Journal of General Microbiology (1988), **134**, 1807–1810. *Printed in Great Britain*

Fixation of N_2 by Bacteroids from Stem Nodules of *Sesbania rostrata*

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(*Received 21 October 1987; revised 16 March 1988*)

Bacteroids, prepared from stem nodules of *Sesbania rostrata* inoculated with *Rhizobium* sp. strain ORS571, in steady-state reactions in which O_2 was supplied in solution with soybean leghaemoglobin or mammalian myoglobin as O_2 carriers, and with succinate as exogenous substrate, fixed N_2 to NH_3 with highest rates at 10 nM free O_2, where O_2 uptake was approximately half-maximal. Higher concentrations (> 100 nM) of free O_2, shown previously to be required for optimum nitrogenase activity in O_2-trained ORS571 grown in continuous culture, inhibited N_2 fixation by stem nodule bacteroids.

INTRODUCTION

Bacteroids from stem nodules of *Sesbania rostrata* increased their O_2 consumption and nitrogenase activity at increasing concentrations of free dissolved O_2 up to 15 nM. At these very low concentrations, sesbania stem nodule leghaemoglobin was better than soybean leghaemoglobin in the facilitation of O_2 flux (Bergersen *et al.*, 1986). However, studies at higher concentrations of free O_2 were prevented because of shortage of stem nodules at that time. The same bacteria grew and fixed N_2 efficiently at 7–10 μM dissolved O_2 in continuous culture, but when grown at < 1 μM-O_2, nitrogenase activity was impaired above 0·1 μM-O_2 (Bergersen *et al.*, 1986). Thus it would be of interest to establish the sensitivity to O_2 of nitrogen fixation by the symbiotic forms of these bacteria. Trinchant & Rigaud (1987) found, with a different experimental system, that stem nodule bacteroids with succinate increased their nitrogenase activity in the range 5–20 nM-O_2; no decline in activity was recorded up to 50 nM. In this paper we report the steady-state relationship between rates of O_2 consumption and N_2 fixation by stem nodule bacteroids, in the range 6–1400 nM free dissolved O_2, using soybean leghaemoglobin and mammalian myoglobin in the reaction solutions.

METHODS

Plants, growth and production of nodules. Stem nodules on *Sesbania rostrata* inoculated with *Rhizobium* sp. strain ORS571, were produced, and bacteroid suspensions prepared from them, as described by Bergersen *et al.* (1986).

Flow chamber methods. The apparatus consisted of a stirred reaction chamber, in which bacteroid suspensions were retained and supplied with a flow of solution containing dissolved air, soybean oxyleghaemoglobin or sperm whale oxymyoglobin and 10 mM-succinate. The absorption spectrum of the effluent solution passing from the chamber through a spectrophotometer flow cell allowed monitoring of haemoglobin deoxygenation in the chamber and thus free O_2 concentration and O_2 consumption rates could be calculated. Except for measurement of N_2 fixation, the apparatus, solutions, analytical methods and methods for recording data and calculation, were all as described previously (Bergersen *et al.*, 1986).

Measurement of nitrogenase activity. Nitrogenase activity was assayed as the production of ammonia in solution (Bergersen & Turner, 1967), rather than by reduction of C_2H_2 to C_2H_4 (Bergersen *et al.*, 1986; Gebhardt *et al.*, 1984; Trinchant & Rigaud, 1987). In the apparatus used, experiments with bacteroids from other legumes, in which dissolved C_2H_2 was progressively replaced by dissolved N_2 (and *vice versa*) as substrates for nitrogenase (F. J. Bergersen & G. L. Turner, unpublished) showed no differences between rates of appearance of C_2H_4 from C_2H_2 and NH_3 from N_2. Rates of N_2 fixation were calculated from the concentration of NH_4^+ in the effluent

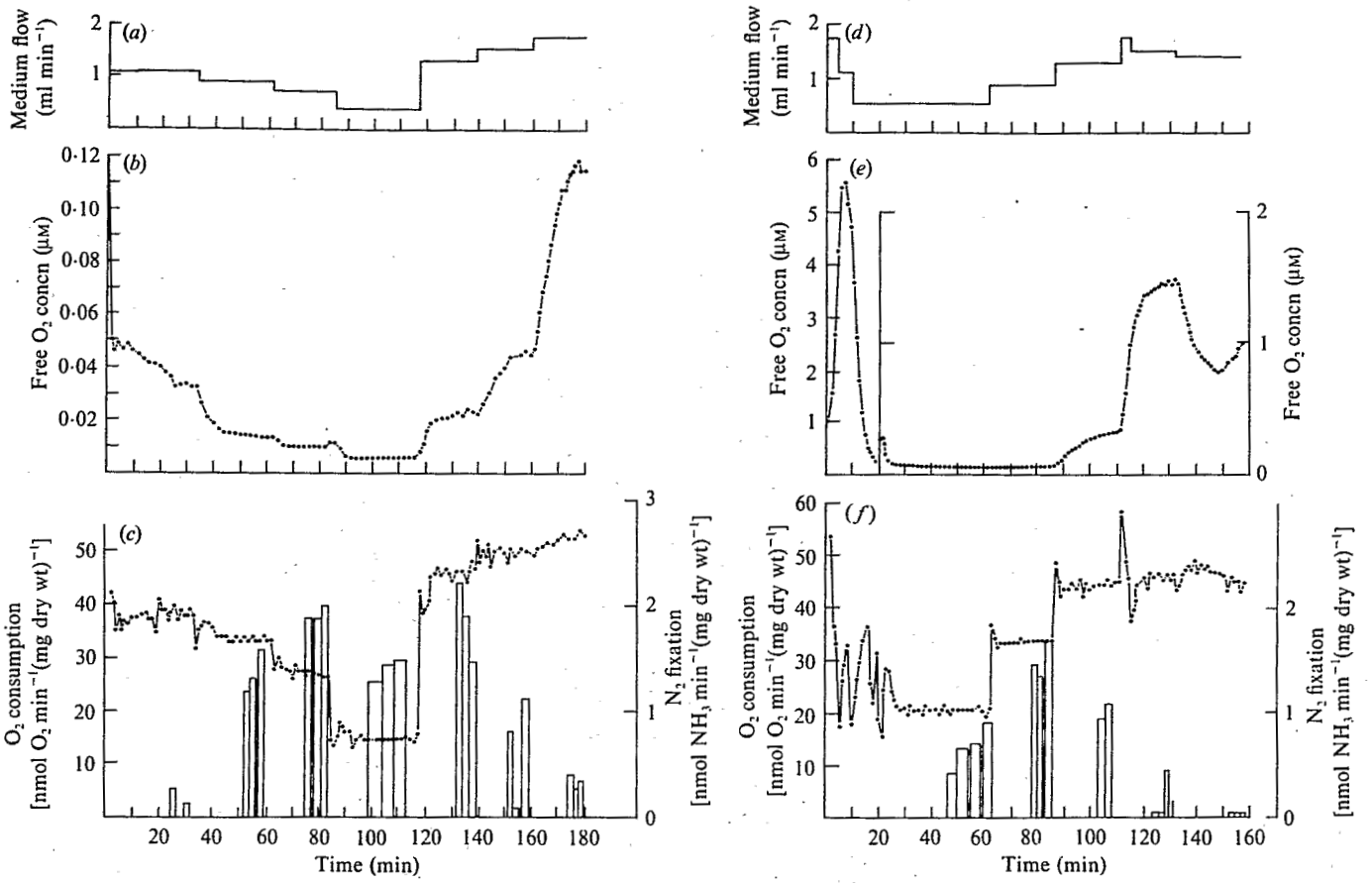

Fig. 1. Time-courses of flow chamber reactions with stem nodule bacteroids from *Sesbania rostrata*. The reaction solution, supplied at rates shown in (*a*), contained 103·4 μM-oxyleghaemoglobin (soybean), 10 mM-succinate, 250·4 μM dissolved O_2, in 25 mM-potassium phosphate buffer, pH 7·4, with 2 mM-$MgSO_4$. Concentrations of free dissolved O_2, calculated from oxygenation of leghaemoglobin in the effluent solution are shown in (*b*) and rates of O_2 consumption (●) and N_2 fixation (bars) in (*c*). The reaction solution, supplied at rates shown in (*d*), was as above but with 96·2 μM sperm whale oxymyoglobin and 244·5 μM free dissolved O_2. O_2 concentrations and reaction rates are shown in (*e*) and (*f*) respectively. The O_2 data are plotted at intervals of 1 min. N_2 fixation rates are calculated from concentrations of NH_4^+ in 2·0 ml samples of effluent solution and plotted as bars of width indicating the period of collection. The chamber contained 9·1 mg dry wt of bacteroids in the chamber for both reactions.

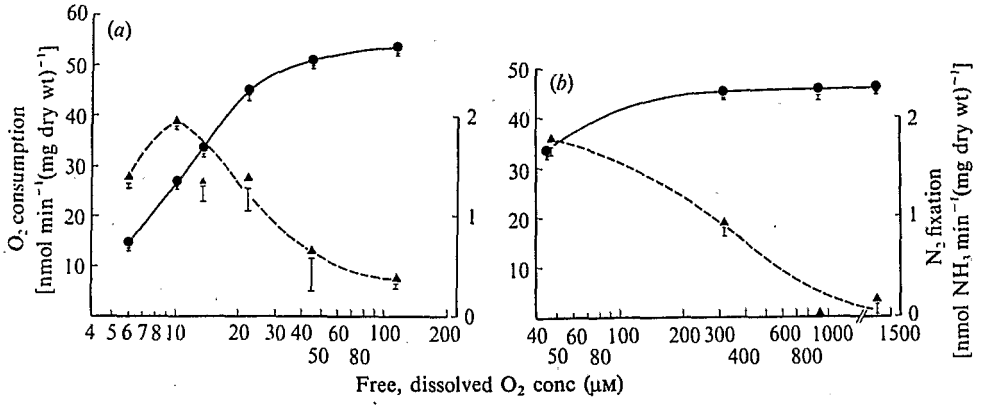

Fig. 2. Steady-state rates of O_2 consumption and N_2 fixation in relation to concentration of free dissolved O_2. Results from the experiments shown in Fig. 1. O_2 consumption (●) and N_2 fixation (▲) are shown with (*a*) leghaemoglobin and (*b*) myoglobin. Vertical bars indicate SEM values of three to four rate measurements. SE values for O_2 concentration during each rate measurement are smaller than the symbols.

solution and the medium flow rate. The concentration of NH_4^+ was measured in samples (0·9 ml) of effluent solution, by the colorimetric method of Chaney and Marbach (Bergersen, 1980), using standards (0·25–6 μg $NH_4^+ - N$) in 0·9 ml of reaction solution.

Steady-state measurements of O_2 consumption and N_2 fixation. Steady rates of O_2 supply were usually established for at least 20 min before collecting three or four samples (2·0 ml, timed by stop-watch) of effluent solution for estimation of NH_4^+. However, at the beginning of experiments more time was allowed for the establishment of the initial steady state, before the first samples were collected.

RESULTS AND DISCUSSION

Data from representative experiments (Fig. 1) show most of the previously reported features of stem nodule bacteroids and of the same bacteria grown in continuous culture at low concentrations of dissolved O_2 (Bergersen *et al.*, 1986). However, in addition, the data include observations over a much greater range of concentration of free dissolved O_2, include measurements made in the presence of oxymyoglobin instead of oxyleghaemoglobins, and present N_2 fixation measurements (production of NH_3 from N_2) rather than C_2H_2 reduction for nitrogenase activity. The following findings have not previously been reported. (I) In each experiment there was an initial, often brief, exposure to concentrations of free O_2 near or above 1 μM and initial rates of N_2 fixation were low. During and immediately following this period, O_2 consumption rates were not closely related to the concentration of free O_2. N_2 fixation was restored after 30–60 min, provided that the O_2 concentration was less than about 100 nM. These effects are seen particularly well in the oscillations in O_2 consumption during the first 30 min (Fig. 1*c*, *f*) and in the increasing N_2 fixation rates between 46 and 62 min (Fig. 1*f*). (II) In agreement with Trinchant & Rigaud (1987), who used a non-steady-state experimental system, steady-state measurement showed that stem nodule bacteroids supplied with succinate have greatest nitrogenase activity near 10 nM free dissolved O_2, where steady-state O_2 consumption rates are near half-maximal (Fig. 2). (III) Steady rates of N_2 fixation were lower at 6 nM free dissolved O_2, where respiration was also much lower (Fig. 2). (IV) When steady-state concentrations of free dissolved O_2 exceeded 20 nM in the presence of oxyleghaemoglobin or 100 nM with oxymyoglobin, O_2 consumption rates approached maximum values and N_2 fixation was inhibited (Fig. 2). In these conditions it seems to be likely that penetration of the bacteroids by O_2 occurs, leading to inactivation of nitrogenase, perhaps by covalent modification of nitrogenase reductase, as described for photosynthetic diazotrophic bacteria (Ludden *et al.*, 1984). This is supported by the comparatively rapid restoration of N_2 fixation following a brief initial exposure to excess O_2 (Fig. 1*f*), although the *de novo* synthesis of new nitrogenase cannot be excluded. (V) If there was a fixed relationship between concentrations of free dissolved O_2,

rates of O_2 consumption and rates of N_2 fixation, as suggested by Fig. 2, the imposition of a changed rate of O_2 supply would cause a predictable change in concentration to a level appropriate for O_2 consumption at that rate of supply. Fixation of N_2 would also change accordingly. Generally, steady-state measurements conformed to the relationships shown in Fig. 2. However, as reported previously for the same bacteria from continuous culture grown at low concentrations of dissolved O_2 (Bergersen *et al.*, 1986), stem nodule bacteroids sometimes consumed O_2 at widely different rates with little or no detectable change in prevailing concentrations of free O_2. This is seen in Fig. 1*e*, *f*, where there was an increasing rate of N_2 fixation between 45 and 62 min, indicating that a steady state was not yet established, despite steady consumption of O_2 and steady concentrations of free O_2 since 30 min. Following an increase in supply of O_2 at 63 min, a new and greater steady rate of O_2 consumption was established within 3 min; N_2 fixation was greater also but there was no discernible change in concentration of free dissolved O_2. Thus, the relationships between respiration rate and concentration of free O_2 shown in Fig. 2 are not invariate. The lack of a fixed relationship between O_2 concentration and O_2 consumption rates and the existence of time-dependent changes in rates during pre-steady-state adjustments in these bacteria, make extremely difficult the interpretation of short-term experiments in which O_2 concentration is continually changing and which attempt to relate apparent differences in optimal O_2 concentrations to utilization of different substrates (Trinchant & Rigaud, 1987). (VI) Finally, it is clear that bacteroids from stem nodules of *S. rostrata* lack the O_2-tolerant N_2-fixing system present in continuous cultures grown at 7–10 μM dissolved O_2. It is therefore unlikely that the presence of apparently active photosynthetic tissue in the cortex of stem nodules (Dreyfus & Dommergues, 1981) results in greater exposure of the symbiotic tissue to O_2 than is the case in root nodules.

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