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Binding of activated isoniazid with acetyl-CoA carboxylase from *Mycobacterium tuberculosis*

Ameeruddin Nusrath Unissa[1]*, Subramanian Sudha[2], Nagamiah Selvakumar[1], Sameer Hassan[3]

[1]Department of Mycobacteriology, National Institute for Research in Tuberculosis (NIRT), Indian Council of Medical Research (ICMR), Mayor V.R. Ramanathan Road, Chetput, Chennai - 600 031, Tamil Nadu, India; [2]Veterinary College, Chennai - 600 031, Tamil Nadu, India; [3]Department of Biomedical Informatics Centre, NIRT, (ICMR), Mayor V.R. Ramanathan Road, Chetput, Chennai - 600 031, Tamil Nadu, India; Ameeruddin Nusrath Unissa - Email: nusrathunissa@gmail.com; Fax: +91-(044)-2836 2528; *Corresponding author

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Abstract:
AccD6 (acetyl coenzyme A (CoA) carboxylase), plays an important role in mycolic acid synthesis of *Mycobacterium tuberculosis* (*Mtb*). Induced gene expression by isoniazid (isonicotinylhydrazine - INH), anti-tuberculosis drug) shows the expression of accD6. It is our interest to study the binding of activated INH with the AccD6 model using molecular docking procedures. The study predicts a primary binding site for activated INH (isonicotinyl acyl radical) in AccD6 as a potential target.

Keywords: *M. tuberculosis,* modeling, docking, AccD6, INH, INADH, acetyl-CoA.

Background:
Tuberculosis (TB) still remains as a major public health problem, despite of significant improvement and advancement made in the field of diagnosis and therapeutics. INH (isonicotinic acid Hydrazide) is the cornerstone of treatment for drug-susceptible TB and it is also widely used to treat latent *Mtb* infection. There has been considerable interest to know the molecular basis of INH resistance, which is less well characterized and mutations in several genes have been associated with it. It is proposed that INH enters *Mtb* as a prodrug by passive diffusion and is activated by catalase-peroxidase, encoded by *katG* **[1]**, to generate free radicals, which then attack multiple targets in the cells. Initial studies utilizing whole cells of *Mtb* demonstrated that INH was principally metabolized to several products, including 4-pyridylmethanol and isonicotinic acid. The authors speculated that INH could be oxidized *in vivo* to yield a number of highly reactive species, including diazenyl, hydrazide, or acyl radicals, which could then go on to oxidize or acylate groups in proteins. The aldehyde, fed exogenously, is quickly reduced to the alcohol **[2]** while in the *in vitro* system with Mn++ presence, the aldehyde has been reported to undergo oxidation to the acid and in the presence of Mn++, the addition of simple amines results in the efficient formation of the corresponding amides **[3]**. One metabolite in vitro from INH conversion in the presence of nicotinamide adenine dinucleotide (NAD+) (and Mn++) is the covalent adduct of the acyl pyridine from INH and the nicotinamide ring system **[4]**. It has been proposed to be important in inhibiting one of the cellular targets associated with mycolic acid synthesis, InhA **[5]**. Studies have shown that an NADH-dependent enoyl acyl carrier protein (ACP) reductase, encoded by *inhA*, and ketoacyl ACP synthase, encoded by *kasA*, are two potential intracellular enzymatic targets for activated INH; and both of these enzymes are involved in the biosynthesis of mycolic acids **[6, 7]**.

The recent microarray approach has generated new insights into the action of INH and shows that INH induces several genes, including *kasA* and *B*, *fabD, efpA* and *accD6*, which are presumably involved in fatty acid oxidation **[8, 9]**. Of which, *accD6*, plays an important role in mycolic acid synthesis. *Mtb* contains six Acyl-CoA carboxylases (ACCase) carboxyltransferase domains, *accD1–6*, whose specific roles in the pathogen is not well defined but they are important for cell

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envelope lipid biosynthesis and that its disruption leads to pathogen death **[11]**. The *Mtb* genome contains three biotin carboxylase subunits (AccA1 to -3) and six carboxyltransferase subunits (AccD1 to -6), with *accD6* located in a genetic locus that contains members of the fatty acid synthase II (FAS II) complex. *accA3*, *accD4*, *accD5*, and *accD6* are expressed at high levels during the exponential growth phases of *Mtb in vitro*. Micro array analysis of *Mtb* transcripts indicated that the transcripts for *accA3*, *accD4*, *accD5*, *accD6*, and *accE* were repressed during later growth stages. The *accD4* and *accD5* have been previously studied, and the function of accD6 designated as a member of the FAS II locus, is a functional carboxyltransferase subunit of the ACCase in *Mtb* **[12]**.

Previous studies using microarray technique have **[8-10]** provided the information regarding the up regulation of AccD6 upon exposure to INH. The present study was aimed to computationally analyze the binding ability of AccD6, via modeling of AccD6 and docking with activated INH products such as isonicotinic acid, isonicotinaldehyde, isonicotinamide and isonicotinyl acyl radical (INADH). The study is first of its kind to provide structural details of AccD6 as a target in addition to InhA and its interaction with the activated INH.

Methodology:

Homology modeling of AccD6:

Modeling was performed using MODELLER 9v3 **[13]** by the following steps: 1. Template Selection: The target protein AccD6 was submitted to protein based Basic Local Alignment Search Tool BLASTp **[14]** from National Center for Biotechnology Information (NCBI) database and was searched against Protein Data Bank (PDB). It showed a percentage identity of 43% with the template protein AccD5 (PDB code-2A7S) that belongs to same group of AccD6. 2. Target-template alignment: The target-template alignment was done using the MODELLER command line with the language python. 3. Model Building: The refined sequence-structure alignment as obtained by MODELLER was used to construct 3D models of the target with the help of the known structures of the template. 4. Model Validation of the models was done by Ramachandran plot **[15]** Combinatorial Extension (CE) **[16]** and Discrete optimized protein energy (DOPE) feature in Discovery Studio (DS) **[17]**. The model was validated to eliminate the structural errors present in the generated model and to improve its quality and stability.

Docking of activated INH with AccD6:

Docking was performed using CDOCKER **[18]**. The ligand with all H atoms was built using DS. The AccD6 model was energy minimized with CHARMm force field before performing docking. The ligands comprises of activated form of INH including INADH, isonicotinic acid, isonicotinaldehyde, isonicotinamide and acetyl-CoA. The CDOCKER protocol is an implementation of the CDOCKER algorithm in the Discovery Studio (DS) environment. It allows running a refinement docking of any number of ligands with a single protein receptor. CDOCKER is a grid-based molecular docking method that employs CHARMm. The receptor is held rigid while the ligands are allowed to flex during the refinement. Docking was done using the default settings. The default speed selection was used to avoid a potential reduction in docking accuracy. There are two methods employed to define the site: a) Finding sites from the receptor cavities (b) Defining the site as the volume of a selected ligand.

Results and Discussion:

Homology Modeling:

In the present study, the structure of AccD6 (an important enzyme for mycolic acid synthesis in *Mtb*) was modeled due to the lack of crystal structure in the PDB. Modeling of AccD6 enables to understand its role in binding of activated INH (INADH). We provided structural details of AccD6 and its interaction with the activated INH. In the results of BLAST search against PDB, 2A7S was identified as the template protein, which had a sequence identity of 43% with AccD6 of *Mtb*. The aligned target and the template sequences were in PIR format. The template sequence was submitted to Pfam, PROSITE databases. The pattern, profiles and the domains obtained were compared with the target sequence. The target sequence was found to contain carboxyl transferase domain, which spans a region of 1-471, and in template the same domain spans a region of 46-546 amino acids. Amino acid sequence of the target was aligned to the template sequence based on the secondary structure information for building an accurate model by ClustalW. The model generated by the above method was subjected to validation using the following softwares: I) Ramachandran Plot using RAMPAGE server: The Ramachandran plot for the modeled structure showed 92% of the residues in the most favored region, 5.8% in the additional allowed region, and 2.3% in the generally allowed region and no residues in the unfavorable region. II) CE (Combinatorial Extension) method: The model obtained was evaluated using CE method. Structurally conserved regions (SCRs) for the target sequence and the templates were determined by superimposition of two structures through sequence alignment. The root mean square deviation (RMSD) score was calculated using the sequence identity and gaps in the alignment displaying RMSD value of 1.0 Å and Z-score of 8.0. III) ERRAT: The structure was submitted to structural analysis and validation server (SAVS), the ERRAT score for the protein was found to be 75.01%. d) DOPE score: Five models were generated by the modeling option in the DS environment for AccD6. Out of these; one of the models with highest dope score of -46172.78 was selected for docking.

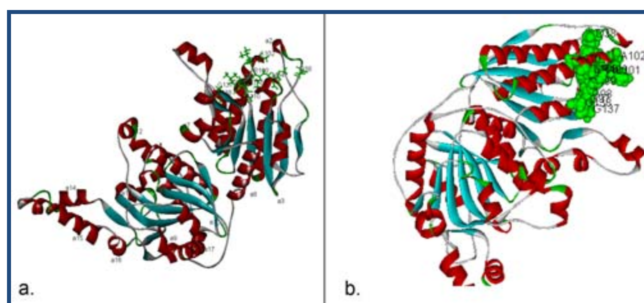

Figure 1: Monomeric structure of AccD6 showing the active site residues (green) in **a)** stick model and helices are labeled, **b)** space filled model.

The initial model was thus generated with the above procedure. As AccD6 is a hexameric protein, modeling all the six chains in a single MODELLER program was tedious so each chain was sequentially modeled as described above. The atom files of all the six chains (ABCDE and F) modeled sequentially was compiled as a single file to obtain the hexameric structure of the protein. The modeled AccD6 was found to contain 25 helices and beta sheets and 51 turns in a single chain. The amino acids

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at the ligand-binding site (i.e. active site) were analyzed to be Met64, Ala67, Gly97, Gly98, Ala99, Arg100, Leu101, Leu108, Gly137 and Gly138 which could be the key residues for its catalytic function **(Figure 1)**.

Docking of AccD6 with activated INH:

The model AccD6 was docked with acetyl-CoA and active forms of INH viz; INADH, isonicotinic acid, isonicotinaldehyde, isonicotinamide using CDOCKER. The values suggest that the ligand INADH could be the correct inhibitor for AccD6 compared to others. We therefore speculate that the higher score (69.92kcal/mol) in case of INADH could be better one on the basis of good-fit, wherein the AccD6 contains sufficient binding space (cleft) enabling the INADH moiety to fit in perfectly. The other smaller molecules (isonicotinic acid, isonicotinaldehyde, isonicotinamide) were unable to produce higher score due to the spatial inconsistency. Supportingly, the obtained score for INADH was similar to acetyl-CoA (substrate) reinforcing the concept of good inhibitor compared to others **(Table 1, see Supplementary material)**. Since INADH has structural complexity similar to acetyl-CoA, also that it produced similar score to that of acetyl-CoA compared to others, which signifies the classical concept of inhibitor design on the basis of substrate similarity **(Figure 2)**.

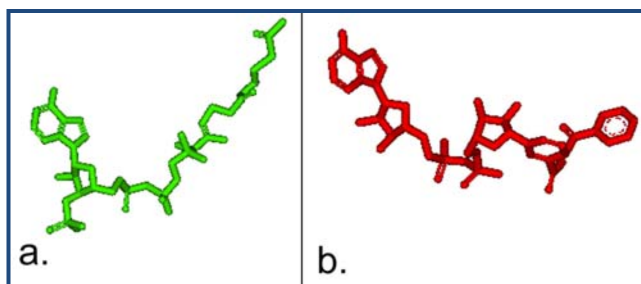

Figure 2: Structural similarity between **a)** Acetyl-CoA and **b)** INADH

The H bond profile at the ligand binding sites of AccD6 with INADH and acetyl-CoA was illustrated in **Figure 3** and **Table 2 (see Supplementary material)**. The H bond profile of AccD6 at the ligand binding sites indicates residues such as Leu101, Ala102, and Gly138 forms 3 H bonds with atoms of INADH molecule whereas the profile for acetyl-CoA indicates 1 H bond formation with residue Ala99. No H bond was found with the other ligands such as isonicotinic acid, isonicotinaldehyde, isonicotinamide. Thus, more number of H bonds in the INADH molecule also indicates that it is a good inhibitor for the target (AccD6). Hence the score and H bond profile suggests that AccD6 has more binding affinity towards INADH, therefore these observations correlates with earlier reports **[4, 5]** that the gene *accD6* is switched on during INH exposure.

INH has multiple effects on the tubercle bacillus, and its activity resulting in cell death is still unclear. One of the first consequences of INH action on *Mtb* to be noticed was the loss of acid fastness, and this was shown to result from inhibition of the synthesis of mycolic acids **[19]**. These long-chain, unsaturated fatty acids, which contribute to the impermeability of the cell envelope, are confined essentially to mycobacteria and are thus a selective target for drugs. The best-known target is the cell wall mycolic acid synthesis pathway **[20]**, where atleast two enzymes, InhA and KasA have been identified as targets for INH activation. However, in the present study, AccD6 is identified as the target for INADH. It was speculated that the active form of INH ranged from production of nonspecifically toxic free radical species to the generation of a range of reactive (both reactive oxygen and organic) radicals, which then attack multiple targets in the tubercle bacillus **[21]**. In relation to which, in the present study, the active species such as isonicotinic acid, isonicotinaldehyde, isonicotinamide and INADH were taken into account.

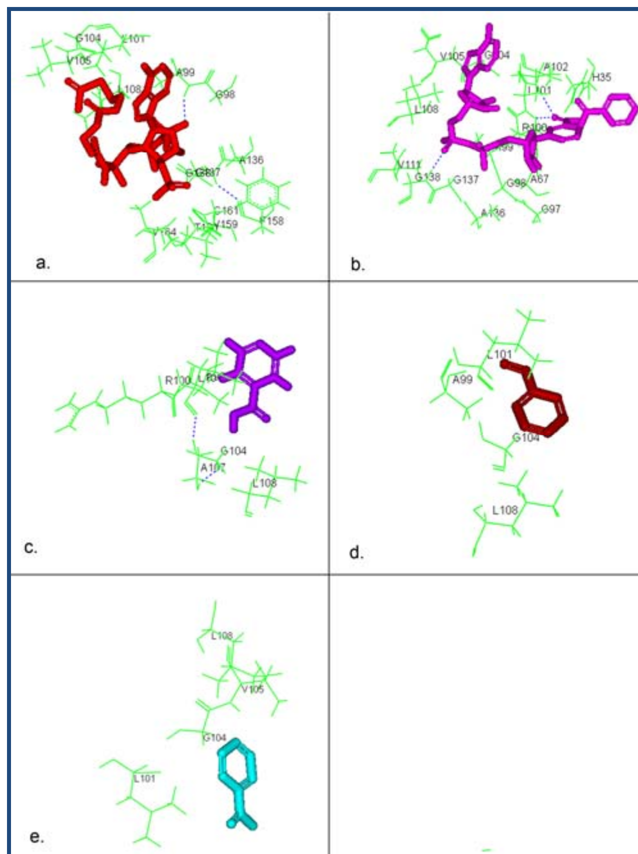

Figure 3: H bond display indicated in blue dotted lines of AccD6 with **a)** acetyl-CoA; **b)** INADH; **c)** Isonicotinamide; **d)** isonicotinaldehyde; **e)** Isonicotinic acid

Conclusion:

In conclusion, we have presented the model structure of AccD6 and predicted its binding ability with INADH, suggesting that AccD6 could also be the target for INADH in addition to InhA. There are still many avenues to be explored using AccD6 as a new target for the structure-based development of novel anti-mycobacterial drugs **[22]** since it has been involved in cell wall synthesis. This study adds new knowledge in the research area of identification of additional targets for activated INH, and is in agreement with the report **[23]** which suggests that the identification of additional targets for activated INH is an important future endeavour.

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Supplementary material:

Table 1: Docking score

Ligands	Score (kcal/mol)
Acetyl-CoA	66.14
INADH	69.92
Isonicotinic acid	11.27
Isonicotinaldehyde	4.69
Isonicotinamide	3.80

Table 2: H bond profile at the ligand binding site

Ligands	No. of Hb	Hb donor	Hb acceptor	Bond distance (Å)
Acetyl-CoA	1	ALA99:HN	aco4:O26	2.10
		ALA107:HN	GLY104:O	2.22
		GLY137:HN	PHE158:O	2.17
INADH	3	LEU101:HN	INADH:O40	2.23
		ALA102:HN	INADH:O40	2.21
		ALA107:HN	GLY104:O	2.22
		GLY138:HN	INADH:O3	2.06
Isonicotinic acid		GLU103:HN	ARG100:O	2.30
		GLY104:HN	LEU101:O	1.98
		ALA107:HN	GLY104:O	2.22
Isonicotinaldehyde		ALA107:HN	GLY104:O	2.22
Isonicotinamide		ALA107:HN	GLY104:O	2.22

Hb = Hydrogen bonds, aco4 = acetyl-CoA, A = Amstrong

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