Oxyleghemoglobin-mediated Hydrogen Oxidation by *Rhizobium japonicum* USDA 122 DES Bacteroids

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DAVID W. EMERICH, STEVE L. ALBRECHT, STERLING A. RUSSELL, TEMAY CHING, and HAROLD J. EVANS

Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

Oxyleghemoglobin was used to supply low concentrations of O2 to H2-oxidizing bacteroids from *Rhizobium japonicum* USDA 122 DES. The H2 oxidation system of these bacteroids was capable of effectively utilizing O2 at the low concentrations of O2 expected to be found in soybean nodules. Apparent Km values of approximately 10 nanomolar O2 have been calculated for the oxyhydrogen reaction. These values include the Km values for both H2 oxidation and endogenous substrate oxidation. Even in the presence of oxyleghemoglobin, H2 additions stimulated C2H2 reduction, reduced the rate of endogenous respiration and maintained the ATP contents of bacteroids. In our reconstituted oxyleghemoglobin and bacteroid system, we estimate that the H2 oxidation system is capable of recycling all of the H2 evolved during the N2 fixation process.

Certain strains of *Rhizobium japonicum* (10) possess a H2 oxidation system that is capable of recycling the H2 that is evolved during the N2-fixing process (11). H2 evolution during N2 fixation is thought to be effectively utilized by the bacteroids. O2 reduction by nitrogenase provides O2 for utilization by the H2 oxidation system in bacteroids, but we do not imply that the interaction of the H2 oxidation system with oxyleghemoglobin is direct.

MATERIALS AND METHODS

Suspensions of washed bacteroids were prepared and amperometric assays for H2 and O2 uptake were conducted as described previously (11). A specifically constructed chamber was used for the simultaneous measurement of the rate of H2 oxidation and the rate of deoxygenation of oxyleghemoglobin (see Fig. 2, inset). This chamber consisted of a cylindrical glass tube (3.5-× 1.2-cm i.d.) connected to the top of a 3-ml rectangular glass cuvette equipped with a side arm (3.2-× 0.2-cm i.d. at an upward angle of 130°). The side arm was attached to the cuvette 1 cm above the base of the cuvette. The H2 or O2 electrode was placed into the cuvette through the cylindrical tube extension of the cuvette and sealed with a piece of butyl tubing by placing it over the end of both the electrode and the cuvette extension. A serum stopper was fitted over the end of the capillary arm of the cuvette and all additions were made by injection through the serum stopper with a hypodermic syringe and needle. The modified cuvette was placed into the sample compartment of a Cary 118C spectrophotometer equipped with a motor driven variable speed stirring magnet (MDC International, Corvallis, Oregon) mounted underneath the sample cuvette. The final volume of the modified cuvette complete with stirring star (Cary Instruments) was 3.1 ± 0.1 ml. The variation in volume was caused by differences in the distance that the electrode was inserted into the cuvette opening. After gassing with N2, through the capillary side arm, oxyleghemoglobin (see legends for concentration) in 50 mM Hepes (pH 7.5) with 2.5 mM MgCl2 and 1.0 mM KH2PO4 (HMP buffer) was added to the cuvette. H2 was added as a dissolved gas in the leghemoglobin-buffer solution. Before the bacteroid suspension was added to the cuvette, the spectrum between 625 and 485 nm was recorded. A suspension of bacteroids in HMP buffer was added to the cuvette to initiate the assay. The assay was stirred constantly during the time course of the reaction. Spectral changes were recorded either...
at 574 or 541 nm. Data collected by measurements at either wavelength yielded essentially identical results. The leghemoglobin spectrum was recorded again after the assay was completed to determine the extent of oxygenation of the leghemoglobin. Na₂S₂O₄ (37 μM) was then added to ensure that complete deoxygenation was obtained.

While preparing this manuscript, a paper by Bergersen and Turner (8) was published in which they described a method of supplying low concentrations of free dissolved O₂ to bacteroids by the deoxygenation of oxyleghemoglobin. Their method is essentially the same as ours although an entirely different apparatus was utilized. We have confirmed the validity of the method and have extended it for investigating the H₂ oxidation system of *R. japonicum* bacteroids. The determination of free dissolved O₂ concentrations from the oxygenation state of leghemoglobin and the extinction coefficient used in the calculations were the same as those reported by Bergersen and Turner (8).

Concurrent assays of C₃H₄ reduction, CO₂ evolution, H₂ oxidation, and ATP content of bacteroids were conducted in nominal 20-ml vaccine bottles containing 2.4 ml HMP buffer. The bottles were evacuated and flushed three times with N₂ that had been passed over BASF catalyst R3-11 (Chemical Dynamics Corp.) to remove traces of O₂. The gas atmospheres over reactions were prepared as described previously (11). The assays were initiated by injection of the anaerobically prepared bacteroid suspensions (100 μl) into the bottles. H₂ oxidation was assayed by withdrawing a 0.5 ml gas sample at 0 and 30 min of incubation at 23 C with shaking (150 cycles/min). H₂ was analyzed gas chromatographically with a Hewlett-Packard model 5830A gas chromatograph which was equipped with a thermal conductivity detector and a 6.4 mm x 2 m column of Molecular Sieve 5 Å operated at 120 C. N₂ served as the carrier gas. C₃H₄ formation and CO₂ evolution were measured after termination of the experiment. The reactions were allowed to run for 30 min then were stopped by the addition of 2.5 ml ice-cold 0.80 M HClO₄. The bottles were immediately placed in ice and stored there until the nucleotide extraction could be done. Samples of 0.5 ml were withdrawn from the assay bottles and C₃H₄ and CO₂ were measured by gas chromatography. C₃H₄ formation was analyzed on a Varian 600D gas chromatograph equipped with a flame ionization detector and a 6.4 mm x 1.5 m column of Porapak N operated at 50 C with N₂ as the carrier gas. Samples were analyzed for CO₂ by use of a Carle gas chromatograph equipped with a thermal conductivity detector and a 6.4 mm x 1.5 m column of Porapak Q operated at 75 C. For these assays, helium was used as the carrier gas. ATP extraction and measurements were carried out as described previously (11).

Leghemoglobin was purified essentially as described by Appleby et al. (4) except that an initial step was included in which the leghemoglobin was removed from the crude nodule extract by a 60-80% (NH₄)₂SO₄ precipitation. The purified leghemoglobin fractions were combined after the final purification step. The concentration of the final product was determined as described by Appleby et al. (4).

Hepes was purchased from Sigma. H₂O, O₂, and N₂ were of the highest purity available from Airco Industrial Gases, Vancouver, Washington. C₃H₄ was generated from CaC₂ and water. Luciferin and luciferase were prepared from Sigma FTE-50 firefly tails. DEAE-cellulose (DE52) was obtained from Whatman. Sephadex G-75 was purchased from Pharmacia Fine Chemicals. All salts used were analytical grade.

**RESULTS AND DISCUSSION**

**Oxyleghemoglobin as O₂ Donor for H₂ Oxidation.** Oxyleghemoglobin functions as a donor of O₂ to the H₂ oxidation system supplying free-dissolved O₂ at concentrations that are not accurately measured by our amperometric method (Fig. 1). A linear rate of H₂ oxidation was maintained for approximately 4 min after all amperometrically detectable O₂ had been consumed (Fig. 1). The rate of H₂ oxidation began to decrease after the 4-min interval and eventually stopped 7 min after exhaustion of the O₂ that could be detected amperometrically. The H₂ oxidation reaction proceeded perceptibly at concentrations of O₂ below 400 nm (Fig. 1).

Several fixed time assays were conducted in an effort to estimate the apparent *Kₘ* for O₂ supplied via oxyleghemoglobin. The assays were initiated in the amperometric chamber (11) and the reaction rate was followed for a period of time. At the desired time, a 500-μl aliquot of the assay was removed as quickly as possible and injected into a sealed cuvette that previously had been flushed with argon and which contained 2.5 ml of argon-sparged HMP buffer and 100 μM KCN. Then the spectrum of the oxyleghemoglobin was quickly recorded. The spectrum was recorded again after addition of sodium dithionite to the cuvette to completely deoxygenate the oxyleghemoglobin. This served as a basis for determining the concentrations of oxyleghemoglobin in the experiments in which oxyleghemoglobin-dependent H₂ oxidation was followed. Several different experiments revealed a roughly linear relationship between the rate of disappearance of oxyleghemoglobin and the rate of H₂ oxidation, but the data obtained varied considerably due to the nature of the experimental manipulations.

**Relationship between Leghemoglobin and H₂ Oxidation.** To establish more accurately the relationship between the rate of leghemoglobin deoxygenation and the rate of H₂ oxidation, a system was devised which allowed simultaneous measurement of H₂ oxidation and the deoxygenation of oxyleghemoglobin (Fig. 2, inset). Spectral changes at either 541 or 574 nm were used as a measure of the extent of oxygenation of leghemoglobin as described by Bergersen and Turner (8). The results of one such experiment is shown in Figure 2. The time course of H₂ oxidation is shown by the hyperbolic curve of Figure 2 (curve a). H₂ oxidation ceased when the supply of oxyleghemoglobin was exhausted as shown by the (a) absence of any further spectral change and by (b) the lack of a spectral change in leghemoglobin after addition of Na₂S₂O₄. The leghemoglobin deoxygenation curve was sigmoidal (curve b, Fig. 2). The sigmoidicity of oxyleghemoglobin utilization through bacteroid respiration in a system.

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**Fig. 1.** Simultaneous measurement of H₂ oxidation and O₂ uptake by *R. japonicum* 122 DES bacteroids supplied with oxyleghemoglobin. Assay was conducted in the dual electrode amperometric chamber (2.8 ml) as described elsewhere (11). Curve a represents the time course of H₂ oxidation and curve b shows the time course of O₂ utilization in the same experiment. Arrows indicate the time that the bacteroid suspensions (equivalent of 0.67 mg dry weight) was injected into the chamber to initiate the reaction. Leghemoglobin concentration was 10.2 μM.
systems, the data indicate the H₂ oxidation system may be capable of utilizing O₂ from some other O₂-carrying protein located within the peribacteroid sacs.

**Estimation of the** \( K_{\text{m}} \)** for O₂.** The O₂ concentrations at various points along the progress curve can be calculated as demonstrated by Bergersen and Turner (8) from the dissociation constant (37.3 nm) for the oxyleghemoglobin complex estimated by Wittenberg et al. (23). The rate of H₂ oxidation also may be obtained at each calculated O₂ concentration and the data then may be used to determine an apparent \( K_{\text{m}} \) for O₂ of the H₂ oxidation system. From the data in Figure 2, the apparent \( K_{\text{m}} \) for O₂ of the H₂ oxidation reaction was 10.8 nm (Fig. 3). Values near 10 nm were typical. From these data it is clear that the H₂ oxidation can function effectively at extremely low concentrations of dissolved O₂. Previous values of the \( K_{\text{m}} \) determined amperometrically were higher (approximately 1 μM) because the method was not sufficiently sensitive to quantitate free-dissolved O₂ concentrations in the nm range. The estimated values of the \( K_{\text{m}} \) for O₂ are apparent values because bacteroid respiration supported by endogenous substrates occurs simultaneously with H₂ oxidation. The \( K_{\text{m}} \) value determined is an upper limit for the oxidation of H₂ by O₂ provided via oxyleghemoglobin. Relative rates of oxyleghemoglobin deoxygogenation in the presence and absence of H₂ are shown in Figure 4. No attempt was made to calculate \( K_{\text{m}} \) values for O₂ on the basis of data obtained where rates of leghemoglobin deoxygogenation due to endogenous respiration were subtracted from rates of leghemoglobin deoxygogenation in presence of H₂. The system is complicated by the fact that saturating levels of H₂ (15) reduce the rate of endogenous respiration up to 60%. At less than saturating concentrations of H₂, there is an inverse relationship between the rate of H₂ oxidation and the rate of endogenous respiration (Emerich, unpublished). The effect of the rate of O₂ utilization on the shape of the curve is shown in Figure 4. H₂ increases the rate of O₂ consumption about 2.5-fold (15, 17) in the absence of leghemoglobin. The increase due to H₂ in the experiment described in Figure 4 is approximately 3-fold.

**Effect of Leghemoglobin on H₂-mediated Bacteroid Metabolism.** H₂ oxidation will support ATP synthesis and decrease the rate of endogenous respiration by suspensions of *R. japonicum* bacteroids lacking added leghemoglobin (11, 15). Table I shows that H₂ oxidation mediated via oxyleghemoglobin also supports ATP synthesis and results in a decrease in endogenous respiration. In addition, O₂ provided via oxyleghemoglobin increases H₂ oxidation in *R. japonicum* 122 DES bacteroids. Oxyleghemoglobin with a no gas phase was predicted by Stokes (18) and recently experimentally demonstrated by Bergersen and Turner (8).

A series of experiments were conducted similar to that described in Figure 2. From these experiments (data not presented) the following was concluded. The magnitude of the decrease in absorption of leghemoglobin (541 or 574 nm) in the sigmoidal portion of the curve is proportional to the concentration of oxyleghemoglobin initially present in the assay. The rate of change at the inflection point (where 50% of the oxyleghemoglobin is consumed) in the sigmoidal curve is proportional to the amount of bacteroids added (0.3–1.7 mg dry weight equivalent, average specific activity of 1.7 μmol h⁻¹ mg⁻¹ dry weight). Furthermore, the inflection point of the change in oxyleghemoglobin concentration coincides with the point which represents an approximate one-half maximum rate of H₂ oxidation. The rate of H₂ oxidation is dependent on the amount of bacteroids present, but independent of the concentration of H₂ (except at extremely low H₂ concentrations). The rate of H₂ oxidation is dependent on the concentration of oxyleghemoglobin and not upon the total concentration of leghemoglobin present. The characteristics observed were those expected when oxyleghemoglobin donates O₂ to the hydrogenase system. Although leghemoglobin may not serve directly as an O₂ donor to the H₂ oxidation system or to the endogenous respiration

**FIG. 3.** Estimation of the \( K_{\text{m}} \) of O₂ for H₂ oxidation by suspensions of *R. japonicum* 122 DES bacteroids in the presence of leghemoglobin. Data were obtained from Figure 2. O₂ concentrations at the indicated rates of H₂ uptake were calculated by a method similar to that of Bergersen and Turner (8). The \( K_{\text{m}} \) value estimated from these data was 10.8 nm. These data are typical of that obtained in 12 separate determinations.
also stimulates C2H2 reduction activity by suspensions of washed bacteroids (5, 7, 9, 24). The addition of both oxyleghemoglobin and H2 stimulated C2H2 reduction by suspensions of washed *R. japonicum* 122 DES bacteroids (Table I). Also, the addition of leghemoglobin caused a dramatic increase in respiration as measured by CO2 evolution. Both in the presence and in the absence of leghemoglobin, the addition of H2 suppressed CO2 evolution, and in agreement with Emerich et al. (unpublished). The extent of suppression seems to be proportional to the amount of H2 added in the assay.

As reported by Appleby et al. (5), oxyleghemoglobin stimulated bacteroid respiration and increased their ATP content. As shown in Table I, the addition of oxyleghemoglobin increased the ATP content of bacteroids about 2.5-fold. A series of experiments, such as the one shown in Table I, were conducted at several different partial pressures of O2. The particular experiment shown in Table I was chosen because the ATP contents of bacteroids incubated with H2 were not significantly different from those incubated without H2. Bacteroids supplied with H2, either with or without leghemoglobin, exhibited significantly higher rates of C2H2 reduction and significantly lower rates of endogenous respiration than those without H2. ATP formed via H2 oxidation effectively supported C2H2 reduction in lieu of ATP formed via endogenous carbohydrates. Marked increases in both H2-stimulated C2H2 reduction and H2-suppressed endogenous respiration were observed from the addition of oxyleghemoglobin in either the presence or absence of H2. Thus, in addition to the stimulatory effects of leghemoglobin, H2 oxidation provides additional benefits to the symbiotic association. H2 oxidation increases C2H2 reduction and reduces endogenous respiration while maintaining the ATP level of the bacteroids. By reducing endogenous respiration, the H2 oxidation system conserves carbohydrate which is ultimately derived from photosynthetic Photosynthate is presumed to limit the nitrogen fixation capacity of the symbiosis. Therefore, H2 oxidation should increase nitrogen fixation capacity. Albrecht et al. (1) have shown that soybeans inoculated with groups of *R. japonicum* strains possessing the oxyleghemoglobin system increase the percent nitrogen and total nitrogen contents of soybeans by 10.3 and 26.2%, respectively, compared to strains of *R. japonicum* lacking leghemoglobin.

![Figure 4. Deoxygenation of oxyleghemoglobin by *R. japonicum* USDA 122 DES bacteroids in the presence and absence of H2. Curve a (right scale) represents the deoxygenation of oxyleghemoglobin in the absence of H2, and curve b (left scale) represents the rate of deoxygenation in the presence of H2. Initial and final concentrations of H2 from curve b were 134 and 62 μM, respectively. The equivalent of 0.76 mg dry weight of bacteroids was present in both assays. The concentration of leghemoglobin was 25.4 μM. Concentration of oxyleghemoglobin was calculated by a method identical to that described by Bergeren and Turner (8). The results are typical of data obtained from more than 25 separate determinations.](image)

![Graph showing the relationship between oxyleghemoglobin concentration and H2 uptake rate.](image)

**Table I. Effect of Oxyleghemoglobin (Lhb) on C2H2 Reduction, CO2 Evolution, H2 Uptake, and ATP Content by *R. japonicum* 122 DES Bacteroids**

<table>
<thead>
<tr>
<th>Initial Partial Pressure of H2 (atm)</th>
<th>C2H2 Formation</th>
<th>CO2 Evolution</th>
<th>H2 Uptake</th>
<th>ATP Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lhb</td>
<td>+Lhb</td>
<td>Lhb</td>
<td>+Lhb</td>
</tr>
<tr>
<td>0.10</td>
<td>70.4 ± 2.0</td>
<td>650 ± 11</td>
<td>465 ± 49</td>
<td>5161 ± 270</td>
</tr>
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<td></td>
<td>650 ± 11</td>
<td>465 ± 49</td>
<td>5161 ± 270</td>
<td>4.92 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>63.4 ± 5.7</td>
<td>657 ± 76</td>
<td>487 ± 41</td>
<td>5692 ± 176</td>
</tr>
<tr>
<td></td>
<td>50.0 ± 2.1</td>
<td>493 ± 14</td>
<td>603 ± 27</td>
<td>6124 ± 133</td>
</tr>
</tbody>
</table>

*Assay bottles and gas atmospheres were prepared as described under "Materials and Methods." The gas atmospheres initially consisted of H2 as indicated, 0.002 atm O2, 0.10 atm C2H2, and sufficient N2 to bring the gas mixture to 1 atm.

*Assays contained the equivalent of 11.7 mg dry weight of anaerobically prepared bacteroids. Each value is the mean of triplicate determinations (±SE of the mean). Leghemoglobin when present, was at a concentration of 106 μM.

*Values were obtained after 30 min exposure to assay conditions as indicated.

**Table II. Effect of the Extent of Leghemoglobin Oxygenation on the Capacity to Recycle Hydrogen**

<table>
<thead>
<tr>
<th>Oxyleghemoglobin</th>
<th>H2 Uptake Ratea/Max H2 Evolution Rate from Nitrogenase</th>
<th>H2 Uptake Rateb/25% Maximum H2 Evolution from Nitrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total leghemoglobin</td>
<td>% of maximum</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>8.1</td>
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<tr>
<td>75</td>
<td>84</td>
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<td>30</td>
<td>48</td>
<td>3.9</td>
</tr>
<tr>
<td>20</td>
<td>33</td>
<td>2.7</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*a To deliberately maximize H2 evolution from nitrogenase the rate of H2 evolution from nitrogenase was set equal to the ratio of C2H2 reduction.*

*b To estimate the recycling capacity of H2 recycling under the assumed normal conditions, the rate of H2 evolution from nitrogenase was set equal to 25% of the rate of C2H2 reduction.

**Fig. 4. Deoxygenation of oxyleghemoglobin by *R. japonicum* USDA 122 DES bacteroids in the presence and absence of H2. Curve a (right scale) represents the deoxygenation of oxyleghemoglobin in the absence of H2, and curve b (left scale) represents the rate of deoxygenation in the presence of H2. Initial and final concentrations of H2 from curve b were 134 and 62 μM, respectively. The equivalent of 0.76 mg dry weight of bacteroids was present in both assays. The concentration of leghemoglobin was 25.4 μM. Concentration of oxyleghemoglobin was calculated by a method identical to that described by Bergeren and Turner (8). The results are typical of data obtained from more than 25 separate determinations.
the oxyhydrogen system.

Estimation of the Extent of H₂ Recycling Capacity. Rates of H₂ oxidation at different extents of leghemoglobin oxygenation were determined and these data were used to estimate the capacity of the H₂ oxidation system to recycle the H₂ evolved by nitrogenase. For this comparison, the H₂ evolution rate from nitrogenase was calculated in two different ways. To deliberately maximize H₂ evolution from nitrogenase it was assumed that the H₂ evolution rate was equivalent to the rate of C₂H₂ reduction. Under such an assumption, the entire electron flow through nitrogenase would be utilized for H₂ evolution. As shown in Table II, at the 10% oxygenation of leghemoglobin, the H₂ recycling capacity through H₂ oxidation is slightly greater than the capacity for H₂ evolution by nitrogenase. At a 20% level of oxygenation of leghemoglobin, which is the normal physiological level according to Appleby, the capacity to recycle H₂ is approximately 3-fold greater than the capacity to evolve H₂.

Normally, only about 25% of the total electron flow through nitrogenase is lost as evolved H₂ and not 100% as assumed in the above comparison (Table II). If we assume that 25% of the total electron flow through nitrogenase is lost as evolved H₂ (13), then bacteroids supplied with 20% oxygenated leghemoglobin would possess a capacity to utilize H₂ that is 10-fold greater than the rate of H₂ evolution.

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