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***Research Article** (Open Access)*

THE THREE-DIMENSIONAL STRUCTURE OF HOMOSERINE DEHYDROGENASE FROM MYCOBACTERIUM TUBERCULOSIS: AN *IN SILICO* STUDY

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ABSTRACT
Tuberculosis (TB), despite comprehensive efforts to culminate it, continues to be a global health crisis. Persistent rise of resistance against approved drugs including the newest one-Bedaquiline, raise an alarming situation. It warrants exploration of newer targets indispensable for the bug. In this context, the enzymes of aspartate biosynthesis pathway, a central metabolic pathway, present attractive targets to combat the causative organism, *Mycobacterium tuberculosis* (*Mtb*). Homoserine dehydrogenase (HSD) a key enzyme in the aspartate pathway, catalyses the reversible conversion of L-aspartate β-semialdehyde to L-homoserine, using NAD(H) or NADP(H) as a coenzyme. This enzyme is important for *Mtb* growth and pathogenicity. We report *in silico* structural characterization of *Mtb*-HSD. The crystal structure of *Mtb*-HSD is not yet available; its three-dimensional structure is predicted using AlphaFold tool. Physicochemical and stereochemical parameters were analysed to determine the accuracy of the AlphaFold predicted model. Sequence analysis and modelled structure revealed *Mtb*-HSD as a monofunctional enzyme containing three domains i.e., NAD-binding domain, catalytic domain, and ACT domain. This study forms the basis for future research pertaining to delineation of the active site of the enzyme and its critical interactions to aid inhibitor design against this enzyme.

Keywords: *Tuberculosis, Mycobacterium tuberculosis, Aspartate biosynthesis, Homoserine dehydrogenase, AlphaFold model*

INTRODUCTION
Tuberculosis (TB) is one of the earliest known diseases which has been a persistent health and economic issue for people worldwide, especially in low- and middle-income nations. Even today, TB is one of the top thirteen leading causes of morbidity and mortality globally (Yelamanchi and Surolia, 2021) and also a second leading infectious killer after SARs-CoV-2 in year 2021 (Global Tuberculosis Report, 2022; Chitale *et al.*, 2022). Year 2021 – 2023 recorded an increasing trend of TB incidences, bringing TB incidence rate of 2023 to the level of 2018 (Global Tuberculosis Report, 2024).
Mycobacterium tuberculosis (*Mtb*), the pathogenic bacteria that causes TB, has evolved a resistance to the existing medications, leading to the emergence of drug-resistant strains that create a major barrier to the advancement of TB control strategies (Brouqui *et al.*, 2017). Bedaquiline, the drug which was approved for treatment of drug resistant TB in 2012, has itself witnessed remarkable resistance in last few years (Satapathy *et al*., 2024). Consequently, there is a pressing need to discover novel drug targets. Recent researches have indicated various central carbon metabolic pathways that are crucial to *Mtb* survival and pathogenicity (Cole *et al.*, 1998; DeJesus *et al.*, 2017; Wellington and Hung, 2018). The enzymes in these metabolic pathways can be targeted for therapeutic intervention (Murima *et al.*, 2014). Moreover, essential amino acid biosynthetic enzymes are largely missing in humans and found only in plants, fungi and bacteria (Wellington, 2018; Berney and Berney-Meyer, 2017; Evans and Mizrahi 2015; Wellington *et al*., 2017)

and hence, can serve as selective targets. *Mtb* synthesizes most of its amino acids (Berney *et al.*, 2015) which makes the enzymes of these biosynthesis pathways promising drug target(s). One such pathway is aspartate pathway, where aspartate is converted into several essential amino acids, including threonine, lysine, methionine, and isoleucine (Berney *et al.*, 2015, Pavelka Jr and Jacobs Jr, 1996).

At first, the enzyme aspartate kinase mediates the phosphorylation of aspartate to aspartyl phosphate which is further transformed into L-aspartate- β -semialdehyde by aspartate semialdehyde dehydrogenase (Figure 1). The second product L-aspartate- β -semialdehyde subsequently undergoes metabolism to yield either L-homoserine (L-HSE) or L-lysine. Homoserine dehydrogenase (HSD), EC 1.1.1.3, is a key enzyme in the aspartate metabolic pathway. This oxidoreductase enzyme catalyses critical branch point i.e. the reversible conversion of L-aspartate- β -semialdehyde to L-HSE which is the third step of the aspartate pathway (Figure 1). L-HSE act as the precursor of L-threonine, L-isoleucine, and L-methionine.

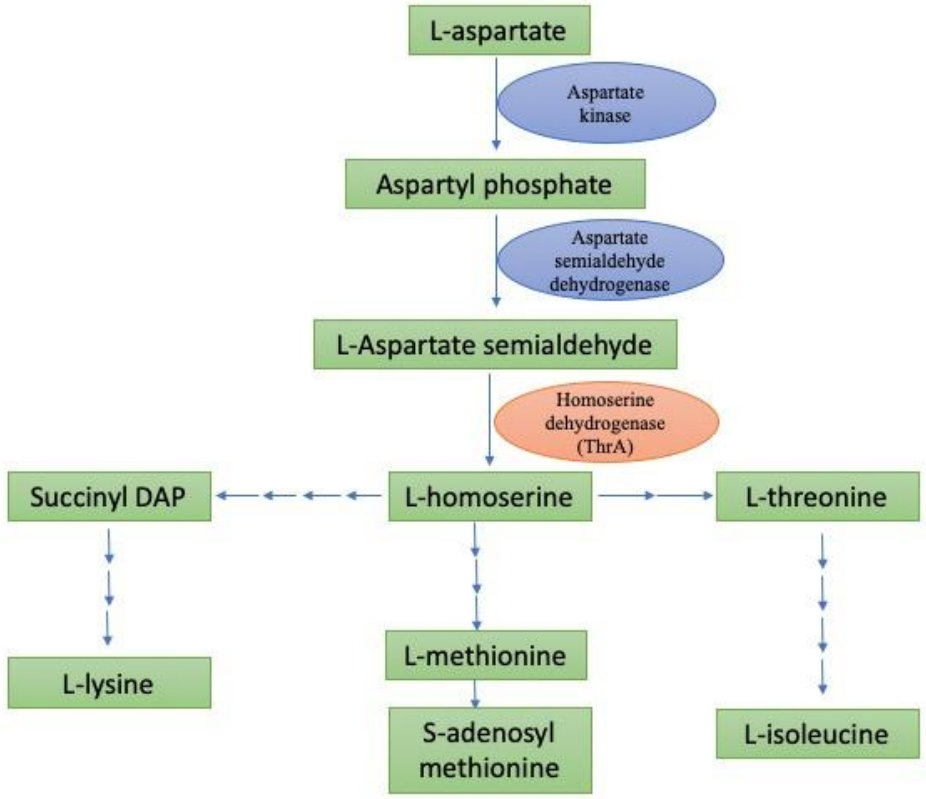

Figure 1: An overview of Aspartate degradation

Threonine and homoserine auxotrophs are bactericidal in *Mtb* (Hasenoehrl *et al.,* 2019) and the accumulation of homoserine in *Mtb* has been shown to be toxic (Yelamanchi and Surolia, 2021). Homoserine dehydrogenase is an established antifungal target (Han *et al.*, 2006; DeLaBarre *et al.*, 2000) and antibacterial target (Tang *et al.*, 2021). Collectively, these researches suggest that aspartate degradation pathway specifically HSD, one of the major regulatory enzymes of this pathway, may prove to be a promising anti-TB target.

Interestingly, this enzyme exists in two forms (Singh and Jha, 2024): monofunctional enzyme having HSD activity in yeast (Jacques *et al*., 2001), some bacteria (Jacques *et al.*, 2001, Kim *et al*., 2020) and archaea (Hayashi *et al.*, 2015) whereas bifunctional enzyme having HSD and additional aspartokinase (AK) activity in few bacteria (Ohshida *et al*., 2018; Omori *et al.,* 1993) and plants (Weisemann and Matthews, 1993; Paris *et al*, 2003). The activity of monofunctional enzyme may or may not be feedback-sensitive depending

upon its domain constitution. The activity of bifunctional enzyme has been reported to be sensitive to feedback inhibition by L-threonine (Navratna *et al*, 2015).
The potential of *Mtb*-HSD as a drug target and the diversity observed in this enzyme among different organisms prompted us to delve further into its three-dimensional (3D) structure which provides the basis for delineation of its active site and corresponding enzyme-substrate interactions that can further be exploited for inhibitor design against it.

MATERIALS AND METHODS

Sequence analysis and physicochemical characterization

The amino acid sequence of Mtb-HSD was subjected to analysis by submitting the sequence in Expasy ProtParam Server (Gasteiger *et al.,* 2005). This analysis provided a range of physicochemical characters of the submitted sequence including number of amino acids, molecular weight, theoretical pI, amino acid composition, number of negatively and positively charged amino acids, atomic composition, extinction coefficients, half-life, instability index, aliphatic index and Grand average of hydropathicity (GRAVY).

Secondary structure prediction

The secondary structure forms the base of protein folding. The secondary structure was predicted using SOPMA (Geourjon & Deleage, 1995) and PSIPred (McGuffin *et al.,* 2000). It provided with details of positions of helix, sheets, turns and other secondary structure elements in the amino acid sequence.

Mtb-HSD 3D structure prediction through AlphaFold AI

The amino acid sequence based predicted 3D structure of Mtb-HSD (AF-P9WPX1-F1) was available in AlphaFold Protein Structure Database (Varadi *et al.,* 2023; Jumper *et al.,* 2021) which hosts structures of over 200 million entries, providing broad coverage of UniProt, and freely accessible to the scientific community.

Structure validation and presentation

The 3D structure downloaded from AlphaFold Protein Structure database was subjected to analysis by employing SAVES v6.0 Structure validation server (https://saves.mbi.ucla.edu/); PROCHECK (Ramachandran plot, Chi1-Chi2 plots, G factors) (Laskowski *et al.,* 2006), ERRAT (Colovos & Yeates, 1993), and Verify3D (Lüthy *et al.,* 1992) programs. Structure presented in Figure 4 is made using PyMOL (Schrodinger, 2015).

RESULTS AND DISCUSSION

Physicochemical characterization of Mtb-HSD

Physicochemical characteristics of the enzyme are summarized in Table 1. Comprising 441 amino acids, the protein is slightly acidic with an isoelectric point (pI) in the acidic range. Its instability index, below 40, indicate a stable protein. An aliphatic index above 100 suggests high thermal stability. A positive Grand Average of Hydropathicity (GRAVY) value indicates polar nature of the protein.

Secondary Structure Prediction

A graphical representation of the secondary structure composition showing, percentage of different secondary structures and number of amino acids involved in forming a specific secondary structure is depicted in Figure 2A and 2B. Alpha helices are significantly more prevalent than beta sheets, while the proportion of random coils is comparable to that of alpha helix. These graphs are representation of the results obtained from SOPMA (Geourjon & Deleage, 1995) predicting three conformational states. Additionally, Figure 2C shows a PSIPred chart (McGuffin *et al.,* 2000) depicting the positions of strand, helices and coils along with prediction confidence. Notably, the confidence of prediction for majority of helices and sheets is significantly high.

Table 1: Physiochemical properties of *Mtb*-HSD

Number of amino acids		441	
Molecular weight		45552.74	
Theoretical pI		4.76	
Amino acid composition			
Amino acid	Abbreviation	Number	Percentage
Alanine	Ala (A)	70	15.9%
Arginine	Arg (R)	28	6.3%
Asparagine	Asn (N)	9	2.0%
Aspartic acid	Asp (D)	27	6.1%
Cysteine	Cys (C)	2	0.5%
Glutamine	Gln (Q)	7	1.6%
Glutamic acid	Glu (E)	30	6.8%
Glycine	Gly (G)	42	9.5%
Histidine	His (H)	5	1.1%
Isoleucine	Ile (I)	21	4.8%
Leucine	Leu (L)	38	8.6%
Lysine	Lys (K)	10	2.3%
Methionine	Met (M)	7	1.6%
Phenylalanine	Phe (F)	7	1.6%
Proline	Pro (P)	18	4.1%
Serine	Ser (S)	26	5.9%
Threonine	Thr (T)	26	5.9%
Tryptophan	Trp (W)	0	0.0%
Tyrosine	Tyr (Y)	11	2.5%
Valine	Val (V)	57	12.9%
Pyrrolysine	Pyl (O)	0	0.0%
Selenocysteine	Sec (U)	0	0.0%
Number of negatively charged residues	(Asp + Glu)	57	
Number of positively charged residues	(Arg + Lys)	38	
Extinction coefficients			
Extinction coefficient*[1]		16515	
Abs 0.1%*[2]		0.363, assuming all pairs of Cys residues form cystines	
Extinction coefficient		16390	
Abs 0.1%		0.360, assuming all Cys residues are reduced	
Estimated half-life			
Mammalian reticulocytes, in vitro		30 hours	
Yeast, in vivo		>20 hours	
Escherichia coli, in vivo		>10 hours	
Instability index (II)		24.98	
Aliphatic index		105.53	
Grand average of hydropathicity (GRAVY)		0.242	

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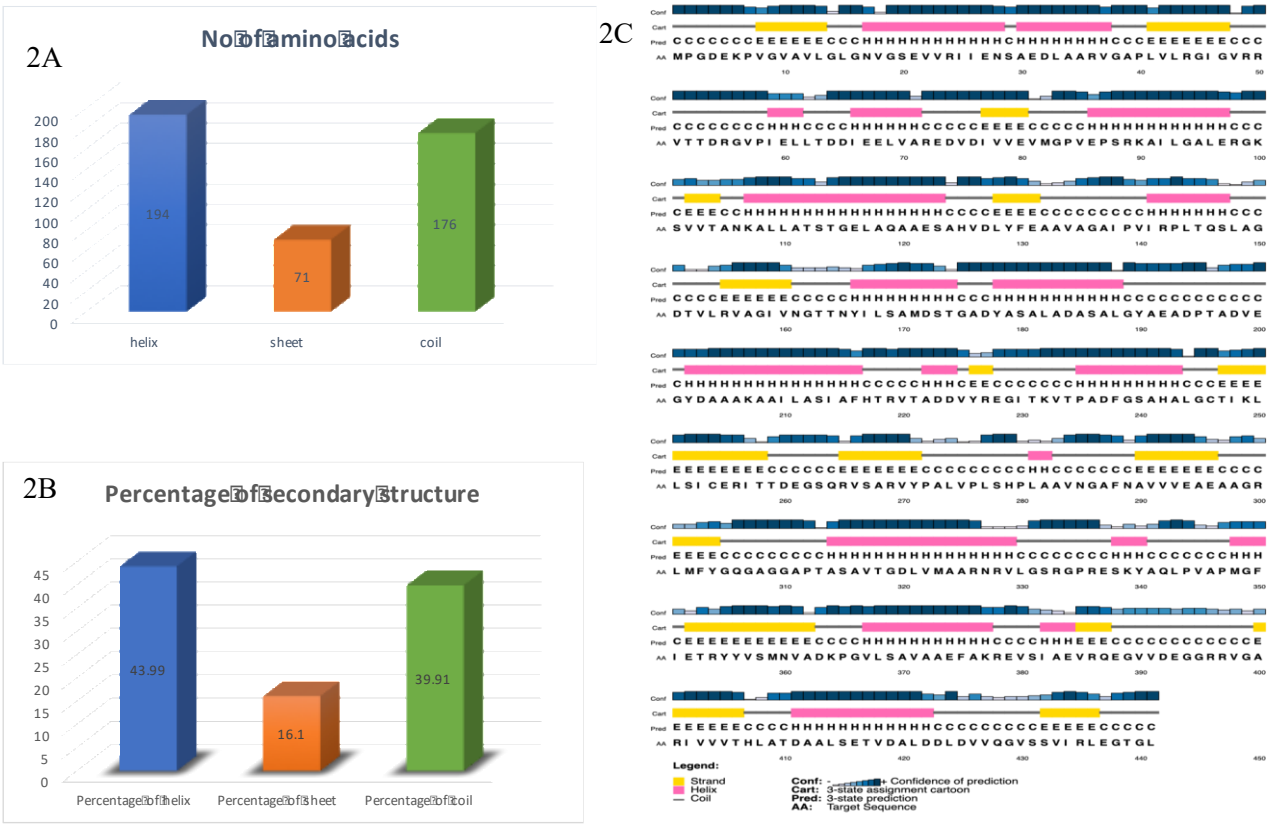

Figure 2: Secondary structure of *Mtb*-HSD. 2A. Number of amino acids involved in forming Helix, sheet and coil. 2B. Percentage of different secondary structures. 2C. Positions of different secondary structures are shown.

3D structure of *Mtb*-HSD

The 3D structure was predicted through the revolutionary AlphaFoldAI (Jumper et al., 2021), developed by Google DeepMind and European Bioinformatics Institute (EMBL-EBI). AlphaFold consistently achieves experimental-level accuracy, especially for proteins with homolog that have experimentally solved structures. A structure for UniProt entry P9WPX1, coresponding to *Mtb* HSD was available in the AlphaFold Protein Structure Database under the label "AF-P9WPX1-F1-model_v4.pdb" (Varadi *et al.*, 2023). The predicted structure was validated using SAVES v6.0 tool (https://saves.mbi.ucla.edu/). At first, Ramachandran plot analysis was performed employing PROCHECK (Laskowski *et al.,* 2012), which evaluates the stereochemical quality of the protein structure on a residue-by-residue basis. Notably, the analysis showed no Ramachandran outliers, indicating a good structure model (Figure 3). The overall quality G-factor was 0.1, signifying an acceptable model (PROCHECK values of the G-factor typically range from 0 to -0.5, with values near zero indicating good models). Since, there were no Ramachandran outliers in PROCHECK stereochemical analysis consequently no bad contacts or abnormal scores were evident for the main-chain or side-chain parameters. Further, ERRAT (Colovos & Yeates, 1993) which measures the quality of non-bonded atomic interactions was employed to assess the model quality. With a significantly high ERRAT score of 95.95, much ahead of the acceptable range (>50), the model can be categorized as a high-quality one. VERIFY 3D (Lüthy et al., 1992) analysis indicated a reasonably good sequence-to-structure agreement as 70.29% of the residues had an averaged 3D-1D score $\geqslant$ 0.1, with only

0.03% residues showing a negative averaged score below -0.05, except one outlier with a score of -0.07. Overall, the model appears to be reasonably accurate.

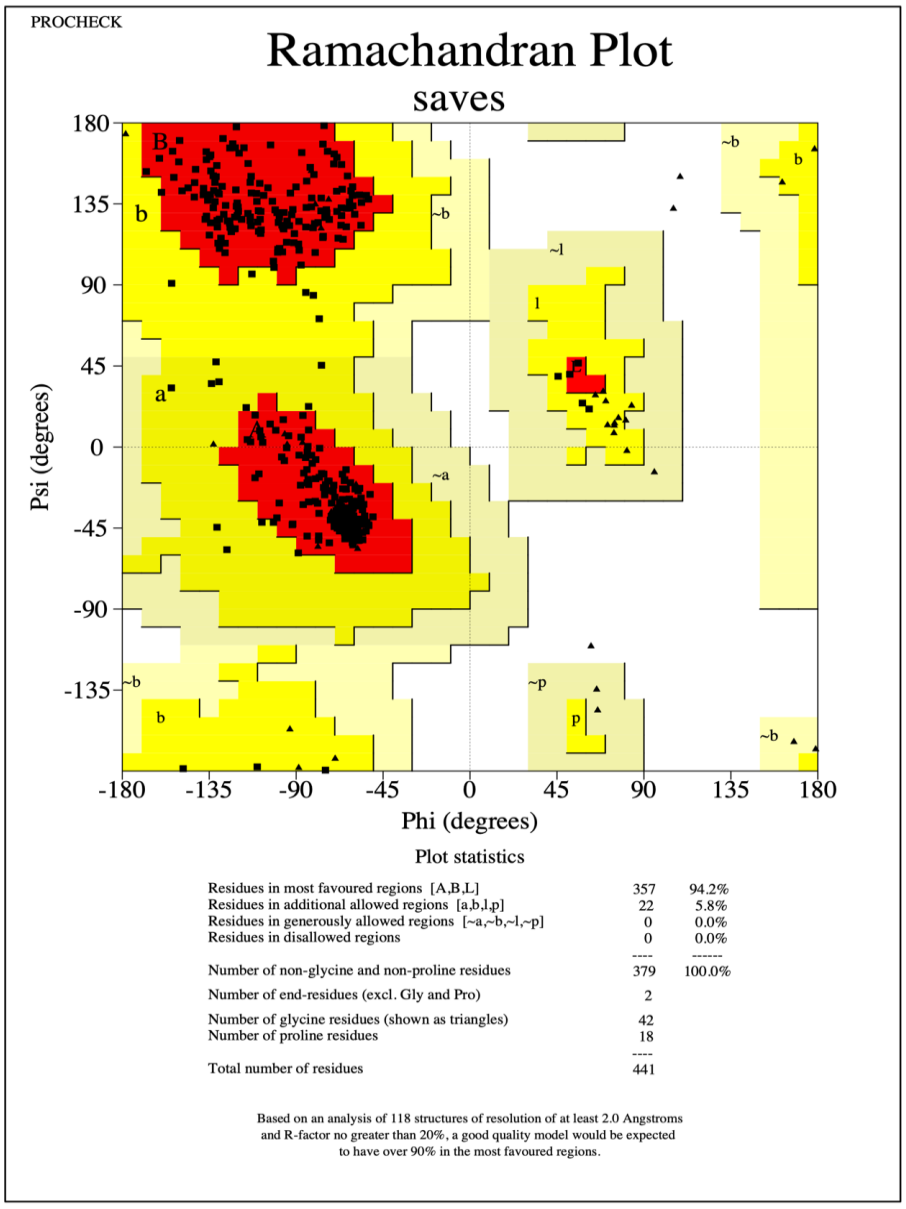

Figure 3: Ramachandran Plot of *Mtb*-HSD structure depicting 94.2% residues in most favoured regions and 5.8% residues in additional allowed regions. No outlier was observed.

Alike reported HSD structures, the three-dimensional structure of *Mtb* HSD also harbours a substrate binding as well as a nucleotide binding domain. Majority of monofunctional HSDs are reported to exist as a homodimer in solution. Nevertheless, the structures predicted through AlphaFold is of a single protein chain and hence, the structure of a monomer of *Mtb* HSD is reported here. However, when we provided an input of two protein sequences in AlphaMultimer (Evans et al., 2021), it built a homodimeric structure suggesting that this might be the case in solution.

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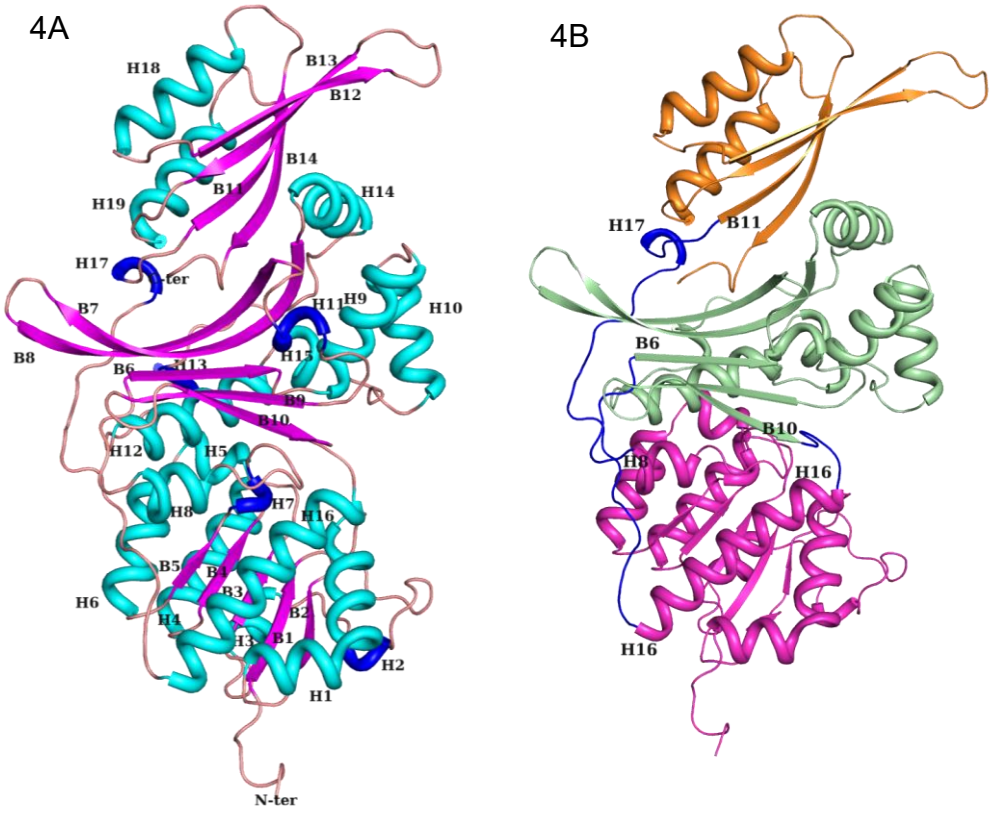

Figure 4: Three-dimensional structure of *Mtb*-HSD. 4A. Three-dimensional structure of *Mtb*-HSD is depicted in cartoon representation. Helices (cyan - α-type; blue - 3_{10}-type) and β-strands (pink) are numbered. Connecting loops are shown. 4B. Three domains of *Mtb*-HSD are shown in different colours: N-ter domain (pink), middle domain (green) and C-ter domain (orange). Connecting regions between the domains are shown in blue. Secondary structures, which share the domain connecting loops, are numbered.

Briefly, the 3D structure (Figure 4A) comprises 19 helices and 14 beta strands, interconnected by loops forming three distinct structural domains: An N-terminal domain, a middle domain and a C-terminal domain. The N-terminal domain – the functional NAD-binding domain is discontinuous (14-131 aa and 306-330). It comprises of five-stranded parallel beta sheet (β2↑β1↑β3↑β4↑β5↑) sanwiched betwwen helices H3, H4 and H6 on one side, and H1 and H16 on the other side (Figure 4A), H16 being the discontinuous segment. Additionally, H2, H5 and H7 flank the sanwiched sheet on the edges. The middle domain (139-322 aa) harbours a five-stranded anti-parallel beta sheet (β8↑β7↓β6↑β9↓β10↑) cupped by helices H8, H12 and H13 on one side and H9, H10, H11, and H14 on the adjacent side. Two 3-10 helices H15 and H17, form the rim of this cup. This domain houses the homoserine dehydrogenase catalytic site (Figure 4A). Finally, the smallest C-terminal domain (355- 437 aa) comprises of helices H18 and H19 situated on top of a four-stranded anti-parallel beta sheet (β12↑β13↓β11↑β14↓) and harbours the ACT regulatory domain (Figure 4A). The characteristic ferredoxin-like fold of ACT, a βαββαβ unit in which four β -strands form an antiparallel β -sheet, while two α -helices are positioned together (Navratna *et al*, 2015) is evident in *Mtb* HSD structure (β11-H18-β12-β13-H19-β14). A dimeric or tetrameric HSD attains

their state through its ACT domains forming a regulatory pocket**.** *Mtb*-HSD, based on the occurrence of ACT domain, is predicted as a feedback-sensitive member of the ACT domain-containing family proteins (Singh and Jha, 2024). The ACT domain appears in various contexts and is perceived to be a conserved regulatory binding fold. It is associated with a broad range of metabolic enzymes whose activity is regulated by amino acid concentrations. (Chipman and Shaanan, 2001), in this case by L-Threonine.
The three domains are shown by different colours in Figure 4B. The discontinuous N-terminal domain is connected to the middle domain through a loop between H8 and β6 and then again through a loop between β10 and H16. The N-terminal domain is connected to the C-terminal ACT domain by a loop-$_{3\text{-}10}$-helix-loop, the connecting region between H16 and B11, which transverse closely to the substrate binding region of middle domain, providing it an opportunity to regulate the catalytic activity. Studies have demonstrated that Corynebacterium HSD, an actinobacterial HSD, contains an ACT domain at the C-terminal end and can be allosterically inhibited by L-Threonine (Archer *et al.,* 1991). Since, *Mtb* is also an actinobacteria, possessing a HSD harbouring an ACT domain, it is likely to be allosterically inhibited by L-Thr. However, this needs to be substantiated by biochemical experiments. The physiochemical parameters categorize the protein as a stable one (Table 1), marking it favourable for overexpression and purification. Further studies exploring the nucleotide binding site and active site of the enzyme are warranted to exploit the potential of this very important enzyme as a target for anti-TB drugs.

CONCLUSION

Mtb-HSD is an essential enzyme for the growth and survival of *Mtb* and is notably absent in mammals, making it a promising selective target for anti-TB therapies. This study investigates the three-dimensional structure of *Mtb*-HSD using an in-silico approach, aiming to explore the different domains of this enzyme. It lays a groundwork for further delineation and characterization of the active site and the co-factor binding site of *Mtb*-HSD. Understanding the overall structure and active site structural features can guide the design of specific inhibitors, contributing to the development of targeted strategies for *Mtb* inhibition.

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