Glutamate as a Carbon Source for N₂-Fixing Bacteroids Prepared from Soybean Root Nodules

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The possibility that glutamate might serve as a source of reducing power, supporting fixation of N₂ to NH₃ by bacteroids in soybean root nodules, was investigated. Suspensions of bacteroids were retained within a reaction chamber supplied with a flow of solution containing dissolved air, buffer, oxyleghaemoglobin and either disodium glutamate (1 or 10 mM) or no added substrate (2 or 20 mM-NaCl). The solution passing out of the chamber was analysed for dissolved NH₃ and proportional oxygenation of leghaemoglobin was determined spectrophotometrically. Rates of production of NH₃ and of consumption of O₂ and the concentrations of free, dissolved O₂ prevailing during steady states were calculated. At 1 mM, glutamate stimulated O₂ demand of the bacteroids and enhanced NH₃ production. However, increased production of NH₃ was shown to arise entirely from deamination of [¹⁵N]glutamate and not from N₂ fixation. At 10 mM-glutamate, the proportion of NH₃ arising from [¹⁵N]glutamate increased with increased steady state concentrations of free, dissolved O₂ in the chamber, but there was also significant and efficient stimulation of N₂ fixation when the concentration of free, dissolved O₂ was between 10 and 70 nM. The kinetics of O₂ consumption by bacteroids using endogenous substrates or 10 mM-glutamate were similar.

INTRODUCTION

The most recent evidence from studies of mutants of various species of *Rhizobium* (Arwas *et al.*, 1985; Finan *et al.*, 1983; Ronson *et al.*, 1981; Streeter & Salminen, 1985) supports results of other studies (e.g. Peterson & LaRue, 1981; Saroso *et al.*, 1984; Tuzimura & Meguro, 1960), which conclude that the tricarboxylic acid cycle acids succinate and malate are the principal energy sources for the symbiotic N₂ fixing bacteroids in the root nodules of legumes. An hypothesis involving glutamate, an important product of N₂ fixation, as a carbon source for bacteroids in nodules has been proposed (Kahn *et al.*, 1985) and this possibility has been supported by Salminen & Streeter (1987) who showed uptake, incorporation and respiration of exogenous [¹⁴C]glutamate by bacteroids from soybean nodules. The present paper presents results which show directly that both O₂ consumption and N₂ fixation by soybean bacteroids may be stimulated by exogenous glutamate, although adjustments must be made for NH₃ arising from deamination of glutamate.

METHODS

Plants, growth and production of nodules and bacteroid suspensions. Seeds of soybean (*Glycine max* Merr. cv. Lincoln) were inoculated with *Bradyrhizobium japonicum* strain CB1809 and grown in a sand/vermiculite mixture supplied with nutrient solution in a glasshouse as described previously (Bergersen & Turner, 1970). Bacteroid suspensions containing 70–100 mg dry wt ml⁻¹ in 50 mM-potassium phosphate buffer, pH 7.4 were prepared anaerobically, essentially as described by Bergersen & Turner (1973) but potassium phosphate buffer (50 mM, pH 7.4) was used instead of Tris/HCl for washing the bacteroids.

Abbreviation: LbO₂, oxyleghaemoglobin.

Oxyleghaemoglobin. Unfractionated oxyleghaemoglobin (LbO₂) was prepared from soybean root nodules and assayed as described by Appleby & Bergersen (1980). The value for the ratio of the association:dissociation constants for oxygenation, used in the calculations of concentration of free, dissolved O₂ from proportional oxygenation values, was $0.04 \times 10^{-6} \text{ mol l}^{-1}$.

Flow chamber methods. The apparatus consisted of a stirred reaction chamber, in which bacteroid suspensions were retained above a microporous membrane filter (Amicon) and supplied with a flow of solution pumped at measured rates from a reservoir. The absorption spectrum of the effluent solution passing from the chamber through a spectrophotometer flow cell allowed monitoring of the state of leghaemoglobin oxygenation in the chamber, which is a sensitive indicator of the concentration of free O₂. The O₂ consumption rates were calculated from the flow rate and the difference between the concentrations of free, dissolved O₂ and LbO₂ in the solutions entering and leaving the chamber. Various concentrations of free O₂ and rates of consumption of O₂ were achieved by varying the flow rate in monitored steps. Except for measurement of N₂ fixation, the apparatus, solutions, analytical methods and methods for recording data and calculation, were all as described previously (Bergersen & Turner, 1985; Bergersen *et al.*, 1986).

The reaction solution was made up in 25 mM-MOPS (Calbiochem)/KOH buffer, pH 7.4, and contained 2 mM-MgSO₄, 0, 1.0 or 10 mM-sodium glutamate (the concentration of Na⁺ was maintained with NaCl in medium with no added glutamate) and 90–100 µM-LbO₂ (added to a final volume of 500 ml as 30 ml of concentrated LbO₂ in 50 mM-potassium phosphate buffer), stirred in air at 23–24 °C. Concentrations of dissolved O₂ in the reaction solution were calculated from tables of the solubility of O₂ in water (Deane, 1979), at the atmospheric pressure and ambient temperature prevailing in the reservoir during each experiment. Within the chamber the temperature was 30 °C.

Measurement of N₂ fixation. This was calculated from the concentration of NH₃ in the effluent solution and the medium flow rate. Concentration of NH₃ was measured in 0.9 ml samples of effluent solution by the colorimetric method of Chaney and Marbach (Bergersen, 1980) using standards of 0.25–6 µg ammonium sulphate-N made up in 0.9 ml of reaction solution. The reference cuvette contained the reagent blank, also prepared in reaction solution. In this way, the effects of the background colour of the solution were eliminated. In a typical analysis $(\mu\text{g NH}_3\text{-N}) = (A_{625} - 0.001)/0.0933$ and minimum A_{625} values of 0.020 were reliably obtained with a Varian DMS90 spectrophotometer. Thus the lower limit for reliable measurement was $0.25 \mu\text{g NH}_3\text{-N ml}^{-1}$.

¹⁵N analysis. In some experiments [¹⁵N]glutamate (90–95 atoms% ¹⁵N; Amersham) was used to enable NH₃-N derived from glutamate to be differentiated from that derived from fixation of N₂ dissolved in the reaction medium. In these experiments, effluent solution was collected from the chamber during steady states prolonged for up to 120 min, in order to collect sufficient NH₃ for mass spectrometer analysis. Leghaemoglobin was removed from the 70–100 ml of solution by filtration through a Diaflo YM10 membrane (Amicon) and NH₃ was recovered and estimated by distillation into 1% (w/v) boric acid, after addition of an excess of NaOH and titration (Bergersen, 1980). Incorporation of ¹⁵N into the bacteroids was determined by recovering the chamber contents, washing the bacteroids by centrifugation and resuspension, and analysis for total N and ¹⁵N. Atoms% ¹⁵N was determined in titrated distillates as described by Bergersen (1980), using a VG Micromass (Middlewich, Cheshire, UK) model 903 isotope ratio mass spectrometer.

RESULTS

Preliminary experiments with ¹⁵N₂ dissolved in reaction solutions established that, in the absence of glutamate, >98% of the NH₃ in the effluent solution arose from N₂. Initial experiments using 1 mM-glutamate showed that bacteroid O₂-demand and production of NH₃ were enhanced compared with rates using no added substrate. Fig. 1 presents the time-course of an experiment in which medium was changed from a solution with no added substrate to 1 mM-glutamate, 83 min after the experiment commenced with the injection of bacteroid suspension (47 mg dry wt) into the chamber. Within 10 min of the change, the steady state concentration of free, dissolved O₂ declined from $20.4 \pm 0.02 \text{ nM}$ to a new steady value of $9.8 \pm 0.1 \text{ nM}$, due to the increased demand for O₂. The O₂ consumption rate increased slightly [from 5.83 ± 0.008 over 20 min preceding the change, to $5.95 \pm 0.04 \text{ nmol O}_2 \text{ min}^{-1} (\text{mg dry wt})^{-1}$ in the 30 min after glutamate supply commenced], due mainly to increased deoxygenation of LbO₂ in the reaction chamber. The flow of solution being constant, the net effect on the rate of O₂ consumption was small. Production of NH₃ increased from 1.45 to $2.34 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$. Fig. 2 shows the relationships between the concentration of free, dissolved O₂ and rates of O₂ consumption and NH₃ production in the presence and absence of 1 mM-glutamate. The contribution of NH₃ from deamination of glutamate was assessed by supplying [¹⁵N]glutamate (1 mM) in the reaction

Fig. 1. Effect of 1 mM-glutamate on O_2 consumption and apparent N_2 fixation by CB1809 bacteroids (47.0 mg dry wt) in a flow chamber reaction, with a constant flow (0.883 ± 0.006 ml min $^{-1}$) of medium containing $113.9 \mu\text{M-LbO}_2$ and $230 \mu\text{M}$ free, dissolved O_2 . Samples for analysis of NH_3 were collected over the time intervals covered by the vertical bars.

Fig. 2. Relationship between concentration of free, dissolved O_2 during steady states, and rates of O_2 consumption (a) and NH_3 production (b). The medium entering the stirred reaction chamber contained no added substrate ($\circ$), 1 mM-glutamate ($\bullet$), $108.5 \mu\text{M-LbO}_2$ and $224\text{--}236 \mu\text{M}$ free O_2 . Steady state concentrations of free, dissolved O_2 were varied by changing the rate of flow of reaction medium through the chamber and were calculated as described in Methods. Measurements were made over periods of 10–20 min, commencing at least 15 min after each change in flow rate. There were 40 min between the change to glutamate and the first succeeding rate measurements. The chamber contained 43 mg dry wt of bacteroids.

solution. The results (Table 1) showed that all of the increase in NH_3 production obtained when 1 mM-glutamate was supplied arose from the glutamate itself; N_2 fixation was unchanged. Analysis of the effluent solution by HPLC revealed no ninhydrin-reactive products other than NH_3 and unused glutamate (M. B. Peoples, personal communication). Because 1 mM-glutamate may be considered to be a low concentration (10 mM-succinate is routinely used in other work

Table 1. *Effects of 1 mM-[¹⁵N]-glutamate and correction of N₂ fixation rates*

The medium contained 112.8 µM-LbO₂, 234 µM free, dissolved O₂ and 49.2 mg dry wt of bacteroids. Conditions prevailing during similar steady states are indicated in (a).

(a) *Conditions during steady state measurements, and apparent N₂ fixation rates (R) calculated from total NH₃ in samples of effluent solution*

	Collection time (min)	Flow (ml min ⁻¹)	Free O ₂ concn (nM)	O ₂ consumption [nmol min ⁻¹ (mg dry wt) ⁻¹]	Apparent N ₂ fixation [nmol NH ₃ min ⁻¹ (mg dry wt) ⁻¹]
No added substrate	79	1.140 ± 0.005	33 ± 0.9	6.84 ± 0.03	1.62 ± 0.03
Glutamate	101	1.184 ± 0.144	40 ± 0.4	7.04 ± 0.09	2.41 ± 0.05

(b) *Analysis of medium and effluent and calculated corrected N₂ fixation rates*

	Medium supplied			Effluent collected			N ₂ fixation [nmol NH ₃ min ⁻¹ (mg dry wt) ⁻¹]
	NH ₃ -N (µg ml ⁻¹)	Glutamate-N (µg ml ⁻¹)	Atoms% ¹⁵ N	Vol. analysed (ml)	NH ₃ -N (µg)	Atoms% ¹⁵ N	
No added substrate	0.29	0	—	87	121.8	0.3802	1.84*
Glutamate	0.29	7.01	1.3265	111	185.1	0.5826	1.86†

* N₂ fixation rate (R) = $F(a - b)/B$, where a and b are nmol NH₃ ml⁻¹ for effluent and medium, respectively; F is the medium flow rate (ml min⁻¹) and B the bacteroid dry wt.

† N₂ fixation rate (corrected) (R') = PR , where R is the apparent fixation rate, P is the proportion of NH₃ from N₂ fixation and $P = 1 - [(c - x)/(d - x)]$, c , d and x being the atoms% ¹⁵N values for effluent NH₃ (glutamate medium), the glutamate supplied and effluent NH₃ (no added substrate) respectively.

Table 2. *Effects of 10 mM-[¹⁵N]glutamate and correction of N₂ fixation rates*

The medium contained 105 µM-LbO₂, 237 µM free, dissolved O₂ and 56.4 mg dry wt of bacteroids. (Calculations and notations are as in Table 1.)

(a) *Conditions during steady state measurements and R*

	Collection time (min)	Flow (ml min ⁻¹)	Free O ₂ concn (nM)	O ₂ consumption [nmol min ⁻¹ (mg dry wt) ⁻¹]	R [nmol NH ₃ min ⁻¹ (mg dry wt) ⁻¹]
No added substrate	(Samples only)	1.237	64 ± 6	6.09 ± 0.03	0.866 ± 0.009
Glutamate	38	1.735	29 ± 2	9.17 ± 0.07	3.676 ± 0.053

(b) *Analysis of medium and effluent and R'*

	Medium supplied			Effluent collected			R' [nmol NH ₃ min ⁻¹ (mg dry wt) ⁻¹]
	NH ₃ -N (mg ml ⁻¹)	Glutamate-N (mg ml ⁻¹)	Atoms% ¹⁵ N	Vol. analysed (ml)	NH ₃ -N (mg)	Atoms% ¹⁵ N	
No added substrate	0.29	0	—	(Samples only — 0.553 µg ml ⁻¹)	—	0.3802	0.87
Glutamate	0.29	70.1	1.3265	49.0	107.12	0.6901	2.80

with bacteroid suspensions), the effects of [¹⁵N]glutamate were examined with a supply concentration of 10 mM. Endogenous activities of N₂ fixation and respiration were lower, but stimulation of O₂ demand and of NH₃ production were much greater (Table 2). Although 32.7% of the effluent NH₃ arose from the glutamate, the corrected N₂ fixation rate in the presence of glutamate [2.8 nmol min⁻¹ (mg dry wt)⁻¹] was much greater than with no added substrate [0.87 nmol NH₃ min⁻¹ (mg dry wt)⁻¹]. All subsequent experiments used 10 mM-glutamate. The proportion of NH₃ arising from glutamate increased with increasing concentrations of free, dissolved O₂, but not in proportion to increased rates of O₂ consumption (Fig. 3).

Fig. 3. Effects of steady state concentrations of free, dissolved O₂ on rates of O₂ consumption (●) and proportion of effluent NH₃ arising from 10 mM-glutamate in the reaction medium entering the chamber (○). This medium contained 103.8 μM-LbO₂ and 231–241 μM free, dissolved O₂. The chamber contained 53.3 mg dry wt of bacteroids.

When steady rates of O₂ consumption and N₂ fixation were plotted against the corresponding steady concentrations of free, dissolved O₂ (Fig. 4), rates with and without glutamate were similar up to 10 nM-O₂ but at 30 nM-O₂, 10 mM-glutamate doubled the rates of N₂ fixation and O₂ consumption compared to those observed without added substrate. At higher concentrations, N₂ fixation declined variably. In one experiment, N₂ fixation with glutamate fell by more than 50% between 40 and 100 nM-O₂.

Rates of O₂ consumption and N₂ fixation were positively correlated up to 40 nM-O₂ in three separate experiments with 10 mM-glutamate ($r = 0.97$; slope = 3.7 O₂/NH₃; $n = 12$). In experiments with no added substrate, between 5 and 70 nM-O₂, similar results were obtained ($r = 0.88$; slope = 3.9 O₂/NH₃; $n = 21$).

The similarity between the effects of exogenous glutamate and endogenous respiratory substrates is also seen in the kinetics of O₂ consumption. Double reciprocal plots of steady O₂ consumption rates (v) and steady concentrations of free dissolved O₂ (s) were curved upwards and plots of v^{-1} against s^{-2} were linear (Segel, 1975), with somewhat similar s^{-2} intercepts ($K' = 8.8$ and 7.7 nM-O₂ in two experiments with 10 mM-glutamate, compared with 5.2 nM-O₂ for endogenous activity). Data from two representative experiments are shown in Fig. 5.

DISCUSSION

Recent studies of substrate utilization by suspensions of root nodule bacteroids have utilized the C₂H₂ reduction assay to assess the coupling between respiratory and nitrogenase activity (e.g. Bergersen *et al.*, 1986; Trinchant & Rigaud, 1987). The experiments described above were intended to evaluate the possibility that glutamate, an important product of N₂ fixation in soybeans (Aprison *et al.*, 1954), may also serve as a carbon source for bacteroids, as proposed by Kahn *et al.* (1985). Therefore it was more appropriate to measure nitrogenase activity as NH₃ produced from N₂; this is more directly related to the reasons given by Kahn *et al.* (1985) when proposing that substrates such as glutamate could be utilized. In this discussion we merely consider the possibility that glutamate may be a carbon source and do not address the merits of the proposal. In our experiments the products of bacteroid activities were swept from the reaction chamber in the effluent medium stream, thus simulating the *in vivo* situation in which NH₃, fixed from N₂, is swept from the bacteroids by rapid assimilatory pathways in the host cytoplasm (e.g. Meeks *et al.*, 1978). According to the hypothesis of Kahn *et al.* (1985), this should favour the utilization of N-containing substrates such as glutamate. The results showed that at 1 mM-glutamate, O₂ demand was increased slightly, but the increase in concentration of NH₃ in the effluent medium was entirely due to the production of NH₃ from deamination of glutamate, N₂ fixation remaining unaltered. In contrast, 10 mM-glutamate stimulated both O₂ demand and reduction of N₂ to NH₃. The concentration of glutamate in the cytosol of soybean nodules has

Fig. 4

Fig. 5

Fig. 4. Effects of 10 mM-glutamate on steady rates of O₂ consumption and N₂ fixation (corrected). Results are from three separate experiments, one having both no added substrate (Δ) and glutamate (○) treatments. Reaction solutions entering the chamber contained 95.3–107.7 μM-LbO₂ and 225–243 μM free O₂. The chamber contained 39.2–53.5 mg dry wt of bacteroids. Dotted lines (Δ, ▲) indicate no added substrate. Continuous and dashed lines (●, ○) indicate 10 mM-glutamate. Bars indicate standard errors, where these exceed the dimensions of the symbols; the latter indicate means of two to five estimates. N₂ fixation rates were determined from concentrations of NH₃ in the effluent solution, after correction for NH₃ arising from glutamate (Fig. 3).

Fig. 5. Plots of v^{-1} against s^{-1} (●) and s^{-2} (○) for steady rates of O₂ consumption and steady concentrations of free, dissolved O₂. (a) Endogenous activity; (b) with 10 mM-glutamate. K' was calculated from the regression: $v^{-1} = a + bs^{-2}$, where a^{-1} is $V_{\max}$ and K' is given by $(a/b)^{-1/2}$. In (a) $V_{\max} = 7.3$ nmol O₂ min⁻¹ mg⁻¹ and $K' = 5.2$ nM; in (b) $V_{\max} = 12.5$ nmol O₂ min⁻¹ mg⁻¹ and $K' = 7.7$ nM.

been estimated to be in the range 1.6–5.5 μmol (g fresh wt)⁻¹ (Reynolds *et al.*, 1982; Minamisawa *et al.*, 1986; Streeter, 1987). Table 3 indicates the calculation of probable limits to the *in vivo* concentrations of glutamate in soybean nodules. The values, 1–11 mM, encompass the range of concentrations used in the reaction chamber experiments. Thus it is possible that the bacteroids in nodule tissue could utilize glutamate as a carbon source with stimulation of N₂ fixation. Salminen & Streeter (1987) reported that 0.5 mM-glutamate stimulated C₂H₂ reduction by soybean bacteroids. This result, different from ours with 1 mM-glutamate, was obtained with a different, non-steady-state system and probably at higher concentrations of free O₂, since

Table 3. Calculation of glutamate concentration in 36-d-old soybean nodules

Parameter	Value	Source
1. Glutamate concentration [$\mu\text{mol (g fresh wt)}^{-1}$]	1.6	Streeter (1987)
	2.3	Reynolds <i>et al.</i> (1982)
	5.5	Minamisawa <i>et al.</i> (1986)
2. Dry wt per nodule (g)	0.007	Bergersen (1982)
3. Nodule fresh wt/dry wt	4.45 ± 0.022	F. J. Bergersen (unpublished)
4. Nodule fresh wt (g)	0.03115	2×3
5. Glutamate content per nodule (μmol)	0.0716–0.1713	1×4
6. Nodule vol. (cm^3)	57.1×10^{-3}	Bergersen (1982)
7. Central tissue vol./total nodule vol.	0.64	Bergersen (1982)
8. Infected cell vol./central tissue vol.	0.77	Bergersen (1982)
9. (Cytoplasm + peribacterial space) vol./infected cell vol.	0.542	Bergersen (1982)
10. Glutamate concn ($\mu\text{mol cm}^{-3}$)		
(i) Distributed throughout nodule	0.9–3.0	5/6
(ii) Confined to cytoplasm of infected cells and the peribacteroid space	3.3–11.2	$5/(6 \times 7 \times 8 \times 9)$

mammalian oxymyoglobin was used in the reaction solution. Both experimental systems support the proposed mechanism of Kahn *et al.* (1985), but do not prove that it operates *in vivo* in nodules because glutamate may not freely cross the peribacteroid membrane, which separates bacteroids from the cytoplasm of infected nodule cells. Proof will require a confirmatory study of the N_2 fixing capacity of nodules produced by mutants of *B. japonicum* which are unable to utilize glutamate.

As prepared in our experiments, soybean bacteroids had substantial endogenous reserves of substrates which sustained respiration and N_2 fixation with little diminution for at least 4 h without added substrates (data not shown). This endogenous respiration was efficient, only 3–4 mols O_2 being consumed per mol NH_3 produced from N_2 . Although respiration and N_2 fixation were stimulated little, if at all, by 10 mM-glutamate when the concentration of free, dissolved O_2 was less than 10 nM both were doubled (compared with endogenous activity) when 10 mM-glutamate was supplied between 15 and 40 nM- O_2 . Thus this substrate was as efficient as endogenous substrates in supporting N_2 fixation. In other experiments, other exogenously supplied substrates were less efficient [unpublished data; also, for 10 mM-succinate, 3.6 mol O_2 (mol C_2H_2 reduced) $^{-1}$, quoted in Bergersen *et al.* (1986), equivalent to 7.2 mol O_2 (mol NH_3) $^{-1}$, compared with 3.7 and 3.9 mol O_2 (mol NH_3) $^{-1}$ with glutamate and endogenous substrates respectively].

The kinetics of steady rates of O_2 consumption were also similar when either endogenous substrates or glutamate were being respired, but the steady state measurements did not show the complex kinetics obtained when non-steady-state rates and succinate were used (Bergersen & Turner, 1980). Although the utilization of the large reserves of poly- β -hydroxybutyrate found in bacteroids has not been demonstrated (Wong & Evans, 1971), no other material would seem to be present in sufficient amount to account for the prolonged activity of bacteroids supplied with no other energy source, as found in our reaction chamber experiments. The similarity between the efficiency of support of N_2 fixation when endogenous substrates and glutamate are being utilized and between the kinetics of O_2 consumption with the respective energy sources may indicate that both are being used through common pathways. Our results do not disclose whether deamination of glutamate, which is clearly not directly related to rates of O_2 consumption, occurs in the medium, in the peribacterial space (i.e. within the free space of the bacterial envelope) or after it is taken up by the bacteroids. Nor do they indicate which of the possible products of deamination support the increased O_2 consumption.

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