

Review Article**TRUNCATED OR 2/2 HEMOGLOBINS : A NEW CLASS OF GLOBINS WITH NOVEL STRUCTURE AND FUNCTION****Amit Kumar^{1#}, Mouprya Nag^{2#} and Soumen Basak^{2*}**¹*Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India*²*Chemical Sciences Division, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Kolkata 700064, India*[#]*Equal contribution*

Abstract: Bright red hemoglobins, the most well-known paradigm in protein science, seem to be ubiquitous in nature. With advances in modern tools and techniques, discovery of new globins at a rapid pace has expanded this family. With every discovery, new insights emerged regarding their novel structure, function and several other characteristics previously not observed for hemoglobins. Even the classical function unanimously assigned to hemoglobins – oxygen transport and storage – needed re-evaluation. The ability of this class of proteins to show responses against various gaseous ligands, even the poisonous ones, indicate that it is obviously as ancient as life. As organisms evolved, hemoglobins also evolved, and accumulated a great degree of diversity in all aspects. The classical globin fold is very unique with 3-on-3 alpha helical bundle as observed in the traditional oxygen-transport hemoglobins like myoglobin, human blood hemoglobin and leghemoglobins in plants. However, a class of the newly discovered hemoglobins, which dominate the superfamily and appears ancient in origin mostly have 2-on-2 fold, commonly termed as “truncated” hemoglobins. These hemoglobins are phylogenetically distinct from their classical counterparts and are often shorter in their polypeptide length by 20-40 residues mainly due to a lack of short A helix, D helix and F helix. However, hemoglobins with 2-on-2 fold were also later found to have polypeptide chain lengths similar in size to classical globins. Disordered pre-F helix region, conserved glycine motifs and other key residues and apolar tunnels through their protein matrix for migration of ligands are some unique characteristics features of these truncated hemoglobins. Some of these are also hexacoordinated at heme iron where an amino acid from within the protein coordinates heme iron in absence of a ligand. These hemoglobins are well known for their high affinity towards ligand and have a diverse mechanism of ligand binding involving tunnel system and operated by “gate” mechanism of conserved residues. These hemoglobins seem to act as good detoxificant or scavenger of poisonous gases to protect the harbouring organism in adverse environmental condition. This mechanism might help pathogens harbouring these globins to evade host defence mechanism during infection. The current review summarizes these findings with regard to truncated hemoglobins in a comprehensive manner providing insight into structure and function relationship of this novel hemoglobin family.

Keywords: Heme; Truncated hemoglobin; 2-on-2 globin fold; 3-on-3 globin fold; hexacoordinate hemoglobin

INTRODUCTION: HEMOGLOBINS – THEIR UBIQUITOUSNESS AND ORIGIN

As we know today, hemoglobins (Hb) are found in all forms of life from archaea to eukaryotes through bacteria, protozoa, algae, plants and of

course animals (Vinogradov *et al.*, 2006). The gene for hemoglobins is as ancient as life. Hemoglobin is ubiquitous and performs various functions not just in the animal world but also in plants as well as in fungi, protists and bacteria. It has been demonstrated that hemoglobins can perform additional functions besides transport of oxygen into/out of tissues, ranging from intracellular oxygen transport to catalysis of redox reactions. The diversity in function of hemoglobin is because

Corresponding Author: **Soumen Basak**
E-mail: soumen.basak@saha.ac.in

Received: May 12, 2013

Accepted: June 24, 2013

Published: July 30, 2013

of the heme protoporphyrin IX containing reactive iron (Fe).

Phylogenetic analysis indicates that the genes for hemoglobin have descended from an ancient, common ancestral gene (Figure 1). Thus, different functions of the hemoglobins explain the acquisition of new roles by a pre-existing structural gene, which requires changes not only in the coding regions but also in the regulatory elements of the genes.

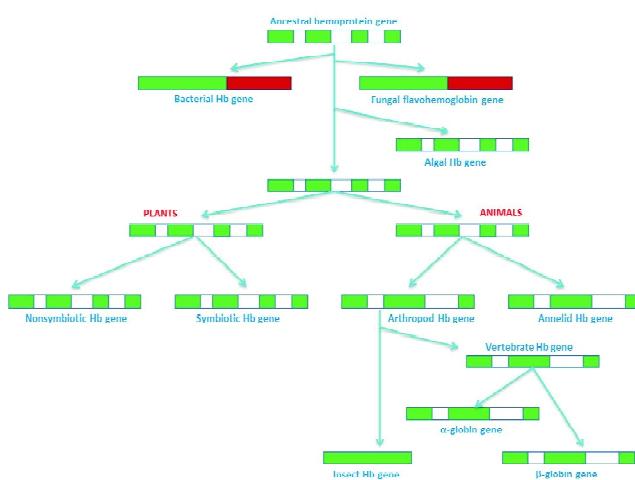


Figure 1: Schematic overview of hemoglobin gene evolution from bacteria to man. Globin-coding exons and genes are shown as colour-filled boxes. This figure was adapted from (Hardison, 1996)

The homology that is found in the hemoglobin gene points to several exciting possibilities which may occur during evolution: many intron insertions in the evolutionary tree, or a good deal of intron sliding, or repeated insertions of introns. The new hemoglobins or hemoglobin-like proteins have several predicted roles including the reversible binding of gaseous ligands. Since many of these heme containing proteins are globins, they are often called hemoglobins and well known as hemoglobin family, even if their overall tertiary structure is very different from that of vertebrate hemoglobin (Hardison, 1996). However, the sole role of heme protein is not restricted to oxygen transport and respiration, and other possibilities are summarized in Table 1.

These new findings with advances in molecular biology and new techniques has changed our view about globins as oxygen (O_2) carriers and added an exciting new twist to the structural and functional relationship of Hb.

Globins

The protein myoglobin, considered as paradigm for this family of proteins, consists of eight α -helical segments that fold around a heme group (Figure 2). The helices building up the globin fold are conventionally labelled A to H according to

Table 1
Diversity of proposed functions and regulation of hemoglobins (Hardison, 1998)

Class	Exemplary genus	Hemoglobin	Function (demonstrated and proposed)
Vertebrate	Homo	HbA	Oxygen transport between tissues.
	Homo	Cytoglobin	May have role in tumor suppressor.
	Homo	Neuroglobin	May have role in neuroprotection.
Plant	Glycine	Lba	May sequester oxygen away from nitrogenase. May transport oxygen to electron transport chain in nodule.
	Glycine	nsHb	Intracellular oxygen movement.
Alga	Arabidopsis	nsHb	Nitric oxide deoxygenase activity.
	Chlamydomonas	CeHbt	Oxygen bound to Ll637 Hb can be reduced. It may serve to accept electrons, sequester oxygen or deliver oxygen inside the organelle.
Fungi	Saccharomyces	YHb (a flavohemoglobin)	Can transfer electrons from NADPH to heme iron. May serve to protect from oxidative stress.
Bacteria	Alcaligenes	FHb (a flavohemoglobin)	Proposed electron transfer. Possible role in anaerobic metabolism, perhaps gas metabolism during denitrification
	Vitreoscilla	VHb	Can serve as terminal electron acceptor during respiration. May scavenge oxygen.
	Mycobacterium	HbN & HbO	Nitric oxide scavenging.
	Thermofida	Tf-trHb	Oxygen binding and sensing proteins.

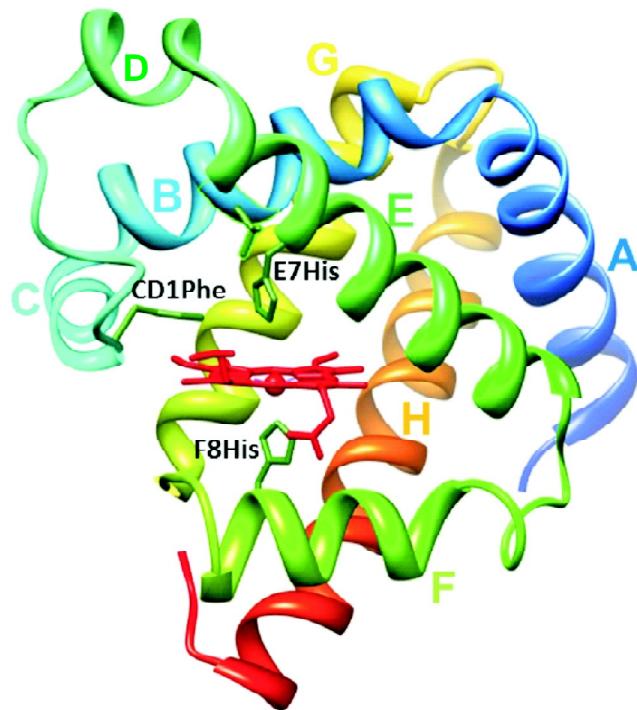


Figure 2: A typical myoglobin fold with heme prosthetic group and key residues in pockets

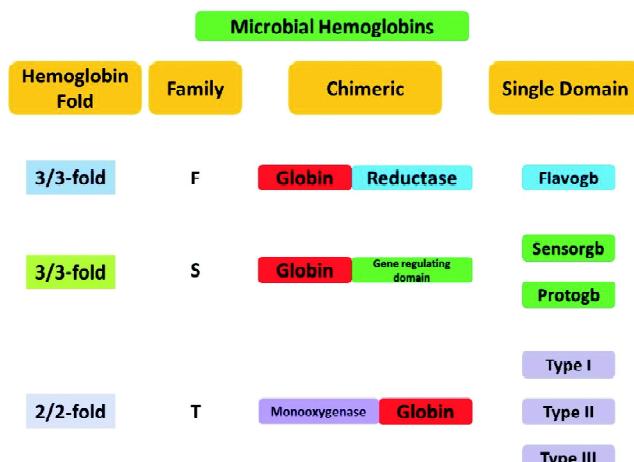


Figure 3: Schematic diagram showing classification of microbial hemoglobins. Figure adapted from (Vázquez-Limón *et al.*, 2012)

their linear sequence order. This globin fold results in formation of a hydrophobic pocket used for housing the heme prosthetic group. The heme iron is the site for ligand binding. The quaternary assemblages of these subunits are very diverse and it is this variation in assembling of the subunits and their mutual interaction that is responsible for playing an important role in ligand binding affinity of multimeric Hb by using its special property of cooperativity.

All the globins identified can be divided into two general classes, namely single chain globins and chimeric globins based on domain assemblies (Vinogradov *et al.*, 2006). The former group (Figure 3) comprises the vertebrate α - and β -globins, myoglobin (Mb), the plant symbiotic hemoglobins (SHbs), non-symbiotic hemoglobins (NsHbs), protoglobins (Pgbs) and the 2/2 Hbs. Chimeric proteins comprise of an N-terminal globin domain and NAD and FAD binding C-terminal domain like in flavohemoglobin or the globin coupled sensors (GCSs) with highly variable C-terminal domains. Thus, all globins belong to one of three globin lineages: the 3/3 FHbs (Family F), 3/3 single domain (SDgbs) of Family S (eukaryote and bacterial Hbs, the GCSs and Pgbs) and the 2/2 truncated Hbs of Family T (Vinogradov *et al.*, 2005). Of these only one of the three globin lineages, the 2/2 Hbs, are represented in all three kingdoms of life (Vinogradov *et al.*, 2006), although truncated hemoglobins are yet to be discovered in animal species.

When different hemoglobins were aligned, it could be noticed that a new group of hemoglobins (Family T), which are evolutionarily distinct, emerged with some unique features and termed as "truncated haemoglobin". These hemoglobins have truncation in N or C terminal (and elsewhere) which results in shortening of polypeptide length (Figure 4). The truncated hemoglobins typically showed a 2-on-2 alpha helical globin fold in their crystal structures, as opposed to the well-known 3-on-3 globin fold in classical hemoglobins (Family F and S). In this review, we discuss the characteristic features of this distinct class of hemoglobins often called truncated hemoglobins, including (a) their phylogenetic relationship with other hemoproteins and (b) description of their structure and ligand binding affinities. The discussions highlight new concepts regarding Hb structure and lead to potentially new functions of members within the Hb superfamily. It should, however, be mentioned that some hemoglobins with 2-on-2 fold did not show truncated polypeptide chains but were almost as long as the classical globins.

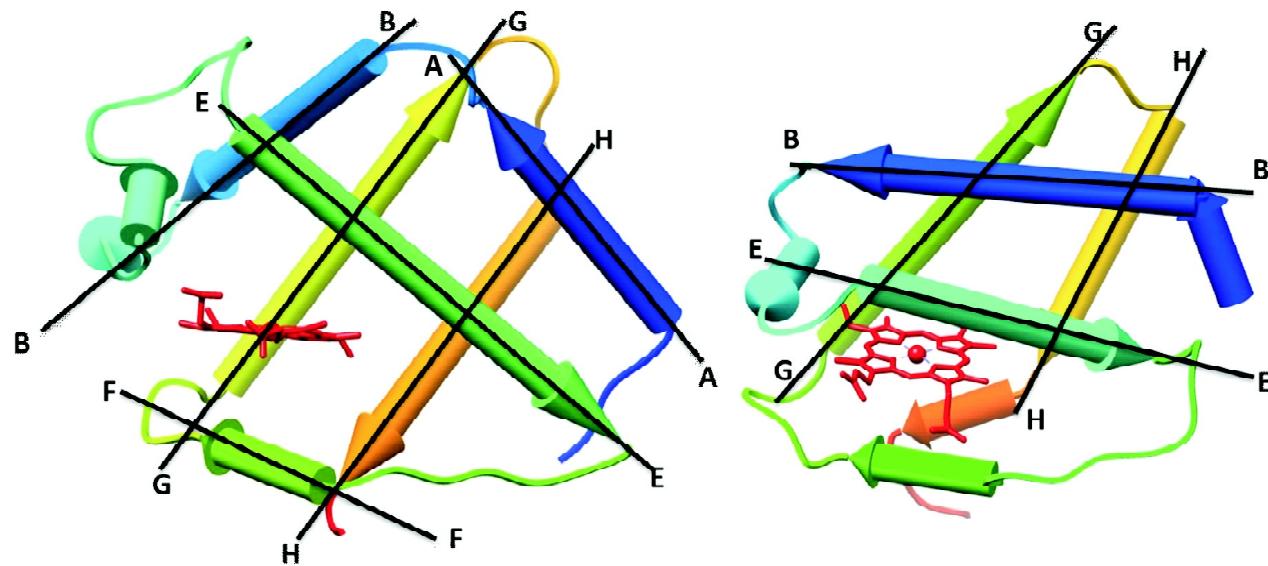


Figure 4: A comparison of 3-on-3 and 2-on-2 globin folds, representative of classical Hbs and truncated Hbs respectively

Truncated Hemoglobins: Phylogenetically Distinct Within Hemoglobin Superfamily

Truncated hemoglobins (trHbs) are small hemoproteins, found in bacteria, higher plants and unicellular eukaryotes, building a distinct phylogenetic group within the globin superfamily (Figure 5). No trHbs have been identified in animals yet (Milani *et al.*, 2005). Usually, their primary structure is 20-40 residues shorter than that of mammalian Hbs (Figure 6), thus resulting in shortened or deleted α -helices as well as modified interhelical loops. The trHb polypeptide chain is not simply a truncated version of a conventionally folded globin. Rather, it owes its conformational stability to residue deletions and substitutions at specific sites, as compared with non-vertebrate globins (Wittenberg *et al.*, 2002). TrHbs are, however, structurally distinct from *Cerebratulus lacteus* miniHb, the shortest Hb known composed of 109 amino acids (Pesce *et al.*, 2002).

Although truncated hemoglobins (trHbs) are found in diverse group, members of this family of 2/2 Hbs (Figure 5) have received a fair degree of attention because of their occurrence in a large number of different microbial species and their unusual globin fold, characterised by a two-over-two α -helical packing (Figure 4) instead of the classical three-over-three helical arrangement typical of vertebrate globins (Milani *et al.*, 2001).

Additionally, in contrast to vertebrate and other non-vertebrate hemoglobins, trHbs are characterised by a remarkable variability in the nature of the residues at the active site, especially in the distal side of the heme pocket (Wittenberg *et al.*, 2002). In turn, this feature may be related to their proposed diverse physiological roles as terminal oxidases (Wittenberg *et al.*, 2002), oxygen sensors and scavengers of oxygen and nitric oxide active species (Milani *et al.*, 2003a).

Crystal structures of truncated hemoglobins show that their fold is based not only on a subset of the “classical” globin fold (so-called 3-on-3 α -helical sandwich) like that of sperm whale myoglobin (Mb). Rather, it is seen that the 2/2 globin folds deviate remarkably from the conventional globin fold, some of them common to all truncated hemoglobins and some specific for different groups of trHbs (Figure 6). TrHb is a 2-on-2 α -helical sandwich (“2/2fold”) based on four α -helices, corresponding to the B-, E-, G- and H-helices of the classical globin fold. The antiparallel helix pairs (B/E and G/H) surround and protect the heme group from the solvent phase. G- and H- helices make up the rear face of the heme pocket, which matches very well with the globin fold topology (Nardini *et al.*, 2007). It is worthwhile to emphasize that the 2-on-2 fold differs from the 3-on-3 classical globin fold at several sites distributed over the whole

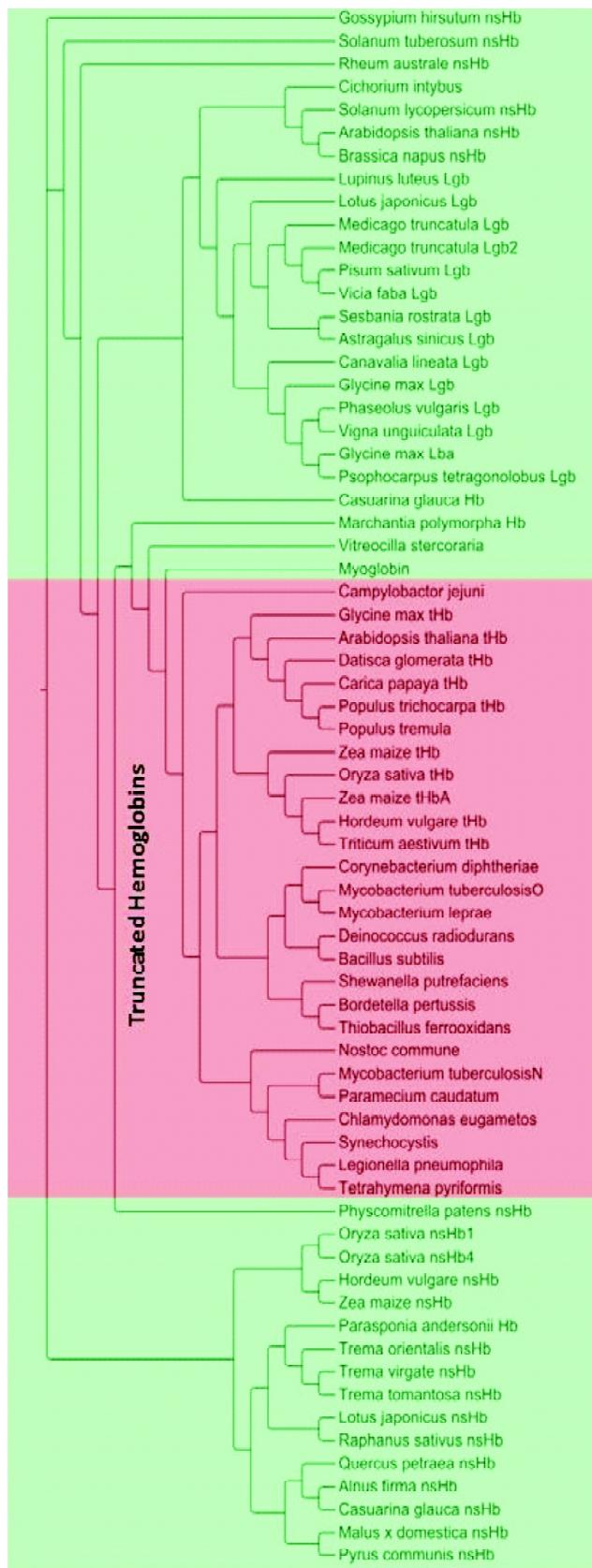


Figure 5: Phylogenetic analysis of hemoglobins shows distinct family of truncated hemoglobin

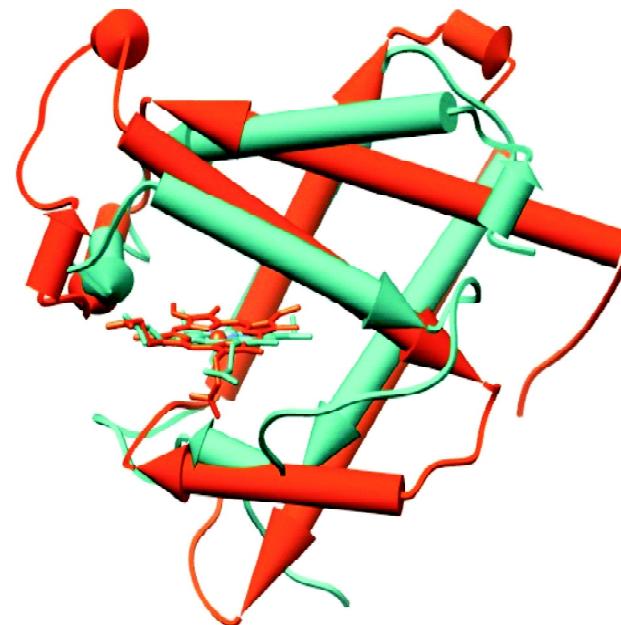


Figure 6: Superposition of two Hbs - myoglobin (5MBN) in red and Mtb trHbN (1RTE) in light sea green showing shortening in polypeptide chain and typical folds

polypeptide chain. The most noticeable differences between them are the drastically shortened A-helix, the absence of a D-helix, a long polypeptide segment (pre-F) in extended conformation, followed by a variable-length F-helix (in groups I and III this is reduced to one-turn helix) that properly supports the heme proximal HisF8 residue, allowing heme iron coordination.

Classification of Truncated Hemoglobins: Phylogeny, Structure and Functional Diversity

Since truncated hemoglobins are found in bacteria, unicellular eukaryotes, and higher plants, and occur with a high frequency in bacteria, it can be proposed that the trHb fold existed long before the vertebrate fold. Further phylogenetic analysis of the increasing number of amino acid sequences have shown that this new globin family (trHb) branches into three groups I (trHbN), II (trHbO), and III (trHbP) (Figure 7). It is observed that trHbs are orthologous within each group and paralogous across the groups (Vuletich and Lecomte, 2006). It is found from the analysis that Group I globins separate into two divergent subgroups. Group II is comparatively homogenous and Group III shows highest level of sequence conservation. An

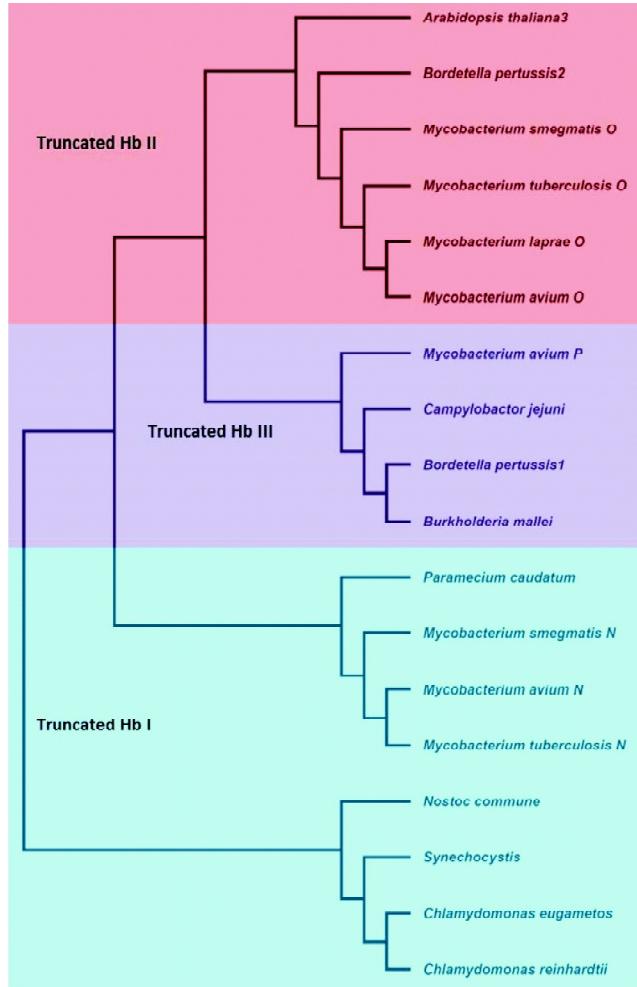


Figure 7: Diversity within truncated hemoglobins

analysis of the phylogenetic trees of each group predicts that the Group II trHb gene was the original gene, and the Group I trHb and Group III trHb genes were obtained via duplication and gene transfer events. Horizontal gene transfer (HGT) is thought to play a major role in the evolution of species. Presence of a Group II globin gene in almost all organisms having genes from more than one group leads to the conclusion that the Group II gene originated prior to the Group I and Group III genes (Vuletich and Lecomte, 2006).

(i) The phylogenetic analysis of group II trHb has major branches, one dominated by the actinobacteria and proteobacteria and the other dominated by the plants and fermicutes (Figure 7). This tree brings out two important inferences. First, the LCA (last common ancestor) of actinobacteria (Vuletich and Lecomte, 2006) and fermicutes appeared after the split from

proteobacteria but it seems that Group II globin genes of actinobacteria and group II globin genes of proteobacteria are more similar to each other than they are those of fermicutes. Second, all of the fermicute sequences come from the bacillales subdivision. These two pieces of evidence suggests that the second major branch of Group II globins was most likely the result of a gene transfer event to the ancestor of bacillales and subsequent transfer to the other divisions. In this view, the transfer originated from either proteobacteria or actinobacteria, and the gene subsequently evolved to perform a specific function in the bacillales. The plant sequences most likely arose from a HGT promoted by infection or a symbiosis event.

(ii) It is seen that genomes that contain a Group III globin gene contain a Group II globin gene as well. Only seven organisms contain solely a Group III globin gene (Vuletich and Lecomte, 2006). Thus, it appears that a gene duplication event occurred in an ancestral proteobacterium and for the most part both Group II and Group III genes were retained. In the seven cases above, the organism most likely lost the need for the Group II globin. Retention of the Group II globin gene suggests that the individual trHb groups have non-identical functions.

(iii) Group I tree contains two subgroups. The Group I gene apparently also originated in proteobacteria, likely before the emergence of Group III globins. It can be proposed that when proteobacteria and cyanobacteria split, cyanobacteria retained the Group I globin and discarded the Group II globin. No proteobacteria have both a Group I and a Group II globin gene, this suggests that two genes must have been in competition in their early history. The ancestor of *Mycobacterium* apparently obtained its Group I globin gene from HGT (Figure 1), after its Group II globin had diverged sufficiently from the incoming Group I globin to perform two necessary and different functions in that organism. The above scenario makes the transfer of the Group I globins to photosynthetic eukaryotes more easily as cyanobacteria could have transferred the gene to *Chlamydomonas* at the time of chloroplast generation.

Thus, from the phylogenetic analysis it is found that trHbIII prefers a high level of sequence conservation as will be discussed later. The phylogenetic analysis further suggests an evolutionary scenario where Group II HbO gene is the ancestral gene, and Group I and Group III genes are the result of duplication and gene transfer events (Nardini *et al.*, 2007).

Since trHbs are abundantly found in bacteria, their origin is thought to be very ancient. Some of the organisms hosting these ancient globins are pathogenic bacteria that perform photosynthesis, fix nitrogen, or have distinctive metabolic capabilities. Truncated Hbs from more than one group have been found to coexist in some bacteria, suggesting a wide diversification of functions. Such postulated functions, include long-term ligand or substrate storage, nitric oxide (NO) detoxification, O₂ /NO sensing, redox reactions and O₂ delivery under hypoxic conditions (Wittenberg *et al.*, 2002).

So far, four Group I trHbs – from *C. eugametos* (Ce trHb) (Pesce *et al.*, 2000), *P. caudatum* (Pc-trHb) (Pesce *et al.*, 2000), *M. tuberculosis* (Mt-trHbN) (Milani *et al.*, 2003a; Milani *et al.*, 2001) and *Synechocystis sp.* (Ss-trHb) (Hoy *et al.*, 2004; Lecomte *et al.*, 2001), *N. commune* (Couture *et al.*,

2000), three Group II trHbs – from *M. tuberculosis* (Mt trHbO) (Milani *et al.*, 2003a), *G. stearothermophilus* (Gs trHb) (Ilari *et al.*, 2007) and *B. subtilis* (Bs trHb) (Giangiacomo *et al.*, 2005), and one Group III trHb – from *C. jejuni* (Cj-trHbP or Cj-2/2HbP) (Nardini *et al.*, 2006; Wainwright *et al.*, 2006) – have been structurally characterized.

Comparative Study of TrhbI, TrhbII and TrHbIII

Structural features of the three classes of truncated haemoglobin are compared in Figure 8. While there are conserved features, some interesting differences do appear. It is seen that in trHbI, H-helix is partly bent with approximately 10 C-terminal residues deletion compared to sperm whale Mb. Moreover the whole CD-D region on the heme distal site is shortened to about 3 residues to bridge between C- and E-helices. Recent studies have shown a 3-7 amino acid insertion located between the C- and E-helices, which is a unique structural feature of Group III 2/2HbPs. Such elongation of the CD region spans the C- and E-helices of one carboxy-terminal and one amino-terminal turn relative to the corresponding helices in group I and group II structures, but does not shift the position of the E-helix relative to the heme distal site (Figure 8B).

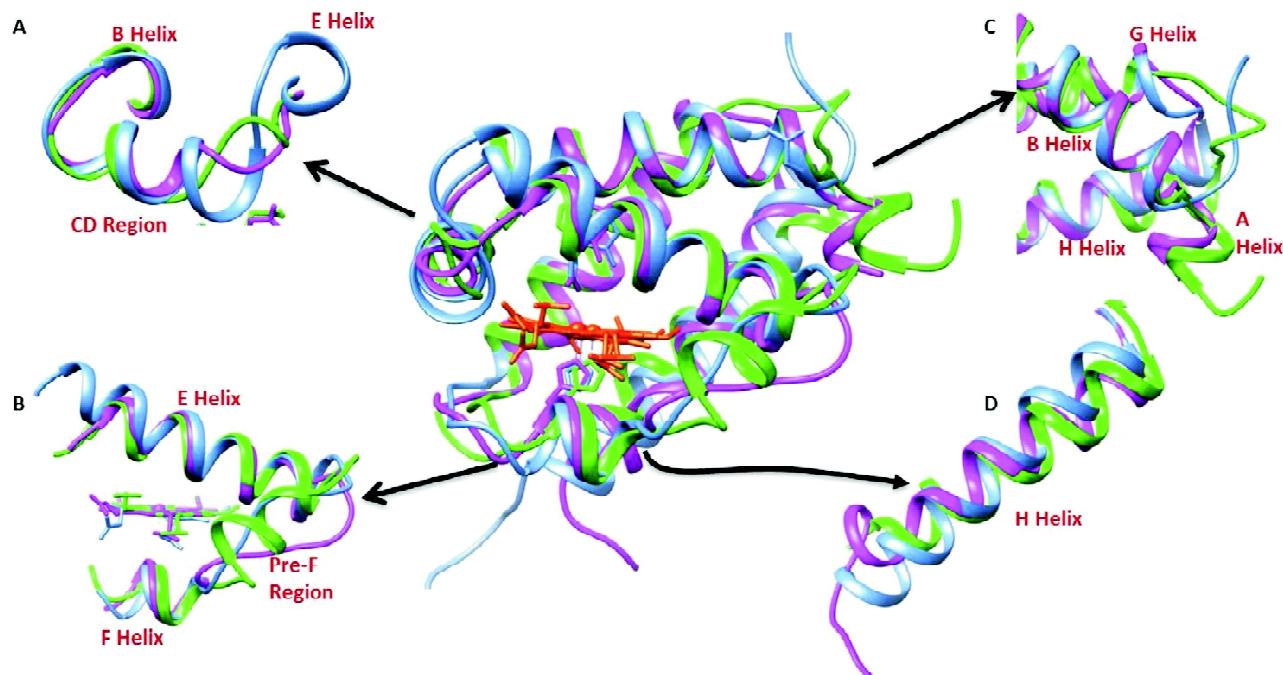


Figure 8: Comparative view of 2/2 globin fold within trHbI (1DLY) in magenta, trHbII (1NGK) in green and trHbIII (2IG3) in cornflower blue. A, B, C and D are the zoomed view of the area where major difference are observed

Additionally, in Group III 2/2HbP the C-helix displays a clear α -helical character, as compared to the 3_{10} helical characters commonly displayed not only by Group I and Group II 2/2Hbs but also by vertebrate globins (Bolognesi *et al.*, 1997).

In order to support the stabilization of the compact 2-on-2 fold, specific sequence motifs have been suggested. Among these, three conserved glycine motifs have been identified in Group I and II. They are located at the AB and EF interhelical corners and just before the short F-helix (the last present also in group III). The Gly-based motifs are the key sequence markers in

group I and II 2/2Hbs, providing the protein backbone flexibility needed to stabilize the short A-helix in a conformation locked onto the B- and E-helices, and supporting the pre-F segment in building a properly structured heme crevice in the context of a very short polypeptide chain. In Group III Cj-2/2HbP, due to the full deletion of the A-helix, the AB Gly-Gly motif is missing. The absence of the Gly-Gly motif at the AB hinge of Cj-2/2HbP does not provide the backbone flexibility, thus forcing the residues preceding the B-helix to extend towards the GH region, a conformation opposite to that found in Group I and II proteins.

Proximal Pocket and Heme Coordination

Table 2
Summary of residues lining the heme pocket in 2/2 globins (Nardini *et al.*, 2007)

	<i>Ce-</i> 2/2HbN	<i>Pc-</i> 2/2HbN	<i>Group I</i>		<i>Ss-</i> 2/2HbN	<i>Mt-</i> 2/2HbO	<i>Group II</i>		<i>Group III</i>
			<i>Mt-</i> 2/2HbN	<i>Nc-</i> 2/2HbN			<i>Bs-</i> 2/2sHbO	<i>Gs-</i> 2/2HbO	<i>Cj-</i> 2/2HbP
	(1DLY)	(1DLW)	(1IDR)	NMR str.	(1S69)b	(1NGK)	(1UX8)	(2BKM)	(2IG3)
C6	Val(29)	Val(29)	Leu(42)	Pro(33)	Ile(31)	Leu(32)	Leu(34)	Leu(33)	Leu(28)
C7	Tyr(32)	Phe(32)	Phe(45)	Val(34)	Phe(34)	Val(35)	Ile(37)	Ile(36)	Ile(31)
CD1	Phe(33)	Phe(33)	Phe(46)	Phe(35)	Phe(35)	Tyr(36)	Phe(38)	Phe(37)	Phe(32)
CD2						Pro(37)	Pro(39)	Pro(38)	
CD3									Ala(35)
CD4									Ile(36)
E2	Thr(36)	Ile(36)	Thr(49)	Thr(38)	Val(38)		Thr(45)	Thr(42)	
E5									
E6			Arg(53)	Lys(42)					
E10	Lys(44)	Lys(44)	Lys(57)	His(46)	His(46)	Arg(47)	Lys(48)	Lys(47)	Lys(49)
E11	Gln(45)	Thr(45)	Gln(58)	Leu(47)	Gln(47)	Leu(48)	Gln(49)	Gln(48)	Ile(50)
E14	Phe(48)	Phe(48)	Phe(61)	Phe(50)	Phe(50)	Phe(51)	Phe(52)	Phe(51)	Phe(53)
EF6	Trp(59)	Trp(59)	Tyr(72)	Tyr(61)	Tyr(61)	Tyr(62)	Tyr(63)	Tyr(62)	Tyr(64)
EF10						Arg(66)		His(66)	
F2	Lys(62)	Arg(62)	Ala(75)	Arg(64)	Arg(64)				
F4	Met(64)	Leu(64)	Met(77)	Met(66)	Met(66)	Leu(71)	Leu(72)	Leu(71)	Pro(68)
F7	Ala(67)	Val(67)	Val(80)	Thr(69)	Ala(69)	Arg(74)	Arg(75)	Arg(74)	Lys(71)
FG3	Leu(71)	Met(71)	Arg(84)		Leu(73)	Phe(78)	Phe(79)	Phe(78)	Leu(75)
FG5		Val(73)	Ile(86)	Leu(73)		Ile(80)	Ile(81)	Ile(80)	
FG6									Phe(78)
FG7	Leu(75)			Leu(75)					
FG9					Leu(79)				
G5	Phe(80)	Phe(78)	Phe(91)	Phe(80)	Phe(84)				Phe(83)
G8	Val(83)	Val(81)	Val(94)	Ile(83)	Val(87)	Trp(88)	Trp(89)	Trp(88)	Trp(86)
H11	Val(108)	Ala(106)	Ile(119)	Val(108)	Ala(112)	Leu(116)	Leu(114)	Leu(113)	Ala(110)
H15						Ala(120)	Ala(118)	Ala(117)	
H16					His(117)				
H18	Val(115)	Val(113)	Val(126)	Met(111)		Leu(123)	Met(121)	Met(120)	Phe(117)
H20					Val(121)				

From Table 2 (Nardini *et al.*, 2007) we can see that the most important residues lining the heme crevice and important for protecting and stabilizing the bound heme group are placed mostly at conserved topological positions all through the 2/2Hb family. Hydrophobic residues at positions C6, C7, CD1, E14, F4, FG3, G8, and H11 provide an effective network of van der Waals contacts to the heme in all 2/2 globins. Other protein-heme interactions may arise from residues located in the CD and FG segments and the amino-terminal part of the H-helix. Further stabilizing contacts are provided by hydrogen bond interactions between the heme and polar residues.

The conformation of the Fe-coordinated proximal F8His (the only residue 100% conserved in globins) is typical of an unstrained imidazole ring that facilitates the heme in-plane location of the iron atom, with the imidazole plane lying in a staggered orientation relative to the heme-pyrrole nitrogen atoms, thus increasing covalency of the Fe-HisF8 bond supporting fast oxygen association and electron donation to the bound distal ligand.

Distal Site and Ligand Binding

The trHb heme distal site cavity, hosting the exogenous ligands, is characterized by a repertoire of unusual residues, as compared to classical (non)vertebrate Hbs and Mbs. The distal side E-helix lies very near to the heme in 2/2 globins, due to the shortened CD region, thus forcing side chain crowding of the distal site residues at the topological positions B10, CD1, E7, E11, E14, E15, and G8 (Table 3). Among these residues some are polar, thus allowing the establishment of a network of hydrogen bonds useful not only for an efficient stabilization of the diatomic heme ligand, but also for the rebinding kinetics of dissociated ligands. The distal B10 residue is almost fully conserved as Tyr (Table 3; the only exception in group I is *N. commune* trHb, hosting a His residue). The CD1 site, invariantly Phe in most (non)vertebrate globins, may host Tyr or Leu. The very short span of the CD-D region (about four residues in all trHbs) locates the E-helix next to the heme distal face. The distal E7 residue, His in most (non)vertebrate Hbs, can be Gln, Thr, His, Ser, Leu or Ala, in trHbs (Table 3). Distal site polarity is expected to favor oxygen

Table 3
Selected residues involved in heme interaction and in ligand binding

Secondary Structure(→) Species(↓)	B10	CD1	E7	E11	E15	G8	
Sperm whale	L	F	H	V	L	I	
<i>V.stercoraria</i> FHb	Y	F	Q	L	V	V	
<i>N.commune</i>	H	F	Q	L	L	I	Group I
<i>P.caudatum</i>	Y	F	Q	T	L	V	
<i>C.eugametos</i>	Y	F	Q	Q	L	V	
<i>Synechocystis sp.</i>	Y	F	Q	Q	L	V	
<i>M.smegmatis</i> N	Y	F	L	Q	F	V	
<i>M.tuberculosis</i> N	Y	F	L	Q	F	V	
<i>M.avium</i> N	Y	F	L	Q	F	V	
<i>T.fusca</i>	Y	Y	A	L	L	W	Group II
<i>M.smegmatis</i> O	Y	Y	A	L	L	W	
<i>M.tuberculosis</i> O	Y	Y	A	L	L	W	
<i>M.avium</i> O	Y	Y	A	L	L	W	
<i>M.leprae</i> O	Y	Y	A	L	L	W	
<i>B.subtilis</i>	Y	F	T	Q	L	W	
<i>C.jejuni</i>	Y	F	H	I	W	W	Group III
<i>M.avium</i> P	Y	F	H	M	W	W	

chemistry in the heme crevice, as in peroxidases (Hiner *et al.*, 2002).

Distal pocket of group I truncated hemoglobins has hydrogen bonding network, which involves residues at B10, E7 and E11. In crystal structure of Mtb HbN, it is clearly seen that B10Tyr forms hydrogen bond to stabilize the ligand and interacts further with E11Gln. Similar observation was made in other group I trHbs like in *Chlamydomonas eugametos* Hb and *Paramecium caudatum* Hbs, where B10Tyr buried in the inner part of heme pocket and properly oriented through hydrogen bonds to E7Gln and E11Gln/Thr. This hydrogen bonding network provides stabilization to heme bound ligands.

Group II trHbs show some variation in distal pocket to adopt a proper size and environment relative to group I. B10Tyr is still involved in direct hydrogen bonding to ligands. E11Gln, G8Trp, and E7Thr complete the polar distal frame, with E11Gln side chain and the G8Trp indolic nitrogen atom at hydrogen bonding distance to the bound ligand. Presence of Tyr at CD1 position instead of Phe drastically modify the hydrogen bonding network in this group and is involved in ligand stabilization in the pocket. Further stabilization is provided by G8Trp instead of E11, which is leucine, a nonpolar amino acid.

Structural and sequence analysis indicated that the property of residues at CD1 and E11 sites are correlated in group II trHbs. They display a Tyr residue at position CD1 and have a nonpolar residue at the E11 site (Giangiacomo *et al.*, 2005; Milani *et al.*, 2003b; Vuletich and Lecomte, 2006). However, when a Phe (a non-hydrogen bonding residue) is found at CD1, a hydrogen bond donor is present at the E11 site (Gln or Ser). Thus, in common one of hydrogen bonding residue involved in ligand stabilization is alternatively located either at E11 or CD1 position.

Also, in group III trHbs, B10Tyr and G8Trp are conserved. In this group, heme distal pocket is decorated with B10Tyr, CD1Phe, E7His, E11Ile, E14Phe, and G8Trp in surrounding of the heme-bound ligand. TrpE15 is also conserved but does not contact directly with the bound ligand. Thus, in group III, the only hydrogen bonding element involved in ligand stabilization, besides B10Tyr, is G8Trp. No further stabilization is provided by

residues at CD1 and E11 positions, nor from E7His. In Cj-2/2HbP E7His is present in two alternate conformations, "open" and "closed" corresponding to the side chain pointing towards the solvent or to the heme distal site, respectively, like in myoglobin.

Ligand Access to the Heme Group through A Tunnel System

X-ray crystal structures have shown that trHb consists of a two-branched protein matrix tunnel system (Couture *et al.*, 2000) which helps in controlling diatomic ligand migration to the heme (Figure 9). There are two main paths for O₂ migration, following a long (~16 Å) and a short molecular tunnel (~10 Å) in the protein matrix perpendicular to each other having an L-shaped path through the protein matrix, respectively (Figure 9). The long tunnel corresponds to that identified by (Milani *et al.*, 2003a) from an analysis of the protein crystal structure. In the same work, these authors proposed that a short tunnel could also be operative for ligand migration. A novel dual-path mechanism has been suggested to drive migration of O₂ and NO to the distal heme cavity. While oxygen migrates mainly by the short path, a ligand-induced conformational change regulates opening of the long tunnel branch for NO, via a phenylalanine (E15Phe) residue that acts as a gate. As shown in Figure 9, the long tunnel connects the heme distal site with the solvent at a location between the B, E, and G

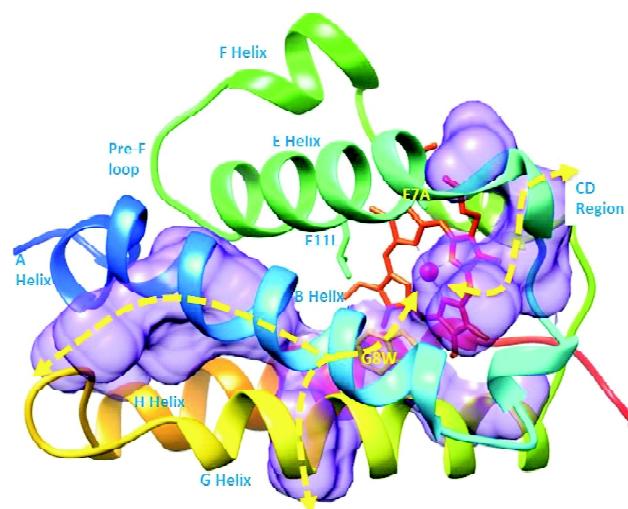


Figure 9: View of the long and short tunnel of trHbO (1NGK). The heme group is shown in red. The long and short tunnels are depicted in purple

helices (Boechi *et al.*, 2008). The relevant residues along the short tunnel are E7Ala, B10Tyr and CD1Phe, and the heme propionate group in trHbN, while Tyr replaces Phe at CD1 in case of trHbO.

Once the oxygen is bound to the Fe atom, a second ligand (i.e., NO) could migrate into the heme distal site of the oxygenated trHb (trHbO-Oxy) and react to yield nitrate (NO detoxification is discussed in detail later). The location and size of the hydrophobic tunnels in Group I trHbs advocate significant roles in controlling diffusion of small ligands to/from the heme distal pocket or in short-term ligand storage and/or accumulation (Milani *et al.*, 2004a; Mishra and Meuwly, 2009; Samuni *et al.*, 2003). Group II globins do not have a well-defined tunnel as found in group I. In fact, the surface of the characterized Group II globins exhibits a shallow depression on the proximal side of the heme. This depression provides partial solvent access to the heme C-pyrrole and is mostly lined with hydrophobic residues, namely E14Phe, E18Tyr, F4Leu, H10Tyr, H14Ala, and H18Leu. Of these positions, E14, F4, and H18 are strongly conserved and H14 is hydrophobic. It has been suggested that this depression may have functional significance, perhaps in redox chemistry. If so, variations at the H10 position may serve to tune the electrostatic environment of the heme group. Whether or not redox chemistry occurs, the hydrophobic depression may serve as a docking site for a reaction partner.

Group III trHb has the largest proportion of highly conserved sequences, most coming from the proteobacteria. As far as heme pocket positions are concerned Group III globins resemble more closely Group II than Group I globins. The pronounced identity level within Group III raises the expectation that characterization of a limited number of such proteins will be helpful to derive a relationship between sequence and activity across the group and contribute new insight into the adaptability of the trHb fold.

Ligand Binding

The ligands O₂, NO, and/or CO combine with the ferrous deoxygenated derivative of trHbN and

trHbO at similar rates (Milani *et al.*, 2005). These ligand-independent combination rates indicate that the electronic factors that give rise to the large ligand-specific differences in most (non) vertebrate Hbs are not operative in trHbs. Ligand binding to trHbs appears to be mainly limited by intra-protein ligand diffusion. Mechanisms involving ligand accumulation in the trHb matrix should also be considered, the trHb tunnel/cavity systems being potentially fit to accommodate several CO molecules (Milani *et al.*, 2004b). Ligand accumulation within the globin matrix may explain heme-catalyzed multiligand reactions (e.g., NO scavenging) as well as ligand binding cooperativity and multiexponential kinetics in monomeric trHbs (Ouellet *et al.*, 2007; Samuni *et al.*, 2003).

Cyanide binding to trHbs

Cyanide is a diatomic ligand capable of binding to ferric & ferrous heme Fe atom. The reactivity of ferric and ferrous (non)vertebrate heme proteins toward cyanide is influenced mainly by the presence of proton acceptor in the heme distal pocket and donor group(s), whose function is to assist the deprotonation of the incoming ligand and the protonation of the outgoing cyanide (Milani *et al.*, 2004b).

The complexes formed between cyanide and ferric heme-proteins are very stable, their stability is primarily determined by the rate of ligand dissociation (i.e., kCN). Kinetics of cyanide dissociation have been shown to be limited mainly by the protonation of the outgoing ligand, reflecting the entirely different hydrogen bond networks that link the heme-Fe-bound cyanide and heme distal residue(s). By contrast, values of the second order rate constant (i.e., k'CN) for cyanide binding to (non)vertebrate globins (characterized by the so-called 'E7 gate', such as sperm whale Mb) (Bolognesi *et al.*, 1982; Perutz, 1989) and to trHbs (characterized by the protein matrix tunnel/cavity system) (Milani *et al.*, 2004a; Milani *et al.*, 2003a; Milani *et al.*, 2001; Pesce *et al.*, 2000; Wittenberg *et al.*, 2002) are closely similar (Table 4) (Milani *et al.*, 2004b). This observation suggests that cyanide binding to the heme Fe-atom is not primarily affected by the protein structural properties defining the overall

diffusion mechanism to the heme distal pocket; rather, it is influenced mainly by the presence of proton acceptor group(s) in the heme distal pocket, whose function is to assist the deprotonation of the incoming ligand (Brancaccio *et al.*, 1994; Milani *et al.*, 2004b). Interestingly, values of $k'CN$ and kCN for cyanide binding to trHbs and sperm whale Mb are similar (Table 4), possibly reflecting a striking case of

convergent evolution (Bellelli *et al.*, 1990; Milani *et al.*, 2004a).

The affinity of cyanide for Cj-trHbP(II) is higher than that reported for any known (in) vertebrate globin by more than three orders of magnitude ($K = 1.2 \times 10^{-6}$ M). The very high affinity of cyanide for Cj-trHbP(II) suggests that this globin may participate in cyanide detoxification (Bolli *et al.*, 2008).

Table 4
Kinetic parameters for CN- binding to ($k'CN$) and dissociation from (kCN) (non)vertebrate globins as well as for reduction of heme-Fe(III)-cyanide complexes

(non) vertebrate globin	k_{on} ($M^{-1} s^{-1}$)	h ($M^{-1/2} s^{-1}$)	k_{off} (s^{-1})
C. eugametos trHb	4.6×10^2	3.5	1.6×10^{-2}
M. tuberculosis trHbN	3.8×10^2	3.1	1.2×10^{-2}
M. tuberculosis trHbO	3.2×10^2	2.8	1.3×10^{-2}
A. limacina Mb	2.0×10^2	$\geq 1 \times 10^2$	2.2×10^{-2}
sperm whale Mb	1.4×10^2	4.4	2.1×10^{-2}
horse heart Mb	1.7×10^2	8.0×10^1	1.5×10^{-1}
P. equorum Hb	n.d.	8.0×10^{-2}	1.1×10^{-2}
S. inaequivalvis HbI	2.3×10^2	0.60	1.9×10^{-2}
human Hb tetramer	1.1×10^2	2.0×10^{-1}	1.2×10^{-1} (R-state) 1.5 (T-state)
human Hb α -chain	1.7×10^2	2.0×10^{-2}	1.7×10^{-1}
human Hb β -chain	1.6×10^2	1.8	1.5×10^{-1}

h is the rate constant for the reduction of trHb(III)-CN- by dithionite to the transient species.

Sulfide binding to trHb

To date, the highest affinity sulfide binding hemoglobin is represented by hemoglobin I (HbI) from the mollusk *Lucina pectinata*, a monomeric protein that binds sulfide (H_2S) in its ferric state (Kraus and Wittenberg, 1990; Kraus *et al.*, 1990). This hemoglobin is one of the few known H_2S carriers in living organisms that have been shown in physiological binding and transporting this toxic gas in the ferric heme iron center. The affinity of hydrogen sulfide for ferric HbI is exceptionally high compared to those for other hemoglobins and is achieved through fast association and very slow dissociation processes. Many bacteria reduce inorganic sulfate to sulfide for the purpose of incorporation into newly synthesized molecules through the so-called pathways of assimilatory sulfate reduction.

Thermodynamics and kinetics of sulfide binding

The data highlights unexpected sulfide binding properties of Bs-trHb and Tf-trHb and demonstrate that these proteins are able to bind sulfide with an affinity constant in the submicromolar range (Nicoletti *et al.*, 2010).

Table 5
Thermodynamic and kinetic constants for sulphide binding to the truncated hemoglobins.
Table extracted from (Nicoletti *et al.*, 2010)

Protein	$K_{(HS)}$ (M^{-1})	$k_{(HS)}$ ($M^{-1}s^{-1}$)	$k'_{(HS)}$ (s^{-1})
Bs-trHb	5.0×10^6	1.3×10^4	0.0026
Tf-trHb	2.8×10^6	5×10^3	0.0018
L. pectinata HbI	1.1×10^7	2.3×10^5	0.00022
L. pectinata HbII	1.7×10^7	1.13×10^4	0.017
L. pectinata HbIII	2.5×10^7	1.4×10^4	0.016

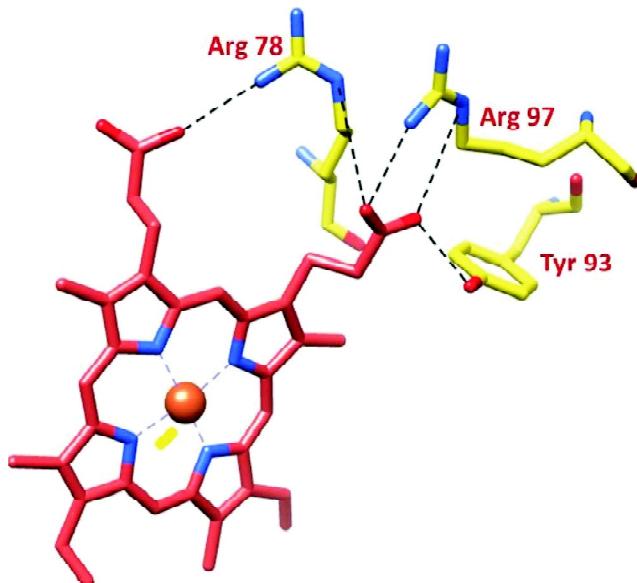


Figure 10: Schematic representation of wtTf-trHb residues involved in stabilization of the heme propionates, when SH bind to heme iron

Sulfide may coordinate to the heme iron in the monoprotonated form, HS-, or in the diprotonated one, H₂S, since the pK for this equilibrium is reportedly ≈ 7 . A comparison between the sulfide binding properties of *L. pectinata* hemoglobins and those of the truncated hemoglobins Bs-trHb and Tf-trHb reveals several analogies but also striking differences (Nicoletti *et al.*, 2010). In all proteins, the kinetic counterpart of the thermodynamic stability of the ferric sulfide adduct is the relatively fast second-order combination kinetics and the very slow sulfide dissociation rate. All proteins are also capable of reacting with sulfide in their ferrous oxygenated forms, a reaction that is most likely accompanied by iron oxidation and release of a superoxide anion. However, *L. pectinata* HbI has been shown to promptly release sulfide upon heme iron reduction, whereas ferric sulfide adducts of Bs-trHb and Tf-trHb are not reduced even by strong electron donors such as sodium dithionite (Nicoletti *et al.*, 2010).

The structural determinants for sulfide binding in bacterial trHbs can be traced back to the properties of the distal heme cavity. The structure of sulfide-bound Hb suggested two different kinds of interactions between the bound ligand and the relevant residues within the distal heme pocket, namely, H-bonding between sulfide

and E7Gln and sulfide-aromatic ring electrostatic interactions with CD1Phe and E11Phe (Nicoletti *et al.*, 2010).

From Figure 10 it can be inferred that a substantial rearrangement of the heme propionate geometry also occurs in Bs-trHb or Tf-trHb upon sulfide binding. In summary, the presence of an aromatic cage and a hydrogen bond donor appear to account for the structural determinants that govern the thermodynamic properties of sulfide binding in hemoglobins.

Truncated Hemoglobins Facilitate Nitric Oxide (NO) Scavenging

Two functions have been suggested for trHbs of plants: NO detoxification similar to that of class I plant Hbs and a function in O₂ transport (Pawlowski, 2008). Assuming a function of trHbs in NO detoxification, it appears plausible that either class I Hbs or TrHbs could be recruited for NO scavenging in nodules since NO is formed during N₂ fixation (Cueto *et al.*, 1996). NO has been suggested to mediate the integration of O₂ uptake, respiratory control and ATP availability. Even well-aerated nodules, like roots, will be frequently challenged with limited O₂ supply. It seems plausible that the basic levels of NO required for the integration of O₂ uptake and respiratory control are higher in a well aerated tissue than in an microaerobic tissue, and that these higher basic levels of NO also necessitate a higher capacity of the NO scavenging system (Ascenzi *et al.*, 2006b). Following are two such examples where it is proved that trHbs do help in scavenging NO:

(i) **Leprosy:** Leprosy is an old, still dreaded infectious disease caused by the slow-growing obligate intracellular bacterium *Mycobacterium leprae*. During infection, *M. leprae* is challenged by the toxic activity of reactive nitrogen species primarily nitrogen monoxide (NO), produced by activated macrophages. The chronic nature of leprosy implies that *M. leprae* has the ability to persist in the host despite the local production of reactive nitrogen species. *M. leprae* truncated hemoglobin GlbO has been proposed to represent merging of both O₂ uptake/transport and scavenging of nitrogen reactive species (Ascenzi *et al.*, 2006b).

Under aerobic conditions, the reaction of the ferrous oxygenated derivative of heme-proteins (heme-Fe(II)-O₂) with NO occurs, reflecting the superoxide character of the heme-Fe(II)bound O₂. The products of this reaction are heme-Fe(III) and NO₃. Under anaerobic conditions, NO has been reported to be converted to N₂O (Herold, 1999; Herold and Fago, 2005). NO scavenging is considered as a 'pseudo-enzymatic process' since it needs a reductase partner(s) to restore heme-Fe(II) and starting a new catalytic cycle (Ascenzi and Visca, 2008; Brunori *et al.*, 2004; Flogel *et al.*, 2001; Frauenfelder *et al.*, 2003; Frey and Kallio, 2005; Gardner *et al.*, 2000; Gow *et al.*, 1999; Herold and Fago, 2005; Poole, 2005; Wu *et al.*, 2003). Since *M. leprae* survives inside macrophages, it is supposed to avoid the deleterious effects of reactive oxygen species (e.g. H₂O₂). Three alternative mechanisms have been proposed to contribute to H₂O₂ resistance in *M. leprae*: (a) the reduced production of H₂O₂ by *M. leprae*-infected macrophages (Marolia and Mahadevan, 1989), (b) the production of alternative (katG-independent) catalase activity (Kang *et al.*, 2001) and (c) the scavenging activity of cell-wall-associated glycolipids (Chan *et al.*, 1989). It is proposed that under anaerobic and highly oxidative conditions, as in the macrophagic environment where *M. leprae* is faced with H₂O₂ (Ascenzi and Visca, 2008; Cooper *et al.*, 2002; Klebanoff and Shepard, 1984; MacMicking *et al.*, 1997; Nardini *et al.*, 2007; Nathan and Shiloh, 2000; Schon *et al.*, 2004; Scollard *et al.*, 2006; Visca *et al.*, 2002; Winrow *et al.*, 1993), the rapid formation of *M. leprae* trHbO-Fe(IV)=O occurs, which in turn facilitates NO scavenging, leading to the formation of heme-Fe(III) and NO₃.

As a whole, *M. leprae* trHbO-Fe(IV)-O facilitates NO detoxification. In turn, NO and NO₂ can serve as antioxidants of the highly oxidizing heme-Fe(IV)=O species. Therefore, *M. leprae* trHbO could be involved in both NO and H₂O₂ scavenging without needing a reductase partner(s) (Ascenzi *et al.*, 2006a; Ascenzi *et al.*, 2008).

(ii) Invasion by *Mycobacterium tuberculosis* in the host body: Tuberculosis remains an urgent global health problem and one third of the world population is infected latently (Norbis *et al.*, 2013;

World Health Organization, 2008). In most healthy individuals, the initial *M. tuberculosis* infection is contained by the immune system, which forces the bacteria to enter into a latent state, often for several years, with probable reactivation later in life (Ulrichs and Kaufmann, 2002). The initial event in this infection involves entry and multiplication within inactivated macrophages. Within the macrophages, the bacteria multiply in a specialized vacuolar compartment called the phagosome. After an early rapid growth phase, infected macrophages are fenced by newly recruited activated macrophages to form the specific granuloma. Nitric oxide (NO) produced by the activated macrophages contributes strongly to induction and maintenance of bacilli latency (Figure 11) (Yang *et al.*, 2009). Advances in free radical research revealed that reactive oxygen or reactive nitrogen intermediates production by immune cells play a crucial role in host defence mechanism against bacterial or viral infections (Yang *et al.*, 2009). Macrophages are believed to play a pivotal role in immune response against the mycobacterium infection by production of NO via the action of enzyme NO synthase (Figure 11). The increase in reactive nitrogen intermediates through reactive nitrogen oxides can modify bacterial DNA (deaminate and direct damage by generating abasic sites and strand breaks) (Richardson *et al.*, 2009). Thus tuberculosis infection is in active balance that teeters for years in a competition between host immunity and *M. tuberculosis* growth, specifying that an endogenous mechanism for NO resistance is operative in the tubercule bacillus (Long *et al.*, 1999). How *M. tuberculosis* manages with NO therefore is a key issue. NO also combines at near diffusion-limited rate with superoxide produced by respiration cells to form the highly oxidizing agent peroxynitrite (Pacher *et al.*, 2007). In response to the host defence, microorganisms have developed various resistance mechanisms by which toxic effects of NO can be evaded. *M. tuberculosis* encodes small, truncated Hbs (trHbs). *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, express two genes, glbN and glbO, encoding distantly related truncated hemoglobins (trHbs), GroupI (trHbN) and Group II (trHbO), respectively (Ouellet *et al.*, 2002). These

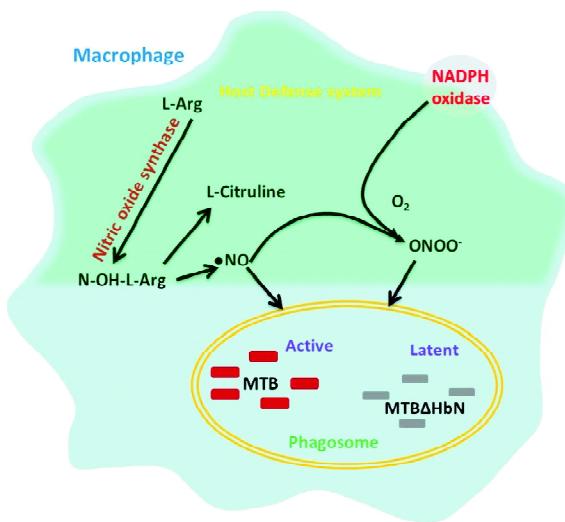
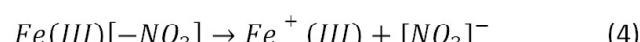
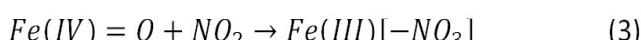
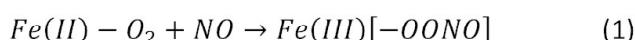


Figure 11: Anti-mycobacterial functions of NO in mycobacterial infection. Produced NO and RNIs can kill intracellular Mtb in the infected cell and trHbN may play a crucial role in scavenging of NO in Mtb and protect it

genes are not crucial for cell growth and its morphology, but are crucial for NO metabolism. It was shown experimentally that B10Tyr is crucial for NO detoxification (Ouellet *et al.*, 2002). TrHbs from *M. tuberculosis* was found to oxidize NO to nitrate *in vitro* and 20-fold faster than Mb. Again, E15Phe residue, which acts as a probable gate at long tunnel, plays a key role in mediating the access of NO to the oxygenated protein as mutation of this residue drastically reduce the nitric oxide dioxygenase (NOD) activity (Oliveira *et al.*, 2012). Thus, it can be concluded that:

- (a) trHbN is critical for NO metabolism *in vivo*.
- (b) trHbN is not essential for cell growth.
- (c) B10Tyr is necessary for NO detoxification by trHbN (deletion of pre-A region from the *M. tuberculosis* HbN drastically reduces its ability to scavenge nitric oxide) (Igarashi *et al.*, 2011; Lama *et al.*, 2009; Mukai *et al.*, 2004).
- (d) trHbN oxidises NO to nitrate (the reaction mechanism is given below (Mishra and Meuwly, 2010).



Thus Mt trHbN, *in situ* in living cells, rapidly removes NO and protects aerobic respiration from NO inhibition under biologically relevant O₂ tensions. In addition, it may reduce the formation of the highly reactive peroxinitrite, thereby preventing the irreversible oxidation and nitration of proteins. trHbN thus might be important to the bacilli under conditions where O₂ supply is limited and NO concentration is elevated such as is anticipated in the case of granuloma or during the reactivation of the bacilli (Ouellet *et al.*, 2002; Poole, 2005). In trHbN, as in other trHbs studied, x-ray and resonance Raman spectroscopy indicated a very crowded pocket in which solvent access through the E7 gate is impaired totally (Ouellet *et al.*, 2002). However, a route for ligand diffusion to and from the heme is present as a hydrophobic tunnel or cavity network through the protein matrix. Such a guiding tunnel may allow efficient diffusion of ligands to the active site. It also may accelerate the NO detoxification reaction by transiently storing several reactants NO molecules. The presence of two different Hb encoding genes in the genome sequence of *M. tuberculosis* indicates that these two hemoglobins may have distinct functions in the oxygen metabolism of the tubercle bacillus (Pathania *et al.*, 2002; Pawaria *et al.*, 2008). Structural analysis and comparison of the biochemical properties of HbN and HbO suggested that these two mycobacterial hemoglobins differ in many ways and may have distinct cellular functions (Pathania *et al.*, 2002). Sequence comparison of various HbN and HbO type trHbs suggests that, despite having structural conservation of the main protein regions thought to be crucial for the stabilization of the trHb fold, there are major differences within the pre-EF and F-loop regions of HbN and HbO (Pathania *et al.*, 2002; Pesce *et al.*, 2000). Therefore, these differences between HbO and HbN may result in differences in their functional properties.

Nitrogen Fixation

NO is a double-edged sword for plants: on the one hand, it is toxic to cells but, on the other hand, together with ROS, it contributes to triggering a hypersensitive response (HR) to prevent the propagation of bio-trophic pathogens (Jokipii-Lukkari *et al.*, 2009). N₂ fixation is catalysed by

the enzyme nitrogenase which is highly sensitive to O₂ and can only function in an O₂- free environment (Bergersen, 1962; Pawlowski, 2008). For aerobic bacteria like rhizobia and frankia strains, this leads to the so-called oxygen dilemma of nitrogen fixation: a high O₂ flux to the respiratory chain is required adjacent to a vanishingly low O₂ concentration at the sites of N₂ fixation (Pawlowski, 2008). In order to solve this problem, external O₂ barriers are combined with high O₂ utilization at the nitrogenase site to obtain a steep O₂ gradient. Only a few prokaryotes can create their own O₂ diffusion barrier that enables them to perform N₂ fixation in air. This group includes frankia strains which produce specialized vesicles whose cell walls show high O₂ diffusion resistance. Frankia strains can perform oxygen protection of nitrogenase themselves by forming specialized cells, vesicles, surrounded by multi-layered envelopes containing bacterial steroid lipids (hopanoids) at the ends of hyphae or short side branches, where nitrogenase can be active under aerobic conditions (Benson and Silvester, 1993). On the other hand, rhizobia rely on the plant host to provide oxygen protection for nitrogenase. Legume nodules are stem-like organs with peripheral vascular bundles in the so-called nodule cortex and the rhizobia-containing cells in the inner tissue (Kraiser *et al.*, 2011). The nodule endodermis, a part of the nodule cortex that is interrupted at the nodule apex, forms the O₂ diffusion barrier, protecting the bacteria-containing tissue while leaving the vascular system well aerated. O₂ concentrations in the inner tissue are very low, in the range of 50 nM. In order to provide a high O₂ flux for respiration, an O₂ binding protein, leghemoglobin (Lb), is formed in the infected cells in mM concentrations (Appleby, 1984). Lb facilitates O₂ diffusion to the N₂ fixing bacteria (bacteroids) and plant mitochondria (Pawlowski, 2008).

Summary

The exciting world of hemoproteins covers all kingdoms of life. Research on plant hexacoordinated non-symbiotic hemoglobins (nsHbs), and especially trHbs, is still in its infancy. In plants, three types of Hbs have been identified: symbiotic, non-symbiotic (nsHb) and truncated

(2/2) Hbs (tHbs) (Ross *et al.*, 2002). Plant Hbs were first identified by Kubo (Kubo, 1939). Kubo's plant Hb was named as leghemoglobin (Lb) by Virtanen and Laine (1946) and is also known as plant symbiotic Hb. Compared to Lbs and nsHbs, little is known about the function of plant 2/2-like Hbs. Since trHbs have only recently been discovered in higher plants (Watts *et al.*, 2001), the number of studies analysing their functional roles is very limited and no information similar to that for some bacterial globins is yet available for trHbs. Structures of plant truncated hemoglobins are yet to be solved. It was postulated that these proteins originated (by HGT) from a bacterial trHb (Vinogradov *et al.*, 2006), so it is likely that properties of plant 2/2-like Hbs and some bacterial trHbs are similar. Several lines of evidence suggest that a function of microbial trHbs is to detoxify NO (Giangiacomo *et al.*, 2005; Milani *et al.*, 2003a; Ouellet *et al.*, 2002; Sarma *et al.*, 2005). Thus, it is of interest to evaluate whether or not plant 2/2-like Hbs participate in the metabolism of NO in plant cells. For this process, it is of interest to identify mechanisms that reduce ferric to ferrous plant 2/2-like Hb (which will subsequently bind O₂ for dioxygenation of NO). It will be interesting to know if corresponding groups of trHbs that have been encountered in bacteria can also be identified in plants. To conclude, many questions still remain unanswered in the fascinating world of haemoproteins, especially in the case of plant nsHbs and trHbs (Jokipii-Lukkari *et al.*, 2009).

The conservation of the B10Tyr in trHbs, and possibly the tunnel, points to a similar function for other trHbs. It will be important to determine whether all trHbs show significant NO scavenging activity and whether some catalyze novel reactions with specialized functions. It is noteworthy that the B10Tyr is also highly conserved in FHbs. Such conservation indicates a very ancient origin for the B10Tyr as well as a conserved function. Apart from a few exceptions like soybean leghemoglobin, B10Tyr is absent in almost all modern animal and plant Hbs. The loss of the B10Tyr and therefore of a less reactive heme-bound O₂ may have been mandatory to evolve a protein capable of transport and diffusion of O₂. The functional roles of trHbs are little known and may be various. The currently

available data indicate that the dramatically simplified 2-on-2 version of the globin fold observed in trHbs may reflect biological functions distinct from O₂ storage or transport. In particular, the combination of a closed distal site with the presence of an elongated protein matrix tunnel hints at internal ligand diffusion mechanisms different from those based on the E7 distal gate in Mb highlighting a previously unpredicted structural plasticity of the globin fold. How the modified fold is related to the functional properties remains to be elucidated and will be of great significance in understanding the selective pressure that maintains the trHbs in these organisms.

Altogether, we are still far from understanding the interplay of oxygen protection of nitrogenase and ROS control in root nodules. An aspect that deserves further examination is the presence and function of bacterial trHbs in symbiosis. An alteration in the dynamical behaviour of the protein skeleton may result in functional difference, which might affect the regulation mechanism that facilitates NO diffusion through the protein matrix. Clearly, untangling the structural basis of the interplay between ligand binding, protein dynamics and ligand migration will shed light on the emerging field of multi-ligand chemistry in heme proteins.

Abbreviations

trHb, truncated haemoglobin; SDgb, single domain haemoglobin; GCS, globin coupled sensors; SHbs, symbiotic hemoglobins; Pgbs, protoglobins; SDgbs, single domain hemoglobins; NsHbs, non symbiotic hemoglobins; YHB, yeast hemoglobin; FHB, flavo-hemoglobin; Mb, myoglobin; HbA, hemoglobin α; Lba, leghemoglobin a; Mtb, *Mycobacterium tuberculosis*; HbN, hemoglobin N; HbO, hemoglobin O; LCA, last common ancestor; HGT, horizontal gene transfer; DNA, deoxyribonucleic acid.

Acknowledgement

Amit Kumar has the pleasure of acknowledging CSIR, Govt of India for a NET fellowship. AK also acknowledges Prof. Anil K. Tyagi for his moral and financial support.

References

- Appleby, C.A. (1984). Leghemoglobin and rhizobium respiration. *Annu Rev Plant Physiol, Plant Mol Biol* 35, 443-478.
- Ascenzi, P., Bocedi, A., Bolognesi, M., Fabozzi, G., Milani, M., and Visca, P. (2006a). Nitric oxide scavenging by *Mycobacterium leprae* GlbO involves the formation of the ferric heme-bound peroxy nitrite intermediate. *Biochem Biophys Res Commun* 339, 450-456.
- Ascenzi, P., De Marinis, E., Coletta, M., and Visca, P. (2008). H₂O₂ and (.)NO scavenging by *Mycobacterium leprae* truncated hemoglobin O. *Biochem Biophys Res Commun* 373, 197-201.
- Ascenzi, P., Milani, M., and Visca, P. (2006b). Peroxynitrite scavenging by ferrous truncated hemoglobin GlbO from *Mycobacterium leprae*. *Biochem Biophys Res Commun* 351, 528-533.
- Ascenzi, P., and Visca, P. (2008). Scavenging of reactive nitrogen species by mycobacterial truncated hemoglobins. *Methods Enzymol* 436, 317-337.
- Bellelli, A., Antonini, G., Brunori, M., Springer, B.A., and Sligar, S.G. (1990). Transient spectroscopy of the reaction of cyanide with ferrous myoglobin. Effect of distal side residues. *J Biol Chem* 265, 18898-18901.
- Benson, D. R., and Silvester, W. B. (1993). Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol Rev* 57, 293-319.
- Bergersen, F. J. (1962). The effect of partial pressure of oxygen upon respiration and nitrogen fixation by soybean root nodules. *J gen Microbiol* 29, 113-125.
- Boechi, L., Marti, M. A., Milani, M., Bolognesi, M., Luque, F. J., and Estrin, D. A. (2008). Structural determinants of ligand migration in *Mycobacterium tuberculosis* truncated hemoglobin O. *Proteins* 73, 372-379.
- Bolli, A., Ciaccio, C., Coletta, M., Nardini, M., Bolognesi, M., Pesce, A., Guertin, M., Visca, P., and Ascenzi, P. (2008). Ferrous *Campylobacter jejuni* truncated hemoglobin P displays an extremely high reactivity for cyanide - a comparative study. *FEBS J* 275, 633-645.
- Bolognesi, M., Bordo, D., Rizzi, M., Tarricone, C., and Ascenzi, P. (1997). Nonvertebrate hemoglobins: structural bases for reactivity. *Prog Biophys Mol Biol* 68, 29-68.
- Bolognesi, M., Cannillo, E., Ascenzi, P., Giacometti, G. M., Merli, A., and Brunori, M. (1982). Reactivity of ferric *Aplysia* and sperm whale myoglobins towards imidazole. X-ray and binding study. *J Mol Biol* 158, 305-315.
- Brancaccio, A., Cutruzzola, F., Allocatelli, C. T., Brunori, M., Smerdon, S. J., Wilkinson, A. J., Dou, Y., Keenan, D., Ikeda-Saito, M., Brantley, R. E., Jr., et al. (1994). Structural factors governing azide and cyanide binding to mammalian metmyoglobins. *J Biol Chem* 269, 13843-13853.
- Brunori, M., Bourgeois, D., and Vallone, B. (2004). The structural dynamics of myoglobin. *J Struct Biol* 147, 223-234.
- Chan, J., Fujiwara, T., Brennan, P., McNeil, M., Turco, S. J., Sible, J. C., Snapper, M., Aisen, P., and Bloom, B.R. (1989). Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc Natl Acad Sci U S A* 86, 2453-2457.
- Cooper, A. M., Adams, L. B., Dalton, D. K., Appelberg, R., and Ehlers, S. (2002). IFN-gamma and NO in

- mycobacterial disease: new jobs for old hands. *Trends Microbiol* 10, 221-226.
- Couture, M., Das, T. K., Savard, P. Y., Ouellet, Y., Wittenberg, J. B., Wittenberg, B. A., Rousseau, D. L., and Guertin, M. (2000). Structural investigations of the hemoglobin of the cyanobacterium *Synechocystis* PCC6803 reveal a unique distal heme pocket. *Eur J Biochem* 267, 4770-4780.
- Cueto, M., Hernandez-Perera, O., Martin, R., Bentura, M. L., Rodrigo, J., Lamas, S., and Golvano, M. P. (1996). Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. *FEBS Lett* 398, 159-164.
- Floegl, U., Merx, M. W., Godecke, A., Decking, U.K., and Schrader, J. (2001). Myoglobin: A scavenger of bioactive NO. *Proc Natl Acad Sci U S A* 98, 735-740.
- Frauenfelder, H., McMahon, B. H., and Fenimore, P. W. (2003). Myoglobin: the hydrogen atom of biology and a paradigm of complexity. *Proc Natl Acad Sci U S A* 100, 8615-8617.
- Frey, A. D., and Kallio, P. T. (2005). Nitric oxide detoxification—a new era for bacterial globins in biotechnology? *Trends Biotechnol* 23, 69-73.
- Gardner, A. M., Martin, L. A., Gardner, P. R., Dou, Y., and Olson, J. S. (2000). Steady-state and transient kinetics of *Escherichia coli* nitric-oxide dioxygenase (flavohemoglobin). The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis. *J Biol Chem* 275, 12581-12589.
- Giangiacomo, L., Ilari, A., Boffi, A., Morea, V., and Chiancone, E. (2005). The truncated oxygen-avid hemoglobin from *Bacillus subtilis*: X-ray structure and ligand binding properties. *J Biol Chem* 280, 9192-9202.
- Gow, A. J., Luchsinger, B. P., Pawloski, J. R., Singel, D. J., and Stamler, J. S. (1999). The oxyhemoglobin reaction of nitric oxide. *Proc Natl Acad Sci U S A* 96, 9027-9032.
- Hardison, R. (1998). Hemoglobins from bacteria to man: evolution of different patterns of gene expression. *J Exp Biol* 201, 1099-1117.
- Hardison, R. C. (1996). A brief history of hemoglobins: plant, animal, protist, and bacteria. *Proc Natl Acad Sci U S A* 93, 5675-5679.
- Herold, S. (1999). Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin. *FEBS Lett* 443, 81-84.
- Herold, S., and Fago, A. (2005). Reactions of peroxynitrite with globin proteins and their possible physiological role. *Comp Biochem Phys A* 142, 124-129.
- Hiner, A. N., Raven, E. L., Thorneley, R. N., Garcia-Canovas, F., and Rodriguez-Lopez, J. N. (2002). Mechanisms of compound I formation in heme peroxidases. *J Inorg Biochem* 91, 27-34.
- Hoy, J. A., Kundu, S., Trent, J. T., Ramaswamy, S., and Hargrove, M. S. (2004). The crystal structure of *Synechocystis* hemoglobin with a covalent heme linkage. *J Biol Chem* 279, 16535-16542.
- Igarashi, J., Kobayashi, K., and Matsuoka, A. (2011). A hydrogen-bonding network formed by the B10-E7-E11 residues of a truncated hemoglobin from *Tetrahymena pyriformis* is critical for stability of bound oxygen and nitric oxide detoxification. *J Biol Inorg Chem* 16, 599-609.
- Ilari, A., Kjelgaard, P., von Wachenfeldt, C., Catacchio, B., Chiancone, E., and Boffi, A. (2007). Crystal structure and ligand binding properties of the truncated hemoglobin from *Geobacillus stearothermophilus*. *Arch Biochem Biophys* 457, 85-94.
- Jokipii-Lukkari, S., Frey, A. D., Kallio, P. T., and Haggman, H. (2009). Intrinsic non-symbiotic and truncated haemoglobins and heterologous *Vitreoscilla* haemoglobin expression in plants. *J Exp Bot* 60, 409-422.
- Kang, T. J., You, J. C., and Chae, G. T. (2001). Identification of catalase-like activity from *Mycobacterium leprae* and the relationship between catalase and isonicotinic acid hydrazide (INH). *J Med Microbiol* 50, 675-681.
- Klebanoff, S. J., and Shepard, C. C. (1984). Toxic effect of the peroxidase-hydrogen peroxide-halide antimicrobial system on *Mycobacterium leprae*. *Infect Immun* 44, 534-536.
- Kraiser, T., Gras, D. E., Gutierrez, A. G., Gonzalez, B., and Gutierrez, R. A. (2011). A holistic view of nitrogen acquisition in plants. *J Exp Bot* 62, 1455-1466.
- Kraus, D. W., and Wittenberg, J. B. (1990). Hemoglobins of the *Lucina pectinata*/bacteria symbiosis. I. Molecular properties, kinetics and equilibria of reactions with ligands. *J Biol Chem* 265, 16043-16053.
- Kraus, D. W., Wittenberg, J. B., Lu, J. F., and Peisach, J. (1990). Hemoglobins of the *Lucina pectinata*/bacteria symbiosis. II. An electron paramagnetic resonance and optical spectral study of the ferric proteins. *J Biol Chem* 265, 16054-16059.
- Kubo, H. (1939). About hemoprotein from the root nodules of legumes. *Acta Phytochim (Tokyo)* 11, 195-200.
- Lama, A., Pawaria, S., Bidon-Chanal, A., Anand, A., Gelpi, J. L., Arya, S., Marti, M., Estrin, D. A., Luque, F. J., and Dikshit, K. L. (2009). Role of Pre-A motif in nitric oxide scavenging by truncated hemoglobin, HbN, of *Mycobacterium tuberculosis*. *J Biol Chem* 284, 14457-14468.
- Lecomte, J. T., Scott, N. L., Vu, B. C., and Falzone, C.J. (2001). Binding of ferric heme by the recombinant globin from the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochemistry* 40, 6541-6552.
- Long, R., Light, B., and Talbot, J. A. (1999). Mycobacteriocidal action of exogenous nitric oxide. *Antimicrob Agents Chemother* 43, 403-405.
- MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu Rev Immunol* 15, 323-350.
- Marolia, J., and Mahadevan, P. R. (1989). Reactive oxygen intermediates inactivate *Mycobacterium leprae* in the phagocytes from human peripheral blood. *Int J Lepr Other Mycobact Dis* 57, 483-491.

- Milani, M., Ouellet, Y., Ouellet, H., Guertin, M., Boffi, A., Antonini, G., Bocedi, A., Mattu, M., Bolognesi, M., and Ascenzi, P. (2004a). Cyanide binding to truncated hemoglobins: a crystallographic and kinetic study. *Biochemistry* 43, 5213-5221.
- Milani, M., Pesce, A., Nardini, M., Ouellet, H., Ouellet, Y., Dewilde, S., Bocedi, A., Ascenzi, P., Guertin, M., Moens, L., et al. (2005). Structural bases for heme binding and diatomic ligand recognition in truncated hemoglobins. *J Inorg Biochem* 99, 97-109.
- Milani, M., Pesce, A., Ouellet, H., Guertin, M., and Bolognesi, M. (2003a). Truncated hemoglobins and nitric oxide action. *IUBMB Life* 55, 623-627.
- Milani, M., Pesce, A., Ouellet, Y., Ascenzi, P., Guertin, M., and Bolognesi, M. (2001). *Mycobacterium tuberculosis* hemoglobin N displays a protein tunnel suited for O₂ diffusion to the heme. *EMBO J* 20, 3902-3909.
- Milani, M., Pesce, A., Ouellet, Y., Dewilde, S., Friedman, J., Ascenzi, P., Guertin, M., and Bolognesi, M. (2004b). Heme-ligand tunneling in group I truncated hemoglobins. *J Biol Chem* 279, 21520-21525.
- Milani, M., Savard, P. Y., Ouellet, H., Ascenzi, P., Guertin, M., and Bolognesi, M. (2003b). A TyrCD1/TrpG8 hydrogen bond network and a TyrB10TyrCD1 covalent link shape the heme distal site of *Mycobacterium tuberculosis* hemoglobin O. *Proc Natl Acad Sci U S A* 100, 5766-5771.
- Mishra, S., and Meuwly, M. (2009). Nitric oxide dynamics in truncated hemoglobin: docking sites, migration pathways, and vibrational spectroscopy from molecular dynamics simulations. *Biophys J* 96, 2105-2118.
- Mishra, S., and Meuwly, M. (2010). Atomistic Simulation of NO Dioxygenation in Group I Truncated Hemoglobin. *J Am Chem Soc* 132, 2968-2982.
- Mukai, M., Ouellet, Y., Ouellet, H., Guertin, M., and Yeh, S.R. (2004). NO binding induced conformational changes in a truncated hemoglobin from *Mycobacterium tuberculosis*. *Biochemistry* 43, 2764-2770.
- Nardini, M., Pesce, A., Labarre, M., Richard, C., Bolli, A., Ascenzi, P., Guertin, M., and Bolognesi, M. (2006). Structural determinants in the group III truncated hemoglobin from *Campylobacter jejuni*. *J Biol Chem* 281, 37803-37812.
- Nardini, M., Pesce, A., Milani, M., and Bolognesi, M. (2007). Protein fold and structure in the truncated (2/2) globin family. *Gene* 398, 2-11.
- Nathan, C., and Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* 97, 8841-8848.
- Nicoletti, F. P., Comandini, A., Bonamore, A., Boechi, L., Boubeta, F.M., Feis, A., Smulevich, G., and Boffi, A. (2010). Sulfide binding properties of truncated hemoglobins. *Biochemistry* 49, 2269-2278.
- Norbis, L., Miotto, P., Alagna, R., and Cirillo, D. M. (2013). Tuberculosis: lights and shadows in the current diagnostic landscape. *New Microbiol* 36, 111-120.
- Oliveira, A., Singh, S., Bidon-Chanal, A., Forti, F., Marti, M. A., Boechi, L., Estrin, D. A., Dikshit, K. L., and Luque, F. J. (2012). Role of PheE15 gate in ligand entry and nitric oxide detoxification function of *Mycobacterium tuberculosis* truncated hemoglobin N. *PLoS One* 7, e49291.
- Ouellet, H., Milani, M., LaBarre, M., Bolognesi, M., Couture, M., and Guertin, M. (2007). The roles of Tyr(CD1) and Trp(G8) in *Mycobacterium tuberculosis* truncated hemoglobin O in ligand binding and on the heme distal site architecture. *Biochemistry* 46, 11440-11450.
- Ouellet, H., Ouellet, Y., Richard, C., Labarre, M., Wittenberg, B., Wittenberg, J., and Guertin, M. (2002). Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. *Proc Natl Acad Sci U S A* 99, 5902-5907.
- Pacher, P., Beckman, J. S., and Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 87, 315-424.
- Pathania, R., Navani, N. K., Rajamohan, G., and Dikshit, K. L. (2002). *Mycobacterium tuberculosis* hemoglobin HbO associates with membranes and stimulates cellular respiration of recombinant *Escherichia coli*. *J Biol Chem* 277, 15293-15302.
- Pawaria, S., Lama, A., Raje, M., and Dikshit, K. L. (2008). Responses of *Mycobacterium tuberculosis* hemoglobin promoters to in vitro and in vivo growth conditions. *Appl Environ Microbiol* 74, 3512-3522.
- Pawlowski, K. (2008). Nodules and oxygen. *Plant Biotechnology, JSPS Sweden Colloquium – The Frontiers of Plant Biotechnology* 25, 291-298.
- Perutz, M. F. (1989). Myoglobin and haemoglobin: role of distal residues in reactions with haem ligands. *Trends Biochem Sci* 14, 42-44.
- Pesce, A., Couture, M., Dewilde, S., Guertin, M., Yamauchi, K., Ascenzi, P., Moens, L., and Bolognesi, M. (2000). A novel two-over-two alpha-helical sandwich fold is characteristic of the truncated hemoglobin family. *Embo Journal* 19, 2424-2434.
- Pesce, A., Nardini, M., Dewilde, S., Geuens, E., Yamauchi, K., Ascenzi, P., Riggs, A.F., Moens, L., and Bolognesi, M. (2002). The 109 residue nerve tissue minihemoglobin from *Cerebratulus lacteus* highlights striking structural plasticity of the alpha-helical globin fold. *Structure* 10, 725-735.
- Poole, R. K. (2005). Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans* 33, 176-180.
- Richardson, A. R., Soliven, K. C., Castor, M. E., Barnes, P. D., Libby, S.J., and Fang, F. C. (2009). The Base Excision Repair system of *Salmonella enterica* serovar typhimurium counteracts DNA damage by host nitric oxide. *PLoS Pathog* 5, e1000451.
- Ross, E. J. H., Lira-Ruan, V., Arredondo-Peter, R., Klucas, R.V., and Sarath, G. (2002). Recent insights into plant hemoglobins. *Rev Plant Biochem Biotechnol* 1, 173-189.
- Samuni, U., Dantsker, D., Ray, A., Wittenberg, J. B., Wittenberg, B.A., Dewilde, S., Moens, L., Ouellet, Y., Guertin, M., and Friedman, J.M. (2003). Kinetic

- modulation in carbonmonoxy derivatives of truncated hemoglobins: the role of distal heme pocket residues and extended apolar tunnel. *J Biol Chem* 278, 27241-27250.
- Sarma, H. K., Sharma, B. K., Tiwari, S. C., and Mishra, A. K. (2005). Truncated hemoglobins: A single structural motif with versatile functions in bacteria, plants and unicellular eukaryotes. *Symbiosis* 39, 151-158.
- Schon, T., Hernandez-Pando, R., Baquera-Heredia, J., Negesse, Y., Becerril-Villanueva, L. E., Eon-Contreras, J. C., Sundqvist, T., and Britton, S. (2004). Nitrotyrosine localization to dermal nerves in borderline leprosy. *Br J Dermatol* 150, 570-574.
- Scillard, D. M., Adams, L. B., Gillis, T. P., Krahenbuhl, J. L., Truman, R. W., and Williams, D. L. (2006). The continuing challenges of leprosy. *Clin Microbiol Rev* 19, 338-381.
- Ulrichs, T., and Kaufmann, S. H. E. (2002). Mycobacterial resistance and immunity. *Front Biosci* 7, 458-469.
- Vázquez-Limón, C., Hoogewijs, D., Vinogradov, S. N., and Arredondo-Peter, R. (2012). The evolution of land plant hemoglobins. *Plant Science* 191-192, 71-81.
- Vinogradov, S. N., Hoogewijs, D., Bailly, X., Arredondo-Peter, R., Gough, J., Dewilde, S., Moens, L., and Vanfleteren, J. R. (2006). A phylogenomic profile of globins. *BMC Evol Biol* 6, 31.
- Vinogradov, S. N., Hoogewijs, D., Bailly, X., Arredondo-Peter, R., Guertin, M., Gough, J., Dewilde, S., Moens, L., and Vanfleteren, J.R. (2005). Three globin lineages belonging to two structural classes in genomes from the three kingdoms of life. *Proc Natl Acad Sci U S A* 102, 11385-11389.
- Visca, P., Fabozzi, G., Milani, M., Bolognesi, M., and Ascenzi, P. (2002). Nitric oxide and *Mycobacterium leprae* pathogenicity. *IUBMB Life* 54, 95-99.
- Vuletich, D. A., and Lecomte, J. T. (2006). A phylogenetic and structural analysis of truncated hemoglobins. *J Mol Evol* 62, 196-210.
- Wainwright, L. M., Wang, Y., Park, S. F., Yeh, S. R., and Poole, R. K. (2006). Purification and spectroscopic characterization of Ctb, a group III truncated hemoglobin implicated in oxygen metabolism in the food-borne pathogen *Campylobacter jejuni*. *Biochemistry* 45, 6003-6011.
- Watts, R. A., Hunt, P. W., Hvitved, A. N., Hargrove, M. S., Peacock, W. J., and Dennis, E. S. (2001). A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. *Proc Natl Acad Sci U S A* 98, 10119-10124.
- Winrow, V. R., Winyard, P. G., Morris, C. J., and Blake, D. R. (1993). Free radicals in inflammation: second messengers and mediators of tissue destruction. *Br Med Bull* 49, 506-522.
- Wittenberg, J. B., Bolognesi, M., Wittenberg, B. A., and Guertin, M. (2002). Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. *J Biol Chem* 277, 871-874.
- World Health Organization (2008). Global Tuberculosis Control 2008: surveillance, planning, financing. Geneva, Switzerland: WHO. ISBN 9789241563543.
- Wu, G., Wainwright, L. M., and Poole, R. K. (2003). Microbial globins. *Adv Microb Physiol* 47, 255-310.
- Yang, C. S., Yuk, J. M., and Jo, E. K. (2009). The role of nitric oxide in mycobacterial infections. *Immune Netw* 9, 46-52.