Characterization of Recombinant Soybean Leghemoglobin $a$ and Apolar Distal Histidine Mutants

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Hargrove, Mark S.; Barry, Jennifer K.; Brucker, Eric Allen; Berry, Michael B.; Phillips, Jr., George N.; Olson, John S.; Arredondo-Peter, Raúl; Dean, Jeanenne M.; Klucas, Robert V.; and Sarath, Gautam, "Characterization of Recombinant Soybean Leghemoglobin $a$ and Apolar Distal Histidine Mutants" (1997). *Gautam Sarath Publications*. 20.

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Characterization of recombinant soybean leghemoglobin a and apolar distal histidine mutants

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Abstract
The cDNA for soybean leghemoglobin a (Lba) was cloned from a root nodule cDNA library and expressed in Escherichia coli. The crystal structure of the ferric acetate complex of recombinant wild-type Lba was determined at a resolution of 2.2 Å. Rate constants for O2, CO and NO binding to recombinant Lba are identical with those of native soybean Lba. Rate constants for hemin dissociation and auto-oxidation of wild-type Lba were compared with those of sperm whale myoglobin. At 37°C and pH 7, soybean Lba is much less stable than sperm whale myoglobin due both to a fourfold higher rate of auto-oxidation and to a ~600-fold lower affinity for hemin. The role of His61(E7) in regulating oxygen binding was examined by site-directed mutagenesis. Replacement of His(E7) with Ala, Val, or Leu causes little change in the equilibrium constant for O2 binding to soybean Lba, whereas the same mutations in sperm whale myoglobin cause 50 to 100-fold decreases in K\textsubscript{O2}. These results show that, at neutral pH, hydrogen bonding with His(E7) is much less important in regulating O2 binding to the soybean protein. The His(E7) to Phe mutation does cause a significant decrease in K\textsubscript{O2} for Lba, apparently due to steric hindrance of the bound ligand. The rate constants for O2 dissociation from wild-type and native Lba decrease significantly with decreasing pH. In contrast, the O2 dissociation rate constants for mutants with apolar E7 residues are independent of pH, suggesting that hydrogen bonding to the distal histidine residue in the native protein is enhanced under acid conditions. All of these results support the hypothesis that the high affinity of Lba for oxygen and other ligands is determined primarily by enhanced accessibility and reactivity of the heme group.

Keywords: soybean leghemoglobin, ligand binding, site-directed mutagenesis

Abbreviations: Lb, leghemoglobin; EPR, electron paramagnetic resonance; Mb, myoglobin

Introduction
Leghemoglobins (Lb) are small monomeric heme proteins found in root nodules of leguminous plants. These proteins have unusually high affinities for oxygen compared to vertebrate myoglobins and hemoglobins. The association rate constants for O2 binding are very large (1 × 10\textsuperscript{8} to 3 × 10\textsuperscript{8} M\textsuperscript{-1} s\textsuperscript{-1}), whereas the dissociation rate constants are in the “normal” range for hemoglobins (5 to 30 s\textsuperscript{-1}; Gibson et al. 1989). High oxygen affinity is required to prevent inhibition of nitrogen fixation, and a moderate dissociation rate constant is necessary for facilitating diffusion of oxygen to a high-affinity bacteroid oxidase (Appleby 1992).

The tertiary folding patterns of lupin and soybean Lb are very similar to those of mammalian myoglobins, and the α and β subunits of human hemoglobin. This pattern consists of seven α-helices (A to H, minus the D helix found in most mammalian myoglobins) wrapped around an iron-protoporphyrin IX prosthetic group that is coordinated to the histidine side-chain of the eighth residue in the F helix. Leghemoglobins also contain a “distal” histidine residue at position E7. Polar interactions involving His-(E7) in mammalian myoglobins...
have been shown to play a key role in regulating ligand binding (Springer et al. 1994). The evidence for hydrogen bonding between bound O$_2$ and the distal histidine residue in leghemoglobin is less clear.

Comparisons of the ligand binding properties of leghemoglobins with those from a wide variety of animal myoglobins and hemoglobins have suggested that the small rates of O$_2$ dissociation from soybean and lupin Lb are due to hydrogen bonding with His(E7) (Appleby et al. 1983; Mims et al. 1983; Stetzkowski et al. 1979; Rohlfis et al. 1988). Initial electron paramagnetic resonance (EPR) experiments with cobalt-containing soybean Lb indicated weak hydrogen bonding at neutral pH and a significant strengthening of this interaction at acid pH (Lee et al. 1993). Fuchsman and Appleby (1979) observed a single narrow IR band at $v_{CO} = 1947$ cm$^{-1}$ for Lba CO at pH 7. This peak corresponds to the main band observed in sperm whale MbCO ($v_{CO} = 1945$ cm$^{-1}$) and indicates a similar electrostatic environment around the bound ligand in both proteins. More recently, (Harutyunyan et al. 1995) reported a distance of 2.7 Å between the second ligand atom and N$^\alpha$ of His(E7) in lupin oxyleghemoglobin II, indicating a hydrogen bond between the imidazole group and bound O$_2$. Thus, hydrogen bonding is thought to occur in leghemoglobins, especially at low pH. However, the strength of this interaction and its exact role in governing the kinetics and equilibria of ligand binding to plant hemoglobins have not been established directly by site-directed mutagenesis.

Like all oxygen binding proteins, leghemoglobins must be in the Fe(II) oxidation state to bind oxygen. Within the root nodule, the protein is kept in the ferrous state by a reductase that catalyses the reduction of metleghemoglobin by NADH (Saari and Klucas 1984; Ji et al. 1994a; and Ji et al. 1994b). However, little is known about the molecular recognition and protein-protein interaction between the enzyme and its substrate. Soybean root nodules contain four leghemoglobin gene products, termed Lba, Lbc1, Lbc2 and Lbc3, and four variants of these four proteins in which the N termini are acetylated (Appleby 1984). The amino acid sequences and ligand binding properties of the four gene products are very similar (Martin et al. 1990). Several studies have shown changes in the relative concentrations of the individual leghemoglobins during plant growth, but the physiological consequences of differential expression and turnover are not well understood (Appleby 1984).

The expression of recombinant hemoglobins and myoglobins has allowed systematic studies of the structural factors that regulate ligand binding to these proteins (Nagai et al. 1987; Mathews et al. 1991; Kloek et al. 1993; Springer et al. 1994). In order to carry out similar mechanistic studies, we have cloned Lba from a soybean cDNA library, inserted it into two expression vectors, and used both constitutive and inducible systems to express large quantities of protein. The crystal structure of recombinant wild-type Lba was determined to 2.2 Å and compared with that of the native protein. The ligand binding, spectral, auto-oxidation and hemin dissociation properties of native and recombinant Lba were also compared in detail. His61(E7) to Ala, Val, Leu and Phe mutations were constructed to examine the role of the distal histidine residue in governing O$_2$ binding. Finally, Tyr30(B10) to Phe and Leu65(E11) to Val mutations were made to examine the structural origin of differences between the rates of O$_2$ binding to lupin LbII and soybean Lba.

Results

Properties of wild-type Lba

As described in Materials and Methods, the N-terminal methionine residue of recombinant Lba is removed post-translationally during constitutive expression in Escherichia coli TB1 cells, making the wild type recombinant Lba chemically identical with native Lba (Figure 1). The absorbance spectra of wild-type metLb, deoxyLb, LbO$_2$ and LbCO are identical with those of the native protein (Table 1). The kinetic parameters for O$_2$, CO and NO binding, hemin loss and auto-oxidation are also the same for the wild-type and native proteins (Table 2, Table 4, and Table 5). These data show that the protein obtained from E. coli TB1 cells is functionally and spectrally the same as native soybean leghemoglobin a. Lbs expressed in the inducible pET28a/BL21DE3 system are only partially processed, yielding a mixed sample of N-met and “native-like” protein. However, the samples prepared from the pET28a/BL21DE3 system are kinetically and spectrally identical with those prepared in TB1. Furthermore, it has been observed that addition of an entire N-terminal S-tag (Novagen) domain has no effect on the kinetic or spectral properties of the Lbs examined here (unpublished results).

Crystal structure of wild type acetomet-Lba

The crystallization system described by Vainshtein et al. (1975) was followed starting with aquomet wild-type Lba. Since 0.5 M ammonium acetate is present in the solution, the acetomet form of the protein is produced in the crystals. The backbone and side-chain positions in the acetate complex of wild-type metLb are very similar to those of the nicotinate complex of native metLb (Ollis et al. 1983 and Ellis et al. 1997). An overlay of the backbone atoms plus the side-chains of the proximal and distal histidine residues of the two structures is shown in Figure 2. The native nicotinate structure is shown in yellow and molecule a of the two molecules in the asymmetric unit of the wild-type structure is shown in blue. The ligands have been removed for clarity.
These data show that the protein obtained from *E. coli* is structurally the same as native soybean Lbα. The most significant differences between the two structures are in the positions of the heme group, distal histidine residue, portions of the CD and FG corners, and part of the F helix including His(F8). These differences appear to be due to the size of the coordinating ligand. In the native structure, the large nicotinate ring forces the distal histidine residue out toward solvent and pushes the heme group downward. The proximal histidine residue, F helix and FG corner rotate in order to compensate for this movement of the porphyrin ring. The CD corner also appears to move away from the bound ligand, but this region is either mobile or disordered in both structures.

Figure 1. The cDNA and amino acid sequence of recombinant soybean Lbα in the pEMBL19* plasmid pLbα. The ribosome binding site lies between the 5′ *Kpn*I and *Nco*I restriction sites. The flanking 5′ *Nco*I and 3′ *Eco*RI restriction sites can be used to move the gene independently of the promoter region. The gene sequence corresponds with that observed for soybean Lbα by Hyldig-Nielsen et al. (1982).
Figure 3 shows electron density around the heme binding site of wild-type Lb. The view is looking into the pocket from solvent with the distal portion of the heme pocket located in the upper half of the drawing. The heme is shown in white, the acetate ligand in yellow and the protein chain in red. Well-resolved density for His61(E7) is seen next to the bound acetate group. Other prominent residues are (counter-clockwise): eu65(E11), located just behind His61(E7); and Phe44(CD1), located to the left of His61(E7), just above the heme plane.

The distal histidine residue in the structure of native soybean metLb appears to be hydrogen bonding with the carbonyl oxygen atom of the nicotinate ligand (Oliss et al. 1983). In the wild-type metLb structure, N^ε of His61(E7) is 2.64 Å and 3.08 Å away from the carbonyl oxygen atom of the acetate group in the two protein molecules found in the asymmetric unit. This proximity suggests that a hydrogen bond is also formed between bound acetate and the distal histidine residue in the wild-type protein. Harutyunyan et al. (1995) have shown that a hydrogen bond occurs between the distal histidine residue and the second coordinated ligand atom in the crystal structure of lupin LbO₂. Thus, in leghemoglobins, the distal histidine residue appears to be quite flexible and is capable of interacting electrostatically with ligands of varying size.

**Role of His61(E7) in O₂ binding**

Hydrogen bonding between bound oxygen and the distal histidine residue in sperm whale myoglobin has been established by X-ray crystallography, and its effect on oxygen affinity has been determined in site-directed mutagenesis studies (Springer et al. 1994). Following the strategy of Springer et al. (1989), we replaced His61(E7) with Ala, Val, Leu and Phe to examine quantitatively how oxygen binding is affected by loss of hydrogen-bonding potential at this position in Lb. Table 2 presents a comparison of the effects of these E7 mutations on the rate and equilibrium constants for O₂ binding to soybean Lb with those observed previously for O₂ binding to sperm whale myoglobin.

In general, the aliphatic replacements have a much smaller effect on O₂ binding to legemoglobin than to myoglobin. These differences are particularly striking when the oxygen dissociation rate constants are exam-
ined. Only the Phe-E7 mutation in Lbα produces a large increase in \( k_{O_2} \), whereas in myoglobin all of the mutations cause at least 100-fold increases in \( k_{O_2} \). These results clearly show that the hydrogen bond between oxygen and His-E7 in leghemoglobin is very weak at neutral pH and not the main determinant of the high affinity of this protein for \( O_2 \), in agreement with the EPR results of Ikeda-Saito et al. (1981) and Lee et al. (1993). The three- to fourfold increases in \( k_{O_2} \) observed for the Val(E7) and Leu(E7) Lbα mutants are balanced by roughly equal increases in \( k_{O_2} \) so there is little net change in overall oxygen affinity. The Ala(E7) and Phe(E7) mutations produce an increase and decrease, respectively, in \( k_{O_2} \) with respect to the wild-type protein. These latter changes seem to imply that the E7 residue may sterically hinder the bound ligand. However, an exact interpretation of these results will require high-resolution crystal structures of the proteins. Regardless, it is clear that the polarity of His(E7) plays a much smaller role in regulating ligand binding to leghemoglobin than to myoglobin.

The effects of pH on oxygen binding to native, wild-type and E7 mutants of soybean leghemoglobin are presented in Table 3. As shown by Appleby et al. (1983), when the pH is decreased from 7 to 5, the rate constant for oxygen dissociation from Lbα decreases twofold with no change in the association rate constant. The resultant increase in affinity at low pH has been attributed to a strengthening of the hydrogen bond between His61 and bound ligand. The apolar mutants of Lbα show no pH dependence (Table 3). These mutagenesis results support the view that His61 plays a role in the pH dependence of \( O_2 \) binding to native leghemoglobin and that hydrogen bonding to the bound ligand is enhanced under acidic conditions.

CO and NO binding

The rate and equilibrium constants for carbon monoxide (CO) and nitric oxide (NO) binding to Lbα, sperm whale myoglobin and the corresponding Leu(E7) mutants are

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**Table 2.** The effects of apolar substitutions at position E7 on the rate and equilibrium constants of \( O_2 \) binding to soybean Lbα and sperm whale Mb at pH 7, 20°C.

<table>
<thead>
<tr>
<th>Residue at 61(E7)</th>
<th>( k_{O_2} ) ( \mu M^{-1} s^{-1} )</th>
<th>( k_{O_2} ) s(^{-1} )</th>
<th>( K_{O_2} ) ( \mu M^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lb</td>
<td>Mb</td>
<td>Lb</td>
</tr>
<tr>
<td>His (wild-type)</td>
<td>130</td>
<td>17</td>
<td>5.6</td>
</tr>
<tr>
<td>Ala</td>
<td>290</td>
<td>53</td>
<td>3.1</td>
</tr>
<tr>
<td>Val</td>
<td>280</td>
<td>110</td>
<td>27</td>
</tr>
<tr>
<td>Leu</td>
<td>400</td>
<td>98</td>
<td>24</td>
</tr>
<tr>
<td>Phe</td>
<td>130</td>
<td>75</td>
<td>280</td>
</tr>
</tbody>
</table>

Data for sperm whale myoglobin were taken from Springer et al. (1994).
presented in Table 4. Replacement of the distal histidine residue with Leu increases the association rate constants for CO and NO binding to both proteins, but again the effects are much greater in myoglobin. The rate constants for CO and NO dissociation are largely unaffected by the Leu(E7) replacement. For both ligands, wild-type Lb has larger association rate constants and smaller dissociation rate constants than wild-type myoglobin. These results point out that with or without a distal histidine residue, leghemoglobin is inherently much more reactive than myoglobin.

**Autooxidation and hemin loss**

A comparison of resistances to auto-oxidation and hemin dissociation is given in Table 5. Brantley et al. (1993) have shown that the rate constants for auto-oxidation of myoglobin mutants correlate inversely with O₂ affinity. Thus, we expected Lb to auto-oxidize slowly due to its high O₂ affinity. However, the opposite effect was observed; k_{autox} for Lb is about fourfold greater than that of wild-type sperm whale Mb. This result does correlate with a much weaker hydrogen bond between bound O₂ and His(E7) in leghemoglobin. Brantley et al. (1993) showed that this polar interaction inhibits protonation of the Fe-O₂ complex and its subsequent dismutation into ferric iron and the neutral superoxide radical. Both myoglobin and leghemoglobin show a marked increase in k_{autox} with decreasing pH, presumably due to protonation of bound O₂.

The rate constant for hemin dissociation from native and wild-type Lb is ~6 h⁻¹ at pH 7.0, 37°C. This rate is 600 times faster than that for myoglobin under the same conditions and, in combination with the increased auto-oxidation rate, results in a much less stable holoprotein (Hargrove and Olson 1996). Lb also has a much larger temperature dependence for hemin dissociation than myoglobin (E_a ≈ 33 kcal/mol for aquometLb versus E_a ≈ 8 kcal/mol for sperm whale aquometMb at pH 7; (Hargrove et al. 1994); and unpublished results). As shown in Table 5, k_{H} for met Lb is ~0.8 h⁻¹ at 20°C, which is roughly tenfold less than the rate observed at 37°C. The rate constant for hemin loss from myoglobin decreases by only a factor of ~2 when the temperature is decreased from 37 to 20°C at both pH 5.0 and pH 7.0 ((Hargrove et al. 1994); and unpublished results).

The rates of hemin loss from the distal histidine mutants of Lb are too fast to be measured conveniently at 37°C. As a result, comparisons between mutant and wild-type leghemoglobins were measured at 20°C. The distal histidine residue of Lb clearly stabilizes bound hemin, as shown by the H61L substitution, which increases the rate constant for hemin dissociation ~50-fold. A similar effect occurs when His(E7) is replaced by Leu in myoglobin. A large part of this effect is probably due to loss of the coordinated water molecule from the ferric H61L protein. Both Leu-E7 metMb and metLb show a broad absorbance band centered at 395 nm, which is characteristic of 5-coordinated ferric proteins (Table 1; Quillin et al. 1993). The pH dependence of hemin dis-

### Table 4. Comparison of the CO and NO binding parameters of soybean Lb and sperm whale Mb at pH 7, 20°C

<table>
<thead>
<tr>
<th>Protein</th>
<th>k_{CO} (µM⁻¹ s⁻¹)</th>
<th>k_{NO} (µM⁻¹ s⁻¹)</th>
<th>K_CO (µM)</th>
<th>K_NO (µM)</th>
<th>K_CO (µM)</th>
<th>K_NO (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Lb</td>
<td>17</td>
<td>0.0078</td>
<td>2200</td>
<td>170</td>
<td>0.00002</td>
<td>9,000,000</td>
</tr>
<tr>
<td>Wild-type Lb</td>
<td>15</td>
<td>0.0084</td>
<td>1800</td>
<td>190</td>
<td>0.00002</td>
<td>9,000,000</td>
</tr>
<tr>
<td>H61L Lb</td>
<td>170</td>
<td>0.0024</td>
<td>72,000</td>
<td>320</td>
<td>0.00002</td>
<td>16,000,000</td>
</tr>
<tr>
<td>Wild-type Mb</td>
<td>0.51</td>
<td>0.019</td>
<td>27</td>
<td>22</td>
<td>0.00010</td>
<td>220,000</td>
</tr>
<tr>
<td>H64L Mb</td>
<td>26</td>
<td>0.024</td>
<td>1100</td>
<td>190</td>
<td>0.00013</td>
<td>1,500,000</td>
</tr>
</tbody>
</table>

Data for sperm whale myoglobin were taken from Springer et al. (1994).
The resistances of both proteins to auto-oxidation and hemin loss are similar at pH 5, whereas at pH 7 sperm whale myoglobin is much more stable.

Comparison with lupin LbII

As shown in Table 6, the association and dissociation rate constants for O₂ binding to lupin LbII are three- to five-fold greater than the corresponding parameters for soybean Lbα. This implies more rapid rates of ligand entry into and exit from the distal pocket of the lupin protein. Both proteins contain His(E7) and Phe(CD1). In lupin LbII the B10 and E11 residues are smaller, Phe and Val, respectively, versus Tyr and Leu in soybean leghemoglobin. However, this decrease in size does not appear to account for the ligand binding differences. Mutation of Leu65(E11) to Val has little effect on O₂ binding to soybean Lbα, and the Tyr30(B10) to Phe substitution causes decreases, not increases, in k₉ and k₈ (Table 6). As suggested by Gibson et al. (1989), the differences in kinetic behavior among leghemoglobins appear to be due to amino acid changes remote from the ligand binding site. The increase in k₋₁ caused by the H61L mutation suggests that the distal histidine residue of Lb stabilizes bound heme by hydrogen bonding to a coordinated water molecule as is observed in myoglobin. The decreased stability of Lbα probably accounts for the lower yields in the constitutive expression (TB1) system relative to that of myoglobin. Purification of leghemoglobin in the presence of CO or other distal ligands prevents heme loss and greatly increases protein yields in both the constitutive and inductive expression systems.

Table 6. Comparison of the rate constants for O₂ binding to soybean Lbα and Lupin LbII at pH 7, 20°C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>k₉ (μM⁻¹s⁻¹)</th>
<th>k₈ (s⁻¹)</th>
<th>k₀₂ (μM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupin LbII</td>
<td>320</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Soybean Lbα</td>
<td>130</td>
<td>5.6</td>
<td>23</td>
</tr>
<tr>
<td>Soybean Lbα (L65V)</td>
<td>180</td>
<td>5.8</td>
<td>30</td>
</tr>
<tr>
<td>Soybean Lbα (Y30F)</td>
<td>79</td>
<td>0.75</td>
<td>100</td>
</tr>
</tbody>
</table>

The rate constants for lupin LbII were taken from Gibson et al. (1989).

Discussion

The crystal structure of recombinant Lbα and the data reported in Table 1 and Table 4 show that the protein expressed in E. coli TB1 cells is functionally and structurally identical with that of native Lbα. It is unclear why the N-terminal methionine residue is removed from this protein when wild-type myoglobin grown under the same conditions is expressed with the N-Met still attached (Springer and Sligar 1987; Phillips et al. 1990). The N-terminal amino acid sequences of the two proteins are similar (Lbα, VAFTE-; Mb, VLSEG-). There is no clear explanation for the difference in post-translational processing during constitutive expression.

Leghemoglobin is much less stable than myoglobin due to a fourfold higher rate of auto-oxidation and a ~600-fold higher rate of hemin loss at pH 7, 37°C. The rate constant for hemin loss from leghemoglobin were measured at 20°C to allow comparison with mutant Lbs, which lose heme too rapidly at 37°C.

Table 5. Comparison of the rate constants for hemin dissociation (k₋₁) from and auto-oxidation (kautoox) of soybean Lbα and sperm whale Mb

<table>
<thead>
<tr>
<th>Protein</th>
<th>kautoox (h⁻¹) at 37°C</th>
<th>k₋₁ (h⁻¹) at 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Lb</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Wild-type Lb</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>H61L Lb</td>
<td>0.3</td>
<td>47</td>
</tr>
<tr>
<td>Wild-type Mb</td>
<td>0.055</td>
<td>0.005(0.01)</td>
</tr>
<tr>
<td>H64L Mb</td>
<td>10</td>
<td>0.094(0.20)</td>
</tr>
</tbody>
</table>

The kautoox values for MbO₂ were taken from Brantley et al. (1993) and the values of k₋₁ for metMb from Hargrove et al. (1996b). Rates of auto-oxidation were measured in the presence of 1mM EDTA, catalase, and superoxide dismutase as described by Brantley et al. (1993). The k₋₁ values in parentheses for metMb were measured at 37°C by Hargrove et al. (1996b) and Hargrove et al. (1994).

* The value of kautoox for Leu(E7) Mb at pH 5 was estimated assuming the same pH dependence as is observed for the Val(E7) Mb mutant by Brantley et al. (1993).

* The rates of hemin loss from leghemoglobin were measured at 20°C to allow comparison with mutant Lbs, which lose heme too rapidly at 37°C.

The rates of hemin loss at 20°C were computed using the k₋₁ value at 37°C and an activation energy of 8.0 kcal/mol. The values in parentheses were measured at 37°C (Hargrove et al., 1996b).
Gibson et al. (1989) also suggested that the Lb rates may be the fastest possible for ligand association to a myoglobin-like protein. The high rates of ligand binding to Lb result in part from a very reactive heme iron atom as has been demonstrated with laser photolysis experiments (Stetzkowski et al. 1985; Gibson et al. 1989). Harutyunyan et al. (1995) have shown that the proximal histidine residue in lupin Lb is much more free to rotate and move toward the heme plane than in myoglobin. These results provide a structural explanation for the high reactivity of leghemoglobins. The heme pocket of Lba is also more flexible and kinetically accessible than those of myoglobin and other heme proteins (Rohlf et al. 1988). This has been demonstrated by many techniques and is exemplified by the ability of Lba to bind large ligands such as imidazole, nicotinate, and long-chain alkyl isocyanides, which do not bind tightly to myoglobin or hemoglobin (Stetzkowski et al. 1979; Kong et al. 1983; Mims et al. 1983).

Even though the bimolecular rate constants are large, His(E7) still appears to offer some resistance to ligand association. The distal heme pocket of the deoxy forms of myoglobin and lupin leghemoglobin both contain a non-coordinated water molecule that hydrogen bonds to His(E7) (Quillin et al. 1993; Harutyunyan et al. 1995). This water molecule must be displaced before other ligands can bind. The increased association rate constants for oxygen binding to the H61A, H61V, and H61L mutants of Lb are probably due to the loss of water in the deoxy forms of these proteins. The same mutations produce even larger increases in $k_{O_2}$ when constructed in sperm whale myoglobin (Quillin et al. 1993; Table 2).

The molecular evidence for a hydrogen bond between bound O$_2$ and His(E7) in leghemoglobin is unequivocal. At pH 7.0. The Ala, Val, and Leu(E7) substitutions do not produce large decreases in $K_{O_2}$ as is observed in myoglobin. However, the pH dependence of $O_2$ dissociation from leghemoglobin does appear to involve the distal histidine residue. Decreasing the pH from 7 to 5 causes a twofold increase in $O_2$ affinity when the E7 residue is histidine but has no effect when the E7 residue is apolar. This result suggests that hydrogen bonding occurs under acid conditions, but even at low pH, the strength of this interaction is still much less than that observed in myoglobin.

The $\beta$ subunit of R-state human hemoglobin shows high rates of ligand association, and replacement of the distal histidine residue with either Gly or Phe has little effect on $O_2$ binding (Mathews et al. 1989). Thus both soybean Lba and R-state human $\beta$ subunits have evolved mechanisms for stabilizing bound $O_2$ and inhibiting auto-oxidation that do not involve a strong hydrogen bond with His(E7). In contrast, strong favorable polar interactions occur in the distal pockets of all mammalian myoglobins and the $\alpha$ subunit of human hemoglobin. However, the distal histidine residue is very important for retaining heme in leghemoglobin and human $\beta$ subunits. Replacing the distal histidine residue with smaller apolar residues causes ≥50-fold increases in the rate of heme loss from metLba and ferric $\beta$ subunits, explaining the high degree of conservation of His(E7) in these proteins (Table 5; Hargrove et al. 1994).

**Materials and methods**

A full-length cDNA for Lba was isolated from a soybean nodule cDNA library using anti-Lba antibodies as a probe (Ji et al. 1994a; Sambrook et al. 1989). Phage (λ-GT11) containing the soybean Lba insert were placed in E. coli, harvested, and the DNA isolated. Lba cDNA was excised and ligated into pBluescript K+ (Invitrogen). To obtain the coding region, polymerase chain reaction (PCR) primers containing a Ncol site at the starting ATG codon and an EcoRI restriction site downstream of the TAA stop codon were used to amplify the Lba gene from the pBluescript-Lba vector. PCR-amplified products were purified by agarose gel electrophoresis and ligated into a pCRII vector (Invitrogen). After transformation, an E. coli colony containing the pCRII-Lba construct was used to obtain purified plasmid with the Ncol-Lba-EcoRI sequence. This gene fragment was removed from pCRII, purified from an agarose gel, and ligated into pACYC184. A duplex oligonucleotide of the constitutive lac promoter used by Springer and Silgar (1987) was constructed with the following sequence of restriction sites: 5′ Bsa636I-KpmI-lac promoter-Ncol 3′. The pACYC184-Lba plasmid was cut with Ncol and Bsa636I, and the oligonucleotide containing the lac promoter was ligated to the Lba cDNA. This lac promoter-Lba construct was removed from pACYC184 and ligated into pEMBL19+ using the flanking KpmI/EcoRI sites.

The initial cDNA product cloned in this manner contained two base-pair differences compared to the Lba sequence reported by Hylde-Nielsen et al. (1982). These changes were probably introduced during the PCR reaction used to amplify the cDNA. The gene was corrected to the native sequence by two rounds of site-directed mutagenesis using the Kunkel (1985) method. The resulting plasmid, pLba, contains the native Lba cDNA sequence in pEMBL19+ under control of the lac promoter (Figure 1). The Kunkel method of mutagenesis was also used to generate the B10, E7 and E11 mutants starting from the “corrected” wild-type Lba gene.

**Expression of wild-type and mutant Lbas**

E. coli TB-1 cells were transformed with pLba and grown in LB medium (with ampicillin) at 37°C. The final yield of purified leghemoglobin (~0.6 mg/l) was roughly one-seventh of that for sperm whale myoglobin expressed under the same conditions (~4 mg/l). H61F, H61V and H61A proteins showed no appreciable expression in the lac promoter-TB1 system. Consequently, the Ncol-Lba-EcoRI fragment was cloned into pET28a (Novagen) for high-level, inducible expression in E. coli BL21DE3 cells. Shake flasks of BL21DE3 cells transformed with the pET28a-Lba vector were grown to an absorbance of ~0.6 in 2 × YT medium at 33°C, induced with 1 mM isopropyl-β-D-thiogalactopyranoside and incubated for an additional four hours. Apo-Lba was found in the supernatant and pellet of the cell lysis mixture. Protein was recovered from the lysis
pellet by dissolution in 6 M guanidinium chloride followed by dialysis into 20 mM Tris (pH 8.0). Holoprotein was reconstituted by the addition of CO-heme or imidazole-hemin to the soluble and pellet fractions of the lysis mixture. The final yield of wild-type or mutant Lbs was ~10 mg/l of medium. Reconstituted holo-Lbs was precipitated with 90% saturated ammonium sulfate for further purification.

Regardless of the method of expression, recombinant Lbs was purified as described by Jun et al. (1994) with the following modifications. The 90% saturated ammonium sulfate Lb pellet was resuspended in 1.8 M ammonium sulfate and loaded directly onto a phenyl Sepharose column, and the protein was not oxidized prior to G-75 chromatography. Purified Lb was in the CO or met-imidazole form (depending on the method of reconstitution) and had a Soret to 280 nm absorbance ratio greater than 4. Native Lbs was purified from soybean root nodules using the method of Jun et al. (1994). N-terminal sequencing of the purified Lbs was performed at the UNL-Protein Core Facility on an ABI-494 Proline sequencer using a manufacturer-supplied protocol.

Crystallography of wild-type Lb

Crystals of the acetate complex of wild-type metLb were grown at room temperature to 0.5 mm × 0.05 mm × 0.05 mm in three to five days using the hanging drop method. The starting concentration of metLb was 1 mM in 0.1 M sodium phosphate (pH 6.0). The precipitant was 2.4 M ammonium sulfate, 0.5 M ammonium acetate at pH 6.8 (Vainshtein et al. 1975). X-ray diffraction data were collected at room temperature on an R-axis IIc imaging plate system using copper Kα radiation from a Siemens rotating anode operating at 50 mA and 90 mA. Crystals were in the P2₁2₁2₁ space group. The data were 90% complete at the limiting resolution of 2.2 Å, with an Rmerge of 10.3%. Phases were determined by molecular replacement using XPLOR (Brunger 1987) starting with the coordinates for the acetate complex of wild-type metLb-nicotinate provided by Dr Paul Ellis (PDB file 1fsl; Ollis et al. 1983; Ellis et al. 1997). Two molecules related by a 2-fold non-crystallographic symmetry axis were present in the asymmetric unit. After several cycles of refinement, manual fitting and solvent addition, the crystallographic R-factor converged to 19.8% (Rfree = 29.7%) with root-mean-square deviations of 0.018 Å in bond lengths, 1.93° in bond angles, 21.36° in dihedral angles and 2.29° in improper angles. Molecule a of the two molecules in the asymmetric unit was used to produce Figures 2 and Figure 3. The coordinates of the structure of wild-type metLb-acetate have been assigned number 1bin in the Protein Data Bank at the Brookhaven National Laboratory.

Spectroscopic and kinetic characterization of wild-type and mutant Lb

Absorbance spectra were measured using a SLM 3000 diode array spectrophotometer. LbCO was prepared by diluting metLb into a cuvette containing 20 mM Tris (pH 8.0) buffer equilibrated with 1 atm CO and an excess of sodium dithionite. MetLb was formed by the reaction of ferrous Lb with potassium ferricyanide followed by chromatography on a Sephadex G-25 column equilibrated in 20 mM Tris (pH 8.0). LbO₂ was produced by the reduction of metLb with sodium dithionite followed by G-25 chromatography in the presence of oxygen. DeoxyLb was produced by the addition of several granules of sodium dithionite to a cuvette containing metLb in anaerobic buffer.

The rate constants for O₂, CO and NO binding were measured using established rapid mixing and laser photolysis methods (Olson 1981; Quillin et al. 1995; Moore and Gibson 1976). O₂ and CO association rate constants at pH 5.0 were measured in 100 mM sodium acetate (pH 5.0). O₂ dissociation at pH 5 was measured by mixing a LbO sample in 10 mM potassium phosphate (pH 7.0) with 100 mM sodium acetate (pH 5.0), equilibrated with 1 atm CO (Appleye et al. 1983).

Rate constants for hemin dissociation and auto-oxidation were measured as described by Hargrove et al. (1994) and Brantley et al. (1993), respectively. Hemin dissociation from HsLb was measured in a Gibson-Dionex stopped-flow apparatus as described by Hargrove et al. (1996a). Mes 2-(N-mopholinio)ethanesulfonic acid was used as the buffer at pH 5 for measurement of hemin dissociation because acetate coordination to metLb markedly inhibits this reaction.

Acknowledgements

This work was supported by United States Public Health Service grants GM35649 and HL-47020, grant C-612 from the Robert A. Welch Foundation, and the W. M. Keck Foundation (J.S.O.), grant 003604-025 from the State of Texas Advanced Technology Program (G.N.P./J.S.O.), a National Institutes of Health Postdoctoral Fellowship AR08355 (E.A.B.), NSF grant OSR-9255225 (R.V.K.), USDA-CREES 95-37305-2441 (R.V.K./G.S.), and the Nebraska Research Initiative (G.S.). R.A.P. was partially supported by DGAFA (Direcccion General de Asuntos del Personal Academico)-UNAM and CoNaCyT (Consejo Nacional de Ciencia y Tecnologia), Mexico. We also thank M. Ikeda-Saito, C. Appleye and A. Wilkinson for reading the original manuscript and making helpful suggestions.

References


