DEREPRESSION OF NITROGENASE IN AZOTOBACTER

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SUMMARY: When nitrogenase in Azotobacter vinelandii 12837 is repressed by ammonia, the derepression is accelerated by endotoxin or cyclic AMP. The phenomenon appears neither to be a consequence of accelerated ammonia utilization nor altered activity of preformed enzyme. This is a unique example of an effect of endotoxin on a procaryotic system.

INTRODUCTION: In studying the nitrogenase activity of Azotobacter mutants it became evident that the induction of this enzyme in wild type strains was sensitive to cultural manipulation. We are reporting here the acceleration of nitrogenase derepression by cyclic AMP and endotoxin. Catabolite repression in bacteria is thought to be mediated by a specific protein which binds cyclic AMP and much work has been done to elucidate the mechanism of cyclic AMP regulation of a number of inducible enzyme systems in bacteria (1). Prusiner, Miller and Valentine (2) have found that several key enzymes involved in ammonia assimilation are regulated by cyclic AMP. When it was found that exogenous cyclic AMP did modify the regulation of nitrogenase in Azotobacter it appeared reasonable to include endotoxins in this study since these substances affect enzyme regulation.

MATERIALS AND METHODS: Azotobacter vinelandii ATCC strain 12837, maintained on Burk's Nitrogen Free-Sucrose (BNF-S) slants (3), was transferred into BNF-S broth and after 18 hours the active culture was inoculated into 500 ml portions of BNF-S fortified with 80 µg/ml of fixed nitrogen as NH_4Cl. All broth cultures were incubated on a reciprocal shaker at 33 C. After 18 hours the cells were harvested by centrifugation, washed once in BNF-S, and resuspended in 400 ml BNF-S with the appropriate NH_4Cl concentration. Endotoxin, cyclic AMP, or AMP was dissolved in 100 ml of BNF-S, filter

sterilized, and added at time "0" to bring total media volumes to 500 ml. Twenty ml aliquots were assayed for nitrogenase activity (NA) by the acetylene reduction method of Dilworth (4) at hourly intervals for the first 4 hours of incubation and at indicated times thereafter. Assays were conducted in 40 ml serum bottles fitted with rubber stoppers into which 0.2 atmosphere of acetylene was injected. After 60 min incubation at 33 C with rotary shaking, 0.5 ml of 5N $H2SO_4$ was injected through the stopper to terminate acetylene reduction. A Tracor Model 550 gas chromatograph equipped with a Porapack N column and flame ionizing detector was used to quantitate the ethylene in 0.5 ml samples from the serum-bottle head space. Plate counts were made at the time of each assay and the results were computed as nM of ethylene produced per hour per 10^8 cells. Control cultures were established in media without cyclic AMP or endotoxin and/or cultures containing AMP at the same molarity of cyclic AMP used in a particular experiment. Ammonia assays were done by the method of Chaney and Marbach (5). Sonication, when required, was effected by 3 bursts of one minute each with an MSE sonicator operating at 1.7 amperes with the cell suspension cooled in an ice-bath. Endotoxin from Salmonella typhimurium SR-11 was prepared by the phenol extraction method of Westphal and Jann (6). Both cyclic AMP and AMP were obtained from Sigma Chemical Co.

RESULTS: The results of an experiment showing the time course of the derepression of nitrogenase activity is shown in Figure 1. It is evident that both in the control culture and in the control supplemented by 10^{-3} M AMP (line I) little enzyme is formed in the first 4 hours. This is an expected result since it takes almost 4 hours for the culture density employed in these experiments to reduce the starting level of 20 µg/ml of fixed nitrogen to below the repressing concentration. Enzyme synthesis does eventually take place in these controls but only after several more hours. In contrast to the controls, in the presence of 10^{-3} cyclic AMP some nitrogenase activity was measurable at 2 hours and a good level was attained after 3 hours (line

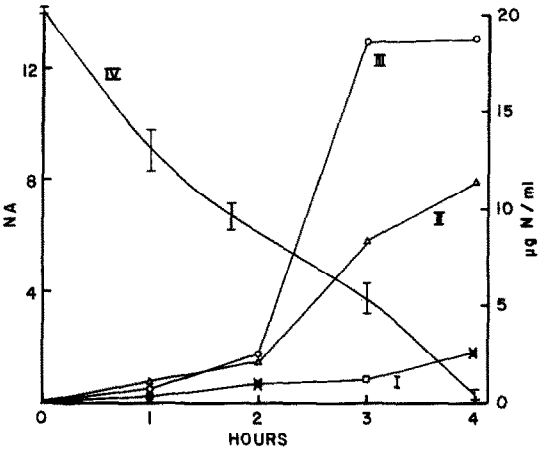

Figure 1. Nitrogenase activity (NA) as nM of ethylene/hour/10^8 cells as affected by nucleotides or endotoxin. I. control (open squares) or 10^{-3} M AMP (x) II. 10^{-3} M cyclic AMP III. 60 μg/ml endotoxin IV. Residual NH_3 nitrogen in the cell suspensions used in I, II, III. Range of measurements indicated by bars and 1 curve represents all measurements.

II). The addition of 60 μg/ml of endotoxin also accelerated the derepression and a high level of enzyme was present after 3 hours (line III). That these results were not due to the accelerated depletion of the NH_3 in the cultures is depicted by line IV which shows the NH_3-N decline which appears to be identical regardless of the presence or absence of endotoxin or nucleotides. The plate counts of all flasks showed neither killing nor growth stimulation.

The experiment was repeated with the repressor concentration increased to 80 μg/ml of fixed nitrogen. With this level of NH_3, the control and the 10^{-3} M AMP were still repressed after 22 hours of incubation. Measurable levels of NA were found in 10^{-3} cyclic AMP cultures after one hour and in the 60 μg/ml endotoxin cultures after 2 hours. The effect of cyclic AMP was assayed on this system at levels ranging from 5 X 10^{-5} M to 2 X 10^{-3} M and optimal derepression was found at 1 X 10^{-3} M. Controls using AMP showed no derepression at any concentration of the nucleotide whether 20 or 80 μg/ml of repressing nitrogen was supplied. The large response with endotoxin at 60 μg/ml suggested the possibility that endotoxin was combining with NH_3 or in

some way dissipating it or rendering it inaccessible to the cell (thus relieving the repression). Ammonia assays run on mixtures of endotoxins and NH_3 gave no indication that the ammonia was tied up in a manner to prevent assay. Furthermore, when chloramphenicol was added to the medium at 100 µg/ml levels, cyclic AMP had no effect in relieving ammonium repression suggesting the effect is on de novo enzyme synthesis.

Two other endotoxin preparations (Difco Salmonella typhimurium W., and Serratia marcescens endotoxin) were tested and both accelerated derepression of the nitrogenase over controls but with not quite as pronounced effect as the S. typhimurium SR 11 endotoxin. The extent of the derepression appeared to parallel the toxic activity for mice reported for the 3 endotoxin samples.

DISCUSSION: The convenient acetylene reduction assay system for nitrogenase activity has lead to its use in testing diverse phenonmena as the effects of pesticides (7) on the nitrogenase system. Azotobacter nitrogenase production is not stimulated by any amount of cyclic AMP which we tested; however, when cultures are grown in sufficient NH_3 to prevent nitrogenase production for a few hours, both cyclic AMP and endotoxin from 3 sources accelerated the rate of recovery from this repression. When protein synthesis was inhibited by chloramphenicol, cyclic AMP was without effect. Neither the mechanism of action of the endotoxins nor that of cyclic AMP on nitrogenase derepression is known. Cyclic AMP is not acting on preformed enzyme but must affect the biosynthetic mechanism of nitrogenase. Ammonia depletion studies show that the phenomenon is not due to accelerated ammonia utilization nor altered permeation of the cells. If the repression of NA by exogenous ammonia is due to the modification of a binding site of the activator protein, cyclic AMP and endotoxins may act on the permeation system by which ammonia gains access to that site. The volume of an Azotobacter cell is ten times that of the usual procaryote and the nitrogen fixing system appears to be accompanied by a complex membrane system.

The finding of the effect of endotoxin might have a significant bearing

on our thinking about the role of Azotobacter in mixed populations of gram-negative bacteria in fertile soil where it was formerly assumed that the presence of a repressed nitrogenase system could not be advantageous to the organism. The possibility of using this system as an assay for endotoxins and the mechanism of action on the unique procaryote is being studied.

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