Leghemoglobin

II. CHANGES IN CONFORMATION AND CHEMICAL REACTIVITY LINKED TO REACTION WITH A DISSOCIABLE LOW MOLECULAR WEIGHT LIGAND, X*

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SUMMARY

Leghemoglobin may be isolated from soybean root nodules as a complex, leghemoglobin a-X of ferric leghemoglobin with a low molecular weight ligand X. That complex dissociates at neutral or alkaline pH and the ligand X may be separated from free leghemoglobin by ultrafiltration. The complex is regenerated by combining ferric leghemoglobin with X. The affinity of ferrous leghemoglobin for X is very much less than the affinity of ferric leghemoglobin for X. The optical spectrum of leghemoglobin a-X closely resembles that of a number of b-cytochromes. The evidence does not distinguish whether X binds to leghemoglobin at the heme iron atom or elsewhere.

Reaction of ferric leghemoglobin a-X with dithionite yields ferrous leghemoglobin a-X. Ferrous leghemoglobin a-X dissociates slowly into free ferrous leghemoglobin and X; first order rate constant = 0.036 s⁻¹. Ferrous leghemoglobin a-X reacts with carbon monoxide only after prior dissociation of the ligand X from leghemoglobin. In this study we show that the spectral entity, Lba-X, is indeed a complex formed by reversible binding of a small ligand, X, to ferric native leghemoglobin.

We show that binding of X is linked to a change in conformation at the heme. We consider the possibility that binding of X at a site remote from the heme changes the conformation near the heme so that the ε-aminogroup of a lysine residue is brought into apposition with the heme iron atom, to which it ligates. Binding of X is linked to a change in the rate of reduction of the ferric protein by dithionite. Binding of X precludes combination of ferrous leghemoglobin with carbon monoxide.

MATERIALS AND METHODS

Leghemoglobin—The preparation of the proteins used has been described (2). The preparation of the Lba-X complex used here was estimated from optical spectra to contain about 60% of the liganded species, Lba-X. Lb concentration was determined as the pyridine hemochromogen (3).

Sodium Dithionite Solutions—Solid sodium dithionite (Mallinckrodt) was dissolved in air-free 1 mM sodium hydroxide and used the same day. Except as noted the concentration was 1 g per liter or approximately 10 mM.

Static Spectra—These were determined with a Cary model 11 recording spectrophotometer.

Reaction Kinetics—A Gibson-Milnes (4) stopped flow apparatus with a 2-cm path in the observation cell was used for all measurements. Wave lengths frequently used were: 430 nm, near the absorption maximum of ferroleghemoglobin, 420 nm near the absorption maximum of the ferroleghemoglobin-X complex at 418.5 nm, 416 nm near the absorption maximum of carbon monoxide leghemoglobin, and 426.5 nm which is isosbestic for the slower of the two kinetic events when leghemoglobin-X complex reacts with dithionite.

Kinetic Difference Spectra—These express the change in millimolar extinction coefficient observed during the course of the kinetic process as a function of the wave length of the observation light.

Conditions of Kinetic Experiments—Experiments were carried...
Buffers were: 50 mM sodium pyrophosphate buffer (Na₄P₂O₇ brought to pH with HCl) at pH 5.3 and 6.8 and 50 mM sodium acetate buffer at pH 5.3. The concentration of Lb was 4.4 or 44 μM for measurements in the Soret and visible regions, respectively. All solutions contained 100 μM EDTA.

Carbon monoxide, when used, was 1 mM in the buffer in which the proteins was dissolved.

Dissociation of X from Leghemoglobin—All operations were performed at 0°. A solution of ferric Lba-X prepared in 13 mM sodium acetate, pH 5.3, was freed from buffer by three times concentrating the solution to a gel over an Amicon UM 2 membrane, and diluting to the original volume, 10 ml, with glass-distilled water. The total Lb concentration was 400 μM, and analysis of the optical spectra of a portion diluted into 0.1 mM MES-NaOH buffer, pH 5.2, indicated that 65% of this was in the form Lba-X and that little or no X was lost during the washing procedure.

Cold sodium hydroxide solution, about 1 ml of 10 mM was added to 9 ml of the Lba-X solution to give a final pH of 9.0. This solution was concentrated to a gel with water, once again adjusted to pH 9.0, and reconcentrated to a gel. The resulting, "stripped" Lba was made to 5.8 ml in 0.1 mM MES-NaOH buffer, pH 5.2, and the optical spectra of both ferrous and ferric forms determined.

The filtrates from the Amicon cell were neutralized to pH 5.2 with HCl, pooled, and freeze-dried. The residue was dissolved in glass-distilled water to a final volume of 1.0 ml. If all of the ligand X, originally bound to the Lba, survived these manipulations, the expected concentration of the final solution would be 2.35 mM X.

Reconstitution of Lba-X—The reaction mixtures, 1.0 ml in 10-mm light path cuvettes at 20° contained: "native" Lba 38.5 μM, or "stripped" Lba, 40 μM; 0.1 mM MES-NaOH buffer, pH 5.2; and 0 to 100 μl of the solution containing X. The spectrum of the ferric protein was recorded 1 min after dilution. Sodium dithionite was then added anaerobically to a final concentration of 1 g per liter, and the spectrum of the ferrous protein recorded after a delay of 3 min to permit relaxation to a stable equilibrium between ferrous Lba, X, and ferrous Lba-X.

Binding of Ligand X to Ferric Lba in Presence of Cyanide—A solution of ferric Lba-X (0.5 pmole) in 10 mM sodium acetate buffer, pH 5.2, containing potassium cyanide (1 mM) was chromatographed on a column, 45 × 1.5 cm, containing Sephadex G-10 (Pharmacia) equilibrated with the cyanide-containing buffer. As a control a similar solution of ferric Lba-X in acetate buffer, but without cyanide, was chromatographed on a similar column in the absence of cyanide. Two control experiments show that the column is adequate to separate Lba from dissociated free X. Ferric Lba-X brought to pH 8.5, and passed over a Sephadex column, is separated into its components ferric Lba and X.

To show the presence of Lba-X in these solutions portions of each were diluted to a total Lba concentration of 50 μM and solid dithionite added. Under these conditions, cyanide dissociates from the heme (1) and ferrous Lba-X is revealed by the appearance of its spectrum with an intense absorption at 554 nm.

RESULTS

Dissociation and Reconstitution of Lba-X—Ultrafiltration of a solution of Lba-X, brought to alkaline pH where the complex dissociates, separated the filterable ligand X from ferric Lba.

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sorbance between an arbitrary time zero and completion of the reaction; $\Delta A_t$ is the change in absorbance at time $t$. $O_2$, carbon monoxide absent, rate = 49.0 $s^{-1}$. $\bullet$, carbon monoxide (1 mM before mixing) present in the hemoglobin solution. Rate = 49.7 $s^{-1}$. 50 mM pyrophosphate buffer, pH 5.3, 20°C.

Fig. 4 (right). Progress of the slower reaction seen when ferric leghemoglobin-X complex reacts with dithionite (1 g per liter). $\Delta A_t$ is the change in absorbance at time $t$. $O_2$, carbon monoxide absent. The accumulated final product is a mixture of ferrous Lba and ferrous Lba-X; rate = 0.036 $s^{-1}$. $\bullet$, carbon monoxide (1 mM before mixing) present in the hemoglobin solution. The accumulated product is carbon monoxide leghemoglobin; rate = 0.037 $s^{-1}$. 50 mM pyrophosphate buffer, pH 5.3, 20°C.

and at 465, 540, 562, and 680 nm in the reactions of ferric and ferrous Lba, respectively, show that only two reactive species, free Lba and its complex Lba-X are present in the solutions. The relation between the extent of spectral change and the amount of ligand X added suggests that formation of ferric Lba-X was substantially complete at the highest concentration of X attained in this experiment, but that formation of ferrous Lba-X fell somewhat short of completion.

Binding of Ligand X to Ferric Lba in Presence of Cyanide—Addition of cyanide (1 mM) converts ferric Lba-X to a spectral species indistinguishable from the cyanide derivative of ferric Lba. This species was passed over a Sephadex column adequate to separate ferric or ferrous Lba from the dissociated, free ligand X. Dithionite, added to the effluent, immediately gives the ferric cyanide derivative of Lba which, in short time, decays by dissociation of cyanide to leave a solution containing ferrous Lba-X, revealed by its characteristic spectrum. Some ferrohemochrome-forming ability, bound X, is lost on the column. The present preparation of Lba-X, chromatographed in the presence of cyanide, retained 40% of its ferrohemochrome-forming ability. Chromatographed in the absence of cyanide it retained 65% of its ferrohemochrome-forming ability. This result implies that the ligand X remains bound to the ferric protein when the distal ligand position of the heme iron atom is occupied by cyanide.

Spectra of Complex Lba-X—The spectrum of the complex ferric Lba-X, presented in Fig. 1, is that of a ferric hemochrome. The optical spectrum of the ferrous complex, Fig. 2, is that of a ferrous hemochrome, closely resembling the spectra of many b-cytochromes, with which it is compared in Table I.
Fig. 7. Direct spectra of the rapidly formed product of dithionite reduction of Lba-X reconstructed by combining kinetic difference spectra with static spectra. ⊗, kinetic difference spectrum of the more rapid reaction plus the spectrum of ferric Lba-X. ●, spectrum of the accumulated mixture of ferrous Lb and ferrous Lba-X minus the kinetic difference spectrum of the slower reaction.

Fig. 8. Direct spectra of the rapidly formed product of Lba-X reacted with dithionite in the presence of carbon monoxide. Spectra reconstructed by combining kinetic difference spectra and static spectra. ⊗, kinetic difference spectrum of the more rapid reaction plus the spectrum of ferric Lba-X. ●, spectrum of carbon monoxide ferrous Lb minus the kinetic difference spectrum of the slower reaction.

DISCUSSION

To understand the role of leghemoglobin in symbiotic nitrogen fixation it is important to know the state of leghemoglobin in the functioning legume root nodule. An early preparation of soybean leghemoglobin, isolated by Ellfolk and Sievers (11) from nodules extracted at pH 5.6 had the low spin signature optical

| Table I |

Spectral constants of ferrous leghemoglobin a-X complex compared with those of other hemeproteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\lambda_{max}$</th>
<th>$\epsilon_{max}$</th>
<th>$\lambda_{max}$</th>
<th>$\epsilon_{max}$</th>
<th>$\lambda_{max}$</th>
<th>$\epsilon_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome $b_5^+$</td>
<td>427</td>
<td>147</td>
<td>530</td>
<td>14.2</td>
<td>559</td>
<td>24.4</td>
</tr>
<tr>
<td>Cytochrome $b_5^-$</td>
<td>425</td>
<td>127</td>
<td>527.5</td>
<td>15.2</td>
<td>557.5</td>
<td>25.1</td>
</tr>
<tr>
<td>Cytochrome $b_5^+$</td>
<td>424</td>
<td>183</td>
<td>528</td>
<td>15.6</td>
<td>556.5</td>
<td>26.9</td>
</tr>
<tr>
<td>Cytochrome $b_5^-$</td>
<td>423</td>
<td>171</td>
<td>526</td>
<td>13.4</td>
<td>556</td>
<td>25.6</td>
</tr>
<tr>
<td>Spirula hemeprotein</td>
<td>423.5</td>
<td>164</td>
<td>538</td>
<td>19.7</td>
<td>558</td>
<td>28.7</td>
</tr>
<tr>
<td>Leghemoglobin a-X</td>
<td>418.5</td>
<td>121</td>
<td>525</td>
<td>16.4</td>
<td>554</td>
<td>32.8</td>
</tr>
</tbody>
</table>

* From Fujita et al. (2).
* From Deeb and Hager (6).
* From Appleby and Morton (7) and Pajot and Groudinsky (8).
* From Strittmatter and Velick (9).
* From Strittmatter and Burch (10).

In this preparation Lb was not completely saturated with the ligand X.

The question arises whether the ligand X is bound to the heme iron atom or elsewhere in the leghemoglobin molecule. A partial answer to this question is given by the experiment in which we demonstrate simultaneous binding of X and of cyanide to ferric leghemoglobin. The optical spectra of the doubly liganded complex show that the cyanide is almost certainly ligated to the heme iron atom. This suggests that the ligand X binds elsewhere than at the heme iron atom. If so the optical spectral change accompanying binding reflects a conformational change near the heme linked to binding of X. On the other hand we cannot neglect the possibility that the ligand is bifunctional, and that cyanide displaces one functional group of X from combination with the heme iron atom, leaving the other functional group attached to the protein.

The optical spectra of the complex, Lba-X, hitherto accessible only in mixtures with aquo ferric Lba (1), are reported here (Table I). They are learned directly by reconstituting the complex Lba-X from leghemoglobin and excess ligand X (Figs. 1 and
and may be discovered by combining kinetic difference spectra with static spectra (Figs. 7 and 8). The spectrum of ferrous Lba-X is very similar to those of a number of b-cytochromes, with which it is compared in Table I. Spectra of this type imply that nitrogenous (or sulfur) ligands occupy the proximal and distal ligand positions of the heme iron atom. Elsewhere we discuss the nature of the ligands to the heme iron atom of Lba-X.

Two kinetic events are seen in the reaction of ferric Lba-X complex with dithionite; a relatively rapid kinetic event is followed by a very much slower subsequent event. The rapid event yields a product which is identified by its optical spectrum (Figs. 7 and 8) as ferrous Lba-X. The rate of this reaction is not affected by the presence of carbon monoxide.

The product of the slow kinetic event is evidently a mixture of ferrous Lba-X and ferrous leghemoglobin. In view of the relatively low affinity of ferrous leghemoglobin for the ligand X, we interpret the slow kinetic event as a relaxation of the complex ferrous Lba-X into an equilibrium mixture of ferrous Lba-X and ferrous leghemoglobin. In the presence of an overriding concentration of carbon monoxide, such that ferrous leghemoglobin is removed by reaction with carbon monoxide 700 times more rapidly than the kinetic event of interest, the rate of the slow kinetic event was not changed, although the product has become carbon monoxide-ferrous leghemoglobin. It follows that the complex, Lba-X, reacts with carbon monoxide at a rate dictated by the rate of dissociation of Lba-X (Reaction 2, below). We conclude that the complex, Lba-X, reacts with carbon monoxide only after prior dissociation to ferrous Lba and X. This sequence of reactions may be formulated:

\[
\begin{align*}
\text{Ferric Lba-X + dithionite} &\rightarrow \text{ferrous Lba-X} & \text{rate} = 51 \text{ s}^{-1} \\
\text{Ferrous Lba-X} &\rightarrow \text{ferrous Lba + X} & \text{rate} = 0.036 \text{ s}^{-1} \\
\text{Ferrous Lba + CO} &\rightarrow \text{LbCO} & \text{rate} = 10^4 \text{ s}^{-1}
\end{align*}
\]

In addition we have measured the rate of reduction of free, unliganded, ferric leghemoglobin by dithionite, Reaction 4:

\[
\text{Ferric Lba + dithionite} \rightarrow \text{ferrous Lba} & \text{ rate} = 24 \text{ s}^{-1}
\]

The rate differs from the rate of reduction of the complex, Lba-X (Reaction 1).

In the presence of carbon monoxide, which removes one product, Reaction 2 is a true first order reaction and the rate gives a measure of the first order rate constant, \(k = 0.036 \text{ s}^{-1}\), for the dissociation of ferrous Lba-X.

If indeed the ligand X binds to leghemoglobin at a site remote from the heme, then we may consider that the configurational change, expressed in the optical spectrum, and the changes in chemical reactivity of the heme toward dithionite (Reaction 4) and toward carbon monoxide are allosterically linked to binding of X. This linked function provides an opportunity for metabolic control of the oxygen affinity of leghemoglobin in situ in the nodule, but we cannot form an opinion as to whether such control is actually exercised until the ligand X is identified and its concentration in the nodule tissue established.

REFERENCES


\[\text{rate} = \frac{1}{100} \text{ s}^{-1}\]