Supply of O₂ regulates demand for O₂ and uptake of malate by N₂-fixing bacteroids from soybean nodules

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Bacteroids, prepared anaerobically from soybean root nodules by fractional centrifugation or by sucrose or Percoll density-gradient methods, were retained within a stirred, flow-through reaction chamber and used to determine rates of respiration and N₂ fixation at various rates of O₂ supply. Liquid reaction solutions containing malate, oxyleghaemoglobin, dissolved N₂ and various levels of dissolved O₂ were passed through the reaction chamber at measured rates of flow. The relative oxygenation of leghaemoglobin in the chamber was determined automatically by spectrophotometry of the effluent solution, and the concentrations of free, dissolved O₂ ([O_{2 free}]) and the rates of O₂ consumption were calculated. N₂ fixation was measured by analysis of fractions of effluent. The principal finding was that stepwise increases in the flow rate (increasing the supply of O₂ and malate) induced an increase in O₂ demand (respiration) resulting in a decrease in [O_{2 free}] and increased N₂ fixation. In some experiments, samples of bacteroids were withdrawn from the flow chamber during steady states and the rates of malate uptake were measured in standard, microaerobic assays. Progressive taking of samples from the flow chamber whilst maintaining constant flow rates (increasing the supply of O₂ and malate per bacteroid) also resulted in increased O₂ demand and declines in [O_{2 free}]. With increased bacteroid respiration, transport of malate into bacteroids (linear with time between 1 and 5 min after starting each assay) increased proportionally. This suggests that the rate of malate transport is tightly coupled with bacteroid respiration. Thus, bacteroid respiration, coupled with malate uptake, must be regulated by the rate of O₂ supply, rather than by the [O_{2 free}] prevailing in the stirred chamber as found or assumed in previous work. These features are discussed in relation to N₂ fixation by anaerobically isolated bacteroids.

Keywords: malate transport, nitrogen fixation, respiration, *Bradyrhizobium japonicum*

INTRODUCTION

The fixation of atmospheric N₂ by the root nodules of legumes is directly correlated with respiration within certain limits of O₂ concentration (e.g. between 10 and 60% O₂ for detached soybean nodules; Bergersen, 1962)

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Abbreviation: [O_{2 free}], free, dissolved O₂.

and is considered to be controlled by the rate at which O₂ from air is able to penetrate into the central tissue, in response to the respiratory demand of the inner nodule tissue (Witty *et al.*, 1986). Penetration of O₂ is restricted by a layer of cells in the inner cortex in which diffusion is impeded by a lack of gas-filled intercellular spaces (e.g. Witty *et al.*, 1986; Parsons & Day, 1990; Witty & Minchin, 1998). It has been found that N₂ fixation by the symbiotic forms (bacteroids) of *Bradyrhizobium japonicum* isolated anaerobically from the root nodules of soybean is supported by C₄-dicarboxylic acids, such as malic acid (Rosendahl *et al.*, 1991), and by respiration

within a narrow range of concentration (0.01–0.1 μM) of free, dissolved O_2 ($[\text{O}_2]_{\text{free}}$) (Bergersen, 1997a). Here again N_2 fixation rates are correlated with bacteroid respiration between certain limits of $[\text{O}_2]_{\text{free}}$ (Bergersen, 1997b).

It is commonly assumed, when constructing simulations of nodule function, that bacteroid respiration rates are governed by the $[\text{O}_2]_{\text{free}}$ to which they are exposed (e.g. Thumfort *et al.*, 1994; Bergersen, 1999). Whilst this assumption is supported by results obtained with isolated bacteroids under certain experimental conditions, when energy-yielding substrates were not limiting (Bergersen & Turner, 1993; Bergersen, 1997b), when no exogenous substrates were supplied, utilization of endogenous reserves by bacteroids was regulated by the rate of supply of O_2 (Bergersen & Turner, 1992), which in turn regulated bacteroid respiration. In the present paper we report experiments with a different strain of *B. japonicum* showing that bacteroid respiratory O_2 demand appears to be regulated by rates of O_2 supply when the bacteroids are supplied with limiting (50 μM) exogenous malate and that there is a parallel regulation of rates of transport of malate into the bacteroids.

METHODS

Nodules, bacteroid suspensions and leghaemoglobin. Nodules aged about 35 d were picked from roots of soybean (*Glycine max* Merr. cv. Stevens) inoculated at planting with *Bradyrhizobium japonicum* strain USDA 110 and grown in pots of sand, supplied twice weekly with McKnight's nutrient solution free of combined nitrogen (Gibson, 1980), or in Leonard jars in a growth cabinet under bacteriologically controlled conditions, as described by Gibson (1980). Nodules were washed with tap water, blotted dry on paper towels and used for the standard bench anaerobic method of preparing bacteroid suspensions as described by Bergersen & Turner (1990), by a sucrose density-gradient method (Waters *et al.*, 1998) or by an anaerobic Percoll (Pharmacia) density-gradient method (adapted from Udvardi *et al.*, 1988). Some nodules were frozen and used later for preparation of purified, unfractionated leghaemoglobin (Appleby & Bergersen, 1980) which was stored frozen at -80°C in 50 mM potassium phosphate buffer (50 mM, pH 7.4) containing 1 mM EDTA.

Flow chamber experiments

Reaction solutions. These were prepared in deionized water and contained MOPS/KOH buffer (25 mM, pH 7.4), 2 mM MgCl_2 , sodium DL-malate (0.05–2 mM) and 10–15 ml leghaemoglobin solution (prepared as above) per 300 ml. The solutions, in reservoir flasks connected to the reaction chamber by a peristaltically pumped capillary line, were stirred at 23°C for 1 h before use, in air (0.21 atm O_2) at 1 atm (93.325–95.992 N m^{-2} or 95.33–98.06 kPa pressure at the elevation of Canberra), or in closed reservoirs under atmospheres containing 0.125 or 0.25 atm O_2 in N_2 . Concentrations of $[\text{O}_2]_{\text{free}}$ in these solutions were calculated from tables of solubility. Actual concentrations of leghaemoglobin were measured by the pyridine haemochromogen method and the proportional oxygenation of leghaemoglobin by spectrophotometry (Appleby & Bergersen, 1980) of the solutions passing into and out of the reaction chamber.

Flow reaction chamber and methods. The flow chamber, associated equipment and methods were as described pre-

viously (Bergersen & Turner, 1985, 1990, 1992, 1993). The water-jacketed (30°C), mechanically stirred conical chamber (volume 12 ml) is closed at its base by a bacteroid-retaining, microporous membrane filter (pore size 0.45 μm), supported on a porous plastic disc. Beneath the disc is an annular collecting space connected to a spectrophotometer flow cuvette by stainless steel capillary (0.3 mm i.d.) tubing. Bacteroids in the chamber are perfused by reaction solution pumped from the reservoirs described above. Spent reaction solution emerging from the cuvette is collected as fractions for determination of NH_3 in solution, considered to be the principal product of N_2 fixation by bacteroids in the chamber (Bergersen & Turner, 1990). The apparatus incorporates facilities for recording automatically, at intervals of 1 min, values of ΔA (the difference between optical absorbances at 576 and 560 nm) of effluent solution and the time of the beginning and end of each effluent fraction collected for analysis. Values of ΔA are converted to concentrations of $[\text{O}_2]_{\text{free}}$ and rates of consumption of O_2 having regard to (a) the ΔA_t values for effluent solution at t min, the ΔA_{oxy} value for fully oxygenated (in air) reaction solution and A_{red} , the value for reaction solution reduced with a few crystals of dithionite; (b) the concentrations of total leghaemoglobin and $[\text{O}_2]_{\text{free}}$ in the reservoir reaction solution; (c) the kinetics of O_2 binding by soybean leghaemoglobin ($K_{\text{eq}} = 0.047 \mu\text{M } \text{O}_2$; Appleby, 1984), and (d) the flow rate of solution through the chamber and the dry mass of bacteroids present there. At t min, the proportional oxygenation of leghaemoglobin in the chamber is:

$$Y_t = (\Delta A_t - \Delta A_{\text{red}}) / (\Delta A_{\text{oxy}} - \Delta A_{\text{red}}) \text{ and} \\ [\text{O}_2]_{\text{free}} = (Y_t \cdot K_{\text{eq}}) / (1 - Y_t)$$

Experimental. The assembled flow chamber and connecting tubes were filled with N_2 before being completely filled with reaction solution which had been degassed under vacuum before storage under N_2 . Bacteroids, prepared as above, were finally resuspended under N_2 in reaction solution which had been degassed under vacuum; 0.5–1.0 ml of the suspension was injected slowly into the stirred chamber. When the chamber contents became visibly purplish (leghaemoglobin partially deoxygenated), the reaction solution pump was started at a flow of about 0.5 ml min^{-1} and data recording commenced. After several periods of different flow rates, the flow was switched to a different reservoir and another series of flow rates was run.

Measurement of malate transport. In preliminary experiments, the effects of method of preparation of bacteroids and of conditions imposed during the assays were examined. In these tests, uptake of [^{14}C]malate was measured essentially as described by Udvardi *et al.* (1988) but with modifications to determine the effects of $[\text{O}_2]_{\text{free}}$ during preparation of bacteroids and during assay of uptake. From these tests, the following protocol was developed for application to bacteroids recovered from the flow chamber.

Samples (3.5 ml) were withdrawn from the flow chamber into argon-flushed syringes at intervals in experiments in which the air-saturated reaction solutions contained only 50 μM malate at a flow rate of 1.0 ml min^{-1} . The first sample in each experiment was taken after the period during which O_2 demand gradually increased (0–20 min; Holtzapffel & Bergersen, 1998). Taking of each sample removed 25.4% of the bacteroids present in the chamber when sampling commenced. Thus with constant flow, after each sampling the O_2 flux to each bacteroid in the chamber increased. The time courses of uptake from 1 mM L-[^{14}C]malate ([1,4(2,3)- ^{14}C]malic acid, 2.04 GBq mmol^{-1} , Amersham; diluted with unlabelled sodium

malate to give suitable concentrations and specific radio-activities) by bacteroids in these samples were measured in standard microaerobic assays in duplicate in rubber-capped argon-flushed tubes, into each of which was injected 1.0 ml of the reaction mixture taken from the chamber by argon-flushed syringe. Into each tube, air-saturated reaction solution (100 µl) plus 10 µl 100 mM [^{14}C]malate was injected (bringing the tube contents to >1.0 mM malate and approx. $10\text{ }\mu\text{M}$ O_2) and timing was started. Samples (150 µl) were taken from the tubes at intervals of 2 min into microfuge tubes, the bacteroids centrifuged through 100 µl of silicone oil into 10 µl 40% (v/v) HClO_4 and radioactivity in the pellets measured by scintillation counting (Udvardi *et al.*, 1988).

Analytical. Ammonia in samples of effluent reaction solution was measured by the Chaney-Marbach method (Bergersen, 1980). Radioactivity was measured by scintillation methods. Calculations of relative oxygenation of leghaemoglobin (Y) and of $[\text{O}_2\text{ free}]$ from Y were as described previously (Appleby & Bergersen, 1980; Bergersen & Turner 1990).

RESULTS

Effect of different preparative methods on bacteroid N_2 fixation and respiration

Bacteroids were prepared by three different methods and their N_2 -fixing activity and respiration were determined in the flow-through chamber. In none of these experiments were more than traces of alanine (Waters *et al.*, 1998) detected in effluent solution by an enzymic method (Walshaw *et al.*, 1997) (data not shown). We relied, therefore, on previous experiments with $^{15}\text{N}_2$ (Bergersen & Turner, 1990) showing that ammonia is the principal product of N_2 fixation by bacteroids in the flow-chamber system. Bacteroids prepared by differential centrifugation in the standard bench method consistently had greater rates of respiration and N_2 -fixing activity than bacteroids prepared anaerobically under argon/ H_2 in a glove box and purified by sucrose density-gradient centrifugation. Typically, respiration by bacteroids prepared in the standard bench method was $0.97\text{--}17.90\text{ nmol O}_2\text{ min}^{-1}\text{ mg}^{-1}$ and N_2 fixation was $0.6\text{--}4.7\text{ nmol NH}_3\text{ min}^{-1}\text{ mg}^{-1}$ (Table 1). For bac-

teroids prepared on sucrose gradients the corresponding values were $0.8\text{--}13.8\text{ nmol O}_2\text{ min}^{-1}\text{ mg}^{-1}$ and only $0.10\text{--}1.39\text{ nmol NH}_3\text{ min}^{-1}\text{ mg}^{-1}$. With similar rates of O_2 supply, respiration of bacteroids prepared by Percoll density-gradient methods was similar to that of sucrose-gradient-prepared bacteroids but N_2 fixation was greater for Percoll-prepared bacteroids (data not shown).

Regulation of bacteroid N_2 fixation and respiration by O_2 supply

When a low flow rate (approx. 0.5 ml min^{-1}) of medium containing low $[\text{O}_2\text{ free}]$ was initially supplied to the bacteroids, bacteroid respiration was low, remaining under $1\text{ nmol O}_2\text{ min}^{-1}\text{ mg}^{-1}$, and $[\text{O}_2\text{ free}]$ declined slowly to approximately 30 nM (Figs 1a and 3a, Table 1). When the flow rate was increased from about 0.5 to 0.75 or 0.8 ml min^{-1} at 40 min , the rate of O_2 and malate supply increased by approximately 50% , $[\text{O}_2\text{ free}]$ in the chamber decreased (Figs 1a and 3a, Table 1), but bacteroid respiration and N_2 fixation increased (Fig. 1c; Tables 1 and 2). With further increases of O_2 supply (increasing the flow progressively), respiration increased and $[\text{O}_2\text{ free}]$ decreased until a limit of respiration was approached, at which point $[\text{O}_2\text{ free}]$ began to rise (e.g. at 3.1 ml min^{-1} ; Fig. 1a, Table 1).

N_2 fixation increased with increased respiration (e.g. Fig. 1b, c). Typically, in one of these experiments, when O_2 supply was increased from 58.6 to 940 nmol min^{-1} (an increase of approx. 16-fold), $[\text{O}_2\text{ free}]$ declined from 0.035 to $0.0098\text{ }\mu\text{M}$ and respiration increased from 0.97 to $17.9\text{ nmol O}_2\text{ min}^{-1}\text{ mg}^{-1}$ (an 18.4-fold increase); N_2 fixation increased from 0.64 to $4.68\text{ nmol NH}_3\text{ min}^{-1}\text{ mg}^{-1}$ (7.4-fold) (Table 1). These effects of increased flow rate were not affected by different soybean growth conditions; similar declines in $[\text{O}_2\text{ free}]$ occurred with bacteroids from glasshouse-grown and growth-cabinet-grown nodules (compare Figs 1 and 2). Sometimes a change of flow rate induced oscillations in respiration rate, which persisted with diminishing am-

Table 1. Data from steady states in the flow-chamber experiment illustrated in Fig. 1

The bacteroids (50 mg dry mass in the chamber) were prepared by the standard bench method. The substrate was 2 mM malate; the reaction solution contained $62.8\text{ }\mu\text{M}$ oxyleghaemoglobin and either $60\text{ }\mu\text{M}$ (A) or $240\text{ }\mu\text{M}$ (B) $[\text{O}_2\text{ free}]$.

Time interval (min)	Flow (ml min^{-1})	O_2 supply* (nmol min^{-1})	$[\text{O}_2\text{ free}]$ (μM) (SD)	<i>n</i>	Respiration ($\text{nmol O}_2\text{ min}^{-1}\text{ mg}^{-1}$) (SD)	N_2 fixation ($\text{nmol NH}_3\text{ min}^{-1}\text{ mg}^{-1}$) (SD)	<i>n</i>
31–42	0.478 A	58.57	0.0350 (0.0437)	12	0.971 (0.044)	0.637 (0.017)	2
47–75	0.747 A	91.54	0.0202 (0.0025)	29	1.610 (0.099)	0.818 (0.092)	7
84–95	0.991 A	121.45	0.0115 (0.0011)	11	2.231 (0.205)	0.978 (0.110)	4
99–113	0.991 B	300.02	0.0129 (0.0009)	15	5.741 (0.150)	1.077 (0.083)	4
119–124	1.441 B	436.33	0.0062 (0.0003)	16	8.483 (0.020)	1.762 (0.277)	7
140–147	1.899 B	575.02	0.0067 (0.0006)	18	11.177 (0.491)	2.306 (0.193)	7
162–172	3.105 B	940.19	0.00981 (0.0002)	11	17.902 (0.711)	4.685 (0.549)	8

* Free dissolved O_2 plus O_2 in oxyleghaemoglobin from the reaction solution at the given flow rate.

Fig. 1. Data from a typical flow-chamber experiment. The bacteroids were prepared by the standard bench method, from glasshouse-grown soybeans. Reaction solutions pumped through the chamber contained 2 mM sodium DL-malate, 63 μM oxyleghaemoglobin and either 60 μM (A) or 240 μM (B) [O₂ free]. (a) Time course of changes in flow rate (continuous line) and concentration of [O₂ free] (Δ) in the chamber. (b) Bacteroid respiration (▼). (c) N₂ fixation by bacteroids measured as evolution of NH₃ (▲).

plitude until a new equilibrium [O₂ free] was reached as long as 30 min after the change (data not shown).

In other experiments, when O₂ supply per bacteroid was increased by progressive removal of bacteroids from the flow chamber whilst maintaining constant flow rates, respiration increased after each sample was taken (Fig. 3a, b). [O₂ free] was almost unchanged through samplings 2, 3 and 4. (Fig. 3a, b). These data also indicated clearly that the supply of O₂ to the bacteroids regulated O₂ demand.

Fig. 2. Effect of increased flow rate from 0.6 to 1.2 ml min⁻¹ on [O₂ free] in the chamber and bacteroid respiration. Bacteroids for these experiments were prepared by the standard bench method, from nodules on soybean plants grown in Leonard jar assemblies in artificially lit growth cabinets. The reaction medium contained 0.5 mM DL-malate, 40 μM oxyleghaemoglobin and 238 μM [O₂ free]. Symbols as in Fig. 1(a, b).

Fig. 3. Flow rates and [O₂ free] (a) and respiration rates of bacteroids (b) in a flow-chamber experiment from which samples were taken (arrows) for measurements of [¹⁴C]malate uptake in standard microaerobic assays. The reaction solution contained 50 μM malate, 140 μM oxyleghaemoglobin and 219 μM [O₂ free]. Symbols as in Fig. 1(a, b).

Fig. 4. The time course of uptake of [^{14}C]malate by bacteroids is affected by the concentration of O_2 during preparation and assay. Bacteroids were prepared anaerobically and shaken vigorously in air (Δ , $\blacktriangledown$) or maintained under argon (∇ , $\blacktriangle$) before uptake of malate was measured in air (Δ , $\blacktriangle$) or microaerobically (see Methods; ∇ , $\triangledown$).

Uptake of malate by bacteroids as affected by method of preparation and assay conditions

The initial rate of uptake of [^{14}C]malate by bacteroids was greatest in aerobically treated bacteroids which were also assayed aerobically (Fig. 4) but the rate declined after 5 min. Usually, rates declined earlier in all aerobic uptake assays than in microaerobic assays. Although rates of uptake were lower for microaerobic than for aerobic assays, they were usually maintained

for at least 9 min. Preparation of bacteroids anaerobically suppressed malate uptake rates, even when uptake was measured in the presence of O_2 . It was considered that the microaerobic assays most closely resembled conditions prevailing in flow-chamber experiments. Other experiments (data not presented) showed that rates of malate uptake by bacteroids recovered from the flow chamber were independent of malate concentration at concentrations up to 1 mM.

Malate uptake by flow-chamber bacteroids

Bacteroid N_2 fixation *in vivo* is supported by respiration, coupled with the consumption of substrates (e.g. malate uptake; Udvardi *et al.*, 1988) provided by the host plant. Also, bacteroids supplied with malate accumulate poly- β -hydroxybutyrate, which serves as an endogenous reserve of respirable substrate. Utilization of this endogenous reserve is regulated in relation to the rate of O_2 supply (Bergersen & Turner, 1992). We investigated uptake of malate by bacteroids taken from the flow chamber and in separate experiments in which batches of bacteroids were exposed simultaneously to different oxygen environments. In the flow-chamber experiments, bacteroids were withdrawn from the flow chamber and uptake of [^{14}C]malate measured in standard microaerobic assays. The results showed that concomitantly with increased bacteroid respiration preceding each sample, maximum (malate un-limited) rates of transport of [^{14}C]malate into bacteroids increased (Table 2). These results suggest that rates of malate uptake may be tightly coupled with bacteroid respiration and thus with N_2 fixation. In other experiments (data not shown), we

Table 2. Steady-state values of [$\text{O}_{2\text{ free}}$] and rates of bacteroid respiration in relation to measurements of [^{14}C]malate uptake by samples withdrawn from flow-chamber experiments

There were two separate experiments (one of which is illustrated in Fig. 4), in which the bacteroids in the flow chamber were supplied with 50 μM malate. Tabulated data are means and (SD) for the 10 min preceding each sampling. Data for duplicate standard microaerobic malate uptake assays in two experiments were pooled and submitted to analysis of variance.

Sample time (min)	Flow chamber conditions:		[^{14}C]Malate uptake ($\text{nmol min}^{-1}\text{mg}^{-1}$)
	[$\text{O}_{2\text{ free}}$] (μM)	Respiration ($\text{nmol O}_2\text{ min}^{-1}\text{mg}^{-1}$)	
16	0.034 (0.002)	0.91 (0.13)	0.68*
	0.011 (0.04)	1.48 (0.28)	
36	0.008 (0.0010)	1.39 (0.04)	2.29*
	0.007 (0.0006)	1.53 (0.09)	
72	0.005 (0.0002)	1.62 (0.04)	2.92*
	0.005 (0.0003)	1.82 (0.05)	
102	0.006 (0.0003)	1.91 (0.02)	3.93*
	0.006 (0.0010)	2.18 (0.13)	

* Analysis of variance showed that, despite small but significant differences between the two experiments, malate uptake increased significantly ($P < 0.001$) with each increase in bacteroid flow chamber respiration.

found that the $V_{\max}$ but not the apparent K_m for malate uptake changed during exposure to higher respiratory demand. These changes were not affected by the inclusion of chloramphenicol (at concentrations shown independently to inhibit protein synthesis) in the flow-chamber medium.

To investigate whether the higher rates of malate uptake were simply the result of an increased demand for malate upon an increase in respiration, bacteroids were prepared anaerobically, incubated either under anaerobic conditions or in air-saturated medium for 1–7 min, and malate uptake measured under both aerobic and microaerobic conditions (Fig. 4). Quite clearly, the exposure to higher O_2 preconditioned the bacteroids to take up malate at a faster rate, regardless of the assay conditions.

DISCUSSION

The flow-chamber data presented here and by Holtzappel & Bergersen (1998) indicate that bacteroid respiratory rate increased as the rate of O_2 delivery increased in the medium flow. Consequently, equilibrium levels of $[O_2]_{\text{free}}$ (6–35 nM; Table 1) were maintained at low levels, or even fell, in the chamber – even when the rate of O_2 supply increased by as much as 25-fold (Figs 1 and 2, Table 1). This suggests that, in flow-chamber experiments with USDA110 bacteroids, respiration is controlled by the rate of O_2 supply, rather than by the $[O_2]_{\text{free}}$ prevailing in the stirred chamber. In other work with bacteroids of *B. japonicum* strain CB1809 (e.g. Bergersen & Turner, 1993), respiration rates were related to $[O_2]_{\text{free}}$ in a Michaelis–Menten fashion. The reasons for this important difference are not clear but could be due to bacterial strain differences. Also, the CB1809 bacteroids received no exogenous substrate during the initial period at low flow rate and during this period showed regulation of endogenous O_2 demand in response to changes in O_2 supply, by mobilizing poly- β -hydroxybutyrate utilization (Bergersen & Turner, 1992). Thus there may be several mechanisms for regulating bacteroid respiration in response to changes in supply of O_2 .

The results also suggest that malate transport is regulated by a related mechanism since rates of malate uptake by bacteroids increased according to respiration rates preceding sampling (Table 2). The rate of supply of malate to bacteroids in the chamber (e.g. 40 nmol min⁻¹ mg⁻¹ at a flow of 1.0 ml min⁻¹; Table 1) greatly exceeded the rate of O_2 consumption (2.2 nmol min⁻¹ mg⁻¹ in the same conditions; Table 1), so it is unlikely that the increase in malate uptake resulted simply from an increase in malate supply to the chamber; this was certainly not the case in the standard uptake assays of Fig. 4. Nor was this simply a case of a stronger sink for malate (resulting from increased respiration) stimulating uptake, because (i) significant differences in rate of uptake were recorded when aerobically and anaerobically prepared bacteroids were

assayed at the same oxygen concentration (Fig. 4). Also, (ii) with successive increases in bacteroid respiration preceding each sampling (Fig. 3), when malate uptake was measured under standard conditions in which respiratory rates were uniform and malate concentrations were non-limiting, specific rates of malate uptake also increased (Table 2). Rather, the respiratory demand during the period preceding the transport assays seemed to feedback and affect the uptake of malate. Since $V_{\max}$ but not K_m of uptake increased upon exposure to higher oxygen supply, it appears that more malate carriers were engaged following the increase in respiratory demand, but the lack of effect of chloramphenicol indicates that this was not the result of synthesis of new carrier proteins. Instead, the respiratory demand seemed to feedback and affect the uptake of malate directly. The nature of this conditioning process is not known but it suggests that both organic acid transport and metabolism in the bacteroid are controlled by a common factor, such as pyridine nucleotide redox poise.

It has long been known that the bacteroids of soybean root nodules have a branched respiratory electron-transport pathway (e.g. Appleby, 1969). Inflected curves of respiration vs $[O_2]$ were interpreted as reflecting differences in the affinities for O_2 of the terminal oxidases of the branches (e.g. Bergersen & Turner, 1975). Respiration at low $[O_2]$ (< 0.1 μ M) was more CO -sensitive and more efficiently coupled to ATP production and N_2 fixation (Appleby *et al.*, 1975; Bergersen & Turner, 1975) than was respiration at $[O_2]$ > 0.5 μ M. Supply of O_2 to bacteroids, when mediated by the presence of partially oxygenated leghaemoglobin, supported respiration in the well-coupled range of $[O_2]$ (Wittenberg *et al.*, 1974; Appleby *et al.*, 1975; Bergersen & Turner, 1975). More recently (Preisig *et al.*, 1996), it has been proposed that there are four terminal oxidases for the electron-transport branches in *B. japonicum* and that in bacteroids it is the *cbb*₃-type cytochrome oxidase that is the principal oxidase operating in the microaerobic environment of soybean nodules. This oxidase has an extremely high affinity for O_2 (K_m = 7 nM). The results of the flow-chamber experiments reported in the present paper show that, when bacteroids were challenged with increasing supply of O_2 , high steady rates of respiration [> 11 nmol O_2 min⁻¹ (mg dry mass)⁻¹] and N_2 fixation [> 2 nmol NH_3 min⁻¹ (mg dry mass)⁻¹] were achieved at about 6 nM O_2 (Table 1, Fig. 1), close to the K_m of the *cbb*₃-type cytochrome oxidase (Preisig *et al.*, 1996). These results imply that $V_{\max}$ for respiration via this oxidase increases with rates of O_2 supply, perhaps up to values as high as 30 nmol O_2 min⁻¹ (mg dry mass)⁻¹ (i.e. twice the rate at the K_m) as transport of malate into bacteroids (Table 2) increases to meet the respiratory demand. In the flow-chamber experiments, the principal product of N_2 fixation was NH_3 in the effluent solution as reported previously with a different strain of *B. japonicum* (Bergersen & Turner 1990). In the present work, no more than traces of alanine (as described by Waters *et al.*, 1998) were found by enzymic

assay or by HPLC (data not presented), even when density-gradient-purified bacteroids were used (Waters *et al.*, 1998). Allaway *et al.* (2000) suggested that in the flow chamber, NH_3 was swept away, thus preventing the concentration from rising sufficiently for alanine to be synthesized in detectable amounts. This seems a reasonable explanation.

Conclusion

The results presented here show that under certain conditions (low substrate and low oxygen supply), bacteroid metabolism, including nitrogenase activity, is regulated by the rate of oxygen supply instead of the concentration of free dissolved oxygen. Regulatory mechanisms apparently exist which coordinate respiratory demand with rates of substrate transport into the bacteroid. This has important implications for maintenance of N_2 fixation rates *in vivo* and needs to be further investigated with intact symbiosomes. However, since bacteroid demand for malate strongly influences the rate of malate uptake into symbiosomes (Ou Yang *et al.*, 1991), it is likely that this regulation will be sensed also at the peribacteroid membrane.

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