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SUMMARY OF THE THESIS

The age old paradigm that hemoglobins (Hb) are exclusive to higher forms of life and perform oxygen transport was brought down by the discovery of hemoglobin in single-celled organisms (Wakabayashi *et al.,* 1986), which have no obvious need for oxygen transport. Hemoglobins were gradually discovered in all five kingdoms of life and are now considered ancient proteins. Hb genes probably predated the split between eukaryotic cells and eubacteria, some 2-4 billion yrs ago. Though globin-like hemoproteins were discovered in early 1950s in yeast and other fungi by Keilin, their investigation has been slow to gain momentum, in part because of the perceived lack of function. Eventually discovery of Hb-like proteins in all forms of life, led to the initiation of investigation on Hb functions beyond oxygen transport and storage.

The discovery of globin genes among prokaryotes has stimulated extensive studies aimed at unraveling the physiological significance of unicellular hbs and understanding the evolutionary relationships that link these proteins to their homologues in higher organisms. Truncated hemoglobins (trHbs), in particular, have gained distinct attention because of their widespread occurrence in 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 (Wittenberg *et al.*, 2002) as terminal oxidases 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, HbP, HbO and HbN. The facultative intracellular pathogen, *M. tuberculosis* has two, HbN and HbO and the obligate intracellular pathogen, *M. leprae*, which has undergone extensive reductive evolution, retains only HbO.

Mycobacterium tuberculosis, the causative agent of tuberculosis, is one of the most notorious and successful pathogens of human. Two genes, *glbN* and *glbO*, encoding the group I trHb (HbN) and the group II trHb (HbO) respectively, were discovered in the complete genome sequence of the virulent *Mycobacterium tuberculosis* (Cole *et al.*, 1998). Recently a type II flavohemoglobin (HMP), encoded by Rv0385, has been discovered in *M. tuberculosis* (Sanjay *et al.*, 2011), in addition to the two trHbs. 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.

Although occurrence of group I HbN is restricted to certain organisms, unraveling of the genome data indicated that the HbN type truncated hemoglobins are present in almost all mycobacterial species (Wittenberg *et. al.,* 2002). Moreover, it has been observed that HbN performs most diverse functions in different organisms, *e.g.* roles in electron transfer chain (in *Nostoc commune)*, photosynthesis (in *Chlamydomonas eugametos*) and in O_2 supply to mitochondria (in *Paramecium caudatum*). These observations led us to investigate whether HbN plays any important function(s) in *M. tuberculosis* survival and pathogenesis.

M. tuberculosis is an obligate aerobe and during infection, it successfully survives inside host macrophages. The pathogenesis of *M. tuberculosis* infection is complicated; however, recent discoveries have attracted great attention due to their

sociation with host-derived and microbial factors. Macrophages are believed to play pivotal role in the immune response against mycobacteria through the production of tokines such as tumor necrosis factor (TNF) - α and interleukin (IL)-1β. TNF-α and -1β, along with interferon (IFN)-γ. (IFN)-γ is produced by T lymphocytes and is nown to induce NO production in macrophages via the action of inducible forms of he enzyme NO synthase (iNOS) (Kuo *et al.*,2000, Lee *et al.*, 1993, Wang *et al.*, 001). NO and related reactive nitrogen intermediates (RNI)s can kill and/or inhibit ntracellular pathogens such as mycobacteria (Adams *et al.*, 1991, Alam, 2002, MacMicking, 1997, Mayer, 1997). This was supported by clinical findings where the 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 considering that the control of the pathogen is imperfect in both species, it can be easily envisaged that tubercle bacilli may have evolved mechanisms for RNI resistance. 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' (HbN), encoded by the *glbN* gene (Couture *et al.*, 1999b; Pathania *et al.*, 2002a). The heterologous expression of *M. tuberculosis* HbN, 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). In addition to relieving NO stress it is also speculated that the high oxygen affinity of HbN (P_{50}~0.01 mm Hg) *in vivo*, 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). Recent finding by our group that HbN is overexpressed inside activated macrophages (Pawaria *et al.*, 2009), further substantiated the belief that HbN plays important role in *M. tuberculosis* survival and pathogenesis.

It has been proposed that the oxygenated HbN (oxy-HbN) could detoxify the macrophage-generated NO, similar to the dioxygenase activity of (flavo)Hbs and myoglobin (Mb), which converts 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). The rapid oxidation of nitric oxide (NO) to nitrate by *M. tuberculosis* HbN (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. This raises the obvious need to identify the redox protein(s) of *M. tuberculosis*, which might be getting associated with HbN for efficient recycling of its active form. Since, the heterologous expression of *M. tuberculosis* HbN, 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), it is expected that HbN is functionally active in heterologous hosts and their protein partner(s) can be identified from them as well. Moreover, identification of the protein partners of HbN in heterologous and native host is expected to help in exploration of HbN functions beyond NO scavenging.

During this study, *in vivo* search for protein partner in heterologous host *E. coli* yielded a 66kDa protein, L-glutamine: D-fructose-6-phosphate aminotransferase (GFAT), which catalyses the formation of D-Glucosamine-6-phosphate using L-glutamine as ammonia source, which is the first step in UDP-*N*-acetyl glucosamine biosynthesis pathway. It has role in amino acid biosynthesis, peptidoglycan biosynthesis and is membrane associated. The relevance of this protein partner is not yet fully understood. However, its involvement in cell wall biogenesis in both *E. coli* and *M. tuberculosis* and discovery as probable partner of HbN suggests that exploring different physiological roles of HbN is crucial. We were not able to identify any redox protein partner from heterologous host *E. coli* or native host *M. tuberculosis*; the reason may be the transient nature of redox interactions or may be because of the expression of these proteins in temporally different space and time. Interestingly, a putative lipoprotein LprI encoded by *lprI* gene was identified as a potent partner protein of HbN in *M. tuberculosis*. This gene is present only in a few pathogenic species of mycobacterium group and is always present upstream to *glbN*. Both HbN and LprI have been found to be co-transcribed. Our results indicate strong interaction between these two proteins in cell-membrane fraction. Association of HbN with cell membrane was a novel finding, and requires further exploration. The physiological relevance of their interaction can only be hypothesized at the moment, because LprI is

still an uncharacterized protein. Based on *in silico* studies the domain MLiC present in LprI suggests its involvement in survival of *M. tuberculosis* by inhibiting lysosomal degradation of mycobacterial membrane. Moreover, the occurence of LprI exclusively in pathogenic strains of mycobacterium complex and availabity of reports which illustrate importance of lipoproteins in mycobacterial survival and virulence (Pennini *et al.*, 2006; Clemens *et al.*, 2011), also suggest its involvement in pathogenesis of the bacillus. It is therefore expected that HbN and LprI may interact to aide in pathogenesis and survival of *M. tuberculosis*. Thus, the protein-protein interactions in heterologous and native host suggest that HbN may have functions associated with cell membrane biogenesis and protection. Our findings therefore suggest that HbN may have functions beyond NO scavenging in *M. tuberculosis*.

Despite the biophysical and structural characterization of HbN 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 HbN. In order to gain an insight into molecular mechanism of NOD activity of HbN, it was imperative to identify its true redox partner. Since, redox protein could not be identified from *in vivo* partner protein search, few redox proteins were selected (which may act as electron donors), to study HbN-reductase interaction *in vitro*. Available microarray data of *M. tuberculosis* gene expression and *in silico* information was extensively analyzed and few reductases which get overexpressed during oxidative and nitrosative stress or which display sequence similarities with reductase part of flavohemoglobin were selected. Protein-protein interactions were studied between HbN and these seleted redox proteins *in vitro*. By *in vitro* crosslinking using paraformaldehyde, it was observed that interaction of HbN with these putative redox partners is somewhat specific, *i.e.*, HbN may interact with more than one redox protein, but not with all of them. This was further confirmed by Surface Plasmon Resonance studies, which indicated that HbN binds very efficiently with some reductases such as mycobacterial flavoreductase encoded by locus Rv3571 and less efficiently with other reductases such as TrxA. Moreover, the interaction of the HbN with the reductase was dependent on the occupancy of its heme pocket with oxygen or CO. This suggests that the interaction is based on the conformation of the HbN, which is very interesting and suggests a novel mechanism of NOD reaction operative solely in the trHbs.

In order to explore whether any specific domain of HbN is involved in redox interactions and in turn in NO detoxification, structure-based sequence alignment of different mycobacterial HbNs was done. This indicated clear conservation of the main regions crucial for the stabilization of the trHb fold. However, a striking difference lied at the N-terminus which showed that HbN of most of the slow growing pathogenic mycobacteria carry a highly charged Pre-A motif that is absent in HbN from the fast growing non-pathogenic mycobacteria. The presence of Pre-A region was initially observed in the crystal structure of *M. tuberculosis* HbN (Milani *et al.*, 2001), which distinguishes it from its homologs present in protozoan, algal and cynobacterial species. Initial experimental studies from our laboratory suggested that Pre-A region is vital for the NOD activity of HbN and it brings about conformational changes in the HbN (Lama *et al.*, 2009). The importance if any, of Pre-A region in HbN-reductase association was evaluated by determining the ability of wild type HbN and Pre-A lacking HbN to interact with different reductases. The results from crosslinking studies exhibited that reductases interact with only oxygen or CO bound form of Pre-A deleted HbN, however, Surface Plasmon Resonance (SPR) studies exhibit that binding efficiency of reductases is greatly reduced in Pre-A deleted HbN as compared to wild type HbN. In addition to this, NO uptake rate and NADH-oxidase activity of wild type HbN and Pre-A deleted HbN in presence of different reductases indicates that presence of some reductases (which display high binding efficiency by SPR), enhances these activities of only wild type HbN. On the other hand, NO uptake rate and NADH oxidase activity of Pre-A deleted HbN was found to be very low and was not affected by presence of any reductase. This was substantiated by other data from our lab which shows that, the increase in the NOD activity of the purified *M. tuberculosis* HbN (carrying Pre-A) on addition of cell extract of *E. coli* cells overexpressing the flavoreductase was 6-fold higher than of the HbN of *M. smegmatis* (lacking Pre-A) (Lama *et al.*, 2009). Moreover, we observed that oxygenated full length HbN can easily oxidise NO, but the deletion of Pre-A region from HbN locks it in such a conformation that NO cannot access Fe core to get oxidised. Overall, these results suggest that HbN can interact with more than one reductase and this interaction is redox state and Pre-A region dependent.

The mechanism by which Pre-A region assists in interaction with reductase is not completely understood. Molecular simulation studies indicate that Pre-A deleted

HbN displays clear cut restriction in the movement of B and E helices as well as EF loop region that may affect functioning of long tunnel branch and PheE15 gate, PheE15 gate is trapped into a closed conformation along the whole trajectory, thus blocking easy access of NO towards the active site. Importantly, the ability of PheE15 gate to switch between open and closed states was recovered when Pre-A region was reinserted into Pre-A deleted HbN of *M. tuberculosis* (Lama *et al.,* 2009). The dynamical alteration observed upon O_2 binding in *M. tuberculosis* HbN (Bidon-Chanal *et al.*, 2006; Bidon-Chanal *et al.*, 2007), which mainly involves displacement of B and E helices along with local rearrangements (primarily the side chain of GlnE11) near the PheE15 gate, is not found in the trajectories run for *M. tuberculosis* Pre-A deleted HbN. Moreover, as in case of flavoHbs (Ermler *et al.*, 1995; Ilari *et al.*, 2002; Frey and Kallio, 2003), the presence of charged residues in Pre-A region, may govern the interaction between the reductase and the HbN 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. To sum up it can be speculated that association of reductase with HbN is modulated by either conformational changes brought about by either redox state of HbN and/or Pre-A region or by the charged residues of the Pre-A region. Moreover, the possibility of interaction with different reductases might provide it functional variability during *in vivo* growth.

Previous studies indicate that expression of HbN is enhanced several fold inside activated macrophages (Pawaria *et al.*, 2008), which in turn indicates that HbN plays crucial part in survival and pathogenesis of *M. tuberculosis*. 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. This 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. At present complete functions of HbN are not understood, except for the efficient NO scavenging activity. It is expected that by virtue of this activity, HbN helps in the intacellular survival of *M. tuberculosis* inside macrophages. Therefore, understanding the effects of HbN overexpression on cell

physiology and proteome might assist in understanding the function(s), if any, of HbN in *M. tuberculosis* pathogenesis and survival. Surprisingly, upon HbN overexpression, it appeared that HbN seems to modulate a large number of factors in cell. To start with when HbN was overexpressed in *M. tuberculosis* cells, the appearance of culture changes remarkably, moreover the cell morphology and acid fast staining patterns also display striking differences. This was further examined by Scanning Electron Microscopy, which exhibited that the mycobacterial cells appeared elongated and beaded upon HbN overexpression. On the other hand, HbN overexpression in *M. smegmatis* cells, did not provide any significant difference to the cell morphology, this may suggest that HbN interacts with certain factors specific to its native host, to bring about the observed changes. Since, lipids and fatty acids are known to be important constituents of mycobacterial cell wall, fatty acid and lipid profiles of HbN overexpressing and wild type cells were generated. There was distinct upregulation of certain anteiso- fatty acids and certain polar lipids in HbN overexpressing cells. Antesio fatty acids are believed to provide membrane fluidity like unsaturated fatty acids but they are less susceptible to oxidation. Therefore, increase in the concentration of anteiso fatty acids suggests that HbN may be involved in maintaining the membrane fluidity and in providing protection against membrane oxidation. Additionally, overexpression of HbN provides growth advantage to mycobacterial cells in the presence of acidified nitrite *in vitro*. Since all these experimental evidences indicated the implications of HbN overexpression *in vitro*, therefore, it was imperative to evaluate the HbN functionalities *in vivo*, during intra-macrophage growth. The HbN overexpressing and wild type *M. tuberculosis* cells were used to infect the macrophage cells and cell lines. The results obtained displayed that HbN assists mycobacterial cells' survival inside activated macrophages. Moreover, the higher number of colony forming units even at the onset of infection, suggest that HbN aides the entry of the bacilli in macrophages and thus collectively it can be concluded that HbN helps the cells in entering the macrophages and then assists them in survival in hostile conditions generated by macrophages. Moreover, during cytokine assays it was obsereved that HbN induces the expressions of pro-inflamatory cytokine IL-6 and TNF-α, and levels of nitric oxide. Additionally, the expression of immunomudulatory cytokine IL-10 is also increased. Collectively these observations suggest that due to the effective modulation of host immune response and efficient NO scavenging HbN drives the cells towards better survival inside host macrophages.

This suggested that HbN, which until now was seen to be beneficial against the *in vitro* generated stress only, was able to defend its host during intacellular survival.

Since, there were prominent differences in lipid and fatty acid profile and in intracellular survival of HbN overexpressing mycobacterial cells; it was explored whether HbN provides any protection against anti-mycobacterial drugs. It was observed that HbN provided distinct growth advantage to cells in the presence of isoniazide and pyrazinamide drugs. According to the available reports isoniazide inhibits the growth of *M. tuberculosis* by providing NO stress. Moreover, it inhibits the mycolic acid production and in turn inhibits cell wall synthesis in mycobacterial cells. On the other hand pyrazinamide is known to affect fatty acid production. It is therefore, believed that HbN provides growth advantage to cells in presence of these drugs because of the changes that it brings about in the fatty acid and lipid biosynthesis.

In brief the overall conclusions based on the present study suggest that HbN is localized in cytoplasm and inner cell membrane in *M. tuberculosis*. It possibly interacts with host specific membrane proteins such as lipoprotein LprI and even causes significant changes in membrane lipids and fatty acids to assist in pathogenicity and survival of the tubercle bacillus. Due to its strong association with flavoreductase (Rv3571), which is alternatively denoted as kshB, and has recently been implicated to be essential for *M. tuberculosis* pathogenesis (Hu *et al.*, 2010), it can be hypothesized that HbN plays significant but unexplored role in pathogenesis of the bacilli. The *in vitro* interaction studies with redox proteins indicate that certain degree of functional flexibility is associated with single domain HbN, because of which it can interact with more than one redox proteins, depending on their availability. Additionally, interaction with redox proteins are conformation dependent, which in turn depends on presence of Pre-A region and/or availability of bound oxygen in heme cavity. The studies on effects of HbN overexpression suggest that HbN may be involved in certain unexplored pathways, other than NO scavenging, which in turn may be responsible for *M. tuberculosis* pathogenesis and survival.

Mycobacterium tuberculosis has evolved several mechanisms to enter the host cell, circumvent host defenses and spread to neighboring cells. Despite extensive research, our knowledge about the virulence factor(s) of *M. tuberculosis* is quite inadequate. Understanding the molecular mechanisms of *M. tuberculosis* pathogenesis

rovide insights into the development of target-specific drugs or effective vaccine dates for the treatment of the disease and to control the tuberculosis pandemic. ıe basis of present study, it is expected that unraveling the HbN functionality and echanism will definetly pave way for better understanding of *M. tuberculosis* gy.