

**EXPRESSION, PURIFICATION, AND FUNCTIONAL
CHARACTERIZATION OF WhiB PROTEINS
OF *Mycobacterium tuberculosis***

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INDIAN INSTITUTE OF TECHNOLOGY DELHI
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by

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DEPARTMENT OF CHEMISTRY

Submitted

in fulfillment of the requirements of the degree of Doctor of Philosophy

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

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Dedicated to

my parents, husband, and my siblings

CERTIFICATE

This is to certify that the thesis entitled, “**Expression, purification, and functional characterization of WhiB proteins of *Mycobacterium tuberculosis***”, being submitted by Ms. **Sonam Kumari** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** in Chemistry is a record of bonafide research work carried out by her. Ms. **Sonam Kumari** has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

Dr. Shashank Deep

Professor

Department of Chemistry

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ABSTRACT

Mycobacterium tuberculosis (*Mtb*) is the causative organism of one of the deadliest infectious diseases worldwide, tuberculosis (TB). It possesses a remarkable feature of entering into a dormant state upon encountering a stressful milieu and emerging to the active state under favorable conditions; furthermore, it senses and adapts to the different environmental conditions experienced during early infection and persistence in the host cell. This capricious nature has made it an uphill battle to target this stubborn bacterium. *Mtb* deploys several sensors and transcription regulators to sense and subvert the host immune response, regulate the expression of some essential genes, eliminate the redox stress and survive inside the host for decades. Iron-sulphur cluster-containing WhiB transcription factors are one such redox-sensing internal sensor and regulator. The engagement of the whiB family of genes as redox sensors and regulators in so many fundamental metabolic pathways of *Mtb* and not showing homology with human proteins makes them an attractive drug target. The thesis entitled '**Expression, purification, and functional characterization of WhiB proteins of *Mycobacterium tuberculosis***' is concerned with the optimization of expression and purification of unstable and insoluble WhiB proteins and comprehending the interaction of WhiB proteins with their binding partners (promoter DNA of crucial genes of *Mtb*) employing biophysical techniques. The findings of this work will help target WhiB proteins for developing new therapeutic drugs.

Chapter 1 (**Introduction**) provides an overview of tuberculosis and the peculiar nature and survival mechanisms adopted by its causative organism, *Mtb*. A detailed review of the WhiB family of proteins of *Mtb* has been presented, including the crucial role they play in the survival of *Mtb* inside the host and cause infection, the structural details of these proteins, and their interaction with promoter DNA of genes of *Mtb*. This chapter further includes a brief description of WhiB-DNA interaction studies and the need to understand WhiB-DNA interaction in detail. The chapter concludes with the origin of the problem in the context of the thesis and the outline of the research problem tackled in the thesis.

Chapter 2 (**Material and methodologies**) provides the details of the procurement of chemicals and reagents used for protein expression, purification, and interaction studies. It further describes the techniques and methods utilized during the investigation. The techniques like gel electrophoresis, fast protein liquid chromatography (FPLC), UV-Visible spectroscopy,

fluorescence spectroscopy, circular dichroism spectroscopy (CD), isothermal titration calorimetry (ITC), and surface-enhanced Raman spectroscopy (SERS), etc. are discussed with their principles.

Chapter 3 (**Optimization of expression and purification of WhiB1, WhiB3, and WhiB6 proteins of *Mycobacterium tuberculosis***) describes in detail the experiments carried out to find optimum conditions for expressing and purifying WhiB proteins. Different strategies used to overcome the instability, insolubility, and aggregation-prone behavior of WhiB proteins are mentioned in this chapter. The low temperature, along with the fusion tags, significantly improved WhiB1 and WhiB3 protein solubility. We used the co-expression of chaperones to rescue WhiB6 from inclusion bodies. We have shown that the coordinated action of a combination of the DnaK-DnaJ-GrpE-GroEL-GroES chaperone network remarkably enhanced the solubility of WhiB6 in soluble fraction during over-expression. Furthermore, the *E. coli* strains like BL21-CodonPlus (DE3)-RIL and BL21 λ (DE3) Rosetta-gami (DE3) cells further improved WhiB proteins expression.

Chapter 4 (**Biophysical Characterization of WhiB6 protein and its interaction with *espA* promoter DNA**) presents a comprehensive investigation of the biophysical characteristics of the WhiB6 protein and the interaction between WhiB6 and *espA* promoter DNA. To get insight into the binding parameters, biophysical techniques were employed. Far-UV CD spectroscopy, Raman spectroscopy, and steady-state fluorescence spectroscopy gave insight into the conformational changes in WhiB6 due to the interaction. We used Raman spectroscopy to get information regarding the residues of WhiB6 involved in the interaction. The thermodynamic parameters and the binding affinity of the WhiB6-DNA interaction obtained from the ITC experiments are also mentioned in this chapter. These findings will provide a better understanding of the interaction of WhiB6 with its binding partners.

Chapter 5 (**Investigation of biophysical characteristics of WhiB3 protein and its interaction with *pks2* promoter DNA**) describes the biophysical characterization studies of WhiB3 protein and the interaction study of WhiB3 with promoter DNA of the *pks2* gene. WhiB3 protein regulates the expression of genes involved in virulence lipid anabolism. Biophysical techniques, including far-UV CD spectroscopy, surface-enhanced Raman spectroscopy, steady-state fluorescence spectroscopy, and ITC, were employed to get detailed insight into the binding parameters of interaction, the conformation changes in protein during the interaction, and the residues involved in the binding of WhiB3-*pks2*. The information

obtained regarding the biophysical characteristics of the protein, the binding affinity, and the thermodynamic parameters of the *WhiB3-pks2* interaction can be taken advantage of in designing drugs to cure TB.

Chapter 6 (**Biophysical Characterization of WhiB1 protein and its interaction with the binding partner: *whiB1* promoter DNA**) deals with the biophysical characterization of WhiB1 protein and its interaction with *whiB1* promoter DNA. WhiB1 binds to its promoter DNA and represses the expression. In this chapter, we used biophysical techniques to understand the interaction. The thermodynamic parameters associated with interaction were obtained from ITC. The details of conformational changes in WhiB1 upon interaction with the DNA was obtained from far-UV CD and steady-state fluorescence spectroscopy.

In Chapter 7 (**Conclusion and Future perspectives**), the salient observations of this study are outlined. The present study has provided insight into the biophysical characteristics of WhiB proteins and unveiled the binding parameters of the interaction of WhiB proteins with the promoter DNA. These findings will further help in understanding the structural and functional behaviors of WhiB proteins. They will help design new drug molecules or small-molecule that could imitate the specificity and binding affinity of WhiB proteins. This idea could be deployed in the research of finding promising drugs to cure TB.

सार

माइकोबैक्टीरियम ट्यूबरकुलोसिस (एमटीबी) दुनिया भर में सबसे घातक संक्रामक रोगों में से एक तपेदिक (टीबी) का प्रेरक जीव है। इसमें एक तनावपूर्ण वातावरण का सामना करने और अनुकूल परिस्थितियों में सक्रिय अवस्था में उभरने पर सुप्त अवस्था में प्रवेश करने की एक उल्लेखनीय विशेषता है; इसके अलावा, यह शुरुआती संक्रमण और मेजबान सेल में दृढ़ता के दौरान अनुभव की गई विभिन्न पर्यावरणीय स्थितियों को महसूस करता है और उनके अनुकूल होता है। इस सनकी प्रकृति ने इस जिद्दी जीवाणु को निशाना बनाने के लिए एक कठिन लड़ाई बना दी है। एमटीबी मेजबान प्रतिरक्षा प्रतिक्रिया को समझने और नष्ट करने के लिए कई सेंसर और ट्रांसक्रिप्शन नियामकों को तैनात करता है, कुछ आवश्यक जीनों की अभिव्यक्ति को नियंत्रित करता है, रेडॉक्स तनाव को खत्म करता है और दशकों तक मेजबान के अंदर जीवित रहता है। आयरन-सल्फर क्लस्टर युक्त WhiB ट्रांसक्रिप्शन कारक एक ऐसा रेडॉक्स-सेंसिंग आंतरिक सेंसर और रेगुलेटर है। एमटीबी के इतने सारे मौलिक चयापचय मार्गों में रेडॉक्स सेंसर और नियामकों के रूप में जीन के व्हिबी परिवार की भागीदारी और मानव प्रोटीन के साथ होमोलॉजी नहीं दिखाना उन्हें एक आकर्षक दवा लक्ष्य बनाता है। माइकोबैक्टीरियम ट्यूबरकुलोसिस के WhiB प्रोटीन की अभिव्यक्ति, शुद्धिकरण और कार्यात्मक लक्षण वर्णन शीर्षक वाली थीसिस अस्थिर और अघुलनशील WhiB प्रोटीन की अभिव्यक्ति और शुद्धिकरण के अनुकूलन से संबंधित है और WhiB प्रोटीन की उनके बाध्यकारी भागीदारों (महत्वपूर्ण जीन के प्रमोटर डीएनए) के साथ इंटरैक्शन को समझने से संबंधित है। बायोफिजिकल तकनीकों को नियोजित करता है। इस काम के निष्कर्ष नई चिकित्सीय दवाओं के विकास के लिए WhiB प्रोटीन को लक्षित करने में मदद करेंगे।

अध्याय 1 (परिचय) तपेदिक और इसके कारक जीव, एमटीबी द्वारा अपनाई गई अजीबोगरीब प्रकृति और उत्तरजीविता तंत्र का एक सिंहावलोकन प्रदान करता है। एमटीबी के प्रोटीनों के व्हिबी परिवार की एक विस्तृत समीक्षा प्रस्तुत की गई है, जिसमें वे मेजबान के अंदर एमटीबी के अस्तित्व में महत्वपूर्ण भूमिका निभाते हैं और संक्रमण का कारण बनते हैं, इन प्रोटीनों के संरचनात्मक विवरण, और एमटीबी के जीनों के प्रमोटर डीएनए के साथ उनकी इंटरैक्शन। इस अध्याय में आगे व्हिबी-डीएनए अन्योन्यक्रिया अध्ययनों का संक्षिप्त विवरण और व्हिबी-डीएनए अन्योन्यक्रिया को विस्तार से समझने की आवश्यकता शामिल है। इस अध्याय का समापन थीसिस के संदर्भ में समस्या की उत्पत्ति और थीसिस में हल की गई शोध समस्या की रूपरेखा के साथ होता है।

अध्याय 2 (**सामग्री और पद्धतियां**) प्रोटीन अभिव्यक्ति, शुद्धिकरण और अन्योन्यक्रिया अध्ययनों के लिए उपयोग किए जाने वाले रसायनों और अभिकर्मकों की खरीद का विवरण प्रदान करता है। यह आगे जांच के दौरान उपयोग की जाने वाली तकनीकों और विधियों का वर्णन करता है। जेल इलेक्ट्रोफोरेसिस, फास्ट प्रोटीन लिक्विड क्रोमैटोग्राफी (एफपीएलसी), यूवी-विजिबल स्पेक्ट्रोस्कोपी, फ्लोरेसेंस स्पेक्ट्रोस्कोपी, सर्कुलर डाइक्रोइज्म स्पेक्ट्रोस्कोपी (सीडी), आइसोथर्मल टाइट्रेशन कैलोरीमेट्री (आईटीसी), और सरफेस-एन्हांस्ड रमन स्पेक्ट्रोस्कोपी (एसईआरएस), आदि जैसी तकनीकों पर चर्चा की गई है। उनके सिद्धांतों के साथ।

अध्याय 3 (**माइक्रोबैक्टीरियम ट्यूबरकुलोसिस के WhiB1, WhiB3, और WhiB6 प्रोटीन की अभिव्यक्ति और शुद्धिकरण का अनुकूलन**) WhiB प्रोटीन को व्यक्त और शुद्ध करने के लिए इष्टतम स्थितियों को खोजने के लिए किए गए प्रयोगों का विस्तार से वर्णन करता है। इस अध्याय में WhiB प्रोटीन की अस्थिरता, अघुलनशीलता और एकत्रीकरण-प्रवण व्यवहार को दूर करने के लिए उपयोग की जाने वाली विभिन्न रणनीतियों का उल्लेख किया गया है। कम तापमान, संलयन टैग के साथ, WhiB1 और WhiB3 प्रोटीन घुलनशीलता में काफी सुधार हुआ। हमने WhiB6 को समावेशन निकायों से बचाने के लिए संरक्षकों की सह-अभिव्यक्ति का उपयोग किया। हमने दिखाया है कि DnaK-DnaJ-GrpE-GroEL-GroES चैपरोन नेटवर्क के संयोजन की समन्वित कार्रवाई ने ओवर-एक्सप्रेसन के दौरान घुलनशील अंश में WhiB6 की घुलनशीलता को उल्लेखनीय रूप से बढ़ाया है। इसके अलावा, BL21-CodonPlus (DE3)-RIL और BL21 λ (DE3) Rosetta-gami (DE3) कोशिकाओं जैसे ई. कोलाई उपभेदों ने WhiB प्रोटीन अभिव्यक्ति में और सुधार किया।

अध्याय 4 (**WhiB6 प्रोटीन का बायोफिजिकल कैरेक्टराइजेशन और espA प्रमोटर डीएनए के साथ इसकी इंटरैक्शन**) WhiB6 प्रोटीन की बायोफिजिकल विशेषताओं और WhiB6 और espA प्रमोटर डीएनए के बीच की इंटरैक्शन की एक व्यापक जांच प्रस्तुत करता है। बाध्यकारी मापदंडों में अंतर्दृष्टि प्राप्त करने के लिए, जैव-भौतिक तकनीकों को नियोजित किया गया था। फार-यूवी सीडी स्पेक्ट्रोस्कोपी, रमन स्पेक्ट्रोस्कोपी, और स्टेडी-स्टेट फ्लोरेसेंस स्पेक्ट्रोस्कोपी ने इंटरैक्शन के कारण WhiB6 में गठनात्मक परिवर्तनों की जानकारी दी। इंटरैक्शन में शामिल WhiB6 के अवशेषों के बारे में जानकारी प्राप्त करने के लिए हमने रमन स्पेक्ट्रोस्कोपी का उपयोग किया। इस अध्याय में आईटीसी प्रयोगों से प्राप्त थर्मोडायनामिक पैरामीटर्स और WhiB6-DNA अन्योन्यक्रिया के बंधन बंधुता का भी उल्लेख किया गया है। ये निष्कर्ष अपने बाध्यकारी भागीदारों के साथ WhiB6 की इंटरैक्शन की बेहतर समझ प्रदान करेंगे।

अध्याय 5 (**WhiB3 प्रोटीन की जैवभौतिक विशेषताओं की जांच और pks2 प्रमोटर डीएनए के साथ इसकी इंटरैक्शन**) WhiB3 प्रोटीन के जैवभौतिक लक्षण वर्णन अध्ययन और पीकेएस2 जीन के प्रमोटर डीएनए के साथ WhiB3 के अन्योन्यक्रिया अध्ययन का वर्णन करती है। WhiB3 प्रोटीन विषाणु लिपिड उपचय में शामिल जीन की अभिव्यक्ति को नियंत्रित करता है। दूर-यूवी सीडी स्पेक्ट्रोस्कोपी, सतह-संवर्धित रमन स्पेक्ट्रोस्कोपी, स्थिर-राज्य फ्लोरोसेंस स्पेक्ट्रोस्कोपी, और आईटीसी सहित बायोफिजिकल तकनीकों को इंटरैक्शन के बाध्यकारी मानकों, इंटरैक्शन के दौरान प्रोटीन में संरचना परिवर्तन, और अवशेषों में विस्तृत अंतर्दृष्टि प्राप्त करने के लिए नियोजित किया गया था। WhiB3-pks2 के बंधन में शामिल है। प्रोटीन की जैवभौतिक विशेषताओं, बाध्यकारी आत्मीयता, और WhiB3-pks2 इंटरैक्शन के थर्मोडायनामिक मापदंडों के बारे में प्राप्त जानकारी का लाभ टीबी को ठीक करने के लिए दवाओं को डिजाइन करने में लिया जा सकता है।

अध्याय 6 (**WhiB1 प्रोटीन का बायोफिजिकल कैरेक्टराइजेशन और बाइंडिंग पार्टनर के साथ इसकी इंटरैक्शन: whiB1 प्रमोटर डीएनए**) WhiB1 प्रोटीन के बायोफिजिकल लक्षण वर्णन और whiB1 प्रमोटर डीएनए के साथ इसकी इंटरैक्शन से संबंधित है। WhiB1 अपने प्रमोटर डीएनए से जुड़ता है और अभिव्यक्ति को दबा देता है। इस अध्याय में, हमने अंतःक्रिया को समझने के लिए जैवभौतिकीय तकनीकों का उपयोग किया है। इंटरैक्शन से जुड़े थर्मोडायनामिक पैरामीटर आईटीसी से प्राप्त किए गए थे। डीएनए के साथ अंतःक्रिया करने पर WhiB1 में संरचनागत परिवर्तनों का विवरण दूर-यूवी सीडी और स्थिर-राज्य प्रतिदीप्ति स्पेक्ट्रोस्कोपी से प्राप्त किया गया था।

अध्याय 7 (**निष्कर्ष और भविष्य के दृष्टिकोण**) में, इस अध्ययन की मुख्य टिप्पणियों को रेखांकित किया गया है। वर्तमान अध्ययन ने WhiB प्रोटीन की जैव-भौतिक विशेषताओं में अंतर्दृष्टि प्रदान की है और प्रमोटर डीएनए के साथ WhiB प्रोटीन की इंटरैक्शन के बाध्यकारी मापदंडों का अनावरण किया है। ये निष्कर्ष आगे WhiB प्रोटीन के संरचनात्मक और कार्यात्मक व्यवहार को समझने में मदद करेंगे। वे नए दवा अणुओं या छोटे-अणु को डिजाइन करने में मदद करेंगे जो WhiB प्रोटीन की विशिष्टता और बाध्यकारी संबंध की नकल कर सकते हैं। इस विचार को टीबी को ठीक करने के लिए आशाजनक दवाएं खोजने के शोध में लगाया जा सकता है।

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Abbreviations

1.	IPTG	β -D-1-thiogalactopyranoside
2.	cAMP	Cyclic adenosine monophosphate
3.	CMR	Cyclic-AMP and redox responsive transcription factor
4.	CRP	Cyclic-AMP dependent regulatory protein
5.	EspR	A virulence associated transcriptional regulator upregulated by PhoP
6.	<i>E. coli</i>	<i>Escherichia coli</i>
7.	<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
8.	ITC	Isothermal Titration Calorimetry
9.	CD	Circular Dichroism
10.	LB Media	Luria Broth Media
11.	SDS- PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
12.	Tris	Tris (hydroxymethyl) Aminomethane
13.	DTT	Dithiothreitol
14.	EDTA	Ethylenediamine Tetraacetic Acid
15.	FPLC	Fast protein Liquid Chromatography
16.	ANS	8-anilino-1-naphthalenesulfonic acid
17.	ΔH	Change in Enthalpy
18.	ΔS	Change in Entropy
19.	ΔG	Change in Free Energy
20.	ΔT	Change in Temperature
21.	GB1	Immunoglobulin binding domain of Streptococcal protein G
22.	Trx	Thioredoxin A