NITROGEN FIXATION IN CULTURED COWPEA RHIZOBIA : INHIBITION AND REGULATION OF NITROGENASE ACTIVITY

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Summary:

Nitrogenase activity in agar cultures of cowpea rhizobia, strain 32H1, was rapidly inhibited by NH_4^+ but this was relieved by increased O_2 tension. Inhibition was more rapid than that caused by inhibitors of protein synthesis and was not relieved by methionine sulfoximine or methionine sulfone. Under conditions where nitrogenase activity was inhibited by NH_4^+, glutamine synthetase and glutamate synthase were substantially unaffected. Glutamate dehydrogenase was undetected in either nitrogenase active or NH_4^+ inhibited cultures. These results indicate that NH_4^+ inhibition of nitrogenase activity in strain 32H1 is not effected through glutamine synthetase regulation of nitrogenase synthesis.

Introduction

Evidence concerning the regulation of nitrogen fixation in *Rhizobium* sp. is scant due to the difficulty of understanding the relative contribution of host and symbiont to the process in legume nodules. In other diazotrophs the immediate product of nitrogen fixation, NH_4^+, has a central role in the regulatory process. NH_4^+ modulates the adenylylation of glutamine synthetase (1), and nitrogenase synthesis in *Klebsiella* requires the presence of biosynthetically active (nonadenylylated) glutamine synthetase (2,3,4). The ammonia assimilating enzymes have been found in cultured rhizobia, legume nodules and bacteroids (5-10) but none of these studies established a role for glutamine synthetase in the regulation of nitrogen fixation in rhizobia.

This report examines the inhibition of nitrogenase activity by NH_4^+, along with the effects of inhibitors of either protein synthesis or the enzymes involved in ammonia assimilation. The activities of the ammonia assimilating enzymes were also examined in cultures either with an active nitrogenase or where nitrogenase activity was inhibited by NH_4^+.

Materials and Methods:

Media and culture transfer sequence. CS8 and CS10 were modifications of CS7 media (11,12). CS8 contained 100 mM arabinose, 12.5 mM Na-succinate and 30 mM KH_2PO_4 (pH 6.0). CS10 (pH 7.0) was similar to CS8 but lacked succinate, glutamine and inositol. Cultures of cowpea rhizobia, strain 32H1, grown on yeast mannitol agar slopes, were suspended in 10 ml sterile water. One ml (5×10^8 cells) of this was dispersed in 10 ml CS8 soft agar and 0.2 ml applied to cellulose acetate filters (Millipore HAWP, 17 mm diameter, 7/plate) on CS8 agar plates which were sealed and incubated at 30°C for 7-10 days.

The development of nitrogenase activity by the bacteria on the filters (disc cultures) was monitored by excising the filter disc plus underlying agar and assaying for acetylene reduction. When test assays yielded greater than 15 nmol C_2H_4/culture/hr the disc cultures (without underlying agar) were transferred to McCartney bottles containing 5 ml fresh CS10 media, and where appropriate, inhibitors of nitrogenase activity (NH_4^+, NO_3^-, glutamine) or protein synthesis (chloramphenicol, rifampicin) or inhibitors of the enzymes of ammonia assimilation (methionine sulfoximine, MX; methionine sulfone, MF). Disc cultures contained approximately 1 mgm whole cell protein (13).

Cell enzyme extracts. The soft agar containing the bacteria was homogenized in 5 ml 50 mM phosphate buffer (pH 7.5) containing 2 mM β-mercaptoethanol, the suspension sonicated in a Bronson B-12 sonicator (6 x 30 sec bursts at 80W) and centrifuged at 20,000 g for 30 min at 4°C.

Enzyme assays. Nitrogenase (EC1.7.99.2) activity in disc cultures was determined by acetylene reduction as previously described (12) but with 0.08 atm C_2H_2 and 0.72 atm Ar in the gas mixture.

Glutamine synthetase (EC6.3.1.2) (GS) activity in cell extracts was determined by the γ-glutamyl transferase assay (14) at pH 7.15 (15); the extent of adenylylation of GS (15) was separately estimated by including 60 mM $MgCl_2$ in the reaction mixture.

Glutamate synthase (EC2.6.1.53) (GOGAT) and glutamate dehydrogenase (EC1.4.1.2) (GDH) activities in cell extracts were determined by recording the rate of oxidation of NADH after the method of Meers *et al.* (16) but in 70 mM phosphate buffer (pH 7.6) (7). Only very low levels of GOGAT and GDH activity were recorded with NADPH as co-factor.

Protein content in the cell extracts was determined by the method of Lowry *et al.* (17).

Results

Nitrogenase activity in disc cultures transferred from CS8 to CS10 was initially low but increased linearly to a maximum (20-40 nmol C_2H_4/disc/hr) after 1-2 hr incubation. Activity was not improved when arabinose was either reduced or replaced by up to 25 mM succinate.

Nitrogenase activity in the disc cultures was inhibited by ammonia, nitrate and glutamine (Fig. 1). The relationship between nitrogenase, nitrate inhibition and nitrate reductase are published separately (18).

Inhibition by NH_4^+ was not progressive with time (Fig. 2). In other experiments disc cultures were transferred to 1 mM NH_4^+ and assayed 12 and 24 mins after transfer. Although nitrogenase activity in these early assays was low (see above), percent inhibition was similar to that for 1 mM

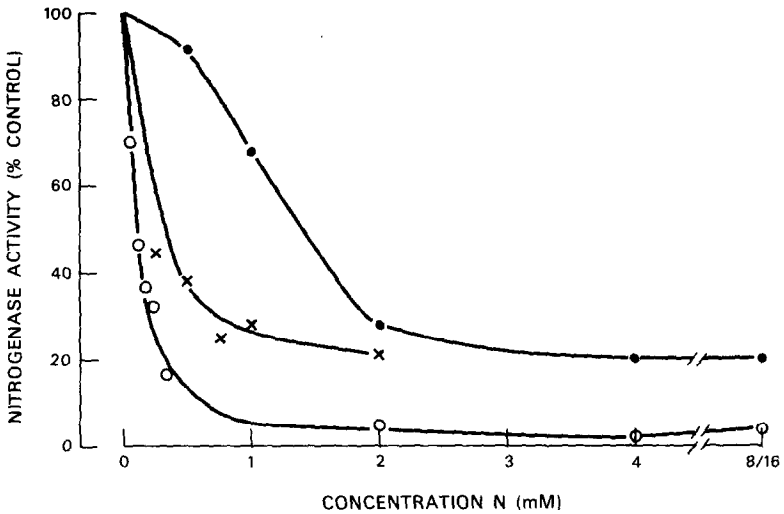

Figure 1. Nitrogenase activity as percent of control (no added N), of 32H1 disc cultures transferred to CS10 media containing NH_4^+(●), glutamine (X) or NO_3^-(O). Mean rates (5 replicates) were determined 1.5 hrs after transfer.

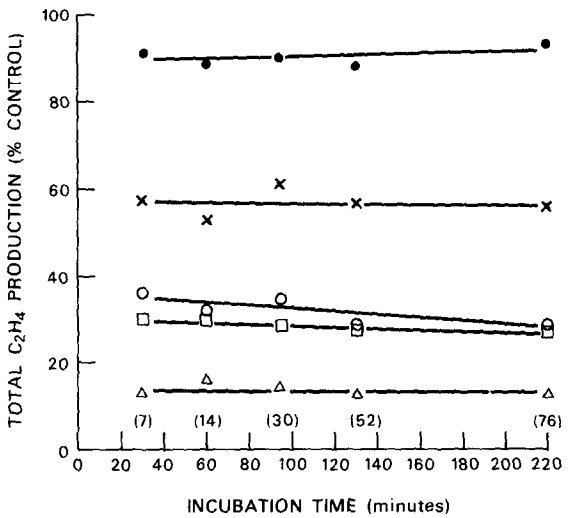

Figure 2. Total ethylene production over time by disc cultures after transfer to CS10 containing 0.5(●), 1.0(X), 1.5(O), 2(□) or 3(Δ)mM NH_4^+. Mean values (6 replicates) are percent of control values (no added NH_4^+) given in parentheses as C_2H_4 produced/culture.

NH_4^+ in Figure 1. Inhibition of nitrogenase activity was not reversed when disc cultures initially exposed to 1 mM NH_4^+ for 30 min were transferred to

TABLE I. Nitrogenase activity (nmol C_2H_4/culture/hr) in disc cultures transferred to CS10 containing 0, 2 or 5 mM NH_4^+ and assayed for C_2H_2 reduction with pO_2 of 0.20, 0.30 or 0.35 atm. Mean rates (5 replicates) determined after 2 hr.

pO_2 (atm)	NH_4^+ concentration (mM) 0	2	5
0.20	38.3	8.5	3.7
0.30	16.0	32.9	15.7
0.35	7.4	26.5	33.0

fresh CS10 and reassayed for C_2H_2 reduction at time periods up to 4 hr after transfer.

Maximum nitrogenase activity in agar grown cultures of strain 32H1 was obtained with 0.2-0.25 atm O_2 in the assay mixture (12). Studies of chemostat cultures of 32H1 indicated that O_2 may effect NH_4^+ inhibition of nitrogenase activity (19). Disc cultures were transferred to CS10 containing 0, 2 or 5 mM NH_4^+ and assayed for nitrogenase activity with gas mixtures containing 0.2, 0.3 and 0.35 atm O_2. The striking result of this experiment (Table I) was that the inhibitory effects of NH_4^+ were not observed at high O_2 tensions. This feature persisted during the 7 hr period these cultures were assayed.

These inhibition studies suggested that NH_4^+ inhibition of nitrogenase activity in strain 32H1 was not effected through repression of nitrogenase synthesis. The following experiments confirm this suggestion.

The inhibition of nitrogenase activity by 1.5 mM NH_4^+ was more rapid than that due to chloramphenicol or rifampicin (Fig. 3). Methionine sulfoximine (MX) and methionine sulfone (MF) relieve NH_4^+ repression of nitrogenase synthesis in *A. vinelandii* and *K. pneumoniae* (20). High concentration of both MX and MF inhibited nitrogenase activity in cowpea rhizobia, 32H1 (Table II). However, there was no relief of NH_4^+ inhibition of nitrogenase activity by either MX or MF.

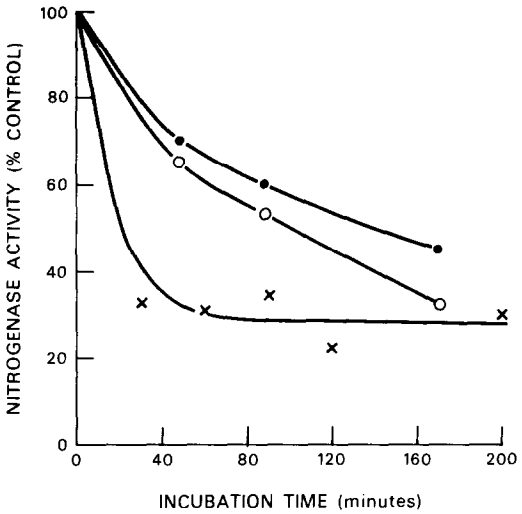

Figure 3. Nitrogenase activity at varying times following transfer of disc cultures to CS10 containing 1.5 mM NH_4^+(X), 200 µgm/ml rifampicin (●) or 100 µgm/ml chloramphenicol (O). Mean values (6 replicate cultures) are percent of control (no added N) values.

TABLE II. Nitrogenase activity (nmol C_2H_4/culture/hr) of 32H1 exposed to methionine sulfoximine (MX) or methionine sulfone (MF) in the presence or absence of 1.5 mM NH_4^+.

	Inhibitor concentration (mM)			
	0	30	60	100
MX	22.6	15.8	12.8	13.4
MX + 1.5 mM NH_4^+	16.5	11.8	10.6	9.7
MF	22.6	20.6	14.9	-
MF + 1.5 mM NH_4^+	16.5	15.2	9.9	-

The activities of the ammonia assimilating enzymes, GS, GOGAT and GDH, were determined in extracts of disc cultures transferred to CS10 or CS10 + 4 mM NH_4^+ (Table III). Under conditions where nitrogenase activity was inhibited the enzyme specific activites of both GS and GOGAT were substantially

TABLE III. Enzyme activities for nitrogenase, glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) of strain 32H1 in the presence and absence of NH_4^+.

Enzyme	CS10	CS10 + 4 mM NH_4^+
Nitrogenase[a]	16.5	6.1
GS[b] - Mg^{2+}	1.21	1.08
+ Mg^{2+}	0.86	0.67
+ Mg^{2+}/-Mg^{2+} (%)	71	62
GOGAT[c]	0.017	0.013
GDH[c]	<0.002	<0.002

(a) nmol C_2H_4/culture/hr. (b) μmol γ-glutamyl hydroxamate/mgm protein/min; -Mg^{2+} measures total GS activity; +Mg^{2+} estimates the unadenylylated form (15). (c) μmol NADH oxidised/mgm protein/min.

the same as those from control cultures (Table III). The GS transferase activity in disc cultures from CS10 was largely in the biosynthetically active form. There was no substantial increase in adenylylation of GS in cultures exposed to NH_4^+ or in cultures grown for 7 days on 15 mM NH_4^+. GDH activity was low or undetectable when assayed over a pH range of 6-8.5, or when substrate and cofactor concentrations were either halved or doubled. Similar relative enzyme activities have been recorded from liquid cell suspensions of 32H1.

Discussion

The most important feature to emerge from our results was the apparently unusual control of nitrogenase activity in agar cultures of strain 32H1. Nitrogenase activity in rhizobia was rapidly inhibited when cultures were exposed to NH_4^+ and no further decline occurred with time (Fig. 1 and 2). This contrasts with classical NH_4^+ repression (21,22) where the decline in activity is initially slow but then increases until all nitrogenase activity is lost. In addition, inhibition of nitrogenase activity was more rapid than

that occasioned by the inhibitors of protein synthesis, rifampicin and chloramphenicol (Fig. 3), and NH_4^+ inhibition could not be relieved by methionine sulfoximine or methionine sulfone (Table III). Under the culture conditions used in these experiments it would appear that NH_4^+ inhibition of nitrogen fixation in strain 32H1 does not occur through repression of nitrogenase synthesis.

In crude cell extracts of cultures where nitrogenase activity was inhibited by NH_4^+, both GS and GOGAT enzyme activities were substantially unaffected and the degree of adenylylation of GS was not significantly increased (Table III). Significant levels of GDH could not be detected even after 24 hr incubation with a high level of NH_4^+. In our experiments the effect of NH_4^+ on these enzymes in cultured 32H1 is similar to that in GS constitutive strains of *K. pneumoniae* where the formation of GDH is repressed (3, 23, 24). Genetic studies are in progress to determine the relationship between nitrogenase and the ammonium assimilating enzymes in 32H1.

Both NH_4^+ and high O_2 tension separately inhibited nitrogenase activity. Our studies also revealed an interaction between O_2 and NH_4^+ (Table I). Apparent inhibition by NH_4^+ was relieved by increasing the O_2 tension. Inhibition by high O_2 tension was mitigated in the presence of NH_4^+. Nitrogenase activity in chemostat cultures of 32H1 is expressed under microaerobic conditions (19). In agar cultures an O_2 gradient within the bacterial mass may result in a microaerobic zone. The paradox in our results may be resolved by assuming that ATP supply, probably generated by oxidative phosphorylation, limits nitrogenase activity. Changing the supply of combined-N could bring about altered O_2-demand or ATP supply through two mechanisms. First, NH_4^+ may increase O_2 consumption near the surface of the bacterial mass, particularly if the culture was N-limited, thereby lowering the O_2 tension (and hence ATP supply) in the active zone. Second, providing exogenous NH_4^+ may stimulate NH_4^+ assimilation and protein synthesis, thus depriving nitrogenase of ATP. Raising the oxygen tension would then alleviate this energy supply problem in

the presence of NH_4^+. In the absence of NH_4^+, at say pO_2 = 0.35 atm, oxygen would inhibit nitrogenase activity. Similar effects to those observed with NH_4^+ have been observed by varying the carbon source (pyruvate, 2-oxoglutarate) and pO_2 (25).

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