THE ROLE OF O_2-LIMITATION IN CONTROL OF NITROGENASE IN CONTINUOUS CULTURES OF RHIZOBIUM sp.

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Received September 20,1976

SUMMARY. Oxygen-limited continuous cultures of the cowpea Rhizobium sp. strain CB756, had high levels of nitrogenase activity, which were not significantly affected by excess ammonium ions or glutamine. When the growth-restricting O_2-limitation was partially relieved, nitrogenase was repressed and this was accompanied by increased adenylylation of glutamine synthetase. It is suggested that the restricted supply of ATP interferes with adenylylation of glutamine synthetase during O_2-limited growth, thus preventing repression of nitrogenase in the presence of excess ammonium ions.

INTRODUCTION

In those strains of the legume root nodule bacteria (Rhizobium spp., rhizobia) whose nitrogenase activity has been studied in liquid culture, the importance of low concentrations of O_2 has been stressed (1-6). However, with cultures on agar media (7) and in continuous culture (5), high gas-phase concentrations of O_2 are tolerated or even required, provided that concentrations of O_2 within the culture itself are sufficiently low. Some reports (8,9) indicate that the rhizobia, when grown in ordinary laboratory culture, possess systems for the assimilation of NH_4^+ which are regulated in a similar fashion to that described for Escherichia coli (10) and Klebsiella aerogenes (11) for example. This system has been implicated in the control of nitrogenase synthesis in Klebsiella spp. (12,13,14). It is believed to involve the adenylylation of glutamine synthetase (10) in the presence of excess NH_4^+. The presence in the bacteria of this inactive form of glutamine synthetase apparently prevents synthesis of active nitrogenase. There have been conflicting

reports about the sensitivity of N_2-fixing cultures of rhizobia to repression of nitrogenase synthesis in the presence of excess NH_4^+ (1,3,4,5,15). Consequently, the role of adenylylated glutamine synthetase in the regulation of nitrogenase activity in these bacteria has been in doubt.

In this paper we report the apparent repression of nitrogenase synthesis in a strain of cowpea rhizobia when NH_4^+ was in excess and when the O_2-limitation which is necessary for high nitrogenase activity, was relieved by increased O_2-solution rates. The steady state concentration of dissolved O_2 remained low and an increase in adenylylation of glutamine synthetase was observed.

MATERIALS AND METHODS

Bacteria. The cowpea strain (Rhizobium sp.) CB756 was used. Induction of nitrogenase in this strain resembles that in the cowpea strain 32H1 used in earlier studies in this laboratory (5,16) but its origin is different. Stock cultures were maintained on yeast extract-mannitol agar (18).

Continuous cultures. The chemostat was used as previously described (5,6) with dilution rates of approximately 0.05/h. The culture volume was 575-600 ml, depending on the stirring rate, which was varied to attain different O_2-solution rates. The head-space gas was air or mixtures of air and N_2, supplied at 100 ml/min. The teflon coil (5) was supplied with 40 ml of air per min. The growth temperature was 28° and cell yield was estimated from a calibration of dry weight of water-washed cells and absorbance at 700 nm. The medium was that used previously (5) but with less succinate (5 mM) and the source of combined nitrogen was glutamine or $(NH_4)_2SO_4$ at various concentrations. The nature of the limitations prevailing in continuous cultures were determined by the criteria of Hill et al (17).

Periodically throughout the work, samples from continuous cultures were plated on yeast extract-mannitol agar and nutrient agar. No contamination was detected.

Assays. Nitrogenase (C_2H_2-reduction) in culture samples was measured in standard shaken assays with added oxyleghaemoglobin (5,6). Respiration of culture samples was measured at various concentrations of dissolved O_2 in an O_2 electrode respirometer (5) and respiration of the entire culture calculated for the prevailing concentration of dissolved O_2 indicated by the electrode immersed in the culture (5). Crude extracts of twice-washed bacteria from 60-90 ml of culture, finally suspended in 1-2 ml of 25 mM K-phosphate buffer, pH 7.5, and containing 2 mM 2-mercapto-ethanol, were made by sonic treatment at ice temperatures, followed by centrifugation at 10,000 g for 10 min. The γ-glutamyl transferase activity of glutamine synthetase (EC6.3.1.2) was measured at pH 7.15 with or without the addition of 60 mM $MgCl_2$ (11). Blanks were included from which ADP and arsenate were omitted. The results are

TABLE 1 Features of some O_2-limited steady states of continuous cultures of CB756.

Culture Conditions				Measurements			
Combined N medium supply	N residual in culture (mM)	Gas-phase (% air)	Stirrer setting	O_2 supply rate (μmol/min)*	Yield (mg/d. wt/h)	Steady state O_2 (μM)	Nitrogenease (nmol C_2H_4/mg d.wt/h)**
2 mM glutamine (control)	0.9	80	5	2.3	3.75	0.2	288 ± 9
1.5 mM NH_4^+	<0.005	100	4.8	5.9	5.0	0.9	0
1.5 mM NH_4^+	0.46	80	5	1.2	4.2	0.1	314 ± 14
34 mM NH_4^+	28.8	80	5	1.2	4.0	0.1	232 ± 3
34 mM NH_4^+	29.4	100	7	4.5	7.8	0.16	9 ± 0.3

* Calculated from cell density, culture volume and respiration of samples measured at the dissolved O_2 concentration prevailing in the culture.

** Measured in the standard shaken assays containing oxyleghaemoglobin (5); means and standard deviations of 4-6 assays.

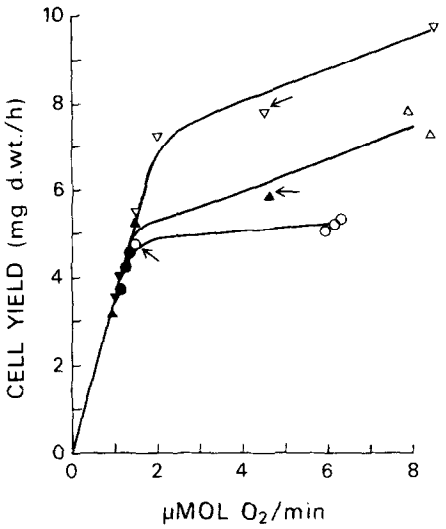

Figure 1. Cell yield and O_2 consumption in continuous cultures of CB756, with NH_4^+ supplied in the medium at 1.5 mM (o,●), 10 mM (Δ,▲) and 34 mM (▽,▼). High nitrogenase activity (> 100 nmol C_2H_4/mg/h) was associated with the steady states denoted by the blacked in symbols and none was detected above the arrowed points.

expressed as relative adenylylation values (activity with 3 mM Mn^{2+}/activity with 3 mM Mn^{2+} and 60 mM Mg^{2+}; ref 9), since the properties of the purified enzyme which are necessary for more precise determination of numbers of sub-units adenylylated, are not known for this organism (11). Glutamate synthase (EC2.6.1.53) and glutamate dehydrogenase (EC1.4.1.2) activities were assayed in extracts at pH 7.6 (8) after determining that in this organism these enzymes are specific for NADH.
All assays were done at 28°. Activities of intact cells are expressed in terms of dry weight of water-washed cells and in extracts in terms of protein determined by the method of Lowry et al (17) with bovine serum albumin standards.

RESULTS AND DISCUSSION

Highest nitrogenase activities in continuous cultures of the cowpea rhizobia (strains 32H1, ref. 6 and CB756, unpublished data) have been attained in O_2-limited steady states (6) but they are insufficient to provide all of the nitrogen needed for growth and a source of combined nitrogen must be provided. In such cultures we consistently failed to observe significant repression of nitrogenase when NH_4^+ was added to a concentration of 5 mM although in the less-active glutamine-limited

cultures, the addition of 5 mM NH_4^+ partially repressed nitrogenase activity (5). Table 1 shows that O_2-limited cultures of CB756, supplied with 34 mM NH_4^+ had 70-80% of the nitrogenase activity of cultures grown with 1.5 mM NH_4^+ or 2 mM glutamine. When the O_2 supply was increased by raised head-gas O_2 and/or increased stirring, there was an increase in cell yield (Table 1; Fig. 1). This was accompanied by greatly diminished nitrogenase activity. The steady-state concentration of dissolved O_2 at 34 mM NH_4^+ remained lower than that of the control culture. It is therefore unlikely that nitrogenase was directly inactivated by O_2, which was never in excess. Increased NH_4^+ in the culture gave increased maximum yields (Fig. 1). Also, with higher NH_4^+ concentration, more stringent O_2-limitation was required for high nitrogenase activity. For example, activities of 100 nmol C_2H_4/h/mg (dry wt.) or more were attained when cell yields were less than 85% of maximum values with 1.5 mM NH_4^+. With 34 mM NH_4^+ such activities were only attained when O_2 supply limited yields to less than 50% of the maximum obtainable with this concentration of NH_4^+. In Table 1, the order shown represents the order in which treatments were imposed. That is, the apparent derepression and repression were fully reversible. However, recovery of activity following a period of increased O_2 supply took 3-4 days (cf. ref. 5).

The relationship between nitrogenase activity, the presence of glutamate synthase, glutamate dehydrogenase and glutamine synthetase and its relative adenylylation were studied under several conditions of combined-N supply and O_2-limitation. Under all conditions, glutamine synthetase (γ-glutamyl transferase with 3 mM Mn^{2+}, 500-700 nmol/min/mg) and glutamate synthase (15-45 nmol NADH oxidized/min/mg) were present. Glutamate dehydrogenase appeared with low activities (2-5 n mol NADH oxidized/min/mg) only when the O_2 limitation was relieved. Relative adenylylation of glutamine synthetase increased with increased rates of O_2 supply in cultures supplied with excess NH_4^+ or glutamine (Fig. 2). In these

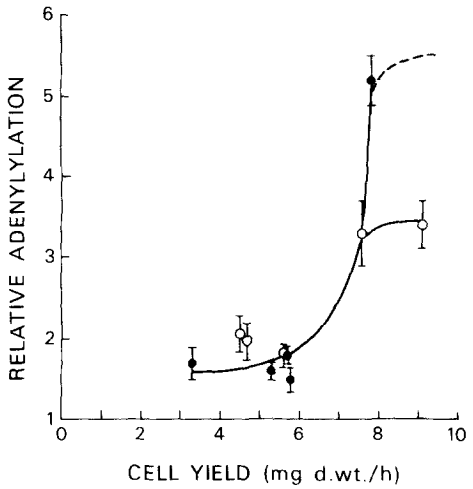

Figure 2. Increased adenylylation of glutamine synthetase with relief of O_2 limitation. Cell yields increased in response to increased O_2 supply. Glutamine (2 mM) (o) NH_4^+ (10 mM) (•). Bars indicate standard deviations.

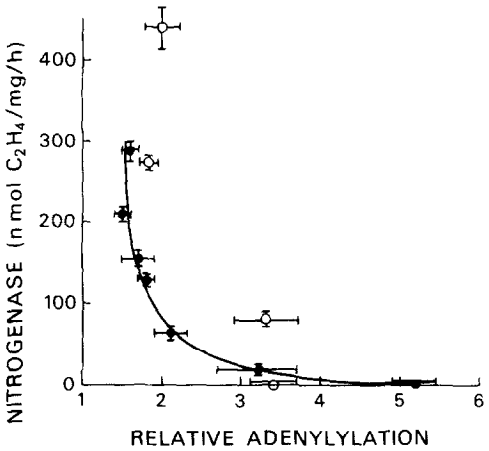

Figure 3. Relationship between relative adenylylation of glutamine synthetase and nitrogenase activity in continuous cultures of CB756 supplied with excess NH_4^+ (•). Data from a culture supplied with 2 mM glutamine are shown for comparison (o). Bars indicate standard deviations. Increased adenylylation was obtained with increased O_2 supply.

steady states, high nitrogenase activity was invariably associated with low relative adenylylation of glutamine synthetase (Fig. 3).

The results indicate that in CB756 there is a mechanism for control of nitrogenase synthesis in the presence of excess NH_4^+ of glutamine, which in

some respects resembles that found in Klebsiella (12,13,14). However, adenylylation of glutamine synthetase and repression of nitrogenase are inhibited when growth is severely restricted by limited supply of O_2. This may result from restricted availability of ATP with consequent failure of adenylylation (11). Alternatively, adenylylation of glutamine synthetase may be controlled by some other system under growth-limiting conditions, in addition to those systems described by Stadtman et al (10). The experiments described resolve the apparent discrepancies reported by Keister and Evans (3) and Evans and Keister (4). They also suggest that the low adenylylation values found in agar cultures of rhizobia in the presence of excess NH_4^+ (14) may be due to restricted supply of O_2 within the colonial mass, as has been reported for these (7) and other bacteria (18).

ACKNOWLEDGEMENTS. Mrs Pat Atkinson provided valuable technical assistance in these experiments.

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