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Effect of Cyclic Guanosine 3',5'-Monophosphate on Nitrogen Fixation in *Rhizobium japonicum*

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The addition of exogenous cyclic guanosine 3',5'-monophosphate (cGMP) at a concentration of 0.1 mM to a free-living culture of *Rhizobium japonicum* 3I1b110 was found to completely inhibit the expression of nitrogenase activity and markedly inhibit the expression of hydrogenase and nitrate reductase activities. The effect was specific for cGMP. Experiments on the in vivo incorporation of radioactive methionine and subsequent analysis of the labeled proteins on polyacrylamide gels showed that the biosynthesis of nitrogenase polypeptides was inhibited. It appears that the time of addition of cGMP is important since the effect was only seen during the early stages of *nif* gene expression. The intracellular level of cGMP was found to respond to physiological changes in the cell, and there was a fall in cGMP concentrations when nitrogenase was induced. Microaerophilic-aerobic shift experiments showed that intracellular levels increased from 0.25 pmol/mg of cell protein under microaerophilic conditions to 2.6 pmol/mg of cell protein under aerobic conditions, suggesting that the cellular pool size of cGMP may be under redox control.

The description of conditions leading to the production of nitrogenase activity by free-living cultures of *Rhizobium* spp. (16, 17, 23, 28, 37) and the increasing knowledge of *Rhizobium* genetics (15, 24) have provided a fresh approach to studies of the regulatory pathways that control symbiotic nitrogen fixation. For example, it has been indicated by the workers cited above that, besides the requirement for low O_2 tension, one of the prerequisites for obtaining maximal nitrogen fixing activity is the choice of the right kind of carbon source. Recent studies have also shown that in *R. japonicum* 110, the formation of the hydrogen uptake system which is believed to recycle H_2 from the nitrogenase reaction (31) is also strongly dependent on the type of carbon source used for growth (S. T. Lim and K. T. Shanmugam, Biochim. Biophys. Acta, in press). In these studies, tricarboxylic acid cycle intermediates such as malate were found to significantly reduce H_2 utilization. In addition, malate was found to repress the utilization of glutamate. Thus, malate seems to have evolved the function of a catabolite-like repressor. The exogenous addition of 1 mM cyclic AMP (cAMP) was found to overcome the malate-mediated inhibition of H_2 utilization. Measurements of the intracellular cAMP pool sizes showed that in a malate medium the internal concentration of this compound was low (0.8 pmol/mg of cell protein). In contrast, in a medium with glutamate as the sole carbon and nitrogen sources, high levels of cAMP were found (30 to 40 pmol/mg of cell protein). Exogenous cAMP did not have any effect on whole-cell nitrogenase activity. Upchurch and Elkan (40) found repression of several NH_4^+ assimilatory enzymes by cAMP in *R. japonicum* grown under aerobic conditions. Recently, Ucker and Signer (39) reported the phenomenon of catabolite-like repression in *R. meliloti*. Here, the addition of succinate to cells grown on lactose resulted in immediate reduction of β-galactosidase synthesis.

From these few reports, it appears likely that the mechanism of catabolite repression of inducible enzyme systems, as known from studies of *Escherichia coli* and other members of the *Enterobacteriaceae* (29, 30), is also operative in *Rhizobium*. Accordingly, cyclic mononucleotides might, therefore, play an important role in the regulation of rhizobial gene expression. The apparent antagonistic role of cGMP versus cAMP in in vitro transcription experiments involving *E. coli gal* or *lac* operons (10, 26) has prompted us to study the effect of cGMP on H_2 uptake and nitrogen fixation in free-living cultures of *Rhizobium* spp.

MATERIALS AND METHODS

Chemicals. cGMP was obtained from Sigma Chemical Co. cGMP from various other sources (Boehringer Mannheim Corp., Calbiochem, and Nu-

tritional Biochemicals Corp.) was also used with similar results. All other cyclic mononucleotides used were obtained from Sigma. Each cGMP preparation was checked for purity and stability by thin-layer chromatography on polyethyleneimine-cellulose F (Merck & Co., Inc.), using 1 M ammonium acetate-95% ethanol (7:13, by volume; pH 9.0) (38) as a solvent. The spots were detected under UV light. All other chemicals used were obtained commercially and were of reagent or analytical grade.

Bacterial strains and growth conditions. *R. japonicum* 3I1b110, 3I1b138, and 3I1b142 were obtained from D. F. Weber, U.S. Department of Agriculture, Beltsville, Md., and *Rhizobium* sp. 32H1 was kindly supplied by J. Tjepkema.

Rhizobial cultures were maintained on agar slants of mannitol-salts-yeast extract medium (20, 27). This medium was also used for the preparation of inocula for the induction experiments described below. Liquid cultures were grown at 30°C starting with a 2% inoculum (absorbance at 420 nm = 0.1). Growth was followed by measuring the absorbance at 420 nm in a Gilford model 300 N spectrophotometer (1-cm light path) and also by the increase in cell protein as previously described (27). Protein was routinely assayed by the procedure of Lowry et al. (21), using bovine serum albumin as a standard. Cellular growth was also monitored by incorporation of L-[^{14}C]leucine (New England Nuclear Corp., 314 Ci/mol) into cellular protein as previously described (27).

Induction of enzymes under microaerophilic growth conditions. The medium used for the induction of nitrogenase, nitrate reductase, and hydrogenase activities in free-living cultures of *R. japonicum* was as described previously (20, 27). The carbon source used was either gluconate or malate (4.0 g/liter). The medium was buffered with 50 mM morpholinopropane sulfonic acid, pH 6.8. In experiments requiring a larger quantity of cells (e.g., for enzyme assays), 250-ml cultures were grown under microaerophilic conditions, using 2.2-liter Büchner flasks. For the determination of nitrogenase and nitrate reductase activities, the gas phase was adjusted to 0.1% oxygen, 3% acetylene, and the remainder, argon. For measuring hydrogenase activity, acetylene was replaced with 2% H_2.

Enzyme assays. Nitrogenase activity was determined by the acetylene reduction procedure (14) with a Varian Aerograph, model 1400, equipped with a flame ionization detector and Poropak R column. Hydrogenase activity in whole cells was determined by following the disappearance of hydrogen from the gas phase using gas chromatography (Varian Aerograph, model 920, equipped with a thermal conductivity detector and molecular sieve column [0.5 nm] with N_2 as the carrier gas) and by the tritium-exchange assay as described previously (20). Nitrate reductase was determined for whole cells under microaerophilic conditions by assaying the conversion of NO_3^- (potassium nitrate, 1.0 g/liter) to NO_2^-, which was determined colorimetrically as described by Nicholas and Nason (25). For glutamine synthetase and malate dehydrogenase assays, cultures were harvested in the late exponential phase of growth and disrupted in a French press (20,000 lb/in^2). The crude extract was centrifuged at 20,000 × g for 20 min, and the supernatant was used immediately for assay. Glutamine synthetase activity was assayed by measuring the amount of γ-glutamyl hydroxymate formed in the presence of Mn^{2+} at 37°C for 15 min, as described by Shapiro and Stadtman (33). Malate dehydrogenase (NAD^+) was assayed, essentially as described by Yoshida (43), by following the reduction of NAD^+ at 340 nm.

The activity of cyclic 3',5'-nucleotide phosphodiesterase was assayed by the procedure described below, as modified by Lee (19) and Chasin and Harris (8). The standard reaction mixture contained, in a total volume of 50 μl: 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgSO_4$, 0.5 mM unlabeled cGMP, [^{3}H]cGMP (17.2 Ci/mmol, ca. 10^5 cpm per reaction mixture), and cell extract. The assay was carried out at 37°C for 10 min, and the reaction was terminated by adding 50 μl of 50 mM EDTA and heating at 100°C for 2 min. The mixture was brought to 37°C, 20 μl (7.2 mg/ml) of 5'-nucleotidase of *Crotalus atrox* venom (Sigma) was added, and the mixture was then incubated for 10 min. The reaction was terminated by heating in a boiling-water bath for 2 min after the addition of 50 μl of 5 mM guanosine solution. The tube was cooled, 1 ml of a 50% (wt/vol) suspension of Bio-Rad resin (AG2-X10, 200 to 400 mesh) was added, and the mixture was shaken and allowed to equilibrate for 10 min before centrifugation to sediment the resin. A 100-μl amount of the supernatant was removed and added to 10 ml of Ready Solv (Beckman Instruments, Inc.) scintillation fluid for counting (Beckman LS 8000 liquid scintillation spectrometer). Boiled samples of crude cell extract were used as controls. The specific activity of the enzyme was expressed as picomoles of cGMP hydrolyzed per minute per milligram of protein.

cGMP assay. Samples of cells (approximately 1 mg of cell protein) for cGMP assay were collected essentially as described by Buettner et al. (5) and rapidly filtered through 0.65-μm membrane filters (Millipore Corp.). The culture medium which passed through the filters was collected in a test tube inside a vacuum flask for the assay of extracellular cGMP levels. The filters were immediately immersed in 0.1 M HCl and kept at 95°C for 10 min. Sampling was completed within 30 s. After cooling, the filters were rinsed, and the extract was lyophilized and suspended in 0.2 ml of 50 mM sodium acetate buffer, pH 6.2. Recovery of cGMP was monitored by the addition of approximately 5,000 cpm of [^{3}H]cGMP (New England Nuclear Corp.). cGMP was determined by the extremely specific radioimmunoassay according to the method of Steiner et al. (35), using the assay kit supplied by New England Nuclear. Acetylation of the samples was carried out before assay to further increase the sensitivity of the assay (6).

cGMP uptake studies. The uptake of [^{3}H]cGMP by intact cells was determined by a modified procedure of Halpern and Lupo (13). The culture was harvested at the required stage of growth and washed twice with growth medium (glutamate-gluconate). The cells were suspended in growth medium to an absorbance at 420 nm of approximately 10. The uptake reaction was started by the addition of 0.5 ml of cell suspension to 1.0 ml of prewarmed growth medium containing the desired concentrations of [^{3}H]cGMP (17.2 Ci/mmol) and incubated at 25°C. Samples (20 μl each) were

removed at different times and transferred to 0.45-μm Millipore filters previously washed with growth medium containing 0.1 mM unlabeled cGMP. The filter was washed twice with 5 ml of the same medium, dried, and counted.

Radiochemical analysis of nitrogenase polypeptide synthesis. Two-milliliter cultures of *R. japonicum* 110 were labeled with 25 μCi of L-[^{35}S]methionine (561.21 Ci/mmol) and incubated for 20 min at 25°C. The labeling was stopped by the addition of 2 mg of unlabeled L-methionine. After a 5-min incubation, the cells were chilled and collected by centrifugation. The pellet was washed once with gluconate-glutamate medium and then three times with 0.9% (wt/vol) NaCl to remove exopolysaccharides (42). Finally, the cells were suspended in 0.2 ml of sodium dodecyl sulfate (SDS) containing "sample buffer" (18) and boiled for 10 min. The radioactivity in 5 μl of the SDS-soluble proteins was determined by counting in a liquid scintillation counter. Samples containing approximately 10^5 cpm were then subjected to one-dimensional SDS-polyacrylamide (10%) slab gel electrophoresis (18, 36). After being stained with Coomassie brilliant blue and destained, the gels were dried and exposed to Kodak X-Omat R films for 8 to 16 h.

On the autoradiogram, the positions of the bands corresponding to the constituent polypeptides (α and β) of component I of the *R. japonicum* nitrogenase complex were detected by comparison with the position of the purified component on the stained gel and by coelectrophoresis with the purified component. The enzyme has been isolated from bacteroids of soybean (*Glycine max* cv. Evans) nodules essentially by the method of Whiting and Dilworth (41) with modifications to be described elsewhere (Scott, Hennecke, and Lim, submitted for publication). The amount of nitrogenase proteins synthesized relative to total SDS-soluble proteins was determined by scanning the autoradiogram strips with an ISCO gel scanner, model 1310, and a Varian Aerograph, model 485, peak integrator.

Immunological techniques. Rabbit antiserum against purified component I of *R. japonicum* was obtained as will be described elsewhere in more detail (Scott, Hennecke, and Lim, manuscript in preparation). The gamma globulin fraction was isolated from the crude serum by three consecutive ammonium sulfate precipitations at 33% saturation (7). Antigen-antibody complex formation was followed in double-diffusion plates (7) which contained 10 mM sodium borate (pH 7.8), 1 mM sodium azide, and 1% agar. Plates were allowed to develop for 24 to 48 h at room temperature.

RESULTS

Effect of cGMP on whole-cell nitrogenase activity. The effect of exogenously added cGMP on nitrogenase activity of a free-living culture of *R. japonicum* 3I1b110 is shown in Fig. 1. Nitrogenase activity as measured by the acetylene reduction assay was more than 70% inhibited in the presence of 0.05 mM cGMP, and almost complete inhibition was obtained at 0.1 mM cGMP. Under similar conditions, cAMP up to 1.0 mM did not block the expression of whole-cell nitrogenase activity. This was not due to a lack of cAMP uptake, as [^{3}H]cAMP was shown to accumulate in the cells. Other cyclic mononucleotides tested (dibutyryl cAMP, cCMP, cTMP, and cUMP) were without effect. Guanosine derivatives used, including GMP, GDP, and GTP (all at 1 mM), were also without any effect on nitrogenase activity. Thus, the observed effect is specific for cGMP. At the end of the incubation period, the culture was assayed for the stability of cGMP by thin-layer chromatography on polyethylenimine-cellulose F. No UV-absorbing material other than cGMP was detected. The material was identified as cGMP by its R_f value and by cochromatography with authentic cGMP and by removal of the spot and analysis for cGMP by radioimmunoassay. cAMP at concentrations up to 1 mM did not overcome the inhibition of whole-cell nitrogenase activity mediated by 0.1 mM cGMP. Higher concentrations were not applied because, under these conditions, cAMP was found to markedly inhibit the growth of *R. japonicum* (40; S. T. Lim and K. T. Shanmugam, Biochim. Biophys. Acta, in press). To test whether the effect of cGMP on nitrogenase activity is unique to strain 110, other strains of *Rhizobium* spp. that are inducible for nitrogenase activity were

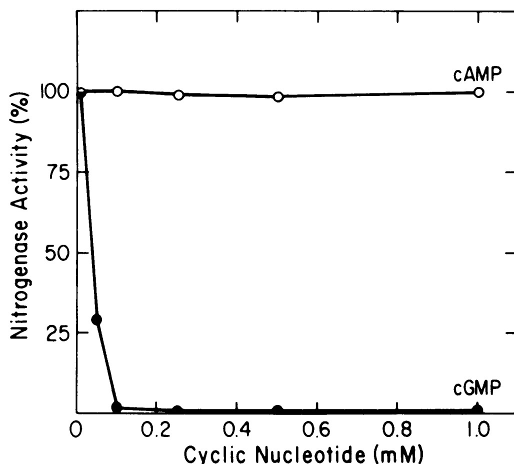

FIG. 1. *Effect of exogenous cGMP on the expression of nitrogenase activity in R. japonicum 3I1b110. cGMP (0.1 mM) was added at the beginning of the experiment, and whole-cell nitrogenase activity was followed over a period of 6 days. The medium contained L-glutamate (1 g/liter) as the nitrogen source and D-gluconate (4 g/liter) as the carbon source. Nitrogenase activity was determined as described in the text. All values are the average of at least two separate experiments.*

assayed in the presence and in the absence of cGMP. In addition to 3I1b110, nitrogen fixation activities of three other strains tested (3I1b138, 3I1b142, and *Rhizobium* spp. 32H1) were similarly affected by cGMP.

Effect of cGMP on biosynthesis of nitrogenase component I proteins. To ascertain whether cGMP exerts its effect only on cellular reactions supporting nitrogenase activity, or also on nitrogenase biosynthesis, the in vivo incorporation of [^{35}S]methionine into cellular proteins was measured. A period of 20 min was found to be an appropriate time for labeling. During this period, the constituent polypeptides of component I were among the major proteins synthesized (% $\alpha + \beta = 7$) in a free-living, N_2-fixing *Rhizobium* culture (Fig. 2b, lane 1). In aerobically grown cells, these peptides were reduced to background levels (Fig. 2b, lane 3) and could not be detected by the criterion of scanning the autoradiogram strip. When cGMP (final concentration, 0.1 mM) was added to an early exponential culture induced for nitrogenase activity, there was a 55 to 60% decrease in de novo nitrogenase biosynthesis within 1 h (lane 2). Under these conditions, the biosynthesis of other as yet unidentified proteins was also repressed or, in a few cases, derepressed. To further substantiate that nitrogenase protein synthesis was affected, the presence or absence of this enzyme in crude cell extracts was determined by immunodiffusion (Fig. 3). Using antibodies against purified component I of *R. japonicum*, we observed a strong cross-reaction with extracts from a microaerophilic culture with a precipitin line fusing into the one obtained with the purified enzyme (Fig. 3a and b). However, only a faint precipitin line was observed with extracts from aerobically grown cells, and there was no cross-reaction from cells grown under microaerophilic conditions in the presence of 0.1 mM cGMP (Fig. 3d and c).

The time of cGMP addition to the culture appeared to be crucial for obtaining maximum inhibition of whole-cell nitrogenase activity. Addition of cGMP at the beginning of the experiment or during the early exponential phase of growth provided maximum inhibition (100% or >95%, respectively). No significant inhibition (<10%) of whole-cell nitrogenase activity or nitrogenase biosynthesis was seen when cGMP was added during the late exponential or stationary phase of growth. After this observation, the initial rate of uptake and the accumulation of [^{3}H]cGMP by *R. japonicum* cells during early and late exponential phases of growth was compared. However, there was no indication of a significant change in cGMP uptake at the two different growth stages. Furthermore, assays of crude extracts for cyclic nucleotide phosphodiesterase activity with [^{3}H]cGMP as the substrate were performed; it was found that the diesterase activity in late-exponential-phase cells (70 U/mg of protein) was not higher compared to the value of early-exponential-phase cells (90 U/mg of protein). Thus, it appears unlikely that the inability of cGMP to repress nitrogenase biosynthesis during the late exponential phase of growth is due to a differential, i.e. decreased, permeability of the cells to cGMP or a differential, i.e. increased, degradation of cGMP by the intracellular phosphodiesterase.

cGMP levels in *R. japonicum* 3I1b110. The results presented above for exogenous cGMP led us to investigate both intracellular and extracellular levels of cGMP throughout the growth cycle. Figure 4A shows the growth cycle in a glutamate-gluconate medium of a culture of *R. japonicum* 3I1b110 grown under microaerophilic conditions in the absence and in the presence of 0.1 mM cGMP, added at the beginning of the experiment. There appeared to be a slightly longer lag phase of growth in the presence of cGMP (about a 12-h difference), but the increase in cell mass during exponential growth was not significantly different. The time course of nitrogen fixation activity is also shown in Fig. 4A. Maximal whole-cell nitrogenase activity occurred in the late exponential phase of growth, and activity was maintained even in the stationary phase. A cGMP-treated culture did not produce nitrogenase activity at any stage during the complete growth cycle. Essentially, the same holds true if nitrogenase polypeptide synthesis is analyzed in such a culture. In a noninhibited culture, the appearance of nitrogenase protein precedes the formation of nitrogenase activity (Scott, Hennecke, and Lim, in preparation). Figure 4B shows both the intracellular and extracellular levels of cGMP during the growth cycle of a parallel culture of *R. japonicum* 3I1b110 grown under microaerophilic conditions. Intracellular cGMP concentrations fell from approximately 0.7 pmol/mg of cell protein after approximately 48 h of incubation (earlier points were not taken due to the difficulty of obtaining enough cells) to about 0.2 pmol/mg of cell protein as nitrogenase began to be induced and leveled off as the culture reached the stationary phase. The extracellular cGMP concentration was low and remained fairly constant after nitrogenase had been induced. In an additional experiment (not shown), a culture growing under microaerophilic conditions (0.1% O_2) to the exponential phase of growth was shifted to aerobic conditions (20% O_2), and growth was followed

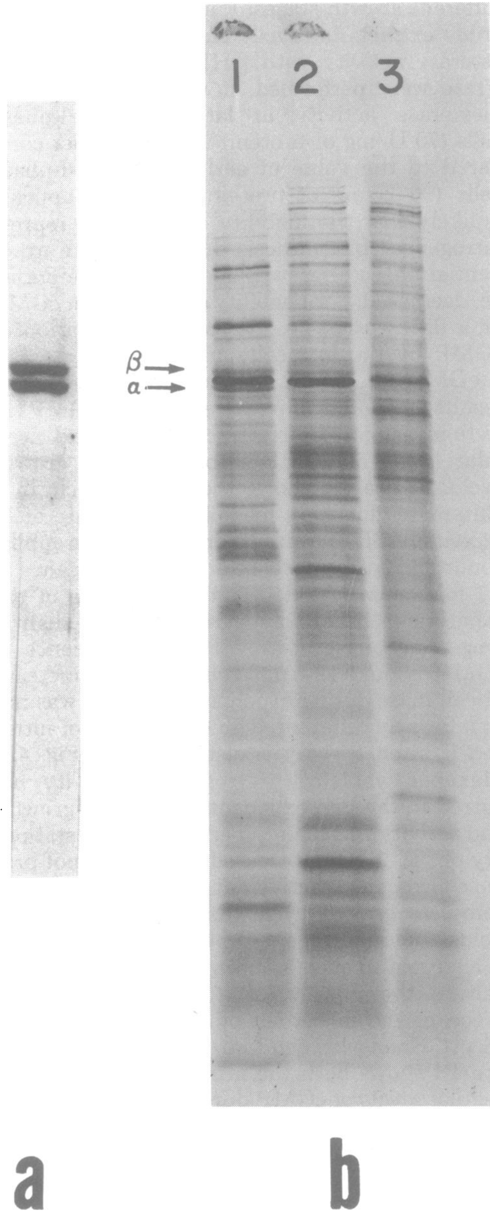

FIG. 2. *(A) Gel electrophoresis of purified component I of R. japonicum in the presence of SDS. A sample containing 5 μg of protein was applied to the gel. The protein was stained with Coomassie brilliant blue. (B) Autoradiogram of the SDS-gel electrophoretic analysis of in vivo-labeled R. japonicum proteins. Arrows indicate the positions of the α and β polypeptides as determined by coelectrophoresis with the purified enzyme component I of R. japonicum. Lanes 1 and 2 show the protein patterns of early exponentially growing cells (65 h after inoculation) under microaerophilic conditions in a glutamate-gluconate medium. Lane 1, Control without cGMP; lane 2, cells labeled 1 h after addition of 0.1 mM cGMP (final concentration); lane 3, aerobically grown cells in a glutamate-gluconate medium.*

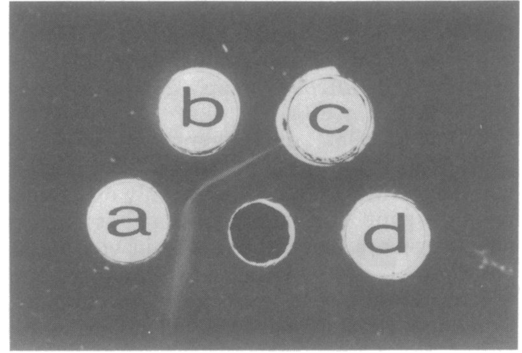

FIG. 3. *Immunological detection of nitrogenase by Ouchterlony double diffusion. The wells contained: (center) antibodies against the purified enzyme; (a) crude cell extract from microaerophilic culture induced for nitrogenase (1 mg of protein); (b) 50 μg of purified nitrogenase; (c) crude cell extract from microaerophilic culture with 0.1 mM cGMP (0.85 mg of protein); (d) crude cell extract from aerobic culture (1.5 mg of protein).*

for 24 h. Intracellular cGMP levels were found to increase from 0.25 pmol/mg of cell protein under microaerophilic conditions to approximately 2.6 pmol/mg of cell protein under aerobic conditions. Cultures grown under aerobic conditions in which nitrogenase was not induced had comparatively higher cGMP levels (averaging about 2.5 to 3 pmol/mg of protein) and did not vary significantly during the growth cycle. cGMP at levels of 0.03 to 0.05 pmol/mg of protein was also detected in bacteroids of soybean nodules infected with *R. japonicum* 3I1b110. The levels were lower than those found in a free-living culture under microaerophilic conditions. However, losses of intracellular cGMP probably occurred during the preparations of the bacteroids.

Another enzyme(s) and enzyme systems inhibited by cGMP. To determine whether cGMP has a similar effect on another enzyme(s) or enzyme systems, the patterns of expression of a variety of rhizobial enzymes were tested. Hydrogenase and nitrate reductase were chosen because these enzymes have been shown to be important in symbiotic nitrogen fixation (9, 31) and are active under conditions of low oxygen tension (9, 20, 31). In addition, glutamine synthetase, an enzyme which assimilates the NH_4^+ produced from N_2 (3, 4, 22, 32), and malate dehydrogenase, an enzyme involved in the production of energy and reducing power (11), were

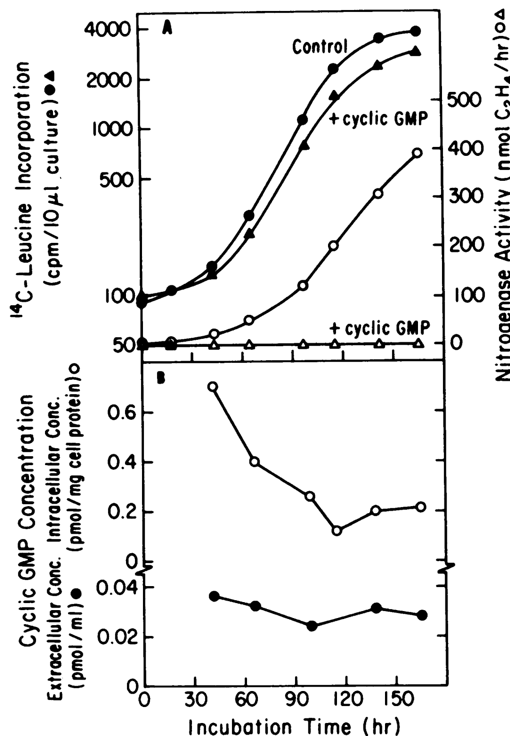

FIG. 4. *(A) Time course of growth of R. japonicum 3I1b110 under microaerophilic conditions with and without 0.1 mM cGMP (final concentration) and time course for induction of nitrogenase activity in these cultures. (B) Levels of cGMP during growth of a parallel culture of R. japonicum 3I1b110. Samples were removed anaerobically at various times during the growth cycle and assayed for both intracellular and extracellular cGMP by radioimmunoassay as described in the text.*

assayed in extracts of cells grown in the presence and in the absence of cGMP. The specific activities of both hydrogenase and nitrate reductase were reduced by about 70 to 75% in the presence of cGMP (Table 1). On the other hand, there was only a slight effect on the levels of malate dehydrogenase and glutamine synthetase.

DISCUSSION

The experimental results presented in this communication (Fig. 1 and Table 1) show that cGMP, when added exogenously at concentrations as low as 0.1 mM to cultures of *R. japonicum* strains, not only completely inhibits the expression of nitrogenase activity, but also leads to a considerable reduction of hydrogenase and nitrate reductase activities, respectively. These enzyme systems are known to be active under microaerophilic growth conditions (9, 20, 27, 32). It should be emphasized that neither the growth

TABLE 1. *Effect of cyclic GMP on other enzyme systems in R. japonicum 3I1b110*

Enzyme system	Specific activity (nmol/min per mg of protein): Without cGMP	Specific activity (nmol/min per mg of protein): With 0.1 mM cGMP
Nitrogenase	1.44	<0.01
Hydrogenase	18.30[a]	5.50[a]
	0.52[b]	0.10[b]
Nitrate reductase	0.53	0.14
Malate dehydrogenase (NAD^+)	510.00	430.00
Glutamine synthetase	840.00	565.00

[a] Determined by assaying for H_2 utilization, using gas chromatography.

[b] Determined by the tritium-exchange method.

nor the overall protein synthesis (Fig. 4) or the activity of other selected enzymes, such as glutamine synthetase and malate dehydrogenase, is significantly influenced. That the cGMP effect is specific for this compound is shown by the fact that a series of other nucleotides does not affect whole-cell nitrogenase activity. In vivo experiments with *E. coli* showed that 5 mM cGMP led to almost complete inhibition of β-galactosidase synthesis in cells growing on glucose or glycerol medium (2), suggesting an antagonistic role of cGMP and cAMP in this organism. In our experiments with *R. japonicum*, a 1 mM concentration of cAMP did not overcome the cGMP-mediated inhibition. Since higher concentrations of cAMP could not be employed due to growth inhibition, this result does not completely rule out the possibility of an antagonistic role of these two compounds in *Rhizobium*.

The experimental results shown in Fig. 2 and 3 show that cGMP affects nitrogenase protein synthesis. However, this does not definitely prove that inhibition of nitrogenase biosynthesis is the primary and only reason that whole-cell nitrogenase activity is affected. For example, the finding that cGMP has only a negligible effect on de novo nitrogenase biosynthesis in an early-stationary-phase culture could indicate that cGMP may not act directly on the level of nitrogenase gene transcription. Therefore, the possibility exists that other effectors or regulatory pathways may participate in or are subject to cGMP-mediated regulation. At present, we do not know the target site for cGMP action inside the cell. However, it is interesting that we have enriched a crude extract of *R. japonicum* by 66-fold for a protein factor that is capable of forming a complex with both cAMP (K_{diss} = 2.5 μM) and cGMP (K_{diss} = 10 μM). The bindings of cAMP

and cGMP are competitive (data not shown). Whether this protein factor is functionally similar to the cAMP receptor protein characterized in *E. coli* (1, 12) remains to be determined.

The best evidence that cGMP may have a regulatory function in *R. japonicum* is the fact that this compound is found naturally in the organism and, above all, that its intracellular level responds to physiological changes imposed on the culture. The radioimmunoassay applied is the most specific and sensitive method available for the determination of cGMP pools. cAMP interfered with the cGMP value only when present at a 10^4-fold-higher concentration. Such a high cAMP concentration in rhizobial extracts has never been encountered (Lim and Shanmugam, in press). Furthermore, the addition of cyclic nucleotide phosphodiesterase to the assay reduced the cGMP values by at least 75%, indicating that cGMP is the major compound that was measured. An increase in nitrogenase activity in the culture coincided with a fourfold drop in cellular cGMP levels (Fig. 4). It is possible that these changes in intracellular concentrations of cGMP may be enough to allow it to function as a regulatory effector. Siegel et al. (34) have shown that in *Pseudomonas* a twofold change in intracellular cAMP concentration is sufficient for it to act as a regulatory effector in catabolite repression. Microaerophilic-aerobic shift experiments with *R. japonicum* have shown that cellular cGMP concentrations increase from 0.25 to 2.6 pmol/mg of cell protein. It is, thus, possible that the levels of cGMP may be under redox control, such that a decrease in oxygen concentration in the medium lowers the cGMP levels, which, in turn, allows the expression of those enzyme systems that have been found to be active under nitrogen-fixing conditions.

The results presented in this paper indicate that the inhibition of nitrogenase biosynthesis by cGMP is apparently not simply competitive inhibition of cAMP because the intracellular concentration of cGMP needed to inhibit nitrogenase is much less than the concentration of cAMP needed to stimulate hydrogenase, an enzyme system important in nitrogen fixation (Lim and Shanmugam, in press). In addition, the K_{diss} of the cAMP receptor protein for cGMP is much higher (fourfold) than that for cAMP. This difference may be suggestive of a unique receptor for cGMP.

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