OVERVIEW

The host pathogen relationships are characterized by the complex interplay between host defense mechanisms and attempts by the microorganisms to circumvent these defenses. From an immunological viewpoint, invasion of pathogenic microorganisms into a susceptible host poses a potential life-threatening situation and thus has to be met with whole arsenal that is available. Reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) can damage DNA and several chemical moieties on which its propagation depends including Fe-S clusters, tyrosyl radicals, hemes, sulphydryls, thioethers and alkenes. ROI are produced by all aerobic cells and RNI by many. High ROI output is the speciality of mammalian phagocytes, with polymorphonuclear leucocytes being major contributor and immunologically activated macrophages being the second highest contributor. However, in terms of production of RNI, macrophages outpace polymorphonuclear leucocytes easily.

A crucial component of host defense mechanism is the macrophage. Macrophages play key roles by recognising, engulfing, and killing microorganisms. The scavenger activity of this cell ensures the uptake and destruction of bacteria in phagolysosomes, on the one hand, and activation of the adaptive component of the immune system through presentation of microbial antigens, on the other hand. Among the microorganisms recognised by macrophages, bacteria are an important and highly diverse class of human pathogens. From the bacterial perspective, entry into a phagolysosome is usually fatal and many pathogens have developed strategies that circumvent the destructive environment of this organelle. Bacterial pathogens that overcome host defenses ensure their ability to survive and propagate. Therefore, a thorough understanding of the normal host response to bacteria provides a foundation to understand bacterial tactics for evading these responses and thus disease prevention.

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Macrophage generated oxygen and nitrogen reactive species control the development of *M. tuberculosis* infection in the host. Nitric oxide (NO), generated in large amounts within the macrophages, controls and restricts the growth of

internalized bacilli. The pathogenicity of mycobacteria is mainly due to the fact that they are extremely well adapted to their host environment, in particular the macrophage. The ability of Mtb to not only avoid being killed by host macrophages, but to survive and replicate within macrophages, is of great interest. It is an established fact that Mtb attaches to macrophages via complement receptors (e.g.C3), mannose receptors, scavenger receptors, and CD14. Mtb appears to alter the maturation of the phagosome. Mycobacterial phagosomes have decreased acidification because they lack the hydrogen-ATPase pump. Some of the strategies that are hypothesized as ways in which Mtb evades the cidal activity of macrophages include prevention of oxidative burst, detoxification of nitric oxide generated in the macrophages, inhibition of phagosome-lysosome fusion and resistance to lysosomal enzymes. The precise survival strategy of Mtb, and the genes required for intracellular survival, are unknown, although several candidate genes have been proposed.

Two genes, *glbN* and *glbO*, encoding truncated hemoglobins (TrHbs), HbN and HbO, respectively were detected in the genome sequence of *Mycobacterium tuberculosis* H37Rv. The computational and sequence analysis of the aminoacid sequence of HbN and HbO has been done which revealed that both the proteins harbour a typical "globin fold" which is characteristic feature of the hemoglobins. A comparative analysis of the main structural features of HbN and HbO with reference hemoglobins *i.e.* sperm whale myoglobin and *Vitreoscilla* hemoglobin indicated distinct structural conservation of the globin fold. The sequence analysis and alignment showed that the key heme binding residues are conserved in both the proteins. HbN and HbO harbour tyrosine at B10 and histidine at F8 positions. The invariant heme binding position, CD1 (phenylalanine) is conserved whereas in HbO it is occupied by tyrosine. In HbN, the potential ligand-binding site is occupied by leucine, whereas HbO harbours alanine.

However, the primary aminoacid sequence of HbN and HbO exhibits all the characteristics of the "TrHb fold" which is a characteristic of a new family of hemoglobins possessing a two over two alpha helical arrangement. In addition to

this, blast search revealed that genome of several pathogenic and nonpathogenic microorganisms encode for HbO as well as HbN like hemoglobins. The two group of hemogobins exhibited distinct homology within themselves but showed little homology with the other group. Hence HbN and HbO constitute a distinct group within themselves.

Why should *M. tuberculosis* evolve two hemoglobins? A preliminary analysis of the structural features of HbO and HbN showed that the two proteins share little sequence similarity and display marked structural differences in their 'E-F' loop regions, suggesting distinct function(s) for these hemoglobins. A detailed study was taken up to characterize the two hemoglobins and arrive at some conclusive functions for them.

The *glbO* and *glbN* genes were fished out from the genomic DNA of *M. tuberculosis* and *M. bovis* (BCG) using PCR and cloned in pBS(KS^+). The sequencing of two genes confirmed that the sequence of *M. bovis glbN* and *glbO* was exactly same as that of *M. tuberculosis.*

The two proteins were further subcloned and expressed independently under the control of T7 promoter in *E. coli* BL21(DE3) which resulted in leaky expression. Therefore, HbN and HbO were expressed under the control of *Vitreoscilla* hemoglobin (*vgb*) promoter resulting in constitutive expression of the two proteins and allowing to monitor the response of HbN and HbO on the physiological aspects of recombinant *E. coli.* For biochemical and biophysical characterization of the two hemoglobins, both HbN and HbO were purified to near homogeneity and the gel filtration analysis revealed both the proteins to be dimeric in nature. The absorption spectrum of the recombinant HbN and HbO was characteristic of a heme protein. The optical absorption spectra revealed that both HbN and HbO are hemoglobins. HbN and HbO from Mtb were further expressed in *M. smegmatis* mc^2 155.

O_2 and NO 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 uptake activity and the oxygen uptake properties of the two proteins were studied.

The NO consumption activities of the two proteins were studied in a flavohemoglobin deficient mutant of *E.coli i.e.* RB9060. HbO conferred NO consumption activity to *E.coli* RB9060. However, the NO uptake by HbO was considerably lower than that of HbN. Thus HbO may not be very efficient in NO detoxification, since another protein (HbN) with higher NO consumption activity is present in the Mycobacterial cells. *E.coli* expressing HbO exhibited nearly 50 fold (P_{50}=0.51) lower oxygen affinity than HbN. 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 HbO did confer a growth advantage to the *E. coli* and *M. smegmatis*. The oxygen uptake rate of HbO carrying *E. coli* and *M. smegmatis* cells was two fold higher in comparison to control or HbN carrying cells.

The membrane association properties of the two proteins were also studied. Around 40-50% of HbO remained associated with the cell membranes and significantly enhanced its respiration in comparison to the membrane fractions of control cells or cells overproducing HbN. Additionally, membrane vesicles prepared from terminal oxidase deficient (*cyo*$^-$, *cyd*$^-$) mutants of *E. coli* did not exhibit significant enhancement in oxygen uptake in the presence of HbO suggesting its interaction(s) with the electron transport chain. Expression of HbO in *M. bovis* BCG, an experimental model of *M. tuberculosis*, was observed (0.2 to 0.5% of total cellular proteins) throughout its aerobic growth. These results provide evidence for the involvement of HbO with the component of aerobic electron transport chain and suggest that its function may be related to facilitation of oxygen transfer during aerobic metabolism of *M. tuberculosis.* The membrane association properties of HbO, thus, may be playing crucial role in sequestering oxygen and facilitating its availability to internalized *M. tuberculosis* (an obligate aerobe) under hypoxic conditions of its intracellular habitat.

Nitric oxide (NO), generated in large amounts within the macrophages, controls and restricts the growth of internalized human pathogen, *Mycobacterium*

tuberculosis H37Rv. The NO consumption activity of HbN was further investigated. It was found that HbN from *M. tuberculosis* exhibits distinct nitric-oxide dioxygenase (NOD) activity and protects growth and cellular respiration of heterologous hosts, *Escherichia coli* and *Mycobacterium smegmatis*, from the toxic effect of exogenous NO and the NO releasing compounds. A flavohemoglobin (HMP) deficient mutant of *E. coli,* unable to metabolize NO, acquired an oxygen-dependent NO consumption activity in the presence of HbN. On the basis of cellular heme content, the specific NOD activity of HbN was nearly 35 fold higher than the single domain *Vitreoscilla* hemoglobin (VHb) but was 7 fold lower than the two-domain flavohemoglobin. HbN dependent NO consumption was sustained with repeated addition of NO demonstrating that HbN is catalytically reduced within *E. coli.* Aerobic growth and respiration of flavohemoglobin (HMP) mutant of *E. coli* was inhibited in the presence of exogenous NO but remained insensitive to NO inhibition when these cells produced HbN, VHb or flavohemoglobin. *M. smegmatis,* carrying a native HbN, very similar to *M. tuberculosis* HbN, exhibited 7.5 fold increase in NO uptake when exposed to gaseous NO, suggesting a NO induced NOD activity in these cells. In addition, expression of plasmid encoded HbN of *M. tuberculosis* in *M. smegmatis* resulted in 100 folds higher NO consumption activity than the isogenic control cells. These results provide strong experimental evidence in support of NO scavenging and detoxification function for the *M. tuberculosis* HbN. The catalytic NO scavenging by HbN may be highly advantageous for the survival of tubercle bacilli during infection and pathogenesis. Further, HbN has a very high oxygen affinity as compared to HbO; binds oxygen efficiently and is expressed in its native host during late stationary phase, thus in the late stationary phase it may be helping the tubercle bacilli to store the oxygen and in tiding over the oxygen deficiency.

Truncated hemoglobins, HbN and HbO conform to a non-conventional two over two alpha helical fold globins. There was no information regarding the presence of a flavohemoglobin (HMP) protein in the *M. tuberculosis* genome. Hu *et al.*(1999) reported the regulation of a gene that they described as encoding a

flavohemoglobin in *M. tuberculosis* H37Rv. The nucleotide sequence cited (EMBL-Genbank Z92774) is predicted to encode a protein having the FAD- and NADH- binding sites of FNR, but not a hemoglobin-like domain. Therefore this protein cannot be considered a homologue of the flavohemoglobins. A search for a conventional three over three a helical globin in the genome of *M. tuberculosis* H37Rv revealed a flavohemoglobin protein encoded by the locus Rv0385 which was composed of a globin domain and a reductase domain. The protein was cloned and expressed in *E. coli.* The absolute absorption spectrum of the *E.coli* expressing Mtb HMP was characteristic of a heme binding protein, however the low intensity of the peak suggested that the protein might exhibit low heme binding. The *E. coli* cells expressing the flavohemoglobin could grow better than the control cells in the presence of sodium nitroprusside *i.e.* under nitrosative stress. However *E. coli* cells expressing Mtb HMP exhibited marginal increase in the NO consumption activity as compared to the control cells. This may be attributed to low heme binding by Mtb HMP in *E. coli* or due to the rapid outburst of the protein expression under the control of T7 promoter in *E. coli* BL21 cells which may lead to the formation of inclusion bodies. A preliminary structural analysis of the CE helix, F region and H helix of the Mtb flavohemoglobin with *Ralstonia eutropha* FHP indicates altered/deleted residues which may lead to weak interactions between the globin domain and the reductase domain, which in turn may lead to low heme binding.

The truncated hemoglobins have been detected in two pathogenic Mycobacterial species viz. *M.tuberculosis* and *M.bovis.* There are several non-pathogenic representatives of the *Mycobacterium* genus. A preliminary investigation was carried out to know the status of genetic loci corresponding to *glbN* and *glbO* in other mycobacterial species. Southern hybridization was carried out on the genomic DNA of the representatives from both pathogenic and non-pathogenic genera.

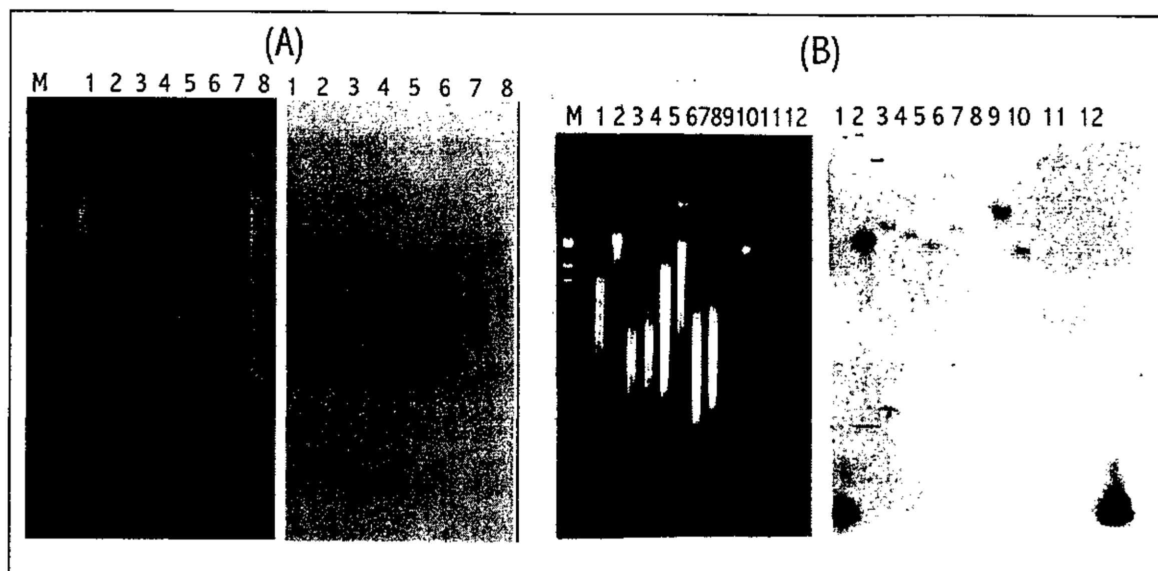

Fig.7.1 Southern blot analysis of various mycobacterial species for the presence of *glbN* and *glbO* encoding loci. Genomic DNA (3-5 ug) from various mycobacterial species was restricted with *Bam HI* and hybridized with radiolabelled *glbO* (A) and *glbN*(B) as probes M- λ/*HindIII* DNA marker, 1. *M.bovis* 2. *M. avium* 3. *M. fortitutum* 4. *M. gastri* 5. *M. kansasii* 6. *M. marinum* 7. *M. microti* 8. *M smegmatis* 9.*M. scrofulaecum(GlbN)*10. *M. smegmatis(GlbN)* 12. glbN *M. tuberculosis* PCR +ve control.

Interestingly, many mycobacterial species showed positive signal when both *glbN* and *glbO* were used as probes (Fig.7.1). However a detailed study needs to be undertaken on various mycobacterial species and should be the subject of further investigation.

The observation that HMP of Mtb provides growth advantage in the presence of SNP takes the candidate genes for NO detoxification to two. Why Mtb should evolve two hemoglobins, one the truncated hemoglobin (HbN) and the other flavohemoglobin (HMP) for similar functions? A deep insight into the fate of Mtb inside the macrophages might help us to arrive at some tangible explanation. The truncated hemoglobin HbN expresses itself in the late stationary phase of mycobacterium lifecycle, whereas it is an established fact that atleast in case of several microorganisms the flavohemoglobin gene is induced by NO. Taking both these facts in consideration, and the observation that HbO also shows marginal NO consumption; the rôle for HbN, HbO and HMP can be

visualized. When the bacillus enters the macrophage, the NO burst may induce the expression of HMP and thus HMP along with HbO (which is expressed throughout the lifecycle of the bacillus) helps in survival of the bacillus during the initial stages. HbO stimulates the respiration of the tubercle bacillus by interacting with the components of electron transport chain (ETC) and thus helps the bacillus to sustain its aerobic metabolism and adopt sufficiently under low oxygen. HbN protects the bacillus in the later stage in the calcified granulomas (Fig.7.2). Hence the presence of more than one TrHb with different oxygen binding and NO consumption activities in addition to a flavohemoglobin may enable the *M. tuberculosis* to cope with varying metabolic demands during different stages in its pathogenicity.

It is well understood that no single killing mechanism is sufficient to kill mycobacteria *in vivo.* However, a substantial body of evidence now exists that implicates a central role for NO in human host defense against Mtb. In this struggle of existence that goes on, Mtb has evolved clever ways to evade toxic effects of reactive nitrogen intermediates. Ehrt and coworkers (1997) showed that a novel *M. tuberculosis* gene, *noxR1*, confers resistance to the toxic effects of RNI, although the precise mechanism is not known. The same group later showed that the *M tuberculosis noxR3* gene also protected bacteria from the toxic effects of ROI and RNI (Ruan *et al.*,1999). Similarly, the *M. tuberculosis* – derived peroxiredoxin gene *ahpc* (alkyl hydroperoxide reductase subunit C) prevented RNI-induced necrosis and apoptosis in human cells (Chen *et al.*,1998). *Ahpc* has also been shown to detoxify $ONOO^-$ to NO_2^- (Bryk *et al.*,2000). NO may also induce the expression of a *M. tuberculosis*-derived 16kDa heat shock protein, a molecule that promotes stationary phase of growth of mycobacteria (Garbe *et al.*, 1999). Thus several workers have implicated different genes which confer resistance to toxic effects of RNI. HbN and HMP genes of mycobacteria add to the list of such candidate genes i.e. *noxR1*, *noxR3*, *ahpc* and the 16 kDa antigen. Such studies, which refine the understanding of the importance and the exact role of NO in defense against TB, may lead to innovative vaccination and treatment strategies.

In the present investigation it has been shown that the truncated hemoglobin HbO is involved with the components of aerobic electron transport chain and thus stimulates the cellular respiration. This property of HbO can be exploited for high-density oxygen intensive fermentation reactions at industrial level where at high cell densities; the oxygen supply to the cells becomes a limiting step for the successful fermentation run. The enhanced energy status of the recombinant cells showed that HbO can be exploited for recombinant protein production and may prove even better than the *Vitreoscilla* hemoglobin for such applications.

The primary sequence comparison of HbO with other hemoglobins revealed a unique feature; the CD1 position is occupied by the tyrosine rather than regular phenylalanine. It will be very interesting to probe why Tyr has evolved at CD1 position? Whether Tyr CD1 is playing a role to make HbO different from HbN? All these questions can be answered using site-specific mutagenesis. Undoubtedly, the solving of three-dimensional structure of HbO will shed light on the function and structure of this unique hemoglobin.

The present study has identified a flavohemoglobin (HMP) encoded by *Mycobacterium* genome. The preliminary characterization has shown that HMP of Mtb provides growth advantage from reactive nitrogen intermediates like SNP, however, the NO consumption activity was low. From the sequence comparison it has been predicted that there may be a loose interaction between globin and the reductase domain in Mtb-HMP. Also there is indication that heme also doesn't bind tightly to the HMP. These can be tested by mutating the residues suspected of involvement in heme-reductase interaction. It will be interesting to undertake a detailed characterization study to search for the functions of HMP and thus throw light on the third hemoglobin in pathogen tubercle bacillus.

The mycobacterial genome harbours three hemoglobins- HbO, HbN and HMP. The discrete functions of these hemoglobins can be unraveled by creating isogenic mutants lacking each of these genetic loci. It will also be interesting to know whether these hemoglobins interact amongst themselves.

On the basis of the results obtained in this study, the following roles of hemoglobins (HbN, HbO and HMP) in mycobacterial biology can be proposed(Fig.7.2);

- HbN acts as a defense molecule for Mtb by scavenging NO in the presence of oxygen and detoxifying its bactericidal activity thus allowing the tubercle bacillus to survive in macrophages.
- HbN may also be playing pivotal role in accessing the oxygen under very low partial pressure of oxygen owing to its very high oxygen affinity and may act as molecule for keeping reserve stock of oxygen to the cell for its survival and ensuring viability of cells under extreme paucity of oxygen and thus leading to reactivation of Mtb after latent phase.
- HbO is involved in facilitating the availability of oxygen to the respiratory apparatus specifically by interacting with cytochrome *O*.
- HbO may also act as an accessory molecule of defense against NO in the initial multiplication stage of tubercle infection when HbN expression is very less.
- HMP may be involved in providing protection against reactive nitrogen intermediates, however other functions for the protein cannot be ruled out and detailed studies are warranted before arriving at definitive conclusions.

This investigation has opened several interesting avenues so as to better understand the *Mycobacterium tuberculosis* biology. The preliminary investigations and database searches have shown that other Mycobacterial species also harbour truncated hemoglobins. It will be interesting to investigate whether HbN and HbO together or separately are restricted to slow growing/pathogenic mycobacteria? This information will prove vital for understanding the evolution of pathogenic mycobacterial species.

So far, *Mycobacterium tuberculosis* is the only pathogen credited with two different truncated hemoglobins viz. HbN and HbO in the genome. The direct involvement of HbN in the nitric oxide detoxification and the importance of nitric

oxide as the major offensive of macrophages contribute towards *Mycobacterium tuberculosis* being the most successful pathogen. Thus HbN may prove to be a potential candidate drug target for tuberculosis cure. Interestingly, due to its direct involvement in the aerobic respiration of *Mycobacterium tuberculosis*, HbO, the second truncated hemoglobin also is a potential drug target. HbO associates with the membrane and expresses throughout the Mtb life cycle, from initial rapid growth phase through caseous granuloma latent phase to the reactivation stage. Any drug targeting HbO will check sequestering of oxygen from the Mtb and thus kill the already stressed internalized bacilli. Thus both HbN and HbO may prove to be promising new drug targets after detailed studies in this direction are undertaken. Thus the tubercle bacillus has evolved multi-tier system to steer clear of NO toxicity and assure the oxygen availability for its survival in the hostile intracellular regime.