The Kinetics of the Reactions of Leghemoglobin with Oxygen and Carbon Monoxide

(Received for publication, September 21, 1971)

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SUMMARY

The values of the kinetic constants for the reactions of legume root nodule hemoglobin (leghemoglobin) with oxygen and carbon monoxide have been determined by stopped flow spectrophotometry. Leghemoglobin a and leghemoglobin c, which differ in amino acid sequence and peptide chain length, have very similar kinetic constants. The oxygen combination rate constant is the largest measured for any hemoglobin. The oxygen dissociation rate constant is similar to that of mammalian myoglobins. The oxygen affinity calculated by combining the kinetic constants agrees with the equilibrium value determined directly. The rate constant for combination with carbon monoxide is large, and is reflected in the very great affinity of leghemoglobin for carbon monoxide.

The kinetic constants for the reactions of leghemoglobin with oxygen are particularly well suited to favor the facilitation of oxygen diffusion by leghemoglobin in the environment of very low mean oxygen pressure existing within the cells of the root nodule. It is suggested that leghemoglobin-facilitated oxygen diffusion serves not only to augment the influx of oxygen, but serves as well to make the oxygen pressure within the nodule or cell everywhere nearly the same.

Leghemoglobin, an oxygen-binding hemoprotein occurring in the nitrogen-fixing root nodules of legumes, was first described by Kubo (2), was the subject of studies by Keilin and Wang (3), Smith (4, 5), and by Thorogood (6), and has been characterized extensively by Ellfolk (e.g. Reference 7), and by Appleby (8). Two major components differing in molecular size and other properties may be isolated from soybean root nodules: leghemoglobin a (Ellfolk’s slow electrophoretic component) of molecular weight 15,400 (9), and leghemoglobin c (Ellfolk’s fast electrophoretic component) of molecular weight 16,800 (9). Two outstanding properties of the protein are its very great affinity for oxygen (9, 10), and the concentration at which it occurs in the root nodule. The concentration in the nodule is 0.2 to 0.5 mm (4), commensurate with the concentration of myoglobin in red skeletal muscles; the concentration in those domains within the nodule to which the protein is restricted must be several-fold greater. The function of leghemoglobin in the nodule remains unknown (for reviews see Evans and Russell (11) and Wittenberg (12)), although it is clear that there is an obligatory relation between the occurrence of leghemoglobin and the ability of a nodule to fix nitrogen (13). One question to which we address ourselves here is whether leghemoglobin may facilitate oxygen diffusion within the nodule.

Leghemoglobin in situ in the nodule is partially oxygenated. The balance is deoxygenated leghemoglobin exhibiting a typical myoglobin-like spectrum characterized by a broad absorption maximum centered near 555 nm (14). Leghemoglobin in situ undergoes reversible oxygenation (14). Leghemoglobin isolated from nodules fragmented into neutral or slightly alkaline media, is in the oxygenated state (6, 14). However leghemoglobin extracted at acid pH may be isolated in a ferrihemochromogen form which may be reduced (e.g. by dithionite) to a typical ferrihemochromogen (8, 15). Appleby (8) has shown that this hemochromogen is formed by reversible combination of leghemoglobin with a small ligand (called X)1 and that formation of leghemoglobin-X complexes is favored at slightly acid pH. Ferric leghemoglobin-X complex does not combine with carbon monoxide2 and may be expected to be unreactive toward oxygen as well.

Acetic acid, and other lower aliphatic mono- and dicarboxylic acids, exert profound effects on the optical spectra, magnetic properties, and chemical reactivity (16, 17) of ferre leghemoglobin, but are without effect on the optical spectrum of the ferrous protein. A final objective of the present study was to discover if acetic acid exerts an effect on the chemical reactivity of the ferroprotein.

1 The abbreviations used are: Lb, Lba, and Lbc, leghemoglobin and leghemoglobins a and c, respectively; Lba-X, the stable complex of leghemoglobin a with a small ligand X. LbO, Lbo, LbcO, the oxygenated and carbon monoxide forms of the ferroproteins.

2 J. B. Wittenberg, C. A. Appleby, and B. A. Wittenberg, in preparation.
MATERIALS AND METHODS

Preparation of Leghemoglobin. Lincoln strain soybeans were grown in a glasshouse at 25–20°C in a sand-vermiculite mixture inoculated with Rhizobium japonicum strain 505 (Wisconsin) and watered twice daily, with the addition of MeKnights's salt solution (18) twice weekly. Root nodules were harvested from 5-week-old plants, the leghemoglobins were extracted from them by grinding in air-equilibrated 0.1 M potassium phosphate buffer (pH 5.5 or 0.8), then centrifuging and fractionating with ammonium sulfate as described previously (8). The resulting mixed leghemoglobin solutions were dialyzed against 100 μM EDTA (pH 6.8), recentrifuged at 20,000 × g for 20 min, concentrated to a small volume by pressure filtration on Amicon UM 10 (Amicon Corp., Lexington, Mass.) membranes, and frozen in liquid nitrogen until chromatographed. For preparation of ferric Lba-X and ferric Lbc, 100 μmoles of the mixed leghemoglobins purified from nodules extracted at pH 5.5 were first passed through a column, 40 × 2.54 cm, of Sephadex G-15 (Pharmacia, Uppsala) equilibrated with 100 μM EDTA (pH 6.8), reconstituted to 15 ml over an Amicon UM 10 membrane, then chromatographed on a column, 28.5 × 5 cm, of DEAE-Sephadex type A-50 equilibrated with 13 mM sodium acetate buffer, pH 5.2, containing 100 μM EDTA, by the procedure of Appleby (8). Ferric Lba-X was eluted with 13 mM sodium acetate buffer, pH 5.2, containing 100 μM EDTA, and ferric Lbc was eluted with 0.05 M sodium phosphate buffer at pH 5.2 containing 100 μM EDTA. These purified fractions were concentrated to small volumes over Amicon UM 10 membranes, and stored in liquid nitrogen. For preparation of LboOa and ferric Lbo, the mixed leghemoglobin fraction from nodules extracted in air-equilibrated 0.1 M potassium phosphate buffer at pH 6.8 was fractionated on a similar column both equilibrated and developed with 13 mM sodium acetate buffer at pH 5.3 containing 100 μM EDTA.

Experimental Conditions—Experiments were performed in 0.05 M sodium pyrophosphate buffer (Na2HPO4 brought to pH with HCl) at pH 5.3 and 6.8, and in 0.05 M sodium acetate buffer at pH 5.3. All solutions contained 100 μM EDTA. The temperature was 20°C except in the determination of the combination rate constant for oxygen. Most measurements of this rapid reaction were made at 10°C; a few measurements were made at 3°C and the value expected at 20°C estimated by graphical extrapolation.

Apparatus—Static spectra were determined in a Cary model 11 recording spectrophotometer.

Reaction kinetics were measured in a Gibson-Milnes (19) stopped flow apparatus with a 2 cm light path in the observation cell.

Oxygen Combination Rate Constant—Anaerobic solutions of initially ferric leghemoglobin (0.5 μM) were reduced by the anaerobic addition of sodium dithionite, with an amount 4 to 7 times greater than that required for reduction of the protein. These solutions were rapidly mixed with solutions of 2.8 μM oxygen in buffer. The dithionite remaining after mixing with oxygen-containing buffer was not sufficient to consume the oxygen present. The reaction was followed at 430 nm. The nature of the product, LboOa, was confirmed by constructing a partial kinetic difference spectrum. Under the conditions used the rate of reaction of the hemoglobin with oxygen is very much greater than the rate of reaction of dithionite with oxygen, and the latter reaction did not interfere. The excess dithionite was ultimately removed by reaction with uncombined oxygen. If the concentration of dithionite (in excess of the initially ferric protein) was deliberately made greater than the final oxygen concentration, a very slow (τ = 30 sec) reduction of the formed oxyhemoglobin by dithionite became evident. The kinetic difference spectrum for this latter reaction is diagnostic for the conversion of LboOa to ferrous Lb and incompatible with any reaction of the ferric protein. This means that the ferric protein did not accumulate during the rapid kinetic process.

The solutions of oxygen in buffer were prepared by adding small amounts of air-equilibrated water to anaerobic buffer. Anaerobic buffer alone, rapidly mixed with deoxyhemoglobin solution, gave no absorbance change, indicating that it and the apparatus were in fact free from significant amounts of oxygen.

Most measurements were made at 10°C. The combination rate constant expected at 20°C was estimated from the determinations made at 10°C, using the temperature dependence of the reaction rate presented in Fig. 1. The mean of several determinations of the rate constant at 10°C is increased by the factor 1.2 to obtain the rate constant at 20°C.

Oxygen Dissociation Rate Constant—The velocity constant for the dissociation of oxygen was measured by two methods, with reasonable agreement between the results obtained.

Solutions of ferric Lb (8.8 μM), were reduced titrimetrically by the stepwise addition of dithionite to a 10% excess. The solution, held in a 30-ml syringe, was deoxygenated by a fine stream of helium bubbled through it; the syringe was closed with a rubber serum stopper, and the course of the titration monitored spectrophotometrically with the syringe barrel placed in the light beam of the spectrophotometer. These solutions were injected into a siliconeized round bottom flask containing oxygen, pO2 = 0.8 mm Hg, in helium, so that the fluid flowed smoothly in a thin layer over the glass surface and by equilibration with the gas.
reached a final free oxygen concentration of 1.5 μM. In this way, the protein was oxygenated without significant denaturation. Solutions of oxyleghemoglobin were rapidly mixed with solutions of dithionite (6 mM) in buffer (when the final pH was 6.8) or in 1 mM NaOH (when the final pH was 5.3), and the reaction followed at a wave length near 414 nm isosbestic for ferric and ferrous Lb. The isosbestic point was determined in the stopped flow apparatus for each protein under each set of experimental conditions. This precaution is required because solutions of LbOz at low oxygen pressure inevitably contain some ferric protein, and the rate of reduction of the ferric protein by dithionite is commensurate with the rate of deoxygenation. The rate of deoxygenation was essentially independent of dithionite concentration from 1.5 to 6.0 mM dithionite after mixing, and the reaction was homogeneous and first order to more than 90% completion.

In other experiments oxyleghemoglobin solutions (8.8 μM LbOz, 1.5 μM free oxygen), were rapidly mixed with solutions of carbon monoxide in buffer, and the reaction followed at 416 nm, a wave length near the absorption maximum for LbCO. The rate was nearly independent of carbon monoxide concentration from 0.5 to 0.8 mM CO after mixing. Rates of oxygen dissociation measured in this way were somewhat slower than those measured in the presence of dithionite, reflecting the limitation to the ratio of carbon monoxide to oxygen pressure achievable in these experiments.

Perreyanide is not suitable to measure the rate of dissociation of oxyleghemoglobin. In the presence of a very large excess of ferricyanide LbOz is converted to the ferrous state, at a rate dependent on the ferricyanide concentration and many-fold less than the rate of dissociation measured by other methods.

Carbon Monoxide Combination Rate Constant—Solutions of ferrous Lb (1 μM) in buffer containing 1 mM dithionite were rapidly mixed with solutions of carbon monoxide in buffer also containing 1 mM dithionite, and the reaction followed at 416 or 432 nm. The combination rate was directly proportional to the carbon monoxide concentration from 2.5 to 15 μM carbon monoxide after mixing.

RESULTS AND DISCUSSION

The rates of the several reactions are collected in Table I. Perhaps the most striking feature of the results is the very great rate of combination of leghemoglobin with oxygen. The rate constant of this reaction is greater than that of any other hemoglobin. The kinetics of the reactions of leghemoglobin are compared with those of other hemoglobins in Table II. On the other hand, oxygen dissociates from leghemoglobin at a moderate rate, which in fact is only about one-half that at which oxygen dissociates from myoglobin, Table II. The exceptionally high affinity of leghemoglobin for oxygen, therefore, is largely a consequence of very rapid combination with oxygen.

The affinity of leghemoglobin for oxygen may be calculated by combining the kinetic constants of the forward and reverse reactions. The affinities so calculated for leghemoglobin a and c agree within a factor of two with the oxygen affinity determined directly, Table III.

The combination of leghemoglobin with carbon monoxide is also very fast, being 20 times that of myoglobin (Table II). The great affinity of leghemoglobin for carbon monoxide (3) reflects the rapid rate of combination.

As has been noted before (27), the relative rates of combination with oxygen and carbon monoxide are similar for all the proteins examined, in the face of a 2000-fold difference between the largest and smallest combination rate constants (Table I).

The two major components, leghemoglobins a and c, differ in molecular size (9), amino acid composition (29), and other properties. The data presented here show that there are no large differences in the kinetic constants of the reactions of these two proteins with oxygen and carbon monoxide. We note that leghemoglobin c does combine somewhat more slowly with oxygen than does leghemoglobin a, and that the lesser oxygen affinity of leghemoglobin c may be ascribed to this difference (Table III).

The rate constants for oxygen dissociation (and for carbon monoxide dissociation) are consistently somewhat greater at pH 6.8 than at pH 5.3. The differences, although not large, exceed the probable experimental error.

No differences were noted in the rate of oxygen dissociation from oxyleghemoglobin isolated as such or regenerated from ferrileghemoglobin.

It is of interest to compare leghemoglobin with animal hemoglobins and as well with oxygen-reactive plant and yeast heme-proteins (Table II). In a general way the properties of leghemo-

### Table I

**Kinetic constants for reactions of leghemoglobin with ligands at 20°C**

| Protein | Buffer anion | pH | Oxygen Combination k' | Oxygen Dissociation k | K/k' × 10^-6 | Carbon monoxide Combination k' |
|---------|--------------|----|-----------------------|-----------------------|-------------|-----------------------------|
| Leghemoglobin a | Pyrophosphate | 6.8 | 118 | 4.4, 4.1^a | 27 | 12.7 |
| | Pyrophosphate | 5.3 | 103 | 2.8 | 37 | 12.6 |
| | Acetate | 5.3 | 110 | 2.9 | 40 | 12.0 |
| Leghemoglobin a isolated as Lba-X complex^b | Pyrophosphate | 6.8 | 119 | 4.8, 3.8^a | 25 | 12.0 |
| | Pyrophosphate | 5.3 | 120 | 3.2 | 38 | 12.8 |
| | Acetate | 5.3 | 104 | 2.9 | 36 | 12.6 |
| Leghemoglobin c | Pyrophosphate | 6.8 | 97 | 4.9 | 20 | 11.8 |
| | Pyrophosphate | 5.3 | 103 | 4.9 | 12.2 | 11.6 |
| | Acetate | 5.3 | 94 | 4.9 | 11.6 | 11.6 |

^a Determined by displacement of oxygen by carbon monoxide.

^b Leghemoglobin-a-X complex reduced with dithionite. The initially formed ferrous Lba-X complex relaxes to an equilibrium mixture containing roughly 75% of a form having a ferromyoglobin-like optical spectrum and roughly 25% of a hemochromogen form. Only the former reacts with carbon monoxide and presumptively also with oxygen.3

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Reactions of Leghemoglobin with Ligands

Vol. 247, No. 2

Comparison of rates of reactions with ligands of leghemoglobin and some other hemeproteins

| Protein                | Oxygen                   | Carbon Monoxide              | Ratio       |
|------------------------|--------------------------|-----------------------------|-------------|
|                        | $p_H$ | On, k' | Off, k | $p_H$ | On, k' | Off, k | $k'/k$ |
| Myoglobin              |        |        |        |        |        |        |         |
| Horse                  | 0.7    | 14     | 11    | 0.019 | 0.5    | 0.017  | 28      |
| Aplysia                | 2.65   | 15     | 70    | 0.025 | 0.5    | 0.02   | 30      |
| Human hemoglobin       |        |        |        |        |        |        |         |
| $\alpha$ chains (--SH) | 0.46   | 50     | 28    | 0.0025 | 4.6 | 0.013  | 11      |
| $\beta$ chains (--SH)  | 0.40   | 71     | 16    | 0.0016 | 4.6 | 0.008  | 15      |
| Tryptophan dioxygenase |        |        |        |        |        |        |         |
| Ascaris                |        |        |        |        |        |        |         |
| Perienteric            | 0.002  | 1.5    | 0.0041 | 0.1       | 0.11  | 0.018  | 8.8     |
| Body wall              | 0.11   | 1.2    | 0.23   | 0.13      | 0.22  | 0.039  | 5.5     |
| Peroxidases            |        |        |        |        |        |        |         |
| Horse radish           | 0.058  | <0.007 |        | 0.0034   | $1.0 \times 10^{-4}$ | < $10^{-4}$ | 17     |
| Cytochrome c$^a$       | 0.040* | 118    | 4.4   | 0.001*   | 12.7 | 9.8    | 8.2     |
| Leghemoglobin $\alpha$ | 0.068* | 97     | 4.9   |          |       |        |         |
| Leghemoglobin $c$      |        |        |        |          |       |        |         |

* Values reported by Antonini (20).
* Values reported by Wittenberg et al. (21).
* Values reported by Brunori et al. (22) and by Noble et al. (23).
* Values reported by Ishimura et al. (24).
* Values reported by Gibson and Smith (25) and Okazaki and Wittenberg (26).
* Values reported by Wittenberg et al. (27) and by Wittenberg et al. (28).
* B. A. Wittenberg and J. B. Wittenberg, unpublished results.
* Values reported by Appleby (10).
* Estimated from values reported by Keilin and Wang (3) and by Appleby (10).

Equilibrium constants for reaction of leghemoglobin with oxygen

Values determined at pH 6.8 or 7.0.

| Protein          | Equilibrium measurement | Kinetic measurement |
|------------------|-------------------------|---------------------|
|                  | $p_H$ | calculated from | $k'/k$ |
|                  | mm Hg | Column 1 | Column 3 |
| Leghemoglobin $\alpha$ | 0.040 | $1 \times 10^8$ | 0.021 | $27 \times 10^4$ |
| Leghemoglobin $c$ | 0.068 | $8.1 \times 10^8$ | 0.028 | $20 \times 10^8$ |

* From Appleby (10).
* These were improperly calculated in the original publication (10).

Leghemoglobin unquestionably plays an important role in the economy of the root nodule, in which it is found in abundance. Virtanen et al. (13), Keilin and Smith (4, 30), and Wilson (31), were among the first to point out the obligatory relation between the occurrence of leghemoglobin and the ability to fix nitrogen. Lund and Wilson (32) have shown that nitrogen fixation in clover nodules is inhibited by CO concentrations about one-tenth those required to inhibit fixation in aerobic free living bacteria such as Azotobacter. This inhibition is often ascribed (33) to a specific effect on nodule leghemoglobin. Nodules respire vigorously at a rate which is diffusion limited (5), and the Rhizobium bacteria are responsible for much of this respiration (34). The uptake of oxygen by thin slices of nodules is halved under conditions of low carbon monoxide pressure where the leghemoglobin is bound in combination with carbon monoxide (35). These facts lead us to enquire whether leghemoglobin, by facilitating oxygen diffusion, may aid the entry of oxygen into the nodule.

The ability of leghemoglobin to facilitate oxygen diffusion has not been tested experimentally. However a protein with similar kinetics and equilibria of oxygen binding, hemoglobin II (Table II, human $\beta$ chains) facilitates oxygen diffusion freely and to the same extent as does normal human hemoglobin A (36). Each protein combines avidly with oxygen, and in each instance the great oxygen affinity is in large part ascribable to very rapid combination with oxygen. Under conditions of adequate oxygen...
pressure the kinetic limitation to the rate of facilitated diffusion is oxygen dissociation (12). The rate of dissociation of oxygen from leghemoglobin is moderately large, about one-half that of myoglobin, and is certainly sufficient to support facilitated diffusion.

The properties of leghemoglobin are ideally suited to favor the facilitation of oxygen diffusion in an environment of very low mean oxygen pressure. Both combination and dissociation rate constants are adapted to this end. The requirements are that the protein be largely saturated with oxygen at the high pressure boundary of the system, near the periphery of the plant cell or root nodule. Simultaneously it must also be largely desaturated at the low pressure boundary; for in the absence of a gradient of oxygenation, the facilitated flux vanishes. Appleby (14), taking advantage of leghemoglobin as an internal indicator of oxygen pressure, estimates that the mean oxygen pressure within a nodule is of the order of 0.01 mm Hg, which is a very low pressure. It is, however, sufficient to support a vigorous oxygen uptake by the Rhizobium bacteroids. The rate constant for the combination of leghemoglobin with oxygen is the largest encountered in any hemoglobin; saturation of leghemoglobin at the periphery of the nodule is thereby assured. The dissociation rate constant, which can be calculated as the rate of oxygen uptake by the nodule is equal to the rate of oxygen dissociation. The mean, volume-averaged, fractional saturation of leghemoglobin within a working nodule is about 20% (14). It is interesting that this is of the same order as the volume-averaged saturation of myoglobin in an operating mammalian heart or red muscle (12). At steady state, the rate of oxygen combination at any point with the cell is equal to the rate of oxygen dissociation. The mean turnover rate (known from the mean fractional saturation, hemoglobin concentration, and the dissociation rate constant), interestingly, is about the same in the root nodule as in mammalian red muscle.

A different set of properties is demanded of muscle myoglobin, and leghemoglobin would be altogether unwieldy to facilitate oxygen diffusion within a muscle. The protein would of course be saturated at the high pressure boundary of the system where the oxygen pressure is 20 mm Hg (12). But, by virtue of the large combination rate constant it would remain saturated all the way to the low pressure boundary of the system at the mitochondrial where the estimated oxygen pressure is 12, although small, is probably not small enough to significantly desaturate leghemoglobin. In the absence of a gradient of oxygen saturation the hemoglobin could make no contribution to the oxygen flux.

Leghemoglobin may serve to meet an unique and very stringent requirement of the nitrogen-fixing system. The potential rate of oxygen uptake by the nodule is very great (5), and much of the oxygen is used by the nitrogen-fixing bacteroids (34). This oxygen must be supplied at a very low pressure because nitrogen fixation by (isolated) bacteroids is abolished by prolonged exposure to oxygen at moderate pressure (37). We offer the suggestion that leghemoglobin, by facilitating the movement of oxygen, may serve to flatten out the gradient of oxygen pressure from periphery to center of the system. The action of leghemoglobin would be to make the oxygen pressure everywhere nearly the same, in a manner reminiscent of the operation of a heat pipe in which molecular motion tends to make the temperature everywhere where the same (36). A glut of oxygen at the periphery and a shortage in the center will be avoided.

Acknowledgments—Part of this work was performed at the Division of Plant Industry, C.S.I.R.O., Canberra, Australia. Soybeans were grown, nodules harvested, and some early steps in the extraction and purification of leghemoglobin were done there. We thank Mrs. L. Grivalds for her help in this phase of the work. We thank Dr. Robert W. Noble for his helpful discussions.

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J. Biol. Chem. 1972, 247:527-531.

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