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Sabarinathan K. Gopalasubramaniam
Laboratorio de Biofísica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Morelos, México

Frank A. Kovacs
University of Nebraska at Kearney, kovacsfa@unk.edu

Fernando Violante-Mota
Laboratorio de Biofísica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Morelos, México

Paul Twigg
University of Nebraska at Kearney, twiggp@unk.edu

Raul Arredondo-Peter
Laboratorio de Biofísica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Morelos, México

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Cloning and characterization of a caesalpinoid (*Chamaecrista fasciculata*) hemoglobin: The structural transition from a nonsymbiotic hemoglobin to a leghemoglobin

Sabarinathan K. Gopalasubramaniam,[1] Frank Kovacs,[2] Fernando Violante-Mota,[1] Paul Twigg,[3] Raúl Arredondo-Peter,[1]* and Gautam Sarath[4,5]

[1] Laboratorio de Biofísica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Morelos, México

[2] Department of Chemistry, University of Nebraska-Kearney, Kearney, Nebraska 66849

[3] Department of Biology, University of Nebraska-Kearney, Kearney, Nebraska 66849

[4] Grain, Forage and Bioenergy Research Unit, USDA-ARS, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0937

[5] Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0937

ABSTRACT

Nonsymbiotic hemoglobins (nsHbs) and leghemoglobins (Lbs) are plant proteins that can reversibly bind O_2 and other ligands. The nsHbs are hexacoordinate and appear to modulate cellular concentrations of NO and maintain energy levels under hypoxic conditions. The Lbs are pentacoordinate and facilitate the diffusion of O_2 to symbiotic bacteroids within legume root nodules. Multiple lines of evidence suggest that all plant Hbs evolved from a common ancestor and that Lbs originated from nsHbs. However, little is known about the structural intermediates that occurred during the evolution of pentacoordinate Lbs from hexacoordinate nsHbs. We have cloned and characterized a Hb (ppHb) from the root nodules of the ancient caesalpinoid legume* Chamaecrista fasciculata. *Protein sequence, modeling data, and spectral analysis indicated that the properties of ppHb are intermediate between that of nsHb and Lb, suggesting that ppHb resembles a putative ancestral Lb. Predicted structural changes that appear to have occurred during the nsHb to Lb transition were a compaction of the CD-loop and decreased mobility of the distal His inhibiting its ability to coordinate directly with the heme-Fe, leading to a pentacoordinate protein. Other predicted changes include shortening of the N- and C-termini, compaction of the protein into a globular structure, disappearance of positive charges outside the heme pocket and appearance of negative charges in an area located between the N- and C-termini. A major consequence for some of these changes appears to be the decrease in O_2-affinity of ancestral nsHb, which resulted in the origin of the symbiotic function of Lbs.

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Key words: Caesalpinoideae; evolution; function; globin; Leguminosae; modeling; origin; spectroscopy; structure; symbiosis.

INTRODUCTION

Nonsymbiotic and symbiotic hemoglobins (Hbs) are O_2-binding proteins that have been identified in plants. Symbiotic Hbs (or leghemoglobins (Lbs) when isolated from legumes) are specifically synthesized in the nodules of N_2-fixing plants. A major function for Lbs is to facilitate the diffusion of O_2 to the actively respiring bacteroids during N_2-fixation.[1,2] Nonsymbiotic Hbs (nsHbs) are synthesized in diverse organs from plants growing in normal and stressed conditions (reviewed in Ross *et al.*[3]).

Nonsymbiotic Hbs are classified into class-1 and class-2 (nsHb-1 and nsHb-2, respectively).[4] The O_2-affinitiy of nsHb-1 and nsHb-2 is very high and high, respectively.[4–6] The very high O_2-affinity of nsHb-1 results from an extremely low O_2-dissociation rate constant, thus it has been proposed that major functions for nsHbs-1 in plant cells are other than O_2-transport, such as to modulate the levels of NO and maintain the energy *status* and redox potential.[7–10] In contrast, the O_2-dissociation rate constant for nsHbs-2 is higher than that of nsHbs-1, thus a likely function for nsHbs-2 is O_2-transport.[4,7]

For many years Hbs were only identified in N_2-fixing plants; however, during the last few decades Hbs have been detected in numerous (non-N_2-fixing) land plants, ranging

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*Correspondence to: Raúl Arredondo-Peter, Lab. de Biofísica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, Col. Chamilpa, 62210 Cuernavaca, Morelos, México.
E-mail: normanac@terra.com.mx.
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from primitive bryophytes to evolved monocots and dicots (reviewed in Garrocho-Villegas *et al.*[11]), indicating their widespread occurrence in land plants. Also, sequence comparison showed that the *hb* gene structure is identical in primitive and evolved nsHbs and Lbs, that is, *nshb* and *lb* genes are interrupted by three introns located at identical positions. This evidence suggests that nsHbs and Lbs evolved from a common ancestor.[11,12]

The detection of nsHb with both symbiotic and non-symbiotic specificities in N_2-fixing plants suggests that symbiotic Hbs and Lbs originated from *nshb* genes.[13–17] Although it is not yet certain that Hb is essential for N_2-fixation in nonlegume plants, such as in *Parasponia andersonii* and actinorhizal plants, Lb is essential for N_2-fixation in nodulating legumes.[18] Thus, a specialization from nsHb to Lb apparently occurred during the evolution of N_2-fixing legumes. Hoy *et al.*[19] analyzed the crystal structure of barley and rice nsHb-1 and soybean Lb*a* and proposed that functional (O_2-transporting) Lbs originated after the stabilization of an open pentacoordinate conformation of nsHbs. However, the structural changes that occurred during the nsHb to Lb transition are largely not known. Also, nsHbs or Lbs that resemble a putative ancestor of Lbs have not yet been identified. This information is of interest to complement our understanding about the evolution of plant Hbs.

The Caesalpinoideae is the oldest subfamily in the Leguminosae family[20,21] and contains non-nodulating and nodulating species.[22,23] Thus, the evolution of a nsHb into a functional Lb probably occurred in a caesalpinoid legume. Here we report the characterization of a Hb (ppHb) from the nodulating caesalpinoid *Chamaecrista fasciculata* (Partridge pea), and describe a number of structural changes that probably occurred during the evolution of its symbiotic function.

METHODS

Plant growth and root nodules harvest

Seeds of Partridge pea (*Chamaecrista fasciculata* (Michx). Green) were obtained from plants grown in a field near Mead, NE. Seeds were scarified by gentle tumbling with coarse sand for 5 min. Scarified seeds were mixed with a rhizobial commercial inoculum (Cowpea type, *Bradyrhizobium* sp. (Vigna), Royal Peat, Becker Underwood, Ames, IA) and planted in 10 inch clay pots in sterilized sand and raised in a greenhouse under natural light with ~32°C day and ~28°C night. After germination, plants were thinned to three seedlings/pot and watered twice weekly with a N_2-free nutrient solution.[24] Plants were otherwise watered with distilled water as needed. Root nodules were harvested from both young and mature (<2 and >5 week old plants, respectively) plants, frozen in liquid N_2, and stored at −80°C until used.

Table I
Sequence Similarity and Identity Between ppHb and Selected nsHbs and Lbs

Plant Hb	Similarity (%)	Identity (%)
Nonsymbiotic Hbs		
Soybean nsHb1	83	68
Parasponia andersonii nsHb1	82	66
Trema orientalis nsHb1	82	67
Tomato nsHb1	78	64
Barley nsHb1	78	57
Rice nsHb1	77	56
Ceratodon purpureus nsHb	71	46
Physcomitrella patens nsHb	69	44
Cotton nsHb2	68	53
Tomato nsHb2	63	48
Arabidopsis nsHb2	62	45
Symbiotic Hbs		
Sesbania rostrata Lb	62	46
Soybean Lba	59	42
Cowpea LbII	59	40
Yellow Lupin Lb	59	42

Sequences of plant Hbs were obtained from the GenBank database (with the accession numbers reported by Garrocho-Villegas *et al.*[11]) and aligned by pairwise with ppHb using the BLAST program. Similarity values show amino acid position with identical polarity (negative, positive, or nonpolar) in aligned sequences. Identity values show identical amino acids in aligned sequences.

cDNA library construction

Library construction was essentially according to Tobias *et al.*[25] Briefly, total RNA was extracted from frozen root nodules using the Concert Plant RNA reagent (Invitrogen). Messenger RNA was purified using the FastTrack 2.0 mRNA isolation system (Invitrogen). First strand cDNA synthesis was primed with a *Not*I-oligo(dT) adapter primer followed by second strand synthesis using the Superscript Plasmid System (Invitrogen). The resulting cDNAs were ligated to *Sal*I adapters, digested with *Not*I and directionally cloned into the pSPORT1 cloning vector (Invitrogen). Plasmids containing cDNA inserts were transformed into Ultramax DH5αFT chemically competent *Escherichia coli* (Invitrogen). One hundred individual clones were randomly isolated and insert DNA was partially sequenced at the University of Nebraska-Lincoln Genomics Core Facility. A total of five clones containing cDNA sharing homology to known plant Hbs were selected and fully sequenced in both directions to assemble the full length cDNA and deduce the amino acid sequence of the Partridge pea Hb (ppHb).

Sequence analysis and prediction of the ppHb structure

Multiple sequence alignment and cluster analysis of ppHb and selected plant Hbs were performed by using the Neighbor Joining Method of the Clustal X program.[26] Sequence alignment was manually verified. Sequence similarity and identity values between ppHb and individual plant Hbs were obtained from pairwise

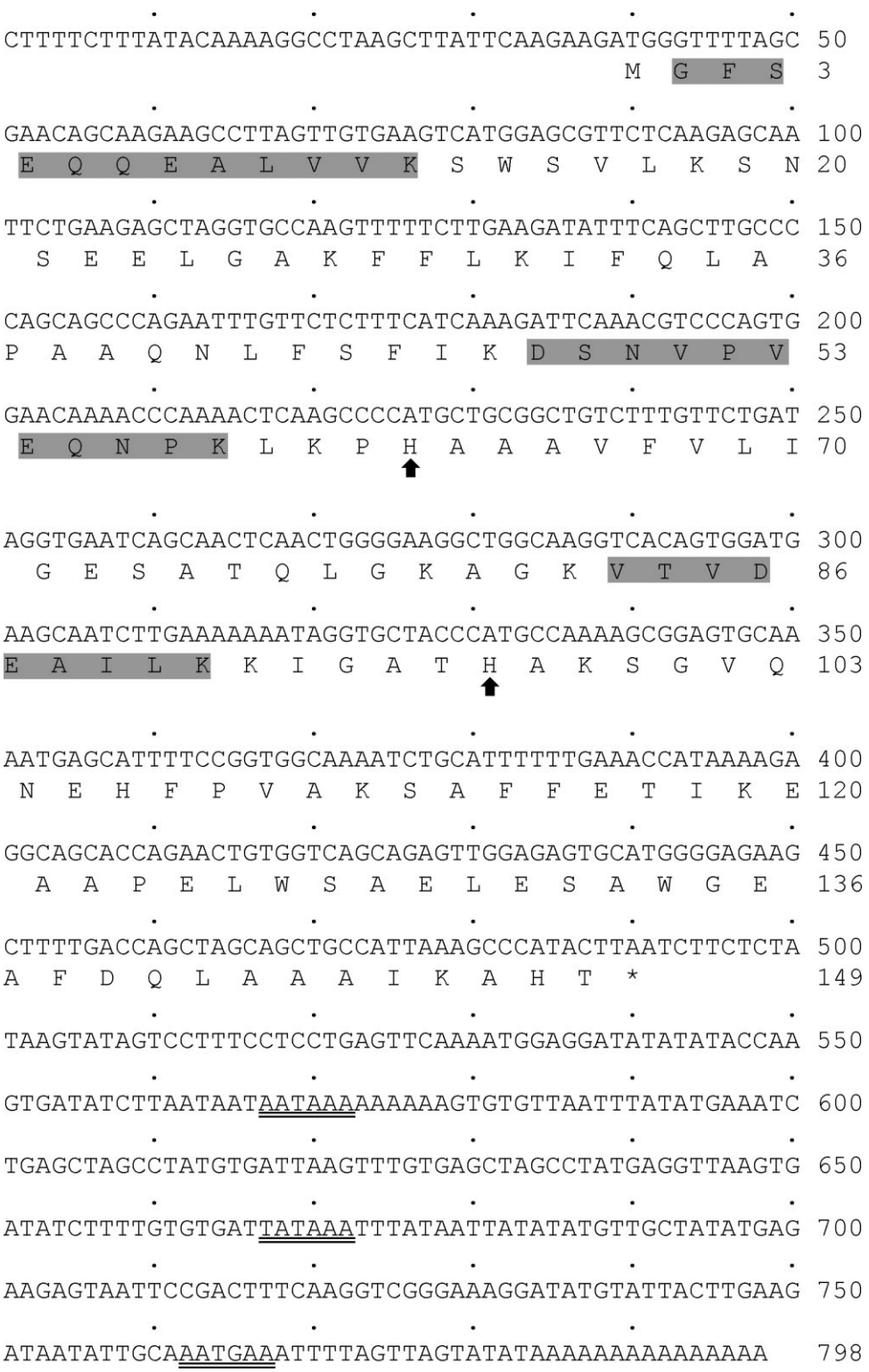

Figure 1

Nucleotide and deduced protein sequences of cDNA clone 11 that codes for C. fasciculata *Hb (ppHb). Arrows show distal and proximal His at positions 62 and 97, respectively. Putative polyadenylation signals are double-underlined. Peptides arising from native ppHb and identified by mass spectrometry are shaded in gray. The ppHb cDNA sequence is deposited in the GenBank database under the accession number EF534200.*

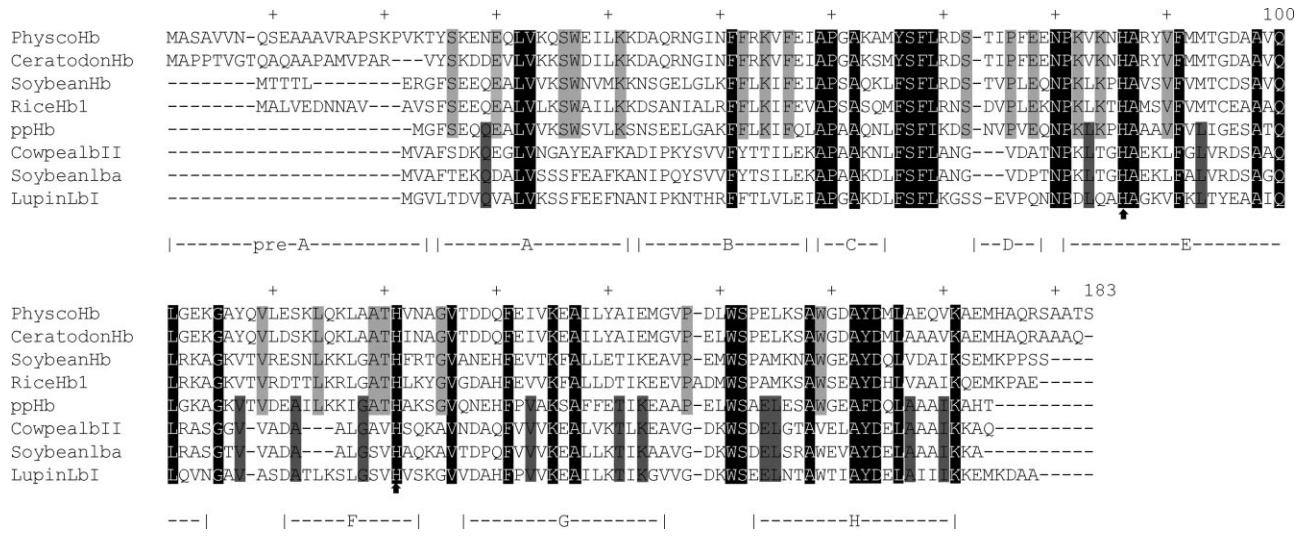

Figure 2

Sequence alignment of ppHb and selected plant nsHbs and Lbs. Arrows show distal and proximal His at positions 86 and 121, respectively. Conserved amino acids in all aligned sequences are shown with black background. Amino acids conserved in aligned ppHb and nsHbs and Lbs are shown with light- and dark-gray background, respectively. Helices are shown with the A to H letters, and were designated (including to prehelix A) according to the position of helices in rice Hb1.[33] Sequences were obtained from the GenBank database using the following accession numbers: Physcomitrella (Physco) Hb: AF218049; Ceratodon Hb: AF309562; soybean Hb: U47143; rice Hb1: U76029; cowpea LbII: U33207; soybean Lba: V00453; lupin LbI: Y00401.

sequence alignments using the BLAST program[27] from the GenBank database (http://www.ncbi.nlm.nih.gov). The tertiary structure of ppHb was predicted by homology modeling[28] using the crystal structure of rice (nonsymbiotic) Hb1 (PDB ID 1D8U) as template. The ppHb and rice Hb1 amino acid sequences (GenBank accession numbers EF534200 and U76030, respectively) were aligned using the Homology module of the Insight II program (Accelrys), and amino acids were automatically substituted. The best rotamer for all side chains was searched automatically using the default parameters of the Homology module. The energy of the whole structure was minimized (100 steps of the steepest descent minimization) using the Discovery force field from Insight II, and the best rotamers for all amino acid side chains were searched again. The refined ppHb model was analyzed using routines available from the SwissPDB-Viewer program (http://ca.expasy.org/spdbv/) (below). Images were edited using the VMD program.[29]

Expression and purification of recombinant ppHb

A cDNA coding for ppHb was subcloned into the expression vector pET28a (Novagen) and transformed into *E. coli* following the manufacturer's instructions. Recombinant ppHb was purified to homogeneity by Nickel-interaction chromatography, followed by ion-exchange chromatography. Protein purification was verified by SDS-PAGE.[30] Total protein was quantitated by using a dye-binding assay (Bio-Rad) and bovine serum albumin as standard, and the recombinant ppHb was quantified on the heme basis using the dipyridine-hemochrome assay.[31]

Spectroscopic analysis of recombinant ppHb

Purified recombinant ppHb was characterized spectrophotometrically using a GBC UV/Vis911A spectrophotometer interfaced to a computer. Ferrous ppHb was oxidized to ferric ppHb by the addition of potassium ferricyanide (final concentration of 200 μM) in 50 mM sodium phosphate buffer (pH 6), and then chromatographed on a PD-10 column (Amersham-Pharmacia) equilibrated with 50 mM phosphate buffer at pH 7. Ferrous ppHb was formed by the addition of few crystals of sodium dithionite (Fluka). Air was bubbled through the ppHb solution to generate the O_2-ligated form of ppHb.

RESULTS AND DISCUSSION

Cloning of a cDNA coding for a caesalpinoid (*C. fasciculata*) Hb

With the exception of a partial characterization of a mimosoid Lb,[32] no work has been done on Lbs other than papilionoid Lbs. Little is known about the properties of Hbs/Lbs from caesalpinoid and mimosoid species, which are ancestral legumes. The analysis of caesalpinoid Hbs/Lbs is of interest because the Caesalpinoideae is the

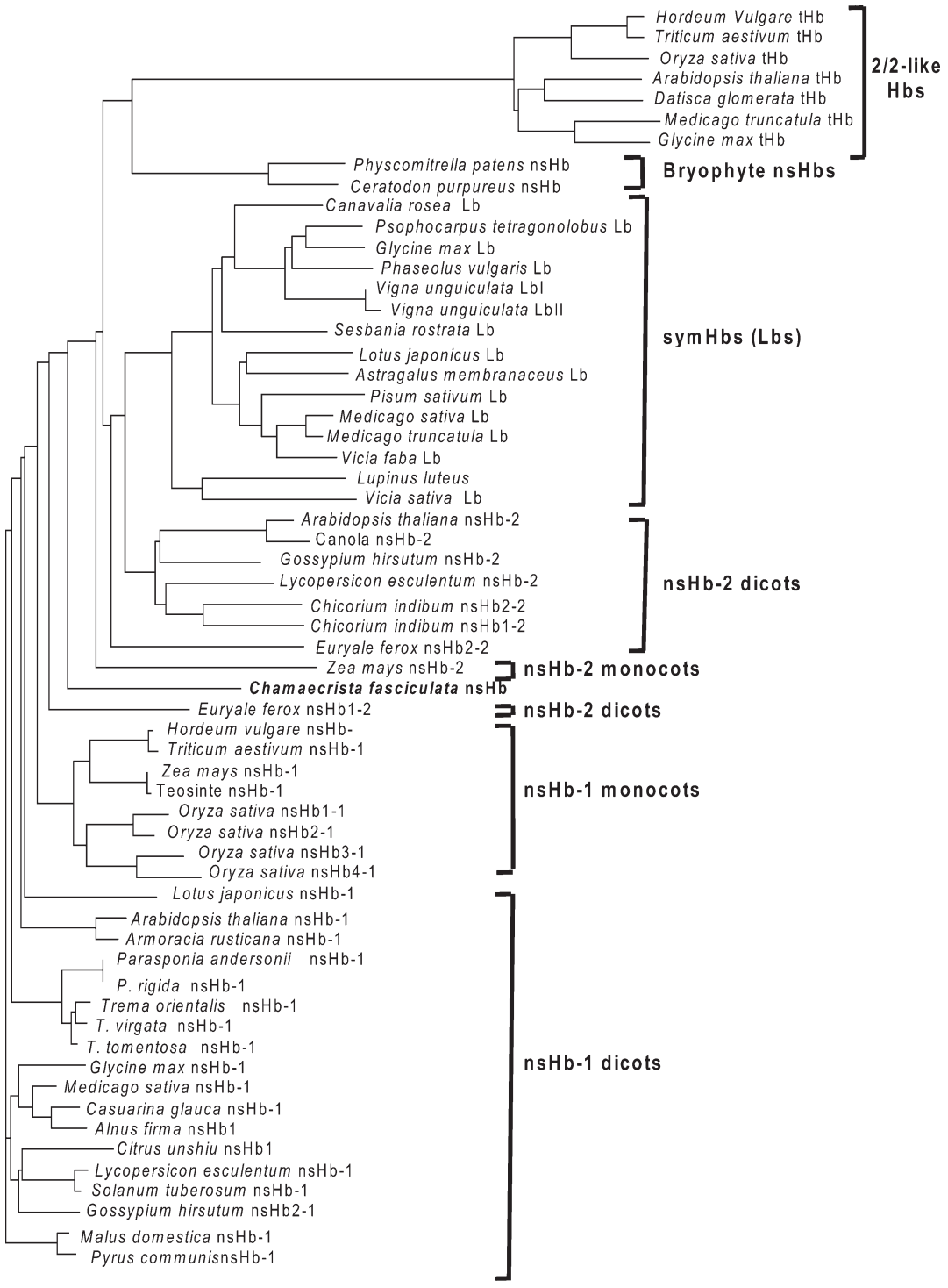

Figure 3

Phenetic relationships between ppHb and selected plant (nonsymbiotic and symbiotic) Hbs. The phenogram was constructed from sequences reported by Garrocho-Villegas et al.[11]

oldest subfamily of the Leguminosae family,[20,21] thus it is probable that a functional (O_2-transporting) Lb originated within a caesalpinoid legume. We isolated one clone (clone 11) from a cDNA library constructed from the caesalpinoid *Chamaecrista fasciculata* (Partridge pea) root nodules. Partial sequencing of 100 cDNA clones

showed that clone 11 is highly abundant (it represents 14% of the sequenced clones), and full sequencing and sequence comparison with sequences deposited in the GenBank database showed that clone 11 is similar to plant Hb sequences (Table I). Predicted protein sequence from clone 11 contains the highly conserved amino acid residues of plant and nonplant Hbs (below). Thus, clone 11 codes for a plant (caesalpinoid) Hb and was named as (Partridge pea) ppHb. The ppHb cDNA clone is 798 bp in length, contains 38 and 407 bp at the 5′-(upstream) and 3′-(downstream) noncoding regions, respectively, and putative polyadenylation signals located at positions 567, 667, and 762, and codes for a predicted polypeptide 149 amino acids in length (see Fig. 1) with a calculated molecular weight of 16,196 Da. Predicted ppHb contains the highly conserved distal and proximal His at positions 62 and 97, respectively.

Because of the high abundance of ppHb cDNA (above) we concluded that ppHb is the major Hb in the *C. fasciculata* root nodules. This was verified by isolating and *de novo* sequencing of the major *C. fasciculata* nodule Hb by gel electrophoresis followed by mass spectrometry. The sequences obtained for several peptides were identical to that of the predicted ppHb protein (highlighted on the amino acid sequence shown in Fig. 1).

Phenetic relationship of ppHb with Plant Hbs

Sequence comparison of the ppHb protein with sequences deposited in databases showed that the ppHb similarity and identity values are higher to plant nsHbs than to Lbs (Table I). This observation showed that ppHb is a nonsymbiotic or nonsymbiotic-like Hb rather than a Lb. Thus, because transcripts coding for ppHb were abundant in the screened library and ppHb is the major nodule Hb (above), it is likely that nonsymbiotic (-like) ppHb, but not Lb, is the functional Hb in the *C. fasciculata* root nodules. Sequence alignment of ppHb with selected plant nsHbs and Lbs showed that highly conserved amino acid residues in nsHbs and Lbs are also conserved in ppHb, and that amino acids conserved in either nsHbs or Lbs are also conserved in ppHb (see Fig. 2). Phenetic analysis showed that ppHb clusters intermediate between nsHbs-1 and nsHbs-2 and Lbs (see Fig. 3). These observations show that ppHb is intermediate between nsHbs and Lbs, and suggest that it is an evolutionary transition from nsHbs to Lbs. Thus, to learn about the properties of ppHb, we performed structural analyses by predicting the structure of ppHb and obtaining the UV/visible spectra of a recombinant ppHb.

Characteristics of the predicted ppHb structure

The tertiary structure of proteins can be predicted with high reliability using *in silico* methods and template structures (i.e., those experimentally elucidated by X-ray crystallography and/or NMR techniques) when the homology between two proteins is ≥30%.[28,34–36] We predicted the tertiary structure of ppHb using rice (nonsymbiotic) Hb1 as a template since ppHb is more similar to nsHbs-1 (∼60%–80%) than to Lbs (∼60%) (Table I). However, to identify variations due to template homologs the structure of ppHb was also predicted using soybean

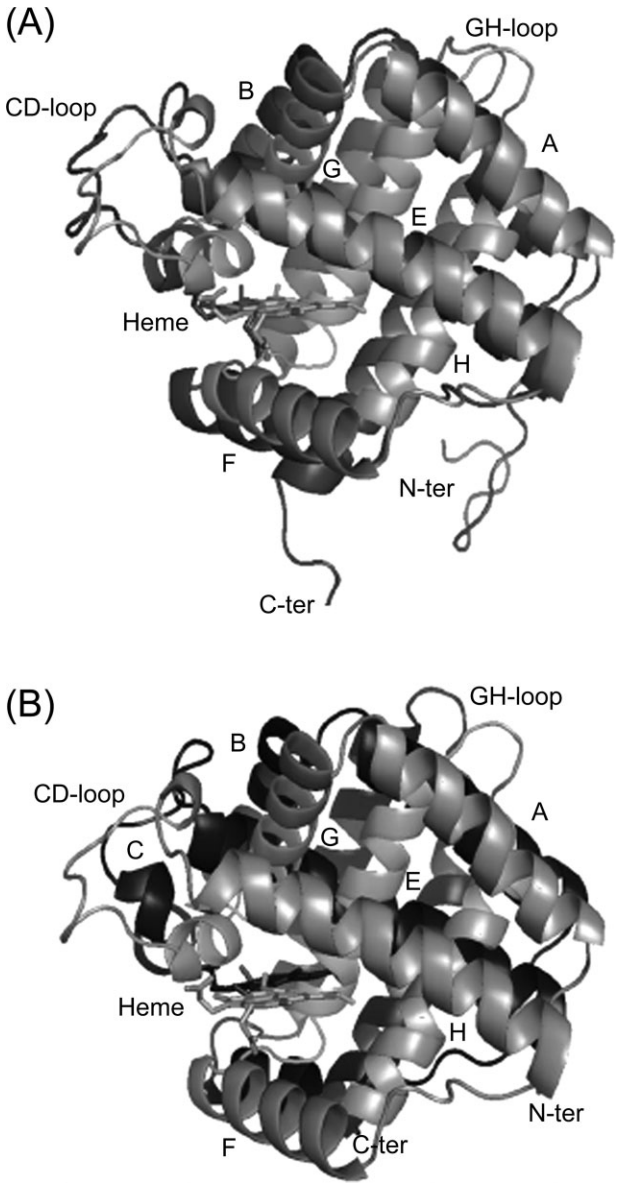

Figure 4

*Overlay of the predicted structure of ppHb (gray) to the native structure of rice Hb1 (**A**) and soybean Lba (**B**) (black). Helices are indicated with letters A–H. Coordinates for the rice Hb1 and soybean Lba structures were obtained from the PDB database with the ID numbers 1D8U and 1BIN, respectively. For experimental details see the Materials and Methods section. The predicted structure of ppHb is deposited in the Protein Model Database (http://mi.caspur.it/PMDB/) under the ID number PM0075011.*

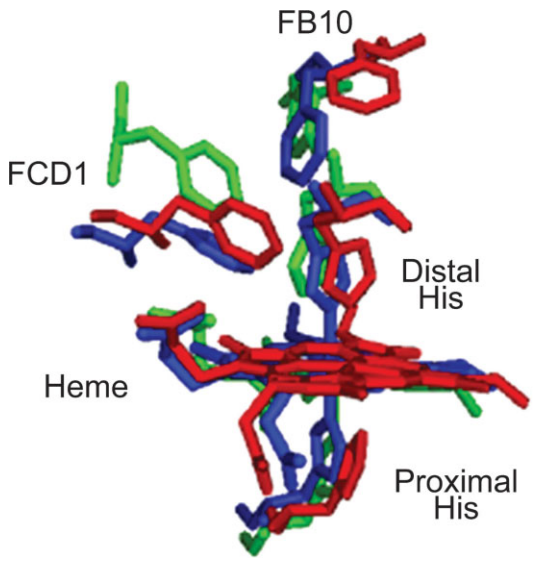

Figure 5

Comparison of the position and orientation of selected amino acids in the ppHb (red), rice Hb1(blue), and soybean Lba (green) heme pocket.

Lb*a* (PDB ID 1BIN) as template. The resulting model of predicted ppHb was identical to that predicted from template rice Hb1 (not shown), thus no variations were identified for the ppHb structure predicted from either rice Hb1 or soybean Lb*a*. Figure 4 shows that predicted ppHb folds into the globin fold and that its structure is more similar to the structure of rice Hb1 than to that of soybean Lb*a*. With the exception of the N- and C-termini, the structure of predicted ppHb is quite similar to the structure of rice Hb1, including the positions of helices E and F, where the distal and proximal His are located, respectively [Fig. 4(A)]. However, a number of differences were identified when the structures of predicted ppHb and soybean Lb*a* were compared [Fig. 4(B)]. Specifically, major differences between ppHb and soybean Lb*a* exist at the CD-loop/helices B and C, GH-loop and position of the heme prosthetic group. A close examination of the amino acids that are essential for binding of ligands to the heme-Fe showed that in ppHb the position of distal His and Phe B10 and CD1 is intermediate between rice Hb1 and soybean Lb*a* (see Fig. 5). An interesting observation is that the distance of proximal His in predicted ppHb is ~3.6 Å farther from the heme-Fe than in rice Hb1 and soybean Lb*a*.

Spectroscopic properties of recombinant ppHb

Spectral analysis showed that the absorption maxima of ferrous and ferric recombinant ppHb are located at 431 and 557 nm and 404, 542, and 633 nm, respectively, similarly to those of soybean Lb*a*. Also, the absorption maxima of ferrous oxygenated recombinant ppHb were located at 413, 540, and 575 nm, similarly to those of oxygenated soybean Lb*a* and rice Hb1 (Table II). This evidence indicates that recombinant ppHb is pentacoordinate and that it binds O_2. Modeling analysis predicted that the position of distal His in ppHb is intermediate between rice Hb1 and soybean Lb*a* (see Fig. 5); however, the spectral analysis showed that the recombinant ppHb is pentacoordinate (Table II). These observations indicate that ppHb is structuraly intermediate between nsHbs and Lbs and suggest that it probably binds O_2 similarly to Lbs and thus functions as an O_2-carrier into the *C. fasciculata* root nodules.

CONCLUSIONS

A prerequisite to the origin of an efficient N_2-fixing symbiosis between rhizobia and legume hosts was the existence of a Lb able to carry and deliver O_2 to the respiring bacteroids. Nonsymbiotic Hbs are widespread in land plants and Lbs are restricted to legumes,[11] thus Lbs originated from nsHbs. It has been postulated that Lbs evolved from either nsHb-1[15] or nsHb-2.[4,12,19] The observation that ppHb sequence similarity is higher to nsHb-1 than to nsHb-2 (77%–83% and 62%–68%, respectively) (Table I) suggests that ppHb could have originated from a nsHb-1 and not from a nsHb-2. This observation also discards the possibility that ppHb is a nsHb-2 and not an evolutionary intermediate between nsHb-1 and Lb.

If ppHb and Lbs evolved from a nsHb-1, it is unlikely that the ancestral nsHb-1 functioned in N_2-fixing nodules by delivering O_2 to bacteroids because of its extremely low O_2-dissociation rate constant. To function as an O_2-carrier structural changes needed to have

Table II

Spectral Characteristics of ppHb, Soybean Lba,[31] and Rice Hb1[6]

	Absorption maxima (nm)					
State/ligand	Søret region		Q region			
ppHb						
Ferric	404		542			633
Ferrous deoxygenated	431			557		
Ferrous oxygenated	413		540		575	
Soybean Lba						
Ferric	404		534			627
Ferrous deoxygenated	427			557		
Ferrous oxygenated	411		541		575	
Rice Hb1						
Ferric	410		540			
Ferrous deoxygenated	424	529		557		
Ferrous oxygenated	412		540		576	

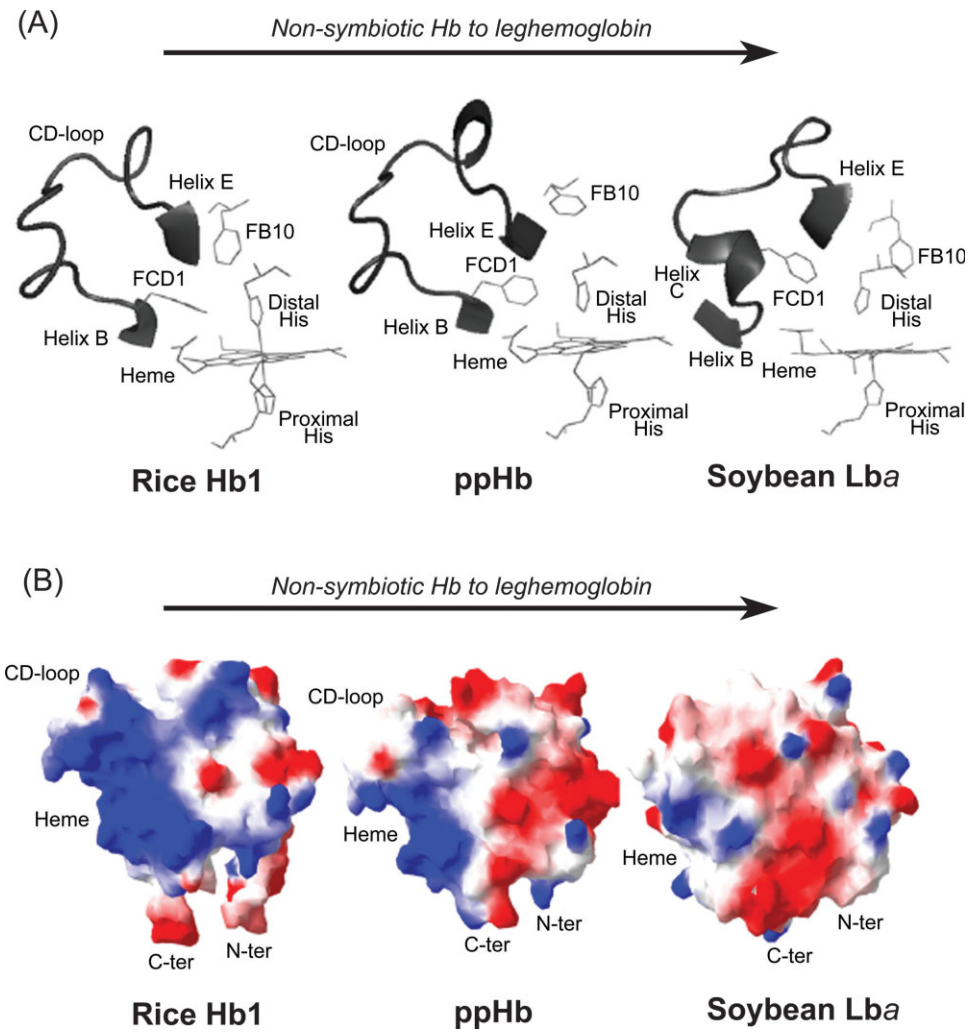

Figure 6

*Comparison of the rice Hb1, ppHb, and soybean Lba CD-loop regions (**A**) and surface charge distribution (**B**). The heme position in (B) is shown as an approximation. Blue and red colors in (B) represent positively and negatively charged amino acids, respectively. The arrows above the figures illustrate the postulated nsHb to Lb evolutionary transition.*

occurred in the ancestral nsHb-1 to evolve into a functional Lb. The comparative structural analysis of nsHb-1 (rice Hb1) and Lb (soybean Lb*a*) with ppHb permitted us to identify the major structural changes that probably occurred during the nsHb to Lb transition. These changes include the following: (i) a hexacoordinate to pentacoordinate transition and changes in the position of residues lining the heme-pocket (see Fig. 5)[19]; (ii) the size decrease and organization of the CD-loop [Fig. 6(A)], which limited the mobility of the helix E and positioned distal His away from the heme-Fe (i.e., in a pentacoordinate position) permiting more efficient O_2-transfer; (iii) the N- and C-termini length decrease resulting in the disappearance of the N/C-pocket region[35] and compaction of the protein into a globular structure; and (iv) the disappearance of positive charges outside the heme pocket and appearance of negative charges in an area located between the N- and C-termini [Fig. 6(B)]. These structural changes probably had consequences in the Hb function into the plant cell, which resulted in the origin of the symbiotic function of Lbs.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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