Recent Insights into Plant Hemoglobins

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Summary

Hemoglobins (Hbs) are proteins that reversibly bind O₂. They are widespread in nature and have been
identified in bacteria, fungi, protozoans, plants and animals. Hbs are predominantly α-helical and are
characterized by the globin backbone containing a non-covalently bound heme. Functionally, all Hbs
can bind small gaseous ligands and small cellular molecules. Although first extracted from soybean
root nodules, it has become apparent that all plants contain Hbs, which are divided in three types: the
truncated, symbiotic, and nonsymbiotic Hbs. The truncated Hbs are localized in the chloroplasts of
algae, but their subcellular localization in higher plants is not known. They are composed of a smaller
α-helical globin fold than that of the symbiotic and nonsymbiotic Hbs, have a lower O₂-affinity than
the nonsymbiotic Hbs, are expressed in both root and shoot tissue and have no assigned function.
Symbiotic Hbs are present in nitrogen-fixing nodules and facilitate the diffusion of O₂ to the respiring
caducous symbiotic bacteria. This type of plant Hbs is well-characterized biochemically, and recent studies
have focused on the cellular mechanisms that control their tissue specific expression and their structure-
function relationships. The nonsymbiotic Hbs are cytoplasmic, exhibit very high affinity for O₂, and are
synthesized during seed germination and in plant vegetative organs, as well as in response to stress.
Based on tissue expression profiles, kinetic properties and cellular Hb levels, it has been suggested
that nonsymbiotic Hbs may play a role in regulating the metabolism of specific plant cells,
such as the aleurone and differentiating xylem.

Key words: hemoglobin, leghemoglobin, nonsymbiotic, symbiotic.

Hemoglobins (Hbs) are proteins that reversibly bind O₂. They are ubiquitous and have been
identified in bacteria, fungi, protozoans, plants and animals (1-3). The existence of Hbs in
such a wide range of organisms suggests that there was a gene that encoded for a common
ancestral Hb, which might have existed about 1.8 billion years ago (4). Hbs can bind several

*This review is dedicated to the memory of Dr. Robert V. Klucas
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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GUS, glucuronidase; Hb, hemoglobin; Lb, leghemoglobin; Mb,
myoglobin; Ns, nonsymbiotic; SNP, sodium nitroprusside; Sym, symbiotic; Tr, truncated.
gaseous ligands such as O₂, CO and NO, as well as other small organic molecules. Ligand binding occurs specifically to an iron atom coordinated within the tetrapyrrole protoporphyrin IX. The Fe-protoporphyrin IX (heme) prosthetic group is found in all Hbs in a one to one molar ratio within the globin backbone. The iron atom of the heme can undergo redox chemistry and will bind O₂ only in the ferrous (Fe²⁺) state. Most Hbs studied, with the exception of the truncated Hbs, fold into the same 3-over-3 α-helical three-dimensional structure, also known as the “globin fold” (5).

Non-plant Hemoglobins

Because of space limitation this review will only describe some characteristics of non-plant Hbs, however the reader is referred to an excellent review by Weber and Vinogradov (3) on this subject. Vertebrates contain four types of Hbs. The most abundant is the O₂-carrying heterotetramer Hb found in the red blood cells, which in adults is composed of two α- and two β-globin polypeptides. The α and β subunits exhibit cooperativity in the binding of O₂ that is pH dependent. Vertebrates also contain a Hb named myoglobin (Mb), which is a monomer found in muscle and cardiac tissues and is involved in O₂ storage and transport (6). Recently, a Hb from brain tissue was discovered and was named neuroglobin (7). There is no known function of neuroglobins, however based on sequence homology and kinetic properties, which are similar to other Hbs, it has been postulated that neuroglobin folds and functions in a manner similar to Mb. The fourth Hb, named histoglobin has just been described (8).

Invertebrates contain somewhat different Hbs as compared to the vertebrate Hbs. Pesce et al (9) have reported crystal structures of a new class of Hbs, the truncated Hbs (trHbs) from the ciliated protozoan Paramecium caudatum and green unicellular algae Chlamydomonas eugametos. These proteins display medium to high O₂-affinity and have substantial deletions in amino-terminal and CD-D α-helical regions of the vertebrate globin fold. They fold in a 2-over-2 α-helical sandwich, which is atypical of most other known Hb structures. Other major differences between trHb and animal Hbs are the E7 residue (helix E, residue 7): in most Hbs, E7 typically is a His residue (distal His), involved in stabilizing the heme bound ligand through H-bonding. The equivalent residue in known trHbs is a Gln.

Bacteria contain two types of Hbs, a Hb that is similar in protein sequence to plant Hbs and a flavohemoglobin (flavoHb). The first type of bacterial Hb was identified in Vitreoscilla (10), and the second type, the flavoHbs, was identified in Alcaligenes eutrophus (11) and Escherichia coli (12, 13). Additionally, yeasts such as Saccharomyces cerevisiae (14) and Candida norvegensis (15) contain flavoHbs. In contrast to the Vitreoscilla-like Hbs, flavoHbs consist of an amino-terminal Hb globin domain, but also a carboxy-terminal flavin domain containing a FAD- and NADP⁺-binding sites. These proteins are related to proteins in the ferredoxin-NADP⁺ reductase family (12). The function of bacterial Vitreoscilla-like Hbs is still under investigation, however it has been argued that these proteins function in O₂ storage and diffusion (16, 17). It seems unlikely that the flavoHbs function in this manner based on
the ability of the protein to undergo oxidation and reduction reactions from the flavin domain (18). It has been proposed that the flavoHbs serve as NO dioxygenases [for example see Poole et al (19), Liu et al (20) and Gardner et al (21)]. FlavoHbs have been also cloned and characterized from the slime mold Dictyostelium discoideum and apparently they are involved in NO signaling (22).

Plant Hemoglobins

Hbs are widely distributed among the plant kingdom, ranging from primitive non-vascular plants to angiosperms. The reader is also referred to Appleby (23), Arredondo-Peter et al (24), Hill (25) and Watts et al (26) for more details and discussion on plant Hbs. Based on analysis of expression patterns and kinetics the plant Hbs can be separated into symbiotic (symHb) and nonsymbiotic (nsHb) types. Based on analysis of gene sequences and protein structures the nsHbs can be separated further into class 1 and class 2 (33), and the newly-recognized truncated Hbs (26). It is thought that the genes for most symHbs (e.g. the legume Lbs and Casuarina Hb 1) were recruited from nsHb class 1 genes and the gene for Parasponia symHb from a nsHb class 2 gene (33). It has been known since 1939 (27) that plants forming nitrogen-fixing symbioses contain a Hb that is present in the nodules of legumes and nonlegumes, henceforth referred to as symHbs because they have not been detected in plant organs other than nodules. These proteins are often named leghemoglobins (Lbs) based on their original identification within the legume root nodules. For many years it was assumed that Lbs were the only types of Hbs present in plants. However, a Hb that was identified in root nodules from Parasponia andersonii (28) indicated that Hbs may be more widely distributed in plant organs. Subsequently, a nsHb was identified in roots of Trematomentosa a non-nodulating close relative of Parasponia (29). Since then nsHbs have been identified and cloned from a number of land plants. TrHbs are a new class of plant Hbs that were originally identified in the chloroplast of Chlamydomonas eugametos (30), and subsequently in organs from Arabidopsis and other higher plants (26).

Based on the analysis of protein sequences it is evident that symHbs and nsHbs are closely related to one another. Both types of plant hb genes contain three introns and four exons, whereas vertebrate Hbs contain two introns (6). The two introns of the vertebrate hb genes are in the same positions as the first and third introns of the plant hb genes. These data indicate that the symHbs and nsHbs are related to vertebrate Hbs and evolved from a common ancestor. Based on a wider distribution in land plants, it would appear that the nsHbs are ancestral to the symHbs, that may have evolved to specifically function in nodules of nitrogen-fixing plants. TrHbs exhibit a different gene structure (26), and thus it is likely that plant TrHbs evolved separately from the ancestor of nsHbs and symHbs (see below).

The overall biochemical features of plant Hbs are similar to those of non-plant Hbs, although some of these proteins exhibit differences in the globin-heme linkage as is evident from deoxyferrous UV/Vis spectra. Deoxyferrous symHbs, animal Hbs and Mbs display a
pentacoordination of the heme, resulting in a broad peak centered at 556 nm (23). The nSHbs exhibit two peaks in the deoxyferrous state at 526 and 556 nm, respectively, indicating that the heme is hexacoordinated (32-34). This absorption spectrum is similar to other hexacoordinated hemeproteins such as cytochrome b (35, 36). Recent spectroscopic analysis showed that a number of other Hbs are also hexacoordinate, including trHbs (26, 37), Synechocystis PCC6803 Hb (38), human neuroglobin (7, 39), and human histoglobin (8). The physiological significance of hexacoordination in these Hbs is not known, but is currently under investigation. Initial studies suggest that hexacoordination could be involved in determining the function of the protein in vivo (34, 39, 40).

Plant trHbs

Truncated hemoglobins (trHbs), found in prokaryotes, protozoa and algae have an unknown function. They fold in a 2-over-2 α-helical pattern. The amino-terminal A helix is almost completely deleted and the CD-D region only consists of approximately 3 amino acid residues in length. TrHbs are not simply a truncated version of the typical globin fold, but are a unique structure based on residue deletions and substitutions that give it conformational stability (41).

A gene in Arabidopsis, glb3, was identified that codes for a protein that is approximately 48% similar to bacterial, protist, and algal 2-over-2 trHbs (26). Arabidopsis GLB3 shares approximately 25% sequence identity with nSHb1 and nSHb2 of the same organism. Northern blot analysis indicated that Arabidopsis glb3 is expressed 4-fold higher in root tissue as compared to shoot tissue. In addition, levels of GLB3 mRNA are not dramatically effected after addition of 2,4-D, cytokinins or abscisic acid, but is upregulated upon auxin treatment as observed by microarray analysis. Expression of glb3 decreases under hypoxic growth conditions (26), which is distinct from the regulation of Arabidopsis nshb1 and nshb2 indicating differential regulation of these genes. Analysis of recombinant Arabidopsis GLB3 indicates that this protein forms dimers when the concentration is higher than 3-30 μM, and it is presumed to be hexacoordinated in the deoxyferrous state, based on the Soret absorbance at 411 nm, which is similar to the nSHbs. The O2-binding rate constant for Arabidopsis GLB3 is 150- and 10-fold higher than Arabidopsis nSHb1 and nSHb2, respectively, but only slightly higher than trHbs of Paramecium (42) and Chlamydomonas (43).

Expressed sequence tags containing a high similarity to Arabidopsis GLB3 were identified in a number of monocots, including barley and rice, and dicots, including soybean, cotton, and tomato (26). These data indicate that it is likely that trHbs are ubiquitous in higher plants. The physiological relevance of the trHbs is not yet understood. In Chlamydomonas, trHB is induced in response to active photosynthesis and localizes to the thylakoid membrane of the chloroplast (30). In Nostoc commune, a trhb gene (formerly named as cyanoglobin) is coexpressed with genes coding for nitrogen fixation (44). Nostoc trHB is also localized on the cytoplasmic side of the cell membrane, but only under anaerobic conditions (45). Additionally,
there is a trHb that is thought to be involved in the protection of bacilli against NO during infection of *Mycobacterium tuberculosis* in humans (21, 46, 47). NO-scavenging could occur by promoting deoxygenation, as observed in oxyHb, oxyMb, and flavoHbs, which converts NO to nitrate (21). In plants, analysis of recombinant *Arabidopsis* GLB3 indicates that this protein is involved in functions other than O₂ storage or transport (26).

The *glb3* gene contains three introns, with the first being located in a similar position as the 3-over-3 Hbs (symHb, nshHb, vertebrate Hb, and Mb). The second and third introns are in different positions as compared to the other plant Hbs. Additionally, none of *glb3*’s introns are in similar positions as the *trh3* genes from *Paramecium* or *Chlamydomonas* (30, 48). Differences in the tertiary structure and position of second and third introns suggest that plant trHbs and symHbs and nshHbs evolved from different ancestors.

**Symbiotic plant hemoglobins**

The symHbs were first identified in soybean root nodules (27), and since then they have been studied and characterized from a number of leguminous [reviewed by Appleby (23)] and nonleguminous species (28, 49-51). SymHbs are primarily involved in the facilitation of O₂ diffusion to the aerobically respiring bacterial symbiont in the infected tissues of nitrogen-fixing nodules of legumes and nonlegumes (23). Its presence is essential for nitrogen fixation in higher plants. SymHbs are found in high concentrations, up to 3 mM in soybean nodules, and have a high affinity for O₂ coupled to a fast O₂-dissociation rate, allowing for the facilitated diffusion of O₂ to actively nitrogen-fixing bacteroids. The most studied symHbs are the Lbs.

**Regulation of symhB genes** – The structural and functional properties of symhB gene promoters have been extensively studied, primarily by Marcker and colleagues, however they are not entirely understood yet. All known symhB gene promoters contain a two-motif nodulin consensus element: AAGAT and CTCTT (52), with a conserved 6-9 nucleotide distance between the two of them (53). These motifs are essential for high level nodule specific expression of symhB genes (54). The 831 bp promoter of the leghemoglobin 1 (*lb1*) gene from lupin contains none of the nodulin-like motifs as a perfect match, but *lb1* expression is similar as in other systems (55). The *lb1* promoter is unlike all of the symhB promoters studied thus far. Therefore, the lupin *lb1* gene promoter was analyzed for functionality by fusion to the reporter gene *gus* and transformed into *Lotus corniculatus* and tobacco (55). GUS activity was detected at the highest levels in root nodules, and in uninfected roots of *L. corniculatus*. In tobacco, GUS activity was localized to roots and leaves, as well as in cultured cells. These results indicate that the lupin *lb1* promoter is not fully nodule specific in transgenic plants. This corroborates previous findings of the *Sesbania rostrata* *glb3* promoter (56) in which activity of the promoter was detected in nodules, stem and leaves of transformed *L. corniculatus*. Therefore, the activity of *lb* promoters in organs other than nodules suggests that possibly *lb* promoters may have functional similarity to *nshB* genes. Nevertheless, despite the detection of the activity of *lb* promoters, no Lb proteins and transcripts have been detected in plant organs.
other than effective (nitrogen-fixing) nodules, which indicates that Lbs are specifically synthesized for the process of symbiotic nitrogen fixation.

In legumes, the Lbs are usually part of a multigene family, and as of yet the specific role(s) of each Lb is not known (23). Lbs and LbIV are the major Lbs in mature soybean and pea nodules, respectively, and have slightly but not significantly higher O$_2$-binding affinities than the other major Lbs (Lbc and Lbl, respectively) (57). Although, the ratios of the different Lbs (Lba/Lbc for soybean and LbIV/Lbl for pea) change during root nodule development, the underlying physiological consequences on root nodule function are unclear. Kawashima et al. (58) characterized two cDNA clones from pea: PsN5, a PsLbA type or LbIV-like Lb, and PsN120, a PsLbB type or Lbl-like Lb. Following analysis of O$_2$-binding affinity, these authors found that recombinant PsN5 had a higher O$_2$-affinity than recombinant PsN120, which was consistent with previous findings (59, 60). In addition, the two lb genes were differentially regulated, with expression of psn5 in the central tissue of effective nodules, and psn120 expression restricted to the region from the infection zone II to the distal part of the nitrogen fixation zone III (58). These results indicate that the PsLbA type protein, having a higher O$_2$-affinity, likely plays a major role in nitrogen fixation and in the O$_2$-consumption of the bacteroids as compared to the PsLbB type protein.

Little is known about the trans-acting factors that regulate lb genes, however work has begun on identifying proteins that function as transcriptional regulators of Lbs. A novel type of DNA-binding protein, CPP1, has been identified to interact with the promoter of a soybean lb gene, gmlbc3 (61). CPP1 is nuclear localized and contains two cysteine-rich regions with 9 and 10 cysteines, respectively. The cpp1 gene is expressed in the distal portion of the central infected tissue during a late phase of nodule development. Transcripts of gmlbc3 are not detected in this region of the nodule, but only in the central infected region. This supports the hypothesis that CPP1 is either a repressor of gmlbc3 gene expression or maintains a repressed state of the lb genes in these cells. Analysis of the expression of the symbiotic bacterial genes in Phaseolus vulgaris nodules showed that the cells at the periphery of the infected zone contain bacteria that have not differentiated into mature nitrogen-fixing cells (62). This suggests that the peripheral cells of the nodule are at a different developmental stage than the central infected cells. Similarly, studies of gmnxs, a homeobox gene, suggested that the soybean nodule has a zone containing less differentiated cells (63). It is therefore possible that genes such as cpp1 are expressed in less differentiated cells and are responsible for the negative regulation of lb genes.

**Structural properties of symHbs** – The crystal structures of lupin Lb (64) and wild type and mutant soybean Lba (65) have been published. Both sets of crystal structure data indicate similar overall folds as observed for other Hbs. The Lbs contain seven $\alpha$-helices in a $3\alpha$-over-3 fold, which enclose an Fe-protoporphyrin IX prosthetic group. They have a distal His in close-proximity to the ligand binding site found in a similar location to the one found in vertebrate Mb and Hbs. This His residue plays a less important role in ligand regulation as compared to Mb and Hbs of vertebrates (65). Lbs are less stable than vertebrate Hbs as a
result of higher levels of heme dissociation and autooxidation (65). While the structural basis for this is not known, it is possible that the distal His is needed to maintain the heme within the globin and thereby influences ligand interactions. This His is conserved among the symHbs (66). It has been demonstrated that soybean Lba, but not animal Mb or Hb, is able to bind large ligands, such as long-chain isocyanides, imidazole and nicotinate (67-69). It is thought that the binding of bulky groups is the result of the free movement of the distal His. Lbs also contain a proximal His (F8) residue that interacts with the heme group, resulting in a pentacoordinate high-spin UV/Vis spectra. Harutyunyan et al (64) reported that this His residue in lupin Lb is free to rotate and move toward the heme plane as compared to Mb. This is similar in soybean Lba (65) and could explain the structural basis for the high degree of reactivity of Lbs, which is a characteristic important to their function as an O2-carrier in root nodules.

Nonsymbiotic plant hemoglobins

The finding of a Hb in uninfected tissues of Trrema tomentosa was the first suggestion that all plants might contain nsHbs. Since that time, nsHbs have been identified from a number of monocots such as maize, rye, and wheat (71), and cloned from the monocots barley (71), rice (31), maize and teosinte (72), and dicots such as soybean (73), Arabidopsis (33), chicory (74), tomato (75) (Garvin et al, personal communication), cotton, citrus, and sugar beet (75), and alfalfa (76). NsHbs have also been identified in primitive land plants, such as mosses (77) and liverworts (75), indicating that nsHbs are widespread in land plants.

NsHB gene families – Most plants have at least two known nshb genes, except for barley in which only one nshb gene has been identified (71). Recently, Li’a-Ruan et al (78) reported the existence of a rice nshb gene family in which four nshb sequences were identified. This family is clustered in two regions: the first contains hb1, hb3 and hb4 and the second contains only hb2. All four genes code for the highly conserved amino acids of plant Hbs (66), including the distal and proximal His, as well as residues located at the dimer interface of rice Hb1 (V50, S53, E123, V124, F127, and A128) as described by Hargrove et al (79). This observation suggested that potential homo and/or heterodimers from nsHb1, 2, 3 and 4 could assemble in rice tissues. Two of the rice nshb genes, hb1 and hb2, have been cloned and their respective recombinant proteins were characterized in detail (31, 79; Ross et al, in preparation). The existence of different promoters upstream of the rice nshb genes (78) suggests that each nshb gene is regulated by separate mechanisms, and is expressed in different organs and/or under different growth conditions. The potential for understanding the differential regulation of rice nshb genes and the presence of novel cis and trans-acting elements can now be elucidated.

Expression of nshb genes – In a number of systems nshb promoters have been analyzed. For example, the activity of nshb promoters of Parasponia andersonii and Trrema tomentosa was localized to the root meristem and vascular cylinder of transgenic tobacco, using gus as a reporter gene (80). Despite these findings, there is no substantial evidence that nsHb
proteins are found in Parasponia roots. Jacobsen-Lyon et al (81) reported that when the Cassarina glauca hb gene was fused to gus and transformed into L. corniculatus, GUS expression was found in the meristem of the root tips, the vascular stele of roots, and the parenchyma internal to the endodermis. These observations showed that nshb genes express in diverse plant organs.

Transcripts for nshbs have primarily been identified in a number of plant organs, including tissues that are metabolically active, such as the stems of soybean (73), suggesting that these proteins are involved during the normal growth of the plant. In monocots, the earliest expression of nshbs is observed in germinating seeds. In barley, nshB transcripts and proteins were detected predominantly in the aleurone cells (71). The presence of nshBs was also reported in excised barley embryos (82), embryo-containing and embryoless half seeds, and aleurone tissue using immunoblotting methods (82, 83). Arredondo-Peter et al (31) showed that under normal growth conditions, hb1 and hb2 are expressed in rice leaves, but that only hb1 is expressed in roots, suggesting differential regulation of this gene family during plant development. Using immunological detection, Aréchaga-Ocampo et al (72) identified a nshB in maize embryos, root and leaf tissues and testis in root and leaf tissues.

Other studies suggest that nshBs are stress-related proteins. For example, under hypoxic conditions the nshb gene is expressed in germinating barley aleurone (25, 71, 84). Nie et al (85) found induction of the barley hb gene under low O2 tension, high levels of CO in aleurone tissue, as well as in the presence of 10 mM nitrate. Two hb genes exist in Arabidopsis which are expressed in plant rosette leaves and roots under normal conditions, however when grown under stress conditions, such as microaerobiosis or low temperature both Arabidopsis nshb are overexpressed as compared to normal growth (33). Arabidopsis nshb1 and nshb2 were fused to gus to determine promoter tissue specificity (75). The nshb1::gus fusion was expressed during germination in the hypocotyl and cotyledons, and nshb2::gus activity was not found in young plants but in the root stem, rosette leaves and roots of mature flowering plants. During an analysis of hormone induction, Arabidopsis nshb1::gus is expressed under hypoxia (5% O2), whereas nshb2::gus is expressed under cytokinin induction (75). Similarly, rice nshb2 promoter was fused to gus and exhibited induction by cytokinins, and is expressed in similar plant organs as Arabidopsis nshb1::gus and nshb2::gus (Ross et al, unpublished results). Lira-Ruan et al (86) reported that nshB levels in rice increase in leaves and roots from plants subjected to darkness and flooding. However, nshB levels did not change when plants were subjected to oxidative (H2O2), nitrosative (SNP) and hormonal (2,4-D) stresses, suggesting that nshB do not participate in a generalized plant response to stress, but rather to specific stress conditions. Analysis of the expression of two tomato nshb genes indicates that hb1 responds to nitrate resupply, addition of phosphate, potassium and iron, whereas hb2 is not significantly induced, suggesting that nshBs may be involved in the responses to nutrient stress, perhaps by participating in signaling mechanisms (Garvin et al, USDA-ARS, University of Minnesota, personal communication).
Organ and tissue localization of nsHbs – Seregélyes et al (76) provided the first immunocytolocalization of the alfalfa nsHb protein. Using cell suspension cultures they were able to detect alfalfa nsHb in the nucleus of cells cultivated under hypoxia. The lack of a nuclear localization signal on the amino-terminus in any of the known nsHbs questions this preliminary data. However, there is a possibility that under particular environmental conditions nsHbs are able to translocate from the cytoplasm to the nucleus. At this time, there is very limited information on the potential partitioning of nsHbs to different cellular compartments. Recently, Ross et al (83) and Lira-Ruan et al (86) investigated the immunolocalization of nsHbs in rice tissues and the synthesis of nsHbs during the development of rice plants, respectively. These authors showed that nsHbs are synthesized in organs from young and mature rice and are localized in the cytoplasm of differentiating cells of the root cap, sclerenchyma, aleurone, and in the vasculature, principally in the differentiating xylem (Fig. 1). The detection of nsHb proteins in vascular regions is consistent with earlier studies (75, 80, 81) that have documented nsHb promoter-driven gus expression in the vascular stele. The existence of nsHbs in terminally differentiating tissue, such as the aleurone, and in terminally differentiating cells, such as the tracheary elements, is particularly interesting. All of these cell types undergo some form of programmed cell death (87, 88) and exhibit tissue-specific metabolism suggesting a potential role for nsHbs in these specific cellular environments.

![Embryonic organs and Vegetative organs](image)

Fig. 1. Detection of nsHbs in rice embryonic (coleoptiles, seminal roots and embryos) and vegetative (2 to 14 weeks old leaves and roots) organs by Western blot, and localization of nsHbs in rice aleurone layer, scutellum, sclerenchyma and root cap tissues by confocal microscopy. NsHbs were detected by using anti-rice Hb1 antibodies. Figures reproduced from Lira-Ruan et al (86) (with permission).
**Structural properties of nsHbs** – The first crystal structure of a nsHb, rice Hb1, was reported by Hargrove et al. (79). Rice recombinant Hb1 is a concentration dependent dimer, as observed by FPLC analysis (40), and crystallizes as such. Rice Hb1 has the same overall structure as other Hbs, the globin fold, and the heme pocket for ferric Hb1 contains a proximal and distal His residue. The unusually high O\(_2\)-affinity (K\(_D\) = 1 nM) of this protein has been explained by the combined effect of the close proximity of the distal His to Fe and the positioning of the Phe40 residue. This latter amino acid is found atypically close to the Cy proximal His as compared to vertebrate and symHbs. This is a highly conserved residue in plant nsHbs and it is likely that it is functionally significant. The unfavorable steric interaction between Phe40 and His73 (distal His) may promote dissociation of the distal His from the Fe atom and permit O\(_2\)-binding. A mutation of Phe40 to Leu results in a His73 dissociation rate decrease of ~10-fold from ~1900 s\(^{-1}\) in wild type Hb1 to 200 s\(^{-1}\) in the mutant (Hargrove and Goodman, unpublished).

Kinetic analysis of ligand binding for the nsHbs shows that this type of Hb has unusually high affinity for O\(_2\). The O\(_2\)-association constant for recombinant rice Hb1 (31), rice Hb2 (Ross et al, unpublished), barley Hb (32) and Arabidopsis nsHb1 (33) (Table 1) is similar to other O\(_2\) transport and storage proteins, such as soybean Lhcb. However, the extremely high O\(_2\)-affinity for many of these proteins is the result of a very low dissociation rate. These rate constants indicate that the unique reactivity is the result of the positioning of amino acids and O\(_2\) within the heme pocket of the protein (79). What is interesting is that the Arabidopsis nsHb2 protein has oxygen binding characteristics more similar to those of the symHbs (Table 1). The functional consequences of differences in ligand-binding kinetics are not known at present. However, the biochemical properties of nsHbs specifically the high affinity for O\(_2\), raises interesting questions about the function(s) of these proteins in plant organs. Some of these possibilities are discussed below.

**Table 1.** Rate and equilibrium constants for the reaction of O\(_2\) from some plant Hbs

<table>
<thead>
<tr>
<th>Protein</th>
<th>K(_{O2}^\prime) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>kO(_2) (s(^{-1}))</th>
<th>KO(_2) ((\mu\text{M}^{-1}))</th>
<th>Reference</th>
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<td>94</td>
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<td>Ross et al</td>
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<tr>
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k\(_{O2}^\prime\) is the O\(_2\)-association constant; kO\(_2\) is the O\(_2\)-dissociation constant; KO\(_2\) (k\(_{O2}^\prime\)/kO\(_2\)) is the O\(_2\)-affinity constant.
Potential functions of nsHbs – While data on the localization, kinetics, regulation and structure of the nsHbs have begun to accumulate, their physiological function still eludes us. Based on their presence under both normal and stressed growth conditions, it is conceivable that these proteins maintain a ‘housekeeping’ function as well as potential cell-specific functions. The very tight binding of O2 by recombinant nsHbs does not readily support an O2-sensor or O2-transport role for these proteins. Appleby et al (89) postulated that under normal aerobic growth conditions, nsHbs would be oxygenated, however under microaerobic or anaerobic conditions nsHbs would be deoxygenated and would trigger an anaerobic response making these proteins involved in O2-sensing. Andersson et al (73) questioned this hypothesis by suggesting that these proteins could function as O2-carriers in metabolically active tissues, such as the stems of soybean, which contain high levels of nsHb transcripts. However, no evidence on nsHb protein levels nor their ligand binding properties were reported for the soybean Hb to critically evaluate this hypothesis.

NsHbs have been implicated as O2-scavengers or in O2-signaling (23). However, as compared to symHbs, which are found in cellular concentrations of 1 to 3 mM (90), nsHbs are found in nM concentrations (25, 83). In addition, given the in vivo tissue localization of nsHbs in metabolically active tissues (e.g. root cap cells and differentiating xylem (83) and aleurone (71, 83)) and the very low O2-dissociation constant of several recombinant nsHbs (Table 1), it is unlikely that they function as O2-scavengers or directly in O2-signaling (24, 25). These proteins may have a number of other functions, including binding and/or transporting ligands, such as CO or NO, and/or may interact with organic molecules and/or proteins under specific conditions (24).

Both, Arredondo-Peter et al (24) and Hill (25) have suggested that plant nsHbs could act as NO-scavengers, in a similar manner to those of the bacterial and yeast flavoHbs, documented to possess NO dioxygenase properties (21, 91, 92). Recently, Wang et al (93) found that a nshb gene from Arabidopsis is transiently induced upon addition of 250 mM nitrate, and sustained induction occurs upon addition of 5-10 mM nitrate, using microarray and gel blot analysis. Additionally, tomato Hb1 is induced under nitrate resupply (Garvin et al, personal communication). These data suggest that the nsHbs have a possible role in nutrient signaling, and particularly in nitrogen signaling.

Based on studies in barley cells (71) and from over and under-expressing barley Hb in maize cells (84) it has been postulated that barley nsHb is involved in ATP metabolism in stressed tissues. Maize suspension cells containing antisense barley nsHb cDNA have considerably lower levels of ATP and total adenylates when grown in hypoxic conditions as compared to the controls or cells that were engineered to overproduce barley nsHb protein. These results have been interpreted as an indication that nsHbs maintain a direct involvement with cellular energy status under O2-limiting conditions. Hill (25) has suggested that barley nsHb functions as an oxygenase by oxidizing NADH in association with other proteins, such as a flavoprotein. He also suggested that barley nsHb is involved in maintaining a proper redox state under hypoxic/anoxic conditions by directly interacting with pyruvate. Localization
data presented by Ross et al. (83) shows that rice nsHbs are synthesized in differentiating cells. Metabolism of these cells is in flux and metabolic redirection occurs in response to specific differentiation signals, one of which is likely to be a change in the cell’s redox state. nsHbs could be involved in redox signaling, especially if the redox state of the heme is functionally important (79; Hargrove, personal communication). Thus, nsHbs may function to sense/or maintain a particular redox environment that promotes cell viability in response to differentiation or environmental cues.

Conclusions

Hbs are very ancient proteins. Apparently their evolution occurred in many directions, with the numerous types of Hbs that have been identified throughout the kingdoms of life: trHb, flavoHb, symHb, nsHb, vertebrate Hb, Mb, neuroglobin, and histoglobin. The biochemical and regulatory properties of Hbs suggest that these proteins may function in organisms according to the metabolic status in the cell, such as a response to changes in pH and/or availability of interacting metabolites. It is very likely that Hbs do not play a single but many roles in different compartments of the cell under particular physiological conditions.

The plant trHbs and symHbs and nsHbs are likely to have evolved independently and function differently in plants today. As discussed above, the symHbs are involved in the facilitation of O$_2$-diffusion in actively nitrogen-fixing organisms (23). However, the physiological functions of the nsHbs and trHbs are still not known. Little information is available about plant trHbs, and thus it is still too premature to propose a function for this group of proteins in plants. In contrast, progress has been made in the analysis of nsHbs and functions for nsHbs in plant organs have been already proposed (24, 25). However, the precise function(s) of nsHbs is still not understood. An intriguing question that may help to understand the current function of nsHbs and their evolutionary relationships is: what was the function of the ancestral nsHb of land plants? An analysis of the nsHbs from the mosses *Physcomitrella patens* and *Ceratodon purpureus* (77) (Arredondo-Peter et al., unpublished observations) aids in answering this question. Sequence alignments of moss Hbs and a number of symHbs and nsHbs, indicate that the size of the Hb polypeptide has decreased over time. This reduction has resulted in the loss of amino acids in the amino-terminal portion of the polypeptide, principally in the pre-A helix region (Fig. 2A). Thus, did this pre-A helix have a function in the past? An interesting possibility is that the pre-A helix functioned as a leader peptide. For example, the moss nsHbs contain a predicted leader peptidase site at position 20 to 25 of the pre-A helix. Additionally, the amino-terminal portion of the moss nsHb has physicochemical properties of a leader peptide, similar to those existing in the *Chlamydomonas* trHb (Fig. 2B), which is translocated from the cell cytoplasm to chloroplasts (30). However, it is not known if the pre-A helix from moss nsHbs is an authentic leader peptide. All other higher plant nsHbs appear to be localized in the cytoplasm. Does this indicate that the ancestor of plant nsHbs was originally translocated to cellular organelles, such as the mitochondria or chloroplast, and that nsHbs became cytoplasmic during evolution? An answer to the above possibilities
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Fig. 2. (A) Sequence alignment of the amino-terminal region from moss (CeriHb and PhyscomHb) Hbs and selected nHbs, and Lds. Alignment was performed by using the Piloup routine of the GCG (Genetics Computing Group) program. Hbs were selected according to rice Hb1 (79) and are indicated with different colours. Note the decrease in the size at the pre-A helix from primitive (moss) to evolved (sym)Hbs and Lds. Hbs, (B) sequence alignment of the amino-terminal region from chloroplastic (Chlamydomonas) and moss Hbs; upper panel shows the homology region with blue background; lower panel shows the amino acid character (red, acidic; blue, basic; green, non-polar; yellow, hydrophobic); "up to bottom" and "bottom to up" oriented arrows show the leader peptide and predicted leader peptide sites in Chlamydomonas and moss Hbs, respectively.
may be obtained from the analysis of Hbs from the algal ancestor to land plants, i.e. by
determining whether cytoplasmic or organelle nsHbs-like exist in Chlorophyta species.
Whatever the function of ancestral nsHbs was, it was probably important for the adaptation
of first plants to a land environment. Moreover, after 360 million years of plant evolution
the function of nsHbs is apparently still important to the adaptation and survival of a large diversity
of present land plants.

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