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In silico Analysis of Isoniazid Resistance in *Mycobacterium tuberculosis*

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***Abstract*—**Altered drug binding may be an important factor in isoniazid (INH) resistance, rather than major changes in the enzyme's activity as a catalase or peroxidase (KatG). The identification of structural or functional defects in the mutant KatGs responsible for INH resistance remains as an area to be explored. In this connection, the differences in the binding affinity between wild-type (WT) and mutants of KatG were investigated, through the generation of three mutants of KatG, Ser315Thr [S315T], Ser315Asn [S315N], Ser315Arg [S315R] and a WT [S315]) with the help of software-MODELLER. The mutants were docked with INH using the software-GOLD. The affinity is lower for WT than mutant, suggesting the tight binding of INH with the mutant protein compared to WT type. These models provide the *in silico* evidence for the binding interaction of KatG with INH and implicate the basis for rationalization of INH resistance in naturally occurring KatG mutant strains of *Mycobacterium tuberculosis*.

Keywords*—Mycobacterium tuberculosis,* KatG, INH resistance, Mutants, Modeling, Docking.

I. Introduction

DESPITE being a controllable, preventable and curable disease, Tuberculosis (TB) still remain as a major public health problem in many parts of the world. The increase in multi-drug resistant (MDR-TB, strains resistant to at least two of the first-line TB drugs - INH and rifampicin) [1] and the recent emergence of extensively drug resistant (XDR-TB, is MDR-TB that is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs [capreomycin, kanamycin, and amikacin]) has further worsened the situation [2]. Drug resistance in TB is essentially a potential threat to the TB control programs. Amongst the drugs used for TB treatment, resistance is reported to all. However, isolates of *M. tuberculosis* resistant to Isoniazid (INH) are seen with increasing frequency (1 in 10^6) compared to other drugs [3].

Isonicotinic acid Hydrazide and Pyridine-4-carbohydrazide are the chemical and IUPAC names of INH respectively. It is the cornerstone of treatment for drug-susceptible TB and it is also widely used to treat latent *M. tuberculosis* infection. There has been considerable interest to know the origin of INH resistance in clinical *M. tuberculosis* isolates at the molecular level. The molecular basis of INH resistance is less well characterized, and mutations in several genes have been associated with it [4]. Mutation in *katG* gene coding for catalase-peroxidase-KatG is a major mechanism of INH resistance in *M. tuberculosis* [5]. KatG plays a key role in activating the prodrug INH - an important drug in the anti-TB therapy. The Ser to Thr mutation (AGC to ACC) at codon 315 of the *katG* gene is considered to be the most prevalent mutation, serving as a reliable marker for the detection of INH resistance [4] and the appearance was most frequent among the MDR strains [6]. However, this mutation was also reported to be associated with intermediate or high levels of resistance to INH (1 to 10 µg/ml) [7]. Clinical *M. tuberculosis* isolates with mutations at codon 315 of the *katG* gene remain virulent [8].

The elucidation of altered INH binding, altered heme binding, or other structural changes due to amino acid substitutions is only in very early stages of detailed study, most of which has been devoted to the commonly encountered KatG mutant -S315T. A number of *in vitro* studies have been undertaken to understand the origins of resistance using the S315T mutant as a model [9]-[21].

Several other studies targeting other mutants of KatG [22]-[25] have clearly shown that the decrease KatG activity observed in the mutant proteins is well correlated with the structural modifications. Till date around 25 different KatG mutants were characterized functionally on varying aspects from all over the world and the number continues to increase each year.

The determination of the crystal structure of *M. tuberculosis* KatG [26] provided a major breakthrough in understanding the molecular mechanism of INH activation and highlighted the remarkable structural similarity both in the overall structures and at the active sites of KatG. Based upon the availability of crystal structure of the WT KatG certain residues in its active site have been postulated to be involved in enzyme-catalyzed activation of INH. Pierattelli et al., 2004 demonstrated the NMR models depicting the interaction between INH-HRP complexes [27]. Bertrand et al., 2004 showed the details about the crystal structure of KatG and the location of residues that may play a role in catalysis including candidates for protein-based radical formation [26]. Metcalfe et al., 2008 provided evidence for the location of the INH binding site in the class I peroxidases [28]. Two other recent reports gave *in silico* evidence for the binding interaction of KatG with INH [29], [30]. Although the above reports has provided extensive kinetic and structural data related to KatG and its mutants, yet structural information regarding the essential clinical mutants occurring at position 315 leading to the cause of INH resistance is still lacking. Therefore, we have made an attempt

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to explore the interactions of KatG mutants with INH to understand the rationale behind the point mutants causing INH resistance. In this process, three dimensional (3D) models for three mutants (Thr, Arg and Asn at position 315 instead of wild Ser) were developed and docked with INH. Thus, the essential clinical mutants occurring at position 315 with the exception of Gly and Iso were created in our study.

II. Methods

A. Homology Modeling of KatG Protein

The KatG proteins, WT (S315) and the three mutants of KatG (S315T, S315R and S315N) were generated using MODELLER programme in Discovery Studio (DS, V-2) Accelrys [31]. The details for model generation were explained with respect to one of the mutant-S315R.

B. Template Selection

In the present study, the target protein sequence obtained from the Tuberculist database, was submitted to protein- Basic Local Alignment Search Tool (BLASTp) and searched against protein data base (PDB). The WT KatG protein from *M. tuberculosis* (PDB code-1SJ2) was considered as template protein displaying maximum percentage identity (99%) with the mutant protein. The heteroatoms such as glycerol and water were removed, maintaining heme (cofactor) in the atom file of the template 1SJ2. As KatG is a conjugate enzyme, heme is required for binding with INH.

C. Model Building

Sequence and structural alignment of 1SJ2 with target sequence was performed using ClustalW. Aligning was done carefully without insertion of any gaps in the conserved secondary structural regions. From the alignment, spatial restraints were derived and used in the 3D model construction with MODELLER in DS.

D. Model Evaluation and Energy Minimization

Validation of the models was performed by dope feature of MODELLER9v3 program, Ramachandran plot and Combinatorial extension (CE) methods. Validation was done in order to eliminate the structural errors and to improve the quality and stability in the generated model.

E. Ligand

The ligand (INH) used in this study was generated from the smiles formula (C1=CN=CC=C1C(=O)NN) of INH from National Center for Biotechnology Information (NCBI) - database. Chemsketch softwareV-10 was used to obtain the 3D (molecule) structure of the ligand and it was saved as a PDB file using DS.

F. Docking Software

The GOLD protocol is an implementation of the Genetic algorithm (GA) wherein the receptor is held rigid while the ligands are allowed to flex during the refinement process. The provision of rotational flexibility (flexible ligand and a protein with flexible hydroxyl groups) for the protein–receptor polar Hydrogen (H) makes it a good choice when the binding pocket contains amino acids that form H bonds with the ligand. GOLD uses a scoring function that is based on force field, favorable conformations, empirical results and weak chemical interactions and includes three terms (a H bonding term, a 4–8 intermolecular dispersion potential, and a 6–12 intramolecular potential for the internal energy of the ligand) [32].

G. Docking protocol

Docking was carried out with the help of software-GOLD. The atom files for both the protein and the ligand were created. Glycerol and other molecules were removed, while retaining three water molecules (7, 11 and 429). H atoms were added to the model and ligand using GOLDMINE before docking followed by energy minimization. The cavity atom file containing the atom number of binding residues was created for both heme (Pro77, Leu78, Arg81, Trp84, Tyr206, Asn208, Pro209, Phe229, Leu242, Ile243, Gly245, His247, Phe249, Gly250, Lys251, Thr252, His253, Thr291, Asn292, Trp298, Leu355, Thr357) and INH (Arg81, Trp84, His85, Tyr206, and Pro209) docking. The binding residues were selected on comparison between binding regions of heme and INH with crystal structures of other molecules (2v2e, 2v2f, 2vcf, 2vcn and 2vcs) similar to KatG. Comparison was done using multiple sequence alignment. Docking was performed including heme. Standardization of docking procedures was done with values in same range with least deviation upon repetition for three times.

Dockings were performed under 'Standard default settings' mode- number of islands was 5, population size of 100, number of operations was 100,000, a niche size of 2, and a selection pressure of 1.1.

Ten docking poses were obtained for each ligand. Poses with highest GOLD score were used for further analysis. The docked poses of the ligands were visualized using Hermes software. The scoring function of GOLD provides a way to rank placements of ligands relative to one another. Ideally, the score should correspond directly to the binding affinity of the ligand for the protein, so that the best scoring ligand pose are the best binders.

III. Results

A. Validation of Models

In this study, KatG proteins were modeled with the help of MODELLER and the models were built based on the WT sequence of *katG* gene. From the results of BLAST search against PDB, 1SJ2 was identified as template protein (Fig. 1). The pattern; profiles and the domains obtained for template and target were similar (data not shown). The models were validated by the following methods:

a) Ramachandran Plot

Evaluation of the models (WT and three mutants) was performed using Ramachandran plot computed with RAMPAGE. Structural evaluation with RAMPAGE showed 96.2% of residues in the most favored region, suggesting a

good quality (Fig. 2 (a)) of the mutant model (S315R) whose crystal structure has not been elucidated in the database.

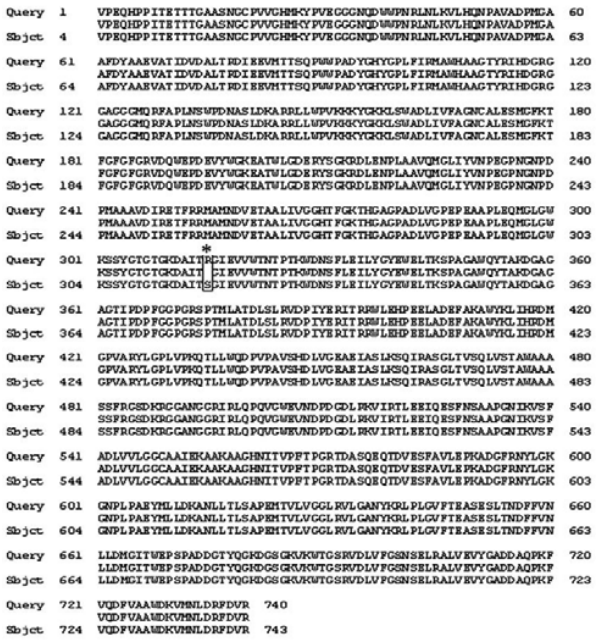

Fig. 1 BLAST Result showing the template and the target similarity score = 1510 bits (3909), expect = 0.0, method= compositional matrix adjust. Identities = 739/740 (99%), positives = 739/740 (99%), gaps = 0/740 (0%)

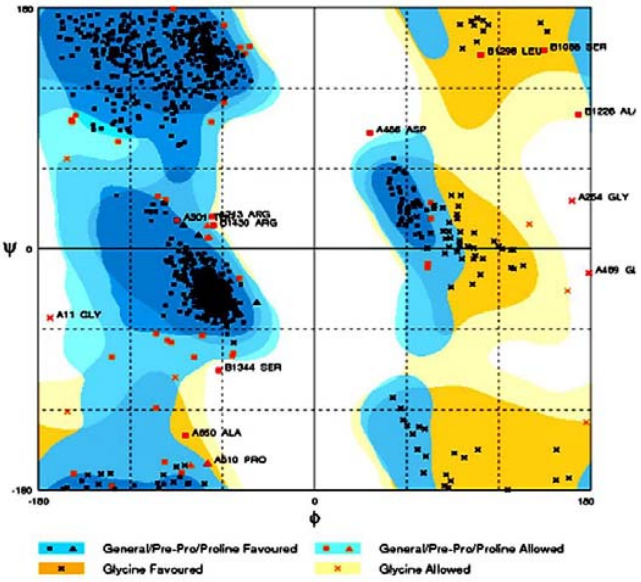

Fig. 2 (a) Ramachandran plot number of residues in favoured region (~98.0% expected): 1375 (96.2%); Number of residues in allowed region (~2.0% expected): 42 (2.9%); Number of residues in outlier region : 13 (0.9%)

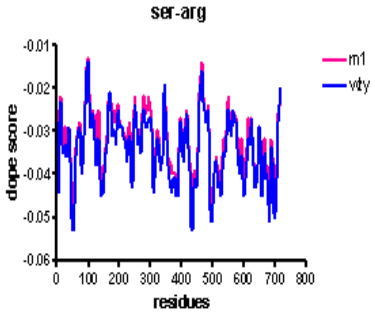

Fig. 2 (b) Dope score for mutant of KatG S315R

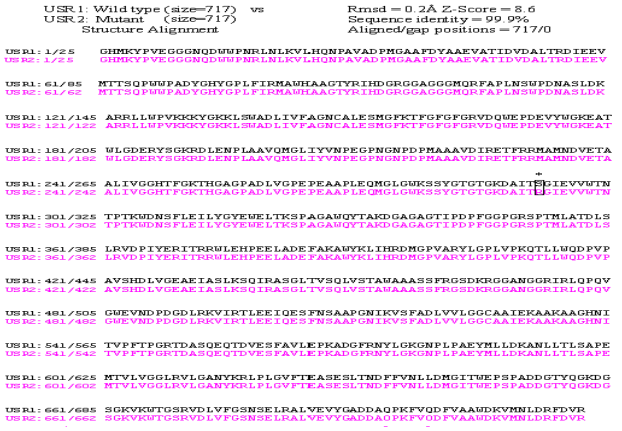

Fig. 2 (c) The structural alignment for the two-modeled domain structure using CE method. In this the * indicates a position that is not conserved (different [S-R]) between WT and mutant KatG

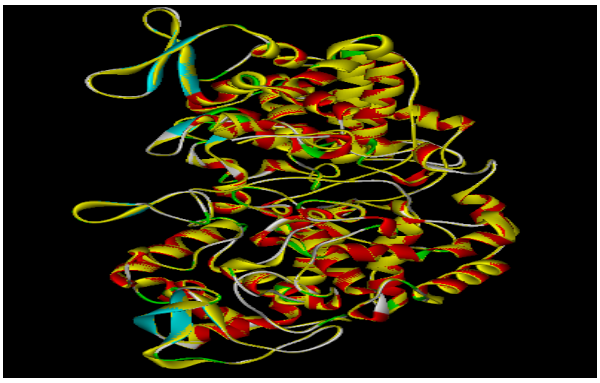

Fig. 2 (d) Superimposed model of template and the target. 1SJ2-WT template (Yellow colored) KatG-mutant target (Red colored) models were viewed in Rasmol (V-2)

b) Dope Score

The graphs (Fig. 2 (b)) based on energy profiles suggest the generated models for each mutant model in MODELLER9v3 was similar to the WT with no significant deviation in the peaks of the graph.

c) CE Method

The structural alignment was performed for the WT and mutant structure (Fig. 2 (c)) and also the 3D structures of WT KatG and mutant (S315R) were superimposed (Fig. 2 (d)).

The root mean square deviation gaps in alignment using the algorithm displayed zero (0.0) Å RMSD and high (8.6) Z-score for the modeled structure, indicative of good structural alignment. Superimposition of WT (S315) and mutant (S315R) models implies that there was a very little degree of deviation from the template; hence the change in single amino acid did not produce any significant change in the overall conformation of the protein (Fig. 3).

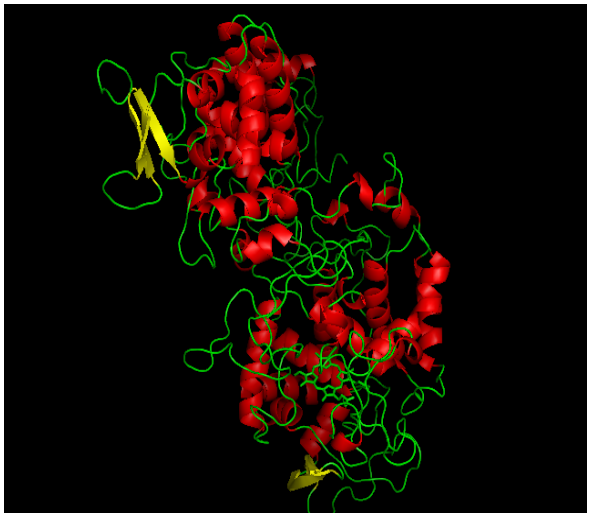

Fig. 3 Structure of mutant-KatG (S315R) generated by MODELLER in DS (Red colored) α helices and βsheets (Green colored) of A chain, (Yellow colored) heme

B. Active Site of KatG

The heme binding site was considered as active site containing an energetically favorable binding site for INH near the *∂-meso* edge of heme regarded as the hydrophobic pocket according to earlier reports 26, 27, 28. A total of 36 amino acids were involved in the ligand (heme) binding interaction with four catalytically important residues such as Arg104, His108, Asp137 and Asn138.

C. Docking of INH-KatGs

The docking of INH with KatGs was performed using the software GOLD. Of the ten poses produced, the best ligand pose was selected based on top GOLD score. Among the three mutants, the high score of 46.47 kcal/mol was obtained for the The INH-KatG complex was visualized using DS in order to get insight into the interaction between the drug and target (Fig. 4). It is evident from the figure that INH drug is located at the active site near one edge of heme -putative oxidizing centre, and is stabilized by H bonding interactions. GOLD score between the INH and mutated models are tabulated (Table I). The binding energy between the enzyme and drug for the WT was found to be 43.82 kcal/mol.

The score suggests the importance of forces such as electrostatic and van der Waals for the interaction of the drug molecule with mutated models for the protein-drug complex formation (Fig. 5).

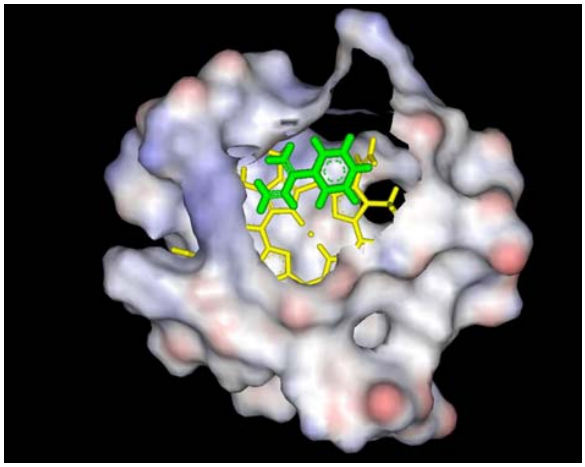

Fig. 4 Surface representation of model (S315R) structure with heme (Yellow colored) and INH (Green colored) docked into the cavity

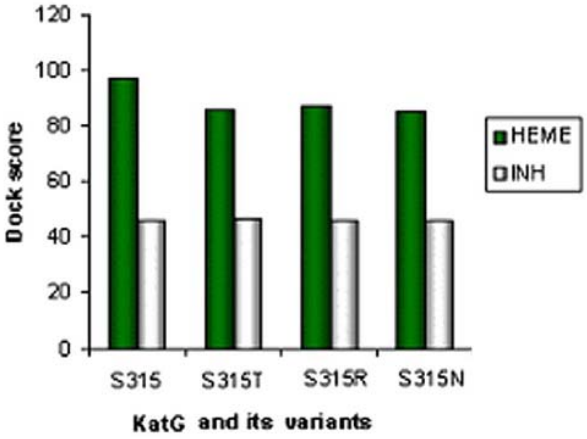

Fig. 5 Docking of Heme and INH with KatG and its mutants

TABLE I
DOCKING VALUES OF HEME AND INH

KatG Models	Heme[a]	INH[b]
S315	97.62	43.82
S315T	85.75	46.47
S315R	85.18	44.87
S315N	63.39	44.07

a = heme docking values; b = INH docking values

D. Hydrogen Bonding Interactions

It is well known that H bonds play an important role in the maintenance of stability, structural integrity and function of biological molecules especially for enzyme catalytic reaction. The mutated models S315T and S315R form five H bonds with the heme group (4 residues and a molecule of water; 3 residues and 2 molecules of water). The model with S315N forms six bonds (5 residues and a ligand).The model structures viz; S315R, S315N, S315T commonly contain His247 in connection with heme moiety to form H bond compared to WT model containing Ser315 and His 276.

The total number of H bond formation was more (8) in the WT compared to other mutants. The H bonds present in the

enzyme-drug complex along with H bond donor and acceptor with their distances are shown in the Table II.

E. RMSD Analysis

Out of these three mutant models, the least RMSD of heme was 5.47 Å for the mutant S315N compared to WT type, among the ten other higher RMSD values. The values were nearly in tune with the heme docking values presenting higher extent of structural deviation for S315N compared to others. The values for the mutants S315R, and S315T were found to be 1.26 Å and 2.30 Å respectively (Fig. 6).

IV. Discussion

KatG is responsible for the transformation of inactive form of INH (isonicotinic acid hydrazide) to its active counterpart (isonicotinic acid). Mutation in *katG* gene coding for KatG is a major mechanism of INH resistance in *M. tuberculosis*.

In this study, KatG mutants were modeled with a view that these mutations at position 315 would also increase steric bulk and further limit access to the binding site.

The rationale behind the mutant selection was on the basis of our previous reports [33]–[37]. Based on the information about the KatG-INH complex in combination with crystal and NMR structures of KatG from reports [26]-[28] it can be suggested that the three mutant models of KatG may affect enzyme activities by different mechanisms.

TABLE II
Hydrogen Bond Formation at the Ligand

KatG Models	No. of Hb	Hb donor	Hb acceptor	Bond distance (Å)
S315	8	HIS276:ND1	HEM1500:O2A	2.90
		HIS276:HN	HEM1500:O1A	2.89
		SER315:N	HEM1500:O1A	2.92
		SER315:OG	HEM1500:O1A	2.55
		Water:HOH7:O	HEM1500:O2A	2.57
		Water:HOH7:O	HEM1500:O1D	2.73
		Water:HOH11:O	HEM1500:O2D	2.67
		Water:HOH492:O	HEM1500:O1D	2.71
S315T	5	HIS247:HE2	HEM1500:NA	2.33
		LYS251:HN	HEM1500:O2D	1.43
		HIS253:HD1	HEM1500:O2A	2.36
		HEM1500:H	THR252:OG1	2.30
		HEM1500:H	HOH492:OH2	2.41
S315R	5	HIS247:HE2	HEM1500:NA	2.42
		LYS251:HN	HEM1500:O2D	2.24
		ARG292:HN	HEM1500:O1A	2.03
		Water:HOH7:OH2	HEM1500:O2A	2.77
		Water:HOH7:OH2	HEM1500:O1D	2.44
S315N	6	HIS247:HE2	HEM1500:NA	2.16
		LYS251:HN	HEM1500:O1D	1.55
		ASN292:HD21	HEM1500:O1A	2.42
		Water:HOH7:OH2	HEM1500:O2A	1.96
		Water:HOH11:H1	HEM1500:O1D	2.41
		ISZ1296:H5	VAL207:O	2.37

ISZ1 = INH, Hb = Hydrogen bond, A° = Angstrom

Each of them impacts consequently on a heme-associated structure, the putative oxidative site. Eventually, the mutations occurring within or close to the regions containing these residues could result in conformational modifications of the active site of KatG and thereby in loss of activity as observed in most of INH resistant strains.

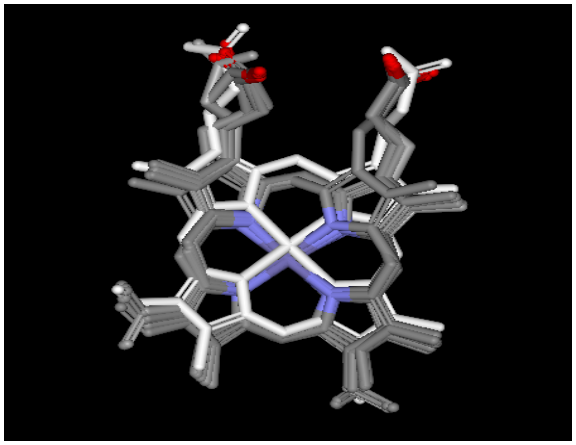

Fig. 6 3D model representation of the final conformations with the lowest RMSD from the centroid of each cluster for heme in WT (White colored) and heme in mutant S315T (Purple colored). Models were viewed in DS (V-2)

On the basis of crystal structure of the mutant S315T (PDB code 2CCD) it was stated that the mutation of S315 or Ser324 (*B. pseudomallei* KatG) to Thr narrows the heme access channel from around 6 to 4.7 Å at its narrowest point in the mutant enzymes from both organisms. These observations and the significantly decreased INH activation in these mutants suggest that drug binding within the heme pocket is a likely requirement for INH activation [38]. In contrast, in the present study the binding affinity of INH for S315T was shown to be more compared to the WT. The possible explanation could be due to the substitution of Ser with Thr, which does not produce much conformational changes as both aminoacids (Ser/Thr) contains the same functional group (i.e) Hydroxyl group.

The mutated model-S315R was functionally characterized in the one of our studies [36], which showed the reduction in the catalytic activity for the mutant compared to WT. Kinetic data [36] was not in concordance with the present *in silico* analysis, showing an increase in INH binding activity for the mutant compared to the WT. On the contrary, similar to the kinetic activity (lesser activity for the mutant compared to WT) the heme docking score showed lesser value for the mutant than the WT.

The S315N mutant displayed an (INH binding) affinity similar to S315R mutant, wherein the heme binding value showed an apparently very significant deviation with 36% reduction compared to the WT. This was in agreement with a report which stated that KatG-S315N was found to be inactive in INH activation [24]. The steric constraints resulting from such substitutions could significantly alter the folding of the

mutant protein and or the positioning of the side chain of the catalytic residue. It will lead to the loss of KatG activity, thereby inhibiting the conversion of INH to its active counterpart. Hence, Ser to Asn substitution may lead to higher degree of INH resistance than Ser to Thr.

In the present study, the INH binding score for the three KatG mutant models exhibited an increment in the binding affinity compared to the WT. However, the heme docking score showed significant high score with WT compared to the mutants. The higher score in case of heme binding could be better explain on the basis of binding space or exact-fit, wherein the KatG protein contain sufficient binding space (cleft) for the heme moiety to fit in perfectly (direct binding) leading to high score. In case of INH binding, (with heme and not the protein directly) the scores were less. Perhaps, due to the binding of INH with ∂-meso edge of heme group rather than the KatG protein directly. It can be presumed that the conformational changes occurring in KatG protein owing to point mutation was clearly reflected by the corresponding changes produced in the heme binding property compared to INH. However, the limitation of this study is non-performance of molecular dynamics (MD) simulations. As MD is a reliable method to determine the protein (KatG) binding activity during dynamic state of the protein as was documented by earlier reports [29], [30]. Nevertheless, these reports [29], [30] did not provide the changes in all the mutant proteins as noted in our study, at position 315 except for the mutant S315T. We are presently involved in the continuation of this study in connection to MD.

V. Conclusions

We have utilized computational techniques to obtain engineered models of KatG and docked with INH. With the help of the predicted models, the conformational changes due to induction of mutation at the residues of active site were determined. If drug (INH) resistance occurs due to mutation at the active site of protein molecule (S315T/R/N), then the binding affinity will be less between the drug and mutant protein than WT (S315). In other words, the affinity will be higher for WT type. As there occurs an inverse relationship between the binding affinity and drug resistance (higher the affinity, lower will be the resistance and vice versa). Based on heme binding score, S315N contribute to very high degree of resistance followed by the mutants, S315R and S315. The model of INH-KatGs complex can be used to rationalize the effects of a number of mutations within the enzyme that confer elevated levels of resistance to INH. Based on INH docking score, it was found that mutants were shown to have higher affinity compared to WT. These data suggest the tight binding of INH with the mutant protein compared to WT. This enables the mutant to hold the inactive form (INH) of drug and inhibiting from the release of active form which is responsible for bactericidal activity, thereby leading to INH resistance.

The interaction between the enzyme and the drug proposed in this study may be useful for better understanding of fundamental mechanism of INH resistance. The data provided basic explanation for the molecular mechanism of mutation-acquired resistance. Overall, these results increase our understanding of the structure and function of this clinically important protein and suggest that further KatG structural studies are needed to elucidate detail mechanisms causing resistance in the mutants.

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