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Regulation of assimilatory nitrate reductase activity in soil by microbial assimilation of ammonium

(L-methionine sulfoximine/glutamine/asparagine)

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ABSTRACT **It is well established that assimilatory nitrate reductase (ANR) activity in soil is inhibited by ammonium (NH_4^+). To elucidate the mechanism of this inhibition, we studied the effect of L-methionine sulfoximine (MSX), an inhibitor of NH_4^+ assimilation by microorganisms, on assimilatory reduction of nitrate (NO_3^-) in aerated soil slurries treated with NH_4^+. We found that NH_4^+ strongly inhibited ANR activity in these slurries and that MSX eliminated this inhibition. We also found that MSX induced dissimilatory reduction of NO_3^- to NH_4^+ in soil and that the NH_4^+ thus formed had no effect on the rate of NO_3^- reduction. We concluded from these observations that the inhibition of ANR activity by NH_4^+ is due not to NH_4^+ *per se* but to products formed by microbial assimilation of NH_4^+. This conclusion was supported by a study of the effects of early products of NH_4^+ assimilation (L amino acids) on ANR activity in soil, because this study showed that the biologically active, L isomers of glutamine and asparagine strongly inhibited ANR activity, whereas the D isomers of these amino acids had little effect on ANR activity. Evidence that ANR activity is regulated by the glutamine formed by NH_4^+ assimilation was provided by studies showing that inhibitors of glutamine metabolism (azaserine, albizziin, and aminooxyacetate) inhibited ANR activity in soil treated with NO_3^- but did not do so in the presence of MSX.**

There is international concern about the potential adverse effects of fertilizer-derived nitrate (NO_3^-) on environmental quality and public health (1–4). This concern has stimulated research on the biological and nonbiological processes that affect the fate of NO_3^- in soils, and it is well established that NO_3^- is the substrate of at least three microbial processes in soils: denitrification, assimilatory NO_3^- reduction, and dissimilatory NO_3^- reduction. Denitrification leads to reduction of NO_3^- to dinitrogen (N_2) and nitrous oxide (N_2O), whereas both assimilatory and dissimilatory NO_3^- reduction lead to conversion of NO_3^- to NH_4^+. Dissimilatory NO_3^- reduction is not affected by NH_4^+ (5), whereas assimilatory NO_3^- reduction is strongly inhibited by NH_4^+ (6).

It is generally assumed that assimilatory NO_3^- reduction is a minor fate of NO_3^- in soil compared with plant uptake, leaching, and denitrification (7–9). This assumption is based largely on studies showing that NH_4^+ is a potent inhibitor of NO_3^- assimilation by soil microorganisms and that NH_4^+ concentrations in agricultural soils are usually greater than those required to inhibit assimilatory NO_3^- reductase (ANR) activity (9–11). Very little information is available, however, concerning assimilatory reduction of NO_3^- in soil and the factors affecting this process. The purpose of the work reported here was to elucidate the mechanism of inhibition of ANR in soil by NH_4^+.

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MATERIALS AND METHODS

The soils used (Table 1) were surface samples (0–15 cm) of Iowa soils that had been sieved (2-mm screen) and stored (4°C) in the field-moist condition. Immediately before use in studies of assimilatory reduction of NO_3^- by soil microorganisms, subsamples of each soil were preincubated at 30°C for 16 hr after treatment with glucose (2.5 mg of carbon per gram of soil) to stimulate microbial activity and assimilation of preexisting NH_4^+ and NO_3^-. They were then treated with KNO_3 (60 μg of nitrogen per gram of soil) and glucose (500 μg of carbon per gram of soil) and shaken with water (3 ml/g of soil) to obtain slurries for the experiments reported.

To continuously monitor the effects of different treatments on ANR activity in soil by use of NO_3^- electrodes, slurries containing 10 g (dry weight) of soil were treated with 14 mg of K_2SO_4 (added to adjust ionic strength), placed on magnetic stirrers in a room maintained at 30°C, and aerated by bubbling with a stream of air. A NO_3^- electrode (Orion model 93-07) coupled with a double-junction reference electrode (Orion model 90-02) containing 0.4 M K_2SO_4 outer filling solution was inserted into each slurry, and the millivolt output was continuously charted by a pen recorder connected to an ion-selective electrode meter. At different times during incubation, soil slurries were treated with one or more of the following amendments: 1.2 mg of nitrogen as $(NH_4)_2SO_4$, 10 μmol of L-methionine sulfoximine (MSX), 100 μmol of D- or L-glutamine or D- or L-asparagine, 10 μmol of azaserine, 45 μmol of aminooxyacetate, and 68 μmol of albizziin.

To study the effect of MSX on NO_3^- reduction and NH_4^+ production in soil, slurries containing 40 g (dry weight) of soil, 2.4 mg of nitrogen as KNO_3, and 20 mg of carbon as glucose were treated with 0 or 40 μmol of MSX and shaken at 30°C. Aliquots (5 ml) of slurry were taken at 40-min intervals, mixed with 5 ml of 4 M KCl, filtered through Whatman no. 5 filter paper, and analyzed colorimetrically for NH_4^+ and NO_3^- by flow injection analysis (Lachat Instruments, Milwaukee). The same experiments were performed using KNO_3 containing 1.0 atom % ^{15}N to confirm that the NH_4^+ produced by incubation of the soil slurries was derived from the NO_3^- added. In these tracer experiments, aliquots of the filtrates used for NH_4^+ and NO_3^- analysis were analyzed as described by Bremner (12) to determine the N-isotope ratios of these forms of nitrogen.

MSX, azaserine, albizziin, aminooxyacetate, and D and L isomers of glutamine and asparagine were obtained from Sigma. The other chemicals used were obtained from Fisher Scientific.

RESULTS AND DISCUSSION

To investigate the role of NH_4^+ assimilation in regulation of ANR activity in soil, we studied the effect of MSX, an

Abbreviations: ANR, assimilatory nitrate reductase; MSX, L-methionine sulfoximine.

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Table 1. Properties of soils used

Soil			Organic carbon,	Total nitrogen,	Sand,	Clay,	CCE,†
Series	Subgroup*	pH	%	%	%	%	%
Harps	TC	7.9	4.2	0.50	9	43	41
Okoboji	CH	7.1	2.8	0.22	19	34	0

*TC, Typic Calciaquoll; CH, Cumulic Haplaquoll.
†CCE, $CaCO_3$ equivalent.

inhibitor of NH_4^+ assimilation by microorganisms, on ANR activity in aerated soils treated with NH_4^+. We found that MSX eliminated the inhibitory effect of NH_4^+ on ANR activity in the soils studied and that its influence on this inhibition was not dependent on the order of addition of NH_4^+ and MSX (Fig. 1). We also found that MSX induced dissimilatory reduction of NO_3^- to NH_4^+ in aerated soils treated with NO_3^- and that the NH_4^+ thus formed did not affect the rate of NO_3^- reduction (Fig. 2). Studies using ^{15}N-labeled NO_3^- showed that most (>90%) of the NH_4^+ formed in these experiments was produced from NO_3^- by ANR activity and not by ammonification of organic nitrogen. These observations indicate that dissimilatory reduction of NO_3^- to NH_4^+ in soil can occur via ANR activity under conditions that inhibit assimilation of NH_4^+.

The experiments reported in Figs. 1 and 2 indicate that the observed inhibition of ANR activity in soil by NH_4^+ was due not to NH_4^+ *per se* but to products formed by microbial assimilation of NH_4^+. This conclusion was supported by a study of the effects of glutamine and asparagine (early products of NH_4^+ assimilation) on ANR activity in soil, because this study showed that the biologically active, L isomers of these amino acids markedly inhibited ANR activity, whereas the D isomers had little effect on ANR activity (Table 2).

Previous workers (9) concluded that NH_4^+ inhibition of ANR activity in soil was not dependent on assimilation of NH_4^+ by soil microorganisms. This conclusion was based largely on the observation that inhibition of ANR activity by NH_4^+ was considerably more rapid than the corresponding inhibition by amino acids known to be formed by assimilation

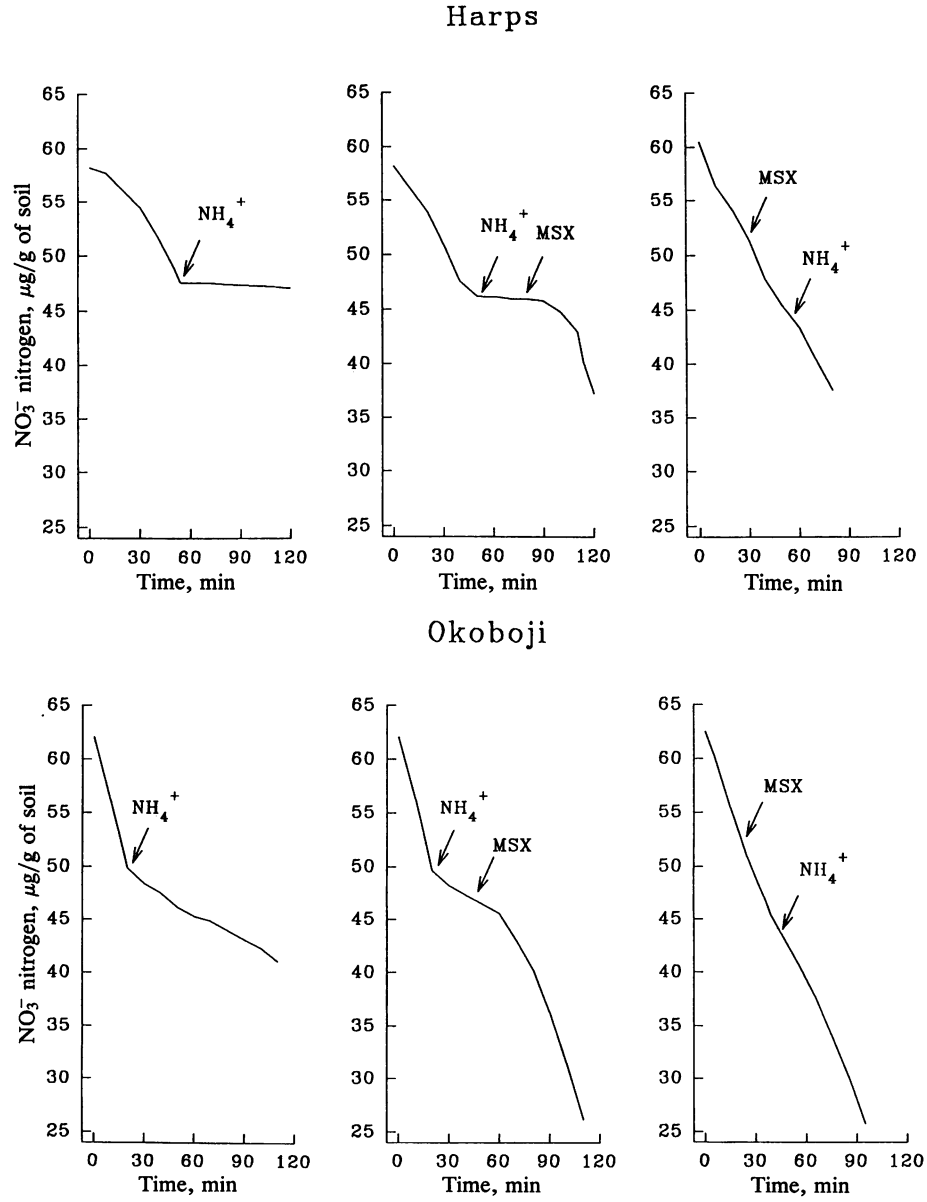

FIG. 1. Effect of MSX on NH_4^+ inhibition of ANR activity in Harps and Okoboji soil. Slurries containing 10 g (dry weight) of soil, 30 ml of water, 0.6 mg of KNO_3 nitrogen, 5 mg of glucose carbon, and 14 mg of K_2SO_4 were incubated at 30°C and treated with 1.2 mg of NH_4^+ nitrogen and 0 or 10 μmol of MSX at the times indicated. The NO_3^- contents of the slurries were monitored by NO_3^- electrodes.

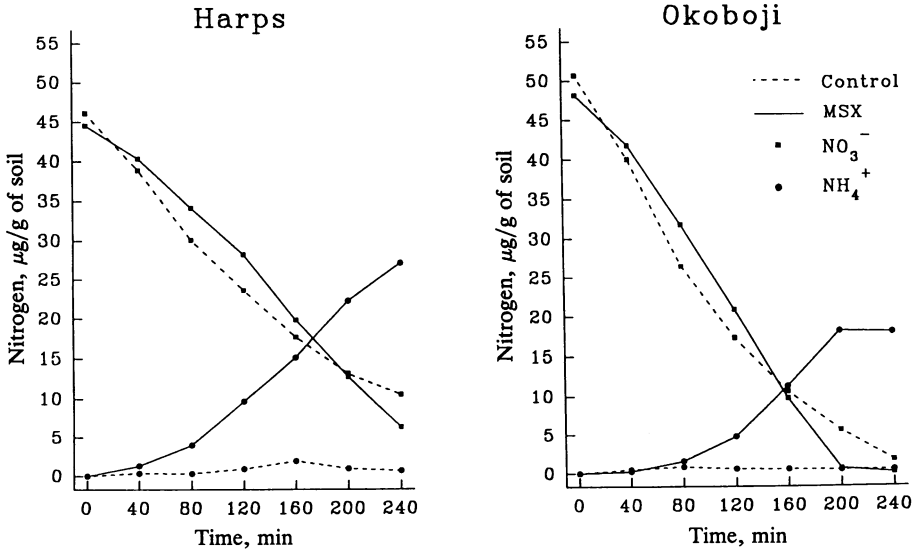

FIG. 2. Effect of MSX on NO_3^- reduction and NH_4^+ production in Harps and Okoboji soil. Slurries containing 40 g (dry weight) of soil, 120 ml of water, 2.4 mg of KNO_3 nitrogen, 20 mg of glucose carbon, and 0 or 40 μmol of MSX were incubated at 30°C, and aliquots of the slurries were taken at 40-min intervals and analyzed for NH_4^+ and NO_3^-.

of NH_4^+. This observation could be explained, however, by differences in the rates of movement of NH_4^+ and amino acids across cytoplasmic membranes. It is well established that NH_4^+ nitrogen in the form of NH_3 can diffuse rapidly across cytoplasmic membranes (13), whereas uptake of amino acids by microorganisms requires specific carriers for transport of amino acids across these membranes and can be considerably slower than uptake of NH_4^+ (14).

There is considerable evidence that microbial assimilation of NH_4^+ in nitrogen-limited environments is primarily due to the activity of glutamine synthetase (15) and that MSX, a specific inhibitor of this enzyme, blocks formation of glutamine in the microbial cytosome (16). Evidence that glutamine synthetase plays a key role in regulation of ANR activity in soil was provided by our finding that whereas MSX eliminated inhibition of ANR activity by NH_4^+, it did not eliminate inhibition of ANR activity by L-glutamine (Fig. 3). Further evidence that ANR activity in soil is regulated by glutamine formed by microbial assimilation of NH_4^+ was provided by studies showing that aminotransferase inhibitors that block metabolism of glutamine inhibited ANR activity in soil treated with NO_3^- but did not do so in the presence of MSX (Table 3).

Methylammonium ($CH_3NH_3^+$) labeled with ^{14}C has been used as an NH_4^+ analog to study the carriers involved in transport of NH_4^+ across cytoplasmic membranes, and experiments using this compound have shown that MSX inhibits its transport of $CH_3NH_3^+$ across these membranes (17–25). This has led several workers to postulate that MSX has a direct inhibitory effect on NH_4^+ transport (17–21), and it has been argued that the inhibition of $CH_3NH_3^+$ (and presumably NH_4^+) transport by MSX is due not to an inhibitory effect of MSX on glutamine synthetase but to the binding of MSX to a regulatory glutamine binding site on NH_4^+ carriers (13). This argument is open to criticism, however, because studies using $CH_3NH_3^+$ have indicated that NH_4^+ transport is tightly coupled to assimilation of NH_4^+ by glutamine synthetase activity (22–25). For example, Rai *et al.* (22) found that uptake of $CH_3NH_3^+$ by a cyanobacterium (*Anabaena variabilis*) was dependent on metabolism of $CH_3NH_3^+$ by glutamine synthetase with formation of the glutamine analog *N*-methylglutamine, and they concluded that uptake of NH_4^+ was similarly dependent on metabolism of NH_4^+ by glutamine synthetase.

Table 2. Effect of L and D isomers of glutamine and asparagine on ANR activity in soil

Amino acid added	% inhibition of ANR activity	
	Harps	Okoboji
L-Glutamine	70.7	75.1
D-Glutamine	4.0	3.9
L-Asparagine	92.0	81.5
D-Asparagine	8.9	5.7

Slurries containing 10 g (dry weight) of Harps or Okoboji soil, 30 ml of water, 0.6 mg of KNO_3 nitrogen, 5 mg of glucose carbon, and 14 mg of K_2SO_4 were incubated at 30°C for 120 min, and their ANR activities were monitored by NO_3^- electrodes. After the first 60 min of incubation, the slurries were treated with 0 or 100 μmol of the amino acid specified. The percent inhibition of ANR activity by the amino acids added was calculated from $[(A - B)/A] \times 100$, where A is the ANR activity before treatment and B is the corresponding activity after treatment.

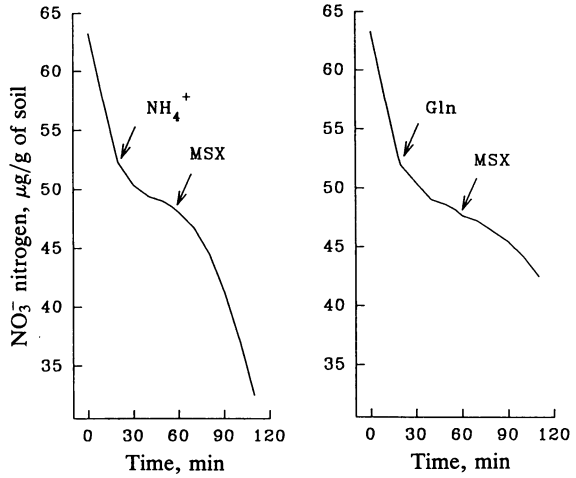

FIG. 3. Comparison of effects of NH_4^+ and L-glutamine (Gln) on ANR activity in Harps soil treated with MSX. Slurries containing 10 g (dry weight) of soil, 30 ml of water, 0.6 mg of KNO_3 nitrogen, 5 mg of glucose carbon, and 14 mg of K_2SO_4 were incubated at 30°C and treated with 10 μmol of MSX and with 1.2 mg of NH_4^+ nitrogen or 100 μmol of glutamine at the times indicated. The NO_3^- contents of the slurries were monitored by NO_3^- electrodes.

Table 3. Effect of aminotransferase inhibitors on ANR activity of soil in the presence or absence of MSX

Aminotransferase inhibitor	MSX	% inhibition of ANR activity
Azaserine	−	72
	+	6
Albizziin	−	46
	+	3
Aminooxyacetate	−	73
	+	4

Slurries containing 10 g (dry weight) of Harps soil, 30 ml of water, 0.6 mg of KNO_3 nitrogen, 5 mg of glucose carbon, and 14 mg of K_2SO_4 were incubated at 30°C for 120 min, and their ANR activities were monitored by NO_3^- electrodes. After the first 45 min of incubation, the slurries were treated with 0 or 10 μmol of MSX, and after the next 15 min of incubation, they were treated with 10 μmol of azaserine, 68 μmol of albizziin, or 45 μmol of aminooxyacetate. The percent inhibition of ANR activity by the aminotransferase inhibitor treatments was calculated as in Table 2.

To determine whether the postulated inhibitory effect of MSX on NH_4^+ transport could account for the ability of MSX to eliminate the inhibition of ANR activity by NH_4^+ observed in the experiments reported in Fig. 1, we repeated these experiments using slurries of Harps soil that had been amended with KOH to increase their pH to 8.8. We found that this increase in pH did not significantly affect the results obtained in these experiments. This indicates that the ability of MSX to eliminate inhibition of ANR activity by NH_4^+ is not related to an effect of MSX on NH_4^+ carriers, because the ratio of NH_3 to NH_4^+ at pH 8.8 is high enough to permit rapid uptake of NH_4^+ by diffusion. This conclusion is consistent with the results reported in Fig. 2 because they demonstrated that the NH_4^+ produced by ANR activity within the microbial cytosome did not inhibit ANR activity in the presence of MSX.

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1. Committee on Nitrate Accumulation (1972) *Accumulation of Nitrate* (Natl. Acad. Press, Washington), p. 106.
2. National Commission on Water Quality (1975) *Staff Report* (GPO, Washington), p. 909.
3. National Research Council (1978) *Nitrates: An Environmental Assessment* (Natl. Acad. Press, Washington), p. 723.
4. Royal Society Study Group (1983) *The Nitrogen Cycle of the United Kingdom* (The Royal Soc., London), p. 264.
5. Tiedje, J. M. (1988) in *Environmental Microbiology of Anaerobes*, ed. Zehnder, A. J. B. (Wiley, New York), pp. 179–244.
6. Sias, S. R. & Ingraham, J. L. (1979) *Arch. Microbiol.* **122,** 263–270.
7. Jones, J. M. & Richards, B. N. (1977) *Soil Biol. Biochem.* **9,** 383–392.
8. Tiedje, J. M., Sorensen, J. & Chang, L. (1981) in *Terrestial Nitrogen Cycles: Processes, Ecosystem Strategies and Management Impacts*, eds. Clark, F. E. & Rosswall, T. *Ecol. Bull. (Stockholm)* **33,** 331–342.
9. Rice, C. W. & Tiedje, J. M. (1989) *Soil Biol. Biochem.* **21,** 597–602.
10. Jansson, S. L. (1958) *Ann. R. Agric. Coll. Swed.* **24,** 101–361.
11. Recous, S. & Mary, B. (1990) *Soil Biol. Biochem.* **22,** 913–922.
12. Bremner, J. M. (1965) in *Methods of Soil Analysis*, ed. Black, C. A. (Am. Soc. Agron., Madison, WI), pp. 1256–1286.
13. Kleiner, D. (1985) *FEMS Microbiol. Rev.* **32,** 87–100.
14. Furlong, C. E. (1987) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington), pp. 768–796.
15. Reitzer, L. J. & Magasanik, B. (1987) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington), pp. 302–320.
16. Brenchley, J. E. (1973) *J. Bacteriol.* **114,** 666–673.
17. Kleiner, D. & Castorph, H. (1982) *FEBS Lett.* **146,** 201–203.
18. Kleiner, D., Alef, K. & Hartman, A. (1983) *FEBS Lett.* **164,** 121–123.
19. Turpin, D. H., Edie, S. A. & Canvin, D. T. (1984) *Plant Physiol.* **74,** 701–704.
20. Singh, D. T., Rai, A. N. & Singh, H. N. (1985) *FEBS Lett.* **186,** 51–53.
21. Singh, D. T., Modi, D. R. & Singh, H. N. (1986) *FEMS Microbiol. Lett.* **37,** 95–98.
22. Rai, A. N., Rowell, P. & Stewart, W. D. P. (1984) *Arch. Microbiol.* **137,** 241–246.
23. Boussiba, S. & Gibson, J. (1985) *FEBS Lett.* **180,** 13–16.
24. Sharak Genthner, B. R. & Wall, J. D. (1985) *Arch. Microbiol.* **141,** 219–224.
25. Kerby, N. W., Rowell, P. & Stewart, W. D. P. (1986) *Arch. Microbiol.* **143,** 353–358.