Kinetics and Thermodynamics of Oxygen, CO, and Azide Binding by the Subcomponents of Soybean Leghemoglobin*

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Leghemoglobin shows extreme high affinity behavior in the binding of both oxygen and CO. We have determined the temperature dependence of the rate constants for ligation of oxygen and CO and from these data the thermodynamics (ΔG°, ΔH°, ΔS°) of ligation for the purified components of soybean leghemoglobin. X-ray crystallography has shown that the heme cavity can easily accommodate ligands the size of nicotinate, and analysis of extended x-ray absorption fine structure data has shown that the Fe atom is in the mean plane of the heme in the leghemoglobin-CO complex. Ligation of oxygen and CO are in accord with this picture in that the Eaq for oxygen binding is that expected for a diffusion controlled reaction and ΔS° for the ligation of both CO and oxygen is consistent with the simple immobilization of the ligand at the Fe, with no evidence for significant conformational changes in the protein or changes in solvation. At 20 °C the rate constants for oxygen and CO binding vary by 26—44% among the eight leghemoglobin components. For azide binding the variation is a factor of 2. These variations appear to arise from amino acid substitutions outside either the heme cavity or the two major paths for ligand entry to the heme. The distribution of leghemoglobin components varies with the age of the soybean nodule during the growing season. The changes in composition alone, however, would only allow the concentration of free oxygen to vary by about 3%. This finding calls into question models that ascribe functional differences between the Lbc and Lbd components and to establish precise values for kinetic and thermodynamic parameters for this model high affinity hemoglobin. Small variations between soybean Lba and unseparated Lbc were observed for oxygen equilibria (7) and for oxygen and CO binding (8, 9). However, the separated Lbc and Lbd components and Lbb were not studied with respect to oxygen and CO kinetics. We have separated soybean Lb into the eight individual components. We report the oxygen and CO association and dissociation rate constants and equilibrium constants, the activation energies, and the standard state changes in free energy, enthalpy and entropy of ligation for the separated components. The rate of azide binding has been shown to be sensitive to differences between the α and β hemes in many hemoglobinins (10), and both the kinetics and equilibria for the binding of this ligand were measured for the eight components of Lb.

MATERIALS AND METHODS

Preparation of Soybean Lb

The planting and growth of soybean plants and harvesting of soybean nodules were performed as described previously (11). Lb was prepared using the method of Appleby et al. (12) with modifications to the procedure described elsewhere (11). The separate Lbs were isolated from one large preparation of Lb derived from 500 g of nodules (fresh weight, 20,000–50,000 nodules). Lbc and Lbd were separated into their respective subcomponents using the isoelectric focusing procedures detailed by Fuchman and Appleby (5). During electrofocusing, nicotinate was used to stabilize the ferric Lb. Ampholytes and nicotinate were separated from the ferric Lb by gel filtration on a 1.5 × 70-cm column of Sephadex G-50 equilibrated with 10 mM Tris-HCl, pH 9.2, containing 0.5 mM KC1 to reduce nonspecific ampholyte binding to the Lb. Lb was exchanged into the buffer of choice by gel filtration on Sephadex G-25.

Preparation of LbO2

The oxygenated samples were prepared from CN-Met-Lb by using the following procedure modified from the method of Imamura et al. (9). A column (2.2 × 25 cm) of Sephadex G-25 was equilibrated with 3.0 m KOH, pH 7, at 4 °C for 4 h. Sodium dithionite (5 mg in 2 ml of Ar-saturated phosphate buffer) was applied to the column. After the dithionite solution had totally entered the column, CN-Met-Lb (2 ml, 0.5 to 2.5 mM) was loaded onto the column, and inactivation. Lb that is isolated from soybean nodules is a family of eight proteins. The major Lb components, Lba, Lbc1, Lbc2, and Lbc3, are probably post-translationally modified to yield the respective minor components: Lbh, Lbd1, Lbd2, and Lbd3 (2, 3). The concentration ratios of Lbc1, Lbc2, and Lbc3 + Lbc3 to Lbh vary with nodule age (4, 5) and functional differences have been proposed for the different Lb components. These changes with age apparently arise from differences in the biosynthetic rates for the components (6). Ligand binding kinetic and equilibrium experiments were carried out to investigate functional differences between the Lb components and to establish precise values for kinetic and thermodynamic parameters for this model high affinity hemoglobin. Small variations between soybean Lba and unseparated Lbc were observed for oxygen equilibria (7) and for oxygen and CO binding (8, 9). However, the separated Lbc and Lbd components and Lbb were not studied with respect to oxygen and CO kinetics. We have separated soybean Lb into the eight individual components. We report the oxygen and CO association and dissociation rate constants and equilibrium constants, the activation energies, and the standard state changes in free energy, enthalpy and entropy of ligation for the separated components. The rate of azide binding has been shown to be sensitive to differences between the α and β hemes in many hemoglobinins (10), and both the kinetics and equilibria for the binding of this ligand were measured for the eight components of Lb.
the protein was eluted, upon exposure to the air, as LbO₂. For all kinetic measurements, the concentration of Lb varied from 5 to 10 μM.

Sephadex was a product of Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), the ampholines were from LKB (Gävle, Sweden), dithionite (Marox brand) was from Holdman and Hardman, Mills Plating, Manchester, United Kingdom. The gases CO, O₂, and Ar were from Union Carbide, Linde Division (Chicago, IL), and NO was from Matheson (East Rutherford, NJ). All gases were CP grade. All samples were covered with a 3-mm layer of mineral oil to reduce the rate of oxidation by exposure to air.

**Determination of kₚₜₜ for the Binding of Azide**

A Cary 219 recording spectrophotometer with thermostated sample and reference holders was used for these determinations. A 3.0-ml aliquot of ferrous Lb in 50 mM KP, buffer, pH 7.0, was added to a cuvette at 22 °C. After the temperature had stabilized, the absorbance at 403 nm was recorded. Subsequently, additions of 1.0 mM NaN₃ in 50 mM phosphate buffer, pH 7.0, were made and the absorbance at 403 nm recorded. The equilibrium constant was determined from a least-squares analysis of a Hill plot. The final protein concentration was 10 μM.

**Instrumentation and Data Acquisition for Kinetics**

The instruments for stopped-flow and laser photolysis have been described elsewhere (14). The Nova 2-10 system was used for monitoring azide association reactions. A Nicolet model 206 digital oscilloscope was used to acquire the data with 0.5-μs resolution for the ferroazide association reactions. Data were transferred on-line to an Apple II computer for smoothing and later transferred to a SAGE II computer for least-squares fitting to mono- and bieponential decay models using the Fletcher-Powell (15) algorithm. For laser photolysis experiments, the cuvette was surrounded by water in a 4- optical port thermostated brass block. All samples in the flash cuvette were covered with a 8-mm layer of mineral oil to reduce the rate of gaseous diffusion during temperature changes. A thermistor located in the cuvette just above the monitoring light beam was used to measure the temperature at the end of a set of measurements as a function of temperature, solutions were returned to the starting temperature. Both kₚₜₜ and the relaxation constant "R" (see below) for a given sample remained unchanged for this cycle of change in temperature, indicating that no significant changes in [O₂] or [CO] occurred during the temperature dependence studies. For CO association, samples were freshly prepared for measurements at different temperatures. For the measurement of CO dissociation determined by NO replacement, a thermostated stopped-flow apparatus was built into the Cary 210 spectrophotometer, allowing double-beam spectrophotometry during the course of this slow reaction. The temperature was varied from 14 to 33 °C. After approximately three half-lives, the reaction was driven to completion by photolyzing the remaining bound CO using a high-energy photographic flash (Wabash Electroflash, type R-1140, Wabash Corp., Brooklyn, NY) also fitted into the Cary sample compartment. The Cary 210 was interfaced to an Apple II+ microcomputer and the absorbance changes as a function of time were fitted to a single exponential decay model. For a given protein, the same NO solution was used for all temperatures.

**Individual Rate Constants**

**Oxygen Combination (k') and Dissociation (k)---Each sample consisted of LbO₂ (10 μM) in 0.1 volumes of CO-saturated phosphate buffer and 0.9 volumes of air-saturated buffer. The concentrations of CO and O₂ were determined from the dilution factors and from their solubilities in aqueous solution under different ambient temperatures and pressures (16). Dye-laser photolysis was used for both O₂ combination and dissociation reactions. The oxygen combination reaction was monitored at 430 nm. The relaxation constant, R, was obtained from the second and much slower phase of the reaction measured on the same sample. The oxygen dissociation rate constant (k) was then obtained from the following equation, using a value for l' (CO association rate constant) obtained in a separate experiment, in some cases, or, at other times, by merely adding sufficient dithionite to remove oxygen from the sample:

\[ k = R(k'[O₂] + l'[CO])/l'[CO], \]

where a term involving the CO dissociation rate constant, l, has been justifiably neglected. This procedure avoided the use of dithionite in the determination of k, and allowed determinations to be made on very small volumes. Errors in k, k'[O₂], and l'[CO]. Our set of measurements was made by flowing oxy-Lb against dithionite in the stopped-flow apparatus. These experiments were carried out to obtain higher precision for Es(k) and ∆Hf for a single Lb component. LbO₂ was prepared from Met-Lb by the addition of an enzyme system (17). The oxygen concentration was raised by blowing argon over the surface of the stock solution (contained in a tonometer) for 20 min, then diluting the sample into argon-saturated buffer to a final concentration of 7.5 μM in heme. The reaction was monitored at approximately 412 nm, the Met-Lb-Lb isosbestic determined in the stopped-flow apparatus just prior to the oxygen dissociation experiment.

**CO Combination Rate Constant (k')**---The sample preparation was similar to that for the oxygen kinetic studies, except that Ar-saturated buffer, with a few granules of solid sodium dithionite, was used instead of air-saturated buffer. The combination reaction following the laser photolysis was monitored at 430 nm.

**CO Dissociation Rate Constant (l**)---This rate constant was determined as a function of temperature for four components by the replacement of CO by NO. The value was confirmed at one temperature for Lba by an independent procedure (b), below. (a) For the NO replacement method, the LbCO solution was prepared in 5 ml of Ar-saturated buffer by adding saturated CO and concentrated Met-Lb (in argon-saturated buffer) to a final concentration 10-15 μM in CO and 10 μM in Lb. A single crystal of dithionite was then added to reduce the Met-Lb to LbCO. The NO solution was prepared in a 50-ml syringe by bubbling NO through argon-saturated buffer for 5 min. The NO gas was passed first through a bubbler containing 2 M KOH. The replacement reaction that followed mixing of the LbCO and NO solutions in the stopped-flow apparatus was monitored at 415 nm. After approximately three half-times, the reaction was driven to completion by photolyzing residual CO from LbCO with a photographic flash (Wabash Electroflash) perpendicular to the observing beam. (b) Owing to some variations in apparent values of l determined with NO for other hemoglobins, an alternative procedure was devised that avoided use of NO. It is clear that a measurement of M, the LbO₂/LbCO partition coefficient (the equilibrium constant for the reaction: LbO₂ + CO ↔ LbCO + O₂) allows one to calculate l, provided one has precise values for k', l', and k. A measurement of M, however, requires that both the ratio of [LbCO]/[LbO₂] = s, and the ratio of [O₂]/[CO] = a, be known, since:

\[ M = ps = K/L = k'[O₂]/k' \]

At a total Lb concentration of 5 μM, for s approximately = 1, it is necessary that [O₂] > 250 μM if the concentration of CO is to be measured reliably, and if [CO] is to be approximately 10 times the Lb concentration for pseudo-first order conditions in the relaxation experiment. In these studies, LbO₂ was prepared by passing Met-Lb over 0.5 ml of a 1% dithionite "plug" in a Sephadex G-25 column. Stock buffers were equilibrated with oxygen and CO, and LbO₂ was equilibrated with oxygen-CO solutions such that the final dissolved gas concentrations were 1075 and 43 μM for oxygen and CO, respectively. The cuvette containing LbO₂-LbCO was sealed with a layer of mineral oil and p was determined from the spectrum of the mixture in comparison with spectra for the same concentration of Lb dissolved in buffers saturated with oxygen and CO. A photographic flash was then discharged in the Cary, photolyzing the bound CO and yielding a slow relaxation back to the initial equilibrium mixture (tLh about 30 s). The relaxation constant, R, is:

\[ R = (k'[O₂] + l'[CO])/l'[CO] + l'[CO] = (l + T/k)/(1 + T/s) \]

where T = l'/k'. Expressions 2 and 3 can be solved for s and then l obtained from l = T/k(ps), yielding:

\[ l = R/(1 + p) = pR/k = R/(1 + p) \]

for our conditions. Thus, one can obtain l from a single relaxation measurement (R) and from spectrophotometric analysis of the final equilibrium mixture, without requiring dissolved gas concentrations. The value calculated for s, however, was within 2% of that calculated from gas solubility tables and dilution factors. The difference between the value of l calculated in this manner, and that determined directly from NO replacement of CO, was 7%.
Azide Association Kinetics—Solutions of Lb (20 \mu M) and sodium azide (200 \mu M) were prepared separately in 0.1 M KP buffer, pH 7. Stopped-flow kinetic measurements were carried out at 23.5 °C with observation at 420 nm. For each measurement, 200 data points were subjected to second order polynomial smoothing in blocks of 10 points to obtain 20 smoothed points at equal time intervals over 95% of the reaction curve. Each measurement was repeated between three and eight times, the data were averaged, and the averaged data were then analyzed for the best-fitting pseudo-first order rate constant by Fletcher-Powell minimization (15). All experiments were completed within 2 h using the same stock azide solution for all samples.

Activation Energies and Thermodynamic Parameters

Activation energies for the four ferrous reactions were calculated from a linear least-squares fit of the rate constants to the Arrhenius equation. Data for each rate constant and each component at a minimum of five temperatures over the range 4–30 °C were employed for each fit, except for CO dissociation, where data at four temperatures, 14–33 °C, were collected. Data reported for 20 °C are from the Arrhenius plot regression line value at that temperature. The standard deviation is the calculated standard deviation of the rate constants from the regression line, which is larger than the standard deviation from the Fletcher-Powell fit at any single temperature. Errors in the thermodynamic parameters derived from rate constants were from the conventional error propagation equation, assuming noncorrelation of variables (18). The partition coefficient, \( M \), was determined as described above for CO dissociation. The temperature was varied from 10 to 30 °C and, because \( s ([O_2])/([CO]) \) remains essentially constant, \( M \) varied directly with \( p \). Thus, a linear least-squares analysis of a van’t Hoff plot using \( \log(p) \) yields \( \Delta H^\circ_p \).

RESULTS

Values for rate constants, equilibrium constants, standard state Gibb's free energy, enthalpy and entropy changes for ligand binding are in Tables I–III for the components of soybean leghemoglobin and for the unfraccionated mixture from which the pure components were obtained. Values for \( M \) are 87 ± 9, 84 ± 4, and 75 ± 6 for, respectively, direct dissociation, dissociation for measurement of \( k' \) and \( l' \), and dissociation for calculations from separate rate constants, using \( k = 5.7 \) and \( k = 5.1 \ s^{-1} \) for Lba. The corresponding values for \( \Delta H^\circ_p \) are –3.5 ± 0.4, –2.6 ± 1.0, and –1.8 ± 0.9 kcal/mol for 1 M standard states in aqueous solution.

The separate Lbs were obtained from a single large preparation of Lbs, and thus are averaged over at least 20,000 nodules. The errors in the rate constants for the gaseous ligands were not obtained from the variance matrix (which, owing to correlations through the time constant, underestimates errors) but from errors (standard deviations) from a regression fit to a van’t Hoff plot using all the data for a given component. For several of the minor Lbs there was only sufficient material for a single set of experiments, which involved at least five determinations at each temperature. The errors given in the table do not include errors from uncertainties in gas concentrations. Since the oxygen solutions for measurement of \( k' \) were 90% air-equilibrated, the error in oxygen must be small. Extensive tests with CO have shown that solutions can be reproduced to within 5%. Since the van’t Hoff analysis errors and the gas concentration errors are independent, one can estimate that the errors in \( k', l', K, \) and \( L \), respectively, should be no more than 5, 5, 6, and 6%. Errors in \( k \) and \( l \) do not depend on errors in gas composition, nor do the errors in activation energies and enthalpy changes. Errors in Gibbs free energies should be increased at most to 0.04 kcal/mol (Table III), but errors in the entropy changes would remain as given, since the major contribution is from the error in the enthalpy change. The errors for the azide association rate constants (Table IV) were from the variance matrix and are probably too small by about a factor of 2 owing to correlations of the residuals. The same azide solution was used for all Lbs, thus the differences do not arise from errors in azide concentration.

The fractions of the various components are known to vary with the age of the nodules (5), and that information can be reduced to three variables, \( A, B, \) and \( C \). Thus, the concentration ratios are as follows: Lbd/Lba = 0.1; Lbc/Lbd = Lbc/Lbs = 0.1; Lba/Lbc = A; Lba/Lbs = A/B; Lba/Lbc = A/C, where the variables \( A, B, \) and \( C \) can be calculated from data in the paper by Fuchsman and Appleby (5), and vary with the age of the nodule. (For instance, for age = 16 days: \( A = 0.29; B = 0.69; C = 0.41 \).) The mole fractions of the various components, \( n_c \), can then be calculated, and the fractional saturation of the total Lb population with oxygen is the following.

\[
Y = \sum n_c K_c [O_2]/(1 + K_c [O_2])
\]

Since values have been determined (Tables I and III) for both \( K \) and \( \Delta H^\circ_p \), \( Y \) can also be calculated as a function of temperature. Both the age of the nodules and the soil temperature vary throughout the growing season. In Table V are listed oxygen concentration values for three selected fractional saturations, for two representative soil temperatures, and as a function of time in terms of nodule age. It is apparent that the entire binding curve moves to the right, i.e. toward higher oxygen concentrations and lower fractional saturation as the growing season progresses and as the nodules age.

It has recently been shown (19) that there is an acid Bohr effect for LHb below pH 7 for oxygen, but not for CO binding. This effect derives entirely from changes in \( k \), such that \( k \) decreases with decreasing pH. Using data from that reference, it can be calculated that the number of Bohr protons (\( n_B \)) is 0.016 at pH 7 (see Ref. 20, where \( X^+ \) is used for \( n_B \)). Let \( K_1 \) and \( K_2 \) be, respectively, oxygen dissociation equilibrium con-

<table>
<thead>
<tr>
<th>Component</th>
<th>( k' ) ( \mu M^{-1} s^{-1} )</th>
<th>( E_a(k') ) Kcal/mol</th>
<th>( k ) s^{-1}</th>
<th>( E_a(k) ) Kcal/mol</th>
<th>( n_B )</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>116 ± 2</td>
<td>4.0 ± 0.4</td>
<td>5.72 ± 0.15</td>
<td>17.5 ± 0.5</td>
<td>49.3 ± 2.2</td>
</tr>
<tr>
<td>a*</td>
<td>113 ± 2</td>
<td>4.0 ± 0.5</td>
<td>5.11 ± 0.07</td>
<td>18.3 ± 0.3</td>
<td>48.0 ± 1.1</td>
</tr>
<tr>
<td>b</td>
<td>124 ± 2</td>
<td>3.6 ± 0.3</td>
<td>5.61 ± 0.15</td>
<td>16.7 ± 1.3</td>
<td>49.6 ± 2.2</td>
</tr>
<tr>
<td>c_1</td>
<td>98 ± 2</td>
<td>4.3 ± 0.4</td>
<td>4.88 ± 0.13</td>
<td>12.8 ± 0.3</td>
<td>39.3 ± 1.7</td>
</tr>
<tr>
<td>c_2</td>
<td>121 ± 2</td>
<td>3.1 ± 0.4</td>
<td>6.13 ± 0.16</td>
<td>15.1 ± 0.6</td>
<td>61.9 ± 2.9</td>
</tr>
<tr>
<td>d_1</td>
<td>130 ± 2</td>
<td>3.9 ± 0.2</td>
<td>5.41 ± 0.14</td>
<td>18.6 ± 0.3</td>
<td>44.7 ± 1.9</td>
</tr>
<tr>
<td>d_2</td>
<td>100 ± 2</td>
<td>4.0 ± 0.2</td>
<td>6.04 ± 0.16</td>
<td>15.1 ± 1.0</td>
<td>46.5 ± 1.9</td>
</tr>
<tr>
<td>d_3</td>
<td>121 ± 2</td>
<td>3.4 ± 0.3</td>
<td>5.23 ± 0.14</td>
<td>14.9 ± 1.1</td>
<td>43.2 ± 1.9</td>
</tr>
<tr>
<td>U</td>
<td>118 ± 2</td>
<td>4.1 ± 0.3</td>
<td>5.93 ± 0.16</td>
<td>15.2 ± 0.1</td>
<td>50.2 ± 2.2</td>
</tr>
</tbody>
</table>

\* Values for \( k \) and \( E_a(k) \) obtained from direct stopped-flow measurement.

\* Unfraccionated Lb.
TABLE II
CO rate constants, activation energies, and calculated equilibrium constants for leghemoglobins at pH 7.0, 20 °C

<table>
<thead>
<tr>
<th>Component</th>
<th>( k' )</th>
<th>( E_h(1) )</th>
<th>( l )</th>
<th>( E_l(1) )</th>
<th>( L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0.0068 ± 0.0001</td>
<td>20.41 ± 0.44</td>
<td>0.59 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.0077 ± 0.0001</td>
<td>19.01 ± 0.47</td>
<td>0.62 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>0.0074 ± 0.0001</td>
<td>21.70 ± 0.41</td>
<td>0.79 ± 0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( k' \) values are in units of \( \mu M^{-1} s^{-1} \)

\( E_0 \) values are in units of Kcal/mol

\( l \) values are in units of s^{-1}

\( E_l \) values are in units of Kcal/mol

\( L \) values are in units of nM

\* Unfractionated Lb.

TABLE III
Values for \( \Delta G^o, \Delta H^o, \) and \( \Delta S^o \) for the association of \( O_2 \) and \( CO \) with leghemoglobins at 20 °C, pH 7.0

Standard state = 1 M. To convert to a standard state of 1 atm, for oxygen, add, respectively, 3.83 Kcal/mol, -3.36 Kcal/mol, and -24.5 eu to \( \Delta G^o, \Delta H^o, \) and \( \Delta S^o \); for \( CO, \) add, respectively, 4.00 Kcal/mol, -3.10 Kcal/mol, and -24.2 eu to tabulated values. To obtain values for unitary free energy and entropy changes, add, respectively, -2.3 Kcal/mol and 8 eu to the tabulated values. Values for \( \Delta G^o \) and \( \Delta S^o \) for \( O_2 \) and \( CO \) dissolved in \( H_2O \) were obtained by fitting solubility data (16) from 2 to 30 °C, correcting for the vapor pressure of \( H_2O \).

<table>
<thead>
<tr>
<th>Component</th>
<th>( \Delta G^o )</th>
<th>( \Delta H^o )</th>
<th>( \Delta S^o )</th>
<th>( \Delta G^o )</th>
<th>( \Delta H^o )</th>
<th>( \Delta S^o )</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>-9.80 ± 0.02</td>
<td>-13.4 ± 0.9</td>
<td>-12 ± 3</td>
<td>-12.38 ± 0.01</td>
<td>-16.0 ± 0.7</td>
<td>-12 ± 2</td>
</tr>
<tr>
<td>b</td>
<td>-9.87 ± 0.01</td>
<td>-14.2 ± 0.6</td>
<td>-15 ± 2</td>
<td>-12.34 ± 0.01</td>
<td>-16.0 ± 0.5</td>
<td>-9 ± 2</td>
</tr>
<tr>
<td>c</td>
<td>-9.80 ± 0.02</td>
<td>-12.8 ± 1.8</td>
<td>-10 ± 6</td>
<td>-12.20 ± 0.02</td>
<td>-16.9 ± 0.7</td>
<td>-16 ± 2</td>
</tr>
<tr>
<td>d</td>
<td>-9.93 ± 0.03</td>
<td>-11.1 ± 1.2</td>
<td>-4 ± 4</td>
<td>-12.14 ± 0.02</td>
<td>-14.0 ± 0.8</td>
<td>-6 ± 3</td>
</tr>
<tr>
<td>e</td>
<td>-9.88 ± 0.03</td>
<td>-11.4 ± 0.4</td>
<td>-5 ± 1</td>
<td>-12.14 ± 0.02</td>
<td>-14.0 ± 0.8</td>
<td>-6 ± 3</td>
</tr>
<tr>
<td>U</td>
<td>-9.79 ± 0.02</td>
<td>-11.1 ± 0.4</td>
<td>-4 ± 1</td>
<td>-12.14 ± 0.02</td>
<td>-14.0 ± 0.8</td>
<td>-6 ± 3</td>
</tr>
</tbody>
</table>

\* Values for \( k \) and \( E_h(k) \) obtained from direct stopped-flow measurement.

\* Unfractionated Lb.

TABLE IV
Azide binding rate constants (\( T = 23.5 °C, 0.1 M KP, buffer \)) and equilibrium constants (\( T = 22 °C, 0.05 M KP, buffer \)) for leghemoglobins, pH 7.0

<table>
<thead>
<tr>
<th>Component</th>
<th>( k' )</th>
<th>( K )</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>36.36 ± 0.05</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>b</td>
<td>26.8 ± 0.07</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>c</td>
<td>22.3 ± 0.03</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>d</td>
<td>19.1 ± 0.01</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>e</td>
<td>17.1 ± 0.02</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>f</td>
<td>22.10 ± 0.05</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>g</td>
<td>19.18 ± 0.06</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>h</td>
<td>18.24 ± 0.04</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>i</td>
<td>20.62 ± 0.09</td>
<td>4.1 ± 0.1</td>
</tr>
</tbody>
</table>

\* Unfractionated Lb.

The rate constants reported here for the unfractionated Lb and for Lba are in good to excellent agreement with previously reported values. Thus, for \( k' \), in units of \( \mu M^{-1} s^{-1} \), the following values for Lba have been reported at 20 °C: 118 (reported here); 118, pH 6.8 (8); 116, pH 7.6 (19). A value of 150, pH 6.5, was reported (9) at 25 °C, which, with our activation energy, adjusts to 134 at 20 °C. For oxygen dissociation, we report here 5.72 s^{-1} by relaxation and 5.1 s^{-1} by direct stopped-flow determination using dithionite. Elsewhere, the following values have been reported, for Lba, in units of s^{-1}: 5.55 (19); 4.6 (quoted in Ref. 19 from previous work (8) at pH 7), and 9.4 (9), which was recognized as being of low precision owing to the presence of Met-Lb which would affect the stopped-flow results (9). For CO association, in units of \( \mu M^{-1} s^{-1} \), we report 11.6 for Lba. Others have reported: 12.7 (19), 12.7 (8),

\* Unfractionated Lb.

TABLE V
Concentration of oxygen \( (nM) \) at three fractional saturations in equilibrium with total leghemoglobin as a function of temperature and nodule age

<table>
<thead>
<tr>
<th>Component</th>
<th>( T = 288 K )</th>
<th>( T = 300 K )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule age</td>
<td>0.1 sat.</td>
<td>0.5 sat.</td>
</tr>
<tr>
<td>days</td>
<td>( nM )</td>
<td>( nM )</td>
</tr>
<tr>
<td>16</td>
<td>3.53</td>
<td>32.0</td>
</tr>
<tr>
<td>20</td>
<td>3.56</td>
<td>32.3</td>
</tr>
<tr>
<td>25</td>
<td>3.59</td>
<td>32.5</td>
</tr>
<tr>
<td>30</td>
<td>3.61</td>
<td>32.7</td>
</tr>
<tr>
<td>35</td>
<td>3.62</td>
<td>32.8</td>
</tr>
<tr>
<td>40</td>
<td>3.62</td>
<td>32.8</td>
</tr>
<tr>
<td>45</td>
<td>3.63</td>
<td>32.9</td>
</tr>
<tr>
<td>50</td>
<td>3.65</td>
<td>33.0</td>
</tr>
</tbody>
</table>

\* Unfractionated Lb.

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and 13.5 (9) at 25 °C, which adjusts to 11.9 with our activation energy. For the CO dissociation rate constant, 1, we report here 0.0068 s⁻¹ for Lba in good agreement with literature values: 0.0078 (19), and with a value of 0.0066 calculated from our activation energy and the value 0.012 reported at 25 °C (9). As reported under “Results,” we find excellent agreement between 1 determined by flash relaxation and by NO replacement. Furthermore, 1 determined by flash relaxation is in reasonable agreement with that determined directly using dithionite, although M and ΔH_M are in better agreement with the values calculated using 1 from the flash relaxation than with 1 determined directly in the stopped-flow apparatus. We conclude, because of the redundancies in the determinations of k and 1 and the agreements of M and ΔH_M with calculated values, as well as the excellent agreement of the values reported here for Lba with those in the literature, that the values for l, l', k, and k' form a consistent set of rate constants.

In Fig. 1 are plotted on a log-log scale values of k versus K for the eight components. The slope is 1, taken from a similar plot for diverse non-cooperative hemoglobins and myoglobins (21). In obtaining Fig. 1, a one-parameter least-squares fit was obtained where only the y intercept varied. This plot confirms, on a smaller scale, the conclusions reported elsewhere (21), that changes in oxygen affinity (K) derive almost exclusively from changes in k, the rate constant for oxygen dissociation. In a single-step model of ligation, the interpretation would be that the transition state closely resembles the reactants (22). The results of Rohlf et al. (23), however, show that at least four steps are required to describe oxygen binding in Lb, which would require a more detailed linear free energy analysis. Nevertheless, it appears that the small structure-function variations for the Lb components are in accord with the much greater differences previously analyzed for diverse hemeproteins (21).

The time course of biosynthesis of the major soybean Lb components varies with age, suggesting a difference in function for the components (4, 5). The ratio of Lba to Lbc Increases greatly in young developing nodules, whereas Lbc/Lbc Increases slightly during initial nodule growth and then levels off. During the lifetime of the nodule, the ratio of Lbc/Lbc Increases very slowly. A functional difference was suggested for the different Lb components (4, 5), and the oxygen binding of Lba and unseparated Lb toward oxygen were shown to differ (7, 8). From values of K and ΔH for all Lb components we can calculate an oxygen binding curve for the unfraccionated Lb as a function of changing composition. From the data in Table V, it can be seen that there is a progressive decrease in oxygen affinity for the total Lb of the nodule as a function of age. If one adopts a simple model for facilitated oxygen transport, such as the steady-state treatment of Wyman (24), then there is a monotonic decrease in the facilitation of oxygen transport by about 3% over the life of the nodule at constant temperature. This is a small effect and raises serious questions about the developmental importance of Lb heterogeneity in oxygen transport (25). These small changes in free oxygen resulting solely from changes in hemoglobin composition would also seem to have but a small effect on the functioning of the nitrogenase system.

Soybean Lbb differs from Lba only in the final two N-terminal residues (2). It is possible that Lbb arises post-translationally from Lba by cleavage of the N-terminal valine of Lba, followed by acetylation of alanine. For oxygen binding, Lba and Lbb appear to be identical, although they clearly differ for azide association, in that Lba binds azide about 30% more rapidly than does Lbb. The Lbd series appears to be derived from the Lbc series by post-translational modifications, in which the N termini are acetylated (3). Here, except for the Lbc/Lbd pair, acetylation does not affect oxygen binding. Lbc and Lbd differed by a factor of 1.5 in K and Lbc and Lbd differed by 6 kcal/mol in ΔH. For CO association, differences between Lbc and Lbd, although small, also appear to be significant. There is also a significant difference between Lbc and Lbd for 1. For oxygen and CO binding, Lbc and Lbd had the most aberrant behavior, having the smallest values for k', the largest for k (and hence the highest values for K), and the lowest values for l', and the highest values for L. The oxy complex of Lbc differs significantly in its visible spectra and titration behavior from oxy-Lba and 1hcr (26). For azide binding, Lba and 1hcr, differed by more than a factor of 2 in association rate constants. For azide affinity, Lba differed from LbcZ, LbcZ, and Lbd by more than a factor of two and differences between LbcZ and corresponding Lbd's were evident.

Sequences have been published for Lba (27, 28), LbcZ, (28), and Lbc and Lbd (29). The sites where there are differences among the four proteins are listed in Table VI together with locations based on the structural homologies of Lesk and Chothia (30) and the x-ray structure of soybean Lb (31). The main ligand path to the heme site is considered to comprise residues 61-65, for which there are no differences among the various Lbs. A second ligand path has been identified in Lb (32) for which the entry is at Lys-36. With the possible exception of the substitution of Val for Ala in LbcZ at location 39, there are no differences among the Lbs for these two residues.

![Fig. 1. A least squares linear free energy plot of log(k) versus log(K) for the eight components of soybean Lb. In accord with previous work (21) the slope was fixed at 1.0.](http://www.jbc.org/)
paths. As shown in the Table, there are only two sites, residues 91 and 97, where there are both heme contacts and differences among the various Lbs (31). In the case of residue 91, all of the Lbc has Ile, whereas Lb has Val. At site 97, Lba and Lbc have Val, whereas Lb has Leu. Thus, on the basis of heme contacts, one would conclude that the properties of Lbc" and Lbc should be the same. It should be recalled that Lbc has the most aberrant rate constants, and that the visible absorption spectrum of the oxy complex of Lbc" differs significantly from those of Lba and Lbc (26). The equilibrium constants for azide binding differ by a factor of 2 for Lbc" and Lbc. Lba and Lbc show significant differences in K and in the azide kinetics and equilibria. Based on proton NMR measurements and a comparison of the sequences of Lba and Lbc, and the structure of Lupin Lb, it was concluded (32) that the heme environments for Lba and Lbc differed. If these differences are attributable solely to heme contact alterations at heme contact sites, then the substitution at site 97 (Val → Ile) can affect absorption spectra, and the same substitution at site 91 can affect NMR spectra, K, and azide kinetics and equilibria. Clearly, however, many of the functional differences between Lbc" and Lbc must derive ultimately from differences outside the heme cavity and outside the two channels for ligand approach in Lb. The kinetic differences found among the Lbs are similar in magnitude to those found for diverse mammalian Mbs (33), for which no substitutions occurred in the heme cavity or along the Case and Karplus (34) ligation path.

Leghemoglobin shows pronounced R-state behavior (21) in both oxygen and CO ligation. For both ligands, a value for ΔS° can be calculated for transferring the ligand from the gas phase to the Fe-binding site. From simple statistical thermodynamic considerations, changes in translational, rotational, vibrational, and electronic entropy can be evaluated, with uncertainty arising from low frequency vibrations (35). For 1-atm standard states, ΔS° should be about -38 e.u. for both ligands. For model heme compounds, binding of both CO and oxygen give very similar values for ΔS°, typically -34 e.u., and ΔH° for CO binding is found to be about 3.5 kcal/mol more negative than for oxygen binding (36). This latter value is in excellent agreement with that reported here from direct measurement of M. Values in the literature for ΔH°M (37) often show large errors when calculated from the appropriate rate constants, or when differences between ΔH° values are taken for K and L. A recent extended x-ray absorption fine structure determination (38) shows that the Fe is essentially in the mean plane of the heme in LbCO. The x-ray structure for Lb shows a large heme cavity that easily accommodates nicotinate as a ligand. These structural findings are in accord with our thermodynamic and kinetic results suggesting that ligand binding in Lb is markedly similar to that in model compounds where little re-orientation of the protein accompanies ligation.

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