JOURNAL OF BACTERIOLOGY, Aug. 1996, p. 4555–4562 Vol. 178, No. 15
0021-9193/96/$04.00+0

Respiratory Control Determines Respiration and Nitrogenase Activity of *Rhizobium leguminosarum* Bacteroids

HUUB HAAKER,[1]* MAGDALENA SZAFRAN,[2] HANS WASSINK,[1]
HANS KLERK,[1] AND MICHIEL APPELS[1]

Department of Biochemistry, Agricultural University, 6703 HA Wageningen, The Netherlands,[1] *and*
Faculty of Commodity Science, Academy of Economics, 60-697 Poznan, Poland[2]

Received 20 December 1995/Accepted 3 June 1996

The relationship between the O_2 input rate into a suspension of *Rhizobium leguminosarum* bacteroids, the cellular ATP and ADP pools, and the whole-cell nitrogenase activity during L-malate oxidation has been studied. It was observed that inhibition of nitrogenase by excess O_2 coincided with an increase of the cellular ATP/ADP ratio. When under this condition the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added, the cellular ATP/ADP ratio was lowered while nitrogenase regained activity. To explain these observations, the effects of nitrogenase activity and CCCP on the O_2 consumption rate of *R. leguminosarum* bacteroids were determined. From 100 to 5 μM O_2, a decline in the O_2 consumption rate was observed to 50 to 70% of the maximal O_2 consumption rate. A determination of the redox state of the cytochromes during an O_2 consumption experiment indicated that at O_2 concentrations above 5 μM, electron transport to the cytochromes was rate-limiting oxidation and not the reaction of reduced cytochromes with oxygen. The kinetic properties of the respiratory chain were determined from the deoxygenation of oxyglobins. In intact cells the maximal deoxygenation activity was stimulated by nitrogenase activity or CCCP. In isolated cytoplasmic membranes NADH oxidation was inhibited by respiratory control. The dehydrogenase activities of the respiratory chain were rate-limiting oxidation at O_2 concentrations of >300 nM. Below 300 nM the terminal oxidase system followed Michaelis-Menten kinetics (K_m of 45 ± 8 nM). We conclude that (i) respiration in *R. leguminosarum* bacteroids takes place via a respiratory chain terminating at a high-affinity oxidase system, (ii) the activity of the respiratory chain is inhibited by the proton motive force, and (iii) ATP hydrolysis by nitrogenase can partly relieve the inhibition of respiration by the proton motive force and thus stimulate respiration at nanomolar concentrations of O_2.

Nitrogen fixation is essentially an anaerobic process. The enzyme nitrogenase, which catalyzes the reduction of N_2 to NH_3, consists of two O_2-labile proteins (16, 25). The physiological electron donors for nitrogenase, flavodoxin and ferredoxin, are auto-oxidizable (35). Aside from an anaerobic environment and a source of reducing equivalents, MgATP is necessary in vitro for nitrogenase activity. MgADP inhibits nitrogenase (30). This introduces a major problem inherent to aerobic nitrogen-fixing organisms, namely that O_2 is essential for ATP synthesis and probably also for electron transport to nitrogenase (14), and on the other hand, O_2 inhibits and inactivates nitrogenase. This paradox has been resolved in the symbiosis of *Rhizobium* or *Bradyrhizobium* species with legumes by the organogenesis of the root nodule. In the central zone of the root nodule a microaerobic environment is created. The microaerobic condition is maintained by a regulation of the influx of O_2 into the central tissue (21), which balances the O_2 uptake of the mitochondria and the bacteroids. Furthermore, a high concentration of the O_2-binding protein leghemoglobin (Lb) is present in the infected cells (3). Experiments with isolated bacteroids showed that O_2-binding proteins stimulated O_2 uptake and nitrogenase activity (13, 34). It was proposed that Lb facilitates O_2 diffusion towards the layer of solution adjacent to the bacteroid surface and thereby increases the concentration of free O_2 near this surface (28, 34). The increased local gradient of the free O_2 pressure stimulates O_2 uptake by a high-affinity terminal oxidase which provides high cellular concentrations of MgATP for the support of nitrogenase activity (5, 8). When the free O_2 concentration increased above 0.1 μM, lower ATP/ADP ratios were found (8). This remarkable behavior has been explained in terms of a branched respiratory chain, with a highly efficient branch terminated by high-affinity oxidase and with an inefficient branch terminated by low-affinity oxidase. Kinetic studies with intact cells indeed indicate the presence of at least two terminal oxidase systems in *Bradyrhizobium japonicum* bacteroids (8, 10, 11). Later, the same investigators showed that *B. japonicum* bacteroids contain a single high-affinity terminal oxidase (apparent K_m value of 20 to 26 nM) and suggested that metabolic effects might have influenced the O_2 uptake kinetics (12). Furthermore, the decrease of the cellular ATP/ADP ratio at higher O_2 concentrations was not observed for *Rhizobium leguminosarum* or *Rhizobium phaseoli* bacteroids (19, 31). Thus, there seems to be a discrepancy between *R. leguminosarum* and *B. japonicum* bacteroids with respect to ATP synthesis and nitrogen fixation at different O_2 concentrations.

Also, the relationship between nitrogenase activity and respiration has been studied with genetic tools. In several members of the family *Rhizobiaceae* the presence of the *fixNOQP* operon has been demonstrated and suggested to be involved in the symbiotic respiratory process (22, 23, 27). With *B. japonicum*, it was demonstrated that the *fixNOQP* gene cluster, coding for a cytochrome *c* oxidase (cytochrome *cbb*$_3$), is essential for an effective symbiosis with soybeans (23). Recently, Preisig et al. (24) have purified the *cbb*$_3$-type oxidase and found a K_m value of approximately 10 nM. For *Azorhizobium caulinodans* it was demonstrated that not the quinol oxidases but the cyto-

---

* Corresponding author. Mailing address: Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands. Phone: 31 317 482868. Fax: 31 317 484801. Electronic mail address: Huub.Haaker@nitro.bc.wau.nl.

chrome *c* oxidases are essential for an effective nitrogen fixation (17). In contrast to that in *B. japonicum* there was no absolute requirement for the cytochrome *cbb*$_3$ oxidase to support nitrogen fixation but another cytochrome *c* oxidase (probably cytochrome *ad*$_3$) was compensating for the loss of the cytochrome *cbb*$_3$ oxidase (22). It was suggested that bacteroids will use a cytochrome *c* oxidase and not a quinol oxidase since a cytochrome *c* oxidase employs the cytochrome *bc*$_1$ complex which augments proton pumping. This will be advantageous under O_2-limiting conditions (17).

In this paper, it will be shown that studies of the rate of consumption of dissolved O_2 by *R. leguminosarum* bacteroids can easily be wrongly interpreted as indicative of multiple terminal oxidases. The oxidases with lower affinities found in intact cells were not established in isolated cytoplasmic membranes. Furthermore, it will be demonstrated that respiration is controlled not only by the kinetic properties of the terminal oxidase system but also by respiratory control. Because of this effect, ATP hydrolysis by nitrogenase stimulates respiration at low free O_2 concentrations and, indirectly, its own activity. This aspect of nitrogenase catalysis has not been recognized previously.

MATERIALS AND METHODS

Growth conditions of plants and isolation procedures. Root nodules were produced under controlled conditions on *Pisum sativum* cv. rondo by inoculation with *Rhizobium leguminosarum* PRE as described previously (1). The bacteroids were isolated from the nodules as described previously (29), and the cytoplasmic membranes vesicles were prepared by sonication four times for 30 s each at an amplitude of 26 μm and frequency of 23 kHz in a medium consisting of 50 mM TES [*N*-tris(hydroxymethyl)methy-2-aminoethanesulfonic acid]-NaOH, 1 mM EDTA, and 5 mM $MgCl_2$ [pH 7.4]. The broken cells were removed by centrifugation for 5 min at 4,000 × *g*. The cytoplasmic membranes were collected by centrifugation for 30 min at 100,000 × *g* and resuspended in the same mixture. To remove residual soluble proteins, the resuspended cytoplasmic membranes were diluted, sonicated, and collected by centrifugation. All isolation procedures were performed under Ar at 4°C. Pea Lb was purified from the soluble nodule fraction as described by Laane et al. (19).

Analytical methods. Whole-cell nitrogenase activity was determined in a reaction mixture containing 50 mM TES-KOH, 5 mM L-malate, 5 mM $MgSO_4$, 480 mM sucrose, 0.2 mM myoglobin (Mb), and 2.5% (wt/vol) fatty acid free serum albumin (final pH 7.4). Mb and pea Lb were reduced as described by Wittenberg et al. (34). The reaction mixture was flushed with argon in a butyl rubber-stoppered assay bottle of a size of 7.9 ml. Different amounts of O_2 (pO_2 of 0 to 0.04 atm) were added to the gas phase of the assay bottle, and the reaction was started by the addition of bacteroids (routinely 0.5 mg of protein) to a final volume of 0.7 ml. The assay bottles were incubated at 30°C and shaken reciprocally with a stroke of 2.5 cm and 160 cycles per min. Gas samples were taken and analyzed for C_2H_4 formation (nitrogenase activity). We used shaking instead of rapid stirring to exchange gasses between the gas and liquid phases, because the O_2 input rate with shaking was about nine times more efficient per milliliter of incubation mixture, yielding lower pO_2 values in the gas phase to obtain the maximal nitrogenase activity. This step is important because higher pO_2 values in the gas phase will lead to more O_2-inhibited bacteroids with a high ATP/ADP ratio (>5) near the gas-liquid interphase and will increase the average ATP/ADP ratio for the bacteroids present in the incubation mixture. Under the optimized assay conditions, nitrogenase activity was linear for at least 20 min, and the ATP/ADP ratio did not change during this incubation period and was not dependent on the protein concentration as long as the concentration did not exceed 1.5 mg/ml. Calibration of the O_2 input rate was done by measuring the amount of NAD^+ formed in the reaction mixture supplemented with 5 mM NADH and 1 mg of *R. leguminosarum* cytoplasmic membranes per ml instead of bacteroids. The rate of formation of NAD^+ was linear under all conditions tested.

The change in absorbance due to the deoxygenation of oxy-Mb (MbO_2) or oxy-Lb (LbO_2) was determined at 30°C in a Gilson oxygraph (volume, 1.65 ml) placed in the light beam of an Aminco-DW2 spectrophotometer. The O_2 and absorbance measurements were detected simultaneously with a two-channel interphase linked to a computer. The deoxygenation of MbO_2 (150 to 200 μM) or LbO_2 (80 to 120 μM) was monitored in a dual wavelength mode at 582 and 590 nm (582/590 nm), with a slit of 3 nm, or at 578/586 nm, respectively. The rate of respiration versus the O_2 concentration was calculated from the time course of an O_2 electrode reading and/or from the absorbance of MbO_2 or LbO_2 as described by Bergersen and Turner (9). The K_d values of MbO_2 and pea LbO_2 used were 0.786 μM (34) and 0.154 μM (32), respectively. The NADH oxidation by cytoplasmic membrane vesicles of *R. leguminosarum* bacteroids was determined in 50 mM TES-NaOH–1 mM EDTA–5 mM $MgCl_2$–2 mM NADH–Mb or Lb, as indicated, (pH 7.4). The redox states of NADH and the cytochrome *b* and cytochrome *c* pools were detected at 340/380, 562/580, and 552/580 nm, respectively.

The cellular ATP and ADP concentrations were measured after the reaction was quenched by the addition of $HClO_4$ (final concentration, 7%). In this study all incubations were quenched after 8 to 10 min. After neutralization of the acidic extract, ADP was converted into ATP with pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate. ATP concentrations were determined by a bioluminescence assay with luciferin and luciferase (EC 1.13.12.7). The intensity of the emitted light which was liberated by the luciferase reaction was directly proportional to the ATP concentration. An internal standard of ATP was employed to correct for inhibition of luciferase and for emission interference by compounds present in the incubation mixture. The protein concentration was determined with the biuret reaction after a precipitation step with deoxycholic acid and trichloroacetic acid (6). Bovine serum albumin was used as the standard.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and Mb (whale skeletal muscle) were obtained from Sigma; myokinase, pyruvate kinase, fatty acid free bovine serum albumin, TES buffer, ATP, and the ATP bioluminescence assay kit were obtained from Boehringer, and L-malate was obtained from Merck. All other chemicals were of the highest analytical grade available. All gasses were purchased from Hoek Loos.

RESULTS

Physiology of O_2 inhibition of whole-cell nitrogenase activity. In obligate aerobic diazotrophs, O_2 plays a dual role. O_2 is necessary for the generation of the proton motive force, but excess O_2 inhibits nitrogen fixation. The dependence of the whole-cell nitrogenase activity in relation to respiration has been studied basically by two methods. Bacteroids in an incubation mixture were brought into contact with O_2 in the gas phase by stirring or shaking (13, 19), or O_2 was delivered by the deoxygenation of an oxyglobin in a closed system (7, 11). Since exchange between the gas and liquid phases allows steady-state measurements, this method has been used to determine steady-state nitrogenase activity.

In Fig. 1A, the effect of the O_2 input rate on nitrogenase activity in *R. leguminosarum* bacteroids is shown. At the end of each experiment the cellular ATP and ADP concentrations were determined. As can be seen, the cellular ATP/ADP ratio was low and increased only slightly with an increasing O_2 input rate. This pattern changed when the nitrogenase activity was inhibited more than 50% by excess O_2. With an increase in the O_2 input rate, from 60 to 80 nmol of $O_2 \cdot min^{-1} \cdot$ mg of protein^{-1}, the cellular ATP/ADP ratio increased from 0.7 to 2.6. A similar observation was made by Trinchant et al. (31) for *R. phaseoli* bacteroids. We also used the no-gas-phase method as described by Bergersen and Turner (7, 8) to study the relationship between nitrogenase activity, the ATP/ADP ratio, and O_2 uptake. No differences in results by the two methods were found: high ATP/ADP ratios in bacteroids with O_2-inhibited nitrogenase activity and a lower ratio when nitrogenase was active or when the O_2 concentration was lower.

To elucidate the physiology of the O_2 inhibition of nitrogenase, we tested whether under the experimental conditions described in the legend to Fig. 1 whole-cell nitrogenase activity was inhibited because of the oxidation of electron carriers or whether nitrogenase was irreversibly inactivated by O_2. For this test the in vivo nitrogenase activity was determined, and hexadecyltrimethylammonium bromide was added to the incubation mixture together with dithionite and an ATP-regenerating system. With this procedure it is possible to determine the in vitro nitrogenase activity inside permeabilized bacteroids (15). It was found that when bacteroids were exposed for less than 10 min at O_2 input rates of <80 nmol of $O_2 \cdot min^{-1} \cdot$ mg of protein^{-1}, nitrogenase was not inactivated (data not shown). It is therefore likely that during the experiments for which the results are shown in Fig. 1A, the inhibition

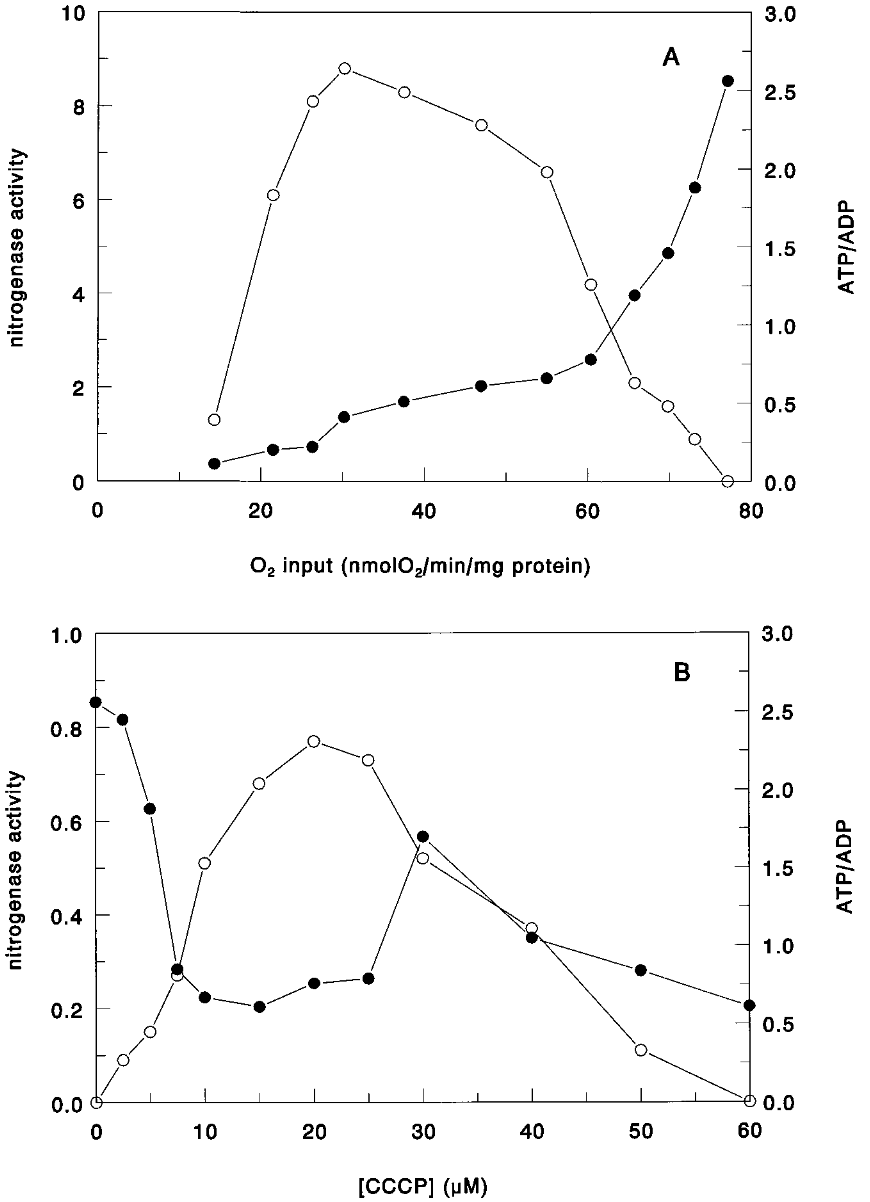

FIG. 1. Effect of O_2 input rate and CCCP on nitrogenase activity and ATP and ADP pools of *R. leguminosarum* bacteroids. The bacteroid protein concentration was 1.1 mg/ml of incubation mixture. ○, nitrogenase activity (nmoles of C_2H_4 formed · minute^{-1} · milligram of protein^{-1}); ●, ATP/ADP ratio. (B) The O_2 input rate was 77 nmol of O_2 · min^{-1} · mg of protein^{-1}.

of nitrogenase is caused by a reversible oxidation of electron donors for nitrogenase or by oxidation of nitrogenase itself.

From studies with isolated nitrogenase, in the absence of reductant, the ATPase activity of nitrogenase is lowered to 10% of its maximal ATPase activity. Thus, by determining the effect of a reductant on the cellular ATPase activity, the contribution of nitrogenase to this activity can be assessed. It was found that in permeabilized bacteroids the cellular ATPase activity was doubled by dithionite. This result is not unexpected since 30% of total cell protein of *R. leguminosarum* bacteroids is nitrogenase (15). Thus, nitrogenase might be a major ATP hydrolyzing system of the cells, and its activity might influence the ATP/ADP ratio. As can be seen from Fig. 1A, inhibition of whole-cell nitrogenase at higher O_2 input rates coincided with an increased ATP/ADP ratio. Is there a relationship between the inhibition of nitrogenase and the increase of the ATP/ADP ratio? It is not likely that the increased ratio will inhibit nitrogenase, since MgATP is a substrate for nitrogenase and MgADP is an inhibitor. But it might be feasible that the increased ATP/ADP ratio reflects a change in another important physiological parameter, namely the magnitude of the proton motive force. If, as in mitochondria, a high proton motive force inhibits respiration, it is possible that in the O_2 input range at which nitrogenase is inhibited, respiration is restrained by respiratory control. This hypothesis was tested. The results are presented in Fig. 1B. With the addition of the protonophore CCCP at an inhibitory O_2 input rate of 77 nmol of O_2 · min^{-1} · mg of protein^{-1}, the cellular ATP/ADP ratio was lowered and nitrogenase activity was stimulated. The sharp increase in the cellular ATP/ADP ratio at approximately 30

TABLE 1. Maximal rates of O_2 consumption and deoxygenation of MbO_2 or LbO_2 by *R. leguminosarum* bacteroids oxidizing L-malate and isolated cytoplasmic membranes with NADH oxidation

Preparation	Mean rate (nmol of $O_2 \cdot min^{-1} \cdot$ mg of protein^{-1} ± SD[a]		
	O_2 consumption	MbO_2 deoxygenation	LbO_2 deoxygenation
Bacteroids			
O_2-inactivated nitrogenase	95 ± 13 (*n* = 6)	49 ± 9 (*n* = 6)	42 ± 3 (*n* = 2)
O_2-inactivated nitrogenase + 50 μM CCCP	110 ± 26 (*n* = 8)	73 ± 7 (*n* = 5)	79 ± 8 (*n* = 2)
Active nitrogenase		78 ± 12 (*n* = 8)	75 ± 9 (*n* = 2)
CCCP (50 μM)-inhibited nitrogenase		93 ± 13 (*n* = 8)	95 ± 10 (*n* = 2)
Cytoplasmic membranes			
Mb	226 ± 25 (*n* = 3)	127 ± 21 (*n* = 3)	
Lb	270 ± 15 (*n* = 2)		290 ± 16 (*n* = 2)
Mb + 5 μM CCCP	442 ± 53 (*n* = 8)	245 ± 41 (*n* = 6)	
Lb + 5 μM CCCP	450 ± 20 (*n* = 3)		502 ± 25 (*n* = 2)

[a] *n*, number of determinations.

μM CCCP was reproducible and is explained in a way similar to that for O_2 inhibition. In this case, CCCP instead of O_2 inhibits electron transport to nitrogenase (19). Higher concentrations of CCCP lower the proton motive force further, keeping the cellular ATP/ADP ratio low despite the inhibition of nitrogenase. It should be realized that the free CCCP concentration in the incubation mixture was lower than the added concentration since fatty acid-free bovine serum albumin bound a considerable amount of CCCP.

Characterization of whole-cell respiration. The experiments for which the results are shown in Fig. 1 indicate that whole-cell respiration might be limited by respiratory control. To obtain more information about the coupling between respiration and the proton motive force, the rate of O_2 uptake in bacteroids under different metabolic conditions was examined by measuring simultaneously the concentration of free O_2 with a polarographic O_2 electrode and the concentration of MbO_2 or LbO_2 by spectroscopy (9). The energy status of the cells was regulated by an active or inactive major energy consumer, nitrogenase, and by the addition of a protonophore. When bacteroids are added to a solution with a high O_2 concentration, nitrogenase is inactive and will be irreversibly inactivated within a few seconds. At O_2 concentrations above 50 μM, the rate of respiration is mainly determined by the change in the free O_2 concentration, which can be measured with an O_2 electrode. At approximately the K_d value of MbO_2 (786 nM), the rate of O_2 consumption is determined only by the rate of deoxygenation of MbO_2. The results of the O_2 uptake experiments are summarized in Table 1. Bacteroids were added to an incubation mixture with a high ($[O_2] > 150$ μM) or a low ($[O_2] < 15$ μM) free O_2 concentration. With a low O_2 concentration, nitrogenase was not inactivated and the effect of an active nitrogenase on the rate of respiration was determined by the rate of deoxygenation of the oxyglobins. Respiratory control was investigated by the addition of CCCP. It was found that respiratory control had no effect on the rate of respiration at higher O_2 concentrations ($O_2 > 50$ μM), but it inhibited respiration below 1 μM O_2 (CCCP stimulated MbO_2 and LbO_2 deoxygenation [Table 1]). Inactivation of nitrogenase inhibited respiration (78 versus 49 nmol of $MbO_2 \cdot min^{-1} \cdot$ mg of protein^{-1} or 79 versus 42 nmol of $LbO_2 \cdot min^{-1} \cdot$ mg of protein^{-1} [Table 1]). When the bacteroids were not exposed to an inactivating O_2 concentration and respiratory control was abolished by CCCP, the rate of respiration at 0.4 μM O_2 was about the same as the maximal rate of respiration at high O_2 concentrations.

We investigated whether the observed inhibition of respiration by respiratory control could be demonstrated with isolated cytoplasmic membranes. It was found that the NADH oxidation was stimulated twofold by CCCP (Table 1). Cytoplasmic membrane vesicles from *R. leguminosarum* bacteroids contain an active succinate oxidase activity (about 90% of the NADH oxidase activity). But, in contrast to the NADH oxidation, succinate oxidation was hardly stimulated by CCCP at high or low free O_2 concentrations. These results confirm the observation by Laane et al. (20) that the coupling between succinate oxidation and phosphorylation (P/O ratio of 0.2) is much less effective than the coupling between NADH oxidation and phosphorylation (P/O ratio of ≈ 1). When both NADH and succinate were oxidized, the maximal rate was the sum of the separate rates, indicating that at saturating O_2 concentrations electron transport to the terminal oxidases causes rate-limiting oxidation.

With intact cells no significant differences were observed between MbO_2 and LbO_2 as O_2 carriers, but with cytoplasmic membranes a difference between the maximal activity of NADH oxidase and the maximal rate of deoxygenation of MbO_2 was observed. Since this difference was not observed with Lb as O_2 carrier, the lower activity was ascribed to a limited increase of the O_2 concentration near the membrane surface by MbO_2 because of its relatively high K_d value compared with that of Lb pea (28, 34).

O_2 consumption experiments have been carried out for *B. japonicum* bacteroids (8, 10, 11). The data were replotted as *v* versus *S* (substrate concentration) and analyzed according to the method of Segel (27), assuming that multiple enzymes catalyze the same reaction. The velocity at any substrate concentration is the sum of the velocities contributed by each enzyme. On the basis of this analysis Bergersen and Turner (8, 10) concluded that *B. japonicum* bacteroids contain low- and high-affinity terminal oxidase systems. When we performed the same analysis, kinetic constants for at least three apparent oxidase systems were obtained (data not shown). However, this interpretation is of course valid only if in the whole substrate range studied, the terminal oxidases are responsible for the rate-limiting step of the series of redox reactions. This condition was verified by determining the redox state of the cytochromes and the cellular NAD(P)H concentration during an

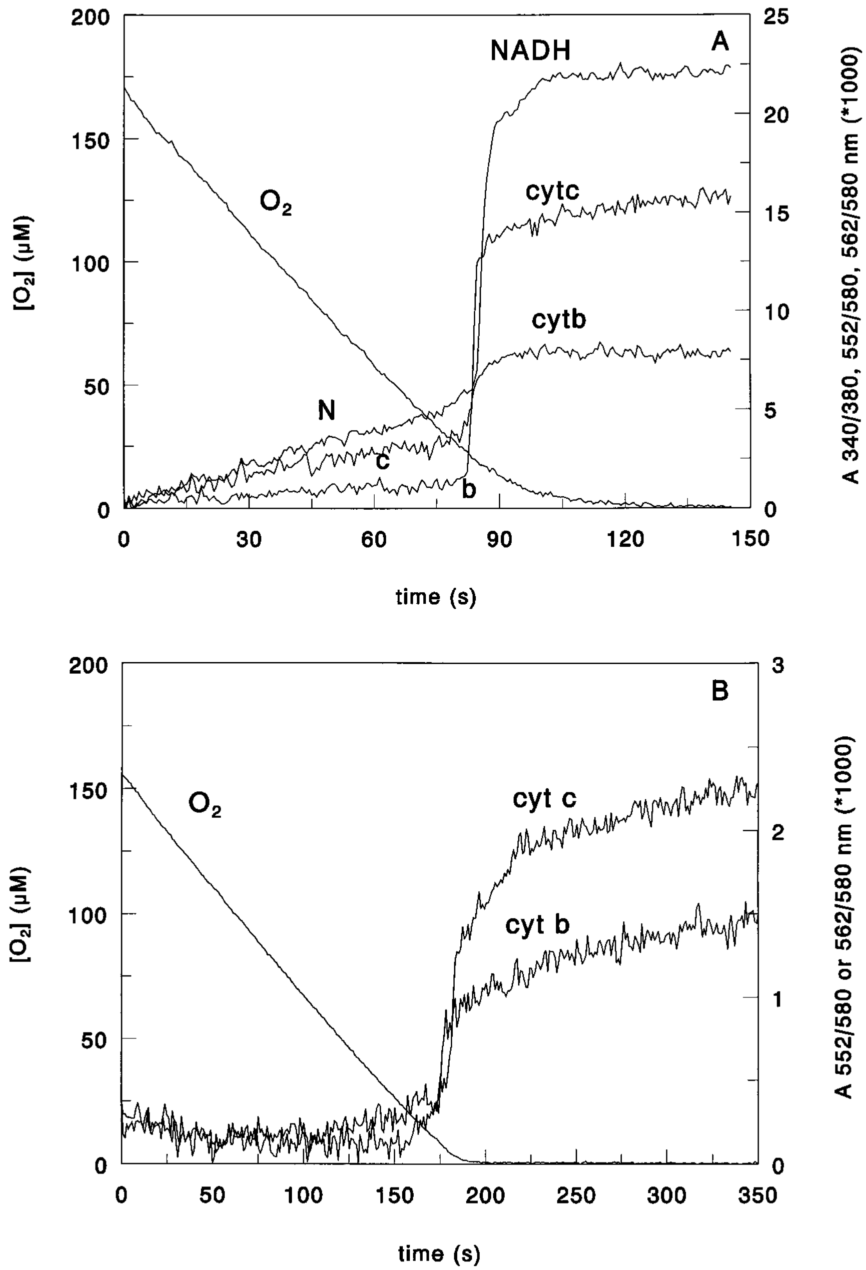

FIG. 2. Time course of concentration of free dissolved O_2 and redox state of NADH (N) and cytochrome (cyt) *b* and cytochrome *c* pools of *R. leguminosarum* bacteroids that oxidize L-malate or cytoplasmic membranes in which NADH is oxidized. Mb was omitted from the standard incubation mixture. The redox states of NADH and the cytochromes *b* and *c* pools were determined at the indicated wavelengths. (A) Bacteroids (0.9 mg of protein/ml); (B) cytoplasmic membranes (0.12 mg of protein/ml plus 5 μM CCCP).

O_2 consumption experiment. The results are presented in Fig. 2. In intact bacteroids, the cytochromes and the pyridine nucleotides switched to the reduced state at an O_2 concentration of approximately 20 μM (Fig. 2A). This switch indicates that at O_2 concentrations above 20 μM, metabolism limits O_2 uptake and not the activity of the terminal oxidases. A similar experiment was performed with isolated cytoplasmic membranes (Fig. 2B). Only at O_2 concentrations below 5 μM were the cytochrome pools reduced, indicating that electron transport towards the terminal oxidases did not cause rate-limiting oxidation. No significant differences were found when succinate alone and succinate plus NADH were used as the electron donors (data not shown). From these experiments it is clear that only the O_2 consumption rate at O_2 concentrations below 5 μM will give meaningful information about the kinetic properties of the terminal oxidases. Since the O_2 consumption rate of whole cells had already declined at approximately 50 μM O_2, an O_2 concentration-dependent factor limited respiration. This factor might be (auto-oxidizable) enzymes or electron-carrying proteins with a relatively low-affinity for O_2 reacting directly with O_2 (2, 4).

Experiments for O_2 consumption and deoxygenation of cytoplasmic membranes were done to determine the kinetics of the terminal oxidases oxidizing NADH or succinate. The re-

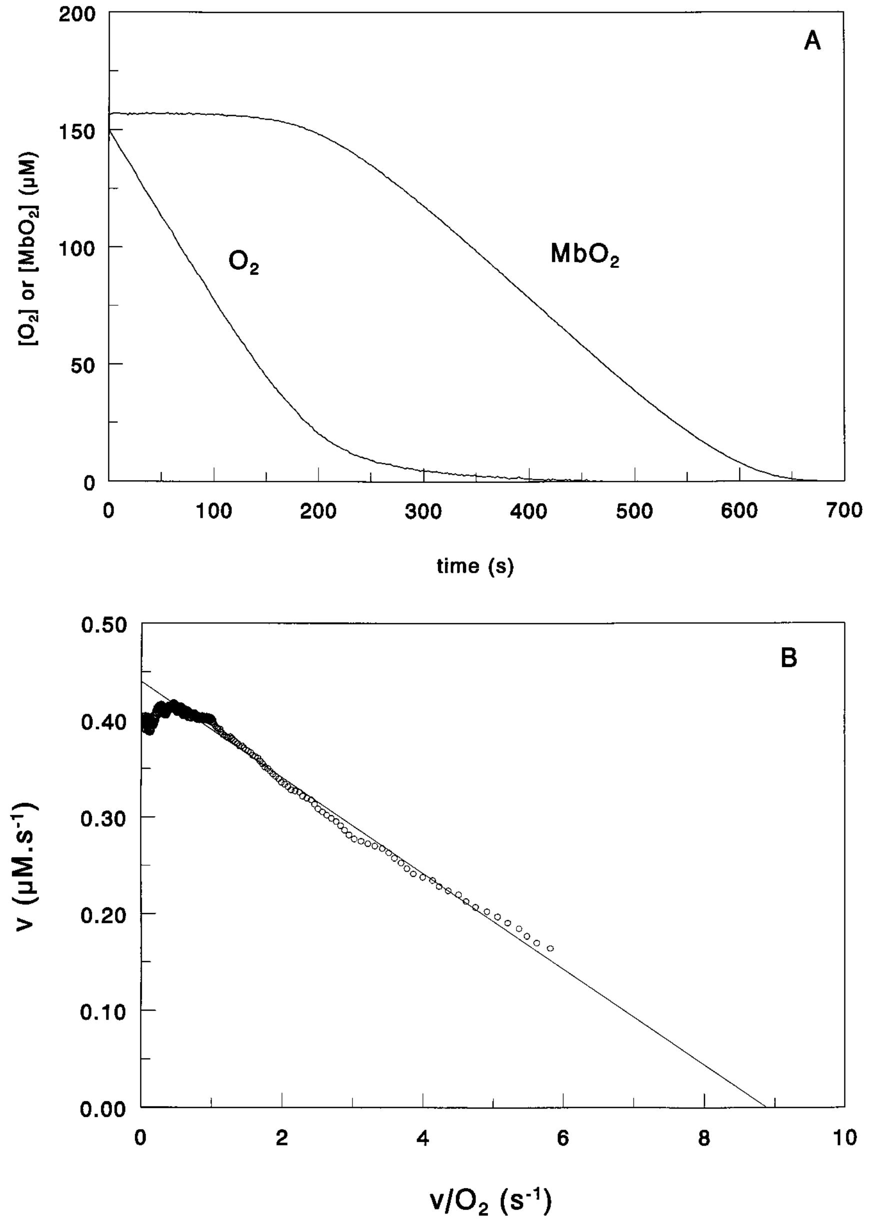

FIG. 3. (A) Time course of concentrations of free dissolved O_2 and MbO_2 in an incubation of cytoplasmic membranes from *R. leguminosarum* bacteroids that oxidize NADH. Concentrations: Mb plus MbO_2, 157 μM plus 5 μM CCCP; membranes, 0.106 mg of protein/ml of incubation mixture. (B) Eady-Hofstee plot derived from deoxygenation kinetics of MbO_2 in panel A. Only the data for $[MbO_2]$ of <115 μM and $[O_2]$ of <4.7 μM have been used in the calculations. The line drawn through the datum points is a linear regression fit of the points with an *x*-axis coordinate of >1. The K_m value calculated was 49 nM.

sults of a typical experiment are shown in Fig. 3A. As indicated above, only data for O_2 concentrations below 5 μM O_2 gave meaningful results. Therefore, the deoxygenation rate of the oxygenated globins was used in the kinetics analysis. Since the MbO_2 deoxygenation rate is maximal up to 450 s, datum points from 450 s ($[MbO_2]$ of 58 μM) to 625 s were used in the kinetics analysis (datum points for less than 2% MbO_2 were also not used because of the uncertainty of the measurements). The data were converted into an Eady-Hofstee plot to determine the K_m value (Fig. 3B). As can be seen, the data are consistent with a terminal oxidase system with an apparent K_m value of 49 nM. When the membrane preparations were not analyzed directly after preparation but were stored frozen, a second K_m value was detected at a higher value (K_m of 130 ± 40 nM [mean ± standard deviation for $n = 11$]), and the maximal activity of this second oxidase was at most 25% of the total activity at V_{max}. The K_m values determined are summarized in Table 2. In contrast to the V_{max} values (Table 1), the metabolic state of the cells or membranes had no effect on the apparent K_m value (inactive or active nitrogenase or with or without CCCP).

The redox spectra of cytoplasmic membranes from bacteroids of the *R. leguminosarum* strain used in this study did not differ from the spectra published by Kretovich et al. (18) and Williams et al. (33) for whole *R. leguminosarum* and *B. japonicum* bacteroids, cytoplasmic membranes, or isolated cytochrome cbb_3-type oxidase (24). In addition, a sodium dodecyl sulfate gel of membranes stained for heme showed two bands

TABLE 2. Apparent K_m values determined from deoxygenation kinetics of globins by *R. leguminosarum* bacteroids oxidizing L-malate and cytoplasmic membranes with NADH oxidation

Preparation	K_m (nM)	
	Mb	Lb
Whole cells	54 ± 16 (n = 9)	49 ± 13 (n = 4)
Cytoplasmic membranes	44 ± 12 (n = 9)	45 ± 8 (n = 3)

[a] K_m values were calculated from Eady-Hofstee plots and are expressed as for the means for the indicated number of determinations (in parentheses) ± standard deviations.

around 30 kDa, as found for the FixP and FixO gene products in *B. japonicum* bacteroids (23). These data indicate that *R. leguminosarum* bacteroids might also contain the cytochrome cbb_3 oxidase.

DISCUSSION

There have been several reports about the cellular ATP/ADP ratio during nitrogen fixation of bacteroids (5, 8, 19, 31). The published ratios vary between 1 and 3.5. In this paper we report ratios of around 0.5 for *R. leguminosarum* bacteroids and show that the ratio increases when nitrogenase switches off. Earlier reports from this laboratory mentioned significantly higher cellular ATP/ADP ratios for *R. leguminosarum* bacteroids during nitrogen fixation (19). These experiments were performed in a reaction chamber with an O_2 electrode with the intention of measuring the free O_2 concentration during an experiment. Because of H_2 production by nitrogenase, the ambient O_2 concentration was much higher than that read from the display of the O_2 electrode. Furthermore, because of the inefficient aeration, the relative high pO_2 concentrations used in the gas phase ($pO_2 > 0.1$ atm [1 atm = 101.29 kPa]) increased the inhomogeneity of the O_2 concentration throughout the incubation mixture and raised the concentration of temporary O_2-inhibited bacteroids (with a high ATP/ADP ratio). Both events contributed to the reported ATP/ADP ratios of ~3 at the maximum activity of nitrogenase (19). Our conclusion is that during nitrogenase activity, the ATP/ADP ratio is less than 1 and increases to 3 to 4 when nitrogenase is O_2 inhibited. Studies with isolated enzyme indicate that MgADP is a strong inhibitor of nitrogenase (30). It is questionable whether nitrogenase activity is possible at the low ATP/ADP ratios found. ADP inhibition of nitrogenase activity was tested in permeabilized bacteroids. In this preparation nitrogenase is present in its in situ environment (high protein concentrations and the presence of its physiological electron donor). It was found that at 5 mM MgATP and 18 mM MgADP, the specific activity is around 20 nmol of C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}, which is about the same as the maximal physiological nitrogenase activity (15). This result indicates that at low ATP/ADP ratios, significant nitrogenase activity is possible.

With respect to the ATP/ADP ratio at higher O_2 concentrations, Bergersen and Turner (8) have reported a deviant behavior for *B. japonicum* bacteroids. When the O_2 concentration increased above 0.1 μM (the O_2 range at which nitrogenase activity became inhibited by excess O_2), the ATP/ADP ratio decreased. This performance has been attributed to a branched respiratory chain terminated by a highly efficient branch with a high-affinity oxidase and with an inefficient branch terminated with a low-affinity oxidase. The experimental evidence came from kinetic analysis of the O_2 consumption of intact cells (8–10). We have shown that it is not possible to use a complete O_2 consumption curve for *R. leguminosarum* bacteroids to determine the kinetic properties of the terminal oxidase systems. At O_2 concentrations as low as 20 μM, metabolism limits oxidation (Fig. 2). Experiments with isolated membranes show that only at O_2 concentrations below 0.4 μM does NADH or succinate oxidation start to decline, indicating that electron transport to the terminal oxidases is no longer rate limiting (Fig. 3). Kinetic analysis of NADH oxidase activity (or succinate oxidase activity) in *R. leguminosarum* bacteroids or cytoplasmic membranes at O_2 concentrations below 500 nM gave results consistent with one terminal oxidase system with an apparent K_m of about 50 nM (Table 2). The spectral data indicate that the oxidase encoded by the *fixNOQP* operon may be present in the cytoplasmic membranes. Our kinetic analysis does not prove that this is the only terminal oxidase that is present, but if other terminal oxidases with significant activity are present, they must have a similarly high affinity for O_2. From the kinetics of O_2 uptake of intact cells at higher O_2 concentrations, it is clear that the bacteroids also contain, in addition to the high-affinity system, low-affinity oxidase activity which is not involved with the respiratory chain. This O_2 uptake system is not relevant for nitrogen fixation because of its low affinity for O_2.

Is there kinetic evidence for a branched respiratory chain operating in *B. japonicum* bacteroids to support the explanation for the decrease of the ATP/ADP ratio at higher O_2 concentrations? Recent data make this interpretation unlikely. Preisig et al. (24) have reported that 85% of the total cytochrome *c* oxidase activity in *B. japonicum* bacteroid membranes is contributed by the cbb_3-type oxidase (K_m of ≈10 nM) and that this oxidase system is also active in the millimolar range. Bergersen and Turner (12) have demonstrated that a single high-affinity terminal oxidase is active in *B. japonicum* bacteroids in the range of O_2 concentrations in which N_2 fixation occurred (10 to 300 nM O_2). Instead of a branched respiratory chain, a change in the type of reductant available for the respiratory chain may explain the lower ATP/ADP ratios at higher O_2 concentrations. If at higher O_2 concentrations, NADH is directed more in the direction of poly-β-hydroxybutyrate formation and nitrogen fixation and less is made available for respiration, then respiration may be more driven by electron donors entering the respiratory chain at the level of coenzyme Q as for succinate or H_2 formed by the action of nitrogenase. This metabolic effect can decrease the stoichiometry of proton translocation during electron transfer through the respiratory chain and may lower the ATP/ADP ratio. An effect of O_2 on the distribution of reducing power between nitrogen fixation and poly-β-hydroxybutyrate formation at different substrate concentrations in *B. japonicum* bacteroids has been proposed (12).

The results of the experiments presented in this paper indicate that a low proton motive force stimulates respiration and that ATP hydrolysis by nitrogenase is an effective way to keep the ATP/ADP ratio low enough to prevent strong inhibition of respiration by respiratory control. If ATP hydrolysis by nitrogenase could be lowered by genetic engineering, the possibility cannot be ruled out that cells with the modified enzyme will have no nitrogenase activity, because respiration at low O_2 concentrations might be inhibited by respiratory control.

ACKNOWLEDGMENTS

This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) and by the Commission of the European Communities in the framework of the programs Energy in Agriculture (contract no. 1915; EEG/Agricultural University) and Stimulation of European Cooperation and Scientific and Technical Interchange [SC1-0179-C(TT)].

We thank P. de Kam for cultivating pea plants and C. Laane for the critical reading of the manuscript.

REFERENCES

1. **Appels, M. A., and H. Haaker.** 1991. Glutamate oxaloacetate transaminase in pea root nodules. Participation in a malate/aspartate shuttle between plant and bacteroid. Plant Physiol. **95:**740–747.
2. **Appleby, C. A.** 1969. Electron transport systems of *Rhizobium japonicum*. I. Haemoprotein P-450, other CO-reactive pigments, cytochromes and oxidase in bacteroids from N_2-fixing root nodules. Biochim. Biophys. Acta **172:**71–87.
3. **Appleby, C. A.** 1984. Leghemoglobin and rhizobium respiration. Annu. Rev. Plant Physiol. **35:**443–478.
4. **Appleby, C. A., P. James, and H. Hennecke.** 1991. Characterization of three soluble c-type cytochromes isolated from soybean bacteroids of *Bradyrhizobium japonicum* strain CC705. FEMS Microbiol. Lett. **83:**137–144.
5. **Appleby, C. A., G. L. Turner, and P. K. Macnicol.** 1975. Involvement of oxyleghaemoglobin and cytochrome *P-450* in an efficient oxidative phosphorylation pathway which support nitrogen fixation in *Rhizobium*. Biochim. Biophys. Acta **387:**461–474.
6. **Bensadoun, A., and D. Weinstein.** 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. **70:**241–250.
7. **Bergersen, F. J., and G. L. Turner.** 1975. Leghaemoglobin and the supply of O_2 to nitrogen-fixing root nodule bacteroids: studies of an experimental system with no gas phase. J. Gen. Microbiol. **89:**31–47.
8. **Bergersen, F. J., and G. L. Turner.** 1975. Leghaemoglobin and the supply of O_2 to nitrogen-fixing root nodule bacteroids: presence of two oxidase systems and ATP production at low free O_2 concentration. J. Gen. Microbiol. **91:**345–354.
9. **Bergersen, F. J., and G. L. Turner.** 1979. Systems utilizing oxygenated leghemoglobin and myoglobin as sources of free dissolved O_2 at low concentration for experiments with bacteria. Anal. Biochem. **96:**165–174.
10. **Bergersen, F. J., and G. L. Turner.** 1980. Properties of terminal oxidase systems of bacteroids from root nodules of soybean and cowpea and of N_2-fixing bacteria grown in continuous culture. J. Gen. Microbiol. **118:**235–252.
11. **Bergersen, F. J., and G. L. Turner.** 1990. Bacteroids from soybean root nodules: respiration and N_2-fixation in flow-chamber reactions with oxyleghaemoglobin. Proc. R. Soc. Lond. Ser. B **238:**295–320.
12. **Bergersen, F. J., and G. L. Turner.** 1993. Effects of concentrations of substrates supplied to nitrogen-fixing soybean bacteroids in flow chamber reactions. Proc. R. Soc. Lond. B **251:**95–102.
13. **Bergersen, F. J., G. L. Turner, and C. A. Appleby.** 1973. Studies on the physiological role of leghaemoglobin soybean root nodules. Biochim. Biophys. Acta **292:**271–282.
14. **Haaker, H., and J. Klugkist.** 1987. The bioenergetics of electron transport to nitrogenase. FEMS Microbiol. Rev. **46:**57–71.
15. **Haaker, H., and H. Wassink.** 1984. Electron allocation to H^+ and N_2 by nitrogenase in *Rhizobium leguminosarum* bacteroids. Eur. J. Biochem. **142:** 37–42.
16. **Kim, J., and D. C. Rees.** 1994. Nitrogenase and biological nitrogen fixation. Biochemistry **33:**389–397.
17. **Kitts, C. L., and R. A. Ludwig.** 1994. *Azorhizobium caulinodans* respires with at least four terminal oxidases. J. Bacteriol. **176:**886–895.
18. **Kretovich, W. L., V. I. Romanov, and A. V. Korolyov.** 1973. *Rhizobium leguminosarum* cytochromes. Plant Soil **39:**619–634.
19. **Laane, C., H. Haaker, and C. Veeger.** 1978. Involvement of the cytoplasmic membrane in nitrogen fixation by *Rhizobium leguminosarum* bacteroids. Eur. J. Biochem. **87:**147–153.
20. **Laane, C., H. Haaker, and C. Veeger.** 1979. On the efficiency of oxidative phosphorylation in membrane vesicles of *Azotobacter vinelandii* and of *Rhizobium leguminosarum* bacteroids. Eur. J. Biochem. **97:**369–377.
21. **Layzell, D. B., L. Diaz del Castillo, S. Hunt, M. Kuzma, O. Cauwenberghe, and I. van Oresnik.** 1993. The regulation of oxygen and its role in regulating nodule metabolism, p. 393–398. *In* R. Palacios, J. Mora, and W. E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
22. **Mandon, K., P. A. Kaminski, and C. Elmerich.** 1994. Functional analysis of the *fixNOQP* region of *Azorhizobium caulinodans*. J. Bacteriol. **176:**2560–2568.
23. **Preisig, O., D. Anthamatten, and H. Hennecke.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc. Natl. Acad. Sci. USA **90:**3309–3313.
24. **Preisig, O., R. Zufferey, L. Thöny-Meyer, C. A. Appleby, and H. Hennecke.** 1996. A high-affinity *cbb*$_3$-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. J. Bacteriol. **178:** 1532–1538.
25. **Robson, R. L., and J. R. Postgate.** 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. **34:**183–207.
26. **Schlüter, A., S. Rüberg, M. Krämer, S. Weidner, and U. B. Priefer.** 1995. A homolog of the *Rhizobium meliloti* nitrogen fixation gene *fixN* is involved in the production of a microaerobically induced oxidase activity in the phytopathogenic bacterium *Agrobacterium tumefaciens*. Mol. Gen. Genet. **247:** 206–215.
27. **Segel, I. H.** 1975. Enzyme kinetics: behaviour and analysis of rapid equilibrium and steady-state enzyme systems. John Wiley & Sons, New York.
28. **Stokes, A. N.** 1975. Facilitated diffusion: the elasticity of oxygen supply. J. Theor. Biol. **52:**285–297.
29. **Szafran, M. M., and H. Haaker.** 1995. Properties of the peribacteroid membrane ATPase of pea root nodules and its effect on the nitrogenase activity. Plant Physiol. **108:**1227–1232.
30. **Thorneley, R. N. F., and A. Cornish-Bowden.** 1977. Kinetics of nitrogenase of *Klebsiella pneumoniae*: heterotrophic interactions between magnesium-adenosine $5'$-diphosphate and magnesium-adenosine $5'$-triphosphate. Biochem. J. **165:**255–262.
31. **Trinchant, J. C., A. M. Birot, M. Denis, and J. Rigaud.** 1983. C_2H_2 reduction, oxygen uptake and cytochrome *c* reduction by bacteroids isolated from french-bean nodules. Arch. Microbiol. **134:**182–186.
32. **Uheda, E., and K. Syono.** 1982. Effects of leghaemoglobin components on nitrogen fixation and oxygen consumption. Plant Cell Physiol. **23:**85–90.
33. **Williams, H. D., C. A. Appleby, and R. K. Poole.** 1990. The unusual behaviour of the putative terminal oxidases of *Bradyrhizobium japonicum* bacteroids revealed by low-temperature photodissociation studies. Biochim. Biophys. Acta **1019:**225–232.
34. **Wittenberg, J. B., F. J. Bergersen, C. A. Appleby, and G. L. Turner.** 1974. Facilitated oxygen diffusion: the role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. J. Biol. Chem. **249:**4057–4066.
35. **Yates, M. G., and C. W. Jones.** 1974. Respiration and nitrogen fixation in *Azotobacter*. Adv. Microbiol. Physiol. **11:**97–135.