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

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INTRODUCTION

Nonsymbiotic and symbiotic hemoglobins (Hbs) are O2-binding proteins that have been identified in plants. Symbiotic Hbs (or leghemoglobins (Lbs) when isolated from legumes) are specifically synthesized in the nodules of N2-fixing plants. A major function for Lbs is to facilitate the diffusion of O2 to the actively respiring bacteroids during N2-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 O2-affinity of nsHb-1 and nsHb-2 is very high and high, respectively.4–6 The very high O2-affinity of nsHb-1 results from an extremely low O2-dissociation rate constant, thus it has been proposed that major functions for nsHbs-1 in plant cells are other than O2-transport, such as to modulate the levels of NO and maintain the energy status and redox potential.7–10 In contrast, the O2-dissociation rate constant for nsHb-2 is higher than that of nsHbs-1, thus a likely function for nsHbs-2 is O2-transport.4,7

For many years Hbs were only identified in N2-fixing plants; however, during the last few decades Hbs have been detected in numerous (non-N2-fixing) land plants, ranging

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The Structural Origin of Leghemoglobin

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 nonsymbiotic specificities in N2-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 N2-fixation in nonlegume plants, such as in Parasponia andersonii and actinorhizal plants, Lb is essential for N2-fixation in nodulating legumes.18 Thus, a specialization from nsHb to Lb apparently occurred during the evolution of N2-fixing legumes. Hoy et al.19 analyzed the crystal structure of barley and rice nsHb-1 and soybean Lba and proposed that functional (O2-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 Caesalpinioideae is the oldest subfamily in the Leguminosae family20,21 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 nsHb into a functional Lb probably occurred in a 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 N2-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 N2, and stored at ~80°C until used.

<table>
<thead>
<tr>
<th>Plant Hb</th>
<th>Similarity (%)</th>
<th>Identity (%)</th>
</tr>
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<tbody>
<tr>
<td>Nonsymbiotic Hbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean nshb1</td>
<td>83</td>
<td>68</td>
</tr>
<tr>
<td>Parasponia andersonii nshb1</td>
<td>82</td>
<td>66</td>
</tr>
<tr>
<td>Trema orientalis nshb1</td>
<td>82</td>
<td>67</td>
</tr>
<tr>
<td>Tomato nshb1</td>
<td>78</td>
<td>64</td>
</tr>
<tr>
<td>Barley nshb1</td>
<td>78</td>
<td>57</td>
</tr>
<tr>
<td>Rice nshb1</td>
<td>77</td>
<td>56</td>
</tr>
<tr>
<td>Ceratodon purpureus nshb</td>
<td>71</td>
<td>46</td>
</tr>
<tr>
<td>Physcomitrella patens nshb</td>
<td>69</td>
<td>44</td>
</tr>
<tr>
<td>Cotton nshb2</td>
<td>68</td>
<td>53</td>
</tr>
<tr>
<td>Tomato nshb2</td>
<td>63</td>
<td>48</td>
</tr>
<tr>
<td>Arabidopsis nshb2</td>
<td>62</td>
<td>45</td>
</tr>
<tr>
<td>Symbiotic Hbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sesbania rostrata Lb</td>
<td>62</td>
<td>46</td>
</tr>
<tr>
<td>Soybean Lba</td>
<td>59</td>
<td>42</td>
</tr>
<tr>
<td>Cowpea Lbb</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td>Yellow Lupin Lb</td>
<td>59</td>
<td>42</td>
</tr>
</tbody>
</table>

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 Fast-Track 2.0 mRNA isolation system (Invitrogen). First strand cDNA synthesis was primed with a NotI-oligo(dT) adapter primer followed by second strand synthesis using the Superscript Plasmid System (Invitrogen). The resulting cDNAs were ligated to Sall adapters, digested with NotI and directionally cloned into the pSPORT1 cloning vector (Invitrogen). Plasmids containing cDNA inserts were transformed into Ultramark DH5α-FT 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.
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 (non-symbiotic) 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. \(^{29}\)

**RESULTS AND DISCUSSION**

**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.

**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 Caesalpinoidae is the
oldest subfamily of the Leguminosae family,\textsuperscript{20,21} thus it is probable that a functional (O\textsubscript{2}-transporting) Lb originated within a caesalpinoid legume. We isolated one clone (clone 11) from a cDNA library constructed from the caesalpinoid \textit{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
Lba (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 Lba. 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 Lba. 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 Lba were compared [Fig. 4(B)]. Specifically, major differences between ppHb and soybean Lba 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 Lba (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 Lba.

**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 Lba. Also, the absorption maxima of ferrous oxygenated recombinant ppHb were located at 413, 540, and 575 nm, similarly to those of oxygenated soybean Lba and rice Hb1 (Table II). This evidence indicates that recombinant ppHb is pentacoordinate and that it binds O2. Modeling analysis predicted that the position of distal His in ppHb is intermediate between rice Hb1 and soybean Lba (see Fig. 5); however, the spectral analysis showed that the recombinant ppHb is pentacoordinate (Table II). These observations indicate that ppHb is structurally intermediate between nsHbs and Lbs and suggest that it probably binds O2 similarly to Lbs and thus functions as an O2-carrier into the *C. fasciculata* root nodules.

**CONCLUSIONS**

A prerequisite to the origin of an efficient N2-fixing symbiosis between rhizobia and legume hosts was the existence of a Lb able to carry and deliver O2 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-115 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 N2-fixing nodules by delivering O2 to bacteroids because of its extremely low O2-dissociation rate constant. To function as an O2-carrier structural changes needed to have
occurred in the ancestral nsHb-1 to evolve into a functional Lb. The comparative structural analysis of nsHb-1 (rice Hb1) and Lb (soybean Lba) 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) permitting more efficient O₂-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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