Scientists have so closely associated hemoglobins (Hbs) with oxygen transport that they have been surprised to discover Hb in organisms that have no obvious need for oxygen transport. Doubly surprising has been the discovery of Hbs in organisms that are only one cell large, such as bacteria. The hemoglobin family of proteins is ancient: Hb genes probably predate the split between eukaryotic cells and eubacteria, some 2-4 billion yrs ago. They are arguably the most studied of all proteins. Recognition that microorganisms (yeast, other fungi, protozoa, bacteria) and higher plants (Wittenberg *et al.*, 2002; Wu *et al.*, 2003) also contain hemoproteins that are unmistakably globin-like is not new (Keilin discovered Hb in yeasts and other fungi in the early 1950s), but their investigation has been slow to gain momentum, in part because of the perceived lack of function. The discovery of new members of the Hb superfamily in diverse and ancient life forms continues to invigorate investigations of Hb functions beyond the classical O_2 storage and transport function.

The amazingly wide distribution of globin genes among prokaryotes has stimulated extensive studies aimed at unraveling the physiological significance of unicellular hemoglobins and understanding the evolutionary relationships that link these proteins to their homologues in higher organisms. Truncated hemoglobins (trHbs), in particular, have received a fair degree of attention because of their occurrence in a large number of different microbial species and their unusual globin fold, characterised by a two-over-two α-helical packing instead of the classical three-over-three helical arrangement typical of vertebrate globins (Milani *et al.*, 2001). TrHbs are characterised by a remarkable variability in the nature of the residues at the active site, especially in the distal side of the heme pocket which in turn, may be related to their diverse physiological roles proposed, that is terminal oxidases (Wittenberg *et al.*, 2002), oxygen sensors and scavengers of oxygen and nitric oxide active species (Milani *et al.*, 2003a). TrHbs are classified into three distinct groups, *viz.* group I, II, and III, whose members are designated as N, O and P, respectively.

Recently, all available mycobacterial genomes, which include *M. tuberculosis, M. smegmatis, M. leprae, M. avium, M. vanbalenii, M. flavescens, M. marinum, M. ulcerans, M. bovis, M. africanum* and *M. microti* were searched for the presence of trHbs (Ascenzi *et al.*,

2007). Within trHbN and trHbO groups, very high identity was observed between groups of phylogenetically related species, including the four pathogenic members of the *M. tuberculosis* complex (*M. bovis, M. africanum* and *M. microti, M. tuberculosis*), the *M. marinum-M. ulcerans* group, and the *M. vanbalenii-M. flavescens* group (Devulder *et al.*, 2005). The occurrence of trHbs in mycobacterial genomes varies depending on the species, and this in part, reflects the ecology of the genus. Species that are commonly found in variable natural environments (soil and water) and cause infection as facultative parasites have all three or at least two trHb types, irrespective of whether they are classified as fast or slow growers. An interesting progression is found in the pathogenic members of the genus *Mycobacterium*. The genome of the opportunistic pathogen, *M. avium* contains one trHb from each of the three trHb groups, trHbP, trHbO and trHbN. The facultative intracellular pathogen, *M. tuberculosis* has two, trHbN and trHbO and the obligate intracellular pathogen, *M. leprae*, which has undergone extensive reductive evolution retains only trHbO.

Mycobacterium tuberculosis, the causative agent of tuberculosis, is one of the most successful pathogens of human. Two genes, *glbN* and *glbO*, encoding the group I trHbN and the group II trHbO respectively, were discovered in the complete genome sequence of the virulent *Mycobacterium tuberculosis* (Cole *et al.*, 1998). In addition to the two trHbs, the bioinformatic analysis of the genome sequence of *M. tuberculosis* revealed the presence of a locus Rv0385, encoding the putative flavohemoglobin (flavoHb or Hmp). The presence of more than one type of Hbs in an organism is not uncommon but the presence of two trHbs and one flavoHb in *M. tuberculosis* suggests evolution of each of these proteins for a distinct function. How these hemoglobins contribute in cellular metabolism and pathogenicity of *M. tuberculsosis* is not known at present.

The heme active site structures as well as the ligand binding properties and temporal expression patterns of trHbN and trHbO are very distinct. It has been argued that the protection of bacilli against nitrogen-reactive species during latency in the granuloma relies on the oxygenated derivative of a homodimeric 'truncated hemoglobin' (trHbN), encoded by the *glbN* gene (Couture *et al.*, 1999b; Pathania *et al.*, 2002a). The heterologous expression of *M. tuberculosis* trHbN, which is identical to the *M. bovis* counterpart, significantly protects *M. smegmatis* and flavohemoglobin mutants of both *Escherichia coli* and *Salmonella enterica*

Typhimurium from nitric oxide (NO) damage through O_2-sustained detoxification mechanism (Pathania *et al.*, 2002a; Pawaria *et al.*, 2007). The trHbO protein across different organisms is more conserved in sequence and is more wide spread in nature as compared to trHbN. Even *M. lepare*, which has undergone reductive evolution, retains a functional trHbO. This suggests that it plays a crucial role in the basic metabolism of the host. Yet there has not been a consensus about its functionalities. Out of the various roles proposed are functioning as a peroxidase, NO detoxification and cellular respiration. In addition to this, trHbO was hypothesized to be endowed with O_2 uptake or delivery properties during mycobacterial hypoxia and latency (Pathania *et al.*, 2002b; Liu *et al.*, 2004). There has been no report on the flavohemoglobin from the *Mycobacterium* genus.

Macrophages from the lungs of patients with tuberculosis express iNOS (Nicholson *et al.*, 1996; Wang *et al.*, 1998) in potentially mycobactericidal amounts (Wang *et al.*, 1998) and can use it to kill mycobacteria *in vitro* (Nozaki *et al.*, 1997). Because reactive nitrogen intermediates (RNI) are essential for the control of murine tuberculosis and are produced in human tuberculosis, and control of the pathogen is imperfect in both species, tubercle bacilli may express mechanisms for RNI resistance. *In vivo*, the high oxygen affinity of trHbN (P_{50}~0.01 mm Hg) may ensure a low but critical level of oxygen, granting survival of *M. tuberculosis* in the granuloma hypoxic environment when the bacilli enter latency (Couture *et al.*, 1999b). The presence of only trHbO in the obligate intracellular pathogen, *M. leprae*, suggests it is indispensable for parasitic lifestyle. All the experimental evidences, which form the basis of the proposed functions of the trHbs have been carried out *in vitro*. Therefore, the evaluation of propensity of trHbN and trHbO to retain their functionalities and ability to provide protection during intra-macrophage growth is imperative. The ability of trHbs of *M. tuberculosis* trHbs to support intracellular survival was determined using a *Salmonella* mutant deficient in growth under nitrosative stress and inside macrophages. The results obtained established that trHbN and trHbO have distinct NO scavenging properties and unlike trHbO, trHbN is capable of retaining significant level of NO metabolizing activity even under low oxygen. The presence of trHbN enhanced the intracellular growth and survival of its heterologous host, *Salmonella*, both in naïve and activated macrophages. This suggested that trHbN, which until now was seen to be beneficial against the *in vitro* generated stress only, was able to defend its host in the

macrophage environment. Since the *Salmonella* strain was specifically attenuated against NO related stress only, it stands to reason that it was the nitric oxide dioxygenase (NOD) activity of trHbN that protected the *Salmonella* cells during intracellular growth. Even if *Salmonella* and *M. tuberculosis* survive in separate compartments inside the macrophages, the employment of NO as defense against these two organisms is undisputable. Therefore, if trHbN protects against the level of RNIs produced against *Salmonella*, it must be capable of protecting its native host, *M. tuberculosis*, also against such NO-related stress. This may also be the probable reason for the inability of trHbO to protect its *Salmonella* host since the NOD function of *M. tuberculosis* trHbO, unlike *M. leprae* trHbO (Ascenzi *et al.*, 2006), is still not clearly established. The results obtained in the present study make this function of trHbO from *M. tuberculosis* less probable at least under the conditions evaluated here. Some other host background *e.g.* those deficient in respiratory components or survival during hypoxia *etc.* need to be evaluated for its contribution during intracellular survival. It is quite possible that the low-reactivity of trHbO in *Salmonella* may be due to the absence of a native partner, whereas, trHbN may be able to use a non-native redox partner efficiently for NO scavenging.

It has been proposed that the oxygenated trHbN (oxy-trHbN) could detoxify the macrophage-generated NO, similarly to the dioxygenase activity of (flavo)Hbs and myoglobin (Mb), which convert NO to nitrate (Gardner *et al.*, 1998b; Liu *et al.*, 2000; Poole and Hughes, 2000; Brunori, 2001; Flogel *et al.*, 2001; Frauenfelder *et al.*, 2001). Despite the biophysical and structural characterization of trHbN and the large number of studies on ligand migration to the heme in related proteins, no detailed information is available on the molecular mechanisms of NO detoxification and the factors controlling it in trHbN. Additionally, nothing is known on how *M. tuberculosis* trHbN regulates ligand access to the heme to achieve NO detoxification and survival of the *Mycobacterium* under the nitrosative stress. Exploration of the current genome data of various mycobacterial species showed the presence of trHbN in fast growing, non-pathogenic mycobacteria, which is unusual if trHbN is expected to play a role in pathogenesis. Structure-based sequence alignment of different mycobacterial trHbN indicated clear conservation of the main regions crucial for the stabilization of the trHb fold. A striking difference lied at the N-terminus which showed that trHbN of most of the slow growing pathogenic mycobacteria carry a highly charged Pre-A motif that is absent in trHbN from the fast

growing non-pathogenic mycobacteria. The presence of Pre-A region was initially observed in the crystal structure of *M. tuberculosis* trHbN (Milani *et al.*, 2001), which distinguishes it from its homologs present in protozoan, algal and cynobacterial species. The three dimensional structure indicated that Pre-A region protrudes out of the compact globin fold and may not contribute significantly to the structural integrity of protein. Initial experimental studies from our laboratory suggested that Pre-A region is vital for the NOD activity of trHbN (Lama *et al.*, unpublished data). The Pre-A motif is composed of highly polar and charged residues with a distinct stretch of 4 positively charged residues (Arg-Leu-Arg-Lys-Arg). The contribution of these charged residues was evaluated by site-directed mutagenesis.

The mutation of all the positively charged residues of Pre-A region completely abolished the ability of trHbN to provide protection against nitrosative stress, thus, clearly establishing the importance of these residues in trHbN function. Two working hypotheses were evaluated to explain the mechanism of involvement of these residues in the NOD reaction cycle of trHbN. The NOD reaction proposed for flavoHbs is:

Overall Reaction: $\mathbf{NO + O_2 + NAD(P)H \rightarrow NO_3^- + NAD(P)^+}$

Reaction Proceeding at heme: $\mathbf{Fe^{2+}\text{-}O\text{-}O + N{=}O \rightarrow [Fe^{3+}\text{-}OONO^-] \rightarrow Fe^{3+} + NO_3^-}$

The Pre-A region, therefore, might regulate the reaction occurring at the heme either directly by modulating the reaction kinetics by some charged interactions or indirectly by influencing the ligand access to the heme active site by some hydrogen bonding interactions. The second possibility arises from the fact that for the catalysis to continue the ferric heme generated during NOD reaction requires to be reduced back to the active ferrous by the involvement of a redox partner. Therefore, the Pre-A region might modulate the interaction of trHbN with such a cognate reductase partner.

Both of these possibilities were evaluated to unravel the role of Pre-A motif in trHbN function. A protein cavity/tunnel system, connecting the protein surface to the heme distal site, has been clearly identified in trHbN (Milani *et al.*, 2001; Milani *et al.*, 2004), which is composed of two roughly orthogonal branches, a 20 Å long tunnel branch and a path of about 8 Å, converging at the heme distal cavity from two distinct protein surface access sites. The O_2 and NO ligands access the heme cavity through the long and the short branches of the tunnel, respectively (Milani *et al.*, 2001; Milani *et al.*, 2004; Crespo *et al.*, 2005; Bidon-Chanal *et al.*, 2006; Bidon-Chanal *et al.*, 2007). The opening of the tunnel long branch, which allows diffusion

of NO, is controlled by PheE15 residue whose side chain mainly populates two conformations (characterized by average C_α-C_β torsional angles of about +40 and –50 degrees). The ability of Pre-A motif in modulating the ligand access to the heme pocket has been proposed to occur through a hydrogen bonding interaction between the Arg-10 residue of Pre-A with a Glu-70 residue lying near the PheE15 gate. The formation and breakage of this hydrogen bond has been proposed to regulate the opening and closing of the PheE15 gate and, thus, Arg-10 of Pre-A could control ligand access (Bidon-Chanal *et al.*, 2006). This hypothesis, however, was ruled out after the Arg-10 to Ala mutant of trHbN showed no distinct difference in its protective ability against nitrosative stress. The individual mutation of the positively charged residues to Ala indicated that Arg-6 and Lys-9 are crucial for the NOD function of trHbN.

The rapid oxidation of nitric oxide (NO) to nitrate by *M. tuberculosis* trHbN (Ouellet *et al.*, 2002) suggests that the ferric heme-iron generated during the reaction cycle is efficiently reduced back to ferrous by the concerted action of a compatible reductase system. Like in case of flavoHbs (Ermler *et al.*, 1995; Ilari *et al.*, 2002; Frey and Kallio, 2003), the presence of charged residues may govern the interaction between the reductase and the trHbN globin domains. Considering this, it is possible that the Pre-A region, due to its high content of charged residues as well as flexibility, might tether the cognate reductase through charged residues and/or mediate electron transfer from the reductase to the heme group. This proposal was evaluated by determining the ability of wild type trHbN and Pre-A lacking trHbN to interact with a mycobacterial flavoreductase encoded by the locus Rv3571. The results showed that both the trHbN species are capable of interacting with the reductase suggesting that Pre-A region is not the sole determinant for maintaining the interaction, which is expected since the contact region between the globin and reductase domains is expected to be much larger in space. However, the increase in the NOD activity of the purified *M. tuberculosis* trHbN (carrying Pre-A) on addition of cell extract of *E. coli* cells overexpressing the flavoreductase was 6-fold higher than of the trHbN of *M. smegmatis* (lacking Pre-A) (Lama *et al.*, unpublished). This suggests that Pre-A might not solely determine the interaction but could be involved in improving the electron transfer rate or the strength of interaction with the reductase.

The interaction of the trHbN with the reductase was based on the occupancy of its heme pocket with oxygen or CO. This suggests that the interaction is based on the conformation of the trHbN, which is very interesting and suggests a novel mechanism of NOD reaction operative

solely in the trHbs. Based on this observation, the role of Pre-A in modulating the reaction kinetics by intra-molecular charged interaction still holds ground. A novel dual-path ligand-induced regulation mechanism is operative in *M. tuberculosis* trHbN, whereby the protein dynamics and the protein matrix tunnel system have evolved to allow the access of O_2 and NO ligands to the heme through distinct migration paths. Strikingly, this ligand migration pathway is drastically different in deoxy-trHbN. The energy barrier for the transition from closed to open state becomes twice as much as in oxy-trHbN, on the other hand, ligand migration through the short tunnel branch becomes more feasible because of lowered energy barrier. The changes in the heme distal site local structure, however, are also linked to alteration of the protein backbone motions. Such conformational changes could be either the cause or the effect of some intra-molecular charged interactions of the Pre-A region. Based on all these observations a working model to explain the molecular mechanism of trHbN function is proposed (Figure 1). TrHbN might interact with a specific reductase or with several different reductases at different times. The latter seems more plausible because if nature was to select a specific partner then its integration within a single polypeptide might have been more beneficial. The interaction with different reductases might provide it functional variability during *in vivo* growth. Additionally, the regulation of the redox domain expression could in turn, regulate the trHbN functionality.

In silico analysis of the mycobacterial genome data, revealed the presence of a locus, Rv0385 encoding the putative flavoHb of *M. tuberculosis*. A growing body of evidence has accumulated in the last few years, demonstrating a role for flavoHbs in the response to the threat posed by nitrosative stress. This general role of flavoHb in NO defense is of great interest for clinical microbiology. Pathogenic microorganisms, such as *M. tuberculosis* and *Salmonella* serovar Typhimurium, might profit from this resistance mechanism upon infection of a potential host. Therefore, the role of this flavoHb was explored, which was more intriguing knowing that a strong NO dioxygenase activity is already contributed by trHbN. The necessary features required to classify the protein encoded by the locus Rv0385 as a putative Hmp, like globin signature residues (HisF8, PheCD1, GlnE7) and potential FAD- and NAD(P)H-binding domains, were found to be well conserved in *M. tuberculosis* Hmp. The Hmp protein from the mycobacterial species seem to make a separate class within the flavoHb family, with more than 70% similarity among themselves but being strikingly different from all other known flavoHbs. The RT-PCR

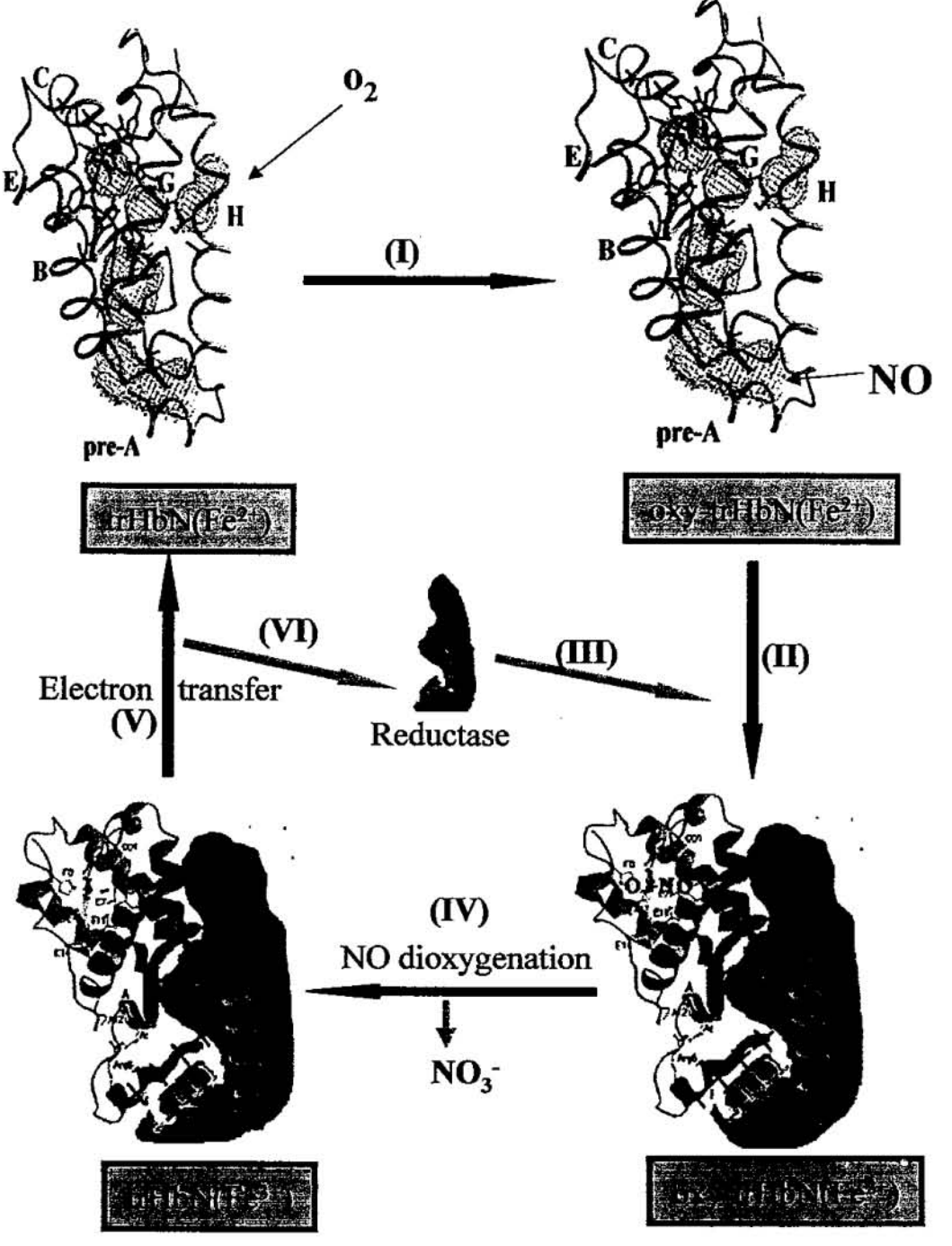

Figure 1: Proposed mechanism of trHbN function: Step (I) involves entry of O_2 through tunnel short branch. This results in opening of the tunnel long branch through conformational changes, which facilitates entry of NO (II). The oxy-trHbN associates with a flavoreductase (III). TrHbN is schematically shown in association with the reductase and the Pre-A region might facilitate this association or even assist in transfer of electrons *via* charged interactions. The bound NO is converted to nitrite (IV) and the residues of Pre-A region might modulate the reaction chemistry by some intra-molecular interactions. The resulting ferric heme-iron genearted in the reaction is reduced back to ferrous heme-iron (V) by the bound reductase. In the absence of bound ligand the flavoreductase dissociates (VI) and oxy-trHbN is ready to catalyse another NOD cycle.

nalysis of *M. tuberculosis hmp* gene showed that it expresses during the early stationary phase f mycobacterial growth.

The spectral analysis of the *E. coli* cells expressing *M. tuberculosis* Hmp showed that it inds oxygen reversibly, reacts with CO and forms a stable oxygenated form which is very imilar to oxyhemoglobin and myoglobin suggesting that it may be able to trap and transfer nolecular oxygen. Interestingly, the over-expression of *M. tuberculosis* Hmp resulted in marked nhancement in the growth of *E. coli*. The observed beneficial effects of Hmp expression on netabolism might be due to increased respiratory activity, *i.e.* higher oxygen uptake, higher pecific activities of terminal oxidases and, therefore, an overall improved cellular energetic harge. The *M. tuberculosis* Hmp failed to protect the *E. coli* host against reactive nitrogen pecies. It displayed cyanide sensitive NO consumption activity like *E. coli* Hmp (Gardner *et al.*, 998b; Hausladen *et al.*, 1998), however, it was >8 fold lower than that of *E. coli* Hmp (Pathania *t al.*, 2002a), which makes a NO detoxification function for *M. tuberculosis* Hmp less plausible. On the contrary, *M. tuberculosis* Hmp was found to have a profound protective effect on the rowth and survival of *E. coli* cells exposed to hydrogen peroxide induced oxidative stress, vhich is unusual because no flavoHb has till date been reported to be protective solely against xidative stress.

The reason for the unique functionality of *M. tuberculosis* Hmp became evident after etailed sequence comparison with *E. coli* and *Ralstonia* flavoHbs. Most of the residues whose unctional implications have been established in the known flavoHbs were either absent or hanged in the *M. tuberculosis* Hmp. The catalytic triad at the heme proximal site was changed rom His-Tyr-Glu to His-Tyr-Met and out of the 3 conserved Tyr residues involved in hydrogen onding at the proximal site, only one is retained. *E. coli* and *Salmonella* flavoHbs when over-xpressed are known to exacerbate oxidative stress by generation of superoxide radicals and H_2O_2 and, thus, inhibit normal aerobic growth in the absence of nitrosative stress (Membrillo-Iernandez *et al.*, 1996; Anjum *et al.*, 1998; Mills *et al.*, 2001). This property was not observed in ase of *M. tuberculosis* Hmp, which in contrast resulted in enhanced growth upon over-xpression. The residues implicated in this property of *Salmonella* are a Tyr-Ser pair whose nutation abrogated this pro-oxidant effect. These residues in the *M. tuberculosis* Hmp are Ala-le. The residues that are involved in electron transfer between the globin and reductase domains re highly polar whose predominantly large side chains originate from all the three domains

(globin, FAD- and NAD(P)H- binding domains). These residues might influence the electrochemical potential of the prosthetic groups, the dielectric constant of the intervening space and finally, the kinetics of the electron transfer process. Most of these residues are different in the linear sequence of *M. tuberculosis* Hmp, however, the adjacent residues are capable of supplementing the charge requirement at those positions. Owing to the vast difference in the overall sequence of *M. tuberculosis* flavoHb and all other known Hmps, a structure-based comparison would be more appropriate in providing a better picture of the residues at these respective positions. It can be presumed that the *M. tuberculosis* Hmp would also form a trilobite structure like the *E. coli* Hmp (Ilari *et al.*, 2002) but slight structure perturbations might bring together residues from different regions of the primary sequence.

The globin domain of *M. tuberculosis* Hmp, when expressed independently of the reductase domain retained its functionality of providing growth advantage as well as protection from oxidative stress to it *E. coli* host. The reductase domain was ineffective under both these conditions. This suggests that it is the globin domain that determines the functionality of the protein and it is capable of interacting with other redox proteins of the cells for the transfer of electrons. The extent of growth advantage provided by the globin domain alone is similar to that provided by the full length Hmp but the ability of the globin domain to protect its host from oxidative stress was ~40% lower than that provided by Hmp. This suggests that the reductase domain functions to enhance the efficiency of the catalytic reaction carried out by the globin domain. Additionally, it can be inferred that even if the residues required for interaction of the globin and the reductase domain don't appear to be conserved, the two domains do interact to give better efficiency.

The comparison of residues that are in van der Waals contacts with the lipid in the *Ralstonia eutropha* flavoHb (FHP) and *E. coli* Hmp proteins with the analogous residues in *M. tuberculosis* Hmp indicated that most residues involved in the formation of the lipid-harboring cavity within the heme domain are conserved. This suggests that *M. tuberculosis* Hmp, like *E. coli* Hmp, may be capable of alkylhydroperoxide reduction (Bonamore *et al.*, 2003) and, therefore, could be involved in the repair of peroxidized membrane phospholipids, thus, reducing hydroperoxides into their corresponding alcohols. This function of Hmp implicates it in protecting the cell membrane against damage from peroxidation during oxidative stress. Currently, it is possible to envision two more roles for Hmp 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. 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 and Hmp, by its NAD(P)H reductase activity, can transfer electrons from NAD(P)H to its heme-Fe and, thus, reducing the Fe^{3+}-O^--O^- or the Fe^{3+}-O^--OH derivative. Alternatively, Hmp may bind ROS and, as such, serve as a proximal sensor for oxidative stress like its *E. coli* counterpart. Further studies with purified Hmp are required to examine these possibilities and determine the precise role of this protein in the oxidative stress.

Ultimately, to understand why virulence genes are regulated the way they are, we must know the whens and the wheres of virulence gene expression. This will depend on the development of techniques and approaches for identifying which genes are expressed *in vivo* during the infection cycle. The together with a more detailed understanding of the mode of action and role in infection of virulence determinants should lead to a full molecular description of pathogenesis. The regulation analysis of the Hbs of *M. tuberculosis* was explored to gain new insight into the properties and functionalities of these novel Hbs. Additionally, comparisons of regulatory regions will enrich the discussions of the evolving functions of proteins.

The analysis of genetic organization of the three *M. tuberculosis* Hbs showed that the flanking genetic loci are quite conserved in case of *glbO* (encoding trHbO) and *hmp* (encoding Hmp) genes and in contrast to which, the upstream and downstream genes of *glbN* (encoding trHbN) exhibited maximum functional variability. In bacteria, gene organization helps in speculating the probable function of the gene based on its positional counterparts, therefore, it is quite possible that trHbN may be playing distinct role(s) in different mycobacterial species and trHbO and Hmp might have a fairly conserved function across species. A SigF binding site was found in the upstream sequence of *hmp* gene, which stressed upon the importance of the Hmp protein under the conditions where SigF is expressed. The transcriptional expression pattern followed the protein expression pattern reported for the trHbs in *M. bovis* (Pathania *et al.*, 2002b; Ouellet *et al.*, 2002), with *glbN* promoter getting up-regulated near the stationary phase and *glbO* promoter being constitutively expressed at all growth phases. The *hmp* promoter was also upregulated at the early stationary phase of *M. smegmatis* growth.

The regulation of the Hb genes during *in vitro* growth correlated well with the functionalities of their encoded proteins. The *glbN* promoter was specifically responsive to

nitrosative stress, NO in particular and trHbN is involved in detoxification of NO, therefore, should be available to the mycobacteria whenever toxic levels of NO are present. The *glbO* promoter responded to general stresses as RNIs and H_2O_2, and trHbO is proposed to be required for aerobic respiration (Pathania *et al.*, 2002b; Liu *et al.*, 2004) and, therefore, it would be required when the bacteria comes across the phagosomal environment characterized by RNIs, ROIs and hypoxia. The up-regulation of *glbO* promoter in response to SNP and expression of *glbN* at a different time of infection suggests that *M. tuberculosis* trHbO, like *M. leprae* trHbO (Fabozzi *et al.*, 2006) may play a role in NO metabolism as well. The *hmp* promoter was maximally up-regulated in the presence of H_2O_2, which again correlates with its deduced functionality of protection against host-generated ROIs.

The promoters of the three *M. tuberculosis* Hbs showed distinct expression profile during growth inside PMA (phorbol 12-myristate 13-acetate) activated THP-1 macrophages, which have been reported to closely model the primary human alveolar macrophages (Tsuchiya *et al.*, 1980; Tuschiya *et al.*, 1982; Leemans *et al.*, 2001; Riendeau and Kornfeld, 2003). The *glbN* promoter showed slow but consistent increase in activity whereas *glbO* promoter activity increased abruptly. On the other hand, the *hmp* promoter activity initially increased and then decreased with time. This increase in *glbO* and *hmp* promoter activities might be due to the induction of some host-specific factor in the *Mycobacterium* itself or else due to the variation in the host environment. This change in activity of the Hb promoters suggests that they respond to the change in macrophage environment owing to the release of cytokines in a time dependent manner, for example, NF-κB (Schaible *et al.*, 1998; Dhiman *et al.*, 2007) and activation of various protection mechanisms, for example, generation of ROIs and RNIs against the *Mycobacterium*.

The overall conclusions based on the structure-function studies suggest that all the three Hbs might be playing very important but distinctly different roles in the physiology and survival of *M. tuberculosis*, which together would form a multi-tier system to help the *Mycobacterium* survive the defense arsenal of its host. The trHbN appears to be evolved for parasitic lifestyle based on its presence in only a limited number of pathogenic organisms, and the selection of this single domain Hb to carry out a function, which would have been carried out more efficiently, by two domain Hb, would give it the flexibility to interact with various partners at different times of growth as well as the functional variability to act as an oxygen carrier, for example, during the

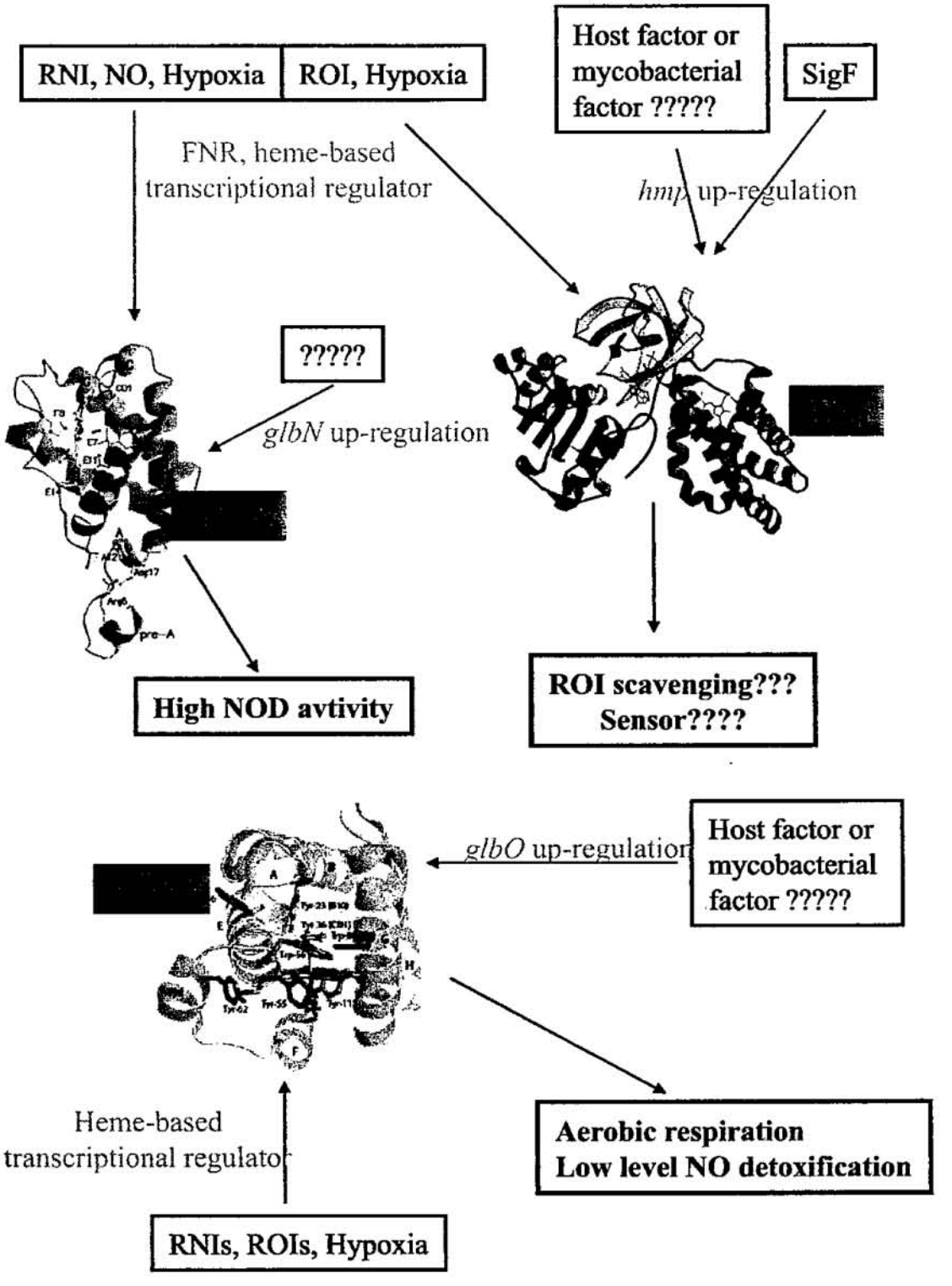

Figure 2: Proposed regulatory and functional characteristics of *M. tuberculosis* hemoglobins. The proposed functions and the regulatory signals are depicted in blue and black, respectively.

latent phase where its high affinity will keep it saturated at low oxygen levels of granuloma and its slow dissociation rate will ensure a slow but steady release of oxygen for the latent phase. The importance of trHbO for intracellular survival is stressed by its up-regulation during growth inside macrophages. trHbO might play a role in respiration in the hypoxic phagosomal environment and also detoxify NO in the absence of trHbN, *i.e.*, when trHbN expression is not induced. Hmp protein on the other hand, would protect the *Mycobacterium* from the oxidative stress, directly by detoxifying the reactive oxygen species (ROIs) or indirectly by acting as a sensor or reversing the damage caused by the ROIs (probably by reducing lipid peroxides).

The renewed interest in TB research has led to a significant burst of activity in exploring the bacilli at cellular and molecular details. Tuberculosis teaches that a balance can teeter for years between host immunity and a pathogen's resistance. A great deal of challenging work remains if humans are to outwit this ancient and clever adversary. Based on the biochemical and physiological studies the proposed regulation and functionality of the *M. tuberculosis* hemoglobins is shown in Figure 2. A better understanding of the *in vivo* activity of the hemoglobin like domain requires more structural and functional experimentation. The trHbN has several unique features (tunnel system, Pre-A), which suggests a novel mechanism of NO detoxification and to understand the mechanism and the role of each of these features its important to ensue a search for oxidoreductase genes in mycobacteria as putative candidates for trHb(III) reduction. The evaluation of the protective mechanism of Hmp would add a wealth of information to the existing reactions carried out by the Hbs. Pathogenic microorganisms, such as *M. tuberculosis* might profit from the hemoglobin assisted resistance mechanism upon infection of a potential host. Bearing in mind the steadily increasing number of antibiotic-resistant pathogens, the Hbs might represent a novel drug target for therapy.

The commercial production of a variety of desirable metabolites and important pharmaceuticals employs the overexpression capacity of oxygen-requiring bacteria, fungi and mammalian cells. The growth advantage provided by the globin domain of Hmp could be exploited for the commercial production of a variety of desirable metabolites and important pharmaceuticals. Protein engineering approaches, either of rational or random origin or based on evolutionary selection, would further help to improve the properties of globin under oxygen-limited growth conditions. In addition to this, the Hmp protein presents a very interesting subject for structure function studies.