Despite the fundamental and biomedical interest in vertebrate Hbs, It has become evident that heme based oxygen carriers are widespread even in the lower phyla of the biosphere. In particular, discovery of Hb, or Hb like proteins, in vertebrates, higher plants, fungi, protozoa and bacteria has invited unexpected views about the functional properties and structural organisation of the representatives of the globin family (Riggs, 1991; Hardison, 1996). Substantial sequence homology in vertebrates and conserved three-dimensional structure of globins, suggests their common evolutionary origin from a monomeric oxygen carrying heme protein (Dickerson & Geis, 1983; Bashford *et al.,* 1987; Pastore & Lesk, 1990; Moens *et al.*, 1996). In contrast to the vertebrate globins, non-vertebrate globins exhibit extensive variability in their structural organisation and cellular functions. The age old perception that hemoglobin is a eukaryotic protein, solely involved in the transport of oxygen and carbon dioxide was challenged when a hemoglobin was discovered in a gram negative bacterium, *Vitreoscilla*. Thus, VHb is the first prokaryotic hemoglobin reported form a microbial system. Since then hemoglobins have been reported from many other genera of prokaryotes. There is tremendous diversity in the structural organization and the postulated functions of non-vertebrate hemoglobins. Their structure may be single chain, one domain, two domain and multidomain globins, chimeric proteins having a globin domain linked to a flavin binding protein and truncated globins (Vinogradov *et al.*, 1993; Pesce *et al.*, 2000). In addition some of these putative globins, particularly from bacteria, unicellular algae and yeast are unique as they perform functions other than oxygen storage and transport. However, exact physiological significance of these microbial globins is not known. Amongst these, VHb from *Vitreoscilla* sp. C1 is unique and the most extensively studied globin of microbial origin.

The gene coding for the *Vitreoscilla* hemoglobin was cloned, sequenced and characterised (Dikshit & Webster, 1988). The gene, *vgb*, is regulated at transcriptional level by oxygen. The relative amount of *vgb* mRNA increases when *E.coli* cells are grown under low oxygen level (Dikshit & Webster, 1989). Analysis of promoter region revealed that there is involvement of FNR or FNR like proteins in modulation of its activity (Joshi & Dikshit, 1998). Under oxygen limited growth conditions, strains engineered to express VHb exhibited an enhanced specific growth rate as well as an increased final cell density. Also, the overall protein content increased by 10-20% (Khosla & Bailey, 1988a). These observations led to the use of VHb in biotechnological processes. Hemoglobin content of *Vitreoscilla* increases about 50-fold when the oxygen concentration of the growth medium falls below 10% of the atmosphere (Boerman & Webster, 1982). This led to the presumption that VHb is functional under low oxygen condition. However the exact function of the protein in native host has eluded the workers. Although primary function of myoglobins and hemoglobins in higher forms of life is oxygen storage and transport, a similar role cannot be envisaged for globins from unicellular organisms.

Recent discovery of a homologue of VHb from another *Vitreoscilla* sp. (Joshi *et al.*, 1998) has evoked interest in this genus. The occurrence of hemoglobins and other heme proteins in other strains of *Vitreoscilla* can further be investigated. The genus seems to have evolved a unique strategy to overcome the oxidative stress. The occurrence of heme proteins and other related protein that may help in releasing oxygen stress could be studied in this species. The objective of the first part of the study was to investigate the relevance of heme proteins in *Vitreoscilla* sp. with the ultimate goal to understand the structure-function relationship of this novel protein. The latter part of the study was done using site directed mutagenesis of the identified hot spots in the VHb. Further; the structural determinants of prokaryotic hemoglobins have been

worked out to understand the phylogenetic relationship of VHb during the course of evolution.

Preliminary studies in *V.stercoraria* and *V.beggiatoides* indicated that both the strains differ with respect to their cellular morphology and antibiotic sensitivity. The former showed good survival under low aeration, whereas, the latter exhibited no growth at all. The growth of *V.stercoraria* under low aeration could be attributed to VHb, whereas *V. beggiatoides* showed accumulation of a dark-tanned pigment, which was indicative of a heme protein. Further analysis showed that this species accumulated high amount of catalase under aerobic conditions. The genetic studies in the native host *Vitreoscilla* were hampered by the lack of a gene transfer system. During the course of this study an efficient gene transfer system was developed for *V.stercoraria* C1. The development of a gene transfer system in *Vitreoscilla* sp. will help in creation of isogenic mutant strains lacking a functional VHb. This will further help in elucidation of function of this protein in the native host. Search for *vgb* homologue in *V.beggiatoides* genome resulted in isolation of clones harbouring the *vgb* like sequences, however, the nucleotide sequencing of the gene revealed a deletion of a single base in the sequence thus perhaps rendering the gene non-functional. Relevance of non-functional *vgb* gene in *V. beggiatoides* is not clear at present. It is quite possible that natural niche of *V. beggiatoides* is not hypoxic like *V. stercoraria,* thus the presence of an oxygen binding protein becomes unnecessary.

The structure-function studies of the VHb were initiated by construction of a homology based model. The model construction coincided with the determination of crystal structure (Tarricone *et al.*, 1997). The homology model of VHb was compared with the crystal structure of VHb. The model of the VHb could predict the structure to a fair degree of accuracy. Further, a high-level expression system for VHb was constructed by introducing a strong CRP site and deleting the *uvr* gene from the parent construct. On the basis of the crystal

structure and homology model, hot spots were identified in VHb that served as a starting point for understanding the structure based functional modulation. A unique feature of all microbial hemoglobin is the E7 position, which is always occupied by a glutamine residue. Along with the E7 glutamine, B10 on the distal side of the heme pocket and F8 histidine on the proximal side were other tagets for site-specific mutagenesis. Replacement of F8 histidine by tyrosine resulted in a mutant protein which showed ability to bind dioxygen and carbon-monooxide. The oxygen affinity of this mutant was lowered as compared to the wild type VHb. This is in contrast to mammalian Hbs where by a replacement of F8 histidine by tyrosine results in ferric form of Hb which is unable to bind the external ligands. The uniqueness of the VHb in globin family is indicated by the fact that the protein could accommodate substantial changes in the proximal heme ligand.

The replacement of B10 tyrosine in the distal pocket with phenylalanine and leucine increased the dissociation rate constant for oxygen; thus, decreasing the oxygen affinity. These observations were substantiated by the increase in the oxygen uptake of the recombinant *E.coli* expressing these mutants. A comparative model of the mutant protein with respect to the native VHb revealed that the increased oxygen uptake observed could be due to the more spacious distal heme pocket in these B10 mutants, which allows external ligand traffic more easily.

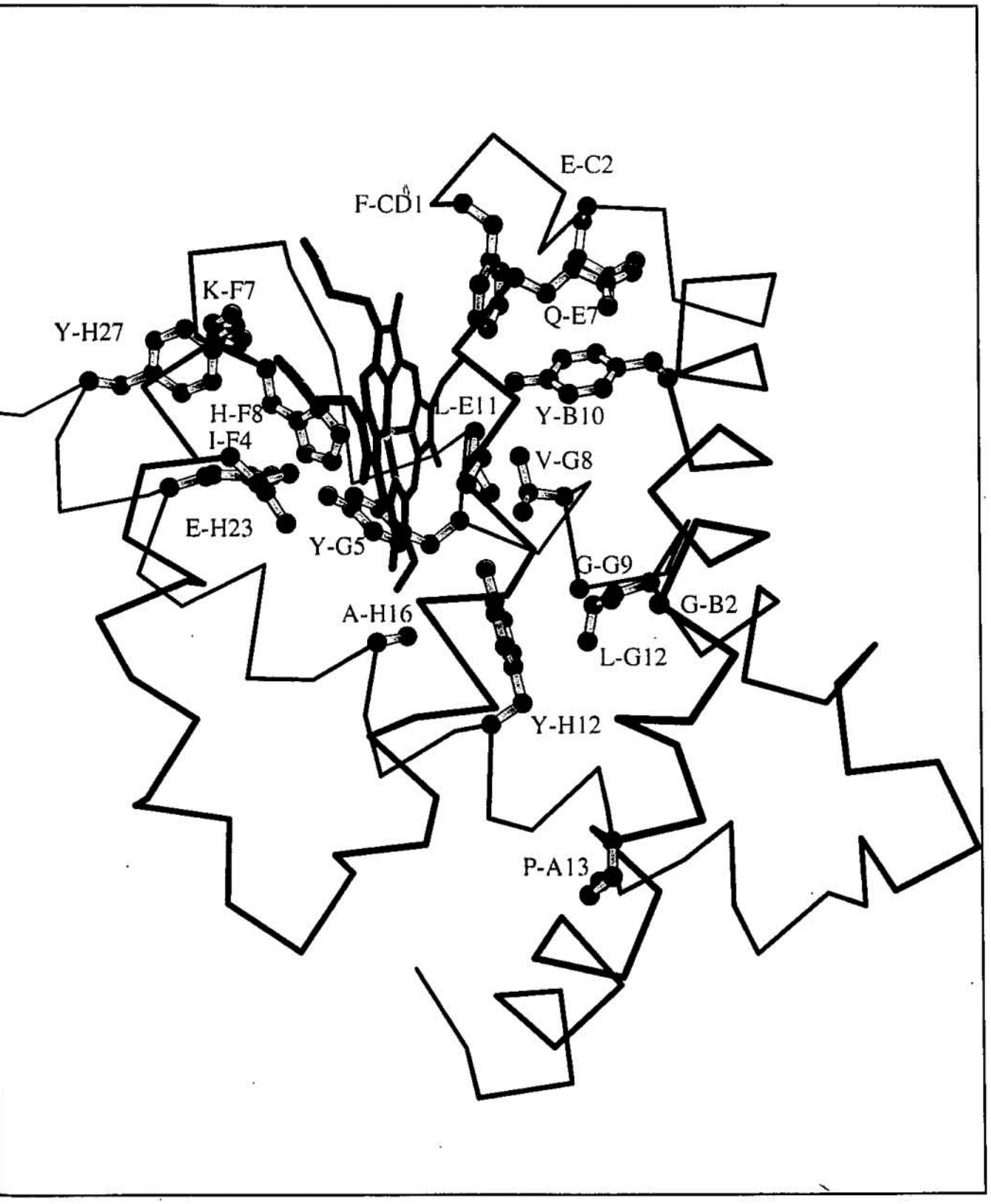
The distal heme pocket from higher eukaryotes has histidine at the E7 position which forms a H-bond with the bound oxygen, thus, stabilising the oxy form of Hb (Perutz, 1979). However E7 position in several invertebrate Hbs, flavohemoglobins and elephant myoglobin is occupied by glutamine. The replacement of E7 glutamine with histidine and leucine was carried out to study the relevance of the presence of the glutamine at E7 position. The glutamine → histidine mutation resulted in a form of VHb which was not able to bind oxygen and, thus, no oxy-form was detectable. An intermediate short-lived oxy-form

could be detected by laser flash photolysis only. However, the glutamine to leucine mutation exhibited an oxy-VHb form that possessed a lower dissociation rate than the wild type VHb. The overexpression of leucine mutant produced a positive effect on the physiology of recombinant *E.coli*. The total energy pool (ATP) of the cells was found to be higher for this mutant. The growth promoting effects of E7 leucine variant of VHb can be exploited for the bioprocesses employing high cell densities such as fed-batch fermentation and immobilised cell systems.

Since the discovery of hemoglobin in *Vitreoscilla*, several Hbs of microbial origin have been reported and many more are expected as a result of several ongoing microbial genome-sequencing projects. Extensive attempts were made to retrieve genetic loci encoding Hb like protein(s) from the genome data available for various microbes and a comparative analysis was done in order to understand the evolutionary history of VHb and its structure-function aspects. A preliminary look at the sequences available reveal tremendous variability in the sequences. Despite of this diversity all the Hbs conform well to the globin fold. The structural determinants of the prokaryotic Hbs were investigated and a prokaryotic Hb template was derived which produced very low scores with Hbs of microbial origin. The Hb or globin like proteins from higher eukaryotes produced high penalty scores thus showing the specificity of the template to resolve the Hbs of microbial origin. This template can serve as a helpful tool for aligning and resolving prokaryotic globin sequences from the data coming out of sequencing projects.

The ancestral radiations of cytochrome b_5 protein in VHb which were investigated in the light of the spectral properties shown by the E7 glutamine → histidine mutant, were correlated with the evidences present in the literature and a hypothesis has been proposed which indicates a possible derivation of microbial globins from cytochrome b_5 like protein. Further, the aminoacid sequences of available prokaryotic Hbs were analysed and phylogenetic trees

Fig. 5.11 α-Carbon trace of VHb showing the conserved residues in prokaryotic hemoglobins



were constructed. A consensus model of prokaryotic Hbs taking VHb structure as base has been depicted in **Fig. 5.11** showing the conserved residues all Hbs reported till date. The trees based on ssu rRNA sequences from similar organisms revealed that evolution of Hbs in lower eukaryotes and prokaryotes has progressed with likely events of horizontal gene transfers. The horizontal gene transfer between yeast *Saccharomyces cerevisiae* and *Vitreoscilla* has been indicated. The presence of Hb in obligate anaerobe *Clostridium* and its high sequence similarity with VHb was surprising. This placed these two (otherwise distant) genera together in the phylogenetic tree. The evidence on the basis of the phylogenetic position of *Clostridium* and *Vitreoscilla* Hb *(vis a vis* rRNA based phylogenetic tree) indicates a possible horizontal transfer of the Hb gene from *Clostridium* to *Vitreoscilla*. The fact that the *Clostridium* Hb is also a single domain globin makes this hypothesis even more compelling for further investigation.