# DEREPRESSION OF NITROGENASE SYNTHESIS IN THE PRESENCE OF EXCESS $NH_4^+$

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SUMMARY

Methionine sulfone and methionine sulfoximine, inhibitors of enzymes involved in $NH_4^+$ assimilation, cause nitrogenase to be synthesized in the presence of excess $NH_4^+$ in Azotobacter vinelandii and Klebsiella pneumoniae. These inhibitors also cause $NH_4^+$ excretion by A. vinelandii. These results indicate that $NH_4^+$ alone is not the actual effector of nitrogenase repression in these organisms.

## INTRODUCTION

Nitrogenase synthesis is normally repressed in cultures growing in the presence of fixed N. When excess $NH_4^+$ is in the medium, the activity of nitrogenase is less than $10^{-4}$ of that found when the bacteria are growing in a N-free medium (1). Sorger (2) has shown that repression of nitrogenase synthesis in A. vinelandii by $NO_3^-$ requires active nitrate reductase (EC 1.9.6.1). Presumably, it is the $NH_4^+$ produced from $NO_3^-$ that is required for repression. We wanted to know if $NH_4^+$ is the actual effector for nitrogenase repression or if some product of $NH_4^+$ assimilation is the effector. To determine if $NH_4^+$ assimilation is required, we added inhibitors of the enzymes required for $NH_4^+$ assimilation. The glutamate analogs, MSX and MSF have been shown (3) to inhibit both glutamine synthetase (EC 6.3.1.2) and glutamate synthetase. The importance of these enzymes in several bacteria under N-limiting conditions has been documented (4,5,6).

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Abbreviations: MSX, L-methionine-DL-sulfoximine;
MSF, L-methionine sulfone

## MATERIALS AND METHODS

The organisms used are A. vinelandii OP (7) and K. pneumoniae M5a1 (8). A description of media, culture conditions and extract preparation for A. vinelandii has been given (9). Extracts of K. pneumoniae were prepared as previously described (10). Acetylene reduction, a convenient assay for nitrogenase activity (11,12) was used. Conditions of the nitrogenase assay have been given (9). Preparation of cell-free extracts for assay of $NH_4^+$ assimilatory enzymes has been described by Brenchley (3). The transferase assay for glutamine synthetase as outlined by Kingdon and Stadtman (13) and the glutamate synthetase and glutamate dehydrogenase (EC 1.4.1.4) assays of Meers et al. (4) were used. All assays were performed at 30°. Protein concentrations were determined by the method of Gornall et al. (14). The amount of $NH_4^+$ in the medium was determined by microdiffusion of the $NH_3$, followed by quantitation with Nessler's reagent. MSF and MSX were obtained from Sigma Chemical Co., St. Louis, Mo.

## RESULTS AND DISCUSSION

Cultures of A. vinelandii were grown with 400 µg N/ml as ammonium acetate in the medium to a cell density of $5\text{-}6\times10^8$/ml. The cells were harvested, resuspended in N-free medium with various additions and incubated 3.5 h before being assayed for $C_2H_2$ reducing activity. The results in Table 1 indicate that nitrogenase synthesis occurs in the presence of $NH_4^+$ only when MSF or MSX is added to the culture. It can be seen that the specific activities of cultures derepressed in $NH_4^+$ free media with these inhibitors exceeds the activity of a culture without an inhibitor. It has been observed that the level of nitrogenase found in steady-state $N_2$-grown cultures of A. vinelandii is less than the maximum amount observed for a period during derepression following growth on limiting $NH_4^+$ (9). It seems that the cells, responding to the N fixed, regulate nitrogenase synthesis so as not to produce excess enzyme. Presumably, this control is overcome by the addition of the inhibitors.

Table 1

Effect of MSF and MSX on Nitrogenase Derepression

| Organism | N-source | Inhibitor added | Specific activity | |
|---|---|---|---|---|
| | | | in vivo* | in vitro** |
| A. vinelandii | $N_2$ | None | 2.20 | 56.5 |
| " | $NH_4^+$ | None | 0.00 | 0.00 |
| " | $NH_4^+$ | MSF(20mg/ml) | 0.72 | 29.9 |
| " | $NH_4^+$ | MSX " | 1.00 | 30.3 |
| " | $N_2$ | MSF " | 1.88 | 76.4 |
| " | $N_2$ | MSX " | 2.41 | 85.3 |
| K. pneumoniae | $N_2$ | None | 4.80 | -- |
| " | $NH_4^+$ | None | 0.00 | -- |
| " | $NH_4^+$ | MSF(2 mg/ml) | 0.23 | -- |
| " | $NH_4^+$ | MSX " | 1.41 | -- |

* nmoles $C_2H_2$ reduced/min x $10^8$ cells
** nmoles $C_2H_2$ reduced/min x mg protein

Cultures of K. pneumoniae were grown to a population density of $3x10^8$/ml in media containing 400 µg N/ml as ammonium acetate. They were then harvested, resuspended in N-free media and shaken aerobically for approximately 2 h. No derepression of nitrogenase occurs during this aerobic incubation (15). Appropriate additions were made and the cultures were incubated anaerobically for 5 h before being assayed. Results of the assay (Table 1) show again that addition of MSF and especially MSX to cultures incubated with excess $NH_4^+$ results in synthesis of nitrogenase, whereas addition of $NH_4^+$ alone yields no nitrogenase synthesis.

Inhibition of $NH_4^+$ assimilation might be expected to cause an organism

to excrete $NH_4^+$ when it is fixing $N_2$. Normally, A. vinelandii does not excrete detectable $NH_4^+$ into the medium, but if 5 mg/ml MSF is added to a culture containing 2 x $10^8$ cells/ml, an increase of 2 μmoles $NH_4^+$/ml per h is found in the medium. Not all of the $NH_4^+$ produced by $N_2$ fixation in the presence of MSF is expected to be excreted because glutamate dehydrogenase is not inhibited by this compound.

The effects of MSF and MSX have been examined by Brenchley (3) in a strain of K. aerogenes that does not fix $N_2$. We observe the same general effects on the $NH_4^+$ assimilatory enzymes with the two $N_2$-fixing strains described in this paper. Glutamate synthetase and glutamine synthetase are inhibited by MSF and MSX in K. pneumoniae. However, the inhibition of glutamate synthetase by MSX is very slight in A. vinelandii. Glutamate dehydrogenase from both organisms is unaffected by MSF or MSX.

These data indicate that $NH_4^+$ alone is not the actual effector for repression of nitrogenase synthesis in A. vinelandii or K. pneumoniae because inhibition of enzymes involved with $NH_4^+$ assimilation relieves repression. It is possible that either glutamate or glutamine is the effector and it also is possible that glutamate synthetase or glutamine synthetase is required to regulate transcription of the nitrogenase genes. Tyler et al. (16) have shown that the active form of glutamine synthetase is required, under certain conditions, for transcription of messenger RNA specifying several enzymes involved with amino acid degradation in K. aerogenes. To determine the actual effector(s), mutant strains defective in $NH_4^+$ assimilation are being studied.

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