SUMMARY

Recent advancement in tuberculosis research has improved our understanding about the disease however, it is still a formidable public health problem in developing countries. Geographical variations among the strains and difference in virulence depending upon the population also make it difficult to find one common vaccine for tuberculosis. Despite the immense information available for *M. tuberculosis*, its complete biology and pathogenesis is still poorly understood. The genome of *M. tuberculosis* has large number of genes with hypothetical functions thus, the present study is an attempt to understand the function of one of those hypothetical genes, which *M. tuberculosis* seem to have acquired and maintained during evolution. It is now established that *whiB* genes are present in all the species of actinomycetes, irrespective of the fact whether they sporulate or not, including *M. leprae*. It has been shown that apart from its possible role in the sporulation of *S. coelicolor* (as originally described), different *whiB*- like genes have been shown to have different functions (Davis and Chater, 1992; Homerova *et al.*, 2003; Gomez and Bishai, 2000; Raghunand and Bishai, 2006a; Styen *et al.*, 2002; Morris *et al.*, 2006; Geiman *et al.*, 2006). The present study also demonstrates that different WhiB proteins will indeed have different functions.

The bioinformatic analysis of WhiB proteins does not provide any indication about the properties of these proteins. Thus, in the present study, the reference point to decipher the biochemical and biophysical properties of WhiB1/Rv3219 was the four conserved cysteine residues and the C-X-X-C motif, which is C-N-R-C in the case of WhiB1/Rv3219 of *M. tuberculosis* H37Rv. Thus, WhiB1 protein was expressed and purified to homogeneity from *E. coli* using Ni^{2+}-NTA affinity chromatography in a single step. Cells from 1 L culture produced ~35 mg of ~98% pure protein. The biochemical characteristics of amino acid cysteine are unique and exceptional due to the presence of thiol group (Giles *et al.*, 2003). Considering redox activity and post-translational modifications, the sulfur of cysteine residues in different proteins can exist in numerous oxidation states. Among them, the thiol and disulfide oxidation states are well characterized. The unique metal binding ability of cysteine due to the thiolate ligand allows it to coordinate with different metal ions. Therefore, the presence of four invariant cysteine residues in WhiB1 makes it an ideal protein to coordinate with specific metal ion. Freshly purified concentrated WhiB1 protein at ~3 mg/ml concentration appeared brownish, an indicative of the presence of a chromophore. The UV-visible range (300-800 nm) spectra displayed two distinct peaks at ~340 nm and ~420 nm with shoulders at ~450 nm and ~550 nm, a feature of [2Fe-2S] cluster containing proteins.

Iron-specific chelation by *o*-bathophenanthroline (OBP) clearly established the coordination of Fe in WhiB1. The iron: WhiB1 ratio varied between 0.13 and 0.15, depending upon the preparation indicating that the cluster was substoichiometric (6.5-8% occupancy for a [2Fe-2S] cluster). The Fe-S cluster of WhiB1 could be reconstituted under semi-anaerobic conditions without using any cofactor. After reconstitution, Fe estimation by OBP method showed that the reconstituted WhiB1 contains 2.85-3.25 iron atoms every WhiB1 monomer. Surprisingly, the UV-visible absorption spectrum of the reconstituted WhiB1 had a single broad peak at 400 nm and did not show any other resolved features at longer wavelength thus, was drastically different from the absorption spectrum of the freshly purified WhiB1. The molar extinction coefficient (ε) at 400 nm for Fe-S cluster of the reconstituted WhiB1 indicated the presence of a [4Fe-4S] cluster, which was then confirmed by electron paramagnetic resonance (EPR) spectroscopy. The mutational studies for ligand identification revealed that the cysteine residues of WhiB1 are critical for the coordination of the iron-sulfur cluster. The Cys^{40}, the C-terminus cysteine of the CNRC motif of WhiB1 was the most critical residue for the coordination of [Fe-S] cluster.

The [4Fe-4S] cluster of WhiB1 is sensitive to air oxidation and the exposure to air oxidizes the [4Fe-4S] core of WhiB1 to [2Fe-2S] core. It is noteworthy that even after a prolonged exposure to air (up to 18 hrs), ~50% of the cluster was retained indicating that the cluster has relatively high affinity with the protein compared to the iron-sulfur cluster of WhiD, which also belongs to WhiB family where exposure to air resulted into complete loss of the cluster (Jakimowicz *et al.*, 2005). This observation was further substantiated by the fact that iron-sulfur cluster of WhiB1 is rather resistant to EDTA chelation where even ~1000-fold molar excess of EDTA did not completely chelate the Fe of iron-sulfur cluster. The [2Fe-2S] cluster bound to IscA2 of *Synechocystis* PCC 6803 was also found to be resistant to EDTA (Morimoto *et al.*, 2003) and was highly sensitive to superoxides and free radicals. Surprisingly, WhiB1 cluster was disintegrated by H_2O_2 in 5 min even when H_2O_2 was present in less than equimolar ratio (100 μM H_2O_2: 120 μM WhiB1). Therefore, the rate of oxidation and cluster removal was directly proportional to the strength of oxidizing environment. The intra-phagosomal concentration of H_2O_2 also usually reaches to 10^{-4} M (Imlay, 2006) thus, WhiB1 iron-sulfur cluster is highly sensitive to superoxides *in vitro*. Glutathione is the most important ingredient of redox buffer of several eukaryotes and prokaryotes including mammals. Its intracellular concentration is approximately 5 mM and it is kept almost completely reduced (the ratio of reduced to oxidized glutathione in *E. coli* is about 200 to1,

Kosower and Kosower, 1978). Thus, it is also important to note that in the presence of oxidized glutathione, the disassembly of WhiB1 cluster was accelerated suggesting its sensitivity towards redox milieu of the cell. Although *M. tuberculosis* redox buffer is not glutathione but a small thiol-containing compound: mycothiol works as redox buffer in actinomycetes (Newton *et al.*, 1996; Bornemann *et al.*, 1997), including *M. tuberculosis*. The iron-sulfur cluster of WhiB1 was resistant to air oxidation in a reducing environment. Therefore, the sensitivity of the iron-sulfur cluster towards the superoxides and free radicals contrary to its stability under the reducing conditions suggests a possible redox dependent regulatory function.

The above assumption was further strengthened by the fact that disassembly of the cluster from WhiB1 led to the exposure of significant ratio of the hydrophobic regions on the surface of the protein as was evidenced from the ANS binding to apo- and holo-WhiB1. The intrinsic tryptophan fluorescence and near-UV CD spectroscopy also confirmed that WhiB1 undergoes substantial conformational changes after the removal of iron-sulfur cluster. Having found that iron-sulfur cluster of WhiB1 is oxidation-labile and cluster disassembly resulted into a conformational change, a question was asked: what could be the significance of redox-sensitive iron-sulfur center and subsequent change in the conformation of the protein upon its removal? The vulnerability of iron-sulfur clusters to oxidative destruction is sometimes associated with the modulation of function of a protein depending upon the redox state of the cell. Reversible cluster rearrangement or disassembly may facilitate alterations in protein structure, which may in turn, regulate its function (Beinert and Kiley, 1999). However, the basic question *i.e.* what is the biochemical function of WhiB1, still remains unanswered.

A possible key to unravel the biochemical function was found from the near-UV CD spectral properties of apo- and holo-WhiB1, where removal of iron-sulfur cluster from the protein allowed cysteine residues to form intramolecular disulfides.

This observation was further substantiated by SDS-PAGE analysis of WhiB1, which showed a conformation dependent mobility differences in the oxidized and reduced apo-WhiB1. TlpA, a thioredoxin-like protein of *Bradyrhizobium japonicum* has been shown to have two intramolecular disulfide bonds where one of them is formed between the cysteines of C-X-X-C motif and another as a structural component (Loferer and Hennecke, 1994). WhiB1 also has a C-X-X-C motif, though we could not show the arrangements of disulfide bonds, as the cysteine double mutants did not express in any condition, the mass spectrometry of the wild type protein under reduced and oxidized conditions clearly showed that the four cysteine residues of WhiB1 form two intramolecular disulfide bonds. It has been shown for

several thioredoxin-like proteins that the disulfide bond formation results into subtle alterations in the conformation of the protein. Intrinsic tryptophan fluorescence studies of WhiB1 also showed change in the conformation due to the reduction of disulfide bonds. The standard redox potential of the disulfides of WhiB1 was determined as –236 mV. The intrinsic redox potential of apo-WhiB1 is comparable to that of other cytoplasmic thioredoxins. Similar to all thioredoxin-like proteins, WhiB1 also catalyzes the reduction of insulin disulfides, though it was relatively less efficient than the *E. coli* thioredoxin, also explained by the fact that the redox potential of WhiB1 is –236 mV, which is much higher than *E. coli* thioredoxin (–269 mV). The secondary structure content and its resistance to thermal denaturation also indicated the presence of a thioredoxin-like fold in WhiB1. Therefore, we conclude that WhiB1 is a protein disulfide reductase similar to many thioredoxin-like proteins.

Two classes of disulfide reductases, ferredoxin:thioredoxin reductase of chloroplast and heterodisulfide reductases of methanogenic archea (Dai *et al.*, 2000; Duin *et al*, 2002; Walter and Johnson, 2004), have been demonstrated which uses an active-site [4Fe-4S] cluster to cleave disulfides in two sequential one-electron steps using novel site-specific cluster chemistry, which involves an intermediate with two thiolate ligands at a unique Fe site. The presence of a regulatory iron-sulfur cluster provides at least two properties to a protein simultaneously: (a) it can sense the redox state of the cell and (b) it can also modulate the protein topology to facilitate the selective interactions in apo- and holo-form to macromolecules. In our study, the disulfide reductase activity was confined to the apo-form, which is not unexpected because in holo-WhiB1 as cysteinyl ligation at a unique Fe site of a [4Fe-4S] cluster would occupy all the free thiols of WhiB1 hence, it is very much obvious that without free thiols, WhiB1 would not participate in the thiol-disulfide exchange reaction. Therefore, we assume that disulfide reductase activity of WhiB1 is elegantly regulated by the presence of iron-sulfur cluster. The cluster disassembly is essential for the conformation change of WhiB1 and subsequent interaction with targets proteins to perform protein disulfide reductase activity. Thus, above observations provided the information that WhiB1 would most likely be active during oxidative stress conditions in *M. tuberculosis*.

After establishing that WhiB1 is a redox-regulated disulfide reductase active during oxidative stress conditions in *M. tuberculosis*, it is important to unravel the *in vivo* cellular targets of WhiB1 to perform its disulfide reductase activity. To identify the regulatory pathway in which WhiB1 may possibly participate, two different strategies were adopted: (a) a genome wide search for the target protein/s (b) a knowledge-based approach to confirm the

hypothetical target/s of WhiB1. The whole genome interaction studies using yeast two-hybrid system led to the identification of α-(1,4)-glucan branching enzyme (GlgB) as an interactor partner of WhiB1. The *in vivo* yeast-two hybrid interaction data was further supported by *in vitro* GST pulldown assay. One important observation of the GST pulldown studies was that the interaction of two proteins occurred only under mild reducing conditions and was specific. Primary sequence analysis of GlgB has shown that *M. tuberculosis* and *M. bovis* GlgB have four cysteine residues. We have also established that WhiB1 is a disulfide reductase. Based on these results, we asked a question whether *M. tuberculosis* GlgB indeed has an intramolecular disulfide bond? The characterization of GlgB protein by CD spectroscopy, proteolytic cleavage and mass spectroscopy revealed that cysteine residues of GlgB form disulfide bond(s), which allowed the protein to exist in two different redox-dependent conformational states (Garg *et al.*, 2006). The fact that these conformations have different surface hydrophobicities was evident from the ANS-fluorescence of the oxidized and reduced GlgB. Homology modeling based on MALDI data also showed that a disulfide bond can form between C193 and C617 of GlgB. Finally, by using the Trx affinity chromatography, it was established that GlgB disulfide is a target for WhiB1, which explains why a mild reducing environment was essential for the interaction in the GST pulldown assay (as mentioned earlier that WhiB1 is a disulfide reductase thus until the WhiB1 disulfide is reduced, it can not transfer its electron to other disulfide). Although, we assumed that WhiB1 would participate in the pathways involved in the defense system against oxidative stress in *M. tuberculosis* but the product of single thioredoxin gene has been implicated in diverse cellular processes. The 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 (apparently proved by molecular modelling) and functionally similar to the 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.*, 1999). Plant β-amylase is reversibly inactivated by 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). Therefore, in the present study, we conclude that WhiB1 play an important role in the regulation of glycogen metabolism in *M. tuberculosis*.

In *M. tuberculosis*, σ factor σ^H (SigH) responds to the oxidative and heat shock. Under normal growth conditions, a Zn^{2+} binding protein: anti-σ^H factor (RshA) regulates the σ^H activity. In a reducing environment, Zn^{2+} coordinated RshA binds σ^H and maintains it in an inactive state; however during oxidative stress, Zn^{2+} release from the RshA facilitates the disulfide bond formation leading to change in the protein conformation, as a consequence, σ^H is released in its active state from the σ^H-RshA complex (Song *et al.*, 2003). The feed back regulation of σ^H activity was achieved by the reduction of structural disulfides of RshA. The gene encoding SigH and RshA and WhiB1 are present in the same locus. It is established that apo-WhiB1 works as a protein disulfide reductase and its activity is fine tuned by virtue of the presence of a regulatory iron-sulfur center. These data prompted us to speculate the oxidative stress associated function for WhiB1. Therefore, we tested whether WhiB1 will work as protein disulfide reductase for RshA. Interaction in a yeast two-hybrid system clearly demonstrated that WhiB1 indeed interacts with RshA. As yeast two hybrid system is prone to produce false positive result, the interaction of RshA and WhiB1 was confirmed by *in vitro* technique "Trx affinity chromatography". This method works only if there is a thiol-disulfide exchange between the two interacting partners, which are WhiB1 and RshA in this particular study. Disulfide bond formation in RshA was demonstrated earlier (Song *et al.*, 2003) and was confirmed further before initiating the experiment. The protein elution pattern as demonstrated by the Trx affinity chromatography clearly showed that RshA and WhiB1 can interact under physiological conditions and the interaction involved a thiol-disulfide exchange. Therefore, we think that in physiological conditions, WhiB1 would respond to the oxidative stress and regulate the function of SigH/Rv3223c by regulating the redox response of the anti-σ^H factor/ RshA/ Rv3221A and in turn, it is likely to be regulated by the SigH as the regulatory region of *whiB1* gene has SigH binding consensus sequence. Finally, it can clearly be said that WhiB1 would function as one of the major redox regulatory protein of *M. tuberculosis.*