

## Update on Biochemistry

# Plant Hemoglobins

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Hbs are ubiquitous proteins in most organisms, including bacteria, protozoans, fungi, plants, and animals (Vinoogradov et al., 1993; Bolognesi et al., 1997). The known and predicted roles of Hbs in each organism encompass functions that include the reversible binding of gaseous ligands and the ability to bind other cellular molecules.

### SYMBIOTIC AND NONSYMBIOTIC PLANT Hbs BELONG TO A LARGE FAMILY OF RELATED PROTEINS

Hbs are widely distributed in higher plants, and by comparing sequences, expression patterns, and ligand-binding properties, it is evident that these fall into two broad groups (Fig. 1; also see Andersson et al., 1996). The symbiotic-type Hbs are predominantly found in the infected cells of nitrogen-fixing nodules of legumes and nonlegumes and function to facilitate oxygen transport. The other group of plant Hbs, nonsymbiotic Hbs, appear to be ancestral to the symbiotic Hbs, are more widespread in the plant kingdom, and generally display a high affinity for oxygen. Their function in plants is the subject of much current inquiry (Appleby, 1992; Andersson et al., 1996; Arredondo-Peter et al., 1997a; Trevaskis et al., 1997; Hill, 1998). Some aspects of plant nonsymbiotic Hb function, particularly that of barley, have been succinctly reviewed by Hill (1998).

### SYMBIOTIC PLANT Hbs ARE PRESENT IN NITROGEN-FIXING TISSUES AND FACILITATE OXYGEN DIFFUSION

First identified in soybean root nodules, symbiotic plant Hbs have been studied extensively and characterized from numerous legume (Appleby, 1992) and nonlegume species (Pathirana and Tjepkema, 1995; Suharjo and Tjepkema,

1995). Appleby (1992) is an excellent review for an in-depth survey of the earlier literature.

The primary role of symbiotic Hbs appears to be the facilitation of oxygen diffusion within infected tissues (Appleby, 1992). This feature is evidenced by a great accumulation of functional Hb in the target tissues with high affinity for oxygen and a relatively fast oxygen-dissociation rate, which permits low cellular concentrations of free oxygen and a reasonable flux of oxygen within tissues actively fixing nitrogen. The symbiotic Hbs from legumes, the Lbs, are the best studied in this group and are discussed below.

Lbs in most legumes belong to multigene families (Brisson and Verma, 1982; Appleby, 1992) and are encoded by genes that have three introns. The first and third introns are positioned similarly to the introns of vertebrate *hb* genes, which suggests that plant and animal Hbs derived from a common ancestor.

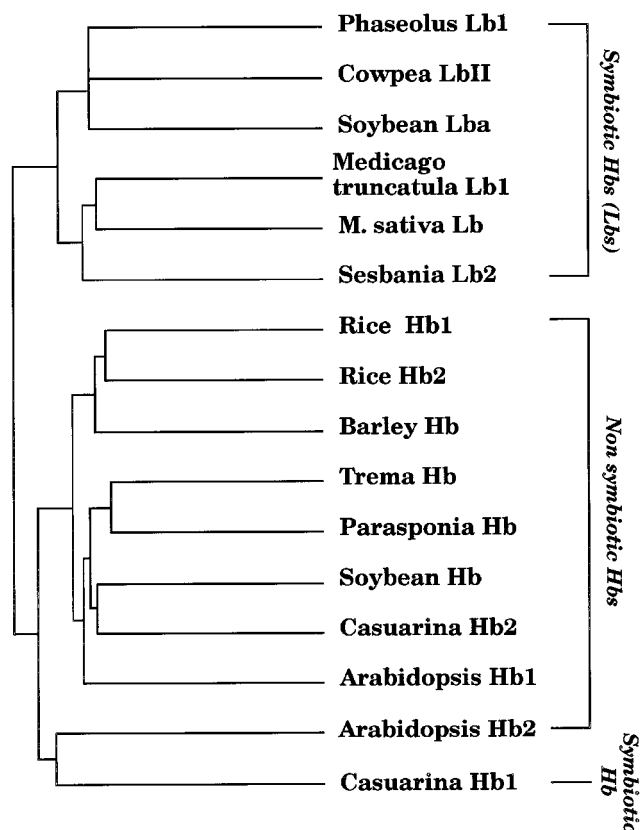
Lbs are the most abundant soluble proteins in root nodules and are frequently found as modified forms in vivo, which generally gives rise to several closely related isoproteins. Modifications include N-terminal acetylation, which does not affect ligand binding (Martin et al., 1990), and some type of modification to the heme (Jun et al., 1994), which prevents the formation of the ferrous-oxygen complex. Heme-modified Lbs have been purified from mature soybean root nodules, although the nature of the heme adduct has yet to be elucidated. The presence of these apparently nonfunctional Lbs in root nodules could be early indicators of the metabolic status of the nodule, and suggest that mechanisms that cause heme modifications exist in nitrogen-fixing root nodules (Jun et al., 1994).

The crystal structures for lupin Lb (Harutyunyan et al., 1995) and wild-type and mutant soybean Lba have been published recently (Hargrove et al., 1997). Crystal structure data indicate a structure very similar to the overall folds observed for other heme proteins such as animal myoglobins and Hbs. There are seven  $\alpha$ -helices that enclose the iron-protoporphyrin IX prosthetic group. All Lbs contain the proximal His that forms a single covalent interaction with the heme group. Lbs also contain a distal His near the ligand-binding site similar in location to the one found in most vertebrate myoglobins and Hbs. However, it appears that this His plays a less critical role in regulating ligand

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Abbreviations: Hb, hemoglobin; Lb, leghemoglobin.



**Figure 1.** Dendrogram that shows the sequence relationships between selected symbiotic and nonsymbiotic Hbs. Amino acid sequences were obtained from the GenBank database, and alignment of the sequences was done using the PILEUP program (Genetics Computer Group, Madison, WI).

binding in Lbs than in other Hbs (Hargrove et al., 1997, and refs. therein). Lbs are inherently less stable than vertebrate myoglobins due to much higher rates of autooxidation and heme dissociation (Hargrove et al., 1997). The structural determinant for these differences in stability is unknown, but might result from the different requirements for ligand binding between these classes of Hbs. However, the ability of the distal His to maintain heme within the protein and to influence its ligand interactions has been suggested to explain the high degree of conservation of this residue in all Hbs (Hargrove et al., 1997).

#### NONSymbiotic PLANT *Hb* GENES ARE STRUCTURALLY SIMILAR TO SYMBIOTIC *Hb* GENES

*Parasponia andersonii*, the only nonlegume known to be infected by rhizobia, contains a Hb that is found in root nodules and roots (Bogusz et al., 1988). This homodimeric protein is encoded by a single gene that contains three introns that are positioned identically to *lb* introns (Landsman et al., 1986) and have oxygen-binding kinetics similar to Lb, suggesting that its primary function in nodules is oxygen transport (Gibson et al., 1989). However, the amino acid sequence of *P. andersonii* Hb differs substantially from the other symbiotic plant Hbs and appears to more closely

resemble the nonsymbiotic Hbs (Fig. 1). Hb genes highly related to the *P. andersonii* *hb* have been cloned from *Trema tomentosa*, a nonnodulating relative of *P. andersonii* (Bogusz et al., 1988), and from *Casuarina glauca* (Jacobsen-Lyon et al., 1995). The *C. glauca* nonsymbiotic *hb* gene also contains three introns, but lacks the nodulin-like regulatory motifs that direct the nodule-specific expression of the symbiotic *hb* genes (Jacobsen-Lyon et al., 1995).

Nonsymbiotic *hb* genes have recently been cloned and sequenced from a number of other higher plants, including Arabidopsis (Trevaskis et al., 1997), barley (Taylor et al., 1994), rice (Arredondo-Peter et al., 1997a), and soybean (Andersson et al., 1996). The *hb* genes from rice have three introns at positions identical to those of known plant *hbs*, suggesting that the ancestral *hb* gene of flowering plants had three introns in an identical location. All of the plants except barley contain two or more nonsymbiotic *hb* genes that are differentially expressed in the plant and code for proteins with potentially differing biochemical properties. For example, the two Arabidopsis Hbs display relatedness to the symbiotic group (AHb2) and the nonsymbiotic group (AHb1), whereas both of the rice Hbs analyzed so far appear to belong to the nonsymbiotic groups of proteins (Fig. 1).

#### NONSymbiotic Hbs ARE EXPRESSED IN DIVERSE PLANT TISSUES

Expression of nonsymbiotic Hbs in plant tissues appears to vary significantly, with the highest levels observed in metabolically active or stressed tissues. Bogusz et al. (1990) found that *P. andersonii* and *T. tomentosa* *hb* gene promoters direct GUS expression in root meristems and in the vascular cylinder of transgenic tobacco, and in root nodules and vascular tissues of mature roots of *Lotus corniculatus*. Similarly, when the promoter for the nonsymbiotic *C. glauca* *hb* gene was fused to a reporter gene encoding GUS and transformed into *L. corniculatus*, this promoter directed low levels of GUS expression in noninfected nodule tissues, but nothing was detected in infected cells (Jacobsen-Lyon et al., 1995). Additionally, GUS activity was detected in meristematic regions of root tips, in parenchyma internal to the endodermis, and in the vascular stele of roots.

Using northern analysis, Andersson et al. (1996) detected Hb transcripts in different parts of soybean plants, with stems exhibiting the highest transcript levels. In Arabidopsis the symbiotic-like *ahb2* gene was expressed at low levels in rosette leaves and was induced by low temperatures, whereas the nonsymbiotic *ahb1* gene was expressed in roots and rosette leaves and levels of expression increased under hypoxic conditions, suggesting that AHb1 is a stress-related protein (Trevaskis et al., 1997), as has been well documented for barley Hb (Taylor et al., 1994; Hill, 1998). There is also some evidence that a signal transduction pathway involving calcium-dependent protein kinase(s) and protein phosphatase(s) may be involved in barley *hb* gene expression (R.D. Hill, personal communication). Results with rice plants grown under normal conditions (Arredondo-Peter et al., 1997a) showed that *hb1* and *hb2* genes were expressed in leaves, but only *hb1* was expressed

in roots, suggesting that rice *hb* genes are regulated by different promoters.

#### POTENTIAL FUNCTIONS OF NONSYMBIOTIC PLANT Hbs: MULTIPLE ROLES IN DIFFERENT TISSUES?

Based on the predicted oxygen-binding kinetics and probable concentration of the nonsymbiotic Hb (approximately 100 nM), Appleby et al. (1988) were the first to suggest that one role of the nonsymbiotic Hbs could be to sense oxygen levels. They suggested that under normal conditions Hb would be oxygenated and under oxygen-limiting conditions deoxyHb would increase, triggering an anaerobic response. Andersson et al. (1996) questioned the oxygen-sensor hypothesis, suggesting instead that Hbs might function as oxygen carriers in metabolically active tissues such as soybean stems, which contain high levels of Hb transcripts. No corresponding data on the levels of Hb proteins or on the affinity of the soybean Hb protein for gaseous ligands were presented, largely due to problems associated with purifying the very small amounts of nonsymbiotic Hbs present in most higher plant tissues.

Such limitations in obtaining purified native protein have been successfully overcome by producing large amounts of recombinant Hbs in *Escherichia coli*. Recombinantly produced Hbs are indistinguishable from their native counterparts (Arredondo-Peter et al., 1997b; Duff et al., 1997; Hargrove et al., 1997) and have permitted detailed studies on the biochemical and biophysical properties of plant Hbs. This allows the study of protein structure-function relationships and the use of recombinant proteins to obtain antibodies that cross-react with the native Hbs in plant extracts (Duff et al., 1998).

Analyses of native and recombinant nonsymbiotic Hbs have shown that these proteins have characteristics that are unique to Hbs capable of reversible oxygen binding. Spectral characterization of the deoxyheme pockets of all of the nonsymbiotic Hbs show that this form of the protein is hexacoordinate. Arredondo-Peter et al. (1997a) used site-directed mutagenesis to show that the side chain of the His residue with homology to the distal His (E7) of vertebrate Hbs is the coordinating ligand. In spite of this, nonsymbiotic Hbs bind oxygen rapidly and with very high affinity (Table I). Furthermore, it is evident from mutagenesis work that the same His side chain is involved in stabilizing

bound oxygen in the oxygenated form of the protein. Therefore, in the presence of oxygen, this His side chain rapidly dissociates from the ligand-binding site and moves into a position from which it can positively interact with bound oxygen. Thus, a striking feature of many of the nonsymbiotic Hbs is the unusually high affinity for oxygen brought about by a moderate association constant that is coupled to an extremely low dissociation constant (see Table I; Arredondo-Peter et al., 1997a; Duff et al., 1997; Trevaskis et al., 1997; Hill, 1998).

These data do not support either the oxygen-sensor or the oxygen-transport hypothesis as functions for the nonsymbiotic Hbs. However, we do not discount the possibility of nonsymbiotic Hbs participating as sensors or oxygen carriers under specific conditions. Clearly, more information regarding their cellular interactions with other proteins and potential ligands is needed to verify these hypotheses. Nevertheless, nonsymbiotic plant Hbs possess the highest reported oxygen affinities (Table I), generating intriguing questions about their function(s) in plant tissues.

Data accumulated from studies with barley Hb, either in barley cells (for review, see Hill, 1998) or expressed in suspension cultures of maize cells (Sowa et al., 1998), suggest that barley Hb is involved in some aspect of ATP metabolism in stressed tissues. Antisense constructs of barley Hb expressed in maize suspension-cultured cells have much lower levels of ATP and total adenylates under hypoxic conditions compared with either control cell lines or lines engineered to overproduce Hb. This has been interpreted as indicating a direct involvement of Hb in maintaining cellular energy status under oxygen-limiting conditions (Sowa et al., 1998). Hill (1998) has also suggested that barley Hb might function as an "oxygenase" when associated with other proteins (see below), and may even participate directly in the oxidation of pyruvate to maintain a proper cellular redox state under anoxic/hypoxic conditions.

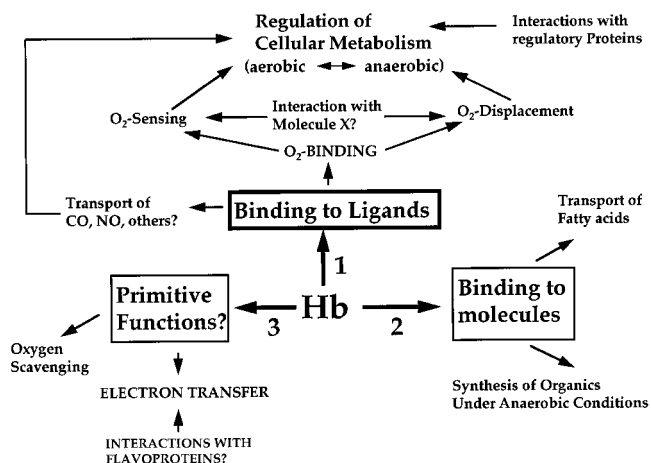
Using a different approach, Holmberg et al. (1997) generated transgenic tobacco expressing a bacterial (*Vitreoscilla*) Hb. Plants containing viable Hb protein, as estimated by western blots, showed enhanced dry matter and chlorophyll and faster germination and flowering times than the wild-type control plants. Transgenic plants also contained higher levels of nicotine and greatly lowered levels of anabasine. These results suggested that plants expressing bacterial Hb experienced a higher cellular oxy-

**Table I.** Rate ( $k'$ ) and equilibrium constants ( $K$ ) for the reaction of oxygen and CO for some representative plant hemoglobins

Protein	$k'O_2$ $\mu\text{M}^{-1}\text{s}^{-1}$	$kO_2$ $\text{s}^{-1}$	$KO_2$ $\mu\text{M}^{-1}$	$k'CO$ $\mu\text{M}^{-1}\text{s}^{-1}$	$kCO$ $\text{s}^{-1}$	$KCO$ $\mu\text{M}^{-1}$
Nonsymbiotic Hbs <sup>a</sup>						
Rice wild-type Hb1	68	0.038	1800	72	0.001	7200
Rice Hb1-H77L	620	51	12	150	0.002	75,000
Barley Hb	2.4	0.028	86	0.21	0.0016	131
Arabidopsis Hb1	74	0.12	617	1.5	0.0012	1250
Arabidopsis Hb2	1	0.14	7	0.25	0.0013	192
Symbiotic Hbs <sup>b</sup>						
Soybean Lba	130	5.6	23	16	0.0084	1900
Lba-H16L	400	2.4	16	170	0.0024	71,000

<sup>a</sup> Data from Arredondo-Peter et al. (1997a), Duff et al. (1997), and Trevaskis et al. (1997).

<sup>b</sup> Data from Hargrove et al. (1997).



**Figure 2.** Possible routes of interactions of nonsymbiotic Hb in plant cells. See text for further discussion.

genation, leading to the observed effects (Holmberg et al., 1997). However, data on the oxygenation conditions of Hb or of the cells in situ were not provided to support this suggestion. A complicating factor is that *Vitreoscilla* Hb possesses a much lower oxygen affinity compared with nonsymbiotic plant Hbs, making it difficult to extrapolate the results observed with transgenic tobacco to any direct predictions about the role(s) of nonsymbiotic Hbs in plants. Another problem in assigning roles for nonsymbiotic Hbs in plants arises from the differences in their biochemical properties and tissue-expression patterns under ambient conditions in plants such as *Arabidopsis*, rice, and soybeans. All of these data would suggest that nonsymbiotic Hbs could have several functions in situ.

In the last few years functions other than oxygen transport have been suggested for Hbs from diverse sources. Animal Hbs are known to possess a wide range of functions: (a) heat transduction through the oxygenation/deoxygenation cycle; (b) modulating erythrocyte metabolism; (c) binding to ion channel proteins; (d) as a source of physiologically active catabolites (for review, see Giardina et al., 1995); and (e) as multifunctional proteins, such as the peritenteric Hb from *Ascaris*, a parasitic nematode, which is probably involved in some initial steps of the sterol synthesis (Goldberg, 1995, and refs. therein). Moreover, there is indirect evidence that the Hb in the bacterium *Vitreoscilla* may function as an oxygen sensor (Joshi and Dikshit, 1994) or as a terminal oxidase (Dikshit et al., 1992). Sequence comparisons indicate that *Vitreoscilla* Hb is more homologous to plant than to animal Hbs (Arredondo-Peter and Escamilla, 1991), suggesting that early plant Hbs could have had functions similar to *Vitreoscilla* Hb. For example, overproduction of *Vitreoscilla* Hb in *E. coli* enhances cell growth by changing flux patterns to the pentose phosphate, TCA, NADH, and ATP pathways, prolonging cell growth during oxygen-limited conditions (Tsai et al., 1996).

It is therefore quite possible that nonsymbiotic plant Hbs could be involved in several metabolic pathways (Fig. 2). For example, route 1 shows possible interactions when nonsymbiotic Hbs bind to small molecules known to be ligands, e.g.

oxygen, CO, and NO. Binding could be influenced through interactions with other cellular molecules that could modify the association or dissociation constants for ligand binding, consequently altering their function as oxygen carriers or oxygen sensors; conversely, such interactions might yield molecules with different activity (e.g. inhibitor/activator-enzyme complexes). Route 2 of Figure 2 shows that Hbs may bind small organic molecules and then function by transporting fatty acids or, under anaerobic conditions, by participating in the synthesis of organic compounds.

Finally, route 3 of Figure 2 indicates that nonsymbiotic Hbs could function as oxygen scavengers, based on their extremely tight binding of oxygen (see Table I). This function might have allowed primitive anaerobic microorganisms to adapt to increasing levels of oxygen as a result of photosynthesis. Additionally, ancestral Hbs might have possessed electron-transport functions. For instance, several bacterial and yeast Hbs are two-domain proteins that contain a globin and a flavin domain (flavoHbs; Zhu and Riggs, 1992; Membrillo-Hernandez and Poole, 1997), which apparently function by transporting electrons (Bolognesi et al., 1997). During evolution the gene coding for flavoHbs could have split and generated a one-domain, *Vitreoscilla*-like Hb, which was the ancestor of plant and animal Hbs. Numerous flavoproteins that reduce bacterial and plant Hbs have been reported (e.g. Jakob et al., 1992; Ji et al., 1994, and refs. therein); therefore, if the gene coding for the flavin domain still exists in plants, the resulting flavoprotein may interact with nonsymbiotic Hbs to function in electron-transfer reactions. It is also possible that mutants of only one of these primitive Hbs was selected within a particular plant family during the evolution of a symbiosis. If this were true, one would expect multiple *hbs* to have evolved with multiple biochemical and physiological functions.

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