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Effect of Carbamoyl Phosphate on Nitrogenase in *Anabaena cylindrica* Lemm

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Carbamoyl phosphate inhibited acetylene reduction by whole cells and cell-free extracts of *Anabaena cylindrica*. Higher levels of both endogenous carbamoyl phosphate and carbamoyl phosphate synthase activity were present in NH_4^+-grown cells (in which acetylene reduction was absent) than in N_2-grown cells (in which acetylene reduction was present). However, inhibition of acetylene reduction was observed also with cyanate, the main initial decomposition product under the conditions used. It is concluded that carbamoyl phosphate or one of its metabolites may act as a physiological regulator of both nitrogenase activity and synthesis, but caution must be used in interpreting effects observed several hours after the addition of carbamoyl phosphate, because these effects may be due to cyanate.

Ammonia, or a product of its metabolism, inhibits nitrogenase synthesis in blue-green algae (cyanobacteria) and may also inactivate nitrogenase already present in the cells (4, 5, 19, 20, 26), although results vary (25). In *Anabaena cylindrica*, it is unlikely that ammonia itself is the regulator of nitrogenase synthesis and activity, since ammonia does not affect nitrogenase activity in vitro (25) and cells grown in the presence of ammonia and L-methionine-DL-sulfoximine, an inhibitor of glutamine synthetase, retain their nitrogen-fixing ability (20, 26). It has been suggested, therefore, that glutamine itself or some of its metabolites may be responsible for in vivo inactivation of nitrogenase and repression of its synthesis (20, 26).

One such metabolite may be carbamoyl phosphate (CP), which is essential for the formation of the significant quantities of labeled citrulline and arginine observed after feeding $^{14}CO_2$ (13, 18), $^{13}NH_4^+$ (16), or $^{13}N_2$ (28) to blue-green algae. Increases in relative pool levels of urea cycle amino acids and enzyme activities were reported when cells were grown on ammonia rather than nitrogen gas (3, 11, 12, 19, 23), suggesting that the supply of external ammonia increased the proportion of newly assimilated nitrogen metabolized through the urea cycle. It was proposed that CP could act as a physiological regulator of both nitrogenase synthesis and activity in *Clostridium pasteurianum*, by binding to nitrogenase (21, 22) and by inhibiting the uptake of MoO_4^{2-} (8); in this bacterium the addition of ammonia does not inactive nitrogenase present in the cells but represses nitrogenase synthesis (6, 7).

This paper reports investigations into the possible role of CP as a physiological regulator of nitrogenase activity and synthesis in *A. cylindrica*. The decomposition of labile CP into cyanate (NCO^-) and then ammonia, carbon dioxide, and phosphate (1) was monitored also to evaluate the importance of artifacts due to the accumulation of NCO^- or NH_4^+ in CP solutions.

MATERIALS AND METHODS

Algae. *A. cylindrica* Lemm. (CU1403/2a) was obtained in pure culture from the Culture Collection of Algae and Protozoa, Cambridge.

Culture conditions. The algae were grown axenically at 25°C in the medium of Allen and Arnon (2) at pH 7.5 in continuous culture volumes of 0.5 or 3.5 liters. Cultures were supplied, if required, with 1 mM or 2 mM NH_4Cl, and had low and no detectable nitrogenase activity, respectively (Table 1). The cultures were magnetically stirred and bubbled with air at 1.0 liter min^{-1} and were illuminated with four 20-W Grolux fluorescent tubes placed 10 cm from the surface of the culture vessels. For cell-free nitrogenase preparations, 3 liters of cells were removed from a culture volume of 3.5 liters and bubbled with Ar-CO_2 (95:5, vol/vol) for 48 h before use.

Acetylene reduction assays. For cell-free assays, the preparation of extracts, reaction mixtures, and assay methods were as described by Haystead et al. (10). Protein was determined by the method of Lowry et al. (14). For whole cells, 7-ml serum bottles contained 1 ml of algae and the gas phase was 10% C_2H_2 in air. The reactions were terminated by the addition

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TABLE 1. *Endogenous levels of CP in N_2-grown and NH_4^+-grown cells from continuous cultures*

NH_4^+ concn (mM) in growth medium	Nitrogenase activity[a]	Endogenous CP[b]	
		nmol per mg of chlorophyll a	mM
0	19.4	0	0
1	3.3	9.4	0.08
2	0	42.9	0.29

[a] Expressed as micromoles of C_2H_2 reduced per milligram of chlorophyll a per hour.

[b] Details of extraction and assay of CP from cells grown in continuous culture are given in the text.

of 0.1 ml of 50% trichloroacetic acid after 30 min of incubation and assayed for C_2H_4 as above.

Uptake of [^{14}C]CP. Uptake of ^{14}C-labeled CP ([^{14}C]CP) was measured under saturating light by filtering through glass fiber filters, and then washing with culture medium, samples of cells taken at various times after the [^{14}C]CP was added, followed by extracting the cells on the filter in 5% chilled trichloroacetic acid and estimating the extracted and unextracted activity separately by scintillation counting.

$^{14}CO_2$ fixation. Fixation of $^{14}CO_2$ was estimated by the method of Lyne and Stewart (15), except that 40 mM $NaH^{14}CO_3$ (10 mCi mol^{-1}) was used, samples were incubated at 10,000 lx with Photoflood 2 bulbs, and scintillation counting was used.

Measurement of endogenous levels of CP. The method was modified from that of Williams et al. (27), and CP was estimated after incorporation into citrulline. Calculation of internal pool concentrations was by the method of Meers et al. (17).

Measurement of CP synthase activity. Cells were broken by sonication for 3 to 4 min, and the CP synthase activities in the supernatant and the pellet from centrifugation at 30,000 × *g* for 30 min were assessed separately. Samples were incubated for 20 min at 30°C in the following reaction mixture: 5 mM ATP, 10 mM glutamine or NH_4Cl, 10 mM $MgCl_2$, 10 mM ornithine monohydrochloride, 10 mM $NaH^{14}CO_3$ (10 mCi mol^{-1}) in a final volume of 1.0 ml in 100 mM Tris-chloride buffer at pH 8.3. The reaction was terminated by heating at 100°C for 5 min, and a sample was chromatographed by thin-layer chromatography using phenol–water (80:20, wt/vol). The spot of radioactive citrulline was located, and the radioactivity was estimated by scintillation counting. Activity was not enhanced by the addition of ornithine transcarbamoylase from *Streptococcus faecalis*, because the endogenous activity was in excess (49 to 82 nmol/min per mg of protein) (3). Results were confirmed using DL-[1-^{14}C]ornithine monohydrochloride and $NaH^{12}CO_3$.

Decomposition of CP and NCO^-. Decomposition of CP was determined enzymatically as citrulline by using the assay of Williams et al. (27). The appearance of NH_3 from NCO^- and CP was determined colorimetrically by the method of Solorzano (24).

Chemicals. [^{14}C]CP (specific activity, 7.99 Ci mol^{-1}) was obtained from New England Nuclear Corp. DL-[1-^{14}C]ornithine monohydrochloride (58 Ci mol^{-1}) and $NaH^{14}CO_3$ (10 mCi mol^{-1}) were obtained from the Radiochemical Centre, Amersham. Ornithine transcarbamoylase from *S. faecalis* was obtained from Sigma Chemical Co., and all other chemicals were purchased from Sigma Chemical Co. or BDH at the highest purity available.

RESULTS

Decomposition of CP under the experimental conditions used. CP decomposed by 20% within 30 min and completely within 12 h (Fig. 1). NH_3 release from both 1 mM CP and NCO^- increased with time to reach 0.12 and 0.05 mM, respectively, after 24 h (Fig. 2). As noted by Allen and Jones (1), release of NH_3 from NCO^- was slower than that from CP.

Acetylene reduction by cell-free nitrogenase extracts. The addition of up to 10 mM CP or NCO^- to crude nitrogenase extracts inhibited acetylene-reducing activity by up to 50 and 75%, respectively (Fig. 3). Inhibition by both CP and NCO^- appeared to be noncompetitive with acetylene. The addition of NCO^- reduced binding of radioactivity from 5 mM [^{14}C]CP to protein while increasing inhibition of acetylene reduction. No inhibition of nitrogenase activity was observed on the addition of up to 10 mM NH_4^+ or glutamine.

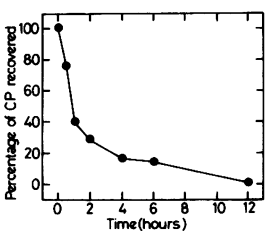

FIG. 1. *Time course of decomposition of CP under the conditions used in experiments with whole cells. Freshly made solutions of 1 mM CP were allowed to stand at 25°C in culture medium (pH 7.5), and samples were assayed at intervals.*

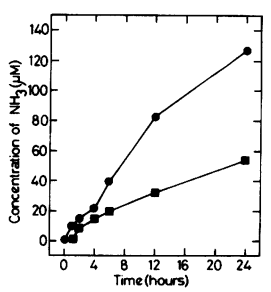

FIG. 2. *Accumulation of NH_3 in solutions originally 1 mM CP (●) and 1 mM NCO^- (■), at 25°C and pH 7.5 in culture medium. Freshly made solutions were allowed to stand, and aliquots were determined at intervals.*

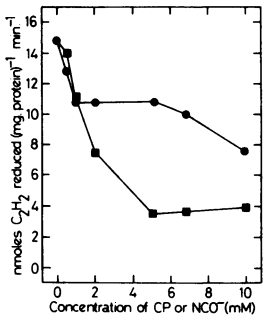

FIG. 3. *Effect on nitrogenase activity, as measured by acetylene reduction, of adding CP (●) or NCO^- (■) to cell-free extracts of A. cylindrica.*

Acetylene reduction by whole cells. When up to 10 mM CP or NCO^- was added to whole N_2-fixing cells, nitrogenase activity declined by up to 90% within 30 min (Fig. 4). However, the addition of only 1 mM CP or NCO^- resulted in total absence of nitrogenase activity after 24 h (Fig. 5). The addition of 1 mM NH_4Cl resulted in a decline of 55% in acetylene-reducing activity within 30 min and a total loss of activity in 24 h (Fig. 5), but the addition of 0.1 mM NH_4Cl had no effect on acetylene reduction over this period. Radioactivity from [^{14}C]CP accumulated on or in the cells, mainly (75%) in trichloracetic acid-insoluble forms. $^{14}CO_2$ fixation, used as a measure of cellular metabolism in N_2-fixing cells, was unaffected by the addition of 1 mM CP or 1 mM NCO^- in the time (2 h) that was sufficient to reduce acetylene reduction by 60%.

As noted by Seto and Mortenson (21) with *C. pasteurianum*, 1 mM CP was also inhibitory to the growth of 2 mM NH_4^+-grown cells, causing 60% inhibition of growth after 24 h, but 1 mM NCO^- had no effect, suggesting that the growth inhibition observed was not due to NCO^- toxicity. Growth on NH_4^+ but not N_2 was unaffected by the addition of 0.25 mM CP in the growth medium.

Endogenous CP levels. Table 1 shows that the internal pool concentration of CP increased from 0 mM in N_2-grown cells, which possessed active nitrogenase, to 0.29 mM in cells grown in 2 mM NH_4Cl, in which nitrogenase activity was undetectable.

Measurement of CP synthase activity. The endogenous pools of CP are presumably produced by the action of CP synthase, and Table 2 shows that the activity of this enzyme in cells grown on 2 mM NH_4Cl was at least eight times that in N_2-grown cells. These rates are low but reproducible and easily detectable by the methods used. Although both glutamine and ammonia were utilized as substrates, activity was greater with ammonia.

DISCUSSION

The experiments reported here with *A. cylindrica* show results similar to those with *C. pasteurianum* (21, 22), from which it was suggested that CP acted as a physiological regulator of both nitrogenase activity and synthesis. Exogenous CP in *A. cylindrica* inhibited nitrogenase activity in vitro, and the increased inhibition observed in vivo suggests that inhibition of ni-

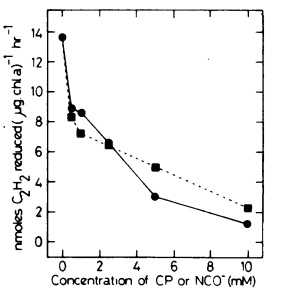

FIG. 4. *Effect on nitrogenase activity, as measured by acetylene reduction, of adding CP (●) or NCO^- (■) to whole cells of A. cylindrica grown in nitrogen-free medium.*

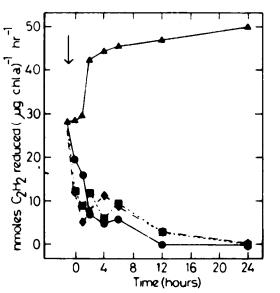

FIG. 5. *Effect on N_2-fixing cells of adding culture medium (▲), 1 mM CP (●), 1 mM NCO^- (■), or 1 mM NH_4Cl (◆) at time arrowed and assaying nitrogenase activity for 30 min immediately (0 h) or after time interval indicated.*

TABLE 2. *CP synthase activity in cell-free extracts of A. cylindrica grown in continuous culture with N_2 or 2 mM NH_4Cl as nitrogen source*

Nitrogen source	Enzyme activity[a] in substrate:	
	Glutamine	NH_4Cl
N_2	0.16	1.05
NH_4^+	2.40	8.60

[a] Details of preparation of cell-free extracts and assay procedures are given in the text. Enzyme activity was measured as nanomoles per milligram of protein per minute.

trogenase synthesis, as well as activity, occurred. This suggestion is supported by the initial sharp decrease and complete decline of nitrogenase activity on the addition of 1 mM CP to whole cells, similar to that observed with 1 mM NH_4Cl and probably due both to inactivation of existing nitrogenase and to inhibition of its synthesis (4, 5, 19, 20, 26).

A physiological role of CP in regulating nitrogenase activity and synthesis is more difficult to assess, but is suggested by the increased levels of both endogenous CP and CP synthase activity in NH_4^+-grown cells, in which nitrogenase activity was absent. Whereas endogenous levels of CP in 2 mM NH_4^+-grown cells appeared to be low by comparison with exogenous CP concentrations used, much of the radioactivity taken up from [^{14}C]CP was insoluble, perhaps in the form of cyanophycin (9, 23), and much of the soluble material would not be [^{14}C]CP due to rapid metabolism in the cells. Although CP synthase in vitro was active with both glutamine and NH_4^+, activity in vivo was probably glutamine dependent, since treatment of whole cells with L-methionine-DL-sulfoximine in the presence of $^{13}NH_4^+$ resulted in the cessation of production of ^{13}N-labeled citrulline and arginine, products of CP metabolism (16). Thus CP appears to be a glutamine-derived metabolite capable of inhibiting nitrogenase activity and synthesis at physiological levels.

Consideration must be given also to three other major possibilities: (i) that not CP itself, but one of its metabolites, is the active inhibitor; (ii) that CP acts indirectly by inhibiting glutamine synthetase; and (iii) that the breakdown products of CP are responsible for the decreases observed in nitrogenase activity and synthesis.

The possibility that a further metabolite of CP, such as one of the urea cycle amino acids, may be the active inhibitor cannot be excluded, since citrulline and arginine were labeled with ^{13}N after only 120 s when $^{13}N_2$ or $^{13}NH_4^+$ was supplied (16, 28). Direct evidence of inhibition of nitrogenase activity by these compounds in vitro or in vivo is lacking, but Bone (4) noted that a combination of arginine and aspartate inhibited nitrogenase synthesis in O_2-inactivated cells of *Anabaena flos-aquae*.

It is unlikely that CP acts indirectly by inhibiting glutamine synthetase activity only (20), and thus nitrogenase activity in vivo, since any isolated decrease in the glutamine pool should be associated with an increase rather than a decrease in nitrogenase activity (20, 26). However, inhibition of glutamine synthetase by CP (20) may explain the inhibition of growth observed in NH_4^+-grown cells in 1 mM but not 0.25 mM CP.

The rapid decomposition of CP into NCO^- and the slower rate of accumulation of ammonia suggest that only results from experiments using CP within 30 min of preparation can be attributed unequivocally to effects of CP rather than those of its decomposition products. Effects seen in longer-term experiments are probably attributable to NCO^- rather than NH_4^+, since NH_4^+ did not inhibit nitrogenase activity in vitro and the concentration of NH_4^+ accumulated by 1 mM CP over 24 h (0.1 mM) did not affect nitrogenase activity or growth in whole cells. The suggestion that NCO^- is responsible for effects of CP seen in longer-term experiments is supported by the similar magnitude of its inhibition of nitrogenase activity in vitro and the rapid and complete decline in nitrogenase activity in whole cells on its addition. That the action of CP is not necessarily only that of NCO^-, however, is suggested by the fact that growth on NH_4^+ is unaffected by NCO^- but inhibited by similar or smaller concentrations of CP.

In summary, the results indicate that addition of CP affects nitrogenase activity and synthesis and that not glutamine itself but glutamine-derived CP may be a physiological regulator of these processes. However, the possibilities of breakdown products or further metabolites being the active inhibitor(s) cannot be excluded due to the rapid breakdown of CP in solution and its rapid metabolism inside the cells. A study of the involvement of urea cycle compounds in the regulation of nitrogenase activity and synthesis might clarify the position with regard to the involvement of further metabolites.

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