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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 carbon monoxide. We have determined the temperature dependence of the rate constants for ligation of oxygen and CO 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 Ea 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 a significant functional role to changes in the distribution of leghemoglobin components in regulating oxygen concentration in the nodule.

The abbreviation used is: Lb, leghemoglobin.

*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 Fuchsman and Appleby (5). During electrofocusing, nicotinate was used to stabilize the ferric Lb. Amyloides 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 M KCl to reduce nonspecific amphyole binding to the Lb. Lb was exchanged into the buffer of choice by gel filtration on Sephadex G-25.

Preparation of LbCo

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 Ar-saturate 0.1 M KPB, pH 7, at 4 °C for 4 h. Sodium dithionite (15 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
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 Pharmaica LKB Biotechnology Inc. (Piscataway, NJ), the ampholines were from LKB (Gaithersburg, MD), dithionite (Manox brand) was from Holdman and Hardman, Miles 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.

Gas bubbling was associated with buffer. The gases were transcribed (13). For equilibration with NO, the gas was first passed through a bubbler containing 2 M KOH. All buffers were 0.05 M potassium phosphate, pH 7, except for CO dissociation, where 0.1 M potassium phosphate, pH 7, was used. All other chemicals were reagent grade.

**Determination of Kₚ 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 ferric Lb in 50 mM KPi, 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 ferrous ligand association reactions. The 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 biexponential decay models using the Fletcher-Powell (15) algorithm. For laser photolysis experiments, the cuvette was surrounded by water in a 4-degree water bath. Dye-laser photolysis was used for both O₂ and CO association reactions. The 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 biexponential decay models using the Fletcher-Powell (15) algorithm.

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For the measurement of CO dissociation determined by 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 of 10-15 μM in CO and 10 μM in LbO₂. A single crystal of dithionite was then added to reduce the Met-Lb to LbCO. The NO solution was prepared in a 50-μl 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 reaction replacement 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. (O) Bending some variations in apparent values of R determined with NO for other hemoglobins, an alternative procedure was devised that avoided use of NO. It is clear that a measurement of R, the LbO₂/LbCO partition constant (the equilibrium constant for the reaction: LbO₂ + CO ↔ LbCO + O₂) allows one to calculate M, 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:

\[ M = ps = KL = k'/(k/l') \]

At a total Lb concentration of 5 μM, for p 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 (tₛ about 30 s). The relaxation constant, R, is:

\[ R = (k'/[O₂] + l'[CO]/[O₂] + l'[CO]) \]

where \( T = t'/T_s \). Expressions 2 and 3 can be solved for s and then l obtained from \( l = T/k(1 + T) \), yielding:

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

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%.
Table I

| Component | k' | E_a(k') | k | E_e(k') | K |
|-----------|----|---------|---|---------|---|
| a         | 116 ± 2 | 4.0 ± 0.4 | 5.72 ± 0.15 | 17.5 ± 0.5 | 49.3 ± 2.2 |
| b         | 113 ± 2 | 4.0 ± 0.5 | 5.51 ± 0.07 | 18.3 ± 0.3 | 48.0 ± 1.1 |
| c         | 124 ± 2 | 3.6 ± 0.3 | 5.61 ± 0.15 | 16.7 ± 1.3 | 49.6 ± 2.2 |
| d         | 98 ± 2  | 4.3 ± 0.4 | 4.88 ± 0.13 | 12.5 ± 0.3 | 39.3 ± 1.7 |
| e         | 121 ± 2 | 3.1 ± 0.4 | 6.13 ± 0.16 | 15.1 ± 0.6 | 61.9 ± 2.9 |
| f         | 130 ± 2 | 3.9 ± 0.2 | 5.41 ± 0.14 | 18.6 ± 0.3 | 44.7 ± 1.9 |
| g         | 100 ± 2 | 4.0 ± 0.2 | 6.04 ± 0.16 | 15.1 ± 1.0 | 46.5 ± 1.9 |
| h         | 121 ± 2 | 3.4 ± 0.3 | 5.23 ± 0.14 | 14.9 ± 1.1 | 43.2 ± 1.9 |
| i         | 118 ± 2 | 4.1 ± 0.3 | 5.93 ± 0.16 | 15.2 ± 0.1 | 50.2 ± 2.2 |

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

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

| Component | $k^\prime$ | $K$ | $E_a$ | $E_l$ | $L$ |
|-----------|------------|-----|-------|-------|-----|
| a         | $11.6 \pm 0.17$ | $4.40 \pm 0.56$ | $0.0068 \pm 0.0001$ | $0.59 \pm 0.01$ | |
| b         | $11.92 \pm 0.17$ | $4.77 \pm 0.08$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |
| c         | $12.41 \pm 0.18$ | $3.99 \pm 0.19$ | $0.0074 \pm 0.0001$ | $0.79 \pm 0.02$ | |
| d         | $9.34 \pm 0.14$ | $4.84 \pm 0.38$ | $0.0074 \pm 0.0001$ | $0.88 \pm 0.02$ | |
| e         | $12.59 \pm 0.18$ | $3.34 \pm 0.21$ | $0.0074 \pm 0.0001$ | $0.88 \pm 0.02$ | |
| f         | $15.45 \pm 0.20$ | $5.02 \pm 0.09$ | $0.0074 \pm 0.0001$ | $0.88 \pm 0.02$ | |
| g         | $9.56 \pm 0.14$ | $4.10 \pm 0.27$ | $0.0074 \pm 0.0001$ | $0.88 \pm 0.02$ | |
| h         | $13.10 \pm 0.19$ | $4.12 \pm 0.25$ | $0.0074 \pm 0.0001$ | $0.88 \pm 0.02$ | |
| U         | $11.33 \pm 0.17$ | $4.15 \pm 0.40$ | $0.0074 \pm 0.0001$ | $0.88 \pm 0.02$ | |

* Unfractionated Lb.

TABLE III
Values for $\Delta G^\circ$, $\Delta H^\circ$, and $\Delta S^\circ$ for the association of O2 and CO with leghemoglobins at 20 °C, pH 7.0

| Component | $\Delta G^\circ$ | $\Delta H^\circ$ | $\Delta S^\circ$ | $\Delta G^\circ$ | $\Delta H^\circ$ | $\Delta S^\circ$ |
|-----------|----------------|----------------|----------------|----------------|----------------|----------------|
| a         | $-9.80 \pm 0.02$ | $-13.4 \pm 0.9$ | $-12 \pm 3$ | $-12.38 \pm 0.01$ | $-16.0 \pm 0.7$ | $-12 \pm 2$ |
| a'        | $-9.87 \pm 0.01$ | $-14.2 \pm 0.6$ | $-15 \pm 2$ | $-12.34 \pm 0.01$ | $-16.9 \pm 0.7$ | $-16 \pm 2$ |
| b         | $-9.80 \pm 0.02$ | $-12.8 \pm 1.8$ | $-10 \pm 6$ | $-12.20 \pm 0.02$ | $-16.9 \pm 0.7$ | $-16 \pm 2$ |
| c         | $-7.02 \pm 0.03$ | $0.1 \pm 0.4$ | $3 \pm 1$ | $-12.14 \pm 0.02$ | $-16.0 \pm 0.8$ | $-6 \pm 3$ |
| d         | $-9.86 \pm 0.03$ | $-10.8 \pm 1.0$ | $-4 \pm 3$ | $-12.20 \pm 0.02$ | $-16.9 \pm 0.7$ | $-16 \pm 2$ |
| e         | $-9.80 \pm 0.03$ | $-11.1 \pm 1.2$ | $-4 \pm 3$ | $-12.20 \pm 0.02$ | $-16.9 \pm 0.7$ | $-16 \pm 2$ |
| f         | $-9.88 \pm 0.03$ | $-11.4 \pm 0.4$ | $-5 \pm 1$ | $-12.20 \pm 0.02$ | $-16.9 \pm 0.7$ | $-16 \pm 2$ |
| g         | $-9.79 \pm 0.02$ | $-11.1 \pm 0.4$ | $-4 \pm 1$ | $-12.20 \pm 0.02$ | $-16.9 \pm 0.7$ | $-16 \pm 2$ |

* Values for $k$ and $E_a(k)$ 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

| Component | $k^\prime$ | $K$ | $E_a$ | $E_l$ | $L$ |
|-----------|------------|-----|-------|-------|-----|
| a         | $36.36 \pm 0.95$ | $4.8 \pm 0.2$ | $0.0068 \pm 0.0001$ | $0.59 \pm 0.01$ | |
| b         | $26.98 \pm 0.07$ | $5.6 \pm 0.1$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |
| c         | $22.32 \pm 0.03$ | $5.2 \pm 0.2$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |
| d         | $19.21 \pm 0.01$ | $3.0 \pm 0.1$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |
| e         | $17.18 \pm 0.03$ | $2.8 \pm 0.1$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |
| f         | $22.10 \pm 0.05$ | $4.6 \pm 0.1$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |
| g         | $18.24 \pm 0.04$ | $3.0 \pm 0.1$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |
| h         | $20.62 \pm 0.09$ | $4.1 \pm 0.1$ | $0.0067 \pm 0.0001$ | $0.62 \pm 0.01$ | |

* 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

| Nodule age | $T = 288 K$ | $T = 300 K$ |
|------------|-------------|-------------|
| days       | nM          | nM          |
| 16         | 3.53        | 3.53        |
| 20         | 3.56        | 3.56        |
| 25         | 3.59        | 3.59        |
| 30         | 3.61        | 3.61        |
| 35         | 3.62        | 3.62        |
| 40         | 3.62        | 3.62        |
| 45         | 3.63        | 3.63        |
| 50         | 3.65        | 3.65        |

* Unfractionated Lb.

DISCUSSION
The rate constants reported here for the unfractionated Lb and for Lba are in good to excellent agreement with previously reported values. Thus, for $k^\prime$, in units of $\mu M^{-1} s^{-1}$, the following values for Lb 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),...
and 13.5 (9) at 25 °C, which adjusts to 11.9 with our activation energy. For the CO dissociation rate constant, $l$, 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 $l$ determined by flash relaxation and by NO replacement. Furthermore, $k$ determined by flash relaxation is in reasonable agreement with that determined directly using dithionite, although $M$ and $\Delta H^-M$ are in better agreement with the values calculated using $k$ from the flash relaxation than with $k$ determined directly in the stopped-flow apparatus. We conclude, because of the redundancies in the determinations of $k$ and $l$ and the agreements of $M$ and $\Delta H^-M$ 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 $l$, $t'$, $k$, 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's 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 decreases 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 Lbc toward oxygen were shown to differ (7, 8). From values of $K$ and $\Delta H^O$ for all Lb components we can calculate an oxygen binding curve for the unfractonated Lb as a function of changing composition. From the data in Table VI, 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 a small effect on the functioning of the nitrogenase system.

Soybean Lb 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 modification, in which the N termini are acetylated (3). Here, except for the Lbc/Lbd pair, acetylation does not affect oxygen binding. Lbc and Lbcl differed by a factor of 1.5 in $K$ and Lbc and Lbcl differed by 6 kcal/mol in $\Delta H^O$. For CO association, differences between Lbcl and Lbcl, although small, also appear to be significant. There is also a significant difference between Lbc and Lbc for $l$. For oxygen and CO binding, Lbc and Lbcl 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 Lb's differs significantly in its visible spectra and titration behavior from oxy-Lba and 1hcr (26). For azide binding, Lba and Lbc, differed by more than a factor of 2 in association rate constants. For azide affinity, Lba differed from Lbcl, LbcZ, and LbcZ by more than a factor of two and differences between Lb's and corresponding Lbd's were evident.

Sequences have been published for Lba (27, 28), Lbc (28), and Lbc (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 Lbs for these two locations based on the structural homologies of Lesk and Chothia (30) and the x-ray structure of soybean Lb (31). The sites where there are differences.

| Residue | Location | Leghemoglobin |
|---------|----------|---------------|
| a       | c1       | c2       |
| 1       | NA EXT   | Val  | Gly   | Gly   |
| 5       | A helix  | Glu   | Glu   | Asp   |
| 8       | A helix  | Asp   | Glu   | Glu   |
| 90      | A helix  | Ala   | Ala   | Thr   |
| 31      | B helix  | Thr   | Asn   | Thr   |
| 39      | C helix  | Ala   | Ala   | Ala   |
| 48      | CD irreg.| Ala   | Ala   | Ser   | Ala   |
| 54      | CD irreg.| Thr   | Thr   | Ser   | Thr   |
| 67      | E helix  | Ala   | Ala   | Gly   | Gly   |
| 78      | E helix  | Ala   | Thr   | Ala   | Ala   |
| 79      | E helix  | Ser   | Asn   | Asn   | Ser   |
| 84      | EF EXT   | Ala   | Ala   | Ala   | Ile   |
| 80      | F helix  | Gly   | Val   | Val   | Gly   |
| 91      | h edge   | Val   | Ile   | Ile   | Ile   |
| 97      | FG irreg.| Val   | Val   | Ile   | Ile   |
| 115     | G helix  | Ala   | Glu   | Glu   | Glu   |
| 119     | GH irreg.| Asp   | Gly   | Asp   | Asp   |
| 120     | GH irreg.| Lys   | Asn   | Lys   | Lys   |
| 127     | H helix  | Ser   | Arg   | Ser   | Ser   |
| 144     | HC irreg.| Phe   | Phe   | Phe   |

![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.](image-url)
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’s have Ile, whereas Lba has Val. At site 97, Lba and Lbc, have Val, whereas Lbc2 and Lbc, have Ile. Thus, on the basis of heme contacts, one would conclude that the properties of Lbc2 and Lbc should be the same. It should be recalled that Lbc2 has the most aberrant rate constants, and that the visible absorption spectrum of the oxy complex of Lbc2 differs significantly from those of Lba and Lbc (26). The equilibrium constants for azide binding differ by a factor of 2 for Lbc2 and Lbc. Lba and Lbc, show significant differences in $K$ and in the heme 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 Lbc2 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 Mb’s (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 $\Delta S^\circ$ 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, $\Delta S^\circ$ should be about $-38$ e.u. for both ligands. For model heme compounds, binding of both CO and oxygen give very similar values for $\Delta S^\circ$, typically $-34$ e.u., and $\Delta H^\circ$ 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 $\Delta H^\circ M$ (37) often show large errors when calculated from the appropriate rate constants, or when differences between $\Delta H^\circ$ values are taken for $K$ and $L$. A recent extended x-ray absorption fine structure determination (38) shows that 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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