Chapter-3
Identification of partner protein(s) of *Vitreoscilla* hemoglobin (VHb): to get an insight in VHb functionality

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Introduction

Bacterial hemoglobins (Hbs) represent a new frontier in the study of hemeproteins due to their widespread occurrence among diverse species as well as their multiple yet unexplained functions (Bolognesi et al., 1997; Imai, 1999). From the structural viewpoint, three different types of bacterial Hbs have been identified: classical 3-over-3 helix Hbs, truncated two-over-two helix Hbs, and flavohemoglobins (Ermler et al., 1995; Tarricone et al., 1997; Pesce et al., 2000).

The hemoglobin from the bacterium Vitreoscilla (VHb) is the first microbial hemoglobin to be identified (Wakabayashi et al., 1986; Webster, 1987). It exists primarily as a homodimer, each subunit of which is 146 amino acids in length (Wakabayashi et al., 1986; Webster, 1987). Each monomer assumes a classic globin fold like eukaryotic hemoglobin, although without an ordered E-helix and with unusual structures in both proximal and distal heme sites (Tarricone et al., 1997). VHb binds oxygen reversibly with a $K_d$ of 6 $\mu$M (Webster, 1987) and is induced roughly 50-fold under conditions of low oxygen (Boerman and Webster, 1982). These properties led to the original hypothesis that, particularly under low oxygen conditions, VHb binds available oxygen and supplies it to the terminal respiratory oxidases, allowing for efficient respiration when oxygen is scarce (Webster, 1987). This is presumably the mechanism, which enables Vitreoscilla to inhabit oxygen poor conditions despite its obligatory aerobic nature (Pringsheim, 1951). This working model is supported by several experimental evidences proposed (Ramandeep et al., 2001; Park et al., 2002). Additional roles for VHb in cellular metabolism have also been identified. These include possible augmentation of oxygenase activities (Fish et al., 2001; Lin et al., 2003; Lee et al., 2004), service as an alternative terminal respiratory oxidase (Dikshit et al., 1992), NO detoxification (kaur et al., 2002), and protection against hydrogen peroxide (Gekil et al., 2003; Kvist et al., 2007). Microarray analysis indicates that the presence of VHb in E. coli significantly affects the transcription of hundreds of genes (Roos et al., 2004); the molecular mechanism, by which this occurs, however, is elusive.

Since the isolation of the VHb gene (vgb) (Dikshit and Webster, 1988; Khosla and Bailey, 1988) many organisms have been genetically engineered to express VHb, leading to enhanced growth and productivity (with respect to synthesis of a variety of secondary metabolites as well as degradation of aromatic compounds) in bacteria, fungi, plants, and even mammalian cells (Frey and Kallio, 2003; Zhang et al., 2007). 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
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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.

To that end we sought to identify further the partner proteins for VHb in *E. coli* expressing *vgb*, and the role of VHb in their functions. *E. coli* has been a very successful surrogate host for investigating both VHb function and control of *vgb* expression, particularly because of the absence of the necessary technology for doing such work in *Vitreoscilla*. Specifically, the wealth of genetic information, sophisticated and easily transformable plasmid vectors, and mutant strains available for *E. coli* are absent in *Vitreoscilla*, while at the same time there are 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).

Among the VHb partners in *E. coli* newly identified in the work reported here are OxyR and Fnr, which are important transcription regulators of genes encoding functions related to oxygen levels. Taken together, the interactions of VHb with Fnr and OxyR are particularly interesting, as they appear to add regulation of transcription to the existing overwhelming list of VHb functions.
Results

3.1 VHb interacts with cellular proteins of *Vitreoscilla* and *E. coli*

In preliminary examination, we expressed VHb in *E. coli* under its native promoter which is functional in *E. coli* as both share common transcriptional machinery (Khosla and Bailey, 1989; Dikshit *et al.*, 1990; Yang *et al.*, 2005). *E. coli* cells carrying plasmid pUC8:16 (containing *vgb* gene along with its promoter) and pUC19 were grown in LB at 37°C. Then cells were harvested after 12 hrs and lysates were prepared. *E. coli* cell lysate with control plasmid and expressing VHb were run on a non-denaturing gel, followed by Western blotting using anti-VHb antibody as a probe. Interestingly, in addition to VHb itself, the antibody identified two other bands that we interpret as representing VHb complexed with one or more partner proteins (Fig. 3.1B). Similarly in *Vitreoscilla* cell extract, we got two additional bands other than VHb in western blot using VHb antibodies (Fig. 3.1A). Above results therefore, suggest that, VHb is able to make complex with some different cellular proteins in its native *Vitreoscilla* and heterologous *E. coli* host.

3.2 VHb interacts with different proteins under different physiological conditions

Our next obvious aim was to identify VHb interacting proteins in *E. coli*. As VHb expresses under hypoxic condition through an oxygen sensitive promoter and is related to cell processes involving oxygen, *E. coli* expressing VHb was 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 in aerobically growing log phase cells (harvested after 6-7 hrs of growth); one of these, corresponding to sizes of approximately 24 kDa, was found appearing consistently. When cells harvested from oxidative stress cultures were tested, a 34 kDa protein was found associated with VHb (Fig. 3.2). A VHb interacting protein of around 29 kDa appeared in cells growing under microaerobic conditions (Fig. 3.2). These results suggest that VHb may interact with distinct proteins of cells depending upon oxygen availability and physiological conditions.

3.3.1 Identification of VHb interacting proteins.

Proteins obtained from co-immunoprecipitation were transferred to PVDF membrane and subjected to N-terminal sequence analysis for identification. The 34 kDa, 29 kDa, and 24kDa proteins, specifically interacting with VHb were identified as OxyR, FNR and CRP respectively after N-terminal sequencing (Table.1). Surprisingly, all three identified proteins
were transcriptional regulators, OxyR controls gene expression of antioxidant genes and FNR is an oxygen sensitive transcriptional regulator.

<table>
<thead>
<tr>
<th>Protein identity (size)</th>
<th>N-terminal Sequence</th>
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<tbody>
<tr>
<td>FNR (29kDa)</td>
<td>MNIRDLEYLVALAEHR</td>
</tr>
<tr>
<td>OxyR (34kDa)</td>
<td>MLKLTNINYLGSRPMP</td>
</tr>
<tr>
<td>CRP (24kDa)</td>
<td>MVLGKPDTEWFL</td>
</tr>
</tbody>
</table>

Table 3.1 N-terminal sequence analysis of VHb interacting proteins

3.3.2 In vitro protein-protein interaction of VHb with OxyR, FNR and CRP

3.3.3 Cloning and expression of OxyR, FNR and CRP

The oxyR, fnr and crp genes were PCR amplified from E. coli genomic DNA using gene specific primers. The forward and reverse primers were designed based on the sequence of the genes which contained NdeI and BamHI sites, respectively. The PCR products of oxyR and fnr were cloned in the pBS KS+ cloning vector at SmaI site. Blue-white screening was done and the clones were screened by digesting the recombinant plasmids with NdeI and BamHI. The clones giving the correct insert size were picked up and designated as pBSOxyR, pBSFNR and pBSCR. The nucleotide sequencing of cloned insert confirmed the authenticity of genes. The plasmid construct, pBSOxyR, pBSFNR and pBSCR were then digested with NdeI and BamHI and the resultant fragments were cloned into NdeI-BamHI site of pET28c and the transformants were selected on LB plates supplemented with kanamycin. The transformants were screened by digestion with NdeI and BamHI and the clones giving the correct size of the insert were designated as pETOxyR, pETFNR and pETCRP. These plasmid constructs were then transformed into E. coli BL21DE3 where the proteins were over-expressed under T7 promoter. Expression of OxyR FNR and CRP were analyzed by running the cell lysate on 12% SDS-PAGE followed by coomassie brilliant blue staining (Fig. 3.3, 3.4 and 3.5).

3.3.4 Isolation and purification of OxyR, FNR and CRP

OxyR, FNR and CRP were expressed as 6XHis-tag proteins in pET28c vector to facilitate their purification through Ni²⁺-NTA column. For purification, cells were frozen at −20 °C for 6 hrs, thawed at 4 °C and disrupted by sonication with 15% intermittent pulses of 20 sec for 10 min in a Heat system sonicator fitted with a micro sonicator probe. The soluble
Figure 3.1(A) Analysis of VHb-partner protein interactions in vivo. Proteins were separated by non-denaturing PAGE, transferred to nitrocellulose membrane, and probed with VHb antibodies. Lanes 1, 2. whole cell extract of *Vitreoscilla*. Lane 3. purified VHb. Presumptive complexe of VHb with partner proteins is indicated by arrows.

Figure 3.1(B) Analysis of VHb-partner protein interactions in vivo. Proteins were separated by non-denaturing PAGE, transferred to nitrocellulose membrane, and probed with VHb antibodies. Lanes 1, purified VHb; 2, whole cell extract of *E. coli*; 3, whole cell extract of *E. coli* expressing VHb. Presumptive complexes of VHb with partner proteins are indicated by arrows.
Proteins precipitated by anti-VHb antibodies from whole cell lysates of VHb-expressing E. coli cells. Lanes 1, protein size standards (sizes kDa noted in left hand margin); 2, pulldown eluant from cells expressing VHb under aerobic conditions; 3, eluant from cells expressing VHb under oxidative stress (in the presence of 1 mM H$_2$O$_2$); 4, eluant from cells expressing VHb under microaerobic conditions; 5, purified VHb.
Figure 3.3  Expression of FNR of *E. coli* under T7 promoter (A) Vector map of pETFNR (B) SDS PAGE showing expression of FNR Lane M: molecular weight marker Lane 1: *E. coli* BL21 negative control Lane 2: *E. coli* BL21 expressing FNR Lane 3, 4: purified protein after Ni-NTA chromatography.
Figure 3.4 Expression of OxyR of *E. coli* under T7 promoter (A) Vector map of pETOxyR (B) SDS PAGE showing expression of OxyR Lane M: molecular weight marker Lane 1: *E. coli* BL21 negative control Lane 2: *E. coli* BL21 expressing OxyR Lane 3, 4: purified protein after Ni-NTA chromatography.
Figure 3.5  
Expression of CRP of *E. coli* under T7 promoter (A) Vector map of pETCRP (B) SDS PAGE showing expression of CRP. Lane M: molecular weight marker Lane 1: *E. coli* BL21 negative control Lane 2: *E. coli* BL21 expressing CRP Lane 3, 4: purified protein after Ni-NTA chromatography.
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Lysate was separated by centrifugation at 17,000 g for 30 min at 4 °C in a sorvall SS-34 rotor and loaded on to a Ni\textsuperscript{2+}-NTA affinity column (4 ml) pre-equilibrated in lysis buffer. To ensure complete binding of the recombinant protein to the affinity column, lysate was passed through the column four times at a flow rate of 1.0 ml/min. To remove the non-specific binding of other proteins, the column was washed with 25 column volumes of wash buffer at a flow rate of 1.0 ml/min. The bound protein was eluted with elution buffer at a flow rate of 1.0 ml/min in ten fractions of 1.0 ml each. Purified proteins were dialyzed against 50 mM Tris HCl, pH 8.0 and 100 mM NaCl with three changes at 4 °C. Purified OxyR, FNR and CRP proteins were used for further study.

3.3.5 In vitro protein-protein interaction of OxyR and VHb

In order to confirm VHb and OxyR interaction, purified VHb and OxyR proteins were mixed and run on a 10% non-denaturing PAGE followed by western blotting using VHb antibody. A distinct complex of VHb with OxyR was detected (Fig. 3.6). To further confirm this interaction pull down assay was done with purified VHb and OxyR using VHb antibody. OxyR was pulled out along with VHb through VHb antibody (Fig. 3.7), these results substantiated interaction of VHb with OxyR in vitro.

3.3.6 In vitro protein-protein Interaction of FNR and VHb

In previous results, we captured FNR-VHb interaction in microaerobic (reducing) conditions indicated redox dependent interaction of these two proteins. To further elucidate the contribution of redox and conformational state of VHb in protein-protein interactions, in vitro binding of VHb was monitored by western blot analysis. In primary experiment we could not trap VHb-FNR interaction on western blot indicating that VHb interaction with FNR may be transient or redox state dependent. To capture these interactions we used paraformaldehyde, a crosslinking agent which crosslink lysine residue in close proximity. First VHb was converted into different redox states. 50 µl of VHb solution (50 µg) was purged with oxygen to prepare oxygen bound hemoglobin preparation. The VHb preparation was reduced by dithionite and oxidized with potassium ferricyanide. 20 µg of VHb, in different states, was mixed with purified FNR protein in the presence of 1% PFA and the resulting mixture was incubated at 37°C for 1 hour. After that 125 mM Glycine was added to this mixture to stop the reaction. The resulting mixture was quantitated using BCA protein assay kit (Pierce) and 10µg of this mixture was loaded on 12% SDS PAGE and was transferred on PVDF membrane and probed with polyclonal anti-VHb antibodies. Interestingly, only reduced form of VHb was found interacting with FNR (Fig. 3.8) suggesting redox specific binding of VHb with FNR.
Figure 3.6  protein-protein interaction of purified VHb and OxyR: purified VHb (5μg) and OxyR (5μg) were run on 10% native PAGE followed by western blotting using anti-VHb antibodies. Lanes 1, purified VHb; 2, purified VHb and OxyR; 3, purified OxyR. In this experiment, VHb was in the oxyform.
Figure 3.7 *In vitro* protein-protein interaction of VHb and oxyR using pull down assay:
Purified VHb and OxyR proteins were mixed and were pulled-out using anti-VHb antibodies conjugated sepharose bead. Bound proteins were eluted from beads using low pH buffer and were run on 12% SDS PAGE. Lane 1: Purified VHb protein alone. Lane 2, 3: Purified VHb and OxyR proteins after pull down with anti-VHb antibodies. Lane 4: purified OxyR.
Figure 3.8  **FNR and VHb interaction in presence of cross linking agent PFA.** Purified VHb was converted into different redox states and was incubated with FNR in presence of PFA. The samples were run on 15% SDS PAGE followed by western blotting using anti-VHb antibodies. In lane 1. VHb oxidized+FNR PFA treated; lane 2. VHb reduced +FNR PFA treated; lane 3. VHb oxygenated +FNR PFA treated; lane 4. VHb without PFA
3.3.7 **In vitro** protein-protein interaction of VHb and CRP

Under aerobic condition, identified protein was CRP; so we checked protein-protein interaction of purified VHb and CRP using native page and pull down under aerobic condition, however, no stable complex of VHb with CRP was detected. Possibility of transient interaction between VHb and CRP was checked using chemical cross-linker PFA. NO specific binding of VHb with CRP was detected *in vitro*. 
3.4 Discussion

Heterologous expression of *Vitreoscilla* hemoglobin (VHb) has been reported to improve cell growth, protein synthesis, metabolite productivity and nitric oxide detoxification. Although these physiological effects are attributed to VHb via enhancement of respiration and energy metabolism by facilitating oxygen delivery, the mechanism of VHb action remains to be elucidated.

Expression of VHb in *Escherichia coli* triggers increased ATP production, improved growth rate and final cell density, and enhanced foreign protein production. A plausible explanation is that the presence of VHb within the respiratory membrane promotes the oxygen flux to one or two terminal oxidases: aerobic terminal oxidase (Cyo) and microaerobic terminal oxidase (Cyd) (Tsai *et al.*, 2002). Such effect is expected to cause an increase in proton-pumping efficiency and concomitantly lead to a remarkable generation of ATP (Andersson *et al.*, 2003). An increased production of translational components (the active 70S ribosomes and tRNA levels) has been detected through asymmetrical flow field-flow fractionation (AFFFF), suggesting another important role of VHb on the protein synthesis machinery. Recently, it has been established that the prosthetic heme group of VHb possesses peroxidase-like activity very similar to that of mammalian hemoglobins (Suwanwong *et al.*, 2006; Kvist *et al.*, 2007). The consensus view that has emerged from these studies clearly indicate that VHb 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. These findings support the hypothesis that VHb not only acts as an oxygen carrier but possesses other important functional roles. However, the underlying mechanism of VHb on cellular catabolic regulation is not yet entirely understood.

A combination of techniques has provided information that physical interactions occur between VHb and global transcriptional regulators, FNR and OxyR. The interaction of these redox sensitive regulators appeared dependent on redox state of cells. VHb expresses under a native oxygen responsive promoter and recombinant expression of VHb substantially modulates redox status of cells, which in turn affects multiple cellular processes. Such pleotropic effects on host physiology by recombinant expression of VHb might be achieved through direct and indirect interaction of VHb with various transcriptional regulators.

The interaction between VHb and FNR occurs under conditions in which VHb is in the reduced, unoxgenated state, which should exist under very low oxygen conditions. In these conditions, FNR remains in active form. FNR, Fumarate and nitrate reduction regulatory proteins are $O_2$ sensors that control the switches between aerobic and anaerobic...
metabolism through the regulation of hundreds of genes. The cellular concentration of FNR is similar under both anaerobic and aerobic growth conditions (Sutton et al., 2004), but its activity is regulated directly by oxygen. Under anaerobiosis, FNR acquires a [4Fe-4S] cluster that causes a conformational change facilitating dimerization and activation of the protein (Moore et al., 2001). The presence of O₂ 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). It has been shown that the active and inactive forms of FNR are interconverted 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 Fe³⁺ to Fe²⁺ form. Although it is as yet speculation, it is possible that reduced (Fe²⁺) VHb, induced to high levels by the low oxygen conditions, could, by binding Fnr, donate an electron to provide the reduction. This would be somewhat similar to the reduction of the VHb Fe³⁺ to Fe²⁺ which is catalyzed by NADH-VHb reductase (originally named NADH-cytochrome o reductase) (Webster and Liu, 1974).

OxyR, another VHb interacting protein, was identified in VHb expressing E. coli cells from stationary phase or 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. The OxyR regulon includes genes involved in peroxide metabolism, peroxide protection, and redox balance (Storz et al., 1990; Zheng et al., 2001; Mongkolsuk et al., 2002). OxyR functions as a reversible cellular redox switch. In the presence of 100 nM H₂O₂, activation occurs by the formation of an intramolecular disulfide bond between Cys199 and Cys208 (Storz et al., 1990; Lee et al., 2004). 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). Further complication however, is that VHb itself can produce peroxide ion, and consequently leads to oxidative stress, under certain physiological conditions (Webster, 1975). Possibly the binding of VHb to OxyR may regulate the latter's activity, perhaps by sequestering it or changing its redox state by production of peroxide ion, in which case VHb-OxyR binding might be of importance in applications in which production of various products by recombinant E. coli is enhanced by VHb (Frey and Kallio, 2003; Zhang et al., 2007). But exact relevance of VHb and OxyR interaction is yet to be unveiled.
Overall results, thus, suggest that vgb expression might be fine tuned very precisely especially in response to changing oxygen levels. It is possible that VHb itself may be involved in controlling the activities of Fnr and OxyR, and thus the expression of other genes which must be regulated by these transcription factors in response to oxygen levels. This might occur by involvement of VHb in determining the oxidation states of Fnr and OxyR or via VHb binding to Fnr or OxyR to modulate their abilities to bind DNA. In either case this might constitute an oxygen sensing function of VHb, as has been observed in other globin/heme based sensors (Hou et al., 2001; Gilles-Gonzalez and Gonzalez, et al., 2005).