EFFECT OF CARBAMYL PHOSPHATE ON THE REGULATION OF NITROGENASE IN CLOSTRIDIUM PASTEURIANUM

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SUMMARY

One mM carbamyl phosphate inhibited the in vitro acetylene reduction activity of nitrogenase 30% whereas at high concentrations a maximum inhibition of 50% was observed. When 1 mM carbamyl phosphate was added to a culture growing on N_2 1) nitrogenase synthesis was completely repressed and 2) after a period of 2.5 hrs in the absence of growth, the specific activity decreased to less than 50% of its activity just before the addition of the inhibitor.

INTRODUCTION

Previous studies have established that there is no detectable nitrogenase or nitrogenase activity when Clostridium pasteurianum is grown in the presence of excess NH_3 (1,2). Similar findings were reported for Azotobacter vinelandii and Klebsiella pneumoniae (3,4,5). The implication that NH_3 is an effector in regulating the dinitrogen-fixing system is suggested by these findings. However, the possibility that NH_3 was a feed-back inhibitor of nitrogenase activity was ruled out since NH_3 had no effect on in vitro nitrogenase activity (1). Because there are no reports of compounds that regulate nitrogenase activity and because information on regulation of nitrogenase synthesis is still incomplete, we undertook a systematic study of this problem in Clostridium pasteurianum. Recently we reported (American Society for Microbiology meetings, 1973) that carbamyl phosphate inhibits the in vitro acetylene reduction activity of nitrogenase. This paper documents the findings of this earlier report as well as new findings that show that carbamyl phosphate not only inhibits in vitro activity but also is involved in control of nitrogenase biosynthesis.

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MATERIALS AND METHODS

Clostridium pasteurianum was grown in media described by Daesch and Mortenson (1). Measurement of acetylene reduction by pure nitrogenase in vitro was described previously (6), except that the gas phase contained 0.85 atm argon and 0.15 atm acetylene. Whole cell acetylene reduction assays were performed by a modification of the method reported by Moustafa and Mortenson (7). A 0.1 ml portion of 0.05 M phosphate buffer, pH 7, containing 2% sucrose was added to 1.9 ml of the culture. Thirty μl gas samples were removed at 2 min intervals and analyzed in a Varian 1520 gas chromatograph (8).

Protein was determined by the biuret method (9).

RESULTS

Carbamyl phosphate inhibited nitrogenase activity (measured by acetylene reduction) at concentrations as low as 1 mM (Fig. 1). A 30% decrease in rate was observed at 1.0 mM but the maximum inhibition attained with concentrations as high as 20 mM was only a 50% decrease in rate.

Two nitrogen-fixing cultures of C. pasteurianum were grown in identical medium. To one, 1.0 mM carbamyl phosphate was added at exponential growth as indicated in the figures 2 and 3. Cell density and acetylene reduction activity were measured. Figure 2b shows that the differential rate of formation of nitrogenase was completely repressed by the addition of 1.0 mM carbamyl phosphate, whereas the rate in the non-supplemented culture increased logarithmically. This repression lasted for the duration of the experiment. Two hours after the addition of carbamyl phosphate the growth rate decreased (Fig. 2a). As seen in figure 3 soon after the addition of 1.0 mM carbamyl phosphate the specific activity of nitrogenase rapidly decreased to about 70% of the uninhibited rate. This rapid decrease in specific activity probably was the result of feed-back inhibition of carbamyl phosphate

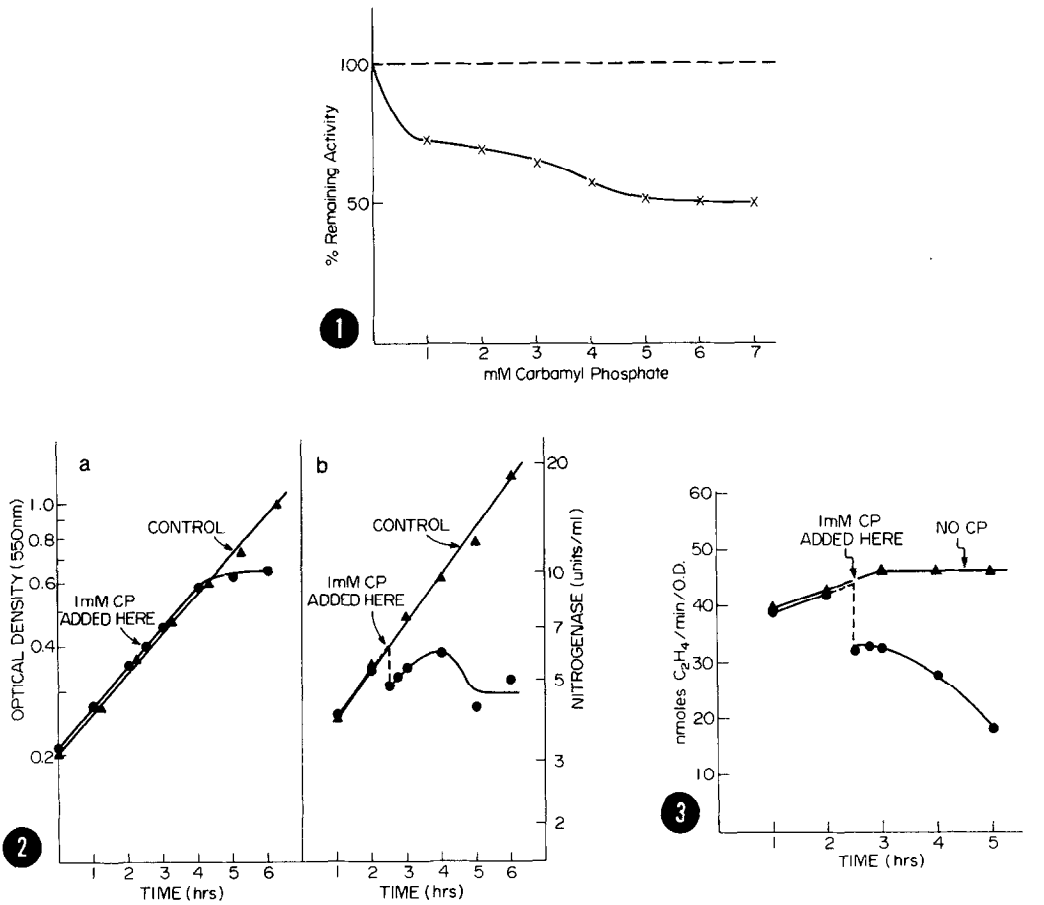

Fig. 1. Inhibition of *in vitro* acetylene reduction activity with various concentrations of carbamyl phosphate. The assay was performed as described (6). Aqueous solution of carbamyl phosphate was freshly prepared, and added to reaction mixture immediately before the start of the reaction. Molybdoferredoxin 0.18 mg and Azoferredoxin 0.35 mg.

Fig. 2a. Effect of carbamyl phosphate on a growing N_2-fixing culture. Two-250 ml cultures were grown under N_2-fixing conditions as described (1). 2 ml samples were removed from each at various times for the acetylene reduction assay and 1 ml samples for $O.D._{550}$ determination. At the indicated time, 1 mM carbamyl phosphate was added to one culture. Growth and acetylene reduction activity were followed.

Fig. 2b. Repression of nitrogenase by 1 mM carbamyl phosphate. Whole cell acetylene reduction assays were performed as described in the methods section.

Fig. 3. Inhibition of acetylene reduction activity by 1 mM carbamyl phosphate. Activity was determined on samples removed from the cultures described in Fig. 2.

as described earlier in the *in vitro* studies (see Fig. 1). In addition carbamyl phosphate caused a significant decrease in the rate of *in vivo* acetylene reduction activity (Fig. 3).

When a 1.0 mM concentration of carbamyl phosphate was added to a culture growing on NH_3, a 20% inhibition of growth was observed. Since growth of an NH_3-grown culture is not dependent on nitrogenase activity, growth inhibition would not be expected unless some other aspect of metabolism were affected by carbamyl phosphate. Experiments with other concentrations of carbamyl phosphate were performed. An external concentration of 0.1 mM carbamyl phosphate brought about a repression of nitrogenase to about 80% that of the non-supplemented culture. With this concentration of carbamyl phosphate no inhibitory effect on growth was observed in an NH_3-grown culture. With concentrations of carbamyl phosphate higher than 1.0 mM, nitrogenase synthesis was also completely repressed but inhibition of growth of an NH_3-grown culture was even more pronounced.

DISCUSSION

Previous observations have suggested some role for NH_3 in the repression of nitrogenase. The experiments described here indicate that the addition of carbamyl phosphate, a product of early NH_3 assimilation, not only represses the formation of nitrogenase but also causes feed-back inhibition of its activity. One might consider carbamyl phosphate an end product of inorganic nitrogen metabolism.

The addition of carbamyl phosphate (1 mM) completely repressed nitrogenase synthesis and inhibited N_2-supported growth. This concentration of carbamyl phosphate also caused some inhibition of growth on NH_3 which suggests that carbamyl phosphate at this higher concentration, in addition to repressing nitrogenase, affected other metabolism, possibly by affecting arginine biosynthesis and/or pyrimidine biosynthesis; it affected RNA synthesis (10,11). Nevertheless, the

experiments described here demonstrate that carbamyl phosphate plays a significant role in nitrogenase regulation. The possibility that it is an indirect role has not been eliminated. Carbamyl phosphate may raise the level of some other nitrogenous metabolite which is the real co-repressor. Experiments designed to measure the binding of carbamyl phosphate to 1) nitrogenase and 2) to other "cytoplasmic" proteins that may be involved in the formation of an active repressor complex, are in progress.

ACKNOWLEDGMENT

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REFERENCES

1. Daesch, G., and Mortenson, L. E., 1972, J. Bacteriol. 110:103.
2. Mortenson, L. E., Morris, J. A., and Jeng, D. Y., 1967, Biochim. Biophys. Acta 141:516.
3. Strandberg, G. W., and Wilson, P. W., 1968, Can. J. Microbiol. 14:25.
4. Mahl, M. C., and Wilson, P. W., 1968, Can. J. Microbiol. 14:33.
5. Parezko, R., and Wilson, P. W., 1970, Can. J. Microbiol. 16:681.
6. Dalton, H., Morris, J. A., Ward, M. A., and Mortenson, L. E., 1971, Biochemistry 10:2066.
7. Moustafa, E., and Mortenson, L. E., 1971, Nature (London) 216:1241.
8. Moustafa, E., and Mortenson, L. E., 1968, Anal. Biochem. 24:226.
9. Gornall, A. G., Bardawill, C. J., and David, M. M., 1949, J. Biol. Chem. 177:751.
10. O'Donovan, G. A., and Neuhard, J., 1970, Bacteriol. Rev. 34:278.
11. Pierard, A., Glansdorff, N., Mergeay, M., and Wiami, J. M., 1965, J. Mol. Biol. 14:23.