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Review

The structure and function of plant hemoglobins

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Abstract

Plants, like humans, contain hemoglobin. Three distinct types of hemoglobin exist in plants: symbiotic, non-symbiotic, and truncated hemoglobins. Crystal structures and other structural and biophysical techniques have revealed important knowledge about ligand binding and conformational stabilization in all three types. In symbiotic hemoglobins (leghemoglobins), ligand binding regulatory mechanisms have been shown to differ dramatically from myoglobin and red blood cell hemoglobin. In the non-symbiotic hemoglobins found in all plants, crystal structures and vibrational spectroscopy have revealed the nature of the structural transition between the hexacoordinate and ligand-bound states. In truncated hemoglobins, the abbreviated globin is porous, providing tunnels that may assist in ligand binding, and the bound ligand is stabilized by more than one distal pocket residue. Research has implicated these plant hemoglobins in a number of possible functions differing among hemoglobin types, and possibly between plant species.

Keywords: Plant hemoglobin; Symbiotic; Non-symbiotic; Truncated; Leghemoglobin; Rice; *Arabidopsis*

1. Introduction

Hemoglobin (Hb) is a familiar protein, composed of a heme *b* prosthetic group held within the alpha helical globular fold of a 100–200 amino acid protein monomer by a covalent bond to a histidine side chain termed the ''proximal histidine''. The heme contains an iron atom with four of the six coordination sites occupied by the heme pyrrole nitrogens. In ''pentacoordinate'' Hbs, the proximal histidine coordinates the fifth site, leaving the sixth open for exogenous ligand binding (Fig. 1A). Exogenous ligands are usually diatomic gases such as oxygen and nitric oxide, and Hbs are commonly associated with the ability to perform ligand transport, sensing, scavenging, detoxification, and electron transfer [5].

Hemoglobin was originally isolated and studied from mammals, but is now known to exist in nearly all organisms including archaeobacteria, eubacteria, and eukaryotes [81]. Three types of hemoglobins exist in plants; the originally identified symbiotic hemoglobins (sHbs) and the more recently discovered non-symbiotic and truncated hemoglobins (nsHbs and trHbs). All plants that have been studied contain one or more Hbs, some with Hbs from all three types, suggesting multiple and varied functions [21].

Only a small number of crystal structures of plant hemoglobins have been solved. However, structural insights have been gleaned from electron paramagnetic resonance, resonance raman, and UV/VIS spectroscopy, as well as from point mutation studies, mass spectrometry, electrochemistry, and computer modeling. This review will explore the background and recent advances in the structural understanding of each of the three plant hemoglobin categories, in addition to current theories about their function.

Abbreviations: Hb, hemoglobin; sHb, symbiotic hemoglobin; nsHb, non-symbiotic hemoglobin; trHb, truncated hemoglobin; Lb, leghemoglobin; Mb, myoglobin; Lba, soybean leghemoglobin; riceHb, rice hemoglobin; barHb, barley hemoglobin; cornHb, corn hemoglobin; AtHb, *Arabidopsis* non-symbiotic hemoglobin; CeTrHb, *Chlamydomonas eugametos* truncated hemoglobin; ESTs, expressed sequence tags; AtTrHb, *Arabidopsis thaliana* truncated hemoglobin; MtTrHb, *Medicago truncatula* truncated hemoglobin; rmsd, root mean square deviation.

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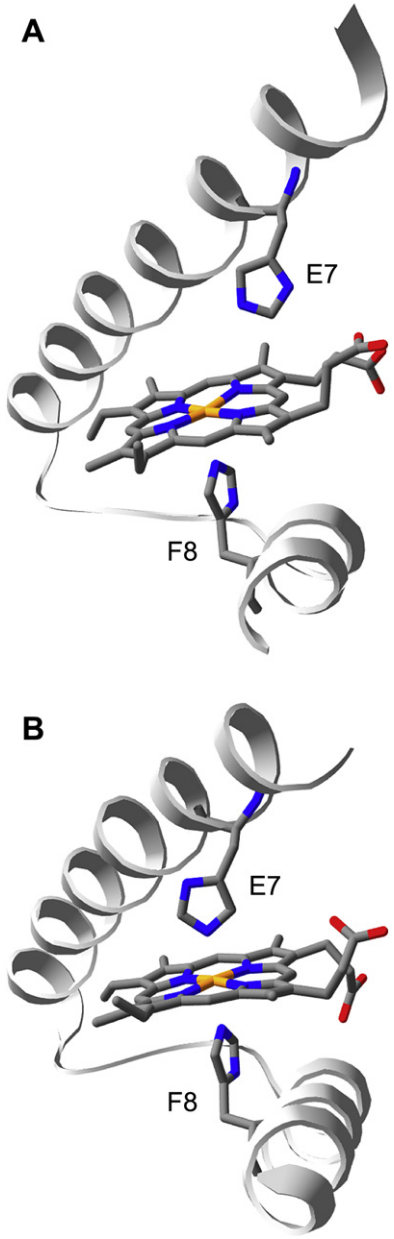

Fig. 1. Pentacoordinate versus hexacoordinate hemoglobins. A) The structure of Lba (1BIN) demonstrates the open distal pocket of pentacoordinate hemoglobins, while B) the structure of riceHb1 (1D8U) demonstrates distal histidine (E7) coordination to the sixth binding site of the heme iron. Shown are the E- and F-helices, the distal and proximal histidines, and the heme.

2. Symbiotic hemoglobin structure and function

SHbs were first discovered by Kubo in 1939. These proteins are found in millimolar quantities in the nodules that form on the roots of plants in symbiosis with the bacterium *Rhizobium* [2]. These plants are usually legumes and therefore these proteins have been called leghemoglobins (Lb); however, sHbs have also been found in the root nodules of non-legumes such as the Ulmaceae *Parasponia andersonii* in symbiosis with *Rhizobium*, and dicotyledonous plants such as *Casuarina glauca* in symbiosis with the actinomycete *Frankia* [3].

The main function of sHb in plants is relatively well understood. The plants benefit from the nitrogen-fixation carried out by their bacterial partners, and in turn carefully regulate oxygen levels in the root nodule for optimal bacterial function. Large quantities of sHb in the root nodule are responsible for facilitating diffusion of the oxygen necessary for nitrogen-fixation, similar to the activity of mammalian myoglobin (Mb) in muscle. However, sHbs must also maintain the low free oxygen concentration required by bacterial nitrogenase enzymes [3]; a role that sHbs are better suited to carry out than Mb due to their higher oxygen affinities. This is achieved by higher oxygen association and lower oxygen dissociation rate constants compared to Mb, but both values are still within the bounds of partial saturation required for binding and release of oxygen along the oxygen gradient within the nodule [46].

After the discovery of an NO regulatory role for mammalian oxygen transport Hbs [29], a subsequent search in plants found nitric oxide production in plants, possibly by nitric oxide synthase, nitrate reductase, or other means [15]. Nitrosyl-leghemoglobin has been found in soybean nodules [50], and arises under normal conditions due to nitrate reduction by the bacteroidal nitrate reductase, according to Meakin et al. [51]. OxyLb has kinetic properties that render it capable of scavenging nitric oxide and peroxynitrite [31]; in addition, ferrylLb and nitrosylLb, two forms found in vivo under certain circumstances but incapable of oxygen binding, can react with nitric oxide and peroxynitrite, respectively, to form metLb [30], which may then react with a known nodule reductase [41] to form ferrous Lb.

Through mutational and kinetic studies, Kundu et al. were able to provide a detailed analysis of both the proximal and distal pockets around the heme of soybean leghemoglobin (Lba). Comparison to Mb demonstrated an opposite mechanism of ligand regulation [43,45]. Ligand regulation in Mb occurs through a ‘‘gating’’ mechanism due to steric hindrance from the distal histidine, and the distal histidine also stabilizes a bound water molecule that must be removed for other ligands to bind. Water is not stabilized in the distal pocket of Lba, contributing to the higher oxygen association rate constant in Lba versus Mb. While ligand stabilization in Mb occurs in the distal pocket via a strong hydrogen bond with the distal histidine, replacement of this histidine with leucine in Lba has a minimal affect on oxygen affinity, suggesting a weak interaction [24].

On the other hand, a serine in the proximal pocket of Mb hydrogen bonds to the proximal histidine limiting movement; the proximal histidine is held in an eclipsed orientation with respect to the heme pyrrole nitrogens [62,76]. In Lba, proximal histidine movement is not hindered and it settles into a staggered orientation, increasing ligand affinity [46] (Fig. 2). Thus, Mb relies on the distal pocket whereas Lba relies on the proximal pocket for ligand regulation. Recent computer simulations by Capece et al. and Marti et al. have been able to mimic the experimental results obtained by Kundu et al., an advance that might enable prediction of ligand binding regulation in other Hbs [12,49].

Crystal structures of sHbs from soybean and lupin have been solved in various oxidation states with many ligands [7,24,26]. Like Mb, the structures are very similar to one

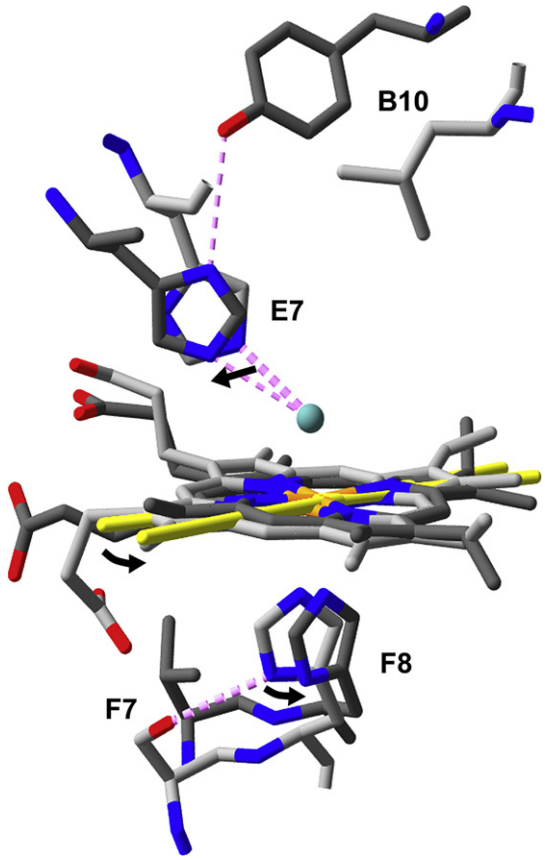

Fig. 2. The heme pocket of myoglobin versus soybean leghemoglobin. Mb (2MBW; light gray) is overlaid with Lba (1BIN as modified according to [42]; dark gray) to compare residues in the distal and proximal heme pockets. On the distal side, the distal histidine, HisE7, is in better position to stabilize a bound ligand (cyan) in Mb, while it is pulled away from the bound ligand by TyrB10 in Lba, and therefore binds with the ligand more weakly. On the proximal side, SerF7 prevents the proximal histidine, HisF8, from rotating in Mb, while ValF7 does not hinder HisF8 motion. The yellow lines passing through the heme plane represent the angle of intersection of the proximal histidine. Proximal pocket interactions in Mb hold the proximal histidine in an eclipsed orientation, with the line of intersection passing through the heme propionate pyrrole nitrogens. Lba can, however, shift to a staggered position allowing a rotation (indicated by arrows) that favors ligand binding.

another and show the typical pentacoordinate heme pocket in both the ferrous and ferric states (Fig. 1A). The distal binding pocket is larger than that of Mb in all of the structures, accounting for high oxygen association, auto-oxidation, and heme dissociation rates, and the ability to bind bulky ligands such as acetate and nicotinate [9]. A comparison of the structures of Lba and Mb also reveals an interaction between the tyrosine at position 10 on the B-helix (TyrB10) and the distal histidine in Lba that orients the distal histidine into a position from which a hydrogen bond with the bound ligand is longer than that in Mb, which does not have such an interaction with LeuB10, thus enabling the stronger distal bond in Mb (Fig. 2).

3. Non-symbiotic hemoglobin structure and function

For decades the only known plant Hbs were the sHbs, which were easily found due to their large quantities in root nodules. It was thought that the sHbs had originated in plants due to horizontal gene transfer from animals, but this postulate became unnecessary as it became evident through sequencing that Hbs were found throughout the plant kingdom [3]. This began with the discoveries that *Parasponia andersonii* Hb is expressed in roots even when not involved in symbiosis, and of a Hb in both *Trema tomentosa* and *Celtis australis*, which are non-nodulating plants not involved in a symbiotic relationship [8]. Additional non-symbiotic hemoglobins were then found in barley, rye, maize, and wheat [77], *Causarina* [40], soybean, clover, alfalfa, and pea [1]. This confirmed their existence in monocots and dicots – including legumes – and strengthened the theory that Hbs have an ancient origin and are ubiquitous in the plant kingdom through vertical evolution.

NsHbs differ from sHbs and mammalian Hb and Mb in that they are generally ‘‘hexacoordinate’’ in both the ferric and ferrous states due to a histidine in the distal pocket that reversibly binds the sixth coordination site of the heme iron [6,20] (Fig. 1B). Two classes (classes 1 and 2) of nsHbs have been distinguished using phylogenetic analysis, and shown to differ in their patterns of expression [78]. Despite hexacoordination, class 1 nsHbs have high oxygen affinities and low oxygen dissociation rate constants [6,20,33] due to stabilization between the distal histidine and the bound ligand akin to that in Mb [6,17]. Class 2 nsHbs have lower oxygen affinities and greater similarity to sHbs than nsHbs, consistent with the observation that most sHbs evolved from class 2 nsHbs [78].

3.1. Class 1 nsHbs

Monocot class 1 nsHbs have been studied extensively in barley and rice, and to a lesser extent in corn. Low concentrations in vivo along with small oxygen dissociation rate constants indicate that these nsHbs are unlikely to function in oxygen transport, while high oxygen affinity and redox potential indicate they are unlikely to function in electron transport [6,32,44]. However, barley hemoglobin (barHb) is expressed in the roots of seedlings, in oxygen-stressed aleurone tissues, and in roots under flooding stress [77]. In addition, barHb expression in aleurone tissue appears to depend not on oxygen availability, but rather on interference with mitochondrial ATP synthesis [55]. Similarly, overexpression of barHb in maize cells increases their ability to maintain ATP levels under low oxygen tension [75]. It was suggested that oxybarHb may function to oxidize NADH to maintain glycolytic ATP synthesis under hypoxic conditions [32].

Since mitochondria release calcium into the cytosol under hypoxic conditions and this affects energy metabolism, Nie et al. have recently tested the effects on barHb expression of compounds that interfere with calcium modulated signal transduction [54]. They found that blocking or chelating cytosolic calcium under anaerobic conditions decreased barHb transcription, and that the effect on barHb was calmodulin independent. They postulate that low oxygen concentration leads to reduced mitochondrial respiration and lower ATP levels, leading in turn to disruption of calcium-ATPases and the efflux transporters that maintain cellular calcium levels. This results

in higher cytosolic calcium levels leading to barHb expression, reestablishment of ATP levels, and restored calcium levels, which would switch off barHb expression.

Overexpression of barHb in alfalfa root cultures maintains root growth, ATP levels, and lowered nitric oxide levels under hypoxia [18], facilitates NADH-dependent nitric oxide metabolism while increasing alfalfa root methemoglobin reductase activity [37], and increases levels of ascorbate, monodehydroascorbate reductase (MDHAR), and hydrogen peroxide scavenging enzymes [38]. Igamberdiev et al. have recently found a barley MDHAR that may assist barHb in NADH-dependent NO scavenging in vitro and in barley root extracts with added ascorbate [36]. This implicates barHb as a possible heme-dioxygenase in addition to its role of maintaining the energy status of plant cells under hypoxic conditions.

Rice contains four nsHbs [81], of which two have been studied in purified recombinant form (riceHb1 and riceHb2). Rice nsHbs are found in roots, leaves, and seeds; particularly in vascular, differentiating, and germinating tissues [6,64]. They are upregulated in plants grown under stress conditions including light deprivation and flooding [47]. In addition, their gene promoters contain regulatory elements implicated in hormone and abiotic stress responses, and the riceHb2 promoter is induced by cytokinins which are involved in differentiation pathways [65]. However, Ohwaki et al. recently found that rice nsHb genes are also induced by nitrate, nitrite, and nitric oxide donors in association with NADH-nitrate reductase [56].

Thus there are currently several postulates for the functions of nsHbs in rice, and it is likely that more than one function is possible among the four nsHbs. Rice nsHb expression in normal plants indicates a role in developmental metabolic functions [64]. Their upregulation in stress response may involve energy maintenance and NADH reduction as hypothesized for barHb [47,65]. On the other hand, the enhanced reduction kinetics found for riceHb1 along with the results of Ohwaki et al., favors a role in electron transport or nitric oxide scavenging and detoxification [56,83].

Corn contains one class 1 nsHb, CornHb1, which is expressed in embryonic organs, with lower levels in leaves and roots [4], and additional root expression under flooding stress [77]. Corn cell cultures have been shown to produce nitric oxide under anoxic stress via nitrate reductase [19]. While overexpression of barHb in corn cells increases their ability to maintain ATP levels under low oxygen tension [75], it also lowers levels of NO under anoxic conditions [19], and decreases ethylene accumulation [48]. More research is required to determine a role for nsHbs in corn; however, similar patterns to barHb and rice nsHbs are emerging.

The first structure of a nsHb was the crystal structure of riceHb1 [25]. While riceHb1 is a partially associated homodimer under physiological conditions [22], it crystallizes as a fully associated dimer. The crystal structure clearly shows the distal histidine hexacoordinated to the heme iron (Fig. 1B), accompanied by a bend in the E-helix, no D-helix, and disorder in the CD-region.

A conserved phenylalanine from the B-helix, PheB10, interacts with the distal histidine. A recent study by Smagghe et al. includes crystallographic examination of B10 mutants in riceHb1; with mutation to leucine, a large cavity is opened in the distal pocket decreasing the interaction with the distal histidine, while in the tryptophan mutant, crowding in the distal pocket forces histidine rotation [73] (Fig. 3). The same study demonstrated that mutation of PheB10 increases the affinity constant for hexacoordination in both the ferrous and ferric states, increases movement of the distal histidine in the carbon monoxide bound state, increases auto-oxidation, and decreases ferric ligand binding, thus indicating a role for this amino acid in destabilizing hexacoordination to promote ligand binding and stabilization of bound exogenous ligands by the distal histidine. This is in agreement with the electrochemistry results of Halder et al., indicating that nsHbs significantly modulate hexacoordination through the protein matrix [23].

The cornHb1 structure was modeled using computational methods, predicting a hexacoordinate model with tertiary structure similar to riceHb1 [66]. The subsequent crystal structure (PDB ID 2R50.pdb; unpublished data, Smagghe, B.J.) is very similar to both the computational model and to riceHb1. As predicted computationally, and as seen in riceHb1, the CD loop region is disordered in cornHb1. However, unlike the computational model, the crystal structure reveals subtle differences in heme pocket amino acid orientations. The structure also reveals a greater number of interhelical hydrogen bonds in cornHb1 than in riceHb1, which may limit flexibility and contribute to kinetic differences between the two proteins [74].

Recently, the crystal structure of ferric barHb in complex with cyanide ligand has been solved by Hoy, et al. [33]. BarHb is a homodimer containing a disulfide bridge between the monomers of the dimer that stabilizes the protein against auto-oxidation [11], and this dimer arrangement and disulfide bridge are clear in the structure. The barHb ligand-bound structure, in comparison to the hexacoordinate structure of riceHb1, reveals that ligand binding requires a large ‘‘piston’’ movement of the E-helix along the helical axis to move the distal histidine out of the heme pocket and position it and PheB10 for favorable ligand stabilization (Fig. 4). This movement is accompanied by stabilization of the CD loop region, including the formation of a small D-helix, and elongation and rearrangement of the EF loop due to unraveling of the C-terminal end of the E-helix and the N-terminal end of the F-helix. Comparison of the barHb ligand-bound structure to Lba indicates that evolution of the pentacoordinate heme pocket found in sHbs likely involved sequence deletions in the CD and EF loop regions that limit flexibility, and selection for amino acid substitutions that have increased interhelical hydrogen bonding and hydrophobic contacts to lock the tertiary structure in the ‘‘open,’’ pentacoordinate conformation.

Much less is known about the structure of nsHbs in dicots than in monocots. Recent characterization of a class 1 nsHb from tomato by Ioanitescu et al., reveals a homodimer with high oxygen affinity, low oxygen dissociation rate, hexacoordination in the ferric form, and a mixture of pentacoordination and hexacoordination in the ferrous state [39]. However, this nsHb appears to provide less ligand stabilization by the distal

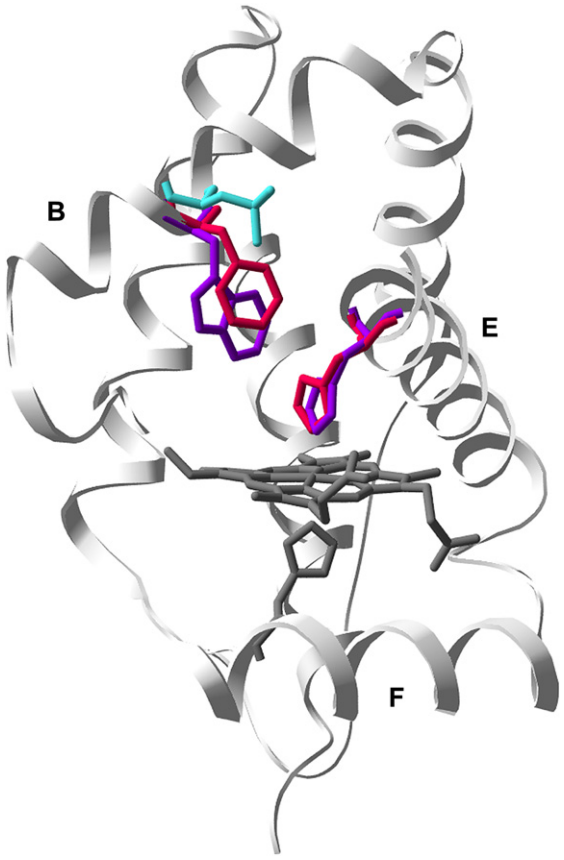

Fig. 3. Rice Hemoglobin B10 mutants. In wild type riceHb1 (1D8U; red), the phenylalanine at position B10 interacts with the distal histidine to regulate hexacoordination and ligand stabilization. Mutation of the B10 amino acid to leucine (2GNV; cyan) opens a large cavity in the distal pocket and the lack of interaction with the distal histidine stabilizes hexacoordination and interferes with ligand binding. Mutation to tryptophan (2GNW; purple) crowds the pocket and forces distal histidine rotation. The CD loop and a portion of the E-helix have been removed for clarity, and the residues shown are B10, E7, and F8, along with the heme.

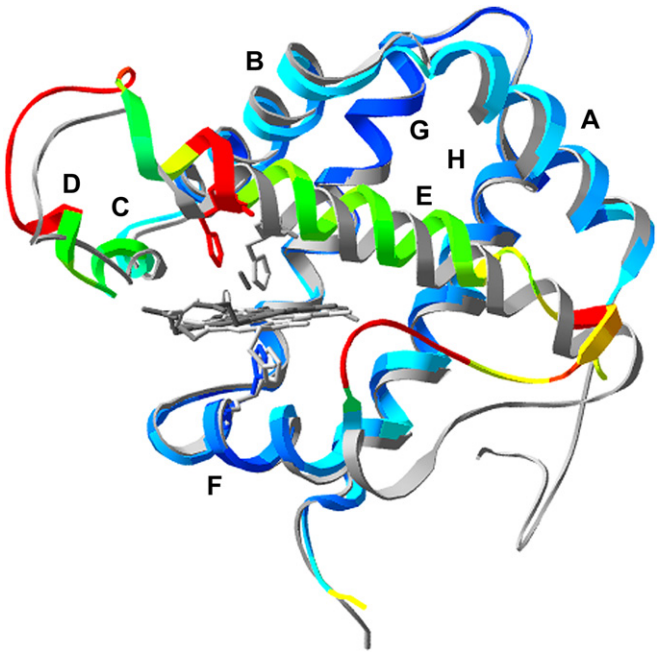

Fig. 4. The tertiary structure of hexacoordinate rice hemoglobin versus ligand bound barley hemoglobin. RiceHb1 (1D8U; gray) is aligned with barHb (2OIF; colored by rmsd from the riceHb1 structure, from blue to red with increasing deviation). The largest change upon ligand binding in this comparison of nsHbs occurs in the E-helix and in the CD and EF loops. The distal histidines and helix labels are included for clarity.

histidine, perhaps due to a change in orientation due to polarity differences in the heme pocket.

Legumes are among the dicots containing nsHbs; soybean, *Lotus japonicus*, and alfalfa are known to contain class 1 nsHbs [21]. In soybeans, nsHb is expressed in stems, seeds, roots, leaves, and at a low level in nodules [1]. In *Lotus japonicus*, nsHb1 is expressed in roots, leaves, stems, and nodules, and expression in roots is repressed by fungal infection [79] but enhanced by hypoxia, cold stress, rhizobial infection, plant hormones, and nitric oxide [72]. *Lotus japonicus* nsHb2 is more closely related to monocot class 1 nsHbs and expression is enhanced by sucrose [72]. The nsHb from alfalfa is found in the nucleus and cytoplasm of root cells and is induced by hypoxia [71]. Overexpression in tobacco plants leads to reduced necrotic symptoms and higher nitric oxide scavenging activity [70]. NsHbs in legumes are therefore implicated in a wide variety of roles and more research is needed to pin down their functions.

In cotton, expression of a class 1 nsHb increases in roots under fungal infection and in response to phytohormones, hydrogen peroxide, and nitric oxide, while overexpression in *Arabidopsis* leads to enhanced disease resistance and nitric oxide tolerance [60,61]. Like *Parasponia andersonii*, *Alnus firma* and *Myrica gale* contain class 1 nsHbs with both symbiotic and non-symbiotic promoters [27,69]. In *Alnus firma* Hb is expressed in leaves, stems, and roots, with enhanced expression in nodules, and is induced by cold stress and nitric oxide [69].

The class 1 nsHb from *Arabidopsis thaliana* (AtHb1) is the most studied dicot nsHb. AtHb1 is induced by hypoxia, sucrose, and nitrite, while AtHb2 (class 2) is induced by low temperature [35,67,78]. Overexpression of AtHb1 promotes vigorous growth and protects *Arabidopsis* plants from the effects of hypoxia, with low oxygen pretreatment increasing survival rates [34]. They are capable of peroxidase-like activity, NADH oxidation, and *S*-nitrosoHb nitric oxide scavenging in vitro [58,67]. Overexpression of AtHb1 in *Arabidopsis* plants results in decreased nitric oxide emission under light deprivation, detection of *S*-nitrosylated AtHb1 in vivo, and decreased hydrogen peroxide levels under hypoxic stress, leading to proposed functions in nitric oxide detoxification and hydrogen peroxide scavenging under hypoxia [58,85].

3.2. Class 2 nsHbs

Very little is known about the biochemistry of class 2 nsHbs. CornHb2 is the only monocot class 2 nsHbs which has been characterized [21]. Non-legume dicots contain both class 1 and class 2 nsHbs [35]. In chicory, a class 2 nsHb is expressed during induction of somatic embryogenesis [28].

A recent study of nsHbs from *Arabidopsis thaliana* (AtHb1, class 1; AtHb2, class 2) by Bruno et al., indicates that both nsHbs are hexacoordinate, though AtHb1 has a large (~40%) pentacoordinate fraction, and there is reduced interaction between the distal histidine and the exogenous ligand in AtHb2 [10]. Modeling from this study implicates a hydrophobic channel and internal ligand docking site that allow rapid ligand migration in AtHb1, while AtHb2 relies on protein fluctuation to regulate the exchange of ligand from solvent to distal pocket.

4. Truncated hemoglobin structure and function

TrHbs are so named because the typical "3-on-3" alpha-helical "sandwich fold" of most globins is truncated to a "2-on-2" fold (2/2), though the full primary sequence is not necessarily shorter in these Hbs, as is the case for higher plant trHbs [81]. They are found in nano- to micromolar concentrations and can be divided phylogenetically into three groups [84]. The first plant trHbs found were group I chloroplast hemoglobins from the unicellular green alga *Chlamydomonas eugametos* (CeTrHb) [13]. Spectroscopic, kinetic, and mutational studies found a pH dependence of coordination state, very low oxygen dissociation, distal glutamine E7 (as opposed to histidine) stabilization of bound ligand assisted by tyrosine B10, and possibly hexacoordination by tyrosine B10 at alkaline pH [14,16].

The subsequent crystal structure of cyanomet CeTrHb revealed the editing of the traditional globin fold (Fig. 5) accompanied by glycine-glycine motifs, and verified ligand stabilization by glutamine E7 and tyrosine B10 [59]. The crystal structure of CeTrHb also exhibits an apolar tunnel connecting the protein surface to the distal heme pocket, and recent xenon crystallography studies found xenon binding sites at the tunnel entries and along the tunnel branches [53]. Such binding in the protein core, along with unusual carbon monoxide rebinding kinetics, implies a role for the tunnel in ligand binding in group I trHbs like CeTrHb [52,68], although their function is still unknown.

A group II trHb from *Arabidopsis thaliana* (AtTrHb) has been characterized, with ESTs of high similarity found in a range of additional vascular and non-vascular plants [82]. This trHb is expressed in roots and shoot tissue, and expression is reduced by hypoxia. The protein is pentacoordinate, but forms a transient hexacoordinate state upon reduction with sodium dithionite, and has a moderate oxygen affinity that does not preclude oxygen transport.

Recently, Vieweg et al. studied two trHbs from the legume *Medicago truncatula* (MtTrHb1 and MtTrHb2), which forms a symbiotic relationship with a mycorrhizal fungi in root arbuscules in addition to symbiosis with *Rhizobium* in root nodules [80]. Both Hbs are induced in response to symbiosis, MtTrHb1 in root nodules with an expression pattern similar to sHbs and MtTrHb2 in root nodule base and vascular tissues and mycorrhizal roots. Again, the function of these trHbs is unknown, but Vieweg et al. propose a function involving the suppression of defense processes against symbioses based on nitric oxide detoxification or scavenging. Pawlowski, et al. have suggested this same role for the recently identified truncated hemoglobin found in the *Frankia*-induced nodules of the actinorhizal plant *Datisca glomerata* [57].

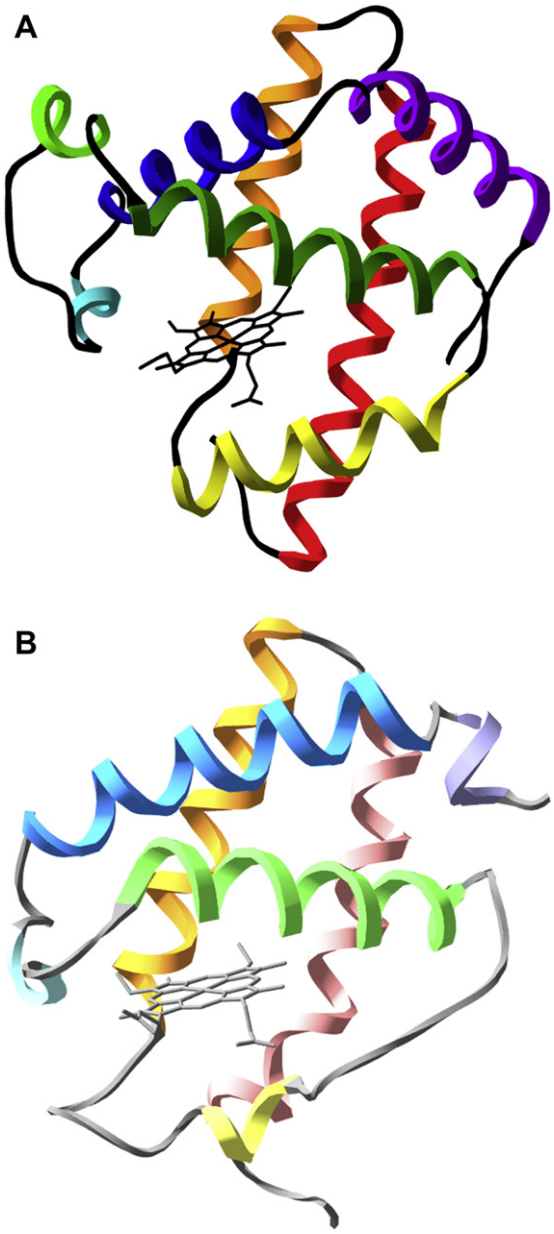

Fig. 5. The tertiary structure of myoglobin versus *Chlamydomonas* truncated hemoglobin. Compared to A) Mb (2MBW), the truncation of the globin fold in B) CeTrHb (1DLY) is evident. Successive helices are individually colored (purple, A; blue, B; cyan, C; green, D/E; yellow, F; orange, G; red, H). Mb is sometimes described as having a 3-on-3 fold (helices A/E/F and B/G/H), while truncated hemoglobins are described as having a 2-on-2 fold (helices B/E and G/H).

5. Conclusion

With the exception of sHbs, study of the structure and function of plant hemoglobins is still in its youth, though it is possible that even the well understood sHbs may be found to have additional functions, as has been the case for mammalian Mb

and Hb. Other plant Hbs have been implicated in metabolic response to hypoxia due to stress or cell differentiation, nitric oxide detoxification, scavenging of hydrogen peroxide and other reactive oxygen species, and hormone mediated signal cascades. The astounding array of endogenous and exogenous factors that induce expression of the non-symbiotic and truncated hemoglobins belies the need for carefully controlled studies designed to elucidate the multiple pathways involving Hbs occurring in specific plant locations and growth stages under various physiological and stress conditions.

Additional structural studies are likewise needed. Currently no structural investigation is available on plant Hbs with both symbiotic and non-symbiotic functions such as the Hb from *Parasponia*. As a transitional Hb, it would be fascinating to compare this structure to known sHb and nsHb structures. Class 2 nsHbs have ligand binding kinetics that differ from class 1 nsHbs, and it would therefore be interesting to compare structures of the ligand bound and unbound forms of these proteins. This would also shed more light on the evolutionary changes that occurred in the specialization of symbiotic hemoglobins. Recent evolutionary trace analysis has raised questions about the roles of a number of highly conserved but thus far unexamined amino acids found throughout nsHbs [63]. On the other hand, a large degree of variation is found in structures of trHbs and no structures have yet been solved of trHbs in higher plants. Structures of trHbs from a monocot such as rice and a dicot such as *Arabidopsis* would facilitate understanding of their modes of ligand binding including the tunnel phenomenon.

Acknowledgements

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