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Molecular regulation of *Mycobacterium tuberculosis* Sigma factor H with Anti-sigma factor RshA under stress condition

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ABSTRACT

Mycobacterium tuberculosis is the causative agent of tuberculosis, the leading fatal infectious disease that claims millions of lives every year. *M. tuberculosis* regulates its stress condition response using its regulatory protein, Sigma Factor H, which binds with its cognate anti-sigma factor RshA in normal conditions, forming a complex inhibiting transcription. During oxidative stress, SigH is released from the complex and binds to RNA Polymerase (RNAP) to initiate transcription. Thus, it is important to understand the molecular conformational state of SigH in complex with different protein partners under different cellular or environmental contexts. This work intends to analyze the SigH-RshA complex, which revealed the variation in SigH shown during complex formation with RNAP and RshA, respectively. Previously, Hydrogen Deuterium Exchange-Mass Spectrometry (HDX-MS) analysis of SigH-RshA interaction provided a detailed insight into the critical residues participating in the interaction. The HDX-MS data were used to dock RshA on the open conformation of SigH from the SigH-RNAP complex structure (PDB: 5ZX2), and closed conformation was obtained from protein modelling. The docking revealed that closed conformation of SigH complexing with RshA in terms of HDX-MS data revealed a major structural shift in SigH while interacting with two different binding partners, RshA and RNAP, under variable environmental conditions. This structural shift of SigH with RshA and RNAP has significance in understanding the stress response of *M. tuberculosis*, and SigH could prove to be a potential drug target.

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1 Introduction

Mycobacterium tuberculosis is the causative agent of one of the most infectious diseases that has ever struck humans. This bacterium's ability to perceive its environment and adapt its physiology accordingly necessitates delicate gene expression regulation for survival in the varying immune signals by the host (Shi et al. 2003; Rohde et al. 2007). Gene expression in bacteria relies on transcription, whose initiation depends on multiple factors, including RNA Polymerase (RNAP), which comprises several subunits, including the sigma factor. Sigma factors, a separable class of RNAP, play a vital role in transcription initiation (Feklístov et al. 2014). They are responsible for linking to the core RNA Polymerase and guiding it through the steps leading to initiation (Manganelli et al. 1999). These include specific recognition of the promoter, separation of the DNA strand and synthesis of the initial nucleotides. This might result in the dissociation of the sigma factor from the RNAP (Borukhov and Nudler 2003).

Among two classes of σ^{70} and σ^{54}, the sigma 70 family includes several primary sigma factors with linked alternative sigma factors. Another group of a phylogenetically unrelated subfamily called the extracytoplasmic function factors, or ECF lies among the Sigma 70 families (Helmann 2002). SigH is one alternative sigma factor found in Mycobacterial species and regulates stress response conditions like heat and oxidative stresses (Raman et al. 2001). It has been demonstrated that SigH is necessary for the stress-induced expression of the genes for SigE and SigB, the two additional alternative sigma factors implicated in the *Mycobacterial* response to various stimuli (Manganelli et al. 2001). Thus, an upregulation in the transcription of auto-regulated (SigH-dependent) promoter causes an increase in SigH expression to adapt to oxidative or heat stress (Raman et al. 2001)

Despite the increased expression of SigH, its enzymatic activities are inhibited by a family of proteins known as the anti-sigma factors. These factors bind SigH and prevent its participation in the RNAP-mediated transcription process. Among these anti-sigma factors, RshA expression is controlled by a gene present in the SigH operon. RshA inhibits SigH-dependent transcription in *M. tuberculosis* (Raman et al. 2001; Song et al. 2003). The autoregulation of the SigH promoter controls RshA function at transcriptional, translational and post-translational levels. During post-translational modification, SigH interacts with its associated anti-sigma factor RshA (Newman et al. 2001; Li et al. 2003). RshA binds to SigH as a redox response signal, and upon oxidation of particular cysteine residues, a conformational change in the sigma factor leads to its release and allows binding with the RNA polymerase holoenzyme. This SigH-RshA complex gets disrupted under oxidizing conditions, allowing SigH to interact with RNAP, leading to transcription initiation. Until SigH rebinds with RshA, a stable transcription of the SigH regulon is maintained (Song et al. 2003). This leads to the activation of the sigmulon (Kang 1999; Song et al. 2003). On reversal of favourable conditions, the reduction state is induced, during which the SigH binding capability of RshA is regained, and thus, expression is inhibited again (Jung et al. 2011).

There are 10 ECFs present in *M. tuberculosis*. Each member of the ECF sigma factors, along with RNAP, identifies and interacts with promoters and σ_4 and σ_2 recognizes the distinct sequence present at -35 and -10 elements, respectively (Campagne et al. 2014). Further, SigH binds to the RNAP-β subunit and creates a channel for the entry of template ssDNA, which binds to the active site of RNAP and initiates the transcription process. The channel exit is destined for the release of transcribed RNA but is blocked by the SigH-RNAP-β initiation complex. A conformation change in SigH dissociates the SigH-RNAP-β complex, opening the channel exit (Zhang et al. 2012; Dolatshahi et al. 2016; Li et al. 2019). Another transcription initiation complex, SigH-Rpo, represents a similar binding interface in SigH-RNAP binding. The active site cleft holds the DNA/RNA hybrid at a post-translational condition while the duplex DNA settles in the main channel (Campagne et al. 2014, 2015). Various interactions involved in the recognition and unwinding of the promoter by SigH-RNAP were also observed in SigH-RPo. A decrease in infectivity occurred in SigC and SigD's deletion, while SigE and SigH's deletion was responsible for virulence (Rodrigue et al. 2006; Sachdeva et al. 2010).

The inhibition of the Sigma factor by its associated anti-sigma factor depends on the reversible protein-protein interaction (ppi) (Duncan and Losick 1993; Hughes and Mathee 1998). In earlier biochemical studies, the purified SigH and RshA were shown to form a stable complex only in reducing conditions (Song et al. 2003; Jamithireddy et al. 2017). Although they interact in reducing conditions, their stability gets affected at temperatures higher than 50ºC, leading to dissociation of complex and release of SigH. Thus, RshA acts as a heat sensor/ oxidative stress sensor to regulate the transcriptional role of SigH (Song et al. 2003).

The disc diffusion assays with diamide showed that mutated SigH is more sensitive than wild-type parental strain, indicating the importance of SigH in bacterial survivability (Fernandes et al. 1999). A wider zone of inhibition was observed in inducing the expression of RshA into the wild-type strain. This may be due to the inhibition of WT SigH for transcription activity in excess RshA (Raman et al. 2001). The sequence analysis of RshA shows that certain portions were conserved among *Mycobacterium sp*. Among these conserved regions, five cysteine residues were tested by swapping them with alanine to study the effect on inhibition of SigH. Three of them (Cys11, Cys41, Cys44) showed decreased effectivity, while two of them

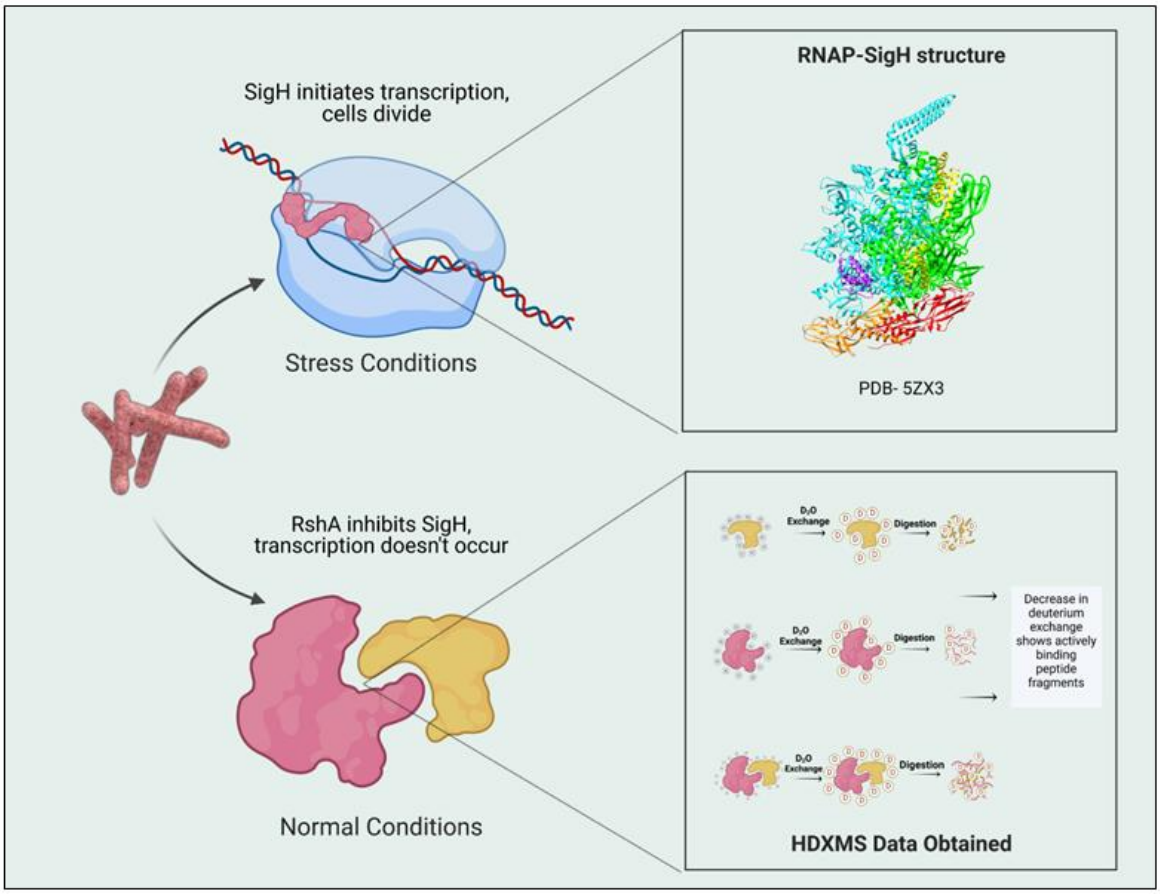

Figure 1 Schematic representation of *Mycobacterium tuberculosis* regulating transcription using Sigma Factor SigH. In stress conditions SigH binds to RNA Polymerase to initiate transcription. In normal conditions, Anti sigma factor RshA binds to SigH and inhibits transcription. The SigH-RshA interaction was analyzed by performing HDXMS (Image Created by Biorender).

(Cys61 & Cys62) showed no effect, which suggested the necessity of cysteine residues for the proper functioning of RshA in inhibiting SigH transcription (Paget et al. 2001).

A comparative study between the survival capacity of wild-type *M. tuberculosis* H37Rv, the SigH mutant strain RH349, and the SigH-complemented mutant strains RH377 and RH395 showed that the SigH mutant has more susceptibility to both diamide and plumbagin (Hassan and Fridovich 1979).

These studies showed the significance of the SigH-RshA complex in regulating the transcription process under both normal and stress conditions of *M. tuberculosis.* Without any molecular structure of this complex, a Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS) study was performed to identify the interface between SigH and RshA. In this study, we analyze the conformation of SigH while forming a complex with RshA compared to a SigH-RNAP complex. Here, we are using previously published HDX-MS data of interaction between SigH and RshA to predict an accurate model of interaction which will decipher the conformation changes associated with SigH in different physiological conditions (Kumar et al. 2012).

2 Materials and Methods

2.1 Amino acid sequence retrieval

The National Centre for Biotechnological Information (NCBI) database was used for retrieval of amino acid sequences in FASTA format for both SigH and RshA proteins. After this, homology modelling was done, followed by further computational analysis.

2.2 Homology Modelling and Validation of 3D models

SWISS-MODEL and DISTILL 2.0 were used to carry out three-dimensional modelling of the protein sequences (Baú et al. 2006; Waterhouse et al. 2018). After creating the models, UCSF Chimera and PyMOL were used to visualize and analyze them (Pettersen et al. 2004). The predicted three-dimensional models were validated using the servers- PROCHECK v.3.5 and ProSA-web (Protein Structure Analysis) (Laskowski et al. 1993; Wiederstein and Sippl

Table 1 List of the values for the Most favoured region and disallowed regions as validated using PROCHECK for Models 1-6

Model	RM Plot	
	Most Favoured Region	Disallowed Region
1	82.6	0.6
2	93.4	0
3	91.1	0
4	75	4.2
5	87	1.3
6	79.6	1.9

1, 2 - Closed and Open conformation of SigH; 3,4 - Closed and Open conformation of RshA; 5,6 – Closed and Open conformation of docked SigH-RshA

2007). Ramachandran plot was also predicted using PROCHECK. Residues were present in disallowed regions, allowed regions, additional allowed regions and most favoured regions. The quality of the analytical structure of these 3-D models were determined by by these values. Additional validation involved the calculation of the Z score of both models using ProSA-web. Protein position is determined by the Z score within experimental NMR and X-ray structure of equal residue length. The model's energy was also obtained from it (Sippl 1995).

2.3 Data from Previous HDX-MS of SigH and RshA

HDX-MS was used for free SigH, free RshA and the SigH-RshA complex. After comparison and analysis of all three, pepsin digest fragments between SigH and the SigH-RshA complex were identified in 32 regions and for RshA and the complex, 20 common fragments were identified. For SigH, 71% of the primary sequence was covered by the pepsin digest fragments, and in the case of RshA, 88% coverage was shown. Observations on carrying out Deuterium exchange for 10 minutes for all three were listed. Excluding two fragments, all showed a decrease in exchange after complex formation. Five of these regions (1-25, 58-69, 90-111, 115-132, 157-171) showed >1 decrease in exchange (Table 2). In the case of RshA, compared to the SigH-RshA complex, only two overlapping sequences (34-49, 35-57) showed a decrease in exchange more than 1 (table 3). This decrease in exchange indicates the involvement of these regions in the RshA-SigH complex formation. Four of the fragments showed an increase in exchange, which could have been a result of a shifted domain due to the binding of RshA with SigH (Kumar et al. 2012).

2.4 Molecular docking analysis

Molecular docking analysis is a basic procedure for characterizing the interaction between two proteins and their binding affinity. The molecular docking for the predicted models of SigH and RshA was done using High Ambiguity Driven protein-protein DOCKing @BonvinLab (HADDOCK 2.4) web server (de Vries et al. 2010). Two input data were provided for the docking analysis: Molecule 1 was SigH, and Molecule 2 was RshA. The active residues were identified from the HDX-MS data of the previous studies as they are predicted to be directly involved in the complex formation. These residues were provided as input parameters. Once the molecular docking was successfully completed, the docked complex was visualized using PyMOL.

Table 2 Peptide fragments showing >1 decrease in Deuterium Exchange

No.	Pepsin digest fragment	Peptide, m/z, Charge state	SigH in complex with RshA	SigH
1	1-25	MADIDGVTGSAGLQPGPSEETDEEL (1259.56), +2	9.360.1	12.060.1
2	58-69	LQETMVKAYAGF (679.35), +2	6.560.1	7.760.4
3	90-111	YINSYRKKQRQPAEYPTEQITD (910.12), +3	7.860.3	10.060.0
4	115-132	ASNAEHSSTGLRSAEVEA (908.43), +2	5.460.1	7.360.0
5	157-171	YYADVEGFPYKEIAE (897.418), +2	8.260.2	9.460.1

Table 3 Peptide fragments showing >1 decrease in Deuterium Exchange

No.	Pepsin digest fragment	Peptide, m/z, Charge state	SigH in complex with RshA	RshA
1	34-49	LDGECTPETRERLRRH (656.664), +3	8.660.4	10.260.7
2	35-57	DGECTPETRERLRRHLEACPGCL (881.084), +3	13.160.0	15.060.1

2.5 Validation of Docked complexes

The HADDOCK score carried out the primary analysis of the docked complex. RMSD from the overall lowest-energy structure and a few other energy parameters were also obtained, including Van der Waals, Restraints violation, Desolvation, Electrostatic energy, Buried Surface Area and Z score. Further validation of the complex was done using PRODIGY (PROtein binDIng enerGY prediction) and also ProFace (Analysis of protein-protein interface)(Saha et al. 2006; Xue et al. 2016). PRODIGY also provided evaluated values for the binding affinity (ΔG) and dissociation constant (kd) values of the SigH-RshA docked complex at 37ºC. The ProFace web server was also used to determine the Interface Area(A) and Patch Area(A^2).

3 Results and Discussion

3.1 Creation of SigH and RshA model

Swiss model was used to predict the model for both SigH and RshA (Model 2 and 4), and Distill 2.0 was used for their closed conformation models (Model 1 and 3). The Swiss model predicted an open conformation for SigH that is very similar to the molecular state in a complex with RNAP (PDB: 5ZX3), while Distill 2.0 predicted a closed conformation of SigH. The Generated 3D models (Figure 2 and 3) were validated using PROCHECK for the Ramachandran plot. The open conformation model of SigH, which resembled its structure in the SigH-RNAP complex for initiating bacterial transcription, did not comply with the HDX-MS data for SigH-RshA interaction. The retrieved data from the previous HDXMS study was used to identify regions actively involved in binding. The regions that showed decreased exchange after complex formation were highlighted on models with the blue colour of both proteins. SigH consists of 5 such regions (table 2), and RshA has 1 (table 3). The multiple sequence alignment of both proteins were performed for three pathogenic strains of bacteria. The blue box highlights the conserved residues participating in the interaction. Except for the N-termini of SigH, all other regions contributing to interaction in both the proteins are largely conserved, implying that the SigH-RshA complex formation may be observed mainly among various species of bacteria.

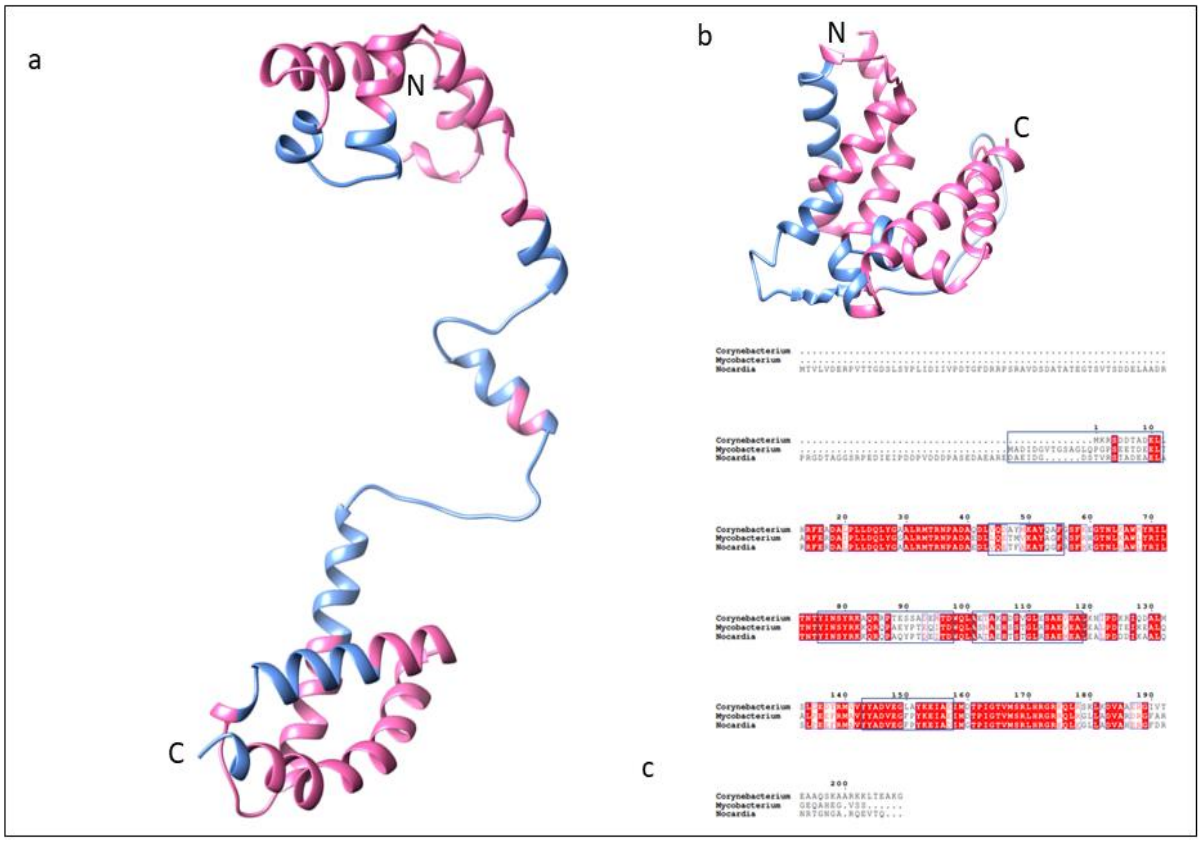

Figure 2 SigH a)The open conformation SigH model (Model 1) was predicted by using the SWISS MODEL server, and b) The closed conformation SigH model (Model 2) was prepared using the Distill 2.0 server. The protein in Pink and blue indicates the regions showing decreased exchange levels upon interaction with its cognate anti-sigma factor RshA. The actively binding sites are listed in Table 2, c) Multiple Sequence Alignment of SigH present across various pathogenic bacterial species shows that the actively binding regions (shown in the blue box) are conserved (shown in red) among them.

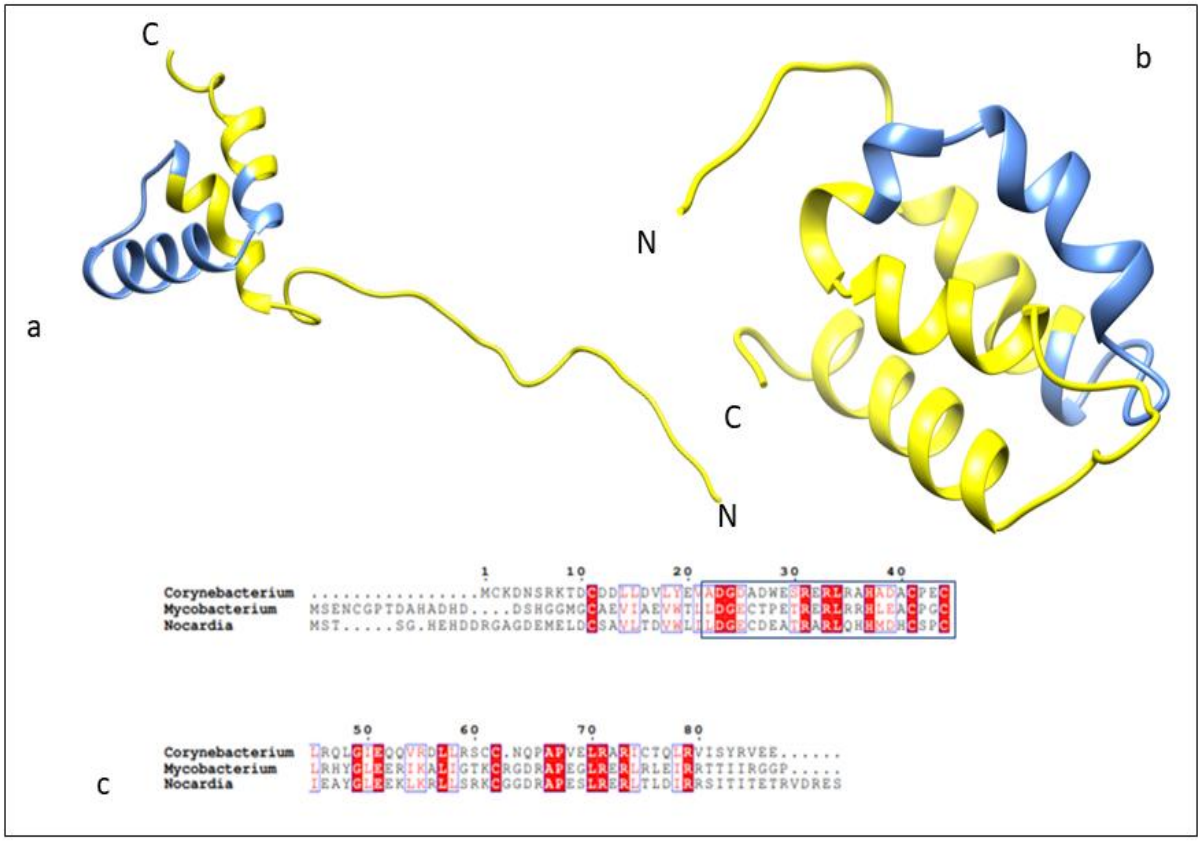

Figure 3 RshA a)The open conformation RshA model with a long loop at N-termini (Model 3) was predicted by using the server Distill 2.0, b) The closed conformation RshA model with a short helix at N-termini (Model 4) was predicted by using SWISS-MODEL, The protein is shown in yellow and blue indicates the regions showing decreased exchange level upon interaction with its cognate anti-sigma factor RshA. The actively binding sites are listed in Table 3, c) Multiple Sequence Alignment of RshA present across various pathogenic bacterial species shows that the actively binding regions (shown in the blue box) are conserved (shown in red) among them.

3.2 Molecular Docking of SigH-RshA

Previously, there was a known structure for SigH-RNAP where SigH encircles the β subunit of RNAP, and its complex unwinds the promoter and initiates transcription. There was still a lack of any known structure of SigH-RshA even though their residual details participating in interaction, complex formation and correspondence were proved through many experimental observations. Molecular docking of the designed protein models and their analysis provided both open and closed conformation models (Model 5 and 6) of the SigH-RshA interaction. Proper binding of the two proteins was not observed in the open conformation as it did not follow the experimental HDX-MS data, although the molecular docking of RshA with the closed conformation of SigH matched the stabilized regions represented by HDX-MS of SigH-RshA complex. So, the closed conformation model was chosen for further analysis. Even though the open conformation of SigH didn't comply with the HDX-MS data, it resembles its conformation in the SigH-RNAP complex ((PDB: 5ZX3). These also represent the structural flexibility of SigH in two different conditions.

3.3 Conformational variations in SigH at different environmental conditions of *Mycobacterium sp.*

Even though SigH has been known to keep an open conformation, as observed from the SigH-RNAP structure (PDB-5ZX3), the same conformation did not follow the HDX-MS data during protein-protein docking of SigH-RshA. Instead, a different closed conformation model of SigH seems to dock with RshA while following the experimental data (Table 2). On comparing the conformation of SigH from the SigH-RNAP crystal structure with the HDX-MS guided docked model of SigH-RshA, it was evident that SigH undergoes a major conformational shift while interacting with two different protein partners. The SigH chain has an open conformation not only when in complex with RNAP but also when it's free and a closed conformation following the interaction with RshA. This conformational change is also evident when the two complexes of SigH-RNAP and SigH-RshA are contrasted. This indicates the presence of certain specific regions, whose accessibility changes due to the conformational change and hence acts as a key to the specific interactions.

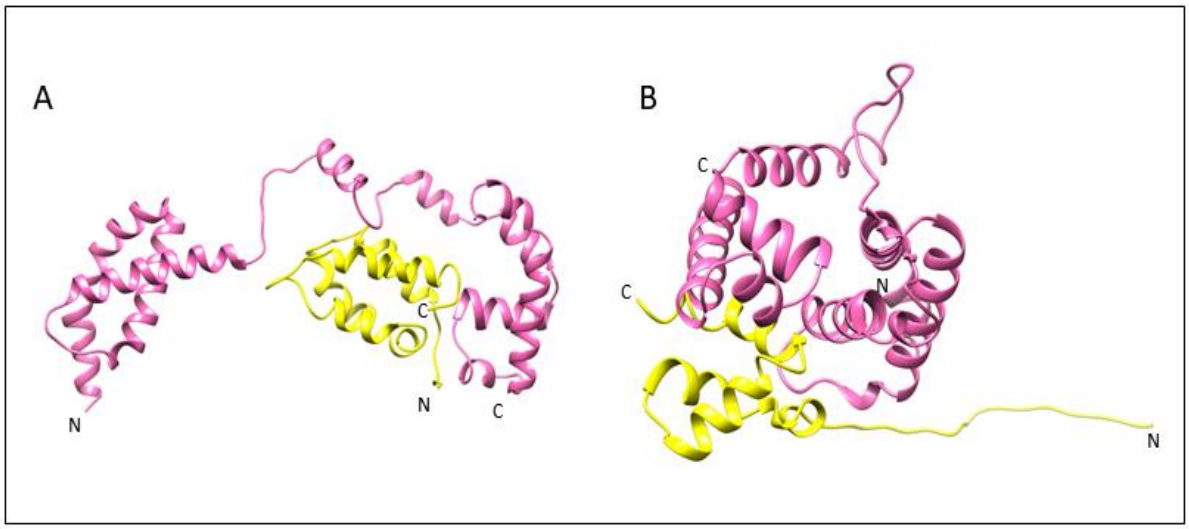

Figure 4 Docked models of SigH-RshA interaction, the models (Model 5 and 6) were prepared by molecular docking using the server PatchDock; SigH is shown in Pink and its partner RshA is shown in yellow; (A) the open conformation models, SigH in its open conformation was obtained from the SigH-RNAP structure (PDB-5ZX2); (B) The closed conformation model, the predicted 3D Model of SigH was used for this model

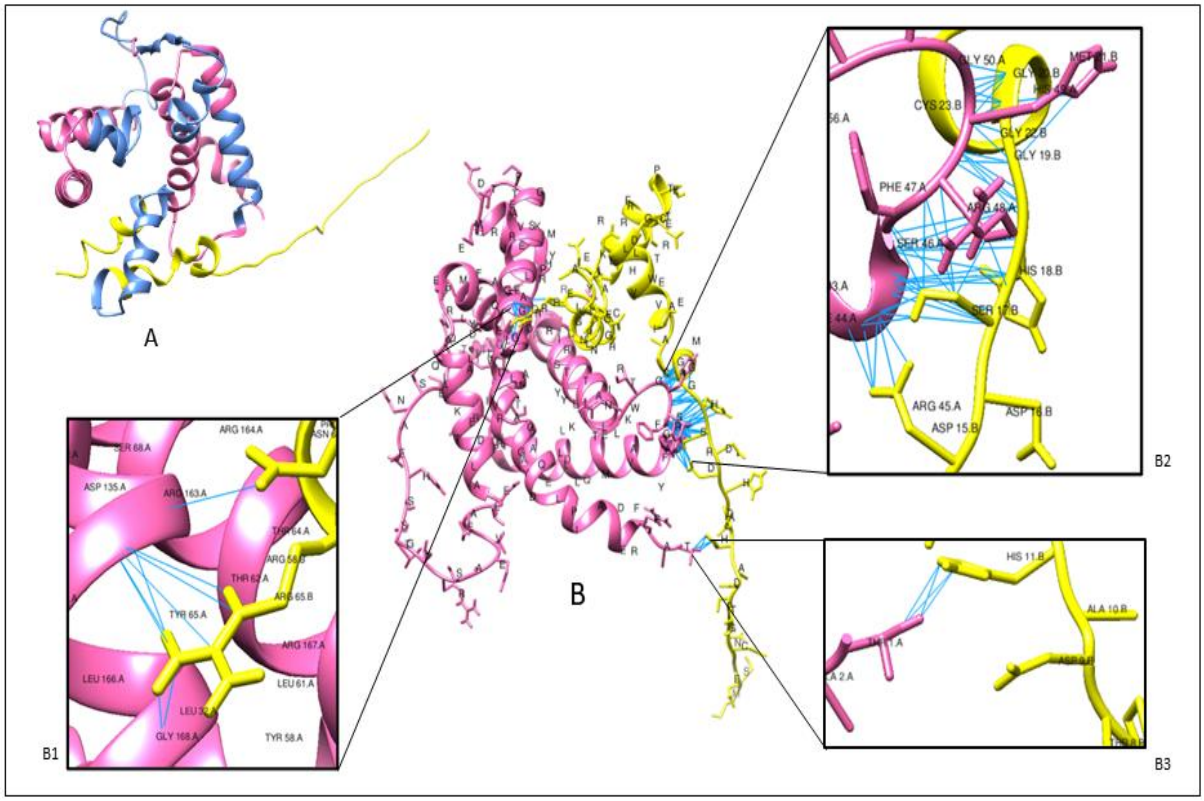

Figure 5 Model of Interaction A) A model for the interaction of SigH-RshA is proposed, which was prepared through docking, using PatchDock; SigH is shown in pink, RshA is shown in yellow and blue represents the regions showing decreased exchange upon interaction; B) The interactions between the two proteins are shown in light blue. These bonds were found to comply with the experimental data previously obtained through HDXMS. These actively binding residues did not resemble the ones involved in SigH-RNA Polymerase structure and hence showed a simultaneous change in accessibility with the conformational change. The binding residues are listed in Table 2 and 3.

3.4 Non-overlapping interacting residues

Previous findings showed that SigH interaction with the β subunit of RNAP unwinds the promoter and creates a channel for the passage of ssDNA, hence initiating transcription (PDB-5ZX2). Without any SigH-RshA structure, the proposed closed conformation model supported by HDX-MS experimental represents a different SigH conformation. The interacting residues were observed using PyMol and paralleled with the ones involved in SigH-RNAP interaction. The residues of SigH in the former case were not identical to the residues that are involved in SigH-RNAP complex formation. Hence, this distinct involvement shows the significance of these particular residues in the protein's specificity towards RshA. The conformational change that the protein goes through between the two complexes can be the reason for the accessibility of these particular residues, which have been observed to play a vital role in the SigH-RshA complex formation.

Conclusion

Mycobacterium tuberculosis, the causative agent of the infectious disease tuberculosis, survives in stress conditions due to the stress response mechanism regulated by the sigma factor SigH. SigH acts during both oxidative stress and heat shock. SigH, in turn, is regulated by RshA, its corresponding anti-sigma factor. In normal conditions of the host cell, SigH stays bound to RshA, which inhibits its transcription and, hence, advances the infection. However, when faced with stress conditions, RshA is released from the complex, and the free SigH binds with RNA polymerase and initiates transcription. SigH mainly interacts with the DNA-directed RNA polymerase subunit beta. The Arg-rich C terminal of the SigH protein interacts with the phosphate backbone and, along with RNAP, unwinds the DNA promoter.

The predicted docked models of SigH-RshA were in both open and closed conformation, but the closed model matched the previously obtained HDXMS experimental data. So, the closed conformation model was chosen for further analysis. It has been observed that there is a major conformational change of SigH when compared between its complexes with RNAP and RshA. SigH shows an open conformation with RNAP and a closed one with RshA. This conformational change was analyzed. It was noted that the C-terminal and N-terminal regions of SigH interact with RNAP β-subunit. However, none of the residues overlapped with the regions that actively interact with RshA. Free SigH, also in an open conformation, shows the significance of the distinct residues involved in the protein-protein interaction. The conformational change leads to the varying accessibility of certain residues, which are vital for the interaction.

Hence, these conformational changes in SigH and the difference in its model of interaction with two different regulatory proteins raise the question of whether SigH can be a potential target for a drug against tuberculosis. To establish SigH as a molecular drug target, an experimental structure of its complex with RshA is necessary, which might be more important to understand the conformation accurately.

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Conflict of Interest

The authors declare that there is no conflict of interest

Financial Discloser

Nil

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