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Repression of Nitrate Reductase in *Neurospora* Studied by Using L-Methionine-DL-Sulfoximine and Glutamine Auxotroph gln-1b

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The effect of L-methionine-DL-sulfoximine, an inhibitor of glutamine synthetase, on the formation of nitrate reductase in the wild-type strain of *Neurospora* in the presence of ammonium ions and of glutamine was studied. Under conditions in which glutamine synthetase was inactivated, it was found that only glutamine could repress nitrate reductase. In a mutant of *Neurospora*, gln-1b, which requires glutamine for growth, only glutamine could repress nitrate reductase. These results suggest a direct role for glutamine as corepressor of nitrate reductase in *Neurospora*.

Nitrate reductase from *Neurospora crassa*, the first enzyme in the assimilation of nitrate, is induced by nitrate (12, 16, 25) and repressed by ammonium (12, 25, 29) by what seems to be a form of nitrogen metabolite repression (1, 20). The mechanism of "ammonium repression" of nitrate reductase in *Neurospora* has been the subject of several recent investigations (6, 9, 19). Dantzig et al. (6) found that in a *N. crassa* mutant lacking NADP-glutamate dehydrogenase, ammonium was unable to repress the formation of nitrate reductase, although certain amino acids could still repress the enzyme. This observation led the authors to suggest that ammonium repression was, in fact, mediated by a nitrogen metabolite(s) other than ammonium ions, an idea that is not new (3, 5, 8). Studies in our laboratory with a glutamine auxotroph of *N. crassa*, identified glutamine (or a close metabolite of glutamine) as a corepressor of nitrate reductase (19). Dunn-Coleman et al. (9) confirmed our results by a similar approach. They hypothesized that octameric glutamine synthetase, the predominant form of the enzyme under conditions of nitrogen sufficiency (9, 18), is the putative repressor.

In the present study, the situation obtained in the glutamine auxotroph, namely the inability to convert ammonia to glutamine, is simulated in the wild-type strain by the use of L-methionine-DL-sulfoximine (MSX). This drug, long known to be an inhibitor of glutamine synthetase (17), is phosphorylated on the enzyme to form methionine sulfoximine phosphate, which binds tightly to the active site (13, 21-23). The data indicate that MSX inhibits the synthetase by acting as an analog of the tetrahedral intermediate or the transition state (15, 23, 30). In this study we attempt to demonstrate that when glutamine synthetase is inactivated in the above fashion, ammonium ions do not repress nitrate reductase in *Neurospora*, whereas glutamine still does repress the reductase. We also propose to demonstrate that in glutamine auxotropic *Neurospora* strain gln-1b glutamine represses the reductase.

MATERIALS AND METHODS

***Neurospora* strains.** All strains except glutamine auxotroph gln-1b came from the fungal genetics stock center at Humboldt State University Foundation, Arcata, Calif. Strain 3-1a (FGSC 935) was used as the wild-type strain. The mutant strain used was kindly provided by R. H. Garrett, University of Virginia, Charlottesville, Va. This mutant recently has been shown to have an impaired glutamine synthetase (7).

Culture conditions. The basic medium without nitrogen source has been described (28) and contained sucrose (20 g/liter) and macro- and micro-elements. One of the following nitrogen sources was included in the medium: ammonium tartrate, 4 g/liter (ammonia medium); glutamine, 2 g/liter (glutamine medium); sodium glutamate, 2 g/liter (glutamate medium); or sodium nitrate, 20 mM (induction medium). The wild-type strain was grown on ammonia medium or glutamine medium as required. Strain gln-1b was grown on glutamine medium. The culture conditions were as described previously (31). When the induction of nitrate was being studied, mycelial pads were grown from a conidial inoculum for 39 h at 27°C in standing culture, washed, subsequently transferred to induction medium (three pads per 30 ml of medium) supplemented or not with the test compound of interest at the required concentration, and then incubated with shaking for the desired time at 27°C. The mycelia were collected, washed with distilled water, blotted dry between paper towels, and either extracted immediately or stored frozen in liquid nitrogen. When the effect of MSX on the decay of nitrate reductase

was studied, the mycelia were grown for 39 h in stationary culture at 27°C, subsequently transferred, and incubated in induction medium with shaking for 3.5 h to permit induction of nitrate reductase. The pads were then washed and transferred to induction medium supplemented with MSX (1 mM) plus 6 mM sodium tungstate (decay medium) and shaken for 3.5 h at 27°C to permit decay of the active nitrate reductase in the absence of the synthesis of active enzyme. The mycelial pads were then harvested, washed with distilled water, blotted dry between paper towels, and extracted immediately or frozen and stored in liquid nitrogen.

Extraction. Mycelial pads were extracted by grinding with an equal weight of silica and 0.1 M phosphate buffer (pH 7.0) containing 1 mM disodium EDTA (2 ml of buffer for every three pads) in an ice-cold mortar. The resulting brei was centrifuged at 12,000 × *g* for 15 min in a Sorvall refrigerated centrifuge. The supernatant was used for enzyme assays.

Enzyme assays. (i) Nitrate reductase. NADPH-nitrate reductase (EC 1.6.6.2) was assayed as described previously (19). One unit of nitrate reductase activity is defined as the production of 1 nmol of nitrite per min at 30°C; specific activity is given in terms of units of activity per milligram of protein.

(ii) Glutamine synthetase. Glutamine synthetase (EC 6.3.1.2) was measured by its transferase activity as described by Ferguson and Sims (10).

Determination of protein. Protein concentrations were determined by using biuret reagent (11) and crystalline bovine serum albumin as standard. In some cases protein concentrations during elution of partially purified preparations from a column were followed by measuring the absorbance at 235 nm.

Determination of ammonia. Ammonia was determined by the Conway microdiffusion technique (4) with Nessler reagent (Paragon, New York, New York) for developing the color and comparing the test color with that of a standard curve.

Materials. NADPH, FAD, bovine serum albumin, ATP, L-methionine-DL-sulfoximine, and Sephadex G-25 and G-200 were from Sigma Chemical Co. Bio-Gel A-1.5m was purchased from Bio-Rad. All other chemicals were of reagent grade and were purchased locally.

RESULTS

The effect of ammonium and glutamine on the rate of formation of nitrate reductase in *Neurospora* in the presence and absence of MSX was studied (Fig. 1). The presence of ammonium ions and glutamine in the induction medium repressed nitrate reductase induction as expected (Fig. 1B). The presence of MSX (1 mM) during the induction decreased the maximum attained specific activity of the reductase by about 20% but had no noticeable effect on the kinetics of induction of this enzyme (Fig. 1B). The reason for the above effect of MSX might be that its presence lowers the quantity of glutamine available for protein synthesis by inhibiting glutamine synthetase. Methionine sulfoximine prevented the repression of nitrate reductase by ammonium ions, but not by glutamine (Fig. 1B). Glutamine synthetase was inactivated in mycelia in vivo by MSX under all the above conditions (Fig. 1A). The foregoing suggests that ammonium ions per se do not repress nitrate reductase and that they must be first converted to glutamine to do so. If MSX prevented the repression of nitrate reductase by ammonium because it prevented the conversion of ammonium to glutamine, the ammonium ions should accumulate intracellularly in the presence of MSX. Such is, in fact, the case in *Neurospora* mycelia (Table 1).

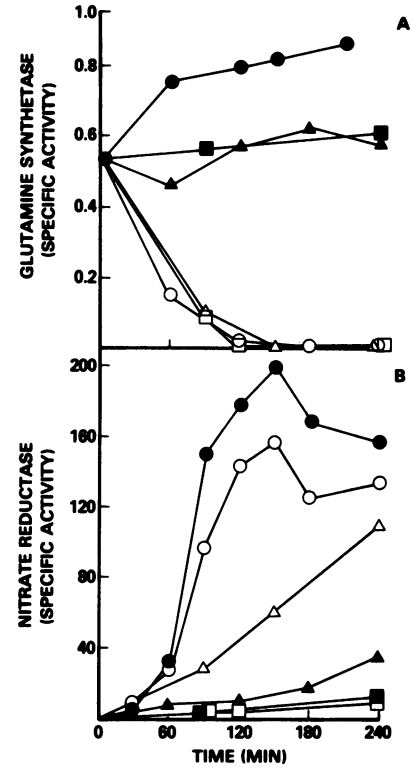

FIG. 1. *Effect of ammonium ions and glutamine on the induction of nitrate reductase in the presence and absence of MSX. The wild-type strain of Neurospora was grown on ammonia medium for 39 h at 27°C as described in the text. After they were washed, the pads were transferred to either induction medium (3 pads/30 ml of medium)* (●), *induction medium containing 1 mM MSX* (○), *induction medium containing 5 mM ammonium tartrate* (▲), *induction medium containing 5 mM ammonium tartrate and 1 mM MSX* (△), *induction medium containing 5 mM glutamine* (■), *or induction medium containing 5 mM glutamine and 1 mM MSX* (□). *These samples were shaken for the indicated time intervals. The pads were harvested, and extracts were made and assayed for nitrate reductase and glutamine synthetase activities as described in Materials and Methods.*

TABLE 1. *Intracellular concentration of ammonia in Neurospora mycelia exposed to induction medium plus or minus MSX*[a]

Addition to induction medium	Ammonia formed (nmol/mg of protein from extract)
None	551
MSX (1 mM)	1,117

[a] Wild-type strain of *Neurospora* was grown in ammonia medium for 39 h as described in Materials and Methods. The mycelial pads were washed and incubated with shaking in basal medium (six pads in 60 ml) containing MSX (1 mM) for 60 min at 27°C. The pads were then washed and incubated in induction medium (three pads in 30 ml) plus or minus MSX (1 mM), with shaking, for 4 h. Mycelial pads were harvested, washed thoroughly with distilled water, and extracted as described in the text. Portions of 1 ml each of the extracts were used for ammonia determinations by the Conway microdiffusion technique.

Nitrate reductase activity was not affected by MSX (1 mM) in vitro (results not shown), suggesting that the apparent effect of MSX on repression of the reductase by ammonium ions is not due to an in vitro artifact, such as an activation of the reductase.

The growth of wild-type mycelia on nitrate, ammonium, or glutamate was inhibited extensively by MSX (1 mM) (Table 2). Growth on glutamine was affected relatively little by MSX (Table 2). The relative ineffectiveness of MSX in inhibiting growth on glutamine is apparently not due to the prevention of MSX uptake by the amino acid, as has been reported in *C. vulgaris* (14) and *K. aerogenes* (2), because glutamine synthetase was inactivated in vivo (Fig. 1A) under the above conditions, indicating that MSX did penetrate the mycelium even in the presence of glutamine.

The apparent inhibition of repression of nitrate reductase by ammonium ions by MSX could conceivably be due to an effect of MSX on the stability of the reductase in vivo or in vitro. If MSX stabilized nitrate reductase, it could lead to the impression that the reductase was being induced. Our results show that MSX had no noticeable effect on the stability of nitrate reductase in vivo (Table 3) or in vitro (Fig. 2). To study the decay of the reductase, the mycelia were induced as described in Materials and Methods and subsequently transferred to decay medium containing sodium tungstate, so that any nitrate reductase synthesized de novo during incubation in the decay medium would be inactive (27). The rate of inactivation of the reductase in vivo was the same in the presence and absence of MSX (Table 3). Nitrate reductase extracted from mycelia induced in the presence or absence of MSX had the same heat inactivation kinetics in vitro at 30°C (Fig. 2).

We previously showed that nitrate reductase is repressed by glutamine, but not by ammonium, in glutamine synthetase-defective strain gln-1a (19). We have now confirmed this observation with another glutamine synthetase-defec-

TABLE 2. *Effect of MSX on growth of wild-type Neurospora*[a]

Growth medium	Wt of mycelium (g)[b]	Growth inhibition by MSX (%)
Ammonia medium (5 mM)	0.0143	
Ammonia medium (5 mM) + MSX (1 mM)	0.0039	73
Glutamine medium (5 mM)	0.0162	
Glutamine medium (5 mM) + MSX (1 mM)	0.0146	10
Glutamate medium (5 mM)	0.0074	
Glutamate medium (5 mM) + MSX (1 mM)	0.0026	65
Glutamate (5 mM) + ammonia (5 mM) medium	0.0216	
Glutamate (5 mM) + ammonia (5 mM) medium + MSX (1 mM)	0.0068	69
Induction medium	0.0087	
Induction medium + MSX (1 mM)	0.0003	97

[a] Wild-type strain of *Neurospora* was grown for 39 h in the medium indicated as described in Methods. Concentrations of nitrogen source are given in parentheses. The resulting pads (three in each case) were washed, pressed between folds of filter paper, placed in aluminum foil, dried overnight at 200°F (92°C), and then weighed.

[b] The values given are the average weight of one mycelial pad and are representative of three measurements.

TABLE 3. *Effect of MSX on the decay of nitrate reductase in vivo*[a]

Addition to decay medium	Nitrate reductase sp act remaining (% control)[b]
None	9.5
MSX (1 mM)	9.1
Ammonia (5 mM)	8.6
Ammonia (5 mM) + MSX (1 mM)	8.7

[a] *Neurospora* strains were grown on ammonia medium as described in Materials and Methods. The pads were washed and incubated in induction medium with shaking at 27°C for 3.5 h; they were finally incubated in decay medium supplemented with the additions noted above for 3.5 h, as described in the text.

[b] The specific activity of nitrate reductase in extracts of mycelia incubated for 0 h in the decay medium was 93 U/mg of protein and designated as 100%. The specific activity remaining in the preparation of mycelia harvested and extracted at the end of incubation in decay medium is expressed as a percentage of this control activity. The results given are representative of three experiments.

tive strain, gln 1b (Table 4), in which the oligomeric structure of glutamine synthetase is affected in a different manner (present predominantly in dimeric form) from that in strain gln 1a (present predominantly in tetrameric form) (7). Strain gln-1b is slightly leaky, hence glutamate and glutamate plus ammonia do repress nitrate reductase to a certain extent. This extent is less than that in the wild-type strain.

DISCUSSION

The results described confirm our earlier report (19), suggesting that glutamine is the main corepressor of nitrate reductase in *N. crassa*. In the wild-type strain, when glutamine synthetase was inactivated by MSX (a specific inhibitor of the synthetase), ammonium ions did not repress nitrate reductase (Fig. 1). Under the same conditions, however, glutamine did repress the formation of the reductase (Fig. 1). The lack of repression by ammonium ions in the presence of MSX was not due to an effect of MSX on the rate of decay of nitrate reductase in vivo (Table 3). Repression by glutamine in the presence of MSX was not due to cellular exclusion of MSX by glutamine, because glutamine synthetase was inactivated in vivo when glutamine and MSX were present in the culture under the above conditions (Fig. 1).

Treatment of mycelia with MSX did not affect the kinetics of decay of nitrate reductase in vitro (Fig. 2), indicating that MSX causes no major structural change in the nitrate reductase molecule itself.

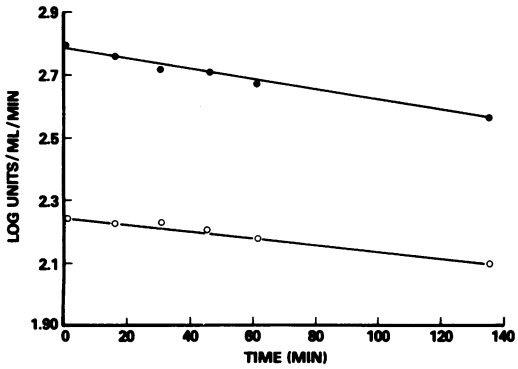

FIG. 2. *Heat inactivation of nitrate reductase from mycelia exposed to induction medium plus or minus MSX. Wild-type Neurospora strains were grown on ammonia medium as described in Materials and Methods, mycelia were subsequently transferred to induction medium plus* (○) *or minus* (●) *1 mM MSX and shaken for 2.5 h at 27°C. Pads were harvested, and extracts were prepared as described in the text. One ml of each extract was incubated at 30°C, and fractions were taken out at desired time intervals and assayed for nitrate reductase activity.*

TABLE 4. *Effect of ammonia and glutamine on formation of nitrate reductase in the wild-type strain and glutamine-requiring mutant, gln-1b, of Neurospora*[a]

Addition to induction medium	Nitrate reductase sp act (% of fully induced control)[b]	
	Wild type	gln-1b
None	100	100
Ammonia (20 mM)	15	74
Glutamine (20 mM)	1	1
Glutamate (20 mM)	24	32
Glutamate (20 mM) + ammonia (20 mM)	1	25

[a] The wild-type strain and mutant gln lb were grown on glutamine medium for 39 h at 27°C, transferred to induction medium plus or minus the test compound at the indicated concentration, and shaken for 3.5 h at 27°C. Extracts from the mycelia were then prepared and assayed for nitrate reductase all as described in Materials and Methods.

[b] Specific activities of nitrate reductase in control cell-free preparations of wild type and gln-lb were 65 and 68, respectively, and each was designated as 100%. Specific activities of preparations from mycelia induced in the presence of the test compounds in the induction medium were expressed relative to these control values. The results given are representative of three different experiments.

Methionine sulfoximine has been reported to cause conformational changes in the glutamine synthetase molecule (30, 32). These changes are not believed to lead to a change in the oligomeric structure of the enzyme.

Glutamine synthetase in mutant gln-1b has been reported to be present either in a tetrameric form or a dimeric form but never in an octameric form (7). Even in this mutant, where the octameric form of the enzyme is reported to be absent (7), glutamine repressed the reductase (Table 4). This also indicates that glutamine per se is involved in repression of nitrate reductase and suggests that the octameric form of glutamine synthetase may not be required for repression.

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