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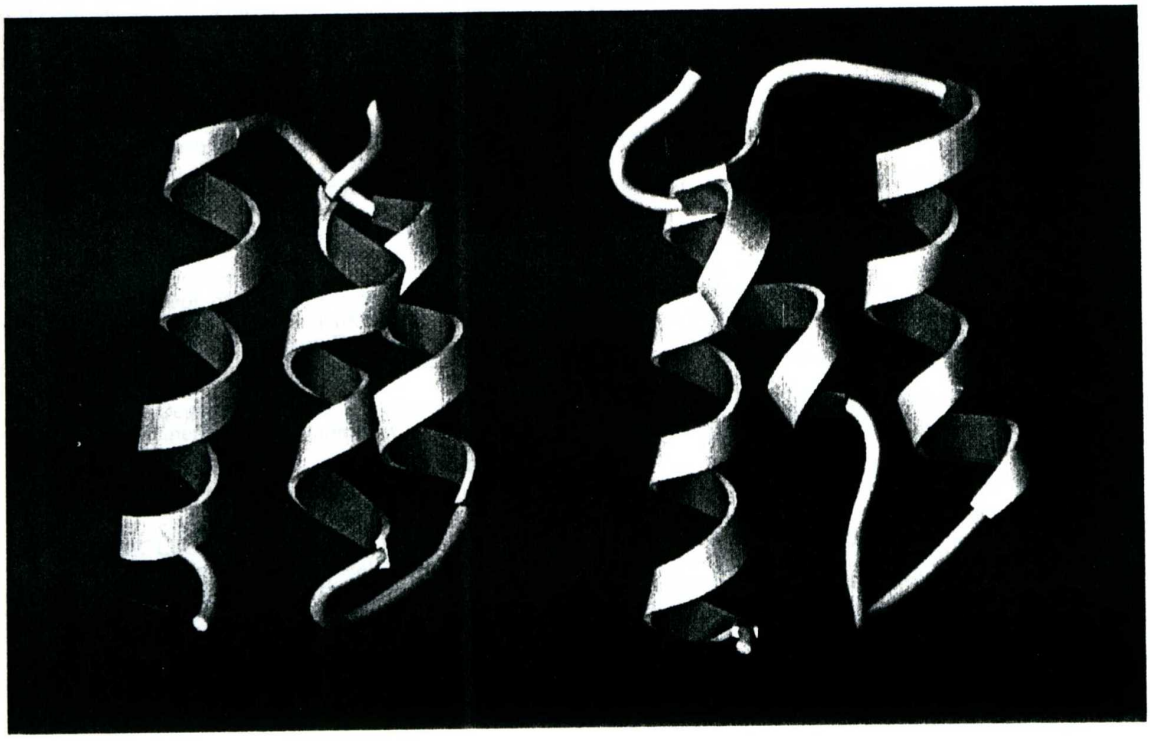

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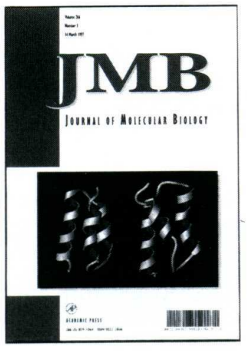

Cover Illustration: Solution structure of the albumin-binding GA molecule—a versatile bacterial protein domain. See paper by Johansson *et al. J. Mol. Biol.* **266,** 859–865.

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

Mark S. Hargrove[1]*, Jennifer K. Barry[1], Eric Allen Brucker[1] Michael B. Berry[1], George N. Phillips Jr[1], John S. Olson[1] Raúl Arredondo-Peter[2], Jeanenne M. Dean[2], Robert V. Klucas[2] and Gautam Sarath[2]

[1]*Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005-1892, USA*

[2]*Department of Biochemistry University of Nebraska-Lincoln Lincoln, NE 68588-0664, USA*

**Corresponding author*

The cDNA for soybean leghemoglobin *a* (Lb*a*) 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 Lb*a* was determined at a resolution of 2.2 Å. Rate constants for O_2, CO and NO binding to recombinant Lb*a* are identical with those of native soybean Lb*a*. Rate constants for hemin dissociation and auto-oxidation of wild-type Lb*a* were compared with those of sperm whale myoglobin. At 37°C and pH 7, soybean Lb*a* 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 O_2 binding to soybean Lb*a*, whereas the same mutations in sperm whale myoglobin cause 50 to 100-fold decreases in K_{O_2}. These results show that, at neutral pH, hydrogen bonding with His(E7) is much less important in regulating O_2 binding to the soybean protein. The His(E7) to Phe mutation does cause a significant decrease in K_{O_2} for Lb*a*, apparently due to steric hindrance of the bound ligand. The rate constants for O_2 dissociation from wild-type and native Lb*a* decrease significantly with decreasing pH. In contrast, the O_2 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 Lb*a* for oxygen and other ligands is determined primarily by enhanced accessibility and reactivity of the heme group.

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

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 O_2 binding are very large (1×10^8 to $3 \times 10^8\ M^{-1}\ s^{-1}$), whereas the dissociation rate constants are in the "normal" range for hemoglobins (5 to 30 s^{-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

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

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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; Rohlfs *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 (Ikeda-Saito *et al.*, 1981). More recent spin echo EPR studies confirmed this observation and further suggested that hydrogen bonding with the distal histidine residue may occur only at low pH (Lee *et al.*, 1993). Fuchsman & Appleby (1979) observed a single narrow IR band at $\nu_{C\text{-}O} = 1947\ cm^{-1}$ for Lb*a*CO at pH 7. This peak corresponds to the main band observed in sperm whale MbCO ($\nu_{C\text{-}O} = 1945\ cm^{-1}$) 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^{ε} 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 & Klucas, 1984; Ji *et al.*, 1994a,b). 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; Kloeck *et al.*, 1993; Springer *et al.*, 1994). In order to carry out similar mechanistic studies, we have cloned Lb*a* 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 Lb*a* 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 Lb*a* 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 Lb*a*.

Results

Properties of wild-type Lb*a*

As described in Materials and Methods, the N-terminal methionine residue of recombinant Lb*a* is removed post-translationally during constitutive expression in *Escherichia coli* TB1 cells, making the wild type recombinant Lb*a* chemically identical with native Lb*a* (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 (Tables 2, 4, and 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-Lb*a*

The crystallization system described by Vainshtein *et al.* (1975) was followed starting with aquomet wild-type Lb*a*. 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*a* are very similar to those of the nicotinate complex of native metLb*a* (Ollis *et al.*, 1983; 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

```
                                                0                                   9
5'          lac promotor                        M  V  A  F  T  E  K  Q  D  A
ggtac'cctaactaaaggagaacaacaac'catggttgctttcactgagaagcaagatgct
c'catgggattgatttcctcttgttgttggtac'caacgaaagtgactcttcgttctacga
3' KpnI                                         NcoI
-30bp                                           0bp

10                                              20                                  29
 L  V  S  S  S  F  E  A  F  K  A  N  I  P  Q  Y  S  V  V  F
ttggtgagtagctcattcgaagcattcaaggcaaacattcctcaatacagcgttgtgttc
aaccactcatcgagtaagcttcgtaagttccgtttgtaaggagttatgtcgcaacacaag
31bp

30                                              40                                  49
 Y  T  S  I  L  E  K  A  P  A  A  K  D  L  F  S  F  L  A  N
tacacttcgatactggagaaagcacctgca'gcaaaggacttgttctcatttctagcaaat
atgtgaagctatgacctctttcgtgg'acgtcgtttcctgaacaagagtaaagatcgttta
91bp                                        PstI

50                                              60                                  69
 G  V  D  P  T  N  P  K  L  T  G  H  A  E  K  L  F  A  L  V
ggagtagaccccactaatcctaagctcacgggccatgctgaaaagctttttgcattggtg
cctcatctggggtgattaggattcgagtgcccggtacgacttttcgaaaaacgtaaccac
150bp

70                                              80                                  89
 R  D  S  A  G  Q  L  K  A  S  G  T  V  V  A  D  A  A  L  G
cgtgactcagctggtcaacttaaagcaagtggaacagtggtggctgatgccgcacttggt
gcactgagtcgaccagttgaatttcgttcaccttgtcaccaccgactacggcgtgaacca
209bp

90                                              100                                 109
 S  V  H  A  Q  K  A  V  T  D  P  Q  F  V  V  V  K  E  A  L
tctgttcatgcccaaaaagcagtcactgatcctcagttcgtggtggttaaagaagcactg
agacaagtacgggtttttcgtcagtgactaggagtcaagcaccaccaatttcttcgtgac
268bp

110                                             120                                 129
 L  K  T  I  K  A  A  V  G  D  L  W  S  D  E  L  S  R  A  W
ctgaaaacaataaaggcagcagttggggacaaatggagtgacgagttgagccgtgcttgg
gacttttgttatttccgtcgtcaacccctgtttacctcactgctcaactcggcacgaacc
327bp

130                                             140
 E  V  A  Y  D  E  L  A  A  A  I  K  K  A  stop
gaagtagcctacgatgaattggcagcagctattaagaaggcataacccggg'aattc
cttcatcggatgctacttaaccgtcgtcgataattcttccgtattgggcccttaa'g
386bp                                                          EcoRI
```

Figure 1. The cDNA and amino acid sequence of recombinant soybean Lb*a* in the pEMBL19$^+$ plasmid pLb*a*. 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 Lb*a* by Hyldig-Nielsen *et al.* (1982).

removed for clarity. These data show that the protein obtained from *E. coli* is structurally the same as native soybean Lb*a*. 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.

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Table 1. Comparison of the wavelength maxima for the Soret absorbance bands of soybean Lb and sperm whale Mb at pH 7, 25°C

Protein	Wavelength peak (nm)				
	Fe(II)	Fe(III)	Fe(II)O_2	Fe(II)CO	Fe(II)NO
Native Lb	428	403	409	416	418
Wild-type Lb	428	403	409	416	418
H61L Lb	425	395	414	419	418
Wild-type Mb	434	409	415	424	421
H64L Mb	434	393	415	425	421

Figure 3 shows electron density around the heme binding site of wild-type Lb*a*. 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): Leu65(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*a* appears to be hydrogen bonding with the carbonyl oxygen atom of the nicotinate ligand (Ollis *et al.*, 1983). In the wild-type metLb*a* 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 LbO_2. 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 O_2 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*a*. Table 2 presents a comparison of the effects of these E7 mutations on the rate and equilibrium constants for O_2 binding to soybean Lb*a* with those observed previously for O_2 binding to sperm whale myoglobin.

In general, the aliphatic replacements have a much smaller effect on O_2 binding to leghemoglobin than to myoglobin. These differences are particularly striking when the oxygen dissociation rate constants are examined. Only the Phe-E7 mutation in

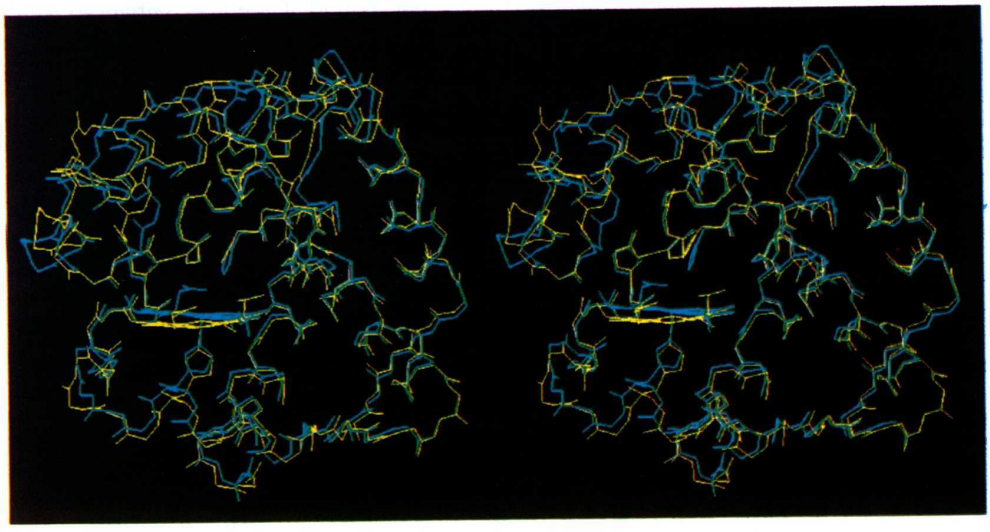

Figure 2. Crystal structure of the acetate complex of recombinant soybean metLb*a*. A stereo overlay of the backbone atoms of the nicotinate complex of native metLb*a* (yellow) and wild-type metLb*a* (blue) including the proximal and distal histidine side-chains. The nicotinate and acetate ligands have been removed for clarity.

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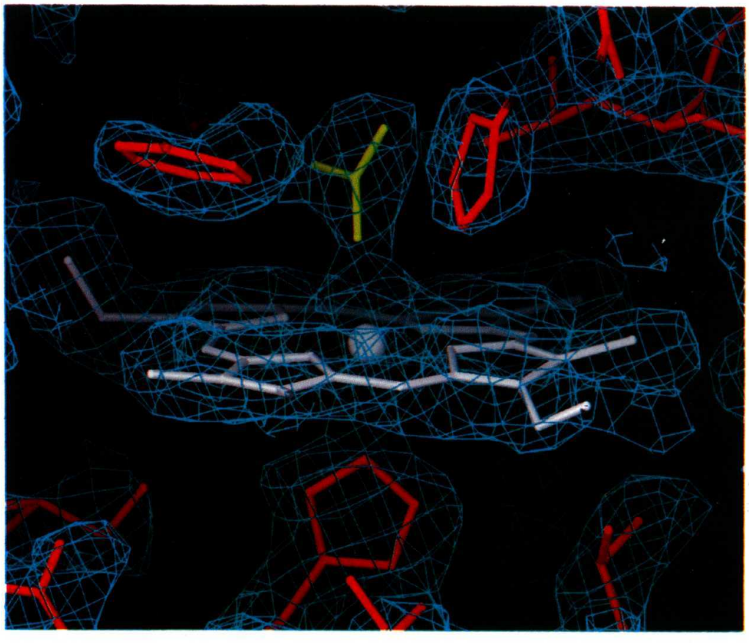

Figure 3. Electron density of the heme pocket in wild-type metLb*a*-acetate. The heme prosthetic group is shown in white, the acetate ligand in yellow and the protein in red. The distal histidine residue is within hydrogen-bonding distance of the carbonyl oxygen atom of the bound acetate group.

Lb*a* 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) Lb*a* 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 Lb*a* 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 Lb*a* 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 Lb*a*, sperm whale myoglobin and the correspond-

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

Residue at 61(E7)	k'_{O_2} $\mu M^{-1} s^{-1}$		k_{O_2} s^{-1}		K_{O_2} μM^{-1}	
	Lb	Mb	Lb	Mb	Lb	Mb
His(wild-type)	130	17	5.6	15	23	1.1
Ala	290	53	3.1	2300	94	0.023
Val	280	110	27	10000	10	0.011
Leu	400	98	24	4100	17	0.023
Phe	130	75	280	10000	0.45	0.0075

Data for sperm whale myoglobin were taken from Springer *et al.* (1994).

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Table 3. Dependence of O_2 binding parameters on pH for native, wild-type, and Leu-E7 soybean Lb*a* at 20°C.

Soybean Lb	pH 7 k'_{O_2} ($\mu M^{-1} s^{-1}$)	pH 7 k_{O_2} (s^{-1})	pH 7 K_{O_2} (μM^{-1})	pH 5 k'_{O_2} ($\mu M^{-1} s^{-1}$)	pH 5 k_{O_2} (s^{-1})	pH 5 K_{O_2} (μM^{-1})
Native	130	5.6	23	140	2.7	51
Wild-type	130	5.6	23	130	2.2	60
H61A	290	3.1	94	250	2.7	92
H61V	280	27	10	280	28	10
H61L	400	24	17	470	25	19
H61F	130	280	0.45	120	230	0.54

ing Leu(E7) mutants are 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*a* 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 O_2 affinity. Thus, we expected Lb*a* to auto-oxidize slowly due to its high O_2 affinity. However, the opposite effect was observed; k_{autoox} for Lb*a* is about fourfold greater than that of wild-type sperm whale Mb. This result does correlate with a much weaker hydrogen bond between bound O_2 and His(E7) in leghemoglobin. Brantley *et al.* (1993) showed that this polar interaction inhibits protonation of the Fe-O_2 complex and its subsequent dismutation into ferric iron and the neutral superoxide radical. Both myoglobin and leghemoglobin show a marked increase in k_{autoox} with decreasing pH, presumably due to protonation of bound O_2.

The rate constant for hemin dissociation from native and wild-type Lb*a* 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 & Olson, 1996). Lb*a* also has a much larger temperature dependence for hemin dissociation than myoglobin ($E_a \approx 33$ kcal/mol for aquometLb*a* *versus* $E_a \approx 8$ 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*a* 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*a* 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*a* 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*a* 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 dissociation from Lb*a* is

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

Protein	k'_{CO} ($\mu M^{-1} s^{-1}$)	k_{CO} (s^{-1})	K_{CO} (μM^{-1})	k'_{NO} ($\mu M^{-1} s^{-1}$)	k_{NO} (s^{-1})	K_{NO} (μM^{-1})
Native Lb	17	0.0078	2200	170	0.00002	9,000,000
Wild-type Lb	15	0.0084	1800	190	0.00002	9,000,000
H61L Lb	170	0.0024	72,000	320	0.00002	16,000,000
Wild-type Mb	0.51	0.019	27	22	0.00010	220,000
H64L Mb	26	0.024	1100	190	0.00013	1,500,000

Data for sperm whale myoglobin were taken from Springer *et al.* (1994).

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Table 5. Comparison of the rate constants for hemin dissociation (k_{-H}) from and auto-oxidation (k_{autoox}) of soybean Lb*a* and sperm whale Mb

Protein	k_{autoox} (h^{-1}) at 37°C		k_{-H} (h^{-1})[b] at 20°C	
	pH 7	pH 5	pH 7	pH 5
Native Lb	0.2	1.6	0.7	1.3
Wild-type Lb	0.2	3.0	0.9	3.0
H61L Lb	0.3	6.6	47	45
Wild-type Mb	0.055	2.4	0.005[c](0.01)	0.4(1.0)
H64L Mb	10	200[a]	0.094[c](0.20)	5.2[c](11)

The k_{autoox} values for MbO_2 were taken from Brantley *et al.* (1993) and the values of k_{-H} 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_{-H} values in parentheses for metMb were measured at 37°C by Hargrove *et al.* (1996b) and Hargrove *et al.* (1994).

[a] The value of k_{autoox} 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).

[b] 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.

[c] The rates of hemin loss at 20°C were computed using the k_{-H} 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).

much smaller than that of myoglobin. 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 O_2 binding to lupin LbII are three- to fivefold greater than the corresponding parameters for soybean Lb*a*. 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 O_2 binding to soybean Lb*a*, and the Tyr30(B10) to Phe substitution causes decreases, not increases, in k'_{O_2} and k_{O_2} (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 affinity due to the Y30F substitution is striking and suggests that an in-

Table 6. Comparison of the rate constants for O_2 binding to soybean Lb*a* and Lupin LbII at pH 7, 20°C

Protein	k'_{O_2} ($\mu M^{-1}s^{-1}$)	k_{O_2} (s^{-1})	K_{O_2} (μM^{-1})
Lupin LbII	320	25	13
Soybean Lb*a*	130	5.6	23
Soybean Lb*a* (L65V)	180	5.8	30
Soybean Lb*a* (Y30F)	79	0.75	100

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

depth study of the role of this residue in ligand binding will be rewarding.

Discussion

The crystal structure of recombinant Lb*a* and the data reported in Tables 1 and 4 show that the protein expressed in *E. coli* TB1 cells is functionally and structurally identical with that of native Lb*a*. 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 & Sligar, 1987; Phillips *et al.*, 1990). The N-terminal amino acid sequences of the two proteins are similar (Lb*a*, 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 of the H61L Lb*a* mutant, ~50 h^{-1}, is in the range of that for H93G sperm whale myoglobin, which lacks a covalent bond between the heme and globin (Hargrove *et al.*, 1996a). The increase in k_{-H} 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 Lb*a* 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 inducible expression systems.

The oxygen association rate constants for leghemoglobins are very fast, of the order of 10^8 M^{-1} s^{-1}, and are close to the rate expected for a diffusion-limited association reaction with an unhindered

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model heme ($k'_{diffusion} \approx 5 \times 10^8\ M^{-1}\ s^{-1}$, Reisberg & Olson, 1980). 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 Lb*a* is also more flexible and kinetically accessible than those of myoglobin and other heme proteins (Rohlfs *et al.*, 1988). This has been demonstrated by many techniques and is exemplified by the ability of Lb*a* 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*a* 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 functional evidence for a hydrogen bond between bound O_2 and His(E7) in leghemoglobin is equivocal 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 β 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 Lb*a* and R-state human β 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 α subunit of human hemoglobin. However, the distal histidine residue is very important for retaining heme in leghemoglobin and human β subunits. Replacing the distal histidine residue with smaller apolar residues causes ⩾50-fold increases in the rate of hemin loss from met-Lb*a* and ferric β 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 Lb*a* was isolated from a soybean nodule cDNA library using anti-Lb*a* antibodies as a probe (Ji *et al.*, 1994a; Sambrook *et al.*, 1989). Phage (λ-GT11) containing the soybean Lb*a* insert were placed in *E. coli*, harvested, and the DNA isolated. Lb*a* cDNA was excised and ligated into pBluescript K+ (Invitrogen). To obtain the coding region, polymerase chain reaction (PCR) primers containing a *Nco*I site at the starting ATG codon and an *Eco*RI restriction site downstream of the TAA stop codon were used to amplify the Lb*a* gene from the pBluescript-Lb*a* 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-Lb*a* construct was used to obtain purified plasmid with the *Nco*I-Lb*a*-*Eco*RI 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 & Sligar (1987) was constructed with the following sequence of restriction sites: 5′ *Bsu*36I-*Kpn*I-*lac* promoter-*Nco*I 3′. The pACYC184-Lb*a* plasmid was cut with *Nco*I and *Bsu*36I, and the oligonucleotide containing the *lac* promoter was ligated to the Lb*a* cDNA. This *lac* promoter-Lb*a* construct was removed from pACYC184 and ligated into pEMBL19+ using the flanking *Kpn*I/*Eco*RI sites.

The initial cDNA product cloned in this manner contained two base-pair differences compared to the Lb*a* sequence reported by Hyldig-Nielson *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, pLb*a*, contains the native Lb*a* 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 Lb*a* gene.

Expression of wild-type and mutant Lb*a*s

E. coli TB-1 cells were transformed with pLb*a* 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 *Nco*I-Lb*a*-*Eco*RI 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-Lb*a* vector were grown to an absorbance of ~0.6 in 2 × YT medium at 33°C, induced with 1 mM isopropyl-β,D-thiogalactopyramoside and incubated for an additional four hours. Apo-Lb*a* was found in the supernatant and pellet of the cell lysis mixture. Protein was re-

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covered 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 Lb*a* was ~10 mg/l of medium. Reconstituted holo-Lb*a* was precipitated with 90% saturated ammonium sulfate for further purification.

Regardless of the method of expression, recombinant Lb*a* was purified as described by Jun *et al.* (1994) with the following modifications. The 90% saturated ammonium sulfate Lb*a* 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*a* 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 Lb*a* 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 Procise sequencer using a manufacturer-supplied protocol.

Crystallography of wild-type Lb*a*

Crystals of the acetate complex of wild-type metLb*a* 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*a* 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 mV and 90 mA. Crystals were in the $P2_12_12_1$ space group. The data were 90% complete at the limiting resolution of 2.2 Å, with an R_{merge} of 10.3%. Phases were determined by molecular replacement using XPLOR (Brünger, 1987) starting with the coordinates for the structure of Lb*a*-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% (R_{free} = 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 3. The coordinates of the structure of wild-type metLb*a*-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*a*

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). LbO_2 was produced by the reduction of metLb with sodium dithionite followed by G-25 chromatography in the presence of oxygen. DeoxyLb*a* was produced by the addition of several granules of sodium dithionite to a cuvette containing metLb in anaerobic buffer.

The rate constants for O_2, CO and NO binding were measured using established rapid mixing and laser photolysis methods (Olson, 1981; Quillin *et al.*, 1995; Moore & Gibson, 1976). O_2 and CO association rate constants at pH 5.0 were measured in 100 mM sodium acetate (pH 5.0). O_2 dissociation at pH 5 was measured by mixing a LbO_2 sample in 10 mM potassium phosphate (pH 7.0) with 100 mM sodium acetate (pH 5.0), equilibrated with 1 atm CO (Appleby *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 H61L Lb*a* was measured in a Gibson-Dionex stopped-flow apparatus as described by Hargrove *et al.* (1996a). Mes (2-(*N*-morpholino)ethanesulfonic acid) was used as the buffer at pH 5 for measurement of hemin dissociation because acetate coordination to metLb markedly inhibits this reaction.

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