Structure and function of leghemoglobins*

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Abbreviation: Lb, leghemoglobin.

ABSTRACT

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Leghemoglobin (Lb) is a myoglobin-like protein of about 16 kDa, which occurs in legume root nodules at very high concentrations. Usually the heme moiety is synthesized by the bacteroids but mitochondria may provide also heme for Lb when bacteria are defective in heme production or perhaps when Lb is produced in uninfected cells of nodules. Lb plays an essential role in the nitrogen fixation process, by providing oxygen to the bacteroids at a low, but constant, concentration, which allows for simultaneous bacteroid respiration and nitrogenase activity. Lb must be in the reduced, ferrous state to carry oxygen. Several factors within the nodules are conducive for Lb oxidation to its ferric, inactive form. During these inactivation reactions free radicals are generated. However, healthy nodules contain around 80% of ferrous Lb and 20% of oxyferrous Lb, but not ferric Lb, which indicates that mechanisms exist in the nodules to maintain Lb reduced; these are the enzyme ferric Lb reductase and free flavins. Lb degradation is a largely unknown process, but several intermediates with modified hemes, presumably by oxidative attack, have been encountered, including modified Lba_m, choleglobin, and biliverdin.

INTRODUCTION

Legumes are unique among crop plants in their ability to fix nitrogen in symbiotic association with bacteria, mostly belonging to the genera *Rhizobium* and *Bradyrhizobium*. Nitrogen fixation by bacteroids (symbiotic form of bacteria) provides ammonia, which is incorporated by the plant into important biomolecules, such as proteins, nucleic acids, porphyrins, and alkaloids. In return, the plant furnishes bacteroids with sugars; these sugars are oxidized to organic acids and used as respiratory substrates by the bacteroids. The symbiosis takes place at the level of root nodules, tumor-like structures formed as a result of the infection of roots by the bacteria. Typical legume nodules possess three structurally and physiologically distinct zones called external cortex, internal cortex, and central region (Fig. 1). The external cortex is separated from the internal cortex by a layer of cells called endodermis which may act as a physical barrier to oxygen diffusion (Witty et al. 1986; King et al. 1988). The central region is formed mainly by infected cells containing the bacteroids. Bacteroids are not free in the cytoplasm of infected cells but are confined inside vesicles called symbiosomes. The membrane of these symbiosomes derives in part from the plant cell plasmalemma. Interspersed among the infected cells there are also uninfected cells whose functions, such as the synthesis of the nitrogenous compounds called ureides, are only now beginning to be understood (Aparicio-Tejo et al., 1993).

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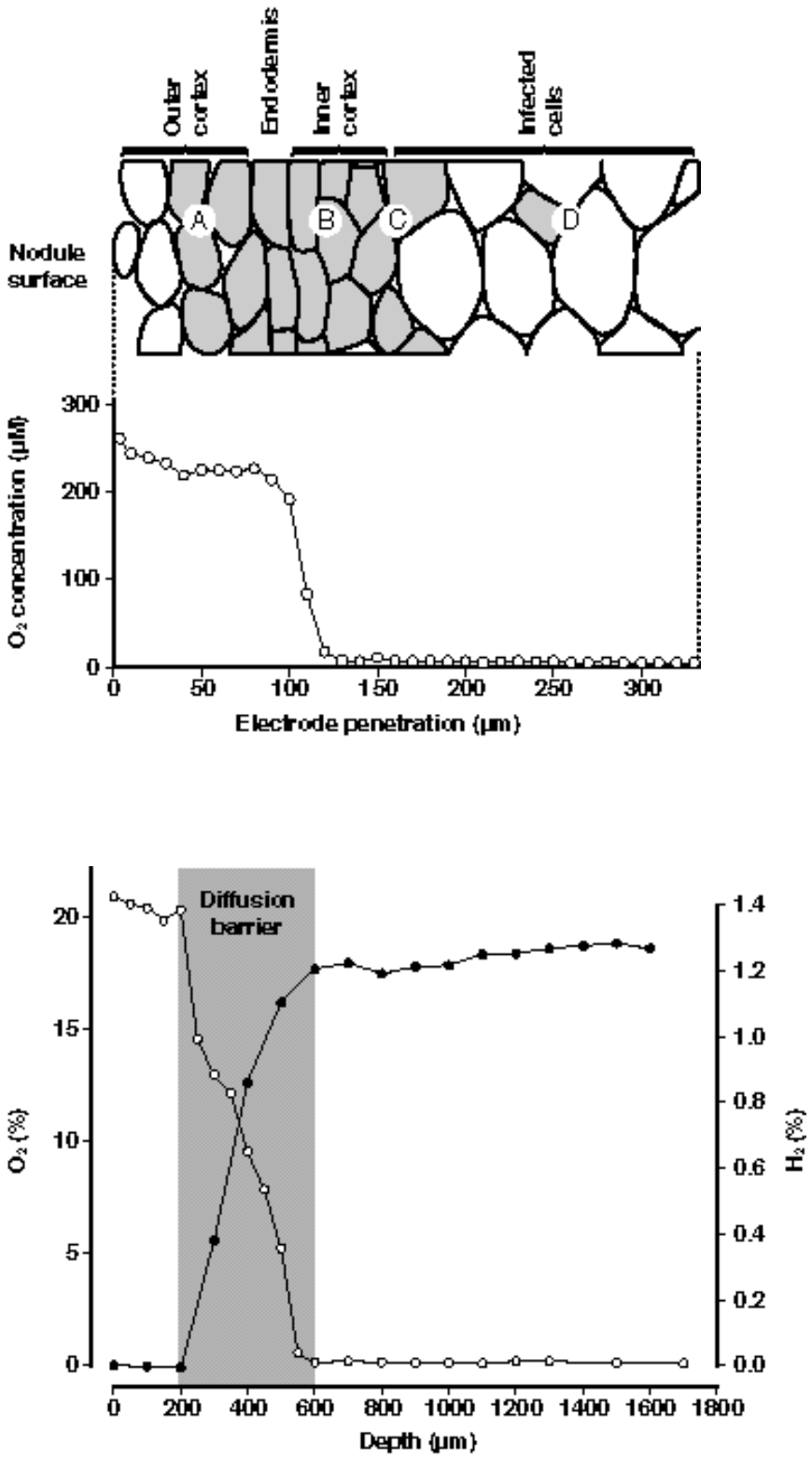

Figure 1. Oxygen diffusion barrier of pea nodules. A marked decrease in oxygen concentration can be observed as the oxygen electrode reaches the central region of nodules. Such a decrease mainly occurs at the level of the endodermis existing between the outer and the inner cortex. After Witty et al. (1986).

SYNTHESIS AND STRUCTURE OF LEGHEMOGLOBIN

To fix nitrogen, legume nodules require a protein called leghemoglobin (Lb). As early as in 1939, the japanese scientist Kubo discovered that soybean nodules contained a red pigment and demonstrated that it was a hemoprotein. Soon this finding was extended to other legume nodules and Lb was found to be constituted by a heme group (protoporphyrin IX) and a single polypeptide (globin) having a molecular mass of 16 kDa (Fig. 2). Under physiological conditions the heme group is synthesized

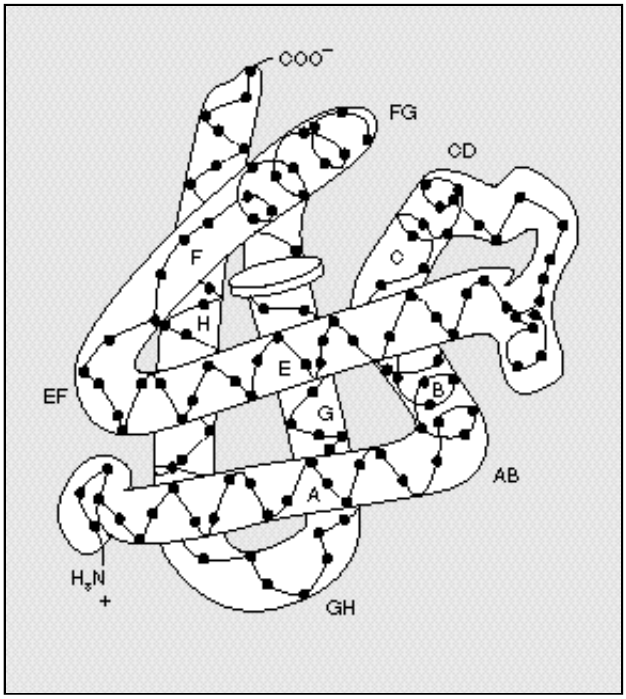

Figure 2. Tertiary structure of lupin Lb at high resolution. Note the localization of the heme and the eight α-helix segments (A, B, ..H) as well as the five non-helix segments (AB, CD,...GH) that are found between the others. The fifth coordination position of Fe (above the heme plane) is occupied by a histidine and the sixth position (below the heme plane) is empty (ferrous Lb) or occupied by a molecule of oxygen (oxygenated ferrous Lb) or water (ferric Lb).

by the bacteroids and the globin by the ribosomes of the infected cells; then, heme and globin are assembled in the plant cell cytoplasm. The synthesis of the heme group is stimulated by the low oxygen concentration existing in the central region of nodules, but this low concentration is not a prerequisite for Lb synthesis. Thus, soybean plants infected with mutant strains of *Bradyrhizobium japonicum* which do not express the *hemA* gene and, consequently, which are deficient in the enzyme δ-aminolevulinic synthase, are still capable of synthesizing Lb (Guerinot and Chelm, 1986). Recently, Sangwan and O'Brian (1991) provided an answer to this question. They found that, in these mutant nodules, the plant supplied δ-aminolevulinic acid to the bacteroids, where the heme was synthesized; the bacteroids then gave the heme back to the plant to form Lb. It was thus shown for the first time that two symbionts were cooperating in the same metabolic pathway, the synthesis of heme. Another series of studies (VandenBosch and Newcomb, 1988) support the possibility that the plant cells themselves, and not the bacteroids, supply the heme; in this case, the heme is presumably synthesized by the mitochondria.

The amino acid sequence of the globin moiety of Lb depends on the legume species. Even within the same species, several isoproteins or Lb components are usually found which differ from each other by a few amino acids. These isoproteins can be isolated by using anion-exchange column chromatography and isoelectrofocusing (Appleby and Bergersen, 1980; Jun et al., 1994a). All the isoproteins appear to have identical oxygen affinity but the fact that their relative proportions vary with nodule aging strongly suggests that the heterogeneity of Lb isoproteins has a physiological significance yet to be discovered (Dilworth and Appleby, 1979; Jun et al.,1994b). On the other hand, the heme moiety is always identical in all Lb molecules and is present, *in vivo*, in the ferrous state. Nevertheless, when Lb is extracted from nodules, a small amount of Lb is inevitably oxidized to the ferric state. This was the reason why some workers erroneously implicated Lb in the transport of the electrons required for reducing nitrogen to ammonia.

FUNCTION OF LEGHEMOGLOBIN

In 1958 Bergersen and Appleby (Appleby, 1984) demonstrated that the ferrous form of Lb combined reversibly with oxygen in the cytoplasm of nodule infected cells. They proposed that the protein carries the oxygen required for bacteroid respiration and, consequently, for maintaining active nitrogen fixation. In 1974 this hypothesis was confirmed (Appleby, 1984), thus solving the so-called "oxygen paradox". According to this paradox, nitrogenase, the enzymatic complex responsible for nitrogen fixation, is rapidly inactivated by oxygen but, at the same time, oxygen is needed for the bacteroids to obtain energy to reduce nitrogen to ammonia. Indeed, this apparent contradiction was explained by the functioning of Lb, which transports oxygen at a low but stable concentration allowing for the simultaneous operation of nitrogenase activity and bacteroid respiration. For this transport (called "facilitative diffusion") to occur in the nodule cells, Lb should exhibit a very high affinity for oxygen and has to very abundant. Likewise, bacteroids should incorporate and consume rapidly the oxygen bound to the hemoprotein.

The facilitative diffusion of oxygen by Lb takes place as follows (Becana and Klucas, 1992a). The ferrous form of Lb has a very high affinity for oxygen. Thus, a 48 nM concentration of free oxygen is sufficient to cause 50%

oxygenation of Lb. For this oxygenated Lb to be active and release oxygen to the respiratory chain of bacteroids, the oxygen affinity of the bacteroid oxidase should exceed (about 10-fold) the oxygen affinity of Lb. Thus, Lb is oxygenated at the plasmalemma level and carries the oxygen to the symbiosome membrane. Because there is no Lb in the peribacteroid space (Robertson et al., 1984), oxygen has to be released from Lb at the level of the symbiosome membrane. Such a release occurs because of the deep oxygen gradient existing between the symbiosome membrane and the bacteroids, which respire oxygen at high rates.

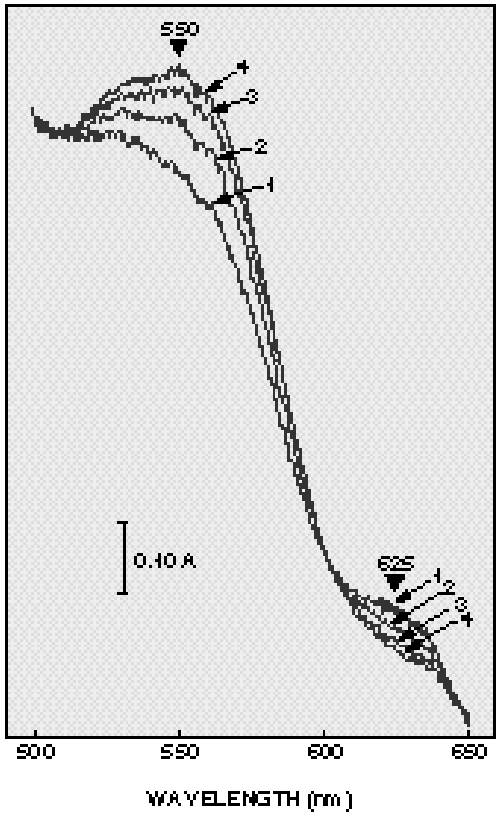

Figure 3. Conversion of ferric Lb into ferrous Lb as measured by direct spectrophotometry of nodule slices. The slice was treated with hydroxylamine and scanned at 5 min (line 1), 10 min (line 2), 20 min (line 3), and 35 min (line 4).

The proportion of oxygenated Lb in the nodules can be estimated by spectroscopy (Fig. 3). Deoxygenated ferrous Lb displays absorption maxima at 427 and 555 nm, and oxygenated ferrous Lb at 411, 541 and 575 nm, whereas ferric Lb exhibits a characteristic peak at 625 nm (Dilworth and Appleby, 1979). Other spectrophotometric techniques, which have been employed to obtain spectra of nodule slices or even of intact nodules attached to the roots, have established that in young nodules there is only ferrous Lb (oxygenated or not) and that in senescent nodules there is also a small amount of ferric Lb (Klucas et al., 1985). On the basis of these spectra the proportion of oxygenated Lb has been assessed in the range of 20-30% for a typical soybean nodule of around 3 mm in diameter (Klucas et al., 1985), but the fractional oxygenation of Lb may vary with nodule age and legume species (King et al., 1988; Monroe et al., 1989; Denison and Layzell, 1991). From this information, along with that obtained from equilibrium reactions, it can be calculated that the concentration of oxygen ligated to the ferrous form is 50,000-fold that of free oxygen and, therefore, that nearly all oxygen required by bacteroids is transported in the form of oxygenated Lb (Becana and Klucas, 1992a).

FUNCTIONAL INACTIVATION OF LEGHEMOGLOBIN

The ferrous form of Lb readily autoxidizes and is converted into the ferric form under the slightly acid conditions of nodules (Puppo et al., 1981). Reactions that inactivate Lb quite often involve free radicals and other activated oxygen species such as hydrogen peroxide (Aviram et al., 1978; Puppo et al., 1981). How is damaging activated oxygen generated? In nodules the superoxide radical and hydrogen peroxide originate from Lb autoxidation and from mitochondrial and bacteroid respiration (Puppo et al., 1981; Becana and Klucas, 1992a). Hydrogen peroxide is not a very reactive metabolite but can act as a cell oxidant because it crosses membranes and gives rise, in the presence of some transition metal ions, to the extremely toxic hydroxyl radical. The hydroxyl radical can be also generated from the attack by hydrogen peroxide on oxygenated Lb (Puppo and Halliwell, 1988). In this case, the heme group is broken down and ferrous iron is released, which in turn reduces hydrogen peroxide to hydroxyl radicals. These radicals are highly reactive, have a lifetime of only 1 ns, and thereby can oxidize nearly all types of molecules provided they are close enough to the location where the radicals are formed. Amongst the biomolecules more prone to oxidative attack by hydroxyl radical are DNA, proteins, and polyunsaturated fatty acids of membranes (Becana and Klucas, 1992b). However, nodules, like animal tissues and other plant tissues, are endowed with various protective mechanisms ("antioxidants") against free radicals and hydrogen peroxide, including superoxide dismutase, catalase, and peroxidases (Dalton et al., 1986, 1993). There is no an scavenging enzyme for hydroxyl radical because it is far too reactive, but the above-indicated antioxidant enzymes can prevent its formation by destroying the superoxide radical and hydrogen peroxide.

MECHANISMS FOR MAINTAINING LEGHEMOGLOBIN ACTIVE

Because of the ease of Lb autoxidation and because of the lack of ferric Lb in healthy nodules, it can be postulated that mechanisms exist in the nodules to maintain Lb in the active, reduced state. This hypothesis was demonstrated by Klucas et al. (1985) using spectrophotometric techniques (Fig. 4). Soybean nodule slices were treated with hydroxylamine to oxidize virtually all Lb within the cells to the ferric form. After washing out hydroxylamine excess, ferric Lb was reduced quickly to ferrous Lb within the infected cells. Several mechanisms can be responsible for this reduction. One of them involves an enzyme, ferric Lb reductase, which we purified from soybean nodules (Ji et al., 1991) This enzyme is different from the partially-characterized enzyme from lupine nodules (Golubeva et al., 1988). It is a flavoprotein of about 100 kDa, consisting of two identical subunits. Subsequently to this discovery, we demonstrated that ferric Lb can also be reduced by free flavins in the presence of NADH or NADPH. This flavin-mediated mechanism, which preserves the activity of Lb and thereby of the whole nitrogen-fixing process, is operative under the extremely low oxygen conditions of nodules (Becana and Klucas, 1990).

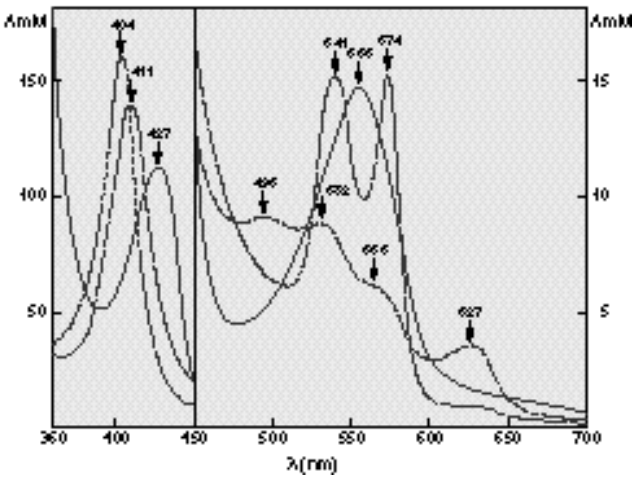

Figure 4. Spectra of soybean Lb in 10 mM K-phosphate buffer (pH 6.4). Ferric Lb (– – –), ferrous Lb (———), oxygenated ferrous Lb (·····).

DEGRADATION OF LEGHEMOGLOBIN

Despite the physiological importance of Lb, little is known about its pathway of degradation. It has been demonstrated that Lb is degraded during aging or stress-induced senescence of nodules (Rigaud and Puppo, 1977; Swaraj et al., 1986; Kanayama and Yamamoto, 1990). This degradation probably requires previous heme oxidation, which would be promoted by free radicals (Becana and Klucas, 1992b). In this respect, several interesting derivatives of soybean Lb isoprotein *a* (called Lba_m) have been isolated which have an intact globin moiety and a modified heme group (Jun et al., 1994a, b).

Metabolic degradation of the Lb apoprotein involves several proteases with high affinity for Lb; these proteases are abundant in nodules, particularly in those entering senescence (Pladys et al., 1988). Degradation of Lb may yield two important pigments, choleglobin and biliverdin (Lehtovaara and Pertilä, 1978). Choleglobin is a Lb derivative having an oxidized heme group but with the iron still attached to it. In biliverdin, however, iron has been lost. These pigments, especially biliverdin, accumulate in senescing nodules. The onset of pigment formation results in a color change of nodules from red to green, a process that can be useful as a marker for Lb inactivation and, therefore, for the loss of nitrogen-fixing activity of leguminous plants.

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