Overview

The exploration of mycobacterial genome sequences in recent years has brought several novel insights into the biology of this fascinating group of organisms, leading to the identification of several previously unidentified genes, including presence of genes for novel hemoglobins (Cole *et al.,* 1998; Cole *et al.,* 2001). Two genes, *glbN* and *glbO*, encoding hemoglobin-like proteins (HbN and HbO, respectively) were discovered in the complete genome sequence of the virulent *M. tuberculosis* and subsequent unraveling of mycobacterial genome data suggested that these Hbs may be ubiquitous in mycobacteria. Three distinct types of trHbs (HbN, HbO and HbP) have been identified within the mycobacterial genome (Wittenberg *et al.,* 2002). The extent of amino acid identity between members of these three groups is less than 18% suggesting that these Hbs are distinct from each other and may be playing different function(s) in the cellular metabolism of mycobacteria. The opportunistic pathogen, *M. avium*, carries all three types of trHbs (HbN, HbO and HbP), whereas, intracellular pathogens like, *M. tuberculosis*, *M. bovis*, *M. marinum etc.* carry two trHbs, HbN and HbO. Interestingly, the obligate intracellular pathogen, *M. leprae*, that has undergone extensive reductive evolution (Cole *et al.,* 2001) and carries a minimal set of genes required for its persistence and pathogenesis, retains at least one hemoglobin (HbO) suggesting that Hb-like proteins may be vital for the intracellular regime of pathogenic mycobacteria. Functions of these mycobacterial Hbs are not very well understood at present and may be diverse.

Studies on mycobacterial Hbs have been mainly concentrated on HbN and HbO of *M. tuberculosis* (Couture *et al.,* 1999; Ouellet *et al.,* 2003; Pathania *et al.,* 2002a; Pathania *et al.,* 2002b; Ouellet *et al.,* 2002). Physiological studies performed on *M. bovis* demonstrated that trHbO is expressed throughout the growth phase, whereas, HbN expression is induced only during stationary phase (Couture *et al.,* 1999; Pathania *et al.,* 2002b) indicating that these oxygen-binding proteins are required at different growth stages of mycobacteria and accordingly, their functions might also be different. HbO of *M. tuberculosis* has been implicated in oxygen uptake and transfer during its aerobic growth (Pathania *et al.*, 2002b). It has been proposed that the presence of HbO in *M. tuberculosis* provides an efficient way of sequestering and competing for oxygen during different stages of intracellular growth to sustain aerobic metabolism. The three dimensional structure of *M. tuberculosis* HbN is characterized by the presence of an extra N-terminal pre-A helical region and extended apolar tunnel/cavity connecting the heme distal pocket to two distinct protein surface sites (Milani *et al.,* 2001). It has been speculated that these unique cavities present in HbN may provide an

alternative port for the diffusion of ligands towards the distal site where solvent access through the classical E7 gate path is completely impaired due to orientation of E-helix and packing of the pocket by side chains of distal site residues (Milani *et al.*, 2001; Milani *et al.*, 2004). HbN is shown to exhibit distinct nitric oxide dioxygenase activity which protects growth and cellular respiration of heterologous hosts, *E. coli* and *M. smegmatis*, from the toxic effects of exogenous NO and the NO-releasing compounds (Pathania *et al.*, 2002a). NO scavenging activity of *M. tuberculosis* HbN suggests an NO detoxification function for the HbN within *M. tuberculosis* and other mycobacterial species. The NO scavenging activity of HbN may be providing a potential defense mechanism to *M. tuberculosis* during infection and its proliferation within the macrophages, where copious amounts of NO are generated, that restricts the survival of the pathogen.

Computational and sequence analysis indicated the presence of these hemoglobins in several other mycobacteria as well, including non-pathogenic, fast growing saprophyte, *M. smegmatis*. Unlike *M. tuberculosis*, which resides within macrophages and avascular calcified granulomas and encounters severe hypoxia and nitrosative stress, *M. smegmatis* neither enter epithelial cells nor persist in professional phagocytes although it has been known to cause soft tissue and bone infections in rare cases (Turenne *et al.*, 2003). In addition, a putative flavohemoglobin gene has also been found to be present in *M. smegmatis* genome. Thus, *M. smegmatis* is also equipped with all the Hbs that are present in pathogenic *M. tuberculosis viz* homologues of two truncated hemoglobins HbN and HbO and a homologue of putative flavohemoglobin. At present, no information on hemoglobins of any fast growing mycobacteria is available in the literature. Studies on these interesting groups of novel hemoglobins present in fast growing mycobacteria and their comparative analysis with Hbs of pathogenic mycobacteria may provide new insights into the functionality of truncated hemoglobins. Therefore, a detailed study was taken up to characterize these Hbs of *M. smegmatis*.

The *MsglbO* and *MsglbN* genes encoding MsHbO and MsHbN, respectively were fished out from the genomic DNA of *M. smegmatis* by PCR and cloned in pBS KS+. The sequencing of the two genes confirmed the authenticity of the genes. The two proteins were further subcloned and expressed independently under the control of T7 promoter in *E. coli* BL21DE3. One of the genes, *MsglbN* did not express under T7 promoter and moreover, there was leaky expression of MsHbO. Therefore, MsHbN and MsHbO were expressed under the control of *Vitreoscilla* hemoglobin (*vgb*) promoter resulting in the constitutive expression of the two proteins and allowing monitoring the response of MsHbN and MsHbO on the

physiology of recombinant *E. coli*. The detailed biophysical and biochemical studies were performed to characterize these novel trHbs. The gel filtration analysis indicated that MsHbN and MsHbO predominantly exist as dimers. Both HbN and HbO interacted with various ligands *e.g.* O_2, CO, NO *etc.* and formed stable oxygenated form that was spectrally very similar to oxyhemoglobins and oxymyoglobins. In its natural host *M. smegmatis*, presence of HbN and HbO was not detectable in aerobically growing cells. Therefore, MsHbN and MsHbO were expressed in their native host, *M. smegmatis* itself to gain insight into their functionality.

Oxygen and nitric oxide are important ligands in the hemoglobin chemistry and since several hemoglobins and flavohemoglobins have been shown to play an important role in NO metabolism/detoxification and oxygen transfer processes, the NO and oxygen uptake properties of the two proteins were studied and compared with the corresponding Hbs of *M. tuberculosis*.

The NO consumption activities of the two proteins were studied in a flavohemoglobin deficient mutant of *E. coli* and compared with the corresponding Hbs of *M. tuberculosis*. MsHbN conferred little NO consumption activity to *E. coli* cells which was almost one-third of the *M. tuberculosis* HbN. Like *M. tuberculosis* HbO, the NO uptake by MsHbO was also lower. Thus both HbO of *M. smegmatis* as well as *M. tuberculosis* may not be very efficient in NO detoxification. *E. coli* expressing MsHbO exhibited lower oxygen affinity almost comparable to *M. tuberculosis* HbO. It is known that the hemoglobins with lower oxygen affinity are beneficial for the host as they bind oxygen efficiently and release it readily. The presence of MsHbO conferred a distinct growth advantage to *E. coli* and *M. smegmatis* like *M. tuberculosis* HbO and the oxygen uptake rates of HbO carrying *E. coli* and *M. smegmatis* cells were higher than the control cells. The far UV-CD spectra of MsHbO and MtbHbO showed similar profile exhibiting troughs at 208 and 222 nm demonstrating no structural differences between the two proteins.

To determine whether the functional expression of MsHbO has any metabolic effect(s) on its *E. coli* host, the growth profile of recombinant *E. coli*, carrying MsHbO with the isogenic strain carrying HbO of *M. tuberculosis* along with the control cells having similar plasmid without any Hb encoding gene were compared. Under high aeration conditions, MsHbO carrying *E. coli* cells outgrew the control cells more or less like MtbHbO carrying cells and the difference became more obvious during late log and stationary phase. The same results were obtained when these Hbs were expressed in its native host. The membrane association properties of MsHbO and its presence throughout the growth phase of

M. smegmatis suggest that it is generally required by the mycobacterial cells to perform some basic function of cell respiration. The function(s) of HbO in mycobacteria is not very clear at present. Its close association with respiratory apparatus of cells and interaction with cytochrome O suggest that one of the functions of HbO may be facilitation of oxygen metabolism by generating an efficient electron transport pathway to sustain aerobic metabolism of host. Several other functions of HbO have also emerged recently. In *M. leprae*, GlbO has been proposed to represent merging of both O_2 uptake/transport and scavenging of nitrogen reactive species (Ascenzi *et al.*, 2006). Recently, trHbO of *M. tuberculosis* has been shown to have peroxidase activity in accord with the presence of typical peroxidase intermediates which may suggest an oxidation/reduction function for trHbO (Ouellet *et al.*, 2007).

Based on the above observations, it has been hypothesized that, since HbO remains in two conformational states in its native host that display distinct cellular localization, a major fraction of MsHbO may remain associated with cell membrane and undergo conformational change into its monomeric form. Under such a situation, it can interact with respiratory apparatus of cells to facilitate electron transfer (through novel/unknown mechanism) by interacting with cytochrome oxidases. The dimeric MsHbO present in the cytosol, may participate in other cellular functions either alone or in conjunction with some interacting partner(s) depending on the cellular environment. This may, thus, allow functional diversity to HbO to participate in multiple cellular functions.

To elucidate whether the functional expression of other hemoglobin, MsHbN had any metabolic effect(s) on its host, the growth and oxygen consumption properties of recombinant *E. coli*, carrying MsHbN and MtbHbN was compared with the control cells having similar plasmid without any Hb encoding gene. Under high aeration, there was no distinct difference in the growth characteristic of *E. coli* carrying any of these two mycobacterial HbN and none of these hemoglobins exhibited any growth advantage over the control cells. However, under low aeration conditions, the cells carrying *M. tuberculosis* HbN outgrew control and MsHbN expressing cells at the late exponential stage and resulted in higher cell mass. These results indicated that MsHbN might not be able to sustain growth and cell viability during hypoxic growth conditions unlike HbN of *M. tuberculosis*. MsHbN exhibits relatively high rate of autooxidation and may not be as beneficial as HbN of *M. tuberculosis*. With the background information that *M. tuberculosis* HbN possesses a potent NO metabolizing activity and can protect its host from the toxicity of NO and its analogs, the NO uptake activity of recombinant *E. coli* carrying these two mycobacterial HbN were compared to check if HbN

of *M. smegmatis* has similar NO scavenging activity. The NO consumption activities of the two proteins were studied in an NO-sensitive flavohemoglobin deficient mutant of *E. coli i.e.* RB9060. In the presence of oxygen, expression of *M. tuberculosis* HbN conferred distinct NO consumption ability to the *hmp* mutant of *E. coli* that was significantly higher than the isogenic strain expressing HbN of *M. smegmatis*. To test whether the presence of *M. smegmatis* HbN provide any protective effect against NO donor and nitrosative stress, the growth profiles and survival pattern of *hmp* mutant of *E. coli* carrying *M. smegmatis* HbN was compared with the isogenic strain having *M. tuberculosis* HbN in the presence of different concentrations of the NO donor, sodium nitroprusside (SNP). Presence of *M. tuberculosis* HbN conferred distinct growth advantage to the *hmp* mutant of *E. coli* in the presence of 1 mM SNP, whereas, *M. smegmatis* HbN carrying cells grew slowly and only marginal growth increase over the control cells, growing under similar conditions, was observed. In the presence of different concentrations of SNP, survival of *M. smegmatis* HbN carrying cells of *E. coli* was 2- to 3-fold lower in comparison to the cells expressing *M. tuberculosis* HbN. These results clearly indicated difference in protective effect of HbN of *M. smegmatis* and *M. tuberculosis* against NO toxicity. To further evaluate the potentiality of *M. smegmatis* HbN against other reactive nitrogen species, the tolerance of hemoglobin expressing cells towards acidified nitrite were tested and similar results were obtained. Taken together, these results demonstrate that the potentiality of *M. smegmatis* HbN to cope with toxicity of NO and nitrosative stress is significantly lower than that of the HbN of *M. tuberculosis* (Lama *et al.*, 2006).

Structure-based sequence alignment of *M. smegmatis* along with other mycobacterial HbN revealed that it is relatively smaller in size than its counterpart present in *M. tuberculosis* and lacks 12-residue long positively charged N-terminal sequence motif constituting the pre-A region of HbN, although structural features *e.g.* TyrB10, PheCD1, LeuE7, PheE14, HisF8, three Gly motifs, crucial for attaining the trHb fold and residues defining the protein matrix tunnel, are all conserved in this mycobacterial Hb. Preservation of trHbs signature sequences in *M. smegmatis* HbN indicates that it can attain two-over-two alpha-helical structure and may function as hemoglobin. The highly polar and charged N-terminal motif is present in all known mycobacterial HbN except *M. smegmatis*, however, its relevance in protein function is currently unknown. Detailed sequence comparison of HbN type trHbs do not carry such additional secondary structure at the N-terminus suggesting that it may not be crucial for the structural integrity of trHb fold and may be specific to certain mycobacterial HbN. To explore whether the presence of 12-residue pre-A region has any

relevance for the functional differences between these two mycobacterial HbN, it was deleted from *M. tuberculosis* HbN and integrated at the N-terminus of *M. smegmatis* HbN. Both of these mutant proteins were expressed in *E. coli* under VHb promoter for their constitutive expression. The spectral analyses of these mutant proteins were consistent with that of typical hemoglobin and myoglobin. To check whether the mutants of MtbHbN and MsHbN also have the potential to metabolize gaseous NO, the NO consumption activity of flavoHb-deficient *E. coli* RB9060, carrying MtbHbN, $MtbHbN^{\Delta 1\text{-}12}$, MsHbN, $MsHbN^{ext1\text{-}12}$ was measured. On a per heme basis, the specific NO consumption ability of *E. coli* expressing *M. tuberculosis* HbN was approximately 3.5-fold higher than that of *M. smegmatis* HbN. This suggests that MsHbN carrying cells are less efficient in NO consumption. Interestingly, *E. coli* expressing N-terminal motif deleted construct, $MtbHbN^{\Delta 1\text{-}12}$ exhibited 2.4-fold lower specific activity for NO uptake in comparison to the wild type carrying *E. coli*. Similarly, the mutant $MsHbN^{ext1\text{-}12}$ carrying *E. coli* cells exhibited approx. 2.7-fold higher NO consumption activity as compared to its wild type.

The growth profile of the wild type and mutant hemoglobins expressing *E. coli* RB9060 cells and the control RB9060 cells carrying plasmid alone was checked in the presence of different concentrations of sodium nitrite. The truncation of pre-A region from the *M. tuberculosis* HbN drastically reduced its NO dioxygenase (NOD) function, whereas significant increase in the NOD activity was gained when the pre-A region was integrated at the N-terminus of *M. smegmatis* HbN. These findings directly attributed the role of pre-A region in HbN function and suggest that *M. tuberculosis* HbN may have novel mechanism for NO scavenging and detoxification.

It has been demonstrated that NO detoxification consumes O_2 and NADH and converts NO and O_2 in equistoichiometric amounts to nitrate as shown below and this activity has been designated as nitric oxide dioxygenase (NOD) activity.

Overall reaction:

$$NO + O_2 + NAD(P)H \longrightarrow NO_3^- + NAD(P)^+$$

Reaction proceeding at heme:

$$Fe^{+2}\text{-}O\text{-}O + N{=}O \longrightarrow [Fe^{+3}\text{-}OONO^-] \longrightarrow Fe^{+3} + NO_3^-$$

Heme-bound peroxynitrite

The catalytic cycling of ferric-to-ferrous heme is necessary for the flavoHbs to function as NO dioxygenases. *M. tuberculosis* HbN efficiently removes and detoxifies NO by dioxygenation. To complete the cycle, the nitrate anion produced during this process should

be removed from the heme cavity and the inactive Fe (III) proton should be reduced back to Fe (II) state that may require concerted action of a compatible reductase system to control the heme-Fe atom redox state. In the presence of oxygen, HbN of *M. tuberculosis* provides substantial NO consumption ability to an *E. coli* flavoHb deficient mutant (Pathania *et al.*, 2002) and enhances NO scavenging activity of *M. smegmatis* (Pathania *et al.*, 2002). The NO metabolizing activity of HbN is sustained in heterologous hosts with repetitive NO, suggesting that heterologous reductases are compatible with HbN of *M. tuberculosis* to some extent. It is quite possible that the presence of a polar and surface exposed charged pre-A allows HbN to generate a high affinity hemoglobin/reductase association to carry out highly enhanced NO detoxification, whereas, efficiency of pre-A lacking HbN may slow down due to low affinity Hb/reductase association. This model is supported by our observation that cell extract of *E. coli* overexpressing *M. tuberculosis* redox protein (Rv3571), having iron-sulfur center and FAD and NAD binding sites, when mixed with purified HbN enhances its NO consumption activity. On the basis of above results, we can conclude that the N-terminal motif modulates the NO scavenging ability of HbN by interacting with reducatse partner protein. Future studies focused at establishing *in vitro* interaction of HbN with different reductases and assessing their coupling efficiency may provide further insight on molecular mechanism of trHbN function in mycobacteria.

Remarkably, the survey of genome databases revealed the existence of flavohemoglobin genes from almost all pathogenic as well as non-pathogenic microbes. The presence of flavoHbs in mycobacterial species is even more surprising as they already harbor two truncated hemoglobins, HbN and HbO, described in previous chapters. These flavoHbs are present in almost all mycobacteria except *M. leprae*, which is known for its reduced genome size. The presence of another NO scavenging protein *i.e.* hmp in *M. tuberculosis* is worth investigation because one of the truncated hemoglobin, HbN is already present for which NO detoxification function has been assigned. Similarly, in a non-pathogenic mycobacterium, *M. smegmatis* also, a flavoHb is present in addition to two truncated hemoglobins. It was interesting to find out whether this genetic locus encoding a putative flavoHb codes for a functional protein and then explore its implications in the biology of the host.

A structure-based sequence alignment putative flavoHbs (MsHMP and MtbHMP) of *M. smegmatis* and *M. tuberculosis* with *Vitreoscilla* hemoglobin and *E. coli* and *R. eutropha* flavoHbs reveals that although there are structural conservation of crucial residues required for attainment of the globin fold, there are some marked differences too which make

mycobacterial flavoHbs unique and different from the conventional ones. Two of the residues lining the heme distal pocket which are conventionally Leu and Val at E11 and G8 positions, respectively, are changed to Phe and Met in case of mycobacterial flavoHb sequences. In *E. coli* flavoHb, the architecture of the heme pocket in the proximal region is dominated by a hydrogen bonding network between His F8, Tyr G5 and Glu H23 that imparts a rigid orientation to the proximal histidine ring with respect to the heme plane (Ilari *et al.*, 2002) and Glu H23 is anchored to Tyr G5 by hydrogen bonding. In case of MsHMP amd MtbHMP, Glu H23 is changed to Met. These changes in the heme proximal and distal pockets may be translated to some structural perturbations in these pockets which may, in turn, affect the ligand binding kinetics. Further investigation of the sequence comparison suggests that the functional regions of globin part important for the interactions with flavoreductase are not conserved in mycobacteria. In FHP, the interactions between the heme and flavin-binding domains mainly occur through H-helix and CE-loop. A salt bridge is formed between the heme and FAD-binding domain of HMP through a basic residue, Lys 84 (F7). In case of MsHMP and MtbHMP, this residue is Asp 84, thus making the interaction between FAD-binding domain and heme less likely. Other functionally important region for reductases interaction is CE region of heme domain. Interestingly, the most important and conserved residues; His/Asn 47 and Gln 48 are not present in MsHMP and MtbHMP. All these differences indicate the possibility of no or very weak interaction between globin and reductase domains.

The primary studies conducted on flavoHb of *M. smegmatis* unraveled several interesting differences between mycobacterial flavoHbs and other conventional flavoHbs suggesting that its function may be distinctly different. This is supported be the fact that MsHMP is devoid of NO dioxygenase activity and is not efficient in relieving the toxicity of NO and nitrosative stress. Interestingly, presence of MsHMP provides stress protection to its host against oxidative stress a function which is not generally assigned to conventional HMPs. This may be due to distinct differences in ligand-binding pockets and subunit interaction properties of MsHMP. Comparison of structural features of MsHMP with known flavoHbs has indicated that there are several distinct differences in the heme proximal and distal pockets which may affect its functional properties. Moreover, the primary studies show that the putative flavohemoglobin from *M. smegmatis* may be involved in protection from oxidative stress rather than nitrosative stress. Currently, it is possible to envision at least two roles for MsHMP in the oxidative stress response. It may function as an antioxidant itself or it may participate in a sensing pathway that responds to oxidative stress. As a

flavohemoprotein, MsHMP may bind ROS and reduce them to water by a mechanism that is analogous to the reduction of oxygen to water by cytochrome *c* oxidase (Babcock and Wikstrom, 1992). For example, it is plausible that superoxide can bind to the Fe^{2+} form of heme to produce the peroxy derivative (Fe^{3+}-O^--O^-) or that H_2O_2 binds to the peroxy form to give the Fe^{3+}-O^--OH derivative. As MsHMP can transfer electrons from NAD(P)H to its heme iron, it is plausible that either the Fe^{3+}-O^--O^- or the Fe^{3+}-O^--OH derivative can then be reduced to water by electrons supplied from NAD(P)H. Alternatively, MsHMP may bind ROS and, as such, serve as a proximal sensor for oxidative stress. Further study with purified MsHMP is required to examine these possibilities and determine the precise role of this protein in the oxidative stress response in its native host as well. Moreover, the possibility that *M. smegmatis* HMP may have a totally different function than other flavohemeglobins can not be ruled out. Detailed studies are warranted before arriving at definitive conclusions.

Overall, in the present work, we have studied three different hemoglobins of *M. smegmatis* which provided new insights into the functionality of mycobacterial Hbs and suggested distinct differences in the structural and functional properties of Hbs from pathogenic and non-pathogenic mycobacteria. HbN of *M. tuberculosis* is very different from HbN of *M. smegmatis* and the difference in their functionality may be attributed to the pre-A region of MtbHbN. The other interesting observation is that HbO of *M. smegmatis* may be vital for the host. The structural and functional properties of HbO of pathogenic and non-pathogenic mycobacteria are more or less similar. It is quite likely that HbO may interact with some components of respiratory apparatus to generate novel electron chain to facilitate oxidative phosphorylation. A distinct class of two domain flavoHb has been found to be present in almost all mycobacteria except *M. leprae*, which is known for its reduced genome size. Structurally and functionally, the flavoHbs of mycobacteria seem to be quite different from other conventional flavoHbs.

The studies on pre-A region of Mtb HbN established that it may be very important for modulating its NO dioxygenase activity. How the pre-A region modulates NO metabolizing activity of HbN or whether it is involved in protein-protein interactions, is not known at present. The answers to these questions would open up several avenues for understanding the molecular mechanism of HbN function. The study on other hemoglobin, HbO has shown that it is more or less similar to HbO of *M. tuberculosis* and is involved in some basic function related to respiration. Moreover, the attempts for generating HbO-knockout strain of *M. smegmatis* have failed even after repeated trials pointing towards the essentiality of this gene. Further studies on the interaction of HbO with membranes would

throw some light on the functional mechanism of its function in the native host *i.e.* mycobacteria. This work also suggested that the two domain flavohemoglobin of *M. smegmatis* and other mycobacteria exhibits some novel characteristics, which make it different from the conventional flavoHbs. Therefore, its detailed understanding with respect to its structure-function studies would provide information about a new class of Hbs having distinct features.