EFFECT OF DIMETHYLSULFOXIDE ON DEREPRESSION OF NITROGENASE IN *SPIRILLUM LIPOFERUM*

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1. Introduction

Spirillum lipoferum can fix N_2 in association with the roots of maize and certain tropical grasses, and partially relieve the dependence of these plants for growth on fixed N [1]. The potentiality of this bacterium, as an agent of associative symbiosis, depends however on its ability to export NH_4^+ outside the cell under the conditions of N_2ase derepression. In general, N_2ase of N_2 fixing bacteria are highly susceptible to ammonia repression [2,3], and N_2ase biosynthesis occurs only when a deficiency of ammonia exists within the cells making it impossible for them to export ammonia synthesized from N_2. On the other hand, the *nif* genes of the most useful symbiotic bacteria belonging to *Rhizobium* spp., when present in the bacteroid form in root-nodules, appear to remain in a derepressed state even in an environment of free ammonia which they export to the cytoplasm of the host cells [3]. It is not clear how ammonia exerts its powerful repression effect on *nif* in most nitrogen fixing bacteria, and why this repression is particularly weak in the bacteroids of *Rhizobium* spp. The repression of *nif* genes by NH_4^+ can however be overcome in *Klebsiella pneumoniae* by certain mutations in *gln* or *gln* regulatory genes [4,5], and in most other N_2-fixing organisms by adding MSX [3,6,7], a structural analogue of glutamine that strongly inhibits GS [8]. As interpretations of these results have been controversial [3,5,9], we looked for an alternative method of constitutive expression of N_2ase by the application of DMSO for a possible clarification of any role of GS in the process of derepression. DMSO is already known to be an effective agent in the induction of several bacterial genes under repressed state [10]. We show here that DMSO can induce biosynthesis of N_2ase in *S. lipoferum* in the presence of NH_4^+ without affecting ammonia repression of GS biosynthesis.

Abbreviations: GS, glutamine synthetase (EC 6.3.1.2); N_2ase, nitrogenase; *nif*, nitrogen fixation genes; *gln*, structural gene for GS; MSX, L-methionine D,L-sulfoximine; CTAB, cetyltrimethyl ammonium bromide; DMSO, dimethylsulfoxide; NF, nitrogen free

2. Materials and methods

2.1. *Bacterial strain*

The strain used was *Spirillum lipoferum* RG which was resistant to streptomycin and formed good colonies within 36 h in the N-free agar medium. This strain was selected from a culture of *Spirillum lipoferum* 81 sent to us by N. R. Krieg.

2.2. *Growth medium*

Nitrogen free medium was composed of salts and C-source as in [11]. AMY medium was made by adding 0.05% NH_4Cl and 0.01% yeast extract to NF medium. GA medium was NF medium containing 0.2% glutamate. For microaerophilic incubation, growth media were made semi-solid by the addition of 0.05% agar.

2.3. *Assay of nitrogenase*

A three step procedure for derepression of nitrogenase and its assay using intact cells of *S. lipoferum* RG was adapted from the method in [11,12]. All incubations during growth and assay period were done at 37°C and A_{590} of cell suspensions was measured:

Step 1. *S. lipoferum* RG was grown overnight with full aeration to stationary phase in AMY medium;

Step 2. Washed cells from step 1 were resuspended

in the semi-solid medium (0.7–1.0 *A* units) and incubated under a stagnant condition (microaerophilic growth) for 24 h. When necessary, addition of DMSO was made in the microaerophilic growth medium after allowing 6 h preincubation without DMSO followed by 18 h incubation with DMSO;

Step 3. Cells collected from step 2 were washed free of any drug or N-source and resuspended in NF medium to 3.2–3.4 *A* units. Each assay vial (9 ml) was sealed with an airtight cap after adding 3 ml cell suspension, pO_2 was reduced to 0.035 atm by withdrawing air with a syringe; 1 ml C_2H_2 was injected into the gas space of the vial and incubation started in a rotary shaker. Amount of C_2H_4 formed during incubation was measured by gas chromatography [11]. DMSO addition was made by injection into the vials, when necessary. % DMSO was calculated as ml DMSO added/100 ml medium.

2.4. *Assay of GS*

Active form of GS was measured by the biosynthetic assay (forward reaction) procedure as in [13,14]. Whole cells were routinely made permeable by treatment with 0.01% CTAB before enzyme assay [13]. One unit of GS activity is defined as the amount of enzyme producing 1 nmol γ-glutamyl hydroxamate/min in the assay mixture [14]. Protein concentration of cell suspension was measured as in [18].

3. Results

In [17], no detectable N_2ase proteins were synthesized when *S. lipoferum* cells were incubated under microaerophilic condition in the presence of ammonia. We found little expression of N_2ase activity when *S. lipoferum* RG was incubated in step 2 under microaerophilic conditions in semi-solid AMY medium, but the presence of DMSO in the same medium resulted in high level expression of N_2ase activity (fig.1). We usually added 8–10% DMSO in the semi-solid medium of step 2 for derepression of *nif* (on adding 10% DMSO, the generation time of *S. lipoferum* RG increased from 125–205 min when tested in a N-rich complex growth medium), but the higher the concentration of the drug used, the longer was the period of lag in the full expression of N_2ase activity in step 3 assay (fig.1). In contrast, *S. lipoferum* RG cells, derepressed in GA semi-solid medium of step 2, showed full expression of N_2ase activity without any lag in step 3 assay, and

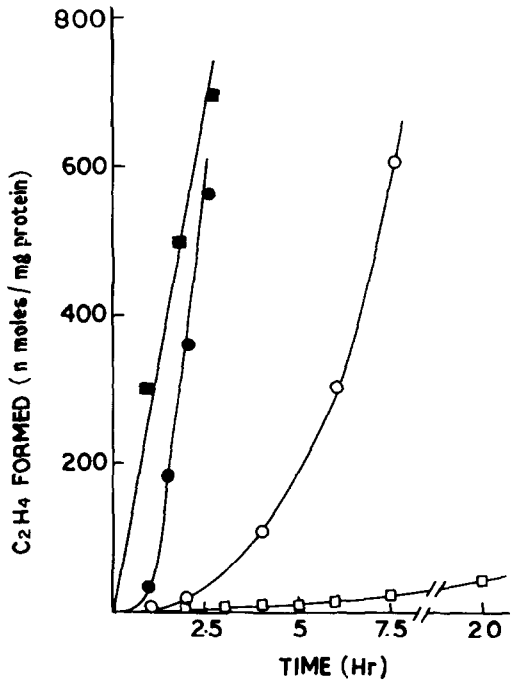

Fig.1. Effect of DMSO on derepression of nitrogenase in presence of ammonia. *Spirillum lipoferum* RG cells were grown, derepressed in semi-solid AMY or GA medium with or without DMSO and assayed for nitrogenase activity as in section 2: (–□–) AMY; (–■–) GA; (–●–), AMY + 8% DMSO; (–○–), AMY + 10% DMSO.

its specific activity (nmol C_2H_4 formed . mg cell protein^{-1} . h^{-1}) was nearly the same as that of the cells derepressed in AMY semi-solid medium containing 8% DMSO but after ~1 h lag (fig.1). As glutamic acid is not known to cause repression of nitrogenase synthesis to any significant extent in bacteria [2,15], it may be assumed that *nif* genes of *S. lipoferum* were maximally expressed under both conditions of derepression. Although maximal N_2ase activity without any lag was shown by the cells derepressed in GA semi-solid medium, an addition of 10% DMSO in the same medium did show a lag in step 3 (fig.2), but this lag was shorter compared to the case where 10% DMSO was added to AMY semi-solid medium. It appears that both DMSO and ammonia in step 2 could be contributory factors for the delay shown by the derepressed cells in the development of maximal N_2ase activity during incubation in the N-free assay medium.

DMSO was also found to be a powerful inhibitor of nitrogenase activity. This is shown in fig.2. It can be observed that when 10% DMSO was injected into

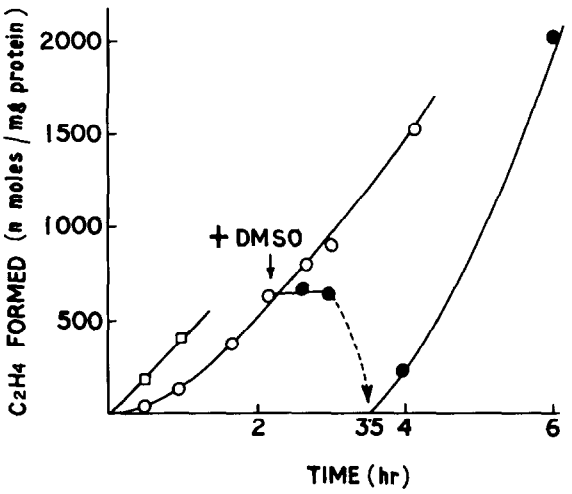

Fig.2. Reversible inhibitory effect of DMSO on nitrogenase activity. *Spirillum lipoferum* RG cells were grown, derepressed in semi-solid GA medium with or without DMSO and nitrogenase assayed as in section 2: (–□–) GA; (–○–) GA + 10% DMSO. At 2 h in step 3, DMSO (10%) was added into the vial containing cells derepressed with GA + DMSO and further incubated for 1 h. The vial was opened, cells washed free of DMSO, resuspended in the N-free medium (3 ml) and step 3 procedure was continued for acetylene reduction starting at 3.5 h. (–●–) DMSO-treated cells during step 3.

assay vials containing derepressed cells, the production of C_2H_4 stopped immediately (experiments not reported here showed that even 2% DMSO could completely inhibit N_2ase activity). Moreover, the DMSO-treated cells, when washed free of DMSO after 1 h of exposure, regained immediately their full N_2ase activity (fig.2). Therefore, the inhibitory effect of DMSO on the N_2ase activity is readily reversible.

In order to determine whether DMSO also affects repression of GS biosynthesis by ammonia, biosynthetic activities of GS were measured at various times during step 3 incubation with *nif* derepressed and repressed cells of *S. lipoferum* RG. As can be seen from table 1, the presence or absence of 10% DMSO in semi-solid AMY medium during microaerophilic incubation of step 2 did not alter the low level of GS biosynthetic activity (expt 1,2) compared to the high derepressed level of the same activity obtained in GA medium (expt 3). In all these experiments maximal GS activities were shown within 60–90 min after the start of step 3 incubation in the N-free medium and thereafter they remained nearly stationary for many hours.

Table 1
GS activities of nitrogenase repressed or derepressed cells of *S. lipoferum* RG

Exp.	Step 2 medium	GS spec. act.[a]		N_2ase spec. act.[b]
		0 min	60 min	
1	AMY	14	30	<10
2	AMY +10% DMSO	8.2	24	150[c]
3	GA	72	90	200

[a] GS specific activities are nmol γ-glutamyl hydroxamate formed . min^{-1} . mg $protein^{-1}$

[b] Nitrogenase specific activities are nmol C_2H_4 formed . h^{-1} . mg $protein^{-1}$

[c] Specific activity was determined after C_2H_4 production became linear with time

CTAB was added to the cells at the indicated time during incubation in step 3 and GS was assayed for biosynthetic activity as in section 2

4. Discussion

GS was implicated as a positive regulatory element in the expression of N_2ase activity based on an analysis of *nif* phenotypes of certain *Klebsiella pneumoniae* strains having mutations within the structural genes of GS [4,16]. Studies with GS regulatory mutants of the same bacteria however suggest that GS may not be absolutely required for *nif* expression [9] nor that GS mediates the repression of *nif* by NH_4^+ [5]. Our results show that the induction of N_2ase synthesis in *S. lipoferum* RG by DMSO in the presence of NH_4^+ was possible without affecting the repressed state of GS. The possibility of constitutive expression of N_2ase in N_2-fixing bacteria in the presence of DMSO would, therefore, be of use in further elucidation of the mechanism of *nif* repression by ammonia.

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