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Review article.

Truncated hemoglobins: A single structural motif with versatile functions in bacteria, plants and unicellular eukaryotes

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Abstract
Truncated hemoglobins (trHbs) constitute a family of oxygen binding heme proteins distributed in eubacteria, cyanobacteria, plants and unicellular eukaryotes like protozoa and algae forming a distinct group within the hemoglobin (Hb) superfamily. Crystallographic studies have shown that trHbs tertiary structure is based on 2-on-2 α-helical sandwich, different from usual 3-on-3 α-helical arrangement in classical hemoglobins and exhibiting hexacoordinate structures compared to traditional mammalian myoglobins (Mbs) and hemoglobins (Hbs) which are pentacoordinate in nature. Phylogenetic studies had shown that trHbs arose from a different clade with an unprecedented editing of the highly conserved globin fold. These unusual globin forms are attributed to carry many versatile functions in living metabolic systems. Very little work has been accomplished that explains the structural plasticity and functional properties of these peculiar globin forms which need appropriate attention.

Keywords: Truncated hemoglobins, trHbs, *A. thaliana glb3*, *N. commune GlbN*, *Frankia*, *Synechocystis* sp. PCC 6808 *SynHb*, *C. eugametos LI637*, *M. tuberculosis trHbN*, *P. caudatum PtrHb*

1. Introduction

Despite the common perception of hemoglobin as a ubiquitous blood protein in higher vertebrates and particularly the eukaryotes, it is now an established fact that this protein is also found in many invertebrates, bacteria, fungi and higher plants (Wittenberg et al., 2002). The ubiquitous nature of hemoglobin distribution in all prokaryotes has been discovered recently as evidenced by fully sequenced archael genomes (Freitas et al., 2004) and earlier reports on many bacterial species (Pesce et al., 2000; Wittenberg et al., 2002). Hemoglobin (Hb) sequences in eukaryotes and prokaryotes are highly variable in amino acid sequences but all the forms have a conserved structure, in the globin fold. The three dimensional structure of classical hemoglobin as observed is a 3-on-3 arrangement of α-helices, is distinct from the 2-on-2 arrangement of truncated hemoglobin fold (trHb).

Truncated hemoglobins (trHbs) are a divergent group of hemoglobins (Hb) identified in many prokaryotes, a few protozoa, eukaryotic algae and higher plants. Phylogenetic analyses indicate that trHbs are distinctively different from bacterial flavohemoglobins, plant symbiotic hemoglobins and also vertebrate hemoglobins (Bolognesi et al., 1982; Anderson et al., 1996; Watts et al., 2001; Frey and Kallio, 2003). These hemoglobins constitute a family of small oxygen binding heme proteins forming a clade within the hemoglobin (Hb) superfamily. Truncated hemoglobins (trHbs) are held to be of very ancient origin and none of the types have ever been detected in the genomes of metazoa (Wittenberg et al., 2002). More than 40 putative trHbs have been identified so far that serve multiple functions in living metabolic systems (Wu et al., 2003).

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2. Truncated Hemoglobins (trHbs) are Characteristically Distinct within the Hemoglobin (Hb) Superfamily

An extreme example of diversity in structure and function had been observed in the family of hexacoordinate truncated hemoglobins (trHbs) in which an endogenous amino acid coordinates the ligand binding site of the heme iron in the absence of exogenous ligands (Das et al., 1999, 2000; Couture et al., 2000). The fact that these trHbs are capable of reversible exogenous ligand binding in hexacoordinate state distinguishes it from classical hemoglobins (Trent III et al., 2001). However, neuroglobins and cytoglobins have also been reported to exhibit hexacoordinated structure whose precise functions are still awaited (Freitas et al., 2004). Truncated hemoglobin (trHb) types display characteristic amino acid sequences of 20–40 residues shorter than the classical hemoglobins and therefore have been proposed to have very little or no sequence similarity (Wittenberg et al., 2002). Molecular crystallographic studies have showed that the tertiary structures of trHbs is based on 2-on-2 α-helical sandwich with a highly conserved globin fold and a hydrophobic tunnel traversing the entire protein molecular matrix to the heme distal site (Pesce et al., 2000). Considerations on structures of trHbs highlight a previously unpredicted structural plasticity of hemoglobin (Hb) forms and focus interests on potentially new functions of the hemoglobin superfamily (Peterson et al., 1997).

3. The 2-on-2 α-Helical Fold in trHbs is Different from 3-on-3 Arrangement in Classical Hemoglobins

High resolution crystal structures of truncated hemoglobins (trHbs) from the protozoon *Paramaecium caudatum*, the unicellular alga *Chlamydomonas eugametos* and other species of gram positive bacteria has revealed an alternate folding pattern with a 2-on-2 sandwich of α-helices that might have probably arose from 3-on-3 hemoglobins (Hbs) by deletion of N terminal α-helix and replacement of the proximal heme binding F helix with an extended non-helical loop (Couture et al., 1994; Das et al., 1999).

Alternatively, it has also been proposed that 2-on-2 trHbs of unicellular organisms may have an origin distinct from 3-on-3 classical hemoglobins (Hbs) i.e. both the hemoglobin types represent separate gene families (Wittenberg et al., 2002). Interestingly, the 2-on-2 trHbs display high sequence conservation and the presence of these genes in bacteria has been attributed to horizontal gene transfer from a common ancestor of protozoa and algae (Pesce et al., 2000). The 2-on-2 trHbs are present in three of the five kingdoms i.e. Monera, Protista and Plantae while both 2-on-2 and 3-on-3 type hemoglobins are found in plants and gram-positive bacteria (Hoy et al., 2004). Based on complete genome sequences studied so far, no 2-on-2 trHbs have been reported in *Coenorhabditis elegans*, *Drosophila melanogaster*, *Homo sapiens* and *Saccharomyces cerevisae* (Bolognesi et al., 1997; Das et al., 2000). Besides, no evidence of 2-on-2 trHbs has been reported till date from fungi and animals which imply either a loss of the gene responsible for expression of trHbs in the fungi/animal lineage or an acquisition of the gene by plants due to horizontal gene transfer (Bolognesi et al., 1997).

4. Truncated Hemoglobins (trHbs) in Plants are Different from Symbiotic and Non-symbiotic Hemoglobins

Plant hemoglobins was previously thought to be restricted to nitrogen fixing root nodules of leguminous plants in highly specialized symbioses with strains of *Rhizobium* or *Bradyrhizobium* species in soil (Appleby, 1992). These hemoglobins were therefore termed as leghemoglobins and the primary function of these hemoglobins is facilitated diffusion of O_2 within the nitrogen fixing root nodules (Appleby, 1984). In contrary to the antiquated belief, it is now confirmed that hemoglobin genes are also present in a wide range of non leguminous plants like *Parasponia*, *Trema*, *Casuarina*, *Hordeum*, *Triticum* and *Zea* (Appleby et al., 1983; Christensen et al., 1991; Taylor et al., 1994; Sowa et al., 1995). The presence of these genes in non-leguminous, nodulating, non-nodulating and other phylogenetically diverse plant genera including some monocots has reinforced the proposal that hemoglobin may be present throughout the plant kingdom.

In plants at least three distinct hemoglobin types have been identified. These have been categorized as symbiotic, non-symbiotic (split into Class I and Class II types), and truncated (Dordas et al., 2003). Symbiotic hemoglobins have been observed to be present in nodules of many plant species and their function has been attributed to O_2 supply during symbiotic nitrogen fixation by nitrogen fixing bacteria (Fleming et al., 1987). Non-symbiotic hemoglobins are believed to exist throughout the plant kingdom and are not involved to symbiotic nitrogen fixation (Jacobsen-Lyon et al., 1995). Furthermore, two sub-classes of non-symbiotic hemoglobins have been classified based on oxygen binding properties. These are referred to as class I and class II non-symbiotic hemoglobins (Dordas et al., 2003). Class II non-symbiotic hemoglobins show similar oxygen binding properties to symbiotic hemoglobins while class I type non-symbiotic hemoglobins exhibit dramatically different oxygen binding properties and are generally induced by hypoxic stress and oversupply of nutrients (Dordas et al., 2003). These stress induced non-symbiotic hemoglobins are expressed in plants in response to specific metabolic stresses and have been

shown to affect plant growth and metabolism under low oxygen tensions (Dordas et al., 2003).

However, the third class of plant hemoglobins i.e. truncated hemoglobins (trHbs) is quite dissimilar in structure and functionally distinct from the former two types (Landsmann et al., 1986; Dordas et al., 2003). Truncated hemoglobins in plants are induced under hypoxic conditions. While hypoxic stress induced hemoglobins are widespread throughout the plant kingdom, yet their overall functions has not been fully elucidated (Gibson et al., 1989; Hunt et al., 2001). It has been proposed that nitric oxide (NO) is an important metabolite regulating hypoxia in plants and therefore one particular function of these trHbs might be ascribed to adaptation of plants during hypoxic stress and modulation of nitric oxide levels inside cells (Hunt et al., 2001).

A good example is the elevated sucrose levels in *Arabidopsis thaliana* that have shown to increase the expression of plant trHb genes (Bogusz et al., 1988; Hill, 1995). It has also been confirmed that the position and number of introns in hemoglobin genes vary considerably among plant species (Hunt et al., 2001). Symbiotic and non-symbiotic class I/ class II plant hemoglobins generally have a 3 intron-4 exon structure with a 3-on-3 α-helical loop exhibiting classical hemoglobin types as compared to 2-on-2 arrangement of α-helices in plant truncated hemoglobins (trHbs) (Appleby et al., 1988; Bogusz et al., 1988; Dordas et al., 2003).

5. *Arabidopsis thaliana* Truncated Hemoglobin *glb3*

Watts et al. (2001) had identified a gene from *Arabidopsis thaliana* that encodes a 2-on-2 globin similar to trHbs of bacteria. The gene was found to be a member of the plant *Glb* family and was named *Glb3* gene. Spectral and kinetic characterization of this *Glb* encoded *glb3* trHb showed similar spectrum to *glb1* and *glb2* non-symbiotic plant Hbs with a strong sequence similarity to a subset of bacterial 2-on-2 trHbs but were less similar to trHbs from *P. caudatum* and *C. eugametos* (Couture et al., 1994; Das et al., 2000; Watts et al., 2001). However, *Glb3* gene expression is different from *Glb1* and *Glb2* plant genes with an appearance of decreased mRNA levels during hypoxia (Watts et al., 2001). The functional characteristics of the protein *glb3* depicted a five member coordinate ring with no bound ligand but binding sites for CO and O_2 that bind to the heme moiety of the protein in a reversible manner (Watts et al., 2001). As evidenced by earlier studies, it is confirmed that the plant genes encoding classical 3-on-3 Hbs (i.e. *Glb1* and *Glb2*) have a 3 intron-4 exon structure with intron positions being absolutely conserved (Trevaskis et al., 1997). Although *A. thaliana Glb3* gene also shows a 3 intron-4 exon structure, interestingly, two of the introns show different positions compared to other plant Hbs and therefore supports a separate evolutionary origin of the *Glb3* genes (Trevaskis et al., 1997). Further comparison of the 2-on-2 *glb3* protein from *A. thaliana* and bacterial 2-on-2 trHbs indicated that the protein resemble closely to *GlbO* gene encoded *trHbO* in gram-positive bacteria (Watts et al., 2001).

On the contrary, the protein is not homologous to *GLBN* gene encoded 2-on-2 *GlbN* of cyanobacteria (Thorsteinsson et al., 1999). Functional properties of *A. thaliana glb3* protein showed a lower O_2 affinity compared to other 2-on-2 trHbs and plant 3-on-3 Hbs. *glb3* protein is dimeric in structure and is an O_2 transport protein with unusual characteristics (Trevaskis et al., 1997). The tertiary structure of the protein shows a longer ef-loop, F2 proline and E7 alanine residues that distinguishes it from *GLBN* encoded *GlbN* in cyanobacteria and probably also contribute to biochemical differences with other characterized 2-on-2 trHbs (Trevaskis et al., 1997; Watts et al., 2001).

6. Truncated Hemoglobins (trHbs) in Symbiotic N_2 Fixing Bacteria and the Cyanobacteria

In recent years, a lot of attention has been focused to understand the mechanism and importance of hemoglobins in nitrogen (N_2) fixing plants (both leguminous and non leguminous) which is a result of symbiotic association with N_2 fixing bacteria (Appleby, 1984; Fleming et al., 1987; Pathirana and Tjepkema, 1995). A good example is the presence of hemoglobin in soyabean (*Glycine max*) which is symbiotic in nature and is even expressed in root nodules of the plant (Anderson et al., 1996). On the other hand, it is proposed that symbiotic hemoglobins might be expressed in all effective N_2 fixing nodules in legumes, which is thought to be essential for the facilitation of O_2 diffusion within the infected cells (Christensen et al., 1991). However, most excitingly, no trHbs have been reported in *Rhizobium* or *Bradyrhizobium* species of bacteria infecting leguminous plants during symbiotic N_2 fixation (Dordas et al., 2003).

In non-leguminous actinorhizal plants, symbiotic N_2 fixation is achieved due to nodulation by another group of gram-positive actinomycete bacteria called *Frankia* that express the occurrence of trHbs (Pathirana and Tjepkema, 1995). Although existence of symbiotic hemoglobins is well established in actinorhizal plants *Casuarina glauca* (Fleming et al., 1987) and *Myrica gale* (Pathirana and Tjepkema, 1995), yet very little is known about the presence of such hemoglobin types and their possible role in many other actinorhizal plants.

Most fascinating is the presence and display of trHbs in most of the *Frankia* strains infecting actinorhizal root nodules (Beckwith et al., 2002). The occurrence of trHbs in *Frankia* (Tjepkema et al., 2002) and the cyanobacterium *Nostoc commune* (Hill et al., 1996) is noteworthy because both of these organisms fix atmospheric N_2 and do so in

specialized cells that protect the enzyme nitrogenase from O_2 during N_2 fixation. The presence of trHbs in five genetically diverse *Frankia* strains representing three of the genetically identified host specificity actinorhizal groups (*Elaeagnaceae*, *Alnus* and *Casuarinaceae*) has been reported (Beckwith et al., 2002). The O_2 binding affinity (k_d) of *Frankia* trHbs is much less compared to hemoglobins from N_2 fixing root nodules of legumes and the non-legume *Parasponia* (Appleby et al., 1983; Wittenberg et al., 1986; Tjepkema et al., 2002). The Hill coefficients for *Frankia* trHbs are significantly less than trHbs of most organisms and therefore display low k_d in a non-cooperative way (Beckwith et al., 2002). Molecular determination of trHb from *Frankia* strain CcI3 has indicated that *Frankia* trHb is monomeric with a molecular mass and O_2 binding properties similar to the cyanoglobin *GlbN* of *N. commune* (Beckwith et al., 2002). High values of O_2 association and dissociation rate constants (k_{on} and k_{off}) of *Frankia* strain CcI3 trHb suggest that the protein is well suited for the facilitation of O_2 diffusion over short distances. Leaving aside the elucidation of functional characteristics of *Frankia* trHbs, no reports pertaining to molecular crystallographic structures and α-helical arrangement of the trHbs from *Frankia* is available which needs further appreciation in order to understand the biological role of these globin forms during symbiotic N_2 fixation.

Structural analysis of monomeric trHb from the cyanobacterium *Nostoc commune* (Hill et al., 1996) has been identified as a 12 kDa hemoprotein termed as *GlbN* and is encoded by a gene abbreviated as *GLBN* (Thorsteinsson et al., 1999). This cyanoglobin molecule binds molecular oxygen reversibly, is the smallest globin subjected to structural analysis and exhibits high O_2 binding affinity and non cooperativity (Hill et al., 1996). The basis of high O_2 affinity of this trHb was investigated through kinetic studies utilizing flash flow spectrophotometry and flash photolysis (Hill et al., 1996). The absorption spectral properties of *GlbN* from *N. commune* differ significantly to leghemoglobin in plants and sperm whale myoglobin despite similar orientation of the heme moieties and ligand binding characteristics (Scott and Gibson, 1997; Trevaskis et al., 1997). When compared to *Frankia* trHb, it was observed that the O_2 dissociation rate (k_{off}) of *N. commune GlbN* was similar to *Frankia* trHb and faster than many other hemoglobins (Tjepkema et al., 2002). Among the trHbs other than *N. commune* and *Frankia*, the only trHb identified with sufficiently high k_{off} value was in *Paramaecium* sp. which suggest an active role of those trHbs in O_2 transport (Table 1).

Most surprisingly trHbs from *N. commune* and another cyanobacterium *Synechocystis* sp. PCC 6808 have been observed to differ in respect to coordination characteristics (Hvitved et al., 2001; LeComte et al., 2001). The truncated globin *GlbN* coded by the gene *GLBN* in *N. commune* is pentacoordinate in the ferrous deoxygenated state whereas the trHb (*SynHb*) from *Synechocystis* sp. PCC 6808 display a hexacoordinate structure in both deoxygenated ferrous and ferric states (Hoy et al., 2004). The association rate constants for the binding of these five ligands of the pentacoordinate structure in *N. commune GlbN* is the highest reported in any naturally occurring hemoglobin; suggesting an unhindered and apolar ligand binding pocket (Hvitved et al., 2000; Scott and LeComte, 2000). Despite structural relatedness of the two trHb types it has been reported that the rate constants for ligand binding in *Synechocystis* sp. PCC 6808 *SynHb* is much smaller than *N. commune GlbN* (Thorsteinsson et al., 1999; Scott and LeComte, 2000). This behavioral discrepancy between the two proteins is quite surprising given their phylogenetic relatedness that can be rationalized considering the effects of intramolecular hexacoordination (Hoy et al., 2004).

7. Truncated Hemoglobins (trHbs) in Mycobacteria, Ciliated Protists and Unicellular Alga

An interesting progression in the expression of trHbs in the genus *Mycobacterium* had been observed (Yeh et al., 2000). The facultative intracellular pathogen infecting man, i.e. *Mycobacterium tuberculosis* expresses two trHb forms termed as *trHbN* and *trHbO* encoded by genes *GlbN* and *GlbO*, respectively (Couture et al., 1999). On the other hand, the obligate intracellular pathogen, *Mycobacterium leprae* is thought to have undergone extensive reductive evolution and retains solely *trHbO* which plays an important role in the survival of the species *in vivo* (Ouellet et al., 2002; Milani et al., 2003). The homodimeric *trHbN* in *M. tuberculosis* display extremely high O_2 binding affinity in a cooperative way with a Hill co-efficient of approximately 2. It is proposed that this binding affinity to O_2 in *M. tuberculosis* ensures the organism a low but critical level of O_2 availability *in vivo*, necessary to afford protection against NO and in return assuring survival of *M. tuberculosis* in the hypoxic environment of the granuloma (Milani et al., 2001, 2003).

Presence of truncated hemoglobins (*PtrHbs*) and hemoglobin genes had been identified in different species of the ciliated protist *Paramaecium* (Das et al., 2000). Most inquisitive is the 1 intron-2 exon conserved structure of the 2-on-2 globin fold of the trHb genes in the genus (Wittenberg et al., 2002). The amino acid sequences of all the species of *Paramaecium* trHbs were found to be more than 87% identical to one another and homologous to trHbs of the ciliated protist *Tetrahymena pyriformis* (Peterson et al., 1997), LI637 trHb of the green alga *C. eugametos* (Couture et al., 1999) and *GlbN* of the cyanobacterium *N. commune* (Hill et al., 1996); all of which exhibit the presence of 120 amino acid residues in the characteristic globin fold. Phylogenetic relationships based on maximum likelihood inference among the trHb encoding genes of all the above mentioned species had confirmed that

Table 1. Kinetic constants for O_2 binding by trHbs from different organisms (Tjepkema et al., 2002).

Organism	Protein	k_{on} ($\mu M^{-1} s^{-1}$)	k_{off} (s^{-1})	$k_d = k_{off}$ k_{on} (nM)
N. commune	*GlbN*	390	79	203
Frankia strain CcI3	*trHb*	206	56	274
P. caudatum	*PtrHb*	30	25	838
M. tuberculosis	*trHbN*	25	0.2	25
C. eugametos	*LI637*	–	0.014	–
Synechocystis PCC 6803	*SynHb*	–	0.011	–
Sperm whale	*Mb*	14	12	857

k_{on} = O_2 association rate constant; k_d = O_2 binding affinity of trHbs; k_{off} = O_2 dissociation rate constant.

Table 2. Important helix position designations and amino acid residues in 2-on 2 trHbs of different organisms.

Protein	B9	B10	CD1	E7	E14	F8
Sperm whale myoglobin	**Ile**	**Leu**	Phe	**His**	**Ala**	His
Vitreoscilla flavohemoglobin	Phe	Tyr	Phe	Gln	**Thr**	His
N. commune GlbN	**Leu**	**His**	Phe	Gln	Phe	His
P. caudatum PtrHb	Phe	Tyr	Phe	Gln	Phe	His
C. eugametos LI637	Phe	Tyr	Phe	Gln	Phe	His
T. pyriformis trHb	Phe	Tyr	Phe	Gln	Phe	His
Synechocystis PCC 6808 *SynHb*	Phe	Tyr	Phe	Gln	Phe	His
M. tuberculosis GlbN	Phe	Tyr	Phe	**Leu**	Phe	His
M. tuberculosis GlbO	Phe	Tyr	**Tyr**	**Ala**	Phe	His
S. coelicolor trHb	Phe	Tyr	**Tyr**	**Ala**	Phe	His
S. aureus trHb	Phe	Tyr	Phe	**Thr**	Phe	His
A. thaliana glb3	Phe	Tyr	Phe	**Ala**	Phe	His

Helical designations are determined by alignment with sperm whale myoglobin mentioned above.

Paramaecium trHb genes had evolved more rapidly than the others (Yang et al., 1995; Pesce et al., 2000).

The presence of three hemoglobin genes responsible for expression of three hemoglobin types has been detected in the chloroplast genome of the unicellular alga *Chlamydomonas eugametos* (Das et al., 1999). Two of these are ferrous recombinant trHb types termed as *LI637* and *LI410*. Phylogenetic analyses based on primary amino acid sequences suggest that *C. eugametos* trHbs share a small gene family with the trHbs of cyanobacteria *N. commune* and *Synechocystis* sp. PCC 6808 and also with the ciliated protozoon's *P. caudatum* and *Tertrahymena pyriformes* (Thorsteinsson et al., 1999; Das et al., 2000; Hvitved et al., 2001). *C. eugametos* trHb *LI637* in chloroplasts is expressed in response to light and require photosynthesis to occur for its full expression (Couture et al., 1999). Several classical hemoglobins from numerous nematodes including *C. elegans* and *A. lumbricoides* share many structural and functional similarities with *C. eugametos* trHb *LI637* including high O_2 affinity and simultaneous presence of distal E7 glutamine and a tyrosine residue in B10 position of the hexacoordinate globin fold (Bolognesi et al., 1997; Peterson et al., 1997; Das et al., 2000). It is also proposed that the O_2 dissociation kinetics of *C. eugametos* trHb *LI637* is very slow (t = 49 s) to support any metabolic function (Das et al., 1999; Tjepkema et al., 2002).

8. Heme Coordination and α-Helical Fold Characterizes the Formation of 2-on-2 trHbs

The trHb polypeptide chain is not merely a truncated version of a conventionally folded globin molecule (Wittenberg et al., 2002). Rather, it owes its conformational stability to amino acid residue deletions and substitutions at specific sites, as compared to other non-vertebrate hemoglobins (Miles et al., 1999). It has been elucidated that specific sequence motifs support attainment of the compact trHb fold supported with frequent heme isomerization (Bolognesi et al., 1997; Das et al., 2001). Very few amino acid residues are strictly conserved throughout the known trHb sequences, the proximal F8 histidine and E14 Phenylalanine being the invariant ones (Table 2) (Hoy et al., 2004). The usual E14 residue in vertebrate hemoglobins and myoglobin is generally an alanine, while in flavohemoglobins of certain bacteria (e.g. *Vitreoscilla* sp.) the usual alanine is replaced by an unusual threonine residue (Zhu and Riggs, 1992; Frey and Kallio, 2003). Traditional mammalian myoglobins (Mbs) and hemoglobins (Hbs) are pentacoordinate in the ferrous, deoxygenated state and exhibit simple bio molecular ligand binding behavior (Suzuki and Imai, 1998; Scott et al., 2001). In vertebrate globins, the bound O_2 molecule is generally stabilized by the distal E7 histidine residue through hydrogen bonding (Das et al., 2001). The E7

residue in most invertebrate globins is replaced by a glutamine while in *M. tuberculosis GlbN* and *GlbO* encoded *trHbN* and *trHbO* this E7 position are occupied by unusual leucine and alanine residues (Couture et al., 1999; Yeh et al., 2000).

In the trHb *glb3* of *A. thaliana* this E7 residue is occupied by an alanine residue (Watts et al., 2001). Intriguingly, the E7 glutamine residue in bacterial trHbs does not seem to play any crucial role during peptide folding, apparently because of conformational constraints on this residue imposed by the polypeptide architecture (Frey and Kallio, 2003; Wu et al., 2003). An important residue position of the globin polypeptide fold is the CD1 region which is generally occupied by a phenylalanine residue, thought to be conserved in almost all vertebrate and non-vertebrate globin forms including trHbs (Wittenberg et al., 2002). Interestingly, this residue in trHbO of *M. tuberculosis* and *Streptomyces coelicolor* is occupied by an unusual tyrosine residue (Milani et al., 2003; Hoy et al., 2004). Crystallographic studies had revealed that the axial tyrosine residue in B10 and highly conserved phenylalanine residue in CD1 positions determine the absolute orientation of the heme moiety including ligand stabilization in invertebrate and bacterial trHbs (Miles et al., 1999). The role of B10 residue in trHbN of *M. tuberculosis* during ligand stabilization of bound O_2 had been clearly established while on the other hand the presence of an unusual histidine residue in the same position in *N. commune GlbN* is highly intriguing (Hill et al., 1996; Couture et al., 1999). The characteristic high O_2 binding affinity in *N. commune GlbN* can be explained due to the presence of distal E7 glutamine residue which provides a hydrogen bond thereby supporting ligand stabilization (Thorsteinsson et al., 1999). The B9 position in bacterial and invertebrate trHbs is occupied by a conserved phenylalanine residue (Bolognesi et al., 1997).

However, the B9 conserved position in *N. commune* is occupied by an unusual leucine residue (Thorsteinsson et al., 1999). This characteristic occurrence in the conserved B9-B10 residue positions in *N. commune GlbN* compared to other bacterial and invertebrate trHbs is highly interesting since *N. commune* is itself a N_2 fixing cyanobacteria and share many functional characteristics with the N_2 fixing actinomycete *Frankia* where detailed studies on molecular crystallography is still awaited with much anticipation (Beckwith et al., 2002). It is therefore felt that further studies on trHbs of N_2 fixing organisms like *N. commune* and *Frankia* might help in elucidating the molecular phylogeny of such organisms thereby highlighting their probable clade of origin.

9. Physiological Significance of trHbs

A variety of functional roles of trHbs has been proposed although much attention needs to be focussed in this direction. The trHb of the unicellular alga *C. eugametos* is induced in response to active photosynthesis and is localized along the chloroplast of thylakoid membranes (Couture et al., 1999; Das et al., 1999). The trHb from cyanobacterium *N. commune GlbN* is thought to play a role similar to leghemoglobin in plants (Hill et al., 1996). Although in gram positive actinomycete *Frankia*, no particular function of trHb has been deciphered; yet it is thought to have similar functions analogous to *N. commune GlbN*, i.e. transport of O_2 under microaerobic conditions that prevent inhibition of nitrogenase activity (Tjepkema et al., 2002). *trHbN* in *M. tuberculosis* responds to detoxification of NO generated by nitric oxide synthetase II generated by human macrophages while trHbO is involved in protection of the bacilli in the granuloma (Milani et al., 2001; Ouellet et al., 2002). In plants, trHbs respond to hypoxic stress (Dordas et al., 2003) while in *Synechocystis SynHb*, no particular function has been ascribed although it is thought to play some role in N_2 fixation (Hoy et al., 2004).

10. Conclusion

An extreme example of diversity in structure and function has been reported in the family of hexacoordinate trHbs (Pesce et al., 2000; Wittenberg et al., 2002). Although high resolution structures of both hexacoordinate and pentacoordiante trHbs have been solved in recent years and several hypotheses regarding functions have been proposed, still a majority of physiological functions of these proteins is yet to be deciphered. Currently available data on the globin fold of 2-on-2 trHbs reflect versatile biological functions in different organisms; distinct from O_2 storage and transport (Frey and Kallio, 2003). Besides, the subsequent heme coordination in the 2-on-2 helical loop with unusual amino acid residues suggests the probable evolutionary relationships, topology and physiological roles of different truncated globin folds in relation to classical hemoglobins (Hbs) (Pesce et al., 2000). The unpredicted structural plasticity of these globin forms needs further appreciation to understand and clarify their possible mechanistic roles in biological systems. Furthermore to the above, special focus on structure function relationships based on molecular crystallographic studies of trHbs in biologically important organisms (including microorganisms like N_2 fixing bacteria) is thought to provide information on undetermined functions never explored or explicated.

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