Chapter-5
Functional Analysis of WhiB1
5.1 Introduction

The role of redox regulatory pathways in signal transduction is well established (Kamata and Hirata, 1999). Thiol-disulfide exchange reactions control the structure and activity of proteins that contain regulatory cysteine residues (Danon, 2002). This reversible disulfide bond formation is mediated by thiol-disulfide oxidoreductases such as thioredoxin and glutaredoxin that exchange reducing equivalents between their active site cysteines and the cysteines of target proteins (Ritz and Beckwith, 2001). In nature, protein disulfide oxidoreductases perform multitude of functions. Thioredoxins from different organisms have been implicated in two totally different functions:

(A) Role in electron transfer—Thioredoxins are known since 1964, when this protein was originally isolated from *Escherichia coli* as an electron donor for ribonucleotide reductase (Laurent *et al.*, 1964). Apart from their oxidoreductase activity, thioredoxins exert control over the activity of its target proteins via reversible thiol-disulfide exchange reactions (Figure 5.1a). In plant chloroplasts, thioredoxin regulates the light-active Calvin cycle by reducing specific regulatory disulfides (Schurmann, 2003). In eukaryotes, thioredoxins regulate the activity of transcription factors such as NF-\(\kappa\)B and AP-1 (Schenk *et al.*, 1994; Hayashi *et al.*, 1993). Thioredoxins also play a pivotal role in the oxidative stress response; peroxiredoxins that catalyze the reduction of \(\text{H}_2\text{O}_2\) are activated by thioredoxin (Tanaka *et al.*, 2000). In *E. coli* during oxidative stress, transient disulfides are formed by the action of reactive oxygen species in proteins such as Hsp33 (Jakob *et al.*, 1999) and the transcription regulator OxyR (Zheng *et al.*, 1998). These bonds are reduced by the thioredoxin and glutaredoxin systems.

(B) Part of structural component—A second regulatory mechanism independent of thiol redox activity depends on the ability of thioredoxin to interact with other proteins to form functional protein complex (Figure 5.1b). *E. coli* thioredoxin is an essential component of a protein complex required for filamentous phage assembly (Russel and Model, 1985). Thioredoxin is also an essential processivity factor for bacteriophage T7 DNA polymerase (Richardson, 1983). In eukaryotes, reduced thioredoxin inactivates the apoptosis signaling kinase-1 (ASK-1) (Liu *et al.*, 2000).

The precise molecular mechanism underlying redox regulation of the organism during stress can be understood by identification and characterization of cellular targets of the thiol-disulfide oxidoreductases.

It has already been demonstrated that *M. tuberculosis* WhiB1 coordinates both [4Fe-4S] or [2Fe-2S] clusters depending upon the redox environment. The [2Fe-2S] cluster of WhiB1 is disassembled in the presence of oxidizing agents such as GSSG, \(\text{H}_2\text{O}_2\) or air. The
Figure 5.1: Regulation of protein activity by thioredoxin. (a) thioredoxin as oxidoreductase. (b) Thioredoxin as a structural component.
disassembly resulted into a change in conformation of the protein and two intramolecular disulfides were formed. The apo-WhiB1 has properties similar to protein disulfide reductases. The insulin disulfide reduction assay of apo- and holo-WhiB1 shows that apo-form of the protein is catalytically active and reduces insulin disulfides. The redox potential of WhiB1 was calculated as -236 mV, which corresponds to the redox potential of many cytoplasmic thioredoxins or protein disulfide reductase-like proteins.

Therefore, to identify the regulatory pathway(s) in which WhiB1 participates, two different strategies were adopted: (a) a genome wide search for the target proteins that interacts with WhiB1 and its subsequent characterization (b) a knowledge based approach to confirm the hypothetical targets of WhiB1. Based on the above two strategies, the present study is divided into two sub parts:

(a) Genome wide search for the target identification of WhiB1

(b) Role of WhiB1 during oxidative stress in *M. tuberculosis*
Chapter 5a
Genome wide target identification of WhiB1

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5a.1 Introduction

The formation and dissociation of complexes involving two or more proteins is a central feature of many biological processes. Complex formation is frequently highly specific; for example, often only one member of a protein family recognizes a particular binding partner despite the fact that all other family members may have an essentially identical fold. Specificity differences are clearly coded on the protein surface, as are the various determinants of binding affinity. Selection and screening methods are powerful tools for studying macromolecular interactions. Examples of such methods include yeast-based two-hybrid systems, bacterial expression-based pulldown assays, immuno-precipitation techniques and other biophysical techniques such as surface plasmon resonance (SPR).

Yeast two-hybrid system (Fields and Song, 1989) is one of the most rapid and sensitive techniques to score the genome-wide protein-protein interactions. It utilizes the genetic power of *Saccharomyces cerevisiae* as host for the analysis of protein-protein interactions (Fields and Song 1989; Chien *et al*., 1991; Durfee *et al*., 1993; Gyuris *et al*., 1993; Vojtek *et al*., 1993). The yeast two-hybrid system artificially mimics the natural process of transcription and is built upon the observation that transcriptional activator Gal4 can be separated into two discrete domains corresponding to DNA-binding domain and activation domain (Brent and Ptashne, 1985; Hope and Struhl, 1986). In the standard yeast two-hybrid approach, the association of an activation domain with a DNA-binding domain is dependent on a bridging function provided by two interacting proteins, fused respectively to the DNA-binding domain and the activation domain. The productive interaction leads to the transcriptional activation of reporter genes whose expression can easily be scored on the basis of colorimetric tests or the ability of the novel trans-activator to transcribe a yeast gene essential for growth. The ease of manipulation and genetic power of yeast allows assessment of a large number of interactions.

The protein pulldown systems are widely used for establishing the interaction of two proteins or library-encoded proteins in vitro. The glutathione S-transferase (GST) pulldown assay is one of the most common and simple processes to determine in vitro protein-protein interactions. Bacterial expressed GST-fused proteins are used to perform direct measure of protein-protein interactions. In far western analysis (Blackwood and Eisenman, 1991; Kaelin Jr. *et al*., 1992), a labeled protein is used to probe the interactions with defined or library-encoded proteins immobilized on a membrane, similar to conventional western analysis. The GST pulldown (Kaelin Jr. *et al*., 1991) is a related technique in which either: (a) a single defined in vitro-expressed protein, (b) an unknown protein present in a pool of proteins in the
cell lysate, or (c) an unknown protein expressed from a pool of in vitro-translated cDNAs is collected by its interaction with a fusion protein composed of the target protein linked to a GST moiety. The complex is then isolated through the binding of the GST moiety to glutathione-coupled beads, followed by purification.

Protein disulfide reductase or thioredoxin mediates the thiol-disulfide exchange reaction and transfer the reducing equivalents to other proteins. First, thioredoxin induces conformational change in the target protein and then facilitates the reduction of disulfide bridge (Brandes et al., 1993; Stumpp et al., 1999). After that, thioredoxin and its target protein form a mixed-disulfide intermediate at the first reactive cysteine, which is the cysteiny1 residue nearest to the N-terminus of the thioredoxin molecule (Brandes et al., 1993). This intermolecular disulfide bond is attacked by the second cysteine of Trx thereby, reducing the target protein and in the process, Trx is oxidized. Substitution of the second cysteine to serine can interrupt this reduction process at the stage of the formation of mixed-disulfide intermediate. This basic concept of the mechanism of the function of Trx was elegantly used by Verdoucq et al. 1999, as a Trx affinity chromatography for the first time to capture the interacting partner proteins of Trx in yeast. Motohashi et al. (2001) applied similar strategy to identify Trx targets in plants. However, with the advancement of understanding, subsequent improvements were incorporated in the technique where Trx mutant was immobilized on the solid phase (matrix) and the matrix was then used for the affinity chromatography (Hisabori et al., 2005). When the protein pool of the cytoplasm was incubated with matrix, potential Trx target proteins with an accessible disulfide bond should form a mixed-disulfide intermediate with the immobilized Trx mutant on the matrix. This intermediate complex is stable under the experimental conditions, i.e. in the absence of any reductant. In order to remove non-specifically bound proteins, the gel was washed with high concentrations of NaCl. After repeated washing, the captured proteins were then eluted from the gel using 10 mM dithiothreitol (DTT), a concentration sufficient to reduce the mixed disulfide bonds (Figure 5a.1). This technique can also be used to establish that the interaction of two protein partners involves a thiol-disulfide exchange. Using purified target protein in the Trx affinity chromatography instead of cytoplasmic extract also serves the purpose.

Therefore, in this part of study, the interacting partner of WhiB1 was searched by yeast two-hybrid method, interaction was confirmed by the GST-pulldown assay and mechanism of interaction has been demonstrated by a Trx affinity chromatography.
5a.2 Methods

5a.2.1 Yeast two-hybrid system

Plasmids pEG202 and pJG4-5 were used for the construction of ‘bait’ and activator fusions, respectively. The whiB1/Rv3219 ORF was PCR amplified by using forward primer 5’ TCC GGA ATT CAT GGA TTG GCG CCA C 3’ and reverse primer 5’ TTT CGC TCG AGG AGT CGT CAG ACC C 3’. To generate a LexA-WhiB1 fusion plasmid pDBD-B1 (Figure 5a.2A), the PCR product was digested with EcoRI-XhoI and inserted into the vector pEG202 at EcoRI-XhoI sites. Genomic DNA was isolated from M. tuberculosis H37Rv by a method described earlier (Raghava et al., 2000). The genomic DNA was digested with EcoRI-XhoI and cloned into the same sites of pJG4-5 vector to express the M. tuberculosis genomic library in fusion with the activation domain of acid blob under gal promoter (pADMtb) (Figure 5a.2B). For initial characterization of the bait in a yeast two-hybrid system, Saccharomyces cerevisiae EGY191 was transformed with following sets of plasmids: (1) pDBD-B1+ pSH18-34 (test) + pJG4-5; (2) SH17-4 + pSH18-34 (positive control for activation) + pJG4-5; (3) RFHMI + pSH18-34 (negative control for activation) + pJG4-5; (4) pDBD-B1 + pJK101; (5) pEG202 + pJK101 and (6) pJK101. The transformants were selected on respective drop out plates and β-galactosidase activity was checked on YNB Gal+Ura-X-gal plates or quantitatively measured by using ONPG as a substrate (Miller, 1972).

To carry out the interactor hunt, following steps were followed:

1. S. cerevisiae containing plasmids pDBD-B1 and pSH18-34 was grown in 300 ml Glu/CM-Ura-His- medium to ~2 x 10^7 cells/ml (OD600 ~0.50).
2. Cells were harvested and washed with sterile water and were resuspended in the 1.5 ml TE buffer/0.1 M lithium acetate.
3. One μg pADMtb DNA and 50 μg high quality sheared Salmon sperm carrier DNA was added to each 30 sterile 1.5 ml microcentrifuge tubes. Fifty μl yeast solution from step 2 was added to each tube.
4. Yeast transformation was carried out as described in “section 2.2.15”. The transformants were selected on 30 Glu/CM-Ura-His-Trp dropout plates. All the plates were incubated at 30 °C for 2 to 3 days.
5. Using sterile spatula, all the yeast cells from each plate were scraped off the plate. Cells were pooled from 30 plates into one single aliquot.
6. Cells were then washed twice with sterile water and pelleted at 1500 g at room temperature. After the second wash, the pellet volume was ~25 ml.
Figure 5a.1: An overview of Trx affinity chromatography

- Gel Bead

OH

SH

Target protein

- Gel Bead

SH

OH

+DTT

- Gel Bead

SH

HS

SH
Figure 5a.2. Plasmids constructs of yeast two hybrid system (A) pEG202 derived “Bait” plasmid (B) pJG4-5 derived “Activation Domain” fusion library plasmid.
7. Cells were resuspended in equal volume of 25% glycerol solution, mixed well and were stored in 1 ml aliquots at -70 °C.

8. One tube of library transformants was removed and diluted to OD ~ 0.5 in a 50 ml Gal/Raff/CM-Ura-His-Trp dropout liquid medium and grown at 30 °C for 6 hrs to induce the library. Cells were harvested by centrifugation at 1500 g for 5 min and washed twice with water and then plated on 10 Gal/Raff/CM-Ura-His-Trp-Leu dropout plates and incubated at 30 °C for 2-5 days.

9. Colony started to appear after 72 hrs and each colony was carefully maintained on a Glu/CM-Ura-His-Trp dropout plates. To check the specific interactions, 10^4, 10^3 and 10^2 cells were spotted on Gal/Raff/CM-Ura-His-Trp-Leu and Glu/CM-Ura-His-Trp-Leu dropout plates and incubated at 30 °C for 72 hours.

10. Clones exclusively growing on Gal/Raff/CM-Ura-His-Trp-Leu dropout plates were selected for identification of probable interactor. Plasmid was prepared from the master Glu/CM-Ura-His-Trp dropout plates as described in “section 2.2.16”. To distinguish library plasmid from other bait and reporter plasmids, preparation was transformed to E. coli KC-8, which only supports the replication of library plasmid.

11. Plasmids were prepared from E. coli KC-8 and transformed to E. coli DH5α. Selected clones were sequenced using 1-YSP primer in an ABI 310 Automated DNA sequencer and analyzed by the Blastn server (NCBI, NIH).

5a.2.2 Cloning of glgB/Rv1326c into pET-29a

To express the Rv1326c/glgB as 6× His-tagged protein in E. coli, entire glgB ORF (2193bp) was PCR amplified from the genomic DNA of M. tuberculosis H37Rv by using Pfu® (Stratagene, Germany). The primers used were forward GlgBF and reverse GlgBR (section 2.1.3). The PCR conditions were as follows: 95 °C for 3 min followed by 25 cycles of 95 °C for 2 min, 66 °C for 1 min and 72 °C for 2 min with final extension at 72 °C for 10 min. Authenticity of the PCR product was confirmed by sequencing the entire fragment with internal primers using an automated DNA sequencer (ABI Prism 310). The PCR product was digested with restriction enzymes BamHI-XhoI and inserted into the BamHI-XhoI digested and dephosphorylated pET-29a expression vector resulting into the recombinant plasmid pET-gl.

5a.2.3 GST pulldown assay

The EcoRI-XhoI digested PCR fragment of whiB1 was cloned into the EcoRI-XhoI sites of pGEX4T-1 vector (Amersham) to generate a fusion plasmid pGEX-B1. The whiB4
gene was amplified using the following primers: forward 5' ATA TAT GAA TTC GTG GTA CGC AGC GTA GAC GCG G 3' and reverse 5' ATA TAT CTC GAG CTA TCC GGC GGT GCC GGT G 3' and cloned into the EcoRI-XhoI digested pGEX4T-1 vector to generate a fusion plasmid pGEX-B4. The recombinants pGEX-B1 and pGEX-B4 were co-transformed with pET-gl into E. coli BL 21(DE3). Protein expression was carried out in LB broth (Difco) for 3 hrs at 30 °C with 0.5 mM IPTG. Cells were harvested and resuspended in 1 ml lysis buffer: 50 mM Tris.HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA with 1/10 tablet of protease inhibitory cocktail (Roche, Germany) per 100 ml of culture. After one freeze-thaw cycle, lysozyme (Sigma, USA) 2 mg/ml was added for 1hr at 37 °C and the cells were sonicated. The supernatant was separated by centrifugation at 17,000 g for 30 min. Crude lysates of co-expressing test proteins (500 µg) were mixed with 3 mg of pre-equilibrated glutathione agarose beads (Sigma, USA). The reaction was carried out in a pulldown buffer (100 mM Tris.HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton-X100, 1 mM DTT) at 4 °C for 3 hrs with continuous mixing on a rocker platform. The beads were washed five times with 1ml of pulldown buffer and the proteins were eluted directly by adding 1X gel loading dye. The bound proteins were analyzed on 10% SDS-PAGE and stained with silver nitrate (Sambrook and Russel, 2001). Co-elution of GlgB along with WhiB1 from the glutathione beads was further confirmed by immunoblot using anti-6× His antibodies (Amersham).

5a.2.4 Expression and Purification of GlgB

The pET-gl was transformed into E. coli BL21 (DE3) competent cells by the heat-shock method and the transformants were named as ECGL. ECGL cells were grown at 37 °C in Luria Bertani broth containing 30 µg/ml kanamycin to the OD600 of 0.6 and expression was induced with 0.3 mM IPTG for 18 hrs, 18 °C at 200 rpm. Cells were harvested by centrifugation at 6000 g at 4 °C, resuspended in 10 ml lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 30 mM Imidazole, protease inhibitor cocktail, pH 8.0) after a wash in the same buffer, stored at −70 °C. The frozen ECGL cells from 1 L culture were thawed at 4 °C and disrupted by sonication. The soluble lysate was separated by centrifugation at 17,000 g for 30 min at 4 °C and loaded on to a Ni2+-NTA affinity column (10 ml; Qiagen, Germany), which was pre-equilibrated with five-column volumes of lysis buffer. The protein purification conditions were as follows: washing in 25 column volumes of buffer (50 mM NaH2PO4, 300 mM NaCl, 40 mM Imidazole, pH 8.0), elution in buffer (50 mM NaH2PO4, 300 mM NaCl and 200 mM Imidazole, pH 8.0) at a flow rate of 1.0 ml/min. Purity level of the protein was tested on a 10% SDS-PAGE (Laemmli, 1970). The Ni2+-NTA column purified GlgB was
dialyzed against buffer A (50 mM Tris.HCl, pH 8.0, 20 mM NaCl) and then loaded on to a 5 ml pre-equilibrated Q-sepharose column in buffer A, at the flow rate of 0.5 ml/min. The bound protein eluted in 5 ml step gradients of NaCl ranging from 100 mM to 0.5 M with an interval of 100 mM, made in the same buffer. Five fractions (1 ml each) were collected from each step at the flow rate of 0.5 ml/min. All the fractions were tested for the elution pattern and purity on a 10% SDS PAGE. The fractions containing purified GlgB were pooled and concentrated in an YM-30 centrifugal concentration device (Millipore, USA). Purified protein was dialyzed against 100 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl with three changes at 4 °C. The effect of reducing agents on GlgB protein was tested in a 6-12% step gradient non-reducing SDS-PAGE. The purified protein was treated with 10 mM dithiothreitol (DTT) or with 3% β-mercaptoethanol for 2 hrs at 25 °C and then mixed with Laemmli gel loading buffer and heated at 60 °C for 10 min before loading on to the gel.

5a.2.5 GlgB enzyme assay

Enzymatic efficiency of the recombinant GlgB protein was determined by using its capacity to hydrolyze amylose. Potato type III amylose (0.2 mg/ml final concentration, Sigma, USA) was used as substrate in all the enzymatic reactions. The reactions were carried out in a 50 mM citrate buffer, pH 7.0 at 30 °C for 30 min with different concentrations: 0.1 μM to 0.5 μM of protein. After 30 min of incubation, the branching product of amylose was detected by iodine staining test. The reaction mixture was mixed with equal volume of KI-I₂ solution and absorbance was recorded at 660 nm in a UV-Visible spectrophotometer (Perkin Elmer, USA) immediately. The decrease in absorbance at 660 nm in relation to the substrate control provides the measure of GlgB activity.

5a.2.6 Mass spectrometry

To investigate the presence of disulfide bond(s) in GlgB, 100 μg purified protein was incubated with 10 mM DTT for 10 hrs at 25 °C. Both DTT treated and untreated proteins were incubated for 2 hrs with 20 mM iodoacetamide at 37 °C in dark and then digested with 2 μg of proteomics grade trypsin (Sigma, USA) for 10 hrs at 25 °C. The peptides were precipitated with 10% TCA in ice for 15 min, washed twice with chilled acetone, air-dried and resuspended in 0.1% TFA. Two μl of each of the sample was mixed with equal volume of matrix and mass spectra were recorded in a MALDI-TOF (Brukers, Ultraflex mass spectrometer). However, in another set of experiments, the GlgB protein was incubated at 45 °C for 4 hrs and then processed further as described.
5a.2.7 Trx affinity chromatography

The method of Motohashi et al 2005 was used to immobilize the WhiB1C40S protein to a cyanogen bromide-activated Sepharose 4B resin with some modifications. Briefly, the method used was as follows: beads to protein ratio of 60 mg: 1 mg. Beads were soaked in 1 mM HCl for 15 min at room temperature (RT) and then washed 10 times with 1 mM HCl at RT. Washed beads were equilibrated in 0.1 M NaHCO₃ and 0.5 M NaCl, pH 9.0 (coupling buffer). The protein (5 mg) dialyzed in the coupling buffer was mixed with the resin and incubated for 15 hrs at 4 °C with continuous mixing. After washing with 5 column volumes of coupling buffer, it was incubated with 5 column volumes of blocking buffer: 0.1 M NaHCO₃; 0.5 M NaCl and 1 M Tris.HCl, pH 9.0 for 13 hr at 4 °C. Beads were then washed with five column volumes of a low pH buffer: 0.1 M acetic acid and 0.5 M NaCl, followed by two more alternating washes with coupling and low pH buffer. After that, the resin was stored at 4 °C in the coupling buffer until used. In our experimental conditions, ~95% protein was bound to the resin. For the interaction, 1 ml of “pre-immobilized WhiB1C40S resin” was equilibrated with 5 column volumes of binding buffer: 50 mM Tris.HCl, pH 8.0, 100 mM NaCl, 0.05% TritonX100, 2 mM EDTA and 10 mM MgCl₂ and then 1 mg of the interacting protein, GlgB was mixed with the binding buffer. The interaction was carried out for 3 hrs at 25 °C in the presence of protease inhibitor cocktail with continuous mixing. Beads were washed with 50 mM Tris.HCl, pH 8.0 and 0.5 M NaCl until the wash was free of the protein and then the bound protein was eluted in 3 ml of buffer: 50 mM Tris.HCl, pH 8.0, 100 mM NaCl containing 10 mM DTT. After TCA precipitation and two washes with chilled acetone, the protein was loaded on to a 10% SDS-PAGE and visualized by Silver staining method.

5a.2.8 Estimation of protein concentration

WhiB1 protein concentration was estimated using molar extinction coefficient (ε_{280nm} = 17550 M⁻¹cm⁻¹) determined by using the software Vector-NTI, which uses amino acid sequence of the protein for analysis. However, protein concentration of whole cell lysates and GlgB were estimated by the Bradford method using BSA as a standard.

5a.3 Results

5a.3.1 WhiB1 interacts with GlgB

The suitability of the use of WhiB1 in a yeast two-hybrid system and that it lacks an intrinsic transcription activation property, reporter assay was carried out with positive (sH17-4) and negative (pRFMH1) control of transcription activation (Figure 5a.3A). Further,
repression assay of WhiB1 in yeast two-hybrid system, using plasmid pJK101 containing an UAS of GAL1 promoter was performed to check that LexA-WhiB1 fusion is expressed and processed by S. cerevisiae transcription machinery. As evident from Figure 5a.3B, the UAS can only exert its effect when LexA operators situated between UAS and GAL1 promoter is free. However, expression of fusion leads to the occupation of LexA operators with fusion protein, which stops the bending of DNA and subsequent activation of GAL1 promoter by UAS. It is clear from the Figure 5a.3 that WhiB1 did not activate the transcription of GAL1 promoter. After establishing that WhiB1 could be studied in the yeast two-hybrid system as a 'bait', the recombinant vector pDBD-B1 was co-transformed with pADMtb (genomic DNA library as a fusion to the activation domain). Therefore, the physical interaction of pDBD-B1 and pADMtb in yeast would result into the activation of LEU2 and β-galactosidase gene and the yeast carrying interacting pair would grow in a medium containing galactose but not glucose. After final screening, sixteen clones were identified and all of them were sequenced (Figure. 5a.4A). All 16 clones had an in-frame fusion of 596 bp 3' end of ORF Rv1326c/glgB, which has been predicted to code for an α-(1-4)-glucan branching enzyme. Since all the clones were identical, it was assumed that the interaction was specific. The interaction between WhiB1 and GlgB was further confirmed where C-terminus region (155 amino acids) of GlgB was fused separately with the activation domain in vector pJG4-5 (pADgT) and was co-transformed with pDBD-B1. Growth of co-transformed cells was reconfirmed on galactose+ leucine- plates (Figure. 5a.4B). These results confirmed that the C-terminus region of GlgB alone could interact with WhiB1.

To further support the in vivo interaction of WhiB1 with GlgB, an in vitro GST pull down assay was carried out. WhiB1 was expressed as a GST fusion (pGEX-B1) while GlgB was expressed as 6× His-tag fusion in the vector pET29a: pETgl. The pGEX-B1 and pETgl were co-expressed in E. coli BL21(DE3) at 30 °C. The whiB4IRv3681c was cloned in to pGEX4T-1 and was co-expressed with GlgB as an internal control. It can be seen from Figure. 5a.4C that WhiB1 specifically interacted with GlgB. In all the pulldown assays, another protein of ~27 kDa was co-purified. N-terminus amino acids sequencing identified the protein as a cleaved GST.

5a.3.2 Sequence analysis of M. tuberculosis GlgB

The amino acids sequence of M. tuberculosis GlgB was compared with that of other glycogen branching enzymes. Protein sequence databases (SWISS-PORT, PIR and GenBank) were searched using the standard search algorithm BLASTP (NCBI, NIH). M. tuberculosis
Figure 5a.3: Characterization of WhiB1 as “bait”. (A) reporter assay to determine that whether WhiB1 has intrinsic property to activate the transcription of reporter gene. (B) Repression assay to establish the expression of LexA-fusion protein in yeast.
Figure 5a.4: Yeast two-hybrid and GST-pull down analyses to show that WhiB1 interacts with GlgB. (A) Interaction of WhiB1 with M. tuberculosis H37Rv genomic DNA library. (B) Direct interaction between WhiB1 and GlgB. pDBD-B1 and pADgl-T (C-terminus of glgB cloned in pJG 4-5) plasmids were transformed to S. cerevisiae EGY191 with the reporter plasmid pSH18-34 containing β-Gal as reporter gene and transformants were selected on Glu+his-ura-trp- plates. To check the interaction, different clones were plated on Gal+his-ura-trp-leu- after 6 hrs of induction in Gal+his-ura-trp- medium. Viable clones were further tested for specific interaction by dilution plating on Gal+his-ura-trp-leu- and Glu+his-ura-trp-leu- plates. (C) WhiB1 interacts with GlgB as shown by GST pull down assay. WhiB1-GST fusion protein was co-expressed with GlgB in E. coli BL21(DE3) and in vitro interaction was carried out at 4 °C in presence of 100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM ATP and 0.1% TritonX-100 using glutathione agarose beads (Sigma).
H37Rv GlgB shared significant sequence similarity with GlgB of *M. bovis* AF2122/97 (P59816) and *M. avium* sub sp. *paratuberculosis* K10 (AAS04751), but showed a deletion of four residues (A/GDHR) closer to the C-terminus compared to GlgB of *S. coelicolor* A3(2) (S70079, Q59832) (Burton et al., 1995). It also has two insertions of four residues near the N-terminus (RSFT) and seven residues (GGVDATD) at the extreme C-terminus. However, significance of these insertions and deletions are not yet clear. The multiple sequence alignment of GlgB of different organisms (Figure 5a.5) showed the presence of four conserved regions in GlgB of *M. tuberculosis* H37Rv. These conserved regions have also been reported in the α-amylase family (Svensson, 1994). However, both *M. tuberculosis* and *M. bovis* BCG GlgBs have four cysteine residues at positions 95, 193, 617 and 658 and are specific to these two species of mycobacteria.

**5a.3.3 Expression and purification of the recombinant GlgB**

To express GlgB with a C-terminal 6× His tag, the PCR product was digested with restriction enzymes *BamHI*-XhoI and inserted into the *BamHI*-XhoI digested and dephosphorylated pET-29a expression vector resulting into the recombinant plasmid pET-gl (Figure 5a.6). Expression of GlgB at 37 °C in the presence of 0.3 mM IPTG for 3 hrs resulted in a very high expression of the protein but all the protein was present in inclusion bodies. The change in IPTG concentration; co-expression of chaperons GroEL/GroES (pKY206) under a constitutive promoter; the decrease of incubation temperature (30 °C/ 25 °C), either in the presence or absence of chaperons, did not affect the solubility. However, a shift to 18 °C for 18 hrs even in the absence of chaperons helped in the solubility of the protein to the extent of ~30%. The Ni²⁺-NTA affinity chromatography resulted in to ~95% pure protein. Therefore, it was further purified on a Q-sepharose anion-exchange column at pH 8.0. The pure GlgB protein eluted at a gradient of 300-400 mM NaCl concentration. The theoretical mass of the recombinant GlgB with N-terminal S-tag and C-terminal His-tag is 86.06 kDa. On a reducing SDS- PAGE, the molecular mass of the recombinant GlgB corresponds to ~ 85 kDa which is in agreement with the theoretical mass of the protein (Figure 5a.7).

The initial characterization of GlgB protein revealed that in solution it is a monomer as estimated by gel filtration chromatography. Enzymatic characterization showed that protein was active and utilized amylose as substrate with a pH and temperature optima of 7.0 and 30 °C, respectively (Garg et al., 2006).

**5a.3.4 Characterization of GlgB protein**

The GlgB protein showed tendency to aggregate at a relatively low concentration (4
Figure 5a.5: Multiple sequence alignment of amino acid sequences of GlgBs of *M. tuberculosis* H37Rv (Q10625), *M. bovis* (P59816), *M. avium* (AAS04751), *S. coelicolor* A3(2) (S70079, Q59832) and *E. coli* (AAC76457). Alignment was carried out using ClustalX and postscript was prepared by ESPript 2.2. Identical and similar amino acids are shown as filled and empty boxes, respectively. The four conserved regions of α-amylase family are underlined. Four cysteine residues of *M. tuberculosis* H37Rv and *M. bovis* are pointed by arrowheads and regions of amino acids either deleted or inserted are empty boxed (A), (B) and (C).
Figure 5a.6: Scheme of cloning of glgB/Rv 1326c in the expression vector pET-29a.
Figure 5a.7 Expression and Purification of the recombinant GlgB in E. coli by Ni\textsuperscript{2+}-NTA affinity and Q-sepharose ion-exchange chromatography. Lanes are labeled on top of the wells. Eluted fractions were separated on 10% SDS-PAGE and stained with coomassie brilliant blue. Immunoblot was carried out with anti-6×His monoclonal antibodies (Amersham Biosciences). Blot was developed with ECL chemiluminescence kit of Amersham Biosciences, as per protocol.
mg/ml). Further, it has been observed in the gel filtration chromatography that protein peak was broad with a wide shoulder indicating a possible presence of soluble aggregates. On a non-reducing SDS-PAGE, the purified GlgB was present as two closely migrating bands however DTT or β-mercaptoethanol treated protein migrated as a single sharp band (Figure 5a.8A). The primary amino acids sequence of *M. tuberculosis* GlgB has four cysteine residues at positions: 95, 193, 617, and 658, therefore there is a possibility of forming intramolecular disulfide bond(s). Cellular environment of all microorganisms is reducing, therefore it will not support disulfide bond formation. Thus, the two closely migrating bands of purified protein on a non-reducing SDS-PAGE might be due to the presence of two populations of different conformations of the same protein: the oxidized and the reduced. The oxidation of free thiols during purification in aerobic conditions cannot be ruled out (Figure 5a.8A). The presence of a single sharp band of high intensity might be attributed to the reduction of intramolecular disulfide(s).

Analysis of secondary structure of GlgB using far-UV CD spectroscopy showed that GlgB is rich in α-helices (Johnson, 1988). The overall shape of the spectra of both the oxidized and reduced GlgB remained identical, suggesting the maintenance of identical secondary structure in both the redox states of the protein (Figure 5a.8B). Near-UV CD spectra of proteins are unique and characteristic of their tertiary structures. Studies on several disulfide-containing proteins such as thioredoxins, TlpA and DsbA, showed that although far-UV CD spectra do not change after reduction, the near-UV CD spectra show significant differences (Hiraoki *et al.*, 1988; Wunderlich *et al.*, 1993; Loferer *et al.*, 1995). Comparison of the near-UV CD spectra of the oxidized and reduced GlgB showed differences in the regions of 255-270 nm. The CD signal changes in this region are due to the changes in the status of the disulfide bonds (Figure 5a.8C). Further, appreciable differences were also observed in the regions of 270-285 nm of the spectra of the oxidized and reduced forms, which is contributed by the aromatic residues. Therefore, the displayed differences in the environment of some of the aromatic residues would have been due to the redox-dependent conformational change of the GlgB. Fluorescence spectroscopy is widely used to study the subtle changes in conformation of a protein (Raman *et al.*, 2001b; Qamara *et al.*, 2004).

The sequence analysis of GlgB showed the presence of 36.54% hydrophobic residues, thus there is a possibility of the exposure of some of the buried hydrophobic patches due to the conformational change after the reduction of disulfide bonds. Exposure of such hydrophobic patches of the reduced and the oxidized GlgB was probed using ANS binding to
Figure 5a.8: Reduction of GlgB leads to a change in the tertiary structure. ((A) Effect of reducing agents on conformation of GlgB. 2 μg Purified protein was treated with 10 mM DTT and 3% β- mercaptoethanol for 2 hrs before loading on to a non-reducing 6-12% gradient SDS-PAGE. (B) Far-UV CD spectra of the oxidized and the reduced GlgB. 7 μM GlgB was treated with DTT and alkylated as described in "materials and methods". (C) Near-UV CD spectra of oxidized and reduced GlgB to show changes in tertiary structure of GlgB upon reduction of disulfide bond (D) Effect of reduction of disulfide on the ANS binding to GlgB. The fluorescence intensity of ANS increased in reduced GlgB compared to oxidized GlgB. For reduction, 8 μM GlgB was treated with 10 mM DTT for 2 hrs at room temperature before the addition of 250 μM ANS. Buffer with ANS was used as control.
such patches. As shown in Figure 5a.8D, fluorescence intensity of ANS was significantly enhanced in the reduced form compared to the oxidized GlgB. The spectra clearly indicated the exposure of some of the hydrophobic surfaces of the GlgB. Similarity in the above results establish a conformational change in GlgB due to the reduction of disulfide bond(s).

5a.3.5 GlgB has intramolecular disulfide bond

Peptide mass finger printing of the tryptic digests of alkylated and non-alkylated GlgB showed that the C95 (LQVTYEGCPEPHTVADAYR, 2109.5 Da) and the C658 (VGSDGSLACVFNFAGAEHR, 2158.0 Da) are present as free thiol, as these two residues were alkylated without any DTT treatment (Figure 5a.9A & B). However, the C617 (CHPALWSLDTPEGYSWIDANDSANVLSFMVR, 3669.033 Da) was alkylated only after incubating the GlgB at 45 °C for 4 hrs and then DTT treatment (10 hrs) followed by a complete trypsin digestion (10 hrs). The results indicated that under normal conditions, the C617 is partially buried and was not available for trypsin digestion. However, even under these conditions, peptide containing C193 (VLPWSGVLWLPDFPCCDGGLYK) was not detected.

Table 5a.1: The expected, iodoacetamide labeled and observed molecular weights of trypsin digested cysteine containing peptides of GlgB.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Expected mass (Da)</th>
<th>Labeled mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQVTYEGCPEPHTVADAYR</td>
<td>2052.5</td>
<td>2109.5</td>
<td>2108.803</td>
</tr>
<tr>
<td>VGSDGSLACVFNFAGAEHR</td>
<td>2100.5</td>
<td>2157.5</td>
<td>2158.138</td>
</tr>
<tr>
<td>CHPALWSLDTPEGYSWIDANDSANVLSFM</td>
<td>3612.3</td>
<td>3669.3</td>
<td>3669.033</td>
</tr>
<tr>
<td>VLPWSGVLWLPDFPCCDGGLYK</td>
<td>2526.2</td>
<td>2583.2</td>
<td></td>
</tr>
</tbody>
</table>

5a.3.6 GlgB disulfide is a substrate for WhiB1

Together with the thioredoxin-like property of WhiB1, its interaction with GlgB under reducing conditions and the presence of an intramolecular disulfide bond in *M. tuberculosis* GlgB indicated a possible role of WhiB1 as disulfide reductase of GlgB. Thus, it was pertinent to probe whether WhiB1/GlgB interaction involves a thiol-disulfide exchange reaction? To address this, the Trx affinity chromatography method, which is based upon the mechanism of thioredoxin-mediated reduction of the substrate disulfide was used. The procedure uses the chemistry of hetero-disulfide formation between the free cysteine residues
Figure 5a.9: MALDI-TOF spectroscopic analysis to show that *Mtb* GlgB has an intramolecular disulfide bond. Purified DTT treated and untreated GlgB was alkylated with 20 mM iodoactamide, digested with trypsin and precipitated with TCA. After washing with chilled acetone the mass of peptide generated were analysed by MALDI-TOF. Panel (A) to show both C95 and C658 are free (1) DTT treated and (2) DTT untreated. Panel (B) to show that C617 is buried and was accessible after pre-incubation of GlgB at 45 °C. (1) 45 °C, DTT treated (2) 45 °C, DTT untreated.
Functional analysis of WhiST

of C-X-X-C motif and its target. In this study, the mutation of WhiB1: C40→S40 has made C37 to possess a thiolate ion for a possible formation of mixed disulfide with any of the Cys residues of GlgB involved in disulfide bond formation. As shown in Figure 5a.10A, the GlgB was eluted in the presence of 10 mM DTT but not in high salt buffer wash. In a control experiment, the wild-type WhiB1 was used in place of C40S mutant. In this condition, most of the input GlgB washed out in high salt buffer (Figure 5a.10A). Thus, the result shows that WhiB1 might act as a reductant of the GlgB disulfide. To rule out the possibility that free cysteine residues, either C95 or C658 of GlgB forming a non-specific mixed disulfide with the free C37 of WhiB1 thus showing the interaction. Therefore, the free cysteine residues of GlgB were alkylated and then used for interaction as described. It can be seen from Figure 5a.10B that the alkylated GlgB also interacted with WhiB1 (C40S) in the Trx affinity column confirming that the interaction was specific to the cysteine residues involved in disulfide bond formation. These results indicated that the WhiB1 is most likely a reductant for GlgB and might also be a possible regulator under certain physiological conditions.

5a.4 Discussion

An attempt to identify the possible substrate protein(s) of WhiB1, whole genome interaction studies with yeast two-hybrid system were carried out which led to the identification of α-(1,4)-glucan branching enzyme (GlgB) as an interactor partner for WhiB1. The genome wide studies culminated in the identification of single interactor partner from whole genomic DNA library of \textit{M. tuberculosis}. The probable reason for getting such a low hit in interactor hunt might be due to the use of genomic DNA library instead of cDNA expression library. The limitations of genomic DNA library were very obvious since it was a complete \textit{EcoRI} and \textit{XhoI} digested library of \textit{M. tuberculosis} genome; therefore, probability of finding a true interactor domain as in-frame fusion with \textit{EcoRI} and \textit{XhoI} site of pJG4-5 is 1: 6. The \textit{in vivo} interaction was further supported by \textit{in vitro} GST-pull down assay, which revealed that the interaction between two proteins could occur only under reducing conditions. Our previous studies had revealed that WhiB1 is a protein disulfide reductase like many thioredoxin-like proteins.

Thioredoxin system is composed of thioredoxin, thioredoxin reductase and NADPH wherein reduction of active site disulfide is governed by a thioredoxin reductase, which accept electrons from NADPH. Thioredoxin-like proteins interact with their substrate proteins and subsequently transfer their reducing equivalents only when cysteines at the active site are in thiol form. Assumption of a similar situation for WhiB1 as thioredoxin-like
Figure 5a.10: Thioredoxin affinity chromatography to show that WhiB1 uses GlgB as a substrate for reductase activity. Both WhiB1 (C40S) and WhiB1 (WT) proteins (5 mg) were immobilized on cyanogen bromide column as described and purified GlgB (1 mg) was used for interaction. After washing with high salt buffer bound protein was eluted in 10 mM DTT and TCA precipitated before loading on to 10% SDS-PAGE. Lanes are as indicated on top and show that wild type WhiB1 failed to interact with GlgB as Cysteine residues of CXXC motif are present as disulfide. (B) Trx affinity chromatography with alkylated GlgB to rule out the nonspecific interaction of WhiB1 (C40S) with free Cys95 and Cys658 of GlgB. Alkylation of cysteine residues were carried out as described in MALDI-TOF analysis without DTT treatment.
protein and the need of reducing conditions for the interaction of WhiB1 and GlgB supports the fact that reduction of the active site disulfide of WhiB1 and subsequent conformational change is essential for the interaction. Further support for WhiB1-GlgB interaction can be derived from the fact that GlgB expression has been demonstrated in the stationary phase (Bruton et al., 1995). Reporter-based analysis of putative promoter of whiB1 in M. smegmatis also showed that it gets activated in stationary to late stationary phase of growth (Lab observations).

Glycogen is a highly branched, starch-like glucose polymer with ~95% α-(1,4)-linkages and ~5% α-(1,6)-branching linkages. Glycogen or other polysaccharides are used as storage compounds throughout the living world (Priess, 1984). In bacteria, three different enzymes are responsible for the catalytic conversion of glucose-1-phosphate to glycogen: (a) ADP-glucose pyrophosphorylase (GlgC), (b) glycogen synthase (GlgA) and (c) α-(1,4)-glucan branching enzyme (glycogen branching enzyme, GlgB) (Priess, 1996). The α-1,4 glucan branching enzyme [1,4-α-D-glucan: 1,4-α-D-glucan 6-D-(1,4-glucano)-transferase [EC 2.4.1.18] catalyzes the cleavage of an α-(1,4)-glucosidic linkage and subsequent transfer of cleaved oligosaccharide to form a new α-(1,6) branch (Borovsky et al., 1976). The presence of glycogen in bacteria serves multiple purposes, but the most crucial one is to store surplus primary carbohydrates, which could be used in later stages of growth depending upon the physiological state of the cell. *Mycobacterium tuberculosis* (*M. tuberculosis*) survives for long periods of time in a latent state, thus glycogen metabolism may play an important role in survival under unfavorable conditions.

CD spectroscopy, proteolytic cleavage and mass spectroscopy analyses of GlgB revealed that cysteine residues of GlgB form disulfide bond(s) which allows protein to exist in two different redox-dependent conformational states. These conformations have different surface hydrophobicities as evident from ANS-fluorescence of the oxidized and reduced GlgB. The change in surface hydrophobicity may have significance during interaction with different cellular protein at different physiological states (Garg et al., 2006). All the above observations suggested the formation of intramolecular disulfide bond in GlgB depending upon the redox state of the cell. To decipher whether disulfide bond(s) formation in *M. tuberculosis* GlgB is structurally feasible, a structural model of *M. tuberculosis* GlgB was generated in collaboration with “Dr. K. V. Radha Kishan, IMTECH”, which is based on *E. coli* GlgB (EcG) structural model (PDB code 1M7X). Generally, branching enzymes are multi-domain proteins due to their catalytic activity in dealing with high molecular weight
sugar molecules. The model shows that *M. tuberculosis* GlgB (MtBG) is likely to be a four-domain (N1, N2, TIM barrel catalytic, and C-terminus) protein, where the cysteines are distributed among all the four domains. As described earlier in the results (section 5a.3.5), when MtBG was subjected to alkylation with iodoacetamide in the oxidized and reduced conditions, C95 (on the N1 domain) and C658 (on the C-terminal domain) were alkylated without DTT treatment. This suggests that C95 and C658 are not involved in disulfide bond formation. In other words, the N1 domain and C-terminal domains possess exposed cysteine residues. Upon reduction, the C617 (present on TIM-barrel domain) was alkylated, while the peptide containing the C193 (present on N2 domain) was not detected. These results suggest that a disulfide bond could link the N2 and TIM-barrel domains and upon reduction, the two domains were separated which exposed the C617 of TIM-barrel domain while the N2 domain might have got reoriented with the C193 being still buried and not accessible for trypsinization. The results also suggest that the relative orientation of the N2 and TIM-barrel domains of MtBG is not similar to the arrangement in EcG. Variations in relative arrangement of domains are not unusual in this class of enzymes. Neopullulanase (NPL), an enzyme with similar function as that of GlgB also has similar domains except the N1 domain (Hondoh et al., 2003). These differences in the relative arrangements would be due to the long linker regions between the domains (Figure 5a.11A & B). For example: the N2 and TIM-barrel domains were separated by 22 residues in MtBG, 20 residues in EcG and 17 residues in NPL. Similarly, the TIM-barrel and C-terminal domains were separated by 10, 11 and 6 residues in MtBG, EcG and NPL, respectively. Therefore, based on the alkylation of cysteine residues in the oxidized and reduced states, followed by MALDI-TOF data, a general scheme for the arrangement of the 4 domains is depicted in Figure 5a.11C.

The previous results indicated a physical interaction between WhiB1 and GlgB, WhiB1 being a protein disulfide reductase, we addressed whether GlgB disulfide is a substrate for WhiB1 as a disulfide reductase. The above assumption was confirmed by the observations that WhiB1 (C40S) mutant was able to form a hetero-disulfide bond with GlgB in Trx affinity chromatography thus concludes that GlgB disulfide is a substrate for WhiB1.

Regulations of glycogen metabolism by thioredoxin-like proteins have been reported from both higher plants and cyanobacteria (Lindahl and Florencio, 2003; Hendriks et al., 2003). Redox regulation of pullulanase-type de-branching enzyme, which is structurally and functionally similar to GlgB, has been proposed in a number of plant tissues, including spinach leaves and the endosperms of barley and maize (Schindler et al., 2001; Cho et al.,
Figure 5a.11: Homology model of Mtb GlgB. (A) The 3 domains are colored differently (N2) domain- orange; TIM-barrel domain - magenta; C-terminal domain - blue. The linker regions between the domains were shown in red. The position of the Cys residues on the individual domains was shown as green sticks. (B) The superposition of neopullulanase structure on the Mtb GlgB model. The color coding for the individual domains on Mtb GlgB was as in A and neopullulanase structure was shown in yellow. The TIM-barrel domain and the C-terminal domains superpose well with each other, however, the N2 domain orientation is different in neopullulanase when compared to Mtb GlgB. (C) The schematic on the domain arrangement based on MALDI-TOF data. The arrangement of domains of E. coli GlgB (N2, TIM-barrel and C-terminal) as seen in the E. coli GlgB structure.
1999). *In vitro* studies with β-amylase also showed reversible inactivation *via* disulfide interchanges (Spradlin and Thoma, 1970). It was shown in *Streptomyces coelicolor* A3(2) that glycogen metabolism is developmentally regulated (Bruton *et al.*, 1995) and a role of *whiB*-like genes in glycogen metabolism has been demonstrated (Yeo and Chater, 2005).

The role of redox-dependent conformational change on the branching enzyme activity of GlgB was studied by alkylating the cysteine residues with iodoacetamide before protein purification. After the modification, cysteine residues are unable to form disulfide bonds. The protein was purified from both alkylated and non-alkylated samples and then tested for the enzymatic activity. It is clear that the change in conformation did not affect the enzyme activity (Figure 5a.12). The result was further confirmed by zymography (Figure 5a.12 inset). These results clearly showed that the redox-dependent conformational change is not directly associated with the branching enzyme activity. However, the data presented here is qualitative and might have not accounted subtle differences in activity due to conformational changes. Although, the conformational change that exposes more hydrophobic regions might facilitate the protein to interact or dissociate from other cellular protein(s), which in turn, may regulate the branching enzyme activity in response to different physiological conditions.

In the present study, we demonstrated using *in vivo* and *in vitro* methods that WhiB1 interacts with α-(1,4)-glucan branching enzyme (GlgB), which is an enzyme of glycogen metabolism. The characterization of recombinant GlgB showed that protein was active in utilizing amylose as a substrate with a pH and temperature optima of 7.0 and 30 °C, respectively. The biophysical characterization of recombinant GlgB revealed that protein exists in two different conformations depending upon the redox state of the cell. The mass spectroscopy and structural model suggested that C193 and C617 are involved in disulfide bond formation, which is responsible for the redox-dependent conformational change. By using thioredoxin affinity chromatography, we showed that disulfide of GlgB is a substrate for WhiB1 disulfide reductase activity.
Figure 5a.12: Redox-dependent conformational change does not affect the branching enzyme activity. Enzyme assay was carried out after alkylation of cysteine residues. To alkylate cysteine residues of GlgB, ECGL cells were re-suspended in lysis buffer containing 2 mM iodoacetamide and protein was purified as described in "section 5a.2.4". A zymogram showing both DTT- treated (1) and untreated (2) GlgBs are active (Inset).
Chapter-5b
Role of WhiB1 during oxidative stress in M. tuberculosis

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5b. Introduction

Prokaryotic core RNA Polymerase (RNAP) is composed of four distinct subunits: β, β', ω and α dimer. The fifth subunit, σ factor reversibly associates with RNAP forming the RNAP holoenzyme, and provides the promoter recognition function. The number of σ factors encoded in a genome is quite variable and ranges from a minimum of one in *Mycoplasma* sp. (Fraser et al., 1995; Himmelreich et al., 1996) to a maximum of 65 in *Streptomyces coelicolor* A3(2) (Bentley et al., 2002). *Mycobacterium tuberculosis* encodes 13 different putative σ factors (Gomez et al., 1997; Cole et al., 1998; Gomez and Smith, 2000). It is generally observed that each σ factor has its own specificity allowing the initiation of transcription of different subsets of genes. Genes belonging to a defined regulon often participates in related cellular functions. Therefore, temporal variation in active σ factor population may represent a powerful way for *M. tuberculosis* to modulate its gene expression profiles in accordance with the physiological requirements and thus achieve a successful infection.

*M. tuberculosis* virulence has been associated with its initial survival within the hostile conditions of macrophages and resistance to reactive oxygen and nitrogen intermediates (Sherman et al., 1995, St John et al., 2001; Storz et al., 1997). To encounter such adverse conditions, *M. tuberculosis* encodes a σ factor responsive to oxidative and heat shock stress known as σ^H^ (SigH). The *M. tuberculosis* sigH gene is induced after treatment with elevated temperature and thiol-specific oxidizing agent diamide (Manganelli et al., 1999; Raman et al., 2001). Using DNA microarray, 48 genes were identified whose expression was elevated after exposure to thiol-specific oxidant diamide; out of these, 39 genes were not induced in sigH null mutant, showing their direct or indirect dependence on σ^H^ (Manganelli et al., 1999; Raman et al., 2001). The genes under σ^H^ control included transcriptional regulators (σ^B^, σ^E^, and σ^H^); proteins involved in thiol metabolism, such as thioredoxin, thioredoxin reductase and enzymes involved in cysteine and molybdopterine biosynthesis (Raman et al., 2001). The immunopathological studies of sigH null mutant showed a distinctive infection phenotype. In resistant C57BL/6 mice, the mutant resulted into a delayed pulmonary inflammatory response. Similarly, in susceptible C3H mice, mutant exhibited diminished immunopathology and did not produce a chronic infection over a long period of time, where life span of infected mice was approximately 3 times to those of infected with wild type bacilli (Kaushal et al., 2002). The complete genome microarray analysis by Kaushal et al. (2000) revealed that σ^H^ mediates the transcription of at least 31 genes directly and modulates the expression of about
150 others. Above literature suggest that *sigH* gene is dispensable for bacterial growth and survival within the host but is required for the immunopathology and lethality.

σ^H^ is very similar to the extra-cytoplasmic σ factor σ^R^ of *S. coelicolor* A3(2). Similar to σ^H^, σ^R^ responds to the heat shock and oxidative stress generated by the formation of disulfide bonds due to the oxidation of cysteine thiol groups (Paget *et al.*, 1998). The σ^R^ activity is regulated at the posttranslational level by a cysteine-containing metalloprotein anti-σ factor (RsrA) whose gene is adjacent to the *sigR*. In a reducing environment, Zn^{2+}-coordinated RsrA binds σ^R^ keeping it in inactive state however, in an oxidizing environment, release of Zn^{2+} from RsrA facilitates the disulfide bond formation, leading to change in the protein conformation and release of σ^R^ in its active form, from the σ^R^-RsrA complex (Paget *et al.*, 2001) (Figure 5b.1). In *M. tuberculosis*, the last gene of *sigH* operon encodes an anti-σ factor of σ^H^ designated as rshA. Similar to σ^R^-RsrA association, RshA also binds to the σ^H^ in redox-dependent manner and this interaction is a means by which σ^H^ is regulated in response to oxidative stress (Song *et al.*, 2003).

The above literature demonstrates a key role of anti-σ^H^ factor in the regulation of σ^H^ during oxidative stress in *M. tuberculosis*. The observation that σ^H^-RshA complex can only form in reducing environment suggests that similar to RsrA, Zn^{2+} coordinated RshA monomer binds to a single molecule of σ^H^ (Song *et al.*, 2003). During oxidative stress, the disassembly of Zn^{2+} atoms from the RshA may be responsible for the conformational changes due to the disulfide bond formation that allows RshA to disassociate from the σ^H^-RshA complex. However, very little is known about the redox regulation of anti-σ^H^. What factors govern the reduction of RshA after oxidative stress is over? For RsrA of *S. coelicolor*, it has been shown *in vitro* that thioredoxin can transfer the reducing equivalents to the protein to reduce it and subsequently reassociate with σ^R^ to provide a feedback mechanism for the regulation of σ^R^ activity through RsrA (Paget *et al.*, 1997). In the Chapter-4 of this study, it has been established that apo-WhiB1 works as protein disulfide reductase. The reductase activity of WhiB1 is fine-tuned by virtue of the presence of a regulatory [Fe-S] center, which prompted us to speculate the oxidative stress associated function for WhiB1. An observation that *whiB1* is situated in the close proximity of *sigH* and *rshA* in the same genetic locus of *M. tuberculosis* also supports this assumption. Therefore, in the light of above information, we asked a question whether WhiB1 being a redox-regulated thioredoxin-like protein, would work as a disulfide reductase for RshA?
Figure 5b.1: A model for the regulation of $\sigma^R$ activity in response to disulfide stress in *S. coelicolor* A3(2) (adapted from Paget and Buttner, 2001).
In the present study, by using yeast two-hybrid system, the physical interaction between WhiB1 and anti-σ^H factor has been demonstrated and by using a Trx affinity chromatography, we ascertained that WhiB1 uses disulfides of anti-σ^H factor as a substrate for disulfide reductase activity.

5b.2 Methods

5b.2.1 Yeast two-hybrid system

The 303 bp rshA ORF was PCR amplified using primers: forward (AsigF) 5’ ATA TAT GAA TTC GTG AGC GAA AAT TGC GGT CCG AC 3’ and reverse (AsigR) 5’ ATA TAT GTC GAC GGG CCC TCC ACG GAT GAT GGT GG 3’ with an EcoRI and Sall sites (underlined). The purified PCR product was digested with EcoRI-Sall and cloned in to the same sites of the vector pEG202 to generate pDBD-AH. The whiB1 gene was cloned in vector pJG-4-5 at the EcoRI-XhoI site to express it in-fusion with the activation domain of acid blob under gal promoter (pAD-B1). To check whether RshA has an intrinsic transcription activation property of the reporter gene, *Saccharomyces cerevisiae* EGY191 was transformed independently as follows: (a) pDBD-AH+ pSH18-34 (test) + pJG4-5; (b) pSH17-4 + pSH18-34 (positive control for activation) + pJG4-5; (c) RFHMI + pSH18-34 (negative control for activation) + pJG4-5; (d) pDBD-AH + pJK101; (e) pEG202 + pJK101 (vector control) and (f) pJK101. The transformants were selected on respective dropout plates and β-galactosidase activity was tested on YNB Gal^Ura_X-gal plates. Plasmids pDBD-AH and pSH18-34 were co-transformed into *S. cerevisiae* EGY191 along with the pAD-B1 while rest of the procedures were as described in the section 5a.2.1.

5b.2.2 Expression and purification of rshA/Rv 3221A

The 303 bp rshA ORF was PCR amplified as described in section 5b.2.1. The purified PCR product was digested with EcoRI-Sall and cloned in to the same sites in vector pET-29a. The correct clone was transformed to the *E. coli* BL21(DE3) strain to express the protein. The full-length RshA protein was expressed in the presence of 0.3 mM IPTG at 16 °C for 18 hrs. Cell lysate was prepared as described for the WhiB1 protein in the section 3.2.4. The purification steps of 6× His-tagged anti-σ^H protein were as follows: after loading the soluble lysate to the column, it was washed with 20 column volumes of the buffer containing 20, 30 and 40 mM of imidazole made in 50 mM NaH_2PO_4 buffer, pH 8.0 and in 300 mM NaCl, successively. The bound protein was eluted in the buffer containing 200 mM imidazole made in the same buffer and dialyzed as required.
For the redox analysis of RshA, 3 μg of purified protein was incubated in degassed 50 mM Tris.HCl, pH 7.5, 100 mM NaCl with GSH (5 and 10 mM) or DTT (1, 10 and 50 mM) or β-ME (3%) in a nitrogen atmosphere at 25 °C for 3 hrs and then 20 mM iodoacetamide (final concentration) was added to the samples to block free thiols and the reaction was incubated for 60 min at room temperature in dark. The samples were run on 15% SDS-PAGE and were visualized by coomassie blue staining.

5b.2.3 Trx affinity chromatography

The method of Motohashi et al. (2005) was used to immobilize the WhiB1C40S mutant protein to a cyanogen bromide activated Sepharose 4B resin with some modifications as described in section 5a.2.7. For WhiB1 and RshA interaction, 1 ml of WhiB1C40S bound resin was equilibrated with 5 column volumes of binding buffer: 50 mM Tris.HCl, pH 8.0, 100 mM NaCl, 0.05% TritonX-100, 2 mM EDTA and 10 mM MgCl2 and then 1 mg of RshA in binding buffer was mixed with the WhiB1C40S bound resin. The interaction was carried out for 3 hrs at 25 °C in the presence of protease inhibitor cocktail with continuous mixing. Beads were washed with 50 mM Tris.HCl, pH 8.0 and 0.5 M NaCl until the wash was free of proteins and then the bound protein was eluted in 3 ml of buffer: 50 mM Tris.HCl, pH 8.0, 100 mM NaCl and 10 mM DTT. After TCA precipitation and two washes with chilled acetone, the protein was loaded on to a 12% SDS-PAGE and visualized by the Silver staining method.

5b.3 Results

5b.3.1 WhiB1 interacts with anti-σH factor (RshA)

Interaction between WhiB1 and RshA was detected under physiological conditions using yeast two-hybrid system. To verify that RshA does not have an intrinsic property to activate the transcription and yeast machinery has processed the rshA gene for protein expression, several controls were used to characterize RshA as a “bait”. The characterization revealed that RshA is suitable to study in yeast two-hybrid system as it did not activate the transcription of the reporter gene and was processed and expressed in S. cerevisiae. Therefore, pDBD-AH and pAD-B1 was co-transformed along with a reporter plasmid pSH18-34 into S. cerevisiae EGY 191. Figure 5b.2 shows that the WhiB1 interacts with RshA in physiological conditions because cells co-transformed with bait and prey plasmids exclusively grew on CM-Gal+Leu- plates. The β-galactosidase plate assay also confirmed the interaction between WhiB1 and RshA. The interaction partners could not be swapped
Figure 5b.2: In vivo yeast two-hybrid system to show that WhiB1 interacts with RshA. pDBD-AH (rshA cloned in pEG202) and pAD-B1 (whiB1 cloned in pJG 4-5) plasmids were transformed to S. cerevisiae EGY191 with the reporter plasmid pSH18-34 containing β-gal as reporter gene. The transformants were selected on Glu+his-ura-trp- plates. To check the interaction, different clones were plated on Gal+his-ura-trp-leu- after 6 hrs of induction in Gal+his-ura-trp- medium. The clones were tested for specific interaction on Gal+his-ura-trp-leu- and Glu+his-ura-trp-leu- plates. β-Galactosidase expression was monitored on Gal+-X-Gal and Glc+-X-Gal plates.
because the vector pJG 4-5 has only EcoRI and XhoI sites for cloning and the rshA gene has two internal XhoI sites.

**5b.3.2 Expression and purification of recombinant RshA**

In order to confirm the *in vivo* interaction of WhiB1 and RshA by an *in vitro* method and to establish other properties of RshA, rshA gene was cloned in the expression vector pET-29a (Figure 5b.3) and expression was carried out in *E. coli* BL21 (DE3) as a C-terminal 6× His tagged protein. To our surprise, no expression was detected at induction temperatures: 37, 30 and 25 °C. However, a shift to 16 °C for 18 hrs made all the difference, where high level of expression was observed and the protein was in the soluble form. Protein was purified by metal affinity chromatography using Ni²⁺-NTA affinity matrix (Qiagen, Germany) and the protein eluted at 200 mM imidazole concentration. Different fractions were checked for the purity on a 15% SDS-PAGE (Figure 5b.4A). Soluble RshA was purified as a single protein band of an apparent molecular weight of ~17 kDa, which is in well agreement with the theoretical molecular weight of the recombinant RshA.

As it has been shown for RsrA of *S. coelicolor* and proposed for RshA of *M. tuberculosis* that in the presence of oxidizing agents, these proteins form intramolecular disulfide bonds. RsrA contains a motif C-X₃-H-X₃₅-C-X₂-C, which is a reminiscent of many zinc-finger proteins. The disulfide bond that is primarily responsible for deforming RsrA is not formed between the proximal cysteine residues within the H-X₃-C-X₂-C motif. Instead, a long-range bonding was formed between C11 and C44. The second, less critical disulfide bond is formed between the C41 and C61. The three (C11, C41 and C44) cysteine residues that form disulfide bonds are the ones essentially required for σ⁸-binding and redox modulation of RsrA *in vitro* and *in vivo* (Bae *et al.*, 2004). The RshA of *M. tuberculosis* has six conserved cysteine residues, like RsrA, suggests the presence of probable Zn²⁺-binding motif and upon oxidation, similar arrangement of disulfide bonds. Therefore, purified RshA was analyzed for the formation of intramolecular disulfide bonds. The SDS-PAGE analysis with DTT, β-ME and GSH revealed that protein migrates slower in the presence of reducing agents than in their absence indicating a possibility of disulfide-mediated conformational change (Figure 5b.4B). The diffused bands in some of the lanes are possible, if the protein samples contain a mixed population of oxidized and partially reduced protein, which is possible during purification.
Figure 5b.3: Scheme of cloning of rshA/Rv 3221A in the expression vector pET-29a.
Figure Sb.4: (A) Expression and Ni²⁺-NTA affinity purification of RshA from *E. coli* BL21 (DE3). Recombinant protein was expressed as 6×His tag protein at 16 °C for 18 hrs after induction with 0.3 mM IPTG at OD₆₀₀ ~0.6. The protein was purified from Ni²⁺-NTA column. (B) RshA forms intramolecular disulfide bonds. Redox analysis of RshA (3.0 µg) was carried out with different reducing agents. (C) Amino acid sequence of RshA showing six cysteine residues in bold, out of which two are present in C-X-X-C motif.
Functional analysis of WhiB1

5b.3.3 Interaction of WhiB1 and RshA involves thiol-disulfide exchange

Having demonstrated the interaction of WhiB1 and RshA by yeast-two hybrid system, we wanted to confirm whether the interaction indeed involves thiol-disulfide exchange between the two proteins? To address this, a Trx affinity chromatography method as described in section 5b.2.3 was used. As shown in Figure 5b.5, the RshA eluted in the presence of 10 mM DTT but not in high salt buffer wash. In a control experiment, the WhiB1WT replaced the WhiB1C40S mutant. In this condition, most of the input RshA was eluted in the high salt wash buffer. Thus, the result showed that WhiB1 also interacts with RshA in vitro and involved in exchange of thiol-disulfide thus, WhiB1 could be a disulfide reductant for RshA under physiological conditions.

5b.4 Discussion

In the present study, a knowledge-based approach was followed to deduce the possible in vivo role of WhiB1 in M. tuberculosis. The most important understanding to unravel the function of WhiB1 came from the biochemical and biophysical studies, which established that WhiB1 is a redox-regulated protein disulfide reductase (as discussed in the chapter-4). The disassembly of Fe-S cluster of WhiB1 during oxidative stress leads to the formation of two intramolecular disulfide bonds, which imparts the disulfide reductase activity to WhiB1. Our assessment that WhiB1 might be involved in redox signaling was further strengthened by the fact that whcE (a Mtb whiB1 homologue) of Corynebacterium glutamicum was found to be involved in both growth adaptation and stress responses. A null mutant of whcE was shown to be susceptible to heat shock as well as thiol-specific oxidant diamide and redox cycling compounds such as menadione and plumbagin (Kim et al., 2005). The most striking observation of Kim et al. (2005) showed that in a sigH mutant strain, the whcE gene was no longer expressed indicating that the sigma factor H is involved in its expression. Similar to C. glutamicum, whiB1 of M. tuberculosis is also present in the close proximity of sigH (Figure 5b.6).

The available information suggests that σ^H of M. tuberculosis may be involved in the regulation of expression of whiB1. The notion is further supported by the fact that the upstream region of whiB1 gene of M. tuberculosis also has a putative consensus sequence for SigH binding, suggesting a possible regulation of whiB1 gene through σ^H.
Figure 5b.5: Thioredoxin affinity chromatography to show that WhiB1 and RshA interaction involved a thiol-disulfide exchange. Both WhiB1 (C40S) and WhiB1 (WT) proteins (5 mg) were immobilized on cyanogen bromide column as described and purified RshA (1 mg) was used for interaction. After washing with high salt buffer the bound protein was eluted in 10 mM DTT and TCA precipitated before loading on to 12% SDS-PAGE. Proteins were visualized through staining with silver ions.
In the present study using yeast two-hybrid analysis, physical interaction between WhiB1 and RshA was established. The in vitro Trx affinity chromatography revealed that WhiB1-RshA interaction involved a thiol-disulfide exchange. As it is mentioned earlier the $\sigma^H$-RshA complex is inactive until oxidative stress or heat shock stress is provided to M. tuberculosis. During oxidative stress, RshA dissociates from $\sigma^H$, which results into disulfide bond formation in RshA. Mutation in Cys$^{52}$ or Cys$^{55}$ abolishes RshA binding to the $\sigma^H$ showing their involvement in the complex formation (Song et al., 2003). After oxidation, the cysteines of RshA have to undergo reduction before it can again coordinate with Zn$^{2+}$ and subsequently bind to $\sigma^H$. The earlier results and interaction data of WhiB1 and RshA led to the suggestion that WhiB1 works as a disulfide reductase for RshA. A probable working model of WhiB1 during oxidative stress conditions has been shown in Figure 5b.7. It is noteworthy that even though RshA possess a C-X-X-C motif, it did not catalyze the reduction of insulin disulfide (lab observations) suggesting that the disulfide reductase activity of WhiB1 is specific and C-X-X-C motif does form an active site disulfide bond unlike RshA where C-X-X-C motif cysteines do not form disulfide bond between them.

To conclude, the genome wide in vivo target identification and knowledge-based approach lead to the identification of two interactor proteins of WhiB1 i.e. an $\alpha$-(1,4)-glucan branching enzyme (GlgB) and an anti-$\sigma^H$ factor (RshA). The physiological roles of both the targets are diverse and they are involved in totally different cellular processes. The GlgB is
Figure 5b.7: Proposed role of WhiB1 during infection and oxidative stress in *M. tuberculosis.*
the last enzyme of glycogen biosynthesis pathway, whereas RshA regulates the activity of heat shock and oxidative stress responsive sigma factor H. WhiB1 has been shown to express in stationary phase by RT-PCR (Geiman et al., 2006). Reporter-based analysis of putative promoter of whiB1 in M. smegmatis also showed that whiB1 gets activated in the stationary to late stationary phase of growth (Lab observations). The stationary phase of growth is often associated with the accumulation of toxic metabolites resulting into an exposure to various stresses including oxidative stress and nutrient starvation. Therefore, the expression of whiB1 in stationary phase validates its involvement in the glycogen metabolism as well as during oxidative stress. Moreover, thioredoxin-like proteins are known to regulate the activity of different proteins involved in different cellular processes. E. coli thioredoxin has been shown to modulate an array of cellular functions by regulating the target protein either through a thiol-disulfide exchange or just being a part of a protein complex as a structural component. Therefore, involvement of WhiB1 as a protein disulfide reductase for different cellular proteins performing diverse functions is not unexpected.