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CHAPTER TWO

C0010 # The Diversity of 2/2 (Truncated) Globins

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Contents

Abstract

Small size globins that have been defined as 'truncated haemoglobins' or as '2/2 haemoglobins' have increasingly been discovered in microorganisms since the early 1990s. Analysis of amino acid sequences allowed to distinguish three groups that collect proteins with specific and common structural properties. All three groups display 3D structures that are based on four main α-helices, which are a subset of the conventional eight-helices globin fold. Specific features, such as the presence of protein matrix tunnels that are held to promote diffusion of functional ligands to/from the haem, distinguish members of the three groups. Haem distal sites vary for their accessibility, local structures, polarity, and ligand stabilization mechanisms, suggesting functional roles that are related to O_2/NO chemistry. In a few cases, such activities have been proven *in vitro* and *in vivo* through deletion mutants. The issue of 2/2 haemoglobin varied biological functions throughout the three groups remains however fully open.

st0015 ABBREVIATIONS

dt0005 **2/2Hb** 2-on-2 globin
dt0010 **Hb** haemoglobin
dt0015 **Mb** myoglobin
dt0020 **(non-)vertebrate Hbs** vertebrate and non-vertebrate Hbs
dt0025 amino acid residues have been labelled using their three-letter codes and the topological site they occupy within the globin fold

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1. INTRODUCTION

The globin family has long been known from studies of vertebrate myoglobin (Mb) and haemoglobins (Hbs), which are haemoproteins typically composed of about 150 amino acids. In the past 30 years, the Hb superfamily has been enriched by the discovery of Hbs and related haemoproteins in virtually all kingdoms of life; among these non-symbiotic Hbs in plants and symbiotic Hbs in plants other than legumes, chimaeric flavoHbs comprised of an N-terminal globin linked to a FAD reductase domain in bacteria and yeasts, neuroglobins and cytoglobins in vertebrates, globin-coupled sensors and protoglobins in eubacteria and in archaea, and globins that fall in the 110–130 amino acid range (per haem), which have been called 'truncated Hbs', in protozoa and in bacteria. Such large variety of Hb types suggests an ancient origin for their genes and stresses the concept that Hbs/globins cover functions that stretch well beyond that of simple oxygen carriers.

In this review, we focus our attention on the truncated Hb family based on the extensive sequence and crystallographic investigations that have performed in various laboratories starting from the early 2000s. The term 'truncated Hb' was first introduced to refer to the size of these small globins. However, structural considerations underline the fact that the tertiary structure of these proteins results from careful editing of the classical globin fold through an evolutionary/engineering process that affects the whole polypeptide chain, rather than just acting through simple truncation of the N- and C-terminal ends. For this reason, the historical term 'truncated Hb' was recently replaced by '2/2Hbs' (read 2-on-2 Hbs) in relation to specific features of their folds (see below).

The 2/2Hbs are small oxygen-binding haemoproteins, identified in bacteria, higher plants, and in certain unicellular eukaryotes, building a clear separate cluster within the haemoglobin superfamily (Nardini, Pesce, Milani, & Bolognesi, 2007; Vinogradov, Tinajero-Trejo, Poole, & Hoogewijs, 2013; Vuletich & Lecomte, 2006; Wittenberg, Bolognesi, Wittenberg, & Guertin, 2002). 2/2Hbs display amino acid sequences that are 20–40 residues shorter than (non-)vertebrate Hbs, to which they are loosely related by sequence similarity (sequence identity to vertebrate Hbs falls well below 20%). Based on amino acid sequence analysis, three 2/2Hbs phylogenetic groups (groups I, II, and III, whose members are designated by the N, O, and P suffixes, respectively) were recognized, proteins being orthologous within each group and paralogous across the groups

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(Vuletich & Lecomte, 2006). Group I and group II can be further separated into two and four subgroups, respectively, whereas group III displays a high level of overall sequence conservation. Despite the conserved small size of 2/2Hbs, sequence identity among proteins from the different groups is low (≤20% overall identity), but may be higher than 80% within a given group. Phylogenetic analyses further suggest 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 transfer events (Vuletich & Lecomte, 2006).

p0045 In some cases, 2/2Hbs from more than one group can coexist in the same organism, indicating a diversification of their functions. In general, members of the 2/2Hb family are monomeric or dimeric proteins characterized by medium to very high oxygen affinities, with cases of ligand-binding cooperativity (Couture, Yeh, et al., 1999). Some of the organisms hosting 2/2Hbs are aggressive pathogenic bacteria; others perform photosynthesis, fix nitrogen, or may display distinctive metabolic capabilities. Although very little is known about their role *in vivo*, possible functions of 2/2Hbs that are consistent with the observed biophysical properties include long-term ligand or substrate storage, nitric oxide (NO) detoxification, O_2/NO sensing, redox reactions, and O_2 delivery under hypoxic conditions (Nardini et al., 2007; Vuletich & Lecomte, 2006; Wittenberg et al., 2002).

p0050 So far, a number of three-dimensional structures belonging to all three groups have been solved by X-ray crystallography and NMR methods, thus providing a clear picture of the structural features specific for each group: six structures from group I 2/2HbNs (from *Chlamydomonas eugametos* (Pesce et al., 2000), *Paramecium caudatum* (Pesce et al., 2000), *Mycobacterium tuberculosis* (Milani et al., 2001), *Synechocystis* sp. (*Synechocystis* 6803: Falzone, Vu, Scott, & Lecomte, 2002; Hoy, Kundu, Trent, Ramaswamy, & Hargrove, 2004; Trent, Kundu, Hoy, & Hargrove, 2004; *Synechococcus* 7002: Scott et al., 2010)), and *Tetrahymena pyriformis* (Igarashi, Kobayashi, & Matsuoka, 2011), five structures of group II 2/2HbOs (from *Mycobacterium tuberculosis* (Milani et al., 2003), *Bacillus subtilis* (Giangiacomo, Ilari, Boffi, Morea, & Chiancone, 2005), *Thermobifida fusca* (Bonamore et al., 2005) *Geobacillus stearothermophilus* (Ilari et al., 2007), and *Agrobacterium tumefaciens* (Pesce et al., 2011)), and one structure from group III 2/2HbPs (from *Campylobacter jejuni* (Nardini et al., 2006)). Additionally, the NMR method was applied to characterize the haem ligand binding site of 2/2HbP from *Helicobacter hepaticus* in solution (Nothnagel, Winer, Vuletich, Pond, & Lecomte, 2011).

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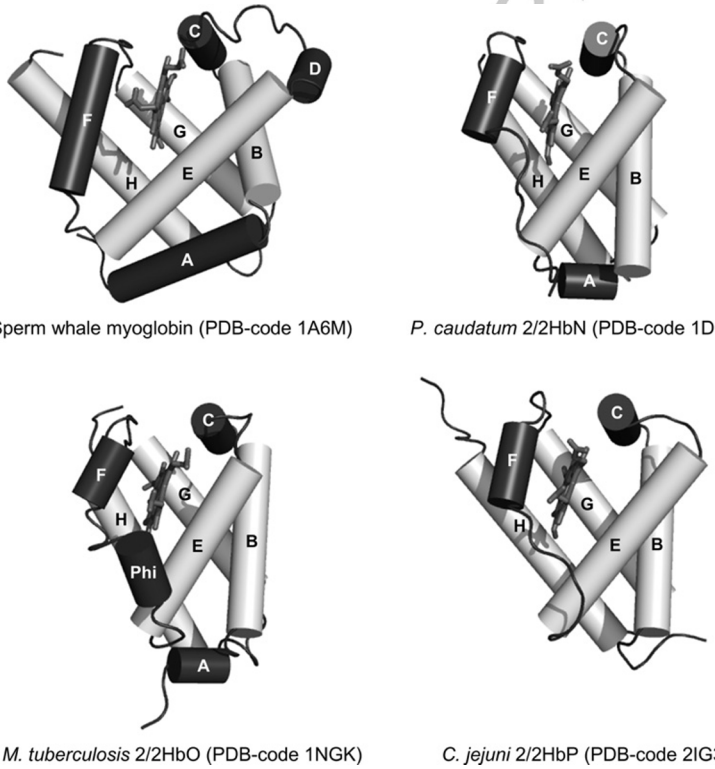
s0010

2. FOLD AND FOLD VARIATION IN 2/2Hb GROUPS I, II, III

p0055 The globin fold of 2/2Hb (Fig. 2.1) has been described as consisting of a simplified version of the 'classical' globin fold (a 3-on-3 α-helical sandwich; Perutz, 1979) typical of sperm whale Mb. The topology of the 2/2Hb fold is characterized by a 2-on-2 α-helical sandwich based on four α-helices, corresponding to the B-, E-, G-, and H-helices of the classical globin fold (Nardini et al., 2007; Pesce et al., 2000). The helix pairs B/E and G/H are arranged each in antiparallel fashion and assembled in a sort of α-helical bundle which surrounds and protects the haem group from the solvent. Although the G- and H-helices generally match the globin fold



f0005 **Figure 2.1** Comparative view of the classical 3/3 globin fold (sperm whale myoglobin) with the 2/2 globin fold in groups I (HbN), II (HbO), and III (HbP). Helices are shown as cylinders and labelled. The helices structurally conserved within 3/3 and 2/2 folds are shown in grey. The haem is shown in stick representation. (For colour version of this figure, the reader is referred to the online version of this chapter.)

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topology, they may be much shorter or bent, as compared to the secondary structure elements in sperm whale Mb. The most striking differences between the 2-on-2 and the 3-on-3 globin folds are (i) the drastically shortened A-helix (completely deleted in group III 2/2HbP), (ii) the absence of the D-helix, (iii) the presence of a long polypeptide segment (pre-F) in extended conformation, and (iv) a variable-length F-helix (reduced to a one-turn-helix in group I and III 2/2Hbs) that properly supports the haem-coordinated proximal HisF8 residue (Pesce et al., 2000; Milani et al., 2003; Nardini et al., 2006) (Fig. 2.1). Structural differences are evident also on the haem distal side, where the 2/2 fold CD–D region differs in length relative to (non-)vertebrate globins.

p0060 Structural superposition of 2/2Hbs of known structure highlights a general good conservation of the α-helical scaffold among the three groups, with the overall fold of group III 2/2HbP equally diverging in its C_α trace from group I and group II 2/2Hbs. Interesting group-specific structural variability/plasticity can be recognized at defined sites of the tertiary structure and correlated to attainment and stabilization of the compact 2/2Hb fold. At the N- and C-termini, the A-helix can be either very short or fully absent (as in group III), while the H-helix is highly variable in length and linearity, being kinked in group I, short in group II, unusually long in group III. Other important structural variations are localized in the core of the protein. For instance, the polypeptide stretch bridging the C- and E-helices is usually trimmed to about three residues in group I 2/2HbNs and group II 2/2HbOs; on the contrary, a 3–7 amino acid insertion is invariantly found in group III 2/2HbPs. Such elongation of the CD region has structural implications on the spanning of the C- and E-helices and on the 3_{10} helical Au1 character of helix C. Indeed, in group III 2/2HbPs from *C. jejuni*, the C- and E-helices are elongated by one additional turn at their C- and N-termini, respectively, relative to the corresponding helices in group I and group II 2/2Hbs, not affecting, however, the position of the E-helix relative to the haem distal site (Nardini et al., 2006). Variable capping interactions in the CE inter-helical region of group III 2/2HbP, however, suggest that secondary structure boundaries may not be conserved and that C- and E-helix lengths, side chain locations, and haem accessibility may differ across the group (Nothnagel, Winer, et al., 2011). Additionally, in group III 2/2HbP, the C-helix displays a clear α-helical character, whereas it is a 3_{10} helix in group I and group II 2/2Hbs and in (non-)vertebrate Au1 globins (Bolognesi, Bordo, Rizzi, Tarricone, & Ascenzi, 1997). Despite the group-specific structural variations, in all 2/2Hbs, the CD region and

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the E-, F-, and G-helices build the protein crevice hosting the haem, which is shielded from the solvent and stabilized by well-conserved polar/electrostatic interactions involving the porphyrin propionates.

p0065 A stable and properly structured haem crevice in the context of such a short polypeptide chain has been correlated to the presence of three glycine motifs, conserved among sequences of group I 2/2HbNs and group II 2/2HbOs. Such Gly motifs are located at the AB and EF inter-helical corners, and just before the short F-helix; they are thought to provide the protein backbone flexibility needed to stabilize the short A-helix in a conformation locked onto the B- and E-helices, and to support the pre-F segment in building the haem pocket (Milani et al., 2001; Pesce et al., 2000). A similar stabilization, however, cannot be achieved in group III 2/2HbPs, where the AB Gly–Gly motif is absent, due to full deletion of the A-helix. As a consequence, the 2/2HbP amino-terminus cannot face the BE inter-helical region, and the protein residues preceding the B-helix extend towards the GH region; such conformation is opposite to that found in group I and II 2/2Hbs. Similarly, a clear Gly–Gly motif cannot be recognized in the EF region of group III 2/2HbP, although scattered Gly residues are present in this region of the sequence. Despite the absence of a clear EF Gly–Gly motif, B- and E-helices in group III 2/2HbPs are oriented as in groups I and II, their stabilization being achieved through group-specific hydrophobic contacts at the B/E helical interface.

p0070 Other group-specific structural variation is localized inside the haem binding pocket, with group III 2/2HbPs displaying a high degree of similarity in sequence and structure of the distal region to group II 2/2HbOs and, simultaneously, sharing a proximal side-extended EF region typically found in group I 2/2HbNs. Structural differences and group-specific residues at the haem distal and proximal sites are correlated to different ligand-binding properties of 2/2Hbs.

s0015 3. THE HAEM ENVIRONMENT

p0075 Despite the trimmed globin fold, the 2/2 helical sandwich provides a minimal scaffold, formed by the E- and G-helices, by the CD region and by the F-helix, that allows efficient incorporation of the haem group within the protein (Fig. 2.1). Besides the HisF8–Fe coordination bond, in all 2/2Hbs, the haem is stabilized by a network of van der Waals contacts with hydrophobic residues at the conserved topological positions C6, C7, CD1, E14, F4, FG3, G8, and H11. Other protein–haem interactions may arise from

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residues located in regions of the 2/2 fold that vary in the three groups, such as the CD and FG segments and the amino-terminal part of the H-helix. Further stabilizing interactions are provided by hydrogen bonds between the haem and polar residues, involving Thr/Tyr at sites E2, E5, and EF6, and by salt bridges involving haem propionates and Arg/Lys residues located at position E10 in all 2/2Hbs, at position F2 in group I 2/2HbN, and at position F7 in group II 2/2HbO (where F7 is invariantly Arg). It should be noted that LysF7, despite being involved in electrostatic stabilization of the haem also in group III *C. jejuni* 2/2HbP, is not conserved in other group III globins (Nardini et al., 2006). Further salt bridge interactions may also derive from sequence-specific substitutions in the surroundings of the haem propionates, as in the case of *M. tuberculosis* 2/2HbN ArgE6, and 2/2HbO ArgEF10. The crystallographic studies on group I 2/2HbNs and group II 2/2HbOs have shown that haem isomerism (insertion of a fraction of the haem groups into the globin structure in an inverted orientation) may be present (Milani et al., 2005).

p0080 The conformation of the Fe-coordinated proximal HisF8 is typical of an unstrained imidazole ring, with the imidazole plane lying in a staggered azimuthal orientation relative to the haem pyrrole nitrogen atoms, thus facilitating haem in-plane location of the iron atom, and supporting fast oxygen association (Bolognesi et al., 1997; Wittenberg et al., 2002) and electron donation to the bound distal ligand.

p0085 The 2/2Hb haem distal site cavity, hosting the exogenous ligands, is characterized by unusual residues as compared to (non-)vertebrate globins. It should be noted that in all 2/2Hbs, the E-helix falls close to the haem distal face due to the 'pulling action' of the shortened CD region, thus causing side chain crowding of the distal site residues at topological positions B10, CD1, E7, E11, E14, E15, and G8. Among these, group-specific selections of residues display polar character and allow the formation of networks of hydrogen bonds functional to the stabilization of the diatomic haem ligand, or implicated in the rebinding of dissociated ligands (Samuni et al., 2003). Distal site polarity is expected to favour oxygen chemistry in the haem crevice, as in peroxidases (Hiner, Raven, Thorneley, García-Cánovas, & Rodríguez-López, 2002).

p0090 There have been a number of experimental studies devoted to the spectroscopic and structural characterization of several diatomic ligands binding (CO, O_2, NO, and cyanide) to the 2/2HbN in *M. tuberculosis* (Couture, Yeh, et al., 1999; Milani, Ouellet, et al., 2004; Ouellet, Milani, Couture, Bolognesi, & Guertin, 2006; Ouellet et al., 2008; Yeh,

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Couture, Ouellet, Guertin, & Rousseau, 2000); these indicate that the ligand binding is largely controlled by a pair of interacting amino acids (GlnE11 and Tyr10) in the haem distal site that participate in hydrogen bonding with the haem-bound diatomic O_2 ligand. Indeed, in 2/2HbN from *M. tuberculosis*, a direct hydrogen bond occurs between TyrB10 side chain and the ligand (O_2 or cyanide, in the ferrous or ferric haem states, respectively), stabilized by GlnE11 that interacts with TyrB10 (Couture, Yeh, et al., 1999; Milani et al., 2001; Milani, Ouellet, et al., 2004; Yeh et al., 2000). It has been shown that the main barrier to ligand binding to deoxy *M. tuberculosis* 2/2HbN is the displacement of a distal cavity water molecule, which is mainly stabilized by residue TyrB10, but not coordinated to the haem iron. As observed in the TyrB10/GlnE11 apolar mutants (TyrB10Phe/Val and GlnE11Val/Ala, respectively), once this kinetic barrier is lowered, CO and O_2 binding is very fast with rates approaching $1-2\times10^9\,M^{-1}s^{-1}$. These large values almost certainly represent the upper limit for ligand binding to a haem protein and also indicate that the iron atom in 2/2HbN is highly reactive (Ouellet et al., 2008). In *P. caudatum* 2/2HbN and in *C. eugametos* 2/2HbN, residue TyrB10, buried in the inner part of the haem pocket, is properly oriented through hydrogen bonds towards residues GlnE7 and Thr/GlnE11, to provide stabilization of the haem-bound distal ligand (Pesce et al., 2000). In *T. pyriformis* 2/2HbN, TyrB10 and GlnE7 are hydrogen bonded to the haem-bound O_2 molecule. Furthermore, TyrB10 is hydrogen bonded to GlnE7 and GlnE11 residues. Mutation of these residues results in fast O_2 dissociation and autoxidation (Igarashi et al., 2011). In all cases, the strongly conserved TyrB10 plays a pivotal role in ligand stabilization through a direct hydrogen bond to the haem ligand. In general, when in group I a hydrogen bonding residue is present at B10, a Gln is located at E7 or E11, or at both these sites, likely completing the distal site hydrogen-bonded network. On the contrary, when a side chain devoid of hydrogen-bonding capabilities is (rarely) hosted at B10, then large hydrophobic residues are coupled at the E7 and E11 sites (Nardini et al., 2007; Vuletich & Lecomte, 2006).

p0095 In group I 2/2Hbs, an example of *bis*-histidine hexacoordination has been reported for group I 2/2HbN from the cyanobacterium *Synechococcus* sp. strain PCC 7002 and PCC 6803 (involving the proximal/distal residues HisF8 and HisE10, respectively), where binding of an exogenous ligand to the haem requires the dissociation of the Fe-coordinated HisE10 from the haem and a large conformational change of the B- and E-helices (Couture

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et al., 2000; Falzone et al., 2002; Hoy et al., 2004; Scott et al., 2002, 2010; Trent et al., 2004; Vu, Nothnagel, Vuletich, Falzone, & Lecomte, 2004).

p0100 The hexacoordinate *Synechocystis* sp. 2/2HbN shows also a unique haem–protein covalent interaction between HisH16 and the 2-vinyl group of the haem. This post-translational modification prevents haem loss and has the potential to modulate the reactivity of the haem group (Couture et al., 2000; Falzone et al., 2002; Hoy et al., 2004; Scott et al., 2002, 2010). In particular, for *Synechocystis* 6803 2/2HbN, it has been shown that the post-translational modification has little effect on the protein structure, perturbing the backbone dynamics only modestly, and that the specificity and rate of the cross-linking reaction depended critically on the nature of the sixth ligand to the haem iron (Nothnagel, Love, & Lecomte, 2009; Nothnagel, Preimesberger, et al., 2011; Pond, Majumdar, & Lecomte, 2012).

p0105 Although the endogenous haem hexacoordination is not a prominent trend for 2/2Hbs, it has been also observed, under specific conditions, in other 2/2Hbs. For instance, the *C. eugametus* 2/2-HbN may also display a six-coordinate haem-Fe atom in its ferric state, while the ferrous derivative displays a five-coordinate high spin haem-Fe atom at neutral pH, and a six-coordinate low spin species at alkaline pH where the sixth ligand to the haem-Fe atom is held to be either TyrB10 or to be stabilized by this residue (Couture, Das, et al., 1999; Couture & Guertin, 1996). Haem hexacoordination has also been observed in the ferrous derivative of group II *M. leprae*-2/2HbO at neutral pH (Visca, Fabozzi, Petrucca, et al., 2002).

p0110 In group II 2/2HbO, specific residue substitutions characterize the distal site environment relative to group I 2/2HbN. In this group, TyrB10 is strictly conserved, and so is TrpG8. The five available structures (Bonamore et al., 2005; Giangiacomo et al., 2005; Ilari et al., 2007; Milani et al., 2003; Pesce et al., 2011) show these residues to be located at the haem distal site, but not necessarily involved in direct ligand binding. Several group II 2/2HbOs display a polar residue, His or Tyr, at the topological position CD1, which in globins was thought to harbour a strictly conserved Phe (Kapp et al., 1995) whose role was to fasten the haem in its binding site (Ptitsyn & Ting, 1999).

p0115 In *M. tuberculosis* 2/2HbO, TyrCD1 is the residue responsible for hydrogen bonding to the diatomic ligand (Milani et al., 2003). Further ligand-stabilizing interactions are provided by TrpG8, whose indole NE1 atom is hydrogen bonded to the haem-bound ligand and to TyrCD1 OH (Boechi et al., 2008; Guallar, Lu, Borrelli, Egawa, & Yeh, 2009; Milani

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et al., 2003; Ouellet et al., 2003, 2007). The crystal structure of *M. tuberculosis* 2/2HbO has also shown that the simultaneous presence of Tyr residues at the B10 and CD1 sites may trigger the formation of a covalent (iso-dityrosine like) bond between the two side chains (Milani et al., 2003), whose functional role is yet unclear.

p0120 A similar network of interaction has also been described in *T. fusca* (Bonamore et al., 2005) and in *B. subtilis* (Boechi, Mañez, Luque, Martì, & Estrin, 2010; Feis et al., 2008; Giangiacomo et al., 2005) 2/2HbOs, where TyrB10, Phe/TyrCD1, and TrpG8 are mainly involved in a hydrogen-bonding network, thus stabilizing the exogenous ligands. In *T. fusca* 2/2HbO, the carbonyl oxygen of the acetate ion ligand is stabilized by hydrogen bonds with residues TyrCD1 and TrpG8 (Bonamore et al., 2005). When instead a Phe residue is present at the CD1 position, as in *B. subtilis* 2/2HbO, TyrB10 assumes the role of hydrogen bond donor for the interactions with the exogenous ligand (Giangiacomo et al., 2005). GlnE11, TrpG8, and ThrE7 complete the polar distal frame with GlnE11 side chain and the TrpG8 indolic nitrogen atom at hydrogen bonding distance to the bound ligand (Giangiacomo et al., 2005). The *G. stearothermophilus* 2/2HbO haem pocket displays a hydrogen bonding network involving TyrB10 and TrpG8 residues similar to *B. subtilis* 2/2HbO (Ilari et al., 2007). Interestingly, when in *M. tuberculosis* 2/2HbO, Au2 TyrCD1 is mutated to Phe, is TyrB10 the hydrogen bonding residue for the haem-bound ligand (Ouellet et al., 2003), thus mimicking in *M. tuberculosis* 2/2HbO what has been observed in *B. subtilis* and *G. stearothermophilus* 2/2HbOs (Giangiacomo et al., 2005; Ilari et al., 2007).

p0125 Recently, *B. subtilis* and *T. fusca*, group II 2/2HbOs have been demonstrated to be able to bind CO in the ferrous state (Droghetti et al., 2010), and sulphide (Nicoletti et al., 2010) or fluoride (Nicoletti et al., 2011) in the ferric state. The architecture of the distal cavities of *B. subtilis* and *T. fusca* 2/2HbOs can be compared with those of the few reported examples of sulphide-binding haem proteins (Rizzi, Wittenberg, Coda, Ascenzi, & Bolognesi, 1996). Molecular dynamics simulation indicates that only TrpG8 residue contributes to the sulphide stabilization through direct hydrogen-bonding interaction, thus accounting for the relatively high affinity for sulphide in these proteins (Nicoletti et al., 2010).

p0130 *A. tumefaciens* 2/2HbO is the first example of a structure where the topological position CD1 is occupied by His. Here, the haem distal site is characterized by the presence of a highly intertwined hydrogen-bonding network, involving residues TyrB10, HisCD1, SerE7, TrpG8, and three

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water molecules. In particular, the haem-coordinated water molecule is directly hydrogen bonded to a distal site intervening water molecule and to TrpG8 (Pesce et al., 2011). Thus, overall, the group II-conserved TrpG8 seems to be the residue playing a crucial/pivotal role in stabilizing the ligand and modulating its escape rate out of the distal pocket (Bonamore et al., 2005; Giangiacomo et al., 2005; Ilari et al., 2007; Milani et al., 2003; Pesce et al., 2011).

p0135 2/2HbOs also tend to contain a small residue at the E7 site, typically Ala, Ser, or Thr. A small residue at the E7 position may suggest the presence of an E7 route entry system to facilitate the accessibility of diatomic ligands to the 2/2HbO haem distal site (see below).

p0140 Group III sequences (all bacterial) display the largest extent of conservation (Wittenberg et al., 2002), all containing PheB9, TyrB10, PheCD1, HisE7, PheE14, TrpE15, and TrpG8. Such a large number of strictly conserved residues near the haem group suggest a narrow range of chemical properties and group III 2/2HbPs form a single homogeneous class. It should be noted, however, that recent sequence analysis based on an expanded and corrected bacterial genome database containing 181 group III 2/2Hbs in eight *phyla* showed group III to be less homogeneous than originally thought and raised the possibility that diverse chemical behaviours may be exhibited by its members (Nothnagel, Winer, et al., 2011).

p0145 In group III *C. jejuni* 2/2HbP, the haem distal pocket residues TyrB10, PheCD1, HisE7, IleE11, PheE14, and TrpG8 surround the haem-bound ligand (Nardini et al., 2006). Similar residues are also conserved in the 2/2HbP from *Helicobacter hepaticus* (Nothnagel, Winer, et al., 2011). Among these, only HisE7 is specific (and fully conserved) in group III (Vuletich & Lecomte, 2006), although the hydrophobic character of the haem pocket distal side is a highly conserved feature of group III globins. Contrary to group II, group III 2/2HbPs display Phe (or hydrophobic) residue at position CD1 and a hydrophobic residue at site E11. In *C. jejuni* 2/2HbP, the only hydrogen-bonding residues involved in ligand stabilization are TyrB10 and TrpG8, while no ligand-stabilizing interactions may be provided by residues at CD1 and E11 positions, nor by HisE7. Mutagenesis and molecular dynamics studies revealed that in the wt protein, the main residue responsible for oxygen stabilization is TyrB10. Bound oxygen is further stabilized by a hydrogen bond from either TrpG8 or HisE7, depending on the orientation of the Fe–O–O moiety, the hydrogen bond to TrpG8 being stronger than to HisE7. Most importantly, the coexistence of multiple conformations for the residues in the distal cavity, each characterized by a

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distinct pattern of hydrogen-bonding interaction, creates differences in the local polarity and affects the stabilization of the haem-bound ligand, the behaviour of each residue being affected by the other residues. Therefore, the oxygen-binding affinity (which in *C. jejuni* 2/2HbP is very high due to a low dissociation rate constant of 0.0041 s^{-1}) is the result of a cooperative property (Arroyo Mañez et al., 2011; Lu, Egawa, Wainwright, Poole, & Yeh, 2007). Furthermore, in the crystal structure of the *C. jejuni* 2/2HbP–cyanide complex, residue HisE7 occurs in two distinct conformations, corresponding to side-chain orientations that point towards the solvent or towards the haem distal site, respectively. Alternative position for the strictly conserved HisE7 suggests a ligand-gating mechanism similar to that described in *P. caudatum* 2/2HbN (Das et al., 2000; Nardini et al., 2006).

s0020

4. TUNNELS AND CAVITIES THROUGH 2/2Hb PROTEIN MATRIX

p0150 Despite the high-density packing of residues in the protein core, inner cavities or tunnels are often found in the protein matrix. Although such residue packing 'defects' may hamper the thermodynamic stability of a folded protein, their presence offers an evolutionary, possibly functional, advantage to the hosting protein. For instance, in enzymes they may provide preferred paths or intramolecular docking stations for the diffusion of substrates and products (Milani et al., 2003; Raushel, Thoden, & Holden, 2003; Weeks, Lund, & Raushel, 2006). In globins, the haem site is often buried inside the protein chain, which prevents direct contact with solvent. Therefore, the ligand has to trace its way to the haem by traversing the globin helical fold. The migration pathways are commonly believed to result from thermal fluctuations of the protein molecular structure, and the ligand access sites are located in (what are held to be) evolutionarily optimized well-defined regions of proteins that can be identified with systematic experimental and computational efforts (Brunori et al., 1999).

p0155 The analysis of the three-dimensional structures of 2/2Hbs have shown that the group I 2/2HbN fold is characterized by the presence of interconnected protein matrix apolar cavities, or a continuous tunnel, which connect the protein surface to an inner region merging with the haem distal site (Milani, Pesce, et al., 2004; Pesce, Milani, Nardini, & Bolognesi, 2008). Such peculiar feature may be related to the orientation of the CD–D region that forces positioning of the E-helix close to the haem distal face, thus preventing ligand access to the distal site cavity through the classical E7.

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p0160 The protein matrix tunnel, linking the protein surface to the haem distal site, appears to be conserved in group I 2/2Hbs, with the exception of the hexacoordinated *Synechocystis* sp. 2/2HbN, likely because of the conformational transitions required in this protein distal site region to achieve haem hexacoordination (Hoy et al., 2004). An alternative haem distal site access through the exposed 8-methyl edge of the haem group and near the propionates has been proposed (Falzone et al., 2002; Scott et al., 2010).

p0165 In agreement with the availability of cavities in the protein matrix, it has been shown that at least three group I 2/2Hbs, from *C. eugametos*, *M. tuberculosis*, and *P. caudatum*, can bind Xe atoms in the crystalline state. The Xe atoms map experimentally at multiple sites and with comparable topology within the tunnel/cavity path in these 2/2Hbs (Milani, Pesce, et al., 2004). In particular, in *C. eugametos* 2/2HbN and in *M. tuberculosis* 2/2HbN, the tunnel is composed of two roughly orthogonal branches converging at the haem distal site from two distinct protein surface access sites. On one hand, a 20-Å long tunnel branch connects the protein region nestled between the AB and GH hinges to the haem distal site. On the other, a short tunnel branch of about 8 Å connects an opening in the protein structure between G- and H-helices to the haem (Fig. 2.2). In *P. caudatum* 2/2HbN, the haem site is connected to the solvent region by a three-cavity system, topologically distributed along the tunnel's long branch described above (Milani, Pesce, et al., 2004). A similar, but more open, tunnel system is also present in *T. pyriformis* 2/2HbN, where the exit of the short branch differs slightly in orientation relative to *M. tuberculosis* 2/2HbN. The tunnel volume of *T. pyriformis* 2/2HbN is about 380 $Å^3$, which is similar to that of *C. eugametos* 2/2HbN (400 $Å^3$), but larger than that of *M. tuberculosis* and *P. caudatum* HbNs (265 $Å^3$ and 180 $Å^3$, respectively) (Igarashi et al., 2011). Although protein cavity volumes vary among 2/2HbNs, these values are not correlated with O_2 association rate constants (Couture, Das, et al., 1999; Couture, Yeh, et al., 1999; Das et al., 2000; Igarashi et al., 2011; Ouellet et al., 2008).

p0170 Residues lining the tunnel branches are hydrophobic and are substantially conserved throughout group I (Vuletich & Lecomte, 2006). PheE15, a well-conserved residue, adopts two conformations in *M. tuberculosis* 2/2HbN (Fig. 2.2). In one, PheE15, the benzene side chain, blocks the longer channel of the tunnel path (the so-called closed state) and in the other it does not (the open state) (Milani et al., 2001; Milani, Pesce, et al., 2004). *M. tuberculosis* 2/2HbN is endowed with a potent nitric oxide dioxygenase activity which allows it to relieve nitrosative stress and enhance *in vivo*

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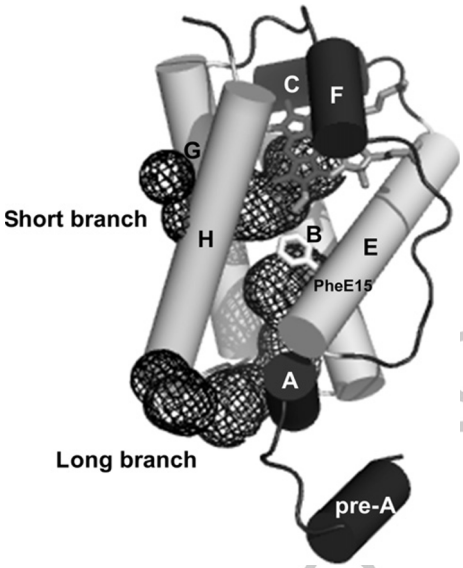

f0010 **Figure 2.2** The protein matrix tunnel (short and long branches) observed in group I *M. tuberculosis* HbN. The tunnel surface, defined by a 1.4 Å radius probe, is portrayed as a mesh. Residue PheE15, causing the main restriction in the diameter of the long branch tunnel, is shown in the close conformation in stick representation and labelled. Helices are shown as cylinders and labelled. The helices structurally conserved within 3/3 and 2/2 folds are shown in grey. The haem is shown in stick representation. (For colour version of this figure, the reader is referred to the online version of this chapter.)

survival of its host through the rapid oxidation of NO to harmless nitrate (Couture, Yeh, et al., 1999; Ouellet et al., 2002; Pathania, Navani, Gardner, Gardner, & Dikshit, 2002). Migration of O_2 and NO to the *M. tuberculosis* 2/2HbN distal haem cavity is driven by a dual-path mechanism. In fact, by long molecular dynamics simulations (0.1 ms), it has been shown that in deoxy 2/2HbN, PheE15 adopts the closed conformation and hence the O_2 ligand enters the protein via the short channel. In the case of oxygenated 2/2HbN, the PheE15 prefers the open conformation, thus facilitating entrance of the second ligand (NO) via the long tunnel branch (Bidon-Chanal et al., 2006; Bidon-Chanal, Martì, Estrin, & Luque, 2007). Recent mutagenesis studies also support this view on the diffusion of small diatomic ligands through the *M. tuberculosis* 2/2HbN protein matrix tunnel system, pointing out the delicate structural balance imposed by the PheE15 gate, which not only regulates ligand migration but also contributes to avoid the collapse of helices B and E, thus preserving the ligand accessibility along the tunnel long branch (Oliveira et al., 2012).

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p0175 In *T. pyriformis* 2/2HbN, the hydrophobic residues Leu11, Ala20, and Leu94 form a gate delimiting the outer solvent space from the inner region, whereas Leu54 and Leu90 are located at the connecting gaps. Leu54 corresponds to the gating residue PheE15 of *M. tuberculosis* 2/2HbN and may contribute significantly to molecular processes that sustain diffusion of diatomic ligands to the haem (Igarashi et al., 2011).

p0180 Strikingly, the *M. tuberculosis* 2/2HbN nitric-oxide dioxygenase activity has been correlated with the presence of the *M. tuberculosis* 2/2HbN-specific pre-A helix (Fig. 2.2). Deletion of pre-A region from the *M. tuberculosis 2/2*HbN drastically reduces its ability to scavenge nitric oxide, whereas its insertion at the N-terminus of pre-A lacking HbN of *M. smegmatis* improved its nitric-oxide dioxygenase activity. Molecular dynamics simulations show that the excision of the pre-A motif results in distinct changes in the protein dynamics, which cause the gate of the tunnel long branch to be trapped into a closed conformation, thus impeding migration of diatomic ligands towards the haem distal site (Lama et al., 2009).

p0185 Other molecular dynamics studies have proposed a different view of NO access to the haem distal site in *M. tuberculosis* 2/2HbN, whereby the ligand molecule preferentially would enter the protein matrix through the tunnel short branch, and once inside the protein, NO diffuses through a series of cavities corresponding to experimental xenon-binding pockets (Daigle, Rousseau, Guertin, & Lagüe, 2009). NO diffusion along the tunnel long branch was found to be hindered by the PheE15 side-chain obstruction, countering the dual-path mechanism proposed previously (Bidon-Chanal et al., 2006). Moreover, NO entering the tunnel long branch would preferentially bypass the PheE15 barrier by means of an additional tunnel located between the E- and H-helices (Daigle et al., 2009). The presence of other ligand migration pathways through the *M. tuberculosis* 2/2HbN matrix, potentially competing with the short and the long tunnel branches, was also proposed for paths located between E- and H-helices or between the C- and F-helices. Both of these paths are surrounded by at least one polar residue and are expected to be the preferred escape channel for removing the products of NO detoxification reaction (such as the nitrate anion) from the protein matrix (Martì et al., 2008; Mishra & Meuwly, 2010).

p0190 Contrary to group I, group II 2/2Hbs do not show an evident tunnel/cavity system connecting the protein surface to the haem distal pocket. The protein matrix tunnel observed in 2/2HbN, is dramatically restricted in 2/2HbO, where different relative orientations of the G- and H-helices, and increased side-chain volumes at topological sites B1, B5, G8, G9, and

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G12, mostly fill the protein matrix tunnel space (Bonamore et al., 2005; Giangiacomo et al., 2005; Ilari et al., 2007; Milani et al., 2003; Pesce et al., 2011). In particular, in 2/2HbOs, the bulky side-chain of the conserved TrpG8 obstructs the short tunnel branch and the deeper part of the distal site pocket typical of 2/2HbNs. The 2/2HbN long tunnel branch retains only two cavities in 2/2HbOs, both fully shielded from solvent contact (Milani et al., 2003). Interestingly, in *M. tuberculosis* 2/2HbO, residue TrpG8 is also responsible for blocking the region corresponding to the 2/2HbN long tunnel and therefore key for ligand entry (Boechi et al., 2008; Ouellet et al., 2007). The restriction of the cavities within the protein matrix becomes extreme in *T. fusca* 2/2HbO, where no internal cavities are detected, due to substitutions with larger residues relative to other 2/2HbOs, or by conformational differences of conserved or similar size residues (Bonamore et al., 2005).

p0195 The substantial absence of a protein matrix tunnel system is mirrored by the conserved presence of a small distal site E7 residue in group II 2/2HbOs (Vuletich & Lecomte, 2006), which does not hinder entrance to the haem distal cavity. Therefore, in 2/2HbOs, diatomic ligands (such as O_2, CO, and NO) may preferably access the haem distal site through an E7 route. Molecular dynamics simulations, however, showed that once the protein is oxygenated, both the E7 route and the path corresponding to the *M. tuberculosis* 2/2HbN tunnel long branch can contribute to ligand entry, because they present similar barriers. This mechanism differs from the case of 2/2HbN, in which each ligand has been proposed to migrate through a separate pathway (Bidon-Chanal et al., 2006). The change in the free energy barrier for the long tunnel is due to the TrpG8 interaction with the haem-bound O_2. The short-tunnel E7 barrier does not change significantly upon oxygenation; consequently, the overall barrier presented by the short-tunnel E7 is similar in the oxygenated and deoxygenated states of the protein. This fact is consistent with the experimental kinetic constants for ligand migration. The results highlight the importance of TrpG8 in regulating ligand migration in 2/2HbO, since not only is it responsible for the high barrier observed in the long tunnel, but it also blocks the short tunnel branch displayed by group I 2/2HbNs. Furthermore, TrpG8 is important in anchoring TyrCD1 and LeuE11 side chains, thereby allowing the stabilization of the haem-bound ligand via hydrogen bonds donated from TrpG8 and TyrCD1. Following its dissociation, the ligand can migrate between three temporary docking sites, which are modulated by the conformational rearrangements of the side chains of several critical distal amino acids,

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including the TrpG8, LeuE11, TyrCD1, and AlaE7. The initial migration of the ligand within the distal pocket leads to its rebinding to the haem iron atom or to its escape into the solvent via a hydrophobic tunnel that coincides with the internal cavities found in the crystallographic structure of 2/2HbO (Guallar et al., 2009). This would indicate that the presence of the conserved residues in group II and group III, but not group I, is responsible for the significantly different migration patterns in 2/2HbO and 2/2HbN.

p0200 Within group II 2/2HbOs, the protein from *B. subtilis* is a peculiar case, as the presence of a Thr residue at position E7 typically blocks the E7 path and the X-ray crystallographic structure does not exhibit a clear tunnel for ligand migration (Giangiacomo et al., 2005). However, O_2 association rate constant k_{on} is higher than that found for *M. tuberculosis* 2/2HbO, and similar to that of *M. tuberculosis* 2/2HbN (Couture, Yeh, et al., 1999; Giangiacomo et al., 2005; Pathania, Navani, Rajamohan, & Dikshit, 2002). The structural and the kinetic data have been reconciled by classical molecular dynamics simulations of the oxy, carboxy, and deoxy proteins which showed that GlnE11 presents an alternate conformation, giving rise to a wide ligand migration tunnel, topologically related to the long tunnel branch found in group I 2/2HbNs. In *B. subtilis* 2/2HbO, residue TrpG8 does not block the tunnel, as generally assumed by inspection of the crystal structure, due to a rearrangement in the distal site involving GlnE11, and the tunnel is open due to the lack of the bulky PheE15, the tunnel gating residue in *M. tuberculosis* 2/2HbN. On the other hand, the results for the CO and O_2 bound protein show that GlnE11 is directly involved in the stabilization of the coordinated ligand, playing a similar role as TyrB10 and TrpG8 in other 2/2Hbs (Boechi et al., 2010). These results underline once more the plasticity and redundancy of several residues within the globin fold that account for the varied ligand-binding kinetics observed.

p0205 Analysis of group III *C. jejuni* 2/2HbP structure shows no evident protein matrix tunnel/cavity system, mostly due to the peculiar backbone conformation of the pre-B helix residues, and to bulky side-chain substitutions (conserved among members of group III) at residues that define the tunnel/cavities walls in group I and II 2/2Hbs (Nardini et al., 2006). Since HisE7 (conserved in group III) adopts two alternate conformations ('open' and 'closed') in *C. jejuni* 2/2HbP, E7 haem-distal-site gating has been proposed to play a functional role for ligand diffusion to the haem, in the absence of a protein matrix tunnel/cavity system (Nardini et al., 2006).

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5. PROPOSED FUNCTIONS FOR THE 2/2Hb FAMILY

p0210 Although the number of the deposited 2/2Hb sequences has grown rapidly over the past years, limited functional information is currently available for these proteins. Examples of proposed functions, consistent with observed biophysical properties, include nitric oxide detoxification, protection from reactive oxygen and nitrogen species, dioxygen scavenging, and recently sulphide binding (Hill et al., 1996; Nicoletti et al., 2010; Ouellet et al., 2002; Parrilli et al., 2010; Scott et al., 2010; Vinogradov & Moens, 2008). However, the diversity of physiological and environmental contexts in which 2/2Hbs are found suggests that additional enzymatic activities and insights into haem chemistry are yet to be discovered. For example, group I 2/2Hb of the unicellular green alga *C. eugametos* is induced in response to active photosynthesis and is localized partly along the chloroplast thylakoid membranes (Couture & Guertin, 1996). Group I 2/2Hb from the ciliated protozoa *P. caudatum* may supply O_2 to the mitochondria (Wittenberg et al., 2002). Moreover, group I 2/2Hb from the *Nostoc* sp. cyanobacterium is thought to protect the nitrogen-fixation apparatus from oxidative damage through O_2 scavenging (Hill et al., 1996).

p0215 Most of the functional analyses have been reported for 2/2Hbs from mycobacterial species, in particular, group I 2/2HbN in *M. tuberculosis*, *M. bovis*, *M. smegmatis,* and *M. avium*, group II 2/2HbO in *M. leprae* and all the above species, while group III 2/2HbP in *M. avium* only (Vinogradov et al., 2006; Vuletich & Lecomte, 2006; Wittenberg et al., 2002). The regression in content of 2/2Hb paralogues from *M. avium* (three 2/2Hbs), through *M. tuberculosis* (two 2/2Hbs), to *M. leprae* (one 2/2Hb) has been proposed to reflect an adaptation from saprophytic lifestyle to obligate intracellular parasitism, which paralleled the loss of functions provided by 2/2HbN and 2/2HbP. These results are consistent with the general notion that a 2/2HbO-like globin provided the ancestor structure from which 2/2HbNs and 2/2HbPs, as well as the classical 3-on-3 structural fold, originated (Nakajima, Álvarez-Salgado, Kikuchi, & Arredondo-Peter, 2005; Vinogradov et al., 2006; Vuletich & Lecomte, 2006).

p0220 Mycobacterial 2/2Hbs have been mostly implicated in scavenging of reactive nitrogen species. During infection, mycobacteria have to face the toxic effects of reactive nitrogen species, primarily NO, produced by activated macrophages expressing inducible NO synthase (Cooper, Adams, Dalton, Appelberg, & Ehlers, 2002; MacMicking et al., 1997; Nathan &

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Shiloh, 2000; Ohno et al., 2003; Schnappinger, Schoolnik, & Ehrt, 2006; Visca, Fabozzi, Milani, Bolognesi, & Ascenzi, 2002). The distinct features of the haem active site structure of NO-responsive mycobacterial 2/2Hbs and their ligand-binding properties (Milani et al., 2001, 2003; Milani, Pesce, et al., 2004; Milani et al., 2005; Visca, Fabozzi, Milani, et al., 2002), combined with co-occurrence of multiple 2/2Hb classes in individual mycobacterial species and their temporal expression patterns *in vivo* (Fabozzi, Ascenzi, Renzi, & Visca, 2006; Ouellet et al., 2002, 2003), suggest that these globins play different physiological functions (Ascenzi, Bolognesi, Milani, Guertin, & Visca, 2007). For instance, *M. tuberculosis* 2/2HbN, is endowed with a potent nitric-oxide dioxygenase activity and has been found to relieve nitrosative stress (Couture, Yeh, et al., 1999; Pathania, Navani, Gardner, et al., 2002) enhancing *in vivo* survival of a heterologous host, *Salmonella enterica typhimurium*, within the macrophages (Pawaria et al., 2007). These findings strongly support the NO scavenging and detoxification roles of HbN, which may be vital for *in vivo* survival and pathogenicity of *M. tuberculosis*. Similarly, a *M. bovis* mutant lacking 2/2HbN does not oxidize NO to NO_3^- and shows decreased respiration upon exposure to NO (Ouellet et al., 2002). Although to a lesser extent, a similar protective effect was also reported for *M. smegmatis* 2/2HbN in the homologous system (Lama, Pawaria, & Dikshit, 2006).

p0225 Detoxification of NO to nitrate is the hypothetical physiological function proposed also for *T. pyriformis* 2/2HbN. Based on oxygen affinity measurements, it has been estimated that *T. pyriformis* 2/2HbN within the cell would be maintained in the Fe(II)–O_2 form, indicating that *T. pyriformis* 2/2HbN does not function as an oxygen transporter. In addition, nitrosative stress mediated by sodium nitroprusside inhibits glyceraldehyde 3-phosphate dehydrogenase activity in *T. pyriformis* (Fourrat, Iddar, Valverde, Serrano, & Soukri, 2007). Therefore, *T. pyriformis* must have acquired a mechanism that senses and protects against nitrosative stress conditions, such as NO exposure (Igarashi et al., 2011).

p0230 Other widely studied member of the group I 2/2HbN are cyanobacterial globins. 2/2HbN from the cyanobacterium *Synechococcus* sp. strain PCC 7002 and PCC 6803 have been characterized structurally and biochemically, focusing mainly on their two unusual structural properties: the bis-histidyl coordination of the haem iron in the absence of an exogenous ligand, and the post-translational covalent attachment of the b haem to the globin by modification of the 2-vinyl substituent (Falzone et al., 2002; Lecomte et al., 2004; Lecomte, Vu, & Falzone, 2005; Pond et al., 2012; Scott

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et al., 2002, 2010; Trent et al., 2004; Vu, Vuletich, Kuriakose, Falzone, & Lecomte, 2004; Vuletich, Falzone, & Lecomte, 2006). The function of *Synecocistis* 2/2HbNs and their relationship to the metabolism of dioxygen, nitric oxide, or various reactive nitrogen and oxygen species are still largely unknown, although comparison to similar 2/2 haemoglobins suggests that reversible dioxygen binding is not its main activity. Recently, *in vitro* and *in vivo* experiments on cyanobacterium *Synechococcus* sp. strain PCC 7002 showed that its transcription profiles indicate that the protein is not strongly regulated under any of a large number of growth conditions and that the gene is probably constitutively expressed. High levels of nitrate, used as the sole source of nitrogen, and exposure to nitric oxide were tolerated better by the wild-type strain than by a 2/2HbN null mutant, whereas overproduction of protein in the null mutant background restored the wild-type growth. The cellular contents of reactive oxygen/nitrogen species were elevated in the null mutant under all conditions and were highest under NO challenge or in the presence of high nitrate concentrations. A peroxidase assay showed that purified 2/2HbN does not possess significant hydrogen peroxidase activity. Taken together, all these evidences suggested for 2/2HbN from cyanobacterium *Synechococcus* sp. strain PCC 7002 a protection role, from reactive nitrogen species, which cells could encounter naturally during growth on nitrate or under denitrifying conditions (Scott et al., 2010).

p0235 The physiological role of *M. tuberculosis* 2/2HbO has been primarily related to O_2 metabolism. 2/2HbO was hypothesized to be endowed with O_2 uptake or delivery properties during mycobacterial hypoxia and latency (Liu, He, & Chang, 2004; Pathania, Navani, Gardner, et al., 2002). This hypothesis is in apparent contrast with the low O_2 association and dissociation rates reported for 2/2HbO (Ouellet et al., 2003), and with its constitutive expression under aerobic conditions during the whole growth cycle of *M. bovis* (Mukai, Savard, Ouellet, Guertin, & Yeh, 2002; Pathania, Navani, Rajamohan, et al., 2002). 2/2HbO (II)–O_2 could still be able to sustain bacterial aerobic respiration by scavenging NO or other reactive species that would block the respiratory chain. In this context, the high stability of 2/2HbO(II)–O_2 would secure the reaction with NO even at very low O_2 tensions, as those that may exist in infected or necrotic tissue (Fabozzi et al., 2006). Interestingly, *M. leprae* 2/2HbO has been proposed to be involved in both H_2O_2 and NO scavenging, protecting from nitrosative and oxidative stress, and sustaining mycobacterial respiration (Ascenzi, De Marinis, Coletta, & Visca, 2008). Under anaerobic and highly oxidative

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conditions, as in the macrophagic environment where *M. leprae* is faced with H_2O_2 (Ascenzi, Bolognesi, & Visca, 2007; Ascenzi & Visca, 2008; Visca, Fabozzi, Milani, et al., 2002), the rapid formation of 2/2HbO—Fe(IV)═O occurs, which in turn facilitates NO scavenging, leading to the formation of haem-Fe(III) and NO_2^-. In turn, NO acts as antioxidant of 2/2HbO—Fe(IV)═O, which could be responsible for the oxidative damage of the mycobacterium. This reaction does not require partner redox enzymes, since the haem–protein oscillates between the haem-Fe(III) and haem-Fe(IV)═O form, being helped by NO in maintaining an efficient H_2O_2 reduction rate. In this framework, it can be understood why *M. leprae* 2/2HbO—Fe(III) does not require a reductase system(s), which indeed has not been identified yet in this elusive mycobacterium (Ascenzi, Bolognesi, & Visca, 2007; Ascenzi & Visca, 2008). The catalytic parameters for NO scavenging by haem-Fe(II)—O_2 and haem-Fe(IV)═O are similar and high enough, suggesting that both reactions could take place *in vivo* (Ascenzi et al., 2008; Ascenzi & Visca, 2008).

p0240 The H_2O_2-induced *M. leprae* 2/2HbO—Fe(IV)═O formation could be relevant for *M. leprae* survival *in vivo* in the presence not only of NO and NO_2^- but also of peroxynitrite (Ascenzi, De Marinis, Visca, Ciaccio, & Coletta, 2009). The formation of peroxynitrite can in fact result from a secondary reaction of NO and the superoxide radical, which is concomitantly produced by activated macrophages. Then, peroxynitrite could rapidly react with CO_2 at the site of inflammation leading to the formation of strong oxidant and nitrating species (Ascenzi, Bocedi, et al., 2006; Goldstein, Lind, & Merényi, 2005). Peroxynitrite detoxification by *M. leprae* 2/2HbO has been shown to be rapid; therefore, 2/2HbO might be an important contributor to such function (Ascenzi, Milani, & Visca, 2006). Furthermore, as reported for NO and NO_2^- (Ascenzi et al., 2008), peroxynitrite acts as an antioxidant preventing the *M. leprae* 2/2HbO—Fe(IV)═O-mediated oxidation of mycobacterial (macro)molecules such as membrane lipids (i.e. lipid peroxidation) (Ascenzi et al., 2009).

p0245 The defence mechanisms against reactive oxygen and nitrogen species represent important components in the evolutionary adaptations, particularly under extreme environmental conditions. In this framework, *in vivo* and *in vitro* experiments have been performed in order to understand the roles of group II 2/2HbO from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (encoded by the *PSHAa0030* gene) in NO detoxification mechanisms (Coppola et al., 2013). The presence of multiple genes encoding 2/2Hbs and a flavohaemoglobin in this bacterium strongly

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suggests that these proteins fulfil important physiological roles, perhaps associated to the peculiar features of the Antarctic habitat (Giordano et al., 2007). Inactivation of the *PSHAa0030* gene renders the mutant bacterial strain sensitive to high O_2 levels, hydrogen peroxide, and nitrosating agents (Parrilli et al., 2010). Furthermore, when the *PSHAa0030* gene was cloned and over-expressed in a flavohaemoglobin-deficient mutant of *E. coli*, unable to metabolize NO, and the resulting strain was analyzed for its growth properties and oxygen uptake in the presence of NO, it was shown that *P. haloplanktis* 2/2HbO indeed protects growth and cellular respiration of the heterologous host from the toxic effects of NO donors. Moreover, the ferric form of *P. haloplanktis* 2/2HbO was shown to catalyze peroxynitrite isomerization *in vitro*, confirming its potential role in scavenging reactive nitrogen species (Coppola et al., 2013).

p0250 Recently, group II 2/2HbOs have been also implicated in metabolic pathways involving physiologically relevant sulphur compounds. *B. subtilis* and *T. fusca* group II 2/2HbOs have been shown to bind sulphide with an affinity constant in the sub-micromolar range such that they are partially saturated with sulphide when recombinantly expressed in *E. coli* (Nicoletti et al., 2010). Thus, these proteins have been proposed to play a direct role as sulphide scavenger under high oxygen growth conditions, due to the oxygen-dependent down-regulation of the competing cysteine synthase B (which is instead a very effective sulphide scavenger under low oxygen conditions). Also, a highly oxidative environment would favour the oxidation of the 2/2Hb haem-Fe atom, thus allowing prompt formation of the high affinity ferric sulphide adduct (Nicoletti et al., 2010). Interestingly, it has recently been demonstrated that the gene encoding *B. subtilis* 2/2HbO (as well as most 2/2Hbs from bacilli and staphylococci) is contained within a thiol redox pathway that is implicated in the bacterial response to the thiol oxidative stress (Larsson, Rogstam, & von Wachenfeldt, 2007). In this framework, it has been proposed that *B. subtilis* 2/2HbO could participate (directly or indirectly) in the complex redox pathway of sulphur metabolism in *Bacillus* sp. (Nicoletti et al., 2010).

p0255 Among group III 2/2HbPs, the protein from the *Campylobacter jejuni* is the most characterized from the structural and biochemical view points (Bolli et al., 2008; Nardini et al., 2006), together with the more recently reported 2/2HbP from *Helicobacter hepaticus* (Nothnagel, Winer, et al., 2011). *C. jejuni*, one of the most important etiological agents of bacterial gastroenteritis worldwide, hosts two Hbs: a single domain globin Cgb and a group III 2/2Hb Ctb. Although both globins are up-regulated by the

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transcription factor NssR (which regulates expression of a small regulon that includes *cgb* and *ctb*) in response to nitrosative stress (Elvers et al., 2005), only Cgb has been proposed to protect the bacterium against nitrosative stress, likely via a NO dioxygenase reaction during the initial stages, followed by a denitrosylase mechanism upon prolonged exposure to NO (Elvers, Wu, Gilberthorpe, Poole, & Park, 2004; Shepherd, Bernhardt, & Poole, 2011). The role of *C. jejuni* 2/2HbP is unclear: it is not directly involved in NO detoxification and it displays an extremely high O_2 affinity, making it unlikely to be an O_2 carrier. Based on the polarity of the haem distal cavity, reminiscent of that found in cytochrome *c* peroxidase, *C. jejuni* 2/2HbP has been proposed to be involved in (pseudo)enzymatic O_2 chemistry (Lu et al., 2007; Nardini et al., 2006; Wainwright, Elvers, Park, & Poole, 2005). Recently, attempts to define a function for *C. jejuni* 2/2HbP have been pursued by examining the effects of a *ctb* mutation on the NO transcriptome and *cgb* gene expression during normoxia and hypoxia. Based on these data, it was proposed that, by binding NO or O_2, *C. jejuni* 2/2HbP dampens the response to NO under hypoxic conditions and limits *cgb* expression, perhaps because Cgb function (i.e. NO detoxification) requires O_2-dependent chemistry (Smith, Shepherd, Monk, Green, & Poole, 2011).

p0260 It is worth noticing also that *C. jejuni* 2/2HbP displays the highest affinity as well as the fastest combination and the slowest dissociation rates for cyanide binding within the Hb superfamily. Thus, it was suggested that this 2/2hbP may act as a cyanide scavenger facilitating *C. jejuni* survival (Bolli et al., 2008).

s0030 6. CONCLUSIONS

p0265 2/2Hbs populate a branch of the Hb superfamily tree, which has been discovered and enriched with data starting in the early 1990s. Our current knowledge on these 'minimal' globins is rather extensive as far as their primary structures are concerned (more than 1000 gene sequences have been identified to date; Vinogradov et al., 2013). Such information allowed to distinguish groups I, II, and III 2/2Hbs and provided insight into their distribution through the evolutionary phyla. Indeed, 2/2Hbs have been mostly found among protozoa and bacteria, although their presence in plants and in some lower eukaryotes has also been reported. Despite the relatively contained size of 2/2Hbs in all three groups, several crystal structures have shown that careful editing of the 'classical' globin (i.e. Mb) 3-on-3 α-helical fold, and the introduction of Gly-based motifs (in groups I and II), provides

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efficient enclosure of the haem in a protein environment that is essentially based on four α-helices (hence the 2/2 helical fold acronym). A key feature emerging from the crystal structures is the presence (at least in groups I and II) of strategic and 2/2Hb-specific protein matrix tunnels or cavities, with conserved topology, which are held to support diffusion of small physiological ligands to/from the haem. The variety of distal site haem cavities, and the properties of the lining amino acids, is substantial and compatible with the display of different (pseudo)enzymatic activities. These are often Au3
related to detoxifying mechanisms devised by a pathogen in response to nitrogen and oxygen reactive species produced by the host. Other roles have been considered, although these are mainly hypothetic rather than proved *in vivo*. Notably, based on affinity of kinetic considerations, in just one case, an intracellular O_2 transport/delivery role has been considered. The haem distal site in 2/2Hbs (as in Mb and Hb) is suited to bind small ligands, likely diatomic gaseous molecules, such as O_2, NO, and CO. Thus, compared to a classical enzyme acting on larger substrates, exploring 2/2Hb functions *in vivo* appears more complex, despite the extensive knowledge available on haem biochemistry. A further complicating factor is related to the pathogenicity of some 2/2Hb carrying microorganisms, or to the limited knowledge we have on their lifestyles and basic microbiology. After more than 15 years in this field, it is felt that one of the main challenges that are still open is a thorough analysis and description of 2/2Hb *in vivo* functions.

REFERENCES

Arroyo Mañez, P., Lu, C., Boechi, L., Martí, M. A., Shepherd, M., Wilson, J. L., et al. (2011). Role of the distal hydrogen-bonding network in regulating oxygen affinity in the truncated hemoglobin III from *Campylobacter jejuni*. *Biochemistry*, *50*, 3946–3956.

Ascenzi, P., Bocedi, A., Bolognesi, M., Fabozzi, G., Milani, M., & Visca, P. (2006). Nitric oxide scavenging by *Mycobacterium leprae* GlbO involves the formation of the ferric heme-bound peroxynitrite intermediate. *Biochemical and Biophysical Research Communications*, *339*, 450–456.

Ascenzi, P., Bolognesi, M., Milani, M., Guertin, M., & Visca, P. (2007). Mycobacterial truncated hemoglobins: From genes to functions. *Gene*, *398*, 42–51.

Ascenzi, P., Bolognesi, M., & Visca, P. (2007). *NO dissociation represents the rate limiting step for O_2-mediated oxidation of ferrous nitrosylated *Mycobacterium leprae* truncated hemoglobin O. *Biochemical and Biophysical Research Communications*, *357*, 809–814.

Ascenzi, P., De Marinis, E., Coletta, M., & Visca, P. (2008). H_2O_2 and •NO scavenging by *Mycobacterium leprae* truncated hemoglobin O. *Biochemical and Biophysical Research Communications*, *373*, 197–201.

Ascenzi, P., De Marinis, E., Visca, P., Ciaccio, C., & Coletta, M. (2009). Peroxynitrite detoxification by ferryl *Mycobacterium leprae* truncated hemoglobin O. *Biochemical and Biophysical Research Communications*, *380*, 392–396.

Comp. by: Saravanan Stage: Proof Chapter No.: 2 Title Name: AMP
Date:25/7/13 Time:18:11:13 Page Number: 72

B978-0-12-407693-8.00002-9, 00002

Ascenzi, P., Milani, M., & Visca, P. (2006). Peroxynitrite scavenging by ferrous truncated hemoglobin GlbO from *Mycobacterium leprae*. *Biochemical and Biophysical Research Communications*, *351*, 528–533.

Ascenzi, P., & Visca, P. (2008). Scavenging of reactive nitrogen species by mycobacterial truncated hemoglobins. *Methods in Enzymology*, *436*, 317–337.

Bidon-Chanal, A., Martì, M. A., Crespo, A., Milani, M., Orozco, M., Bolognesi, M., et al. (2006). Ligand-induced dynamical regulation of NO conversion in *Mycobacterium tuberculosis* truncated hemoglobin-N. *Proteins*, *64*, 457–464.

Bidon-Chanal, A., Martì, M. A., Estrin, D. A., & Luque, F. J. (2007). Dynamical regulation of ligand migration by a gate-opening molecular switch in truncated hemoglobin-N from *Mycobacterium tuberculosis*. *Journal of the American Chemical Society*, *129*, 6782–6788.

Boechi, L., Mañez, P. A., Luque, F. J., Martì, M. A., & Estrin, D. A. (2010). Unraveling the molecular basis for ligand binding in truncated hemoglobins: The trHbO Bacillus subtilis case. *Proteins*, *78*, 962–970.

Boechi, L., Martì, M. A., Milani, M., Bolognesi, M., Luque, F. J., & 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., et al. (2008). Ferrous *Campylobacter jejuni* truncated hemoglobin P displays an extremely high reactivity for cyanide—A comparative study. *FEBS Journal*, *275*, 303–315.

Bolognesi, M., Bordo, D., Rizzi, M., Tarricone, C., & Ascenzi, P. (1997). Nonvertebrate hemoglobins: Structural bases for reactivity. *Progress in Biophysics and Molecular Biology*, *68*, 29–68.

Bonamore, A., Ilari, A., Giangiacomo, L., Bellelli, A., Morea, V., & Boffi, A. (2005). A novel thermostable hemoglobin from the actinobacterium *Thermobifida fusca*. *FEBS Journal*, *16*, 4189–4201.

Brunori, M., Cutruzzolà, F., Savino, C., Travaglini-Allocatelli, C., Vallone, B., & Gibson, Q. H. (1999). Structural dynamics of ligand diffusion in the protein matrix: A study on a new myoglobin mutant Y(B10) Q(E7) R(E10). *Biophysical Journal*, *76*, 1259–1269.

Cooper, A. M., Adams, L. B., Dalton, D. K., Appelberg, R., & Ehlers, S. (2002). IFN-gamma and NO in mycobacterial disease: New jobs for old hands. *Trends in Microbiology*, *10*, 221–226.

Coppola, D., Giordano, D., Tinajero-Trejo, M., di Prisco, G., Ascenzi, P., Poole, R. K., et al. (2013). Antarctic bacterial haemoglobin and its role in the protection against nitrogen reactive species. *Biochimica et Biophysica Acta*http://dx.doi.org/10.1016/j.bbapap.2013.02.018, pii: S1570-9639(13)00081-2. [Epub ahead of print]. Au4

Couture, M., Das, T. K., Lee, H. C., Peisach, J., Rousseau, D. L., Wittenberg, B. A., et al. (1999). Chlamydomonas chloroplast ferrous hemoglobin. Heme pocket structure and reactions with ligands. *The Journal of Biological Chemistry*, *274*, 6898–6910.

Couture, M., Das, T. K., Savard, P. Y., Ouellet, Y., Wittenberg, J. B., Wittenberg, B. A., et al. (2000). Structural investigations of the hemoglobin of the cyanobacterium *Synechocystis* PCC6803 reveal a unique distal heme pocket. *European Journal of Biochemistry*, *267*, 4770–4780.

Couture, M., & Guertin, M. (1996). Purification and spectroscopic characterization of a recombinant chloroplastic hemoglobin from the green unicellular alga *Chlamydomonas eugametos*. *European Journal of Biochemistry*, *242*, 779–787.

Couture, M., Yeh, S. R., Wittenberg, B. A., Wittenberg, J. B., Ouellet, Y., Rousseau, D. L., et al. (1999). A cooperative oxygen-binding hemoglobin from *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 11223–11228.

Comp. by: Saravanan Stage: Proof Chapter No.: 2 Title Name: AMP
Date:25/7/13 Time:18:11:14 Page Number: 73

B978-0-12-407693-8.00002-9, 00002

Daigle, R., Rousseau, J. A., Guertin, M., & Lagüe, P. (2009). Theoretical investigations of nitric oxide channeling in *Mycobacterium tuberculosis* truncated hemoglobin N. *Biophysical Journal*, *97*, 2967–2977.

Das, T. K., Weber, R. E., Dewilde, S., Wittenberg, J. B., Wittenberg, B. A., Yamauchi, K., et al. (2000). Ligand binding in the ferric and ferrous states of *Paramecium* haemoglobin. *Biochemistry*, *39*, 14330–14340.

Droghetti, E., Nicoletti, F. P., Bonamore, A., Boechi, L., Arroyo Mañez, P., Estrin, D. A., et al. (2010). Heme pocket structural properties of a bacterial truncated hemoglobin from *Thermobifida fusca*. *Biochemistry*, *49*, 10394–10402.

Elvers, K. T., Turner, S. M., Wainwright, L. M., Marsden, G., Hinds, J., Cole, J. A., et al. (2005). NssR, a member of the Crp-Fnr superfamily from *Campylobacter jejuni*, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin. *Molecular Microbiology*, *57*, 735–750.

Elvers, K. T., Wu, G., Gilberthorpe, N. J., Poole, R. K., & Park, S. F. (2004). Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in *Campylobacter jejuni* and Campylobacter coli. *Journal of Bacteriology*, *186*, 5332–5341.

Fabozzi, G., Ascenzi, P., Renzi, S. D., & Visca, P. (2006). Truncated hemoglobin GlbO from *Mycobacterium leprae* alleviates nitric oxide toxicity. *Microbial Pathogenesis*, *40*, 211–220.

Falzone, C. J., Vu, B. C., Scott, N. L., & Lecomte, J. T. (2002). The solution structure of the recombinant hemoglobin from the cyanobacterium *Synechocystis* sp. PCC 6803 in its hemichrome state. *Journal of Molecular Biology*, *324*, 1015–1029.

Feis, A., Lapini, A., Catacchio, B., Brogioni, S., Foggi, P., Chiancone, E., et al. (2008). Unusually strong H-bonding to the heme ligand and fast geminate recombination dynamics of the carbon monoxide complex of *Bacillus subtilis* truncated hemoglobin. *Biochemistry*, *47*, 902–910.

Fourrat, L., Iddar, A., Valverde, F., Serrano, A., & Soukri, A. (2007). Effects of oxidative and nitrosative stress on *Tetrahymena pyriformis* glyceraldehyde-3-phosphate dehydrogenase. *The Journal of Eukaryotic Microbiology*, *54*, 338–346.

Giangiacomo, L., Ilari, A., Boffi, A., Morea, V., & Chiancone, E. (2005). The truncated oxygen-avid hemoglobin from *Bacillus subtilis*: X-ray structure and ligand binding properties. *The Journal of Biological Chemistry*, *280*, 9192–9202.

Giordano, D., Parrilli, E., Dettaï, A., Russo, R., Barbiero, G., Marino, G., et al. (2007). The truncated hemoglobins in the Antarctic psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC125. *Gene*, *398*, 69–77.

Goldstein, S., Lind, J., & Merényi, G. (2005). Chemistry of peroxynitrites and peroxynitrates. *Chemical Reviews*, *105*, 2457–2470.

Guallar, V., Lu, C., Borrelli, K., Egawa, T., & Yeh, S. R. (2009). Ligand migration in the truncated hemoglobin-II from *Mycobacterium tubercolosis*. *The Journal of Biological Chemistry*, *284*, 3106–3116.

Hill, D. R., Belbin, T. J., Thorsteinsson, M. V., Bassam, D., Brass, S., Ernst, A., et al. (1996). GlbN (cyanoglobin) is a peripheral membrane protein that is restricted to certain *Nostoc* spp. *Journal of Bacteriology*, *178*, 6587–6598.

Hiner, A. N., Raven, E. L., Thorneley, R. N., García-Cánovas, F., & Rodríguez-López, J. N. (2002). Mechanisms of compound I formation in heme peroxidases. *Journal of Inorganic Biochemistry*, *91*, 27–34.

Hoy, J. A., Kundu, S., Trent, J. T., 3rd., Ramaswamy, S., & Hargrove, M. S. (2004). The crystal structure of *Synechocystis* hemoglobin with a covalent heme linkage. *The Journal of Biological Chemistry*, *279*, 16535–16542.

Igarashi, J., Kobayashi, K., & Matsuoka, A. (2011). A hydrogen-bonding network formed by the B10-E7-E11 residues of a truncated hemoglobin from *Tetrahymena pyriformis* is

Comp. by: Saravanan Stage: Proof Chapter No.: 2 Title Name: AMP
Date:25/7/13 Time:18:11:14 Page Number: 74

B978-0-12-407693-8.00002-9, 00002

critical for stability of bound oxygen and nitric oxide detoxification. *Journal of Biological Inorganic Chemistry*, *16*, 599–609.

Ilari, A., Kjelgaard, P., von Wachenfeldt, C., Catacchio, B., Chiancone, E., & Boffi, A. (2007). Crystal structure and ligand binding properties of the truncated hemoglobin from *Geobacillus stearothermophilus*. *Archives of Biochemistry and Biophysics*, *457*, 85–94.

Kapp, O. H., Moens, L., Vanfleteren, J., Trotman, C. N., Suzuki, T., & Vinogradov, S. N. (1995). Alignment of 700 globin sequences: Extent of amino acid substitution and its correlation with variation in volume. *Protein Science*, *10*, 2179–2190.

Lama, A., Pawaria, S., Bidon-Chanal, A., Anand, A., Gelpí, J. L., Arya, S., et al. (2009). Role of Pre-A motif in nitric oxide scavenging by truncated hemoglobin, HbN, of *Mycobacterium tuberculosis*. *The Journal of Biological Chemistry*, *284*, 14457–14468.

Lama, A., Pawaria, S., & Dikshit, K. L. (2006). Oxygen binding and NO scavenging properties of truncated hemoglobin, HbN, of *Mycobacterium smegmatis*. *FEBS Letters*, *580*, 4031–4041.

Larsson, J. T., Rogstam, A., & von Wachenfeldt, C. (2007). YjbH is a novel negative effector of the disulfide stress regulator, Spx, in *Bacillus subtilis*. *Molecular Microbiology*, *66*, 669–684.

Lecomte, J. T., Vu, B. C., & Falzone, C. J. (2005). Structural and dynamic properties of *Synechocystis* sp. PCC 6803 Hb revealed by reconstitution with Zn-protoporphyrin IX. *Journal of Inorganic Biochemistry*, *99*, 1585–1592.

Lecomte, J. T., Vuletich, D. A., Vu, B. C., Kuriakose, S. A., Scott, N. L., & Falzone, C. J. (2004). Structural properties of cyanobacterial hemoglobins: The unusual heme-protein cross-link of *Synechocystis* sp. PCC 6803 Hb and *Synechococcus* sp. PCC 7002 Hb. *Micron*, *35*, 71–72.

Liu, C., He, Y., & Chang, Z. (2004). Truncated hemoglobin O of *Mycobacterium tuberculosis*: The oligomeric state change and the interaction with membrane components. *Biochemical and Biophysical Research Communications*, *316*, 1163–1172.

Lu, C., Egawa, T., Wainwright, L. M., Poole, R. K., & Yeh, S. R. (2007). Structural and functional properties of a truncated haemoglobin from a food-borne pathogen *Campylobacter jejuni*. *The Journal of Biological Chemistry*, *282*, 13627–13636.

MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K., & Nathan, C. F. (1997). Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 5243–5248.

Martì, M. A., Capece, L., Bidon-Chanal, A., Crespo, A., Guallar, V., Luque, F. J., et al. (2008). Nitric oxide reactivity with globins as investigated through computer simulation. *Methods in Enzymology*, *437*, 477–498.

Milani, M., Ouellet, Y., Ouellet, H., Guertin, M., Boffi, A., Antonini, G., et al. (2004). 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., et al. (2005). Structural bases for heme binding and diatomic ligand recognition in truncated hemoglobins. *Journal of Inorganic Biochemistry*, *99*, 97–109.

Milani, M., Pesce, A., Ouellet, Y., Ascenzi, P., Guertin, M., & Bolognesi, M. (2001). *Mycobacterium tuberculosis* hemoglobin N displays a protein tunnel suited for O_2 diffusion to the heme. *EMBO Journal*, *20*, 3902–3909.

Milani, M., Pesce, A., Ouellet, Y., Dewilde, S., Friedman, J., Ascenzi, P., et al. (2004). Heme-ligand tunneling in group I truncated hemoglobins. *The Journal of Biological Chemistry*, *279*, 21520–21525.

Milani, M., Savard, P. Y., Ouellet, H., Ascenzi, P., Guertin, M., & Bolognesi, M. (2003). A TyrCD1/TrpG8 hydrogen bond network and a TyrB10TyrCD1 covalent link shape the heme distal site of *Mycobacterium tuberculosis* hemoglobin O. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 5766–5771.

Comp. by: Saravanan Stage: Proof Chapter No.: 2 Title Name: AMP
Date:25/7/13 Time:18:11:15 Page Number: 75

B978-0-12-407693-8.00002-9, 00002

Mishra, S., & Meuwly, M. (2010). Atomistic simulation of NO dioxygenation in group I truncated hemoglobin. *Journal of the American Chemical Society*, *132*, 2968–2982.

Mukai, M., Savard, P. Y., Ouellet, H., Guertin, M., & Yeh, S. R. (2002). Unique ligand-protein interactions in a new truncated hemoglobin from *Mycobacterium tuberculosis*. *Biochemistry*, *41*, 3897–3905.

Nakajima, S., Álvarez-Salgado, E., Kikuchi, T., & Arredondo-Peter, R. (2005). Prediction of folding pathway and kinetics among plant hemoglobins using an average distance map method. *Proteins: Structure, Function, and Bioinformatics*, *61*, 500–506.

Nardini, M., Pesce, A., Labarre, M., Richard, C., Bolli, A., Ascenzi, P., et al. (2006). Structural determinants in the group III truncated hemoglobin from *Campylobacter jejuni*. *The Journal of Biological Chemistry*, *281*, 37803–37812.

Nardini, M., Pesce, A., Milani, M., & Bolognesi, M. (2007). Protein fold and structure in the truncated (2/2) globin family. *Gene*, *398*, 2–11.

Nathan, C., & Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 8841–8848.

Nicoletti, F. P., Comandini, A., Bonamore, A., Boechi, L., Boubeta, F. M., Feis, A., et al. (2010). Sulfide binding properties of truncated hemoglobins. *Biochemistry*, *49*, 2269–2278.

Nicoletti, F. P., Droghetti, E., Boechi, L., Bonamore, A., Sciamanna, N., Estrin, D. A., et al. (2011). Fluoride as a probe for H-bonding interactions in the active site of heme proteins: The case of *Thermobifida fusca* hemoglobin. *Journal of the American Chemical Society*, *133*, 20970–20980.

Nothnagel, H. J., Love, N., & Lecomte, J. T. (2009). The role of the heme distal ligand in the post-translational modification of Synechocystis hemoglobin. *Journal of Inorganic Biochemistry*, *103*, 107–116.

Nothnagel, H. J., Preimesberger, M. R., Pond, M. P., Winer, B. Y., Adney, E. M., & Lecomte, J. T. (2011). Chemical reactivity of *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 hemoglobins: Covalent heme attachment and bishistidine coordination. *Journal of Biological Inorganic Chemistry*, *16*, 539–552.

Nothnagel, H. J., Winer, B. Y., Vuletich, D. A., Pond, M. P., & Lecomte, J. T. (2011). Structural properties of 2/2 hemoglobins: The group III protein from *Helicobacter hepaticus*. *IUBMB Life*, *63*, 197–205.

Ohno, H., Zhu, G., Mohan, V. P., Chu, D., Kohno, S., Jacobs, W. R., Jr., et al. (2003). The effects of reactive nitrogen intermediates on gene expression in *Mycobacterium tuberculosis*. *Cellular Microbiology*, *5*, 637–648.

Oliveira, A., Singh, S., Bidon-Chanal, A., Forti, F., Marti, M. A., Boechi, L., et al. (2012). Role of PheE15 gate in ligand entry and nitric oxide detoxification function of *Mycobacterium tuberculosis* truncated hemoglobin N. *PLoS One*, *7*, e49291.

Ouellet, Y. H., Daigle, R., Lagüe, P., Dantsker, D., Milani, M., Bolognesi, M., et al. (2008). Ligand binding to truncated hemoglobin N from *Mycobacterium tuberculosis* is strongly modulated by the interplay between the distal heme pocket residues and internal water. *The Journal of Biological Chemistry*, *283*, 27270–27278.

Ouellet, H., Juszczak, L., Dantsker, D., Samuni, U., Ouellet, Y. H., Savard, P. Y., et al. (2003). Reactions of *Mycobacterium tuberculosis* truncated hemoglobin O with ligands reveal a novel ligand-inclusive hydrogen bond network. *Biochemistry*, *42*, 5764–5774.

Ouellet, Y., Milani, M., Couture, M., Bolognesi, M., & Guertin, M. (2006). Ligand interactions in the distal heme pocket of *Mycobacterium tuberculosis* truncated hemoglobin N: Roles of TyrB10 and GlnE11 residues. *Biochemistry*, *45*, 8770–8781.

Ouellet, H., Milani, M., Labarre, M., Bolognesi, M., Couture, M., & Guertin, M. (2007). The roles of Tyr(CD1) and Trp(G8) in *Mycobacterium tuberculosis* truncated hemoglobin

Comp. by: Saravanan Stage: Proof Chapter No.: 2 Title Name: AMP
Date:25/7/13 Time:18:11:15 Page Number: 76

B978-0-12-407693-8.00002-9, 00002

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., et al. (2002). Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 5902–5907.

Parrilli, E., Giuliani, M., Giordano, D., Russo, R., Marino, G., Verde, C., et al. (2010). The role of a 2-on-2 haemoglobin in oxidative and nitrosative stress resistance of Antarctic *Pseudoalteromonas haloplanktis* TAC125. *Biochimie*, *92*, 1003–1009.

Pathania, R., Navani, N. K., Gardner, A. M., Gardner, P. R., & Dikshit, K. L. (2002). Nitric oxide scavenging and detoxification by the *Mycobacterium tuberculosis* haemoglobin, HbN in Escherichia coli. *Molecular Microbiology*, *45*, 1303–1314.

Pathania, R., Navani, N. K., Rajamohan, G., & Dikshit, K. L. (2002). *Mycobacterium tuberculosis* hemoglobin HbO associates with membranes and stimulates cellular respiration of recombinant *Escherichia coli*. *The Journal of Biological Chemistry*, *277*, 15293–15302.

Pawaria, S., Rajamohan, G., Gambhir, V., Lama, A., Varshney, G. C., & Dikshit, K. L. (2007). Intracellular growth and survival of *Salmonella* enterica serovar *Typhimurium* carrying truncated hemoglobins of *Mycobacterium tuberculosis*. *Microbial Pathogenesis*, *42*, 119–128.

Perutz, M. F. (1979). Regulation of oxygen affinity of hemoglobin: Influence of structure of the globin on the heme iron. *Annual Review of Biochemistry*, *48*, 327–386.

Pesce, A., Couture, M., Dewilde, S., Guertin, M., Yamauchi, K., Ascenzi, P., et al. (2000). A novel two-over-two alpha-helical sandwich fold is characteristic of the truncated hemoglobin family. *EMBO Journal*, *19*, 2424–2434.

Pesce, A., Milani, M., Nardini, M., & Bolognesi, M. (2008). Mapping heme-ligand tunnels in group I truncated(2/2) hemoglobins. *Methods in Enzymology*, *436*, 303–315.

Pesce, A., Nardini, M., Labarre, M., Richard, C., Wittenberg, J. B., Wittenberg, B. A., et al. (2011). Structural characterization of a group II 2/2 hemoglobin from the plant pathogen *Agrobacterium tumefaciens*. *Biochimica et Biophysica Acta*, *1814*, 810–816.

Pond, M. P., Majumdar, A., & Lecomte, J. T. (2012). Influence of heme post-translational modification and distal ligation on the backbone dynamics of a monomeric hemoglobin. *Biochemistry*, *51*, 5733–5747.

Ptitsyn, O. B., & Ting, K. L. (1999). Non-functional conserved residues in globins and their possible role as a folding nucleus. *Journal of Molecular Biology*, *291*, 671–682.

Raushel, F. M., Thoden, J. B., & Holden, H. M. (2003). Enzymes with molecular tunnels. *Accounts of Chemical Research*, *36*, 539–548.

Rizzi, M., Wittenberg, J. B., Coda, A., Ascenzi, P., & Bolognesi, M. (1996). Structural bases for sulfide recognition in *Lucina pectinata* hemoglobin I. *Journal of Molecular Biology*, *258*, 1–5.

Samuni, U., Dankster, D., Ray, A., Wittenberg, J. B., Wittenberg, B. A., Dewilde, S., et al. (2003). Kinetic modulation in carbonmonoxy derivatives of truncated hemoglobins: The role of distal heme pocket residues and extended apolar tunnel. *The Journal of Biological Chemistry*, *278*, 27241–27250.

Schnappinger, D., Schoolnik, G. K., & Ehrt, S. (2006). Expression profiling of host pathogen interactions: How *Mycobacterium tuberculosis* and the macrophage adapt to one another. *Microbes and Infection*, *8*, 1132–1140.

Scott, N. L., Falzone, C. J., Vuletich, D. A., Zhao, J., Bryant, D. A., & Lecomte, J. T. (2002). The hemoglobin of the cyanobacterium *Synechococcus* sp.PCC7002: Evidence for hexacoordination and covalent adduct formation in the ferric recombinant protein. *Biochemistry*, *41*, 6902–6910.

Scott, N. L., Xu, Y., Shen, G., Vuletich, D. A., Falzone, C. J., Li, Z., et al. (2010). Functional and structural characterization of the 2/2 hemoglobin from *Synechococcus* sp. PCC 7002. *Biochemistry*, *49*, 7000–7011.

Comp. by: Saravanan Stage: Proof Chapter No.: 2 Title Name: AMP
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B978-0-12-407693-8.00002-9, 00002

Shepherd, M., Bernhardt, P. V., & Poole, R. K. (2011). Globin-mediated nitric oxide detoxification in the foodborne pathogenic bacterium *Campylobacter jejuni* proceeds via a dioxygenase or denitrosylase mechanism. *Nitric Oxide*, *25*, 229–233.

Smith, H. K., Shepherd, M., Monk, C., Green, J., & Poole, R. K. (2011). The NO-responsive hemoglobins of *Campylobacter jejuni*: Concerted responses of two globins to NO and evidence in vitro for globin regulation by the transcription factor NssR. *Nitric Oxide*, *25*, 234–241.

Trent, J. T., 3rd., Kundu, S., Hoy, J. A., & Hargrove, M. S. (2004). Crystallographic analysis of *synechocystis* cyanoglobin reveals the structural changes accompanying ligand binding in a hexacoordinate hemoglobin. *Journal of Molecular Biology*, *341*, 1097–1108.

Vinogradov, S. N., Hoogewijs, D., Bailly, X., Arredondo-Peter, R., Gough, J., Dewilde, S., et al. (2006). A phylogenomic profile of globins. *BMC Evolutionary Biology*, *6*, 31–47.

Vinogradov, S. N., & Moens, L. (2008). Diversity of globin function: Enzymatic, transport, storage, and sensing. *The Journal of Biological Chemistry*, *283*, 8773–8777.

Vinogradov, S. N., Tinajero-Trejo, M., Poole, R. K., & Hoogewijs, D. (2013). Bacterial and archaeal globins—A revised perspective. *Biochimica et Biophysica Acta*. http://dx.doi.org/10.1016/j.bbapap.2013.03.021, pii: S1570-9639(13)00134-9. [Epub ahead of print]. Au4

Visca, P., Fabozzi, G., Milani, M., Bolognesi, M., & Ascenzi, P. (2002). Nitric oxide and *Mycobacterium leprae* pathogenicity. *IUBMB Life*, *54*, 95–99.

Visca, P., Fabozzi, G., Petrucca, A., Ciaccio, C., Coletta, M., De Sanctis, G., et al. (2002). The truncated hemoglobin from *Mycobacterium leprae*. *Biochemical and Biophysical Research Communications*, *294*, 1064–1070.

Vu, B. C., Nothnagel, H. J., Vuletich, D. A., Falzone, C. J., & Lecomte, J. T. J. (2004). Cyanide binding to hexacoordinate cyanobacterial hemoglobins: Hydrogen-bonding network and heme pocket rearrangement in ferric H117A *Synechocystis* hemoglobin. *Biochemistry*, *43*, 12622–12633.

Vu, B. C., Vuletich, D. A., Kuriakose, S. A., Falzone, C. J., & Lecomte, J. T. (2004). Characterization of the heme-histidine crosslink in cyanobacterial hemoglobins from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002. *Journal of Biological Inorganic Chemistry*, *9*, 183–194.

Vuletich, D. A., Falzone, C. J., & Lecomte, J. T. (2006). Structural and dynamic repercussions of heme binding and heme-protein cross-linking in *Synechococcus* sp. PCC 7002 hemoglobin. *Biochemistry*, *45*, 14075–14084.

Vuletich, D. A., & Lecomte, J. T. (2006). A phylogenetic and structural analysis of truncated hemoglobins. *Journal of Molecular Evolution*, *62*, 196–210.

Wainwright, L. M., Elvers, K. T., Park, S. F., & Poole, R. K. (2005). A truncated haemoglobin implicated in oxygen metabolism by the microaerophilic food-borne pathogen *Campylobacter jejuni*. *Microbiology (Reading, England)*, *151*, 4079–4091.

Weeks, A., Lund, L., & Raushel, F. M. (2006). Tunneling of intermediates in enzyme-catalyzed reactions. *Current Opinion in Chemical Biology*, *10*, 465–472.

Wittenberg, J. B., Bolognesi, M., Wittenberg, B. A., & Guertin, M. (2002). Truncated hemoglobins: A new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. *The Journal of Biological Chemistry*, *277*, 871–874.

Yeh, S. R., Couture, M., Ouellet, Y., Guertin, M., & Rousseau, D. L. (2000). A cooperative oxygen binding hemoglobin from *Mycobacterium tuberculosis*. Stabilization of heme ligands by a distal tyrosine residue. *The Journal of Biological Chemistry*, *275*, 1679–1684.

Comp. by: Saravanan Stage: Proof Chapter No.: 2 Title Name: AMP
Date:25/7/13 Time:18:11:16 Page Number: 78

B978-0-12-407693-8.00002-9, 00002

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