Arvind Anand (2009). ***Identification of cellular partner of VHb and studies on protein-protein interactions to explore novel function(s) of vitreoscilla hemoglobin.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
Supervisor: Dr. K.L.Dikshit **Acc. No. : Th-215**

SUMMARY OF THE THESIS

Discovery of hemoglobin in *Vitreoscilla*, a gram negative bacterium, (Wakabayashi *et al.*, 1986) has brought down the age old paradigm that hemoglobin is exclusive to higher forms of life. Subsequent mining of large number of genomes from different organisms has confirmed its ubiquitous occurrence in all forms of life. It was a common assumption that unicellular organisms are small enough to allow oxygen to diffuse through them and thus, avoid burden of auxiliary mechanisms. The presence of hemoglobin (Hb) in a bacterium strongly suggested an early prokaryotic origin of the globin gene. Since then, the presence of Hb-like proteins has been reported in various microbial systems indicating their widespread occurrence.

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. Single domain hemoglobin with classical three-over-three globin fold has gained distinct attention not because of its eukaryotic Hbs like globin fold rather due to their rare occurrence and unique functionality. Till date, presence of single domain hemoglobin with classical three-over-three globin fold has been reported only in few species.

The bacterial hemoglobin, (VHb) from *Viteroscilla* is one of the interesting examples among bacterial hemoglobin that carries several unique structural and functional properties. VHb shows 25% sequence identity with lupin leghemoglobin. The protein contains 153 amino acid residues and is probably functional as a dimer. Its discovery prompted speculation on the evolutionary origin of hemoglobins (Perutz 1986; Appleby *et al.*, 1990), which had been previously assumed to have exclusive eukaryotic origin. Prior to 1986, the *Vitreoscilla* protein was referred as a soluble cytochrome o (Webster 1987), with the unusual property of forming stable oxygenated species. Although formation of stable oxygenated forms by this hemoprotein does not preclude an oxidase function since similar intermediates are formed by cytochrome *bd* (Poole *et al.*, 1983), nevertheless, it became clear that this bacterium also contains a membrane-bound cytochrome o, similar to the *E. coli* complex, as the dominant oxidase, and that the hemoglobin-like protein, being soluble, was unlikely to be the major respiratory terminal oxidase. However, when a Cyo- Cyd- mutant of *E. coli* was transformed with a plasmid containing the cloned *Vitreoscilla vgb* gene, aerobic growth on succinate or lactate was restored. Association of VHb with membranes is required for restoration of respiration under hypoxic conditions (Dikshit *et al.*, 1992; Chi *et al.*, 2007). This fact is

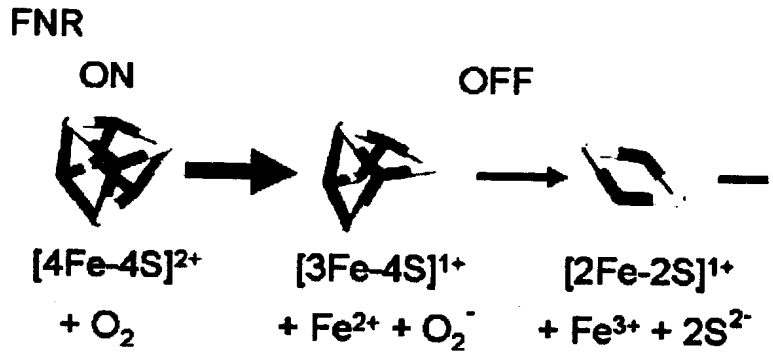
substantiated by the observations that VHb remains localized and concentrated near the periphery of the cytosolic face of the cell membrane in both *Vitreoscilla* and *E. coli* (Ramandeep *et al.*, 2001), and specifically interacts with cytochrome *o* (Ramandeep *et al.*, 2001; Park *et al.*, 2002).

From the earliest work on the cloning and expression of the *vgb* gene in heterologous hosts (Dikshit *et al.*, 1988), it was recognized that VHb might improves their growth and protein production. Consequently, VHb was expressed in variety of bacteria, eukaryotic microbes, plants and even in mammalian cell lines (Pendse and Bailey, 1994). Since then VHb has been expressed in numerous microorganisms for improvement in growth properties or metabolite production. In order to better exploit the attributes of VHb, there has been an ongoing effort to define its functions under a variety of conditions, and to determine to what extent any of the VHb roles mentioned above may be involved in a particular situation. Optimization of this strategy will also require determination of whether VHb has additional undiscovered functions mediated through interactions with other "partner proteins". Among these partners might be enzymes/proteins involved in any process in which oxygen is involved.

E. coli has been a very successful surrogate host for investigating both VHb function and control of *vgb* expression, particularly because of apparent similarities between transcriptional control mechanisms and protein structure and function in *E. coli* and *Vitreoscilla* (Kaur *et al.*, 2002; Khosla and Bailey, 1989; Dikshit *et al.*, 1990; Yang *et al.*, 2005; Chung *et al.*, 2006). Specifically, the wealth of genetic information, sophisticated and easily transformable plasmid vectors, and mutant strains available for *E. coli* are absent in *Vitreoscilla.*

The *vgb* gene expressed under its native oxygen responsive promoter in *E. coli.* As VHb expression is regulated by dwindling level of oxygen in *Vitreoscilla* and its recombinant expression modulate the redox status of its heterologous host (Tsai *et al.*, 1995), *E. coli* expressing VHb were grown under different oxygen levels and to different physiological states e.g aerobic, microaerobic and oxidative stress conditions. Cell lysates were prepared from cells exposed to these conditions and co-immunoprecipitation was done with VHb antibody to screen proteins specifically interacting with VHb. Interestingly, several proteins were found to be associated with VHb under different physiological conditions. However, two of these proteins were found appearing consistently and were identified as global transcriptional regulators, FNR and OxyR. FNR was found to be associated with VHb under microaerobic conditions and on the other hand OxyR was found to be associated under

oxidative stress. The interaction of these redox sensitive regulators appeared to be dependent on redox state of cells. *In vitro* interaction of VHb and FNR was transient in nature and was captured after PFA crosslinking. FNR, Fumarate and nitrate reduction regulatory protein is an O_2 sensor that controls the switches between aerobic and anaerobic metabolism through the regulation of hundreds of genes. It senses oxygen through [4Fe-4S] which helps in dimerization and activation of FNR under microaerobic conditions. The presence of O_2 results in inactivation of FNR via oxidation of this [4Fe-4S] cluster into the [2Fe-2S] cluster (Sutton *et al.*, 2004; Khoroshilo *et al.*, 1997) and the disassembly of the dimer (Lazazzera *et al.*, 1996) and subsequent conversion [2Fe-2S] as shown below.



The overall reaction is shown as following:

$$[4Fe\text{-}4S]^{2+} + O_2 \rightarrow [2Fe\text{-}2S]^{2+} + Fe^{2+} + Fe^{3+} + 2S^{2-} + O_2.^-$$

It has been shown that the active and inactive forms of FNR are inter-converted *in vivo* (Dibden and Green, 2005). Conversion of [2Fe-2S] cluster into the [4Fe-4S] FNR is activated by conversion of its bound iron from the Fe^{+3} to Fe^{+2} as shown above. It is possible that reduced (Fe^{+2}) VHb, induced to high levels by the low oxygen conditions, could, by binding FNR, donate an electron to facilitate the reduction. Thereby VHb may activate FNR and induce its own expression consequently. These finding are rather preliminary in nature to explain exact mechanism underneath. Therefore, a detail exploration would be required to unveil the relevance of redox sensitive, VHb-FNR association.

Other identified protein was OxyR, which was found to be associated with VHb under oxidative stress conditions. *In vitro* interaction of VHb and OxyR indicates stable VHb-OxyR complex. OxyR "oxidative stress regulator," is the transcriptional dual regulator for the expression of antioxidant genes in response to oxidative stress, in particular, under elevated levels of hydrogen peroxide. OxyR is a redox sensitive transcriptional regulator and function as reversible cellular redox switch. VHb is known to be protective against hydrogen peroxide when expressed in *E. coli* or *Enterobacter aerogenes*, both enhancing catalase levels

and having, itself, strong peroxidase activities (Geckil *et al.*, 2003; Kvist *et al.*, 2007). But at the same time VHb itself can produce peroxide ion, and consequently leads to oxidative stress, under certain physiological conditions. Possibly binding of VHb with OxyR may regulate the latter's activity. The exact mechanism and relevance of VHb and OxyR interactions were explored and are explained in detail in following paragraphs.

In several cases the presence of VHb provides the ability to cope better with oxidative stress by altering the status of antioxidant enzymes. For example, *Enterobacter aeruginosa*, *Streptomyces lividens* and *Arabidopsis endogenesis* (Geckil *et al.*, 2003; Kim *et al.*, 2007); and VHb expressed in the plant has been correlated with higher levels of antioxidants such as ascorbate and higher tolerance to photo-oxidative damage (Wang *et al.*, 2009). To gain an insight into the involvement of VHb in oxidative stress responses, both *Vitreoscilla* and recombinant *E. coli* expressing VHb under its native promoter were grown in the presence of sub lethal concentration of H_2O_2. The RT-PCR and protein profiling of VHb expressing cells revealed 40-50% reduction of VHb expression in both *Vitreoscilla* and *E. coli.* The results obtained suggested that the biosynthesis of VHb is down regulated under oxidative stress in *Vitreoscilla* and *E. coli*. Further *E. coli* strains NC4936 (*oxyR* positive) and NC4112 (*oxyR* mutant) were used to study the expression of VHb under oxidative stress. Growth profiles of VHb expressing NC4936 and NC4112 strains and control strains were tested under increasing concentrations of H_2O_2. Interestingly, the VHb-expressing *oxyR* mutant (NC4112) displayed significantly slower growth than its counterpart not expressing VHb, while VHb-expressing NC4936 (o*xyR* positive) displayed distinct growth protection than the control strain, suggesting that the VHb mediated protection from H_2O_2-induced oxidative stress relies on the presence of OxyR.

To Further investigate the role of OxyR in VHb regulation, cellular level of VHb was measured in *E. coli* strains NC4963 (wild type) and NC4112 (*oxyR* negative), transformed with *vgb*. In stationary phase cells the VHb level of NC4112 (*vgb*) was 2.2-fold higher as compared to NC4963(*vgb*) suggesting OxyR negatively regulates VHb expression. Subsequent reexamination of *vgb* promoter region indicated presence of an OxyR binding site which overlaps the FNR, CRP and ArcA binding sites identified earlier (Khosla and Bailey 1989; Yang *et al.*, 2005; Joshi and Dikshit 1994). To confirm involvement of OxyR in VHb regulation, gel shift assay was performed with OxyR and *vgb* promoter region. The binding of OxyR with *vgb* promoter provided direct evidence of OxyR regulation. Since OxyR is redox sensitive transcriptional regulator and remains in reduced and oxidized state depending upon cell environment. OxyR is known to be both a positive and negative regulator of

transcription, acting positively only when oxidized but acting negatively in either oxidized or reduced form (Zheng and Storz, 2000; Storz *et al.*, 1990). To check which form of OxyR binds with *vgb* promoter, $OxyR^r$ mutant (OxyR locked in reduced form) was generated and gel shift assay was performed with $OxyR^r$ or oxidized OxyR with *vgb* promoter. Only oxidized OxyR was able to bind *vgb* promoter suggesting that oxidized form of OxyR negatively regulates VHb expression.

The putative OxyR binding site within the *vgb* promoter overlaps with the FNR, ArcA and CRP binding sites, we checked whether OxyR and FNR compete for binding site by competitive DNA binding study with both of these proteins. The results showed that FNR has more affinity for *vgb* promoter than the OxyR and it dislodges OxyR from its binding site in concentration dependent manner suggesting that OxyR interacts with the *vgb* promoter only when FNR is not operative. Under aerobic conditions, high oxygen concentration is known to generate reactive oxygen species which results in significant peroxide stress and is known to activate OxyR, however, the same condition inactivate FNR. The gel shift assay indicated that OxyR binds to the *vgb* promoter only in its oxidized form in absence of FNR. Possibly the higher expression of VHb generates more oxidative stress within the cell, possibly due to generation of higher reactive oxygen species due to higher respiratory activities in the presence of VHb and disrupts the association of FNR with the *vgb* promoter and activate OxyR to alleviate the situation by down regulating VHb biosynthesis. Multi regulatory *vgb* promoter region could allow for subtle tuning of *vgb* expression so that the levels of VHb produced are optimized for the particular set of environmental conditions and the corresponding VHb functions needed.

As investigated earlier, VHb was found to be associated with OxyR under stationary phase and oxidative stress. In order to explore relevance of VHb-OxyR association, interaction of VHb with both forms of OxyR was studied. Interestingly, VHb was found to interact with only reduced form of OxyR. Further, the role of VHb in altering the redox status of OxyR was examined by comparing redox state of OxyR in the presence of VHb and H_2O_2. VHb converted the reduced OxyR to oxidized OxyR in the presence of NADH just like H_2O_2. These observations suggest that VHb associates only with the reduced state of OxyR and is able to convert it into oxidized form *in vitro*, but only in the presence of NADH; under similar conditions VHb has been shown to generate H_2O_2 (Webster, 1975). VHb may then regulate the redox state of OxyR directly by transmitting the H_2O_2 stress signal.

It has been demonstrated that the expression of VHb activates antioxidant systems in various heterologous hosts, e.g., the *kat* and *sod* genes in *E. aerogenes* (Kvist *et al.*, 2007)

flavohemoglobin. Despite lacking covalently attached reductase domain, VHb has been found functional in various heterlogous hosts suggesting the presence of some compatible redox system in cellular ambience of its heterologous hosts. In its native host, *Vitreoscilla*, a FAD containing reductase, NADH methemoglobin reductase usually co-purified along with VHb (Gonzales and Webster, 1980) indicating its close association with VHb and is required for the functional activity and interactions with the ligands (O_2, CO, NO etc). Non-covalent interaction with reductase may provide flexibility to VHb to interact with more than one reductase depending upon their presence under particular physiological condition; thereby allowing VHb to perform function other than facilitating oxygen transfer.

In present investigation, flavoreductase domains from flavohemoglobin of *E. coli*, *Salmonella*, *Staphylococcus* and one oxidoreductase from *M. tuberculosis* were fused with VHb to explore the role of reductase association in modulation of VHb properties. These fusion constructs were expressed under *vgb* promoter in *E. coli* and their growth profiles were checked under microaerobic and aerobic conditions. All these VHb-Reductase proteins conferred distinct growth advantage in recombinant *E. coli* as compared to isogenic strain with control vector. Interestingly, recombinant expression of VHb-Reductase proteins was found to protect cellular respiration of *E. coli* from NO toxicity more efficiently and effectively than VHb but level of protection was variable in case of each VHb-Reductase protein. The observed effect might be due to integration of reductase domain with VHb as a result of which VHb became more efficient in combating NO mediated inhibition of cellular respiration.

Expression of VHb and VHb-Reductase proteins conferred protection to *E. coli* from oxidative stress generated by H_2O_2 and VHb-Reductase proteins were found more efficient than the VHb since VHb-OxyR interactions were found to be relevant for protection against oxidative stress interaction of VHb-Reductase proteins were tested for their implication under oxidative stress. Surprisingly, VHb-Reductase proteins were not able to interact with OxyR, probably due to masking of interacting surface by fused reductase domain, but still were able to provide protection from reactive oxygen. Thus, in VHb-Reductase proteins the above mentioned mechanism may not be applicable. Alternatively, VHb can directly scavenge superoxide anion and peroxides and protect its host from oxidative stress. It is plausible that due to presence of fused reductase domain VHb-Reductase proteins can more efficiently scavenge superoxide ion and peroxides, hence provide better protection. According to another hypothesis VHb generates superoxide and peroxide anion, resulting in the activation of SoxRS system and the cascade of regulatory processes that result in induction of

and *S. lividens* (Kim *et al.*, 2007). Transcript analysis of VHb carrying cells also revealed distinct increases in the expression of *katG* and *sodA* compared to non-VHb expressing cells. This result suggest that the protective effect of VHb found in several heterologous hosts might also be due to activation of OxyR by VHb and subsequent induction of the antioxidant genes.

The overall results from this part of the study are summarized in the proposed model (figure 6.1): Under hypoxic conditions transcription of *vgb* is upregulated several fold by FNR (Yang *et al.*, 2005), resulting in production of a high level of VHb that may facilitate oxygen availability and enhance the respiratory activity of cells. Accumulation of VHb to high levels within the cell is likely to generate elevated levels of peroxide due to the inherent ability of VHb to produce H_2O_2 in the presence of NADH (Webster, 1975). This may be detrimental to cells, particularly during externally imposed oxidative stress. Accumulation of VHb within the cell may also lead to close association of VHb with reduced OxyR so that the peroxide released by VHb is readily available to activate OxyR. A subsequent conformational change due to the change in the redox state of OxyR may disrupt the OxyR-VHb association and allow OxyR binding to the *vgb* promoter. Simultaneously, the oxidizing conditions created due to the accumulation of VHb may also disrupt the iron-sulfur cluster of FNR and thus it's binding with the *vgb* promoter. This would help clear the region to allow OxyR binding to down-regulate VHb production and thus, reduce the level of VHb-generated peroxide ion. The redox change in OxyR mediated by VHb may also allow OxyR to activate its regulon and thus provide more widespread protection from the oxidative stress to VHb expressing hosts. In addition to this mechanism, VHb also induces *sodA* transcription in heterologous cells indicating that VHb may also aid in protection from superoxide ion. Since *sod*A is regulated by SoxRS and not by OxyR, it is possible that SoxRS is also activated due to an increase in H_2O_2 level occurring during VHb overexpression. Similarly the oxidized environment of VHb-expressing cells may activate SoxR by univalent oxidation of the $[2Fe\text{-}2S]^+$ cluster of the protein, which, in turn, can activate SoxS and switch on the SoxRS regulon. Thus, it is likely that the overall protective effect of VHb against oxidative stress is a dual effect of these two antioxidant regulons.

Reductase has been inseparable part of hemoglobin since discovery of methemoglobin reductase which keeps human hemoglobin in functionally active state. Reductase also plays important role in oxygen chemistry of hemoglobin. VHb is a single domain hemoglobin having classical three-over-three globin fold but without any fused reductase domain. However, it shows close sequence and structural similarity with globin domain of

antioxidant genes that protect cell from oxidative stress by scavenging superoxide and peroxide anion. VHb-Reductase proteins possibly generate more superoxide anion due to presence of fused reductase which causes prompt action of regulatory cascade against oxidative stress. Thereby VHb-Reductase proteins may more effectively rescue the cells from oxidative stress. Further studies are required to examine these possibilities and determine the precise role of VHb-Reductase proteins in the oxidative stress management.

It is a well established fact that many organisms employ flavohemoglobins (flavoHbs) to metabolize NO and moreover, these enzymes form the first line of defense against NO toxicity. Like flavohemoglobins, VHb has also been involved in providing protection to its heterologous host against nitrosative stress (Kaur *et al.*, 2002; Frey and Kallio, 2000). Effect of VHb association with different reductase was monitored in *E. coli* (RB9060), a flavoHemoglobin (Hmp) deficient strain of *E. coli* under nitrosative stress conditions. VHb-Reductase proteins conferred distinct protection to *E. coli* (RB9060) that display higher sensitivity under nitrosative stress however, the level of protection provided by VHb-Reductase proteins was significantly higher than VHb. Nitrosative stress management depends on NO detoxification by Hbs and FlavoHbs. 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

Alternatively, another mechanism is likely to exist where FlavoHb binds NO at heme iron, and effectively reduce it to nitroxyl anion. Heme-bound nitroxyl then reacts with O2 to form nitrate as shown in below and the resultant mechanism has been termed as O2 Nitroxylase (denitrosylase) (Hausladen *et al.*, 2001).

$$HbFe^{+2} + NO \longrightarrow [HbFe^{+2}NO]$$
$$[HbFe^{+2}NO^-] \longleftrightarrow [HbFe^{+3}NO^-]$$
$$[HbFe^{+3}NO^-] + O_2 \longrightarrow HbFe^{+3} + NO_3^-$$

In both of these mechanisms, prosthetic heme Fe(II) gets converted in to Fe(III), which is an inactive form and cannot bind oxygen. Therefore, concerted action of compatible

reductase is required to reduce inactive Fe(III) in to active Fe(II) for catalytic cycle to continue. To explore the role of fused reductase domain, NO consumption rate and NOD activity of VHb-Reductase proteins were measured with Nitric oxide analyzer (World Precision Intrument). NO consumtion rates and NOD activities of VHb-Reductase proteins were found significantly higher than VHb alone. VHb-MtR fusion protein exhibited maximum NO consumption rate and NOD activity suggesting that the reduction efficiency of MtR reductase is maximum and NOD activity varies accordingly in case of other VHb-Reductase proteins, but was more than VHb which has no fused reductase domain. Interestingly, NO consumption rates of VHb-Reductase proteins were matched in terms of level of protection provided by them to *E. coli* (*Δhmp*) under nitrosative stress. Therefore, VHb-Reductase protein having higher NO consumption rate can protect its host more efficiently under nitrosative stress.

It has been demonstrated that NOD activity of Hbs is limited by rate of reduction of heme iron and depends upon efficiency of reductase in converting inactive Fe(III) in to active Fe(II) (Smagghe *et al.*, 2008). The efficiency of enzymatic reduction of Fe(III) VHb with independent reductase proteins was measured in term of reduction velocity. We observed 2-4 fold difference in catalytic efficiency of each reductase which suggests that VHb might interact with different reductase(s) with variable efficiency. Interestingly, NO consumption rates of VHb in the presence of these reductases were directly proportional to their reduction velocities suggesting that the efficiency of reduction plays a crucial role in functionality of VHb under different physiological conditions. VHb enhanced growth and metabolic activities of its host, however, the extent of its benefit was found to be different in different recombinant hosts (Lin *et al.*, 2003; Khosla and Bailey, 1988; Holmberg *et al.*, 1997; Pendse and Bailey, 1994). Therefore, on the basis of these results we could explain the extent of benefit provided by VHb in particular host may depend upon a cognate reductase present in intra-cellular ambience of that host.

From this part of the study it could be argued in favor of multiple functions of evolution of VHb as a single domain protein would give it the flexibility to interact with various partners at different times of growth and under various physiological conditions to carry out diverse functions. Integration of reductase domain with VHb makes it more efficient in combating different physiological stress conditions. Thus, this VHb-Reductase fusion approach would be more relevant in comparing various physiological processes those are affected by recombinant expression of VHb in different heterologous hosts.

Taken together, present study revealed the novel protein-protein interaction of VHb with different global transcriptional regulators under different physiological conditions which not only explains VHb mediated pleotropic effects in various heterologous hosts but its unique ability to interact with different proteins. Interaction of VHb with global transcriptional regulators affects the expression of multiple pathways of cells, like energy metabolism, central intermediary metabolism and protein synthesis and exhibits diverse effects on physiology of its host. Detail studies on VHb and partner protein interactions would further illuminate our understanding on multiple functionalities of microbial hemoglobin.

The present biochemical and physiological studies on VHb and OxyR interactions unveil a novel mechanism of VHb autoregulation and its involvement in oxidative stress management in heterologous host. Thereby, VHb maintains a fine balance between the beneficial level of oxygen and detrimental levels of reactive oxygen species within the cell by autoregulating its own expression and induction of antioxidant genes through OxyR. This would greatly aid our ability to exploit VHb applications at its full potential. Considering the beneficial roles of VHb in several biotechnological processes, e.g., fermentation technology, bioremediation, plant biotechnology etc. our approach of VHb-Reductase fusion will help in screening an effective and efficient VHb-Reductase chimera, which would be more beneficial to its heterlogous host in various biotechnological processes.