpE in *M.tuberculosis* is not found. To understand how DnaK, DnaJ and GrpE interact in *M. tuberculosis* during the chaperon cycle, here we present a detailed structure of three chaperons and their biological communication. Toward this goal, the interface residues which are essential for these biological communications between these three proteins are determined here. By utilizing bioinformatics analysis we have built the model of GrpE and DnaJ; docked GrpE, DnaJ with DnaK to know the functional significance of the chaperone system in *Mycobacterium tuberculosis.*

II. MATERIALS AND METHODS

STRING Database: STRING (*Search* Tool for the Retrieval of Interacting Genes/Proteins) is a database of predicted functional associations between proteins (Mering *et al.*, 2003). This new version 9.1 of STRING is more efficient interolog prediction. The interactions include direct (physical) and indirect (functional) associations of proteins. The database was used here to know the interacting partner of DnaK during folding action. The DnaK (Rv0350) protein sequence of *M. tuberculosis* was given as input. Interacting partners of the protein were given as output with confidence scores. Identified partner with the highest confidence score was considered for further analysis.

***BLAST*: B**asic **L**ocal **A**lignment **S**earch **T**ool (BLAST) is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA. The tool is widely used for searching protein and DNA databases for sequence similarities in homologues. Since we are dealing with protein, BlastP was used for our study. BlastP was searching against Protein Data Bank (PDB) amino acid sequences of DnaK (Rv0350), DnaJ (Rv0352) and GrpE (Rv0351)

from *Mycobacterium tuberculosis* in FASTA format as input. Structural homologous of the three proteins were given as output with E-value, percentage identity and query coverage.

***SWISS-MODEL*:** It is a web-server dedicated homology modeling of protein 3D structures (Guex *et al*., 2009). Homology modeling is currently the most accurate method to generate reliable three-dimensional protein structure models and is routinely used in many practical applications. Homology (or comparative) modeling methods make use of experimental protein structures ("templates") to build models for evolutionary related proteins ("targets"). As the three dimensional structure of GrpE and DnaJ of *M. tuberculosis* is not deciphered, their sequences were given as input while the templates were chosen from the predicted structural homologue with significant E-value of BlastP.

PROCHECK: It analyses and provides an idea of the stereo chemical quality of all protein chains in a given PDB structure. They highlight regions of the proteins which appear to have unusual geometry and provide an overall assessment of the structure as a whole. PDBsum uses version 3.6.2 of PROCHECK. We get Ramachandranran plot of the modeled protein from PROCHECK. We validated the model of GrpE (Rv0351) and DnaJ (Rv0352) and judged the stereochemical quality of the modeled side chains of the proteins.

PatchDock: This algorithm is inspired by object recognition and image segmentation techniques used in Computer Vision (Schneidman-Duhovny *et al*., 2005). Protein–protein docking was used to understand the possible mode of interaction of DnaK with GrpE. Here GrpE (RV0351) and DnaJ (RV0352) were chosen as ligand and DnaK was chosen as the receptor. Protein–protein docking was performed using receptor and ligand combination in the PATCHDOCK server.

FireDock*:* It is an efficient method for refinement and re-scoring of rigid-body protein-protein docking solutions. The FireDock server addresses the refinement problem of protein-protein docking solutions. The method simultaneously targets the problem of flexibility and scoring of solutions produced by fast rigid-body docking algorithms. Given a set of up to 1000 potential docking candidates, FireDock refined and scored the protein complex DnaK and GrpE according to an energy function, spending about 3.5 seconds per candidate solution. At first, we uploaded specific PDB files of two docked proteins DnaK- GrpE and provided a list of 1000 transformations file for refinement.

HOTREGION: HOTREGION is a database of predicted hotspot cluster of interacting proteins. Protein-protein interactions are critically dependent on just a few 'hot spot' residues present at the interfaces of the protein (Cukuroglu *et al.*, 2012). HotRegion stores all available protein–protein interfaces which are extracted from Protein Data Bank (PDB) (Berman et al., 2000). Here, we used the database 'HotRegion' in order to illustrate hot spot co-operativity information at protein–protein interfaces**.** Mtb Dnak – Mtb GrpE; MtbDnak– MtbDnaJ, during client protein binding and client protein releasing after folding process in *M. tuberculosis***.**
***Clustal W*:** ClastalW is a multiple sequence alignment program. It is used to find conserved residues in a protein. Multiple sequence alignments (MSA) of DnaK and GrpE proteins with numerous genera were generated using ClustalW2. It was used here to align the amino acid sequence of DnaK and GrpE from different species such as *Geobacillus kaustrophilus, Escherichia coli, Bos taurus, Rattus norvegicus,* including *Mycobacterium tuberculosis* to identify the conserved functional residues of the proteins.

***EsPript*:** We edited the alignment file obtained from ClustalW using this EsPript database. The main input was an alignment file (.aln) of the pre aligned sequence which we got from ClustalW. EsPript was then used to calculate a similarity score for each residue of aligned sequence. Optional files allow further output showed the aligned sequences, similarities and consensus.
***PDBePISA*:** PDBePISA (Protein interface surface assemblies) is a Web-based interactive tool, used to investigate stability of formation of macromolecular complex (protein,DNA/RNA and ligand). PDBePISA was used for interface analysis of tetrameric proteins assembly in complex. This tool was used in order to identify the, amino acids involved in ATP binding of chaperon protein Mtb's DnaK nucleotide binding domain.
***PIC*:** Protein Interactions Calculator (PIC) is a server which recognizes various kinds of interactions; such as disulphide bonds, hydrophobic interactions, ionic interactions, hydrogen bonds, aromatic- aromatic interactions, aromatic-sulphur interactions and cation - π interactions within a protein or between proteins in a complex (Tina *et al*., 2007). The input was in (.pdb) format. In this study PIC was used to identify various interactions of the identified hotspots and other residues of Mtb DnaK-GrpE and DnaK-DnaJ complex. Protein-protein main chain main chain, side chain side chain bond distances were majored.

***DSSP*:** The DSSP program was designed by Wolfgang Kabsch and Chris Sander to standardize secondary structure assignment. DSSP is a database of secondary structure assignments (and much more) for all protein entries in the Protein Data Bank (PDB). In 2011 Maarten Hekkelman has written new software that produces the same output as the original DSSP, but that deals better with the many exceptions life and the PDB throw at us. The docking complexes of DnaK-DnaJ and DnaK-GrpE in PDB format were taken as input. The output from the new version (2.1.0) was H = α-helix B = residue in isolated β-bridge E = extended strand, participates in β ladder G = 3-helix (3_{10} helix) I = 5 helix (π-helix) T = hydrogen bonded turn S = bend for the interacting hotspots of DnaK-DnaJ and DnaK-GrpE complex.

III. RESULTS

Functional Networking Partners of *Mtb* DnaK

Proper chaperon function of the heat shock protein DnaK (PDB ID 4RTF/Rv0350) in Mtb requires interaction of numerous heatshock co-chaperon proteins. The interacting partners of Mtb DnaK have been predicted by STRING data base (Mering *et al*., 2003). The interacting network of functional proteins with DnaK (PDB ID 4RTF/Rv0350) has been represented in Fig.1 two interacting partners identified were GrpE and DnaJ with the highest confidence scores were considered for further study.

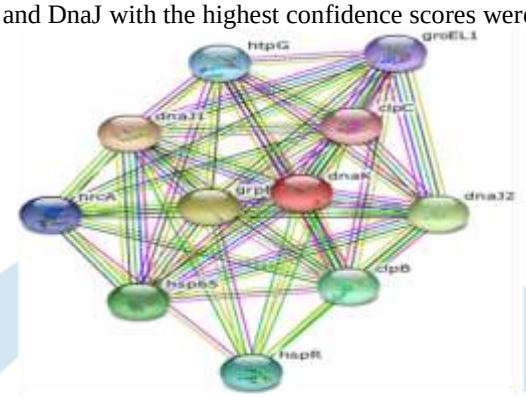

Figure 1: Two interacting partners identified were GrpE and DnaJ with the highest confidence scores were considered for further study

Input: molecular chaperon DnaK;(625amino acids)		**Output**
dnaJ1	molecular chaperone DnaJ; Participates actively in the response to hyperosmotic and heat shock [...] (395 aa)	0.999
grpE	heat shock protein GrpE; Participates actively in the response to hyperosmotic and heat shock b [...] (235 aa)	0.999
dnaJ2	molecular chaperone DnaJ; Participates actively in the response to hyperosmotic and heat shock [...] (382 aa)	0.999
hsp65	molecular chaperone GroEL; Prevents misfolding and promotes the refolding and proper assembly o [...] (540 aa)	0.996
clpB	endopeptidase ATP binding protein; Part of a stress-induced multi-chaperone system, it is invol [...] (848 aa)	0.978
hspR	HEAT shock protein transcriptional repressor HspR (126 aa)	0.974
htpG	heat shock protein 90; Molecular chaperone. Has ATPase activity (By similarity) (647 aa)	0.972
hrcA	heat-inducible transcription repressor; Negative regulator of class I heat shock genes (grpE- d [...] (343 aa)	0.971
groEL1	molecular chaperone GroEL; Prevents misfolding and promotes the refolding and proper assembly o [...] (539 aa)	0.972
clpC	ATP-dependent protease ATP-binding subunit ClpC1 (848 aa)	0.965

Blast Results

The amino acid sequences of DnaK (PDB ID 4RTF/Rv0350), DnaJ (Rv0352) and GrpE (Rv0351) (heat shock proteins) from Mtb H37Rv were obtained from Tubercu List data base and for further study this sequences were used. This result in Fig.2 showed that the number of amino acids of *M. tuberculosis* DnaK (PDB ID4RTF/Rv0350),GrpE(Rv0351)and DnaJ (Rv0352) were 625, 235 and 395, molecular weight were 66.830kDa,24.500kDa and 413.45kDa the theoretical Isoelectric point were 4.5884, 4.1657 and 8.2096 respectively. The closest homology sequences for *Mtb*DnaK, *Mtb*GrpE and *Mtb*DnaJ were obtained by protein BLAST (BLAST P), which showed that DnaK and GrpE from *G. kaustrophilus* have highest homology of 82% and 36% respectively with the target proteins and DnaJ of *Klebsicella pneumonia* has highest homology 96% with Mtb DnaJ.

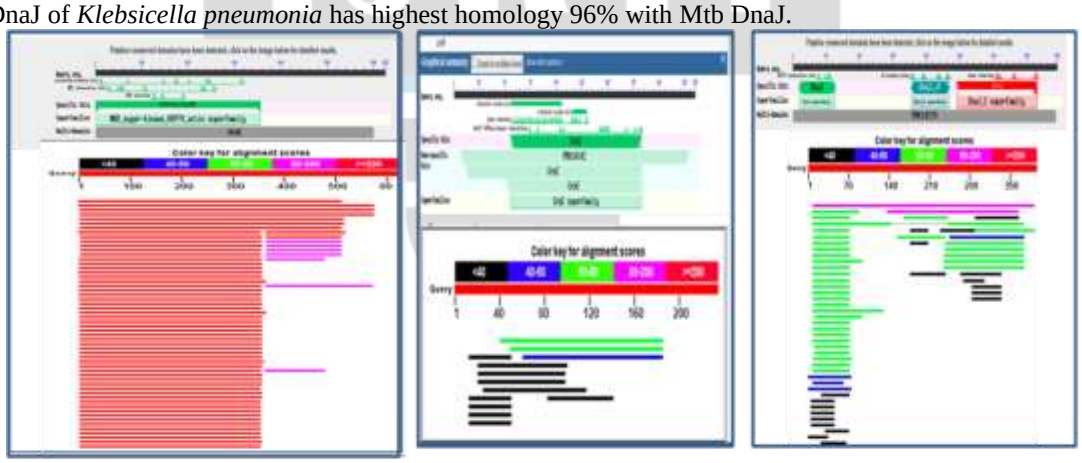

Figure 2: BLAST results of DnaK (left), GrpE (middle) and DnaJ (right).

Homology Modeling of Mtb GrpE and Mtb DnaJ and validation of the models

Structure analysis of Mtb GrpE:

The amino acid sequence of *Geobasillus Kaustrophilus* chaperone GrpE (PDB ID 4ANI) protein was used as a template for modeling the three dimensional structure of *M. tuberculosis* GrpE (target). It (Gk GrpE) had highest homology of 36% with the

target. We successfully modeled the structure of *Mtb* GrpE by using swissmol server. The overall structure of Mtb GrpE composed with long N-terminal α-helix (disordered region).The length of the template sequence was 213 amino acid residues and target sequence was 235 amino acid residues. The modeled structure was visualized using PyMOL (Fig.3A) and validated through PROCHECK (DeLano, 2002).Using PROCHECK, we found that 82.5% of residues were under most favorable region and 0.0% residue was present under disallowed region (Fig.3B).

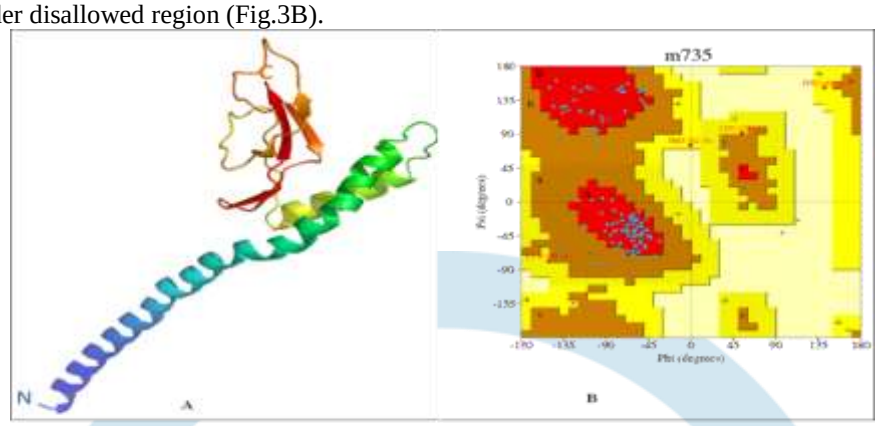

Figure 3: (A) 3D model structure of MtbGrpE using PyMol. Cartoon representation of Mycobacterium tuberculosis GrpE 3D modeled structure overlaid with the template structure (PDB ID: 4ANI). The α-helix extending from the N-terminal portion of the model shown in blue color and C- terminal β-sheet shown in light orange color. The diagram was drawn in PyMOL., visualization software. (B) Ramachandran Plot of Mtb DnaJ. According to the plot 82.5% residues under most favorable region and only 0.0 % residues under the disallowed region.

Structure analysis of Mtb DnaJ:

The amino acid sequence of *Thermus thermophilus* DnaJ chaperone (PDB ID 4J80) protein having sequence identity 39% was used as a template for modeling the three dimensional Mtb DnaJ (Rv0352) (Barends *et al*., 2013). SWISS-MODEL gave 3 pdb files as output with equal sequence identity. Among them one model was taken as the 3D structure of DnaJ. The overall structure of Mtb DnaJ composed with two monomeric subunit containing 351 amino acids residues one is Chain A and another is Chain B shown in the (Fig. 4A). The modeled structure was visualized using PyMOL as shown in figure and validated through PROCHECK. Using PROCHECK, we found that 90% of residues were under most favorable and 0.3 % residues were present under disallowed region (Fig. 4B).

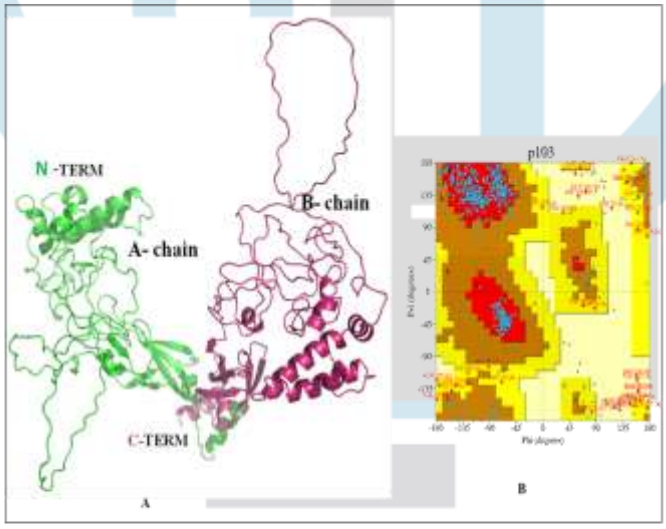

Figure 4: (A) 3D model structure of Mtb DnaJ using PyMol. Cartoon representation of Mycobacterium tuberculosis DnaJ 3D modeled structure overlaid with the template structure (PDB ID: 4J80).This is a dimeric protein containing A and B chain. The α-helix extending from the N-terminal portion of the model shown in green color and C- terminal α-helix shown in light magenta color. The diagram was drawn in PyMOL, visualization software. (B) Ramachandran plot of Mtb DnaJ. According to the plot 90% residues under most favorable region and only 0.3% residues under the disallowed region.

Docking of Mtb DnaK-GrpE:

The long N-terminal GrpE α-helices stabilize the linker of DnaK in the complex of Nucleotide binding domain and substrate binding domain. The Mtb DnaK-GrpE complex was modeled by utilizing Firedock server. An interface is the contact region between two interacting proteins. The interacting interface conformations of both proteins are shown in (Fig. 5).It has been proposed that, in addition to the nucleotide (ATP) exchange mechanism, the N-terminal disordered region of GrpE can trigger substrate release from DnaK (Brehmer *et al*., 2004). The probable hotspots are identified with the help of HotRegion server (Cukuroglu et al., 2012). PyMOL was used to visualize the complex (DnaK-GrpE) (Fig. 6) (DeLano, 2002).

Name of the protein	Hot point name	Hot point number	Chain
DnaK	Leu	153	D
Dnak	Ala	154	D
Dnak	Tyr	155	D
Dnak	His	309	D
Dnak	Asn	336	D
Dnak	Val	339	D
Dnak	Leu	351	D
GrpE	Asp	101	A
GrpE	Arg	104	A
GrpE	Val	150	A
GrpE	Gln	151	A
GrpE	Met	168	A
GrpE	Arg	180	A

Table 1. Hotpoint's result of DnaK and GrpE from HotRegion database:

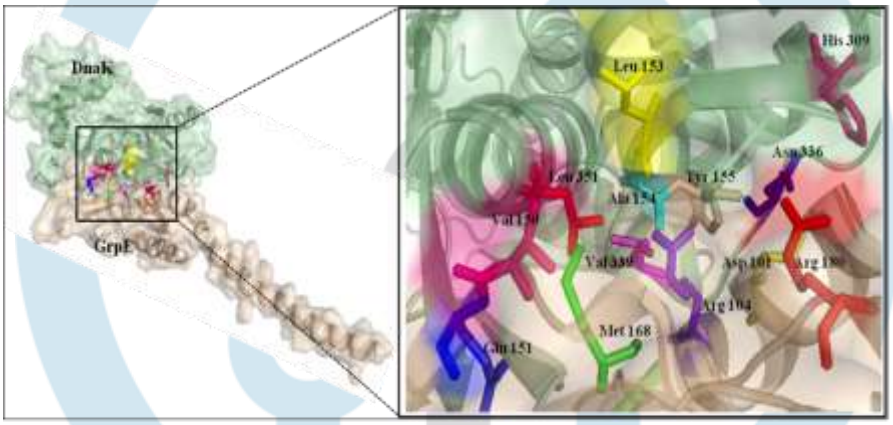

Figure 5: Complex structure of DnaK (pale green color) and GrpE (wheat color). Hotspot residues are shown in right-hand diagram (Black box).

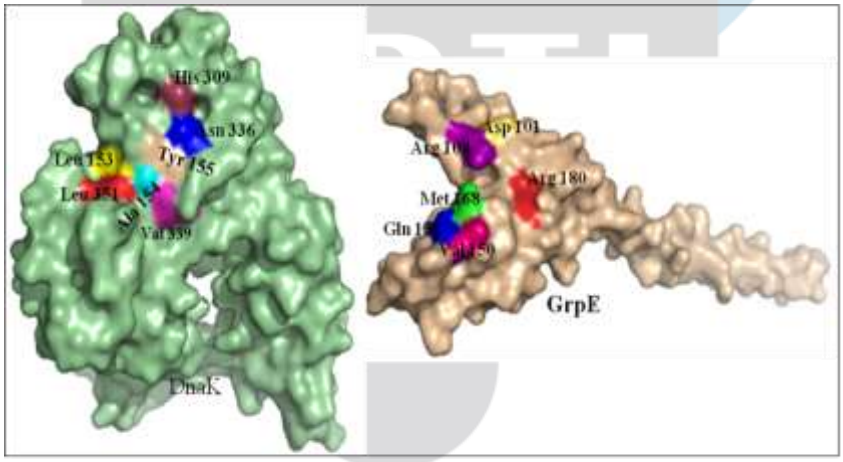

Figure 6: Surface representation of DnaK (pale green) and GrpE (wheat) with different docking residues (Hotspots).

Docking of Mtb DnaK-Mtb DnaJ:

It was reported that ATP-dependent *Mycobacterium tuberculosis* DnaK (PDB ID4RTF/Rv0350) (Hsp70) chaperones whose function is controlled in part by the nucleotide exchange factor co-chaperon GrpE (Rv0351) and DnaJ (Rv0352) (Hsp40 family) (Liberek *et al.*, 1991;McCarty *et al.*, 1995).The interaction between Mtb DnaK and DnaJ control the client protein binding phenomenon in chaperon cycle. The energy minimized complex structure DnaK-DnaJ obtained from FireDock server was visualized using PyMOL (Fig.7). The modeled structure of DnaJ contains two chains such as chain A and chain B (Fig. 4B) .Among them DnaK interacts with the A-chain of DnaJ but not with B-chain shown in the fig. Among all of the complex structures the lowest global energy -56.20 structure was taken as DnaK –DnaJ complex. Hotspots contribute to major stability of the complex were shown (Cukuroglu et al., 2012).

Table 2. Hotpoint's result of DnaK and DnaJ from Hot Region database:

Name of the protein	Hot point name	Hot point number	Chain
Dnak	Ala	129	D
Dnak	Val	145	D
Dnak	Thr	149	D
Dnak	Leu	153	D
Dnak	Val	188	D
Dnak	Val	190	D
Dnak	Leu	351	D
Dnak	Glu	352	D
DnaK	Leu	356	D
DnaJ	Gly	223	A
DnaJ	Ser	224	A

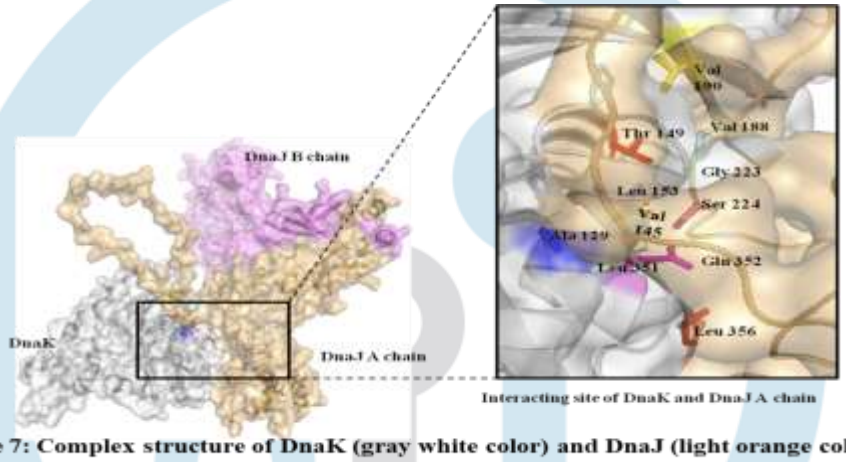

Figure 7: Complex structure of DnaK (gray white color) and DnaJ (light orange color). Hotspot residues are shown in right-hand diagram (Black box).

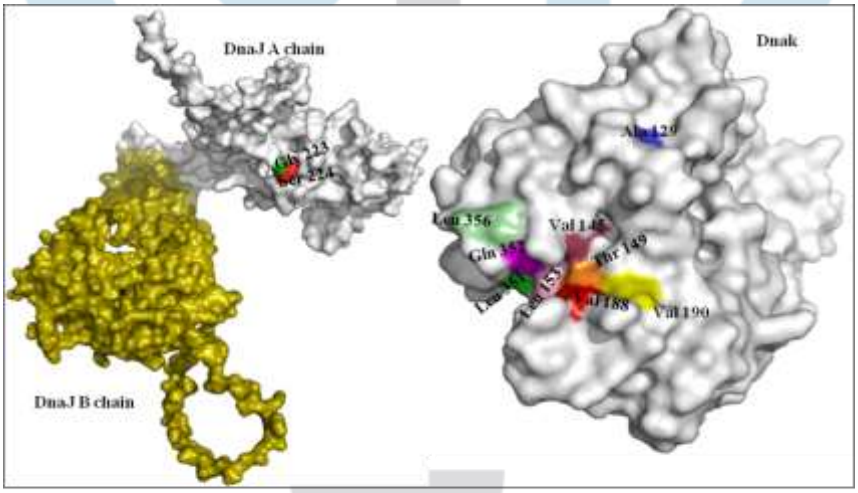

Figure 8: Surface diagrams of DnaJ and DnaK. Different hotspots residues of DnaK-DnaJ interacting complex are shown in different color.

Secondary structure assignment of the Hotspot of DnaK-GrpE and DnaK-DnaJ complex:
The data were represented in Table 3 and 4.

Table 3: DSSP result for secondary structure assignment (DnaK and GrpE hotspots)

Name of the protein	Hot point name	Hot point number	Chain	Secondary structure assignment of the Hotpoint
DnaK	Leu	153	D	α -helix
Dnak	Ala	154	D	α -helix
Dnak	Tyr	155	D	Hydrogen bond turn
Dnak	His	309	D	Bend
Dnak	Asn	336	D	Hydrogen bond turn
Dnak	Val	339	D	α -helix
Dnak	Leu	351	D	α -helix
GrpE	Asp	101	A	α -helix
GrpE	Arg	104	A	α -helix
GrpE	Val	150	A	Extended β ladder
GrpE	Gln	151	A	Extended β ladder
GrpE	Met	168	A	Extended β ladder
GrpE	Arg	180	A	Extended β ladder

Table 4: Result for secondary structure assignment (DnaK and DnaJ hotspots)

Name of the protein	Hot point name	Hot point number	Chain	Secondary structure assignment of the Hotpoint
Dnak	Ala	129	D	α -helix
Dnak	Val	145	D	Extended β ladder
Dnak	Thr	149	D	Hydrogen bond turn
Dnak	Leu	153	D	α -helix
Dnak	Val	188	D	Extended β ladder
Dnak	Val	190	D	Extended β ladder
Dnak	Leu	351	D	α -helix
Dnak	Glu	352	D	α -helix
DnaK	Leu	356	D	α -helix
DnaJ	Gly	223	A	Random coil
DnaJ	Ser	224	A	Random coil

Interacting aminoacid residues of DnaK-GrpE; DnaK-DnaJ complex:

Two main chain main chain interactions are shown below. Mainly three interacting hotspots of Mtb DnaK such as Leu 153, Ala 154 and Tyr 155 form hydrogen bonds with another hotspot residue Arg 104 of Mtb GrpE to give the complex stability (Fig. 9A, 9B).

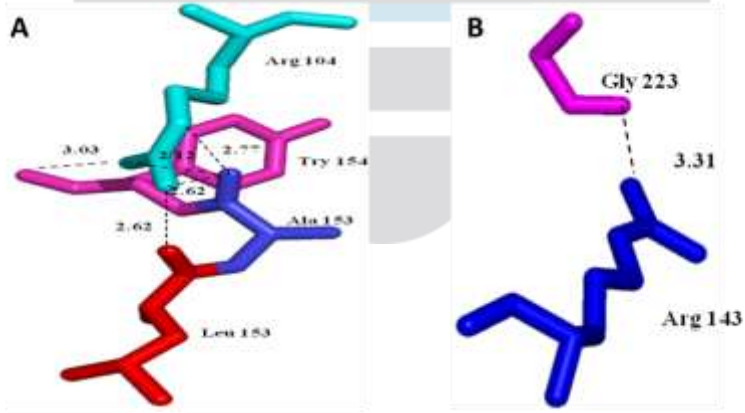

Figure 9: Amino acid residues Leu 153, Ala 154 and Tyr 155 of Mtb DnaK forming hydrogen bonds with Arg 104 of Mtb GrpE are represented as dashed line and the length are shown in angstroms these residues participate in main chain-main chain interaction of DnaK-GrpE during nucleotide exchange (A). Amino acid residues Arg 143 of Mtb DnaK forming hydrogen bonds with Gly 223 of Mtb DnaJ are represented as dashed line and the length is shown in angstroms and these two residues participate in main chain-main chain interaction of DnaK-DnaJ (B).

Hydrophobic Interactions between DnaK and GrpE with in 5 Angstroms:

The following hydrophobic residues obtained from PIC, take part in hydrophobic interaction between two proteins during the complex structure of DnaK-GrpE formation (Fig. 10).

DnaK			GrpE		
Protein	Residues	Chains	Protein	Residues	Chains
19	LEU	D	168	MET	A
351	LEU	D	168	MET	A
355	VAL	D	150	VAL	A
355	VAL	D	168	MET	A

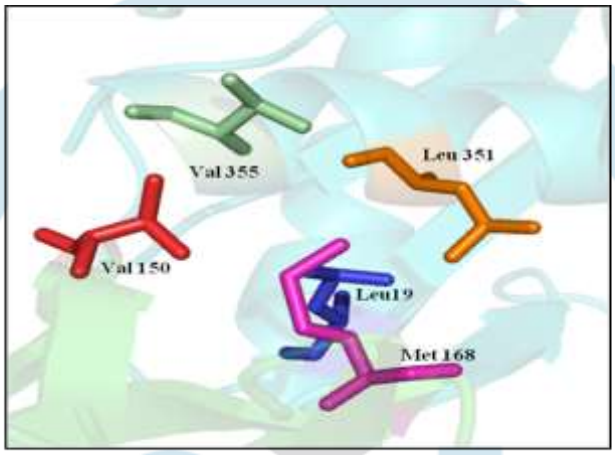

Figure 10: Hydrophobic interaction between DnaK and GrpE

Hydrophobic Interactions between DnaK and DnaJ within 5 Angstroms:

Same as the above the interacting hydrophobic residues are shown below for the complex of DnaK- DnaJ hydrophobic interaction (Fig. 11).

DnaK			DnaJ		
Position	Residue	Chain	Position	Residue	Chain
153	LEU	D	226	ILE	A
187	VAL	D	225	ILE	A
188	VAL	D	225	ILE	A
351	LEU	D	226	ILE	A
355	VAL	D	226	ILE	A
356	LEU	D	141	PRO	A

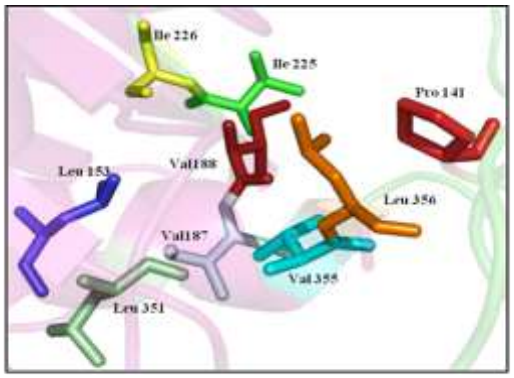

Figure 11: Hydrophobic interaction between DnaK and DnaJ

Multiple sequence alignment:

Sequence analyses have provided a better insight into the function of a particular protein. Hence, we have performed the sequence analysis of one important chaperone proteins DnaK, and two co-chaperone proteins GrpE (Rv0351) and DnaJ (Rv0352). The sequence of DnaK (PDB ID 4RTF/Rv0350), GrpE (Rv0351) and DnaJ (Rv0352) of *Mycobacterium tuberculosis* were separately retrived and subjected in PDB-BLAST to find the homologues sequence with known structure. The search gives total sequences for DnaK (PDB ID 4RTF/Rv0350) and 13 sequences for GrpE (Rv0351).A significant alignment is observed with Hsp70 of *Geobasillus kastrophilus* (Gk) (PDB ID: 2V7Y) with E value corresponding to and other alignment was observed with GrpE protein in *Geobasillus kastrophilus* (PDB ID:4ANI) with E-values corresponding to 2e-16. We also performed multiple sequence alignment using ClustalW and Espript, resulting in a significant sequence similarity among all sequences (Fig.10,11).By multiple sequence alignment DnaK(PDB ID 4RTF/Rv0350) of *M. tuberculosis* containing active site residues Leu153,Ala154,Tyr155,Val339,Leu351 were conserved but there were two specific amino acid residues Asn336 and His309 which are important for *M. tuberculosis* DnaK (PDB ID 4RTF/Rv0350)during the interaction with co-chaperone GrpE(Rv0351) were not conserved. Protein-protein interaction analysis is essential to understand the precise function of particular residues in the binding, and hence function.

Long N-terminal α-Helices of *Mtb*GrpE just like *Gk*GrpE, *Eco*GrpE facilitate the nucleotide (ADP) exchange from Mtb DnaK by forming a ternary complex structure Mtb DnaK-GrpE and help to release the client protein (Brehmer *et al*., 2004) (Bogdanov & Dowhan, 1998). The interaction between the long Mtb GrpE N-terminal α-helices and DnaK is also therefore important to substrate processing for Mtb DnaK.

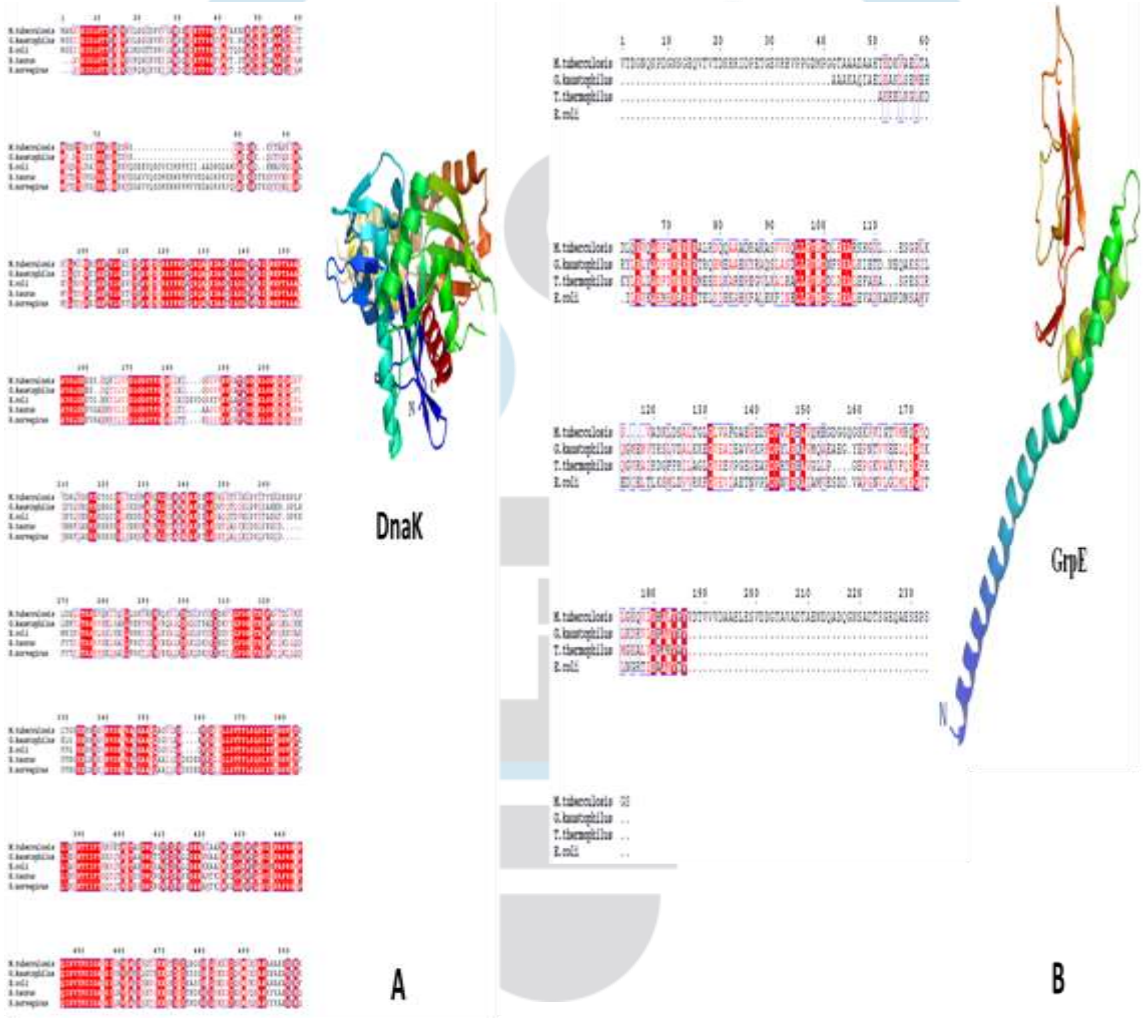

Figure 12: Multiple sequence alignment result of Mtb DnaK. (A) The conserved residues are shown in red color and the highly conserved residues are shown in deep red color throughout five different species (*M. tuberculosis*, *G. kaustrophilus*, *E. coli*, *B.* taurus, *R. novergicus*). Multiple sequence alignment result of Mtb GrpE. (B) The conserved residues are shown in red color and the highly conserved residues are shown in deep red color throughout four different species (*M. tuberculosis*, *G. kaustrophilus*, *T. thermophilus* and *E. coli*).

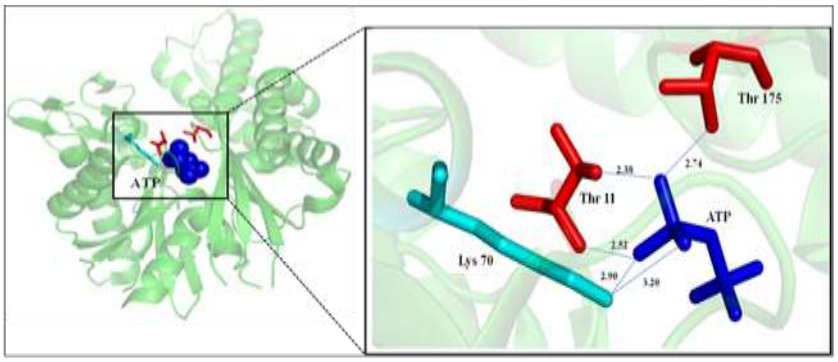

Figure 13: ATP bound state of DnaK (Cartoon diagram) and ATP bound amino acid residues shown in right-hand box. Hydrogen bonds between the bound nucleotide (ATP) and Mtb DnaK amino acid residues are represented as dashed line with length shown in angstroms. Interacting residues are shown in sticks form. Three residues Thr 11 (red), Lys 70 (cyan), Thr 170 (red) bind with phosphate group of ATP.

Atp binding state of Mtb DnaK:
As shown in Fig 13, the nucleotide binding cavity of DnaK in *M. tuberculosis* (NBD) binds with ATP by using following residues Thr 11, Lys 70 and Thr 175 facilitates substrate release, and substrate binding (Fig.13). It is well stated that hydrogen bonds play an important role for structure and function of proteins (Chu *et al*., 2009). Hence, we analyzed number of hydrogen bonds between ATP and chaperone Mtb DnaK (PDB ID 4RTF/Rv0350) containing amino acids. The complex structure shows that the complex between Mtb DnaK-ATP is stabilized by five hydrogen bonds between ATP and Thr 11, Lys 70 and Thr 175 present at the nucleotide binding pocket. These three residues play an important role and are highly conserved throughout different species.

IV. DISCUSSION

Stress response plays a major role in the survival of intracellular pathogens such as *M. tuberculosis* in various ways such as: (a) protects the bacteria within the hostile environments of the host, thereby allowing them to grow rapidly; (b) the stress proteins may themselves contribute to pathogenesis; and (c) many stress proteins, being immunogenic, contribute to the generation of host immunity.

In this piece of work, using several computational approaches we have identified interacting patterns of DnaK in mycobacterium with two co-chaperon proteins DnaJ and GrpE during heat shock response in chaperone cycle. Structures of DnaK-GrpE and DnaK-DnaJ complexes of Mtb are not deciphered till date. Our findings show that Mycobacterium DnaK is a central hub of the mycobacterium chaperone network with co-chaperon protein DnaJ and GrpE. The interacting partners communicate among themselves utilizing specific amino acid residues (Table1 and Table 2). The structure of Mtb DnaK has been deciphered. In order to understand the binding modalities of DnaK with GrpE and DnaJ, we need a complex structure of these proteins. Hence we modeled the co-chaperonins GrpE and DnaJ as shown in Figure (Fig 3A and 4A).DnaJ in several organisms seen to interact with DnaK as a dimer (Ahmad *et al*., 2011and Barends *et al*., 2013).So in order to function properly in Mtb ,we expect that it would also interact with DnaK as dimer. Thus using SWISS MODEL the DnaJ was modeled as a dimer and was docked to get the protein complex. To release all the restrain are energy minimized the complex. We identified the hotspot residues which are essential to maintain the stability of the complex (Fig. 11 and 12). The functional importance of Leu 153, Ala 154 and Tyr 155 residues of Mtb Dnak and Arg 104 of GrpE (Fig. 9) during interaction of Dnak-GrpE has been shown in our study and these residues are highly conserved across Hsp70s (Into *et al*., 2012 and Qi *et al*., 2013) .These interacting residues are near about similar to those in *G. kaustrophilus* and *E. coil's* DnaK-GrpE. We identified for the first time, a large set of hotspot residues in Mtb DnaK, GrpE and DnaJ are highly conserved. Depends upon the data shown in Table3 and Table 4 the hot residues of Mtb DnaK, GrpE and DnaJ are mainly in α –helix, extended β –ladder and random coil form. GrpE(Mtb) modeled structure (Fig.3A) generated in this study indicate that the long α-helices of the GrpE is flexible (Papers & Doi, 2008). On the other hand, the NEF (Nucleotide Exchange factor) induces ADP replacement with ATP, by significantly increasing the ADP dissociation rate (Kabani, 2009) and enhances the folded protein releasing rate. ATP hydrolysis by DnaK leads to the reformation of the stable ternary complex, thus, preventing the aggregation of those protein molecules that are unable to proceed rapidly to the native state. This allows for multiple rounds of refolding. In the present study, it was also shown that amino acid residues Thr 11, Lys 70 and Thr 170 (Fig. 13) (corresponding to residues Thr 11, Lys 68, Thr 172 of Gk Dnak) of NBD binds with ATP in Mtb DnaK during client protein binding and client protein releasing (Liberek *et al*., 1991). Hence it would be expected that Mtb GrpE stimulates ADP-ATP exchange in Mtb DnaK. We therefore reasoned that the requirement for GrpE in the refolding reaction may be to release bound nucleotide to permit substrate protein release. During the course of this interaction process the active side residues of three proteins are much more important. The Dnak-DnaJ-GrpE mediated chaperone cycle of Mtb may be blocked by specific drug targeting towards the active site of any interacting partner before forming a complex (DnaK-GrpE/DnaK-DnaJ).Thus inhibition of chaperone cycle may be occurred.

V. CONCLUSION

As DnaK-DnaJ-GrpE interfaces are important for carrying out chaperone function. Their interfaces serve as an attractive drug target. Blocking their interfaces with small molecule would hamper the chaperone cycle. Hence identification of the interface residues are essential. We can design a small molecule to target towards the interface of bacteria by inhibiting the Chaperon system.

VI. ACKNOWLEDGEMENT

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VII. Conflict of Interest

The authors confirm that there is no conflict of interest.

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