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EFFECTS OF L-METHIONINE-DL-SULFOXIMINE AND β-N-OXALYL-L-α,β-DIAMINOPROPIONIC ACID ON NITROGENASE BIOSYNTHESIS AND ACTIVITY IN *RHODOPSEUDOMONAS CAPSULATA*

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ABSTRACT : Inhibitors of glutamine synthetase cause derepression of nitrogenase biosynthesis in the presence of NH_4^+ in the photosynthetic bacterium *Rhodopseudomonas capsulata*. A new derepressor of nitrogenase biosynthesis, β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), is here compared with the widely used L-methionine-DL-sulfoximine (MSX). With both compounds, a quantitative correlation has been observed between inhibition of glutamine synthetase and derepression of nitrogenase biosynthesis. We also find that both MSX and ODAP inhibit nitrogenase activity *in vivo* in *R. capsulata*. The latter effect seems to be indirect and related to the previously reported reversible inhibition of nitrogenase activity *in vivo* by NH_4^+. As a control it was observed that neither NH_4^+ nor MSX nor ODAP inhibit nitrogenase activity *in vivo* in *Clostridium pasteurianum*.

INTRODUCTION

Nitrogenase biosynthesis is repressed in the presence of ammonia in all free living diazotrophs studied so far. It has been suggested that the repression of nitrogenase synthesis is not caused by NH_4^+ itself, but rather by an interaction of glutamine synthetase with one or several genes involved in nitrogen fixation (1). This view is supported by several lines of evidence, among which is the observation that inhibitors of glutamine synthetase derepress nitrogenase biosynthesis in the presence of ammonia (2). The latter observation has been extended to include several species of diazotrophs (3, 4, 5, 6) by using the following inhibitors of glutamine synthetase : L-methionine sulfone, L-methionine-DL-sulfoximine (MSX) (2), and 5-hydroxylysine (6). In the present report we have established that nitrogenase biosynthesis is subjected to the same type of regulation in the purple non sulfur photosynthetic bacterium *Rhodopseudomonas capsulata*. For this purpose we have used MSX and β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), a neurotoxin (7) that has only briefly been mentioned as a derepressor of nitrogenase biosynthesis in blue-green algae (8).

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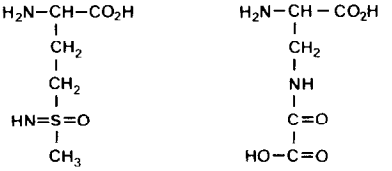
MATERIALS AND METHODS

R. capsulata strain B10 was a generous gift from the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington, IND. *Clostridium pasteurianum* W5 was purchased from the American Type Culture Collection. *R. capsulata* (9) and *C. pasteurianum* (10) were grown according to previously described methods. Nitrogenase, ammonia, and cell mass determinations have been described elsewhere (9). For nitrogenase derepression experiments, cells (30-40 ml of suspension) growing on lactate-ammonium were taken in mid-exponential phase and transferred into sterile 100 ml Erlenmeyer flasks ; the inhibitors were added as indicated, the flasks were stoppered with sterile rubber septa, gassed with argon, and shaken at 30°C in an illuminated water bath (9). For cell free extract preparations, washed cell suspensions were sonicated for 5 minutes with a W185D sonifier (Heat Systems Ultrasonics Inc., Plainview, N.Y.), set at a power of 75W ; the sonicated suspensions were subsequently centrifuged (25,000 x g, 30 min) to remove cell debris. Glutamine synthetase activity was assayed by measuring the formation of γ-glutamylhydroxamate from glutamate and hydroxylamine in the presence of ATP (11). L-methionine-DL-sulfoximine was purchased from Sigma. Synthetic β-N-oxalyl-L-α,β-diamino-propionic acid (12) was a generous gift from Dr. S.L.N. Rao, Hindustan Lever Ltd, Bombay, India.

RESULTS AND DISCUSSION

MSX and ODAP are structural analogs of glutamate and glutamine :



MSX **ODAP**

They both cause derepression of nitrogenase biosynthesis in the presence of ammonia (Figure 1A, Table 1), but their respective effects show several differences : First, MSX derepresses nitrogenase synthesis at much lower concentrations (10 μM) than ODAP (5 mM). Second, above the threshold concentration, the effect of ODAP remains unchanged, whereas increasing the concentration of MSX results in an apparent decrease in nitrogenase biosynthesis (Table 1). As will be shown below (Table 2), this is most likely due to an indirect inhibitory effect of MSX on nitrogenase activity. Third, during derepression in the presence of ODAP, nitrogenase activity increases continuously over a period of 50 hours, whereas in the presence of MSX the activity reaches a maximum after ca. 25 hours and decreases afterwards (Figure 1A). Fourth, MSX markedly inhibits NH_4^+ assimilation, whereas in the presence of ODAP NH_4^+ is taken up as rapidly as in the control flask (Figure 1B). It should be emphasized that after 20 hours of

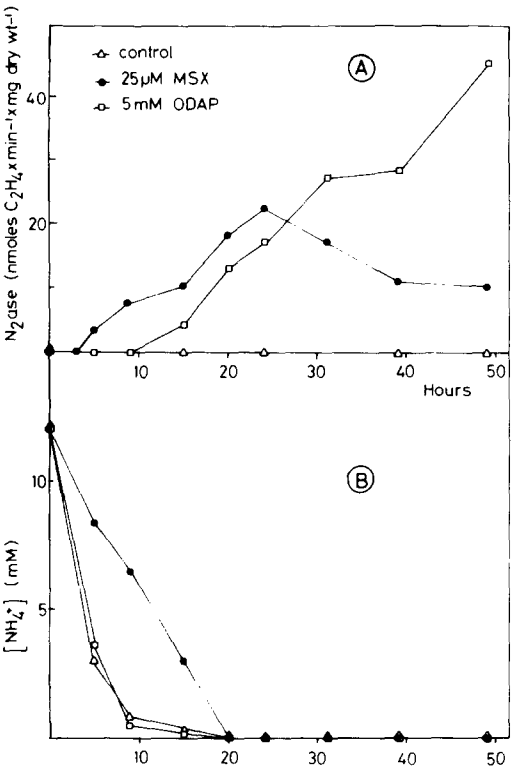


Figure 1. Nitrogenase derepression and ammonia uptake by cells of *R. capsulata* in the presence of optimal concentrations of MSX or ODAP.

A culture growing on lactate-ammonium was taken in mid-exponential phase and split in three 30 ml fractions. These were incubated in 100 ml erlenmeyer flasks (see Methods) gassed with argon. The inhibitors were added at time zero, and 2 ml samples were withdrawn as indicated for nitrogenase assay, dry weight and ammonia determinations.
A. Nitrogenase activity. B. Ammonia concentration.
(Δ) Control, (●) 25 μM MSX, (□) 5 mM ODAP. In the control flasks the inhibitors were either absent or present together with chloramphenicol (40 μg/ml).

incubation the concentration of NH_4^+ in the cell suspensions, although low (10-100 μM), is sufficient to repress nitrogenase biosynthesis in the control flask during at least 50 hours. Occasionally, cultures grown on lactate-ammonium develop some nitrogenase activity after 60-70 hours.

The fact that ODAP does not inhibit NH_4^+ assimilation (Figure 1B) would seem to suggest that this compound does not inhibit glutamine synthetase, since *R. capsulata* assimilates NH_4^+ mainly by the glutamine synthetase-glutamate synthase pathway (13). Therefore we have investigated

Table 1

Derepression of nitrogenase synthesis in *R. capsulata* by MSX and by ODAP

Inhibitor	Concentration (mM)	Nitrogenase activity (nmoles C_2H_4/min x mg dry weight)
Control	-	0
MSX	0.003	0
	0.01	25
	0.05	25
	0.62	18
	5	12
	50	3
ODAP	2	0
	5	45
	50	50

Conditions as described in Materials and Methods. 1 ml aliquots were drawn from the incubation flasks for the assay of nitrogenase. In the presence of MSX, nitrogenase activity was measured after 25 hours of incubation, when it reached a maximum (see Figure 1). In the presence of ODAP, nitrogenase activity was measured after 50 hours of incubation.

Table 2

Effects of ammonia, MSX and ODAP on nitrogenase activity in resting cells of *R. capsulata* and *C. pasteurianum*

Inhibitor	Concentration (mM)	Inhibition (%) of the nitrogenase activity in resting cells of:	
		R. capsulata	*C. pasteurianum*
NH_4^+	1	95	n.t.*
	10	95	0
MSX	0.01	44	n.t.
	0.05	60	n.t.
	0.5	81	n.t.
	5	95	n.t.
	10	n.t.	0
ODAP	1	9	n.t.
	10	12	n.t.
	50	33	0

*n.t. : non tested

Resting cells of *R. capsulata* (2.2 mg dry weight in 1 ml final volume) were suspended in a mineral medium (9) supplemented with DL-lactate (30 mM). Resting cells of *C. pasteurianum* (2.55 mg dry weight in 1 ml final volume) were suspended in phosphate buffer (50 mM, pH 6.8) supplemented with sucrose (2 %). The cells were preincubated for 5 minutes in the presence of the inhibitors in 4 ml flasks stoppered with rubber septa and gassed with argon. Acetylene (0.3 ml) was then injected, and ethylene production measured as described (9).

the effects of MSX and ODAP on glutamine synthetase activity in soluble extracts of *R. capsulata* (Figure 2). We found that MSX is a powerful inhibitor of glutamine synthetase, thus confirming results previously

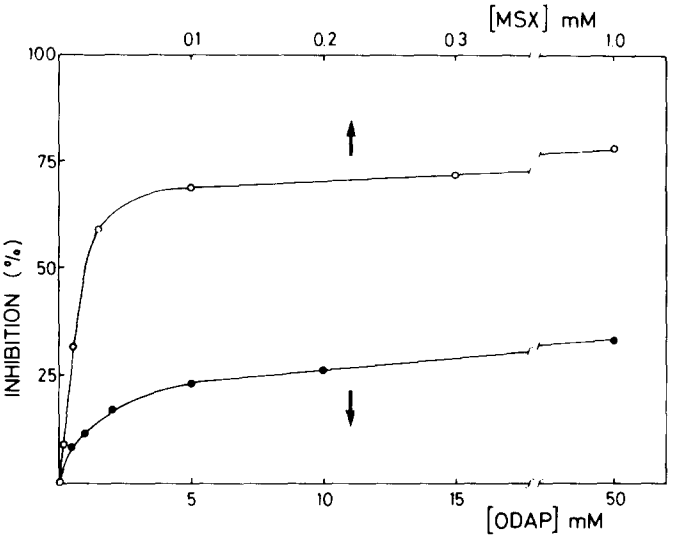


Figure 2. Inhibition of glutamine synthetase by MSX and ODAP in cell free extracts of *R. capsulata*.

Cell free extracts (2.5 mg protein per assay) from cells grown on lactate-glutamate were preincubated in the presence of the inhibitors for 5 minutes at 35°C ; the glutamine synthetase reaction medium was then added and the assay carried out as described by Wellner and Meister (11). No magnesium was added to the assay medium, therefore the total glutamine synthetase activity was measured here (adenylylated + deadenylylated) (○) MSX, (●) ODAP.

obtained with enzymes from other origins (14). ODAP also inhibits glutamine synthetase, although to a lesser extent and at much higher concentrations than MSX. This is, to our knowledge, the first time that ODAP is reported to be an inhibitor of glutamine synthetase. This compound was known to inhibit several glutamate-linked functions with a broad specificity at millimolar concentrations (see 17, 18). It is of note that for both MSX and ODAP half maximum inhibition of glutamine synthetase and derepression of nitrogenase synthesis occur at similar concentrations : These are 15 µM and 10 µM, respectively, for MSX ; 2 mM and 5 mM, respectively, for ODAP. Such quantitative correlations between the abilities to inhibit glutamine synthetase on one hand, and to derepress nitrogenase biosynthesis on the other, provide further evidence that glutamine synthetase might play a role in the regulation of nitrogenase biosynthesis.

In the course of this investigation we have evidenced an additional and previously unreported effect of MSX and ODAP on nitrogenase function : They inhibit nitrogenase activity *in vivo*. Indeed, upon addition of these inhibitors to resting cells of *R. capsulata* containing nitrogenase (e.g. cells

grown on glutamate as a nitrogen source), one observes an inhibition of nitrogenase activity (Table 2). Nitrogenase activity in cell free extracts is not inhibited by MSX nor by ODAP (not shown), therefore inhibition of nitrogenase activity *in vivo* by these compounds is presumably not due to their interacting with nitrogenase. From the following lines of evidence we suggest that inhibition of nitrogenase activity *in vivo* by MSX and ODAP results from glutamine synthetase inhibition and subsequent accumulation of NH_4^+ : First, nitrogenase activity *in vivo* is inhibited by the same concentrations of MSX and ODAP as those that inhibit glutamine synthetase (Table 2 and Figure 2). Second, we have measured large increases of the NH_4^+ level in resting cell suspensions of *R. capsulata* upon addition of MSX (in the presence of 10 µM MSX, the concentration of NH_4^+ increases from less than 1 µM up to 27 µM in 10 minutes), and we had previously reported that NH_4^+ inhibits *in vivo* nitrogenase activity in this organism (15). Third, nitrogenase activity *in vivo* in *C. pasteurianum*, which is known to be insensitive to NH_4^+ (16), is not inhibited by MSX nor by ODAP (Table 2). The strong inhibitory effect of MSX on nitrogenase activity *in vivo* provides a likely explanation for the observation that MSX, at concentrations higher than ca. 0.1 mM causes an apparent decrease of the derepression of nitrogenase biosynthesis (Table 1) : Inhibition of glutamine synthetase by MSX causes an increase in the NH_4^+ concentration which in turn causes a decrease in nitrogenase activity. As ODAP is a much weaker inhibitor of glutamine synthetase than MSX, it causes less NH_4^+ to be produced (5-10 µM in the presence of 10 mM ODAP) and therefore derepressed levels of nitrogenase activity remain unchanged over a wide range of ODAP concentrations (Table 1).

For practical purposes (e.g. photoproduction of NH_4^+, see 4), the high specificity and efficiency of MSX would seem to favor its usage as a derepressor of nitrogenase biosynthesis. According to our data, however, ODAP appears to be more advantageous than MSX for several reasons : It does not significantly inhibit nitrogenase activity *in vivo*, it derepresses nitrogenase biosynthesis over long periods of time, and eventually the derepressed levels of nitrogenase are higher in the presence of ODAP than in the presence of MSX.

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