Vitreoscilla Hemoglobin: Engineering of Oxygen affinity and studies on its Role in Cellular Physiology

Overview

Despite the fundamental and biomedical interest in vertebrate Hbs, it has become evident that heme based oxygen carriers are widespread in almost all the phyla of biosphere. The substantial amino acid homology in vertebrates and the conserved three-dimensional structure of globins suggest their common evolutionary origin from a monomeric oxygen carrying heme protein (Dickerson and Geis, 1983; Bashford *et al.*, 1987; Pastore and Lesk, 1990; Moens *et al.*, 1996). In contrast to the vertebrate globins, non-vertebrate globins exhibit extensive variability in their structural organization and cellular functions. The discovery of the first bacterial hemoglobin in a bacterium, *Vitreoscilla*, changed the perception that hemoglobin is prerogative of only the eukaryotes. Since then, hemoglobins have been reported from many genera of prokaryotes and it has been speculated that these microbial Hbs perform functions other than the ones generally ascribed to them i.e., oxygen transport and storage. Prokaryotic globins are either present as single domain proteins (conforming to the classical three-over-three fold or the truncated two-over-two fold) or they are present as two domain proteins in which the globin fold is appended to the redox active domain at its C-terminus. With the availability of information on the genomes of other microorganisms, it has become clear that in a single genome, more than one globins may be present; a single domain and a two-domain globin. The simultaneous existence of more than one globin in a single genome points towards an important role of these globins in the cellular metabolism. Amongst the prokaryotic Hbs, VHb (from *Vitreoscilla* sp. C1) remains the most extensively studied globin.

The gene coding for VHb has been cloned, sequenced and characterized (Dikshit and Webster, 1988). The gene, *vgb*, is regulated at the transcriptional level by oxygen (Dikshit and Webster, 1989). Analysis of the promoter region revealed that FNR or FNR like proteins in conjunction with CRP modulate of its oxygen regulation (Joshi and Dikshit, 1998). Under the oxygen limited growth conditions, strains engineered to

express VHb have been shown to exhibit an enhanced specific growth rate, an increased final cell density and a 10-20% increase in total protein content (Khosla and Bailey, 1988b). These observations indicate towards the significance of this protein in biotechnological applications. Heme content of *Vitreoscilla* increases about 50-fold when the oxygen concentration of the growth medium falls below 10% of the atmosphere (Boerman and Webster, 1982). This led to the presumption that VHb is functional under low oxygen condition. However, the exact function of VHb in its native host is still elusive. A recent discovery of a homologue of the VHb from another *Vitreoscilla* sp. (Joshi *et al.*, 1998) and from the strictly anaerobic *Clostridium* (Shimizu *et al.*, 2002) has evoked further interest in VHb.

To get an insight into the structure-function relationship of VHb, site directed mutagenesis of certain key residues was carried out. Apart form the role of the residues involved, this study was also aimed at obtaining a low or a high affinity mutant(s) of VHb that could obscure or enhance the effect of expression of VHb on the metabolism in the heterologous host. With the availability of the crystal structure of VHb, it was clear that this hemoglobin has unique structure at distal site; the residues from E7 to E10 do not conform to the α-helix. For all other globins, for which the structures are known, this region has been found to be present in an α-helix. This unique feature of VHb was selected for the study and an attempt was made to change the set of residues (E7 and E8) to the ones that could impart a canonical "globin conformation" to VHb at the distal site. An earlier study had indicated that by changing the E7 Gln to His, VHb predominantly existed in the cells in oxidized form, and the oxygenated form was short-lived which could be detected only by laser flash photolysis. VHb having Leu at E7 instead of Gln did not have any marked effect on the structure of VHb though it conferred some positive physiological attributes to the heterologous host. With this information, E7 Gln was altered to the amino acid residues having variable polarity and volume of their side chains, i.e., Tyr, Phe, Gly and Glu. It was noticed that the incorporation of these residues at E7 position of VHb rendered the protein in the oxidized form. Further, when the helix-

distorting residue, E8 Pro was changed to Ala (the residue having a high propensity to be in an α-helix), or to a smaller and flexible Gly, the mutant VHb existed in the oxidized form in the cell. In nutshell, amino acid residues incorporated in place of the wild type E7 Gln and the E8 Pro did not have any drastic effect on oxygen binding pocket of VHb as the mutant VHb could interact with CO and gave the characteristic Soret peak as exhibited by the wild type protein, though a minor perturbation in the heme pocket could be there that is indicated by a slight shift in the Soret peak and change in the oxygenated form of VHb mutants *in vivo*.

As in all the globins, proximal pocket of VHb has conserved His residue at F8 position. An important aspect of VHb (and the other microbial flavohemoproteins) is that His F8 (which is coordinated to heme iron), is H-bonded to Glu-137 and Tyr95 (G5). The latter two residues are also H-bonded. It has been proposed that Fe-His-Glu-Tyr interaction can modulate the redox properties of heme iron, transforming a globin from a storage protein to a constituent of an electron-transferring enzyme. To explain the relevance of conservation of Tyr 95 (G5) in microbial gobins, this residue was mutated to His, Leu and Phe. There was no drastic effect on the mutant protein, which is evident from its spectral characterization and CO-binding. Also, the expression of the VHb mutated at G5 site did not confer any additional advantages to the host.

The other amino acid that is conserved in all the microbial globins is Tyr 126 (H12). As it also participates in the network of H-bonding at the proximal site, it was mutated to Leu, Phe and His. When subjected to the screen of physiological monitoring, H12Leu VHb showed some promise in imparting the positive physiological traits to the cells. The cells expressing H12Leu VHb showed enhanced final cell density and the increased levels of the recombinant protein production. The static equilibrium constants (K_d) for oxygen for VHb and H12Leu VHb are 7.11 μM and 35.8 μM respectively. It is possible that the lower oxygen affinity of this VHb mutant (as compared to the wild type VHb) is better suited to facilitate oxygen transfer in the cell and the altered oxygen transfer characteristics are manifested in enhanced cell density achieved by the

expression of H12Leu VHb in the heterologous host. The growth promoting effects of H12Leu VHb can be exploited for the bioprocesses employing high cell densities such as fed-batch fermentation and immobilized cell systems. The properties conferred on the heterologous host upon expression of this mutant made it an attractive candidate to carry out further studies on the structural aspect of this mutant VHb. Structural analysis revealed that in H12Leu VHb, a cavity is formed in vicinity of heme (Fig. 6.1 and Fig. 6.2), based on which it is predicted that this cavity acts as an alternate port of entry for oxygen.

At present, VHb is the only single domain bacterial Hb known that lacks a flavoreductase domain but exhibits close sequence homology with the globin domain of flavoHbs. Recently available genome sequence of *Clostridium* has indicated the possibility of presence of VHb homologue but there is no available information on the structural or the functional aspect of Hb from this anaerobe. Therefore, to explore the functional relevance of VHb in existing as a single domain globin, the reductase domain of the flavohemoglobin from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) was appended to the C-terminus of VHb. These studies provided new insight into the functional role of VHb; in nitrosative stress response. This role is in line with the emerging roles that have been ascribed to the other flavohemoglobins. Though functions performed by the eukaryotic globins (other than oxygen delivery) have emerged, interactions of microbial globins with similar ligands is being explored. It was proposed herein that VHb can perform two functions. As a dimer, it can act as oxygen-transfer protein and it can interact with the reductase analogous to the two domain flavohemoglobins and functions in relieving nitrosative stress in the cells. So, it was proposed that VHb exists in two states in the host. Under low oxygen conditions, the promoter is maximally induced leading to accumulation of VHb to a high concentration in the cells where it exists predominantly as a dimer and plays a role in the facilitation of oxygen transfer to the terminal respiratory apparatus. Whereas, under the conditions of high oxygen, where the promoter activity is less, the protein accumulates to a low

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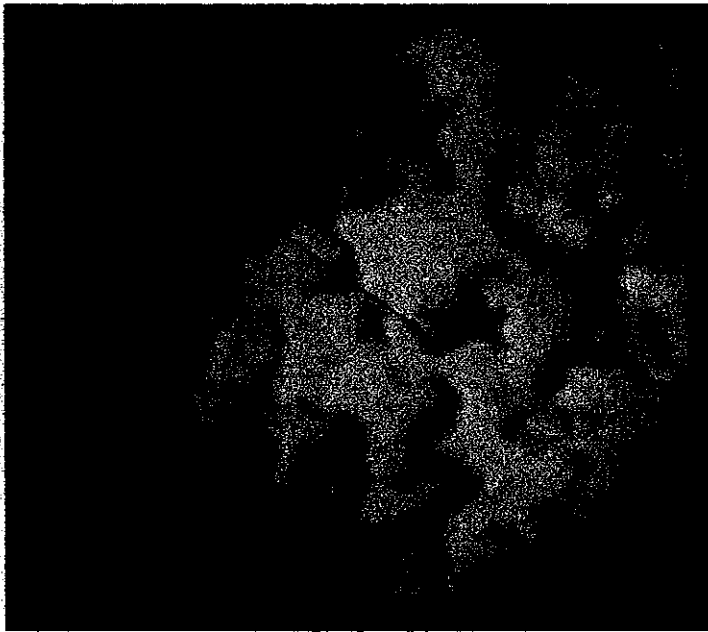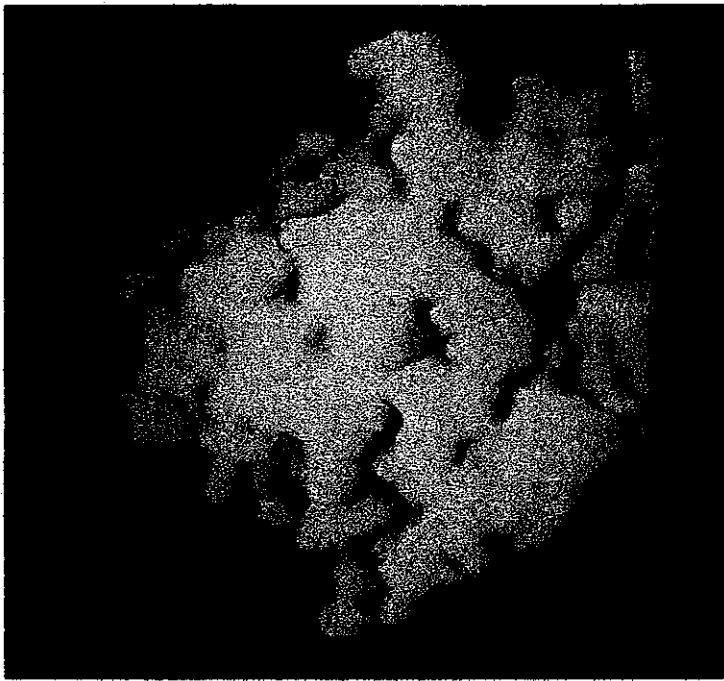
Fig. 6.1 Surface view of VHb (H12) Tyr (126)→ Leu; cavity is formed in the mutant VHb (lower panel) In wild type VHb, no such cavity is formed (upper panel).
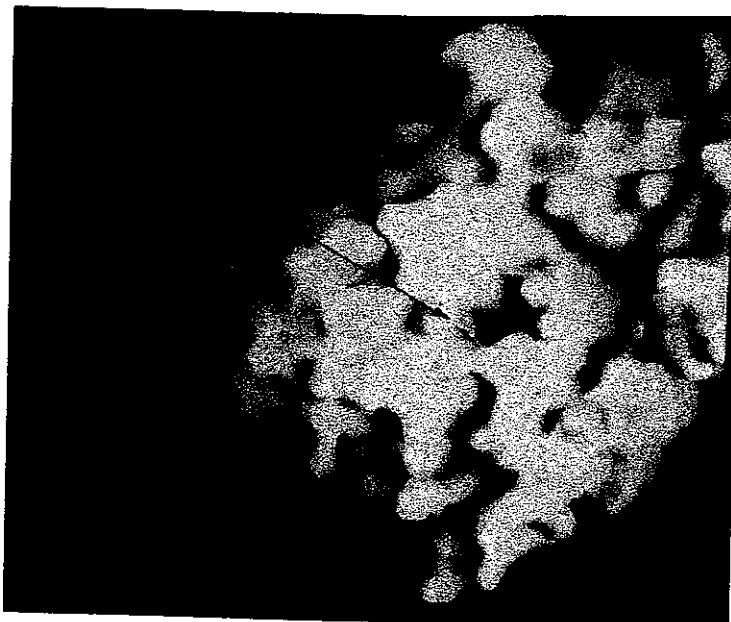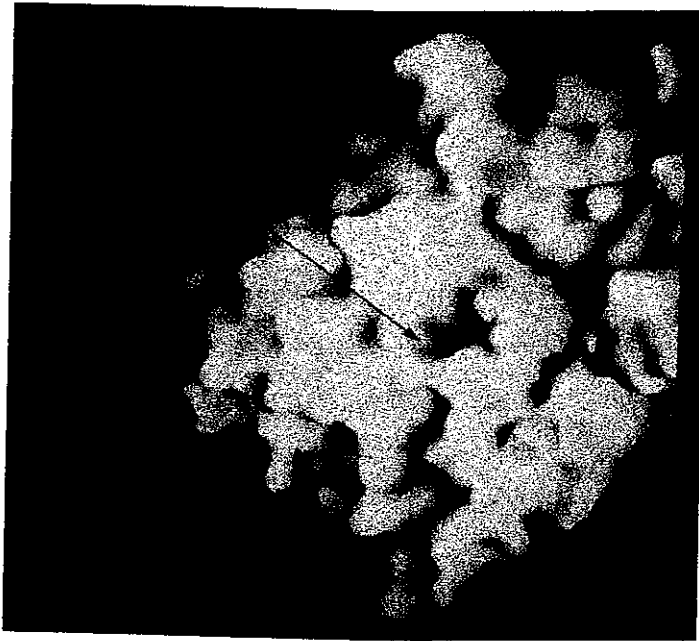
Fig. 6.2 Surface view depicting the presence of native H12 Tyr in the cavity (lower panel) created due to the H12 Leu mutation (upper panel) in VHb.

concentration in the cells. In this case, it interacts with its native reductase and protects the cells from damaging free radicals generated by the oxidation-reduction systems.

As different functions were ascribed to this protein, it became imperative to verify the exact localization of this protein. An inconclusive and unconvincing evidence was available from an earlier biochemical study (Kosla and Bailey), which had indicated that ~40% of VHb localizes in the periplasmic space. Based on the same study, it was also deciphered that the N-terminus of VHb acted as a transport signal (though it would be an unconventional signal as compared to the signal sequences known for *E. coli*) to transport it out in the periplasmic space. To have the direct proof of the localization of this protein in the cell, immunolocalization study was carried out. The study indicated that VHb localizes in the cytoplasmic compartment, both in the native host and in the heterologous host (*E. coli*). This localization of VHb was cytoplasmic, even if the protein was expressed to the higher concentration in the cells. On further analysis, it was found that, within the cytoplasmic compartment, VHb showed localization in proximity to the inner membrane. This specific localization points towards its interaction with the components of the respiratory machinery (terminal oxidase) in the cells. VHb was transported out into the periplasm by appending it to the *E. coli* transport signal at N-terminus. The signal could transport the protein (~50% of the total VHb) into the periplasmic space, which implies that the structure of VHb is no hindrance to the transport of VHb across the inner membrane and it also denounces the role of N-terminus as a signal sequence.

In conclusion, the major findings of this study are:

- Lowering of oxygen affinity by altering Tyr126 (H12) residue in VHb results in a variant of VHb that is superior to wild type VHb for biotechnological applications.
- VHb interacts with flavoreductase *in vivo* and can perform the function analogous to the other flavoHbs when it associates with the reductase domain.

- VHb localizes in the cytoplasmic compartment in the cell in close proximity to the inner membrane, which is consistent with its role in facilitation of oxygen transfer to the terminal respiratory apparatus under hypoxic conditions.