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Review

Plant hemoglobins: What we know six decades after their discovery☆

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Abstract

This review describes contributions to the study of plant hemoglobins (Hbs) from a historical perspective with emphasis on non-symbiotic Hbs (nsHbs). Plant Hbs were first identified in soybean root nodules, are known as leghemoglobins (Lbs) and have been characterized in detail. It is widely accepted that a function of Lbs in nodules is to facilitate the diffusion of O_2 to bacteroids. For many years Hbs could not be identified in plants other than N_2-fixing legumes, however in the 1980s a Hb was isolated from the nodules of the non-legume dicot plant *Parasponia*, a *hb* gene was cloned from the non-nodulating *Trema*, and Hbs were detected in nodules of actinorhizal plants. Gene expression analysis showed that *Trema* Hb transcripts exist in non-symbiotic roots. In the 1990s nsHb sequences were also identified in monocot and primitive (bryophyte) plants. In addition to Lbs and nsHbs, Hb sequences that are similar to microbial truncated (2/2) Hbs were also detected in plants. Plant nsHbs have been characterized in detail. These proteins have very high O_2-affinities because of an extremely low O_2-dissociation constant. Analysis of rice Hb1 showed that distal His coordinates heme Fe and stabilizes bound O_2; this means that O_2 is not released easily from oxygenated nsHbs. Non-symbiotic *hb* genes are expressed in specific plant tissues, and overexpress in organs of stressed plants. These observations suggest that nsHbs have functions additional to O_2-transport, such as to modulate levels of ATP and NO.

Keywords: History; Leghemoglobin; Non-symbiotic; Truncated

1. Introduction

Hemoglobins (Hbs) are hemeproteins that reversibly bind O_2. The most common function of Hbs is associated with the transport of O_2, however Hbs also bind other gaseous ligands, such as NO (Cooper, 1999; Poole and Hughes, 2000; Moller and Skibsted, 2002; Dordas et al., 2003b; Milani et al., 2003; Frey and Kalio, 2005), and organic molecules (Ollesch et al., 1999; Bonamore et al., 2003; D'Angelo et al., 2004; Rinaldi et al., 2006), which suggests that they are multifunctional proteins in living organisms. Phylogenomic analysis showed that Hbs are widespread in organisms, as they have been identified in genomes of archaeobacteria, eubacteria and eukaryotes, including plants (Vinogradov et al., 2005, 2006). In plants three types of Hbs have been identified: symbiotic, non-symbiotic (nsHb) and truncated (2/2) Hbs (tHbs) (Ross et al., 2002). This review describes contributions to the study of plant Hbs from a historical perspective with emphasis on nsHbs. The authors refer interested readers to reviews by Appleby (1992), Fuchsman (1992), Arredondo-Peter et al. (1998), Hill (1998), Ross et al. (2002) and Kundu et al. (2003) for detailed discussion and relevant references on the structure, function and molecular biology of plant Hbs.

2. The discovery of plant Hbs

Plant Hbs were first identified by Kubo (1939). This author analyzed the red pigment of soybean root nodules. Nodules form in legumes after root infection by soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium* and *Sinorhizobium* (collectively know as rhizobia). Nodules

Abbreviations: Hb, hemoglobin; HGT, horizontal gene transfer; Lb, leghemoglobin; Mb, myoglobin; nsHb, non-symbiotic hemoglobin; NO, nitric oxide; tHb, truncated (2/2) hemoglobin.

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are red in color and are the plant organs where nitrogen fixation (by symbiotic rhizobia) occurs. Spectral examination of red pigment isolated by Kubo showed absorption bands that are characteristic of deoxy-ferrous, O_2-ferrous, CO-ferrous, and ferric Hbs. Kubo also crystallized hemin from this pigment and showed that crystals are identical to those of hemin from horse Hb. From this evidence Kubo concluded that the red pigment isolated from soybean root nodules was a hemeprotein.

To identify the physiological role of this hemeprotein in nodules, Kubo incubated nodule bacteria with it and observed stimulation of bacterial O_2-consumption. From this observation he concluded that the physiological role of the hemeprotein in nodules is to stimulate the assimilation and transport of O_2. The nodule hemeprotein discovered by Kubo has been characterized in detail by others. Biochemical, biophysical and molecular biology analyses have shown that Kubo's hemeprotein is a plant Hb with similar (*i.e.* structural) properties to animal Hbs (Fig. 1). Kubo's plant Hb was named as leghemoglobin (Lb) by Virtanen and Laine (1946) and is also known as plant symbiotic Hb.

3. The discovery of the function of Lb in root nodules

The role of Lb in N_2-fixing nodules was elucidated by Wittenberg, Bergersen, Appleby and Turner in 1974 (Wittenberg et al., 1974). These authors observed that the addition of Lb to suspensions of bacteroids enhanced the rate of O_2-uptake and nitrogenase activity (measured as the reduction of acetylene to ethylene), and promoted the formation of ethylene with increasing air O_2 concentrations. Ferric Lb, ferric horseradish peroxidase and ferric cytochrome *c* peroxidase were ineffective, however twelve O_2-binding proteins other than Lb also increased O_2-uptake and acetylene reduction by bacteroids. From these observations the authors concluded that oxyLb and other O_2-binding proteins facilitated the diffusion of O_2 to bacteroids in suspension, and that the function of Lb in nodules is to facilitate the diffusion of O_2 to bacteroids at an internal concentration too low to inhibit or destroy their O_2-sensitive nitrogenase. This concept of high O_2-flux at low free O_2 in the bacteroid vicinity is now generally accepted.

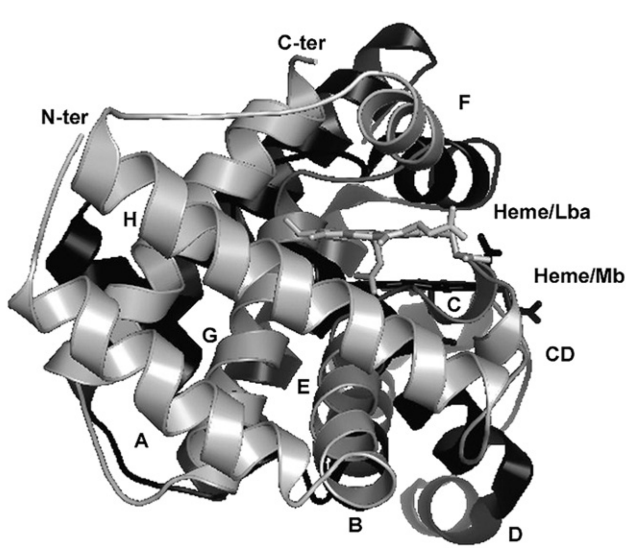

Fig. 1. Tertiary structure of Kubo's nodule hemeprotein (soybean leghemoglobin *a*) (gray) overlaid on the structure of spermwhale myoglobin (Mb) (black). Coordinates for the Lb*a* and Mb were obtained from the Brookhaven Protein Database using the identification numbers **1BIN** and **2MYC**, respectively, and structures were displayed using the PyMol program (http://www.pymol.org/funding.html). Helices (and CD loop in Lb*a*) are shown with the A–H letters.

4. The hypothesis for the origin of Lbs by horizontal gene transfer

For many years Hbs were identified in N_2-fixing legumes, but not elsewhere in the plant kingdom. Early attempts to detect Hbs in non-legumes using biochemical and molecular biology approaches were unsuccessful, so plant Hbs were apparently restricted to N_2-fixing legumes, and thus its origin was a mystery. In 1982 Jeffreys hypothesized that plant Hbs originated by a unique act of horizontal gene transfer (HGT) from a phytophagous insect to a primitive legume *via* a viral vector (Jeffreys, 1982). For this hypothesis to be correct the gene structure of insect *hb* and plant *lb* genes should be identical. Leghemoglobins are encoded by genes that are interrupted by three introns (Jensen et al., 1981; Hyldig-Nielsen et al., 1982). After Jeffreys' HGT hypothesis it was revealed that *hb* gene from the insect *Chironomus* contains no introns (in Appleby et al., 1988), thus the HGT hypothesis for the origin of plant Hbs was discarded, and the vertical evolution hypothesis of plant Hbs was favored. It is now known that other insect *hb* genes do contain introns, some in the exact positions found for legume *lb*s (Kloek et al., 1993; Hankeln et al., 2002; Hoogewijs et al., 2004); even so, other evidence in favor of vertical rather than horizontal evolution of plant *hb* genes soon emerged (see Section 5). An implication of the vertical evolution hypothesis is that Hbs might exist in all (legume and non-legume) land plants.

5. The vertical evolution of plant Hbs: discovery of plant non-symbiotic Hbs

A discovery that supported the vertical evolution hypothesis of plant Hbs was the identification of a Hb in the N_2-fixing root nodules of the non-legume dicot plant *Parasponia* (Appleby et al., 1983). *Parasponia* is a member of the Ulmaceae family, and the only non-legume known to be nodulated by *Rhizobium* strains. *Parasponia* Hb was extracted under anaerobic conditions and final purification was achieved by preparative isoelectric focusing, which produced a single major component of oxyHb (with pI of 6.28), and small amount of ferric Hb (with pI of 6.67). The absorption spectra of (deoxy-ferrous, O_2-ferrous and CO-ferrous) *Parasponia* Hb are similar to those of Lbs and animal Hbs and kinetic constants (O_2-affinity and O_2-association and -dissociation constants) are also similar in *Parasponia* Hb and soybean and other legume Lbs (Gibson et al., 1989). This evidence suggested that the function of Hb in *Parasponia* nodules is similar to function of Lb in legume nodules, *i.e.* to facilitate the diffusion of O_2 to bacteriods.