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Inhibition of assimilatory nitrate reductase activity in soil by glutamine and ammonium analogs

GREGORY W. MCCARTY AND JOHN M. BREMNER*

Department of Agronomy, Iowa State University, Ames, IA 50011-1010

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ABSTRACT **Recent work in our laboratory indicated that the inhibitory effect of ammonium (NH_4^+) on assimilatory nitrate reductase (ANR) activity in soil is not due to NH_4^+ *per se* but to glutamine formed by microbial assimilation of NH_4^+. To test this conclusion, we studied the effects of eight analogs of L-glutamine (L-glutamic acid γ-methyl ester, L-glutamic acid γ-hydrazide, L-glutamic acid γ-hydroxamate, L-glutamic acid γ-ethyl ester, L-glutamic acid dimethyl ester, L-asparagine, L-aspartic acid β-methyl ester, and L-aspartic acid β-hydroxamate) and two analogs of ammonium (hydroxylamine and methylamine) on ANR activity in soil slurries. The studies with the L-glutamine analogs showed that all except L-glutamic acid dimethyl ester inhibited ANR activity in soil. The sharp contrast observed between the strong inhibitory effect of L-glutamic acid γ-methyl ester on ANR activity and the complete lack of an inhibitory effect with the corresponding dimethyl ester suggests that only the free-acid form of glutamine effectively inhibits ANR activity. The studies with hydroxylamine and methylamine showed that both of these ammonium analogs inhibited ANR activity in soil and that this inhibition was dependent upon glutamine synthetase activity. This dependence indicates that inhibition of ANR activity by hydroxylamine and methylamine was due to formation of the glutamine analogs L-glutamic acid γ-hydroxamate and L-glutamic acid γ-methylamide, respectively. These observations support the conclusion that the inhibitory effect of NH_4^+ on ANR activity in soil is due to glutamine formed by microbial assimilation of NH_4^+.**

Most of the fertilizer nitrogen (N) applied to soils to increase crop production is converted to nitrate (NO_3^-) by the nitrifying microorganisms in soils, and there is international concern about the potentially adverse effects of this NO_3^- on environmental quality and public health (1, 2). Briefly, the concern is that intensive use of fertilizer N will lead to increased NO_3^- levels in ground and surface waters and that this, in turn, will lead to increased eutrophication of water resources and to health hazards to humans (particularly infants) through NO_3^- enrichment of drinking water. This concern has stimulated research on the fate of fertilizer-derived NO_3^- in soils, and it is well established that NO_3^- is the substrate for at least three microbial processes in soils; namely, assimilatory reduction of NO_3^- to ammonium (NH_4^+), dissimilatory reduction of NO_3^- to NH_4^+, and denitrification [dissimilatory reduction of NO_3^- to dinitrogen (N_2) and nitrous oxide (N_2O)].

It is generally assumed that most of the fertilizer-derived NO_3^- in soil disappears via plant uptake, leaching, and denitrification and that very little of this NO_3^- is reduced to NH_4^+ through assimilatory NO_3^- reductase (ANR) activity by soil microorganisms (3–5). This assumption is based largely on studies showing that, in contrast to dissimilatory reduction of NO_3^- to NH_4^+, assimilatory reduction of NO_3^- is strongly inhibited by NH_4^+ (6, 7) and that NH_4^+ concentrations in soils usually exceed those required to inhibit ANR activity (5, 8, 9). Very little is known, however, about factors affecting ANR activity in soils, and there is divergence in the literature concerning the mechanism of inhibition of ANR activity in soil by NH_4^+. Some workers have concluded that the inhibitory effect of NH_4^+ on ANR activity is due to NH_4^+ *per se* and is not dependent on assimilation of NH_4^+ by soil microorganisms (5), but we recently reported studies leading us to conclude that the inhibitory effect of NH_4^+ on ANR activity in soil is due to glutamine formed by microbial assimilation of NH_4^+ (10). The purpose of the work reported here was to validate this conclusion by determining the effects of glutamine and ammonium analogs on ANR activity in soils.

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.

The effects of different compounds on ANR activity in soil were studied by use of NO_3^- electrodes. In these studies, 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 charted by a pen recorder connected to an ion-selective electrode meter. The slurries were incubated at 30°C for 120 min, and their ANR activities were monitored continuously by NO_3^- electrodes during this time. After the first 60 min of incubation, the slurries were treated with different amounts of test compound. The percentage inhibition of ANR activity by the test compound was calculated from $(A - B)/A \times 100$, where A is the ANR activity before treatment with test compound and B is the corresponding activity after treatment. L-Methionine sulfoximine (MSX) (1 μmol/g of soil) was added to some slurries to inhibit glutamine synthetase activity (11).

MSX, methylamine hydrochloride, and the amino acids used were obtained from Sigma. The other chemicals were obtained from Fisher Scientific.

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

*To whom reprint requests should be addressed.

Table 1. Properties of soils used

Soil			Organic	Total	Sand,	Clay,	
Series	Subgroup	pH	C, %	N, %	%	%	CCE*
Harps	Typic Calciaquoll	7.9	4.2	0.50	9	43	41
Okoboji	Cumulic Haplaquoll	7.1	2.8	0.22	19	34	0

*$CaCO_3$ equivalent.

RESULTS AND DISCUSSION

Previous work in our laboratory (10) showed that MSX, an inhibitor of L-glutamine production via assimilation of NH_4^+ by soil microorganisms, eliminated the inhibition of ANR activity in soil by NH_4^+ and that azaserine, an inhibitor of L-glutamine metabolism by soil microorganisms, inhibited ANR activity in soil treated with NO_3^- but did not do so in the presence of MSX. We concluded from these results that ANR activity in soil is regulated by the L-glutamine formed by NH_4^+ assimilation (10).

To test this conclusion, we studied the effects of eight analogs of L-glutamine on ANR activity in soil. The results (Table 2) showed that all compounds tested, except L-glutamic acid dimethyl ester, inhibited ANR activity in soil slurries. The sharp contrast between the strong inhibitory effect of L-glutamic acid γ-methyl ester on ANR activity and the complete lack of an inhibitory effect with the corresponding dimethyl ester suggests that only the free-acid form of L-glutamine effectively inhibits ANR activity. Our finding that L-asparagine and other derivatives of L-aspartic acid strongly inhibited ANR activity (Table 2) indicates that these compounds are functional analogs of L-glutamine and inhibit ANR activity by binding regulatory sites designed to bind L-glutamine. The assumption that these sites would normally bind L-glutamine gains support from reports that large increases in L-glutamine concentration occur in the cyanobacterium *Anacystis nidulans* within 30 sec after addition of NH_4^+ (12) and that the inhibition of ANR activity in soil by NH_4^+ also occurs within 30 sec after addition of NH_4^+ (5).

Table 2. Effects of various compounds on ANR activity in soil

Compound	Amount added, μmol/g of soil	% inhibition of ANR activity	
		Harps soil	Okoboji soil
L-Glutamine	1	37	9
	2	54	62
	5	78	74
	10	88	75
L-Glutamic acid γ-methyl ester	1	36	15
	2	55	30
	5	73	72
	10	81	79
L-Glutamic acid γ-hydrazide	1	34	<1
	2	61	13
	5	62	26
	10	67	31
L-Glutamic acid γ-hydroxamate	1	17	29
	2	44	38
	5	60	57
	10	63	58
L-Glutamic acid γ-ethyl ester	1	10	1
	2	14	4
	5	24	7
	10	29	15
L-Glutamic acid dimethyl ester	1	<1	<1
	2	<1	<1
	5	<1	<1
	10	<1	<1
L-Asparagine	1	59	51
	2	70	78
	5	79	79
	10	81	81
L-Aspartic acid β-methyl ester	1	67	35
	2	77	49
	5	80	62
	10	79	62
L-Aspartic acid β-hydroxamate	1	24	16
	2	25	26
	5	45	31
	10	63	39

Slurries containing 10 g (dry weight) of Harps or Okoboji soil, 30 ml of water, 0.6 mg of KNO_3-N, 5 mg of glucose-C, and 14 mg of K_2SO_4 were incubated at 30°C for 120 min. After the first 60 min of incubation, the slurries were treated with 1, 2, 5, or 10 μmol of the compound specified per g of soil.

Studies reported in Table 3 showed that both hydroxylamine and methylamine inhibited ANR activity in soil but that the inhibitory effects of these NH_4^+ analogs were considerably smaller than that of NH_4^+ (particularly the effect of methylamine). It is well established that methylamine and hydroxylamine are substrates of glutamine synthetase and are converted by this enzyme to L-glutamic acid γ-methylamide and L-glutamic acid γ-monohydroxamate, respectively (13–15). To confirm that the inhibition of ANR activity observed with these ammonium analogs was due to their assimilation with formation of L-glutamine analogs, we studied the effect of MSX on their ability to inhibit ANR activity in soil. We found that MSX eliminated the inhibitory effect of hydroxylamine (Fig. 1) and methylamine (Fig. 2) on ANR activity. This indicates that inhibition of ANR activity by these NH_4^+ analogs was dependent upon glutamine synthetase activity and was due to formation of the L-glutamine analogs L-glutamic acid γ-hydroxamate and L-glutamic acid γ-methylamide, respectively.

Previous workers have demonstrated that L-glutamic acid γ-methylamide is a very poor substrate for glutamate synthase, the enzyme primarily responsible for metabolism of glutamine in bacteria (16). It seems unlikely, therefore, that the inhibition of ANR activity in soil by methylamine was due to metabolism of L-glutamic acid γ-methylamide formed by glutamine synthetase activity.

There are reports of amino acids other than glutamine and asparagine inhibiting ANR activity in microorganisms (5, 17), but it seems likely that their influence is indirect. Romero *et*

Table 3. Effects of different concentrations of ammonium, hydroxylamine, and methylamine on ANR activity in Harps soil

Compound	Conc., mg of N per g of soil	% inhibition of ANR activity
Ammonium sulfate	1	26
	3	68
	10	82
	25	86
Hydroxylamine	10	3
	25	47
	50	72
Methylamine	10	0
	50	0
	100	2
	250	19
	500	32
	1000	57
	2000	73

Slurries containing 10 g (dry weight) of Harps soil, 30 ml of water, 0.6 mg of KNO_3-N, 5 mg of glucose-C, and 14 mg of K_2SO_4 were incubated at 30°C for 120 min. After the first 60 min of incubation, the slurries were treated with different amounts of the compound specified. Conc., concentration.

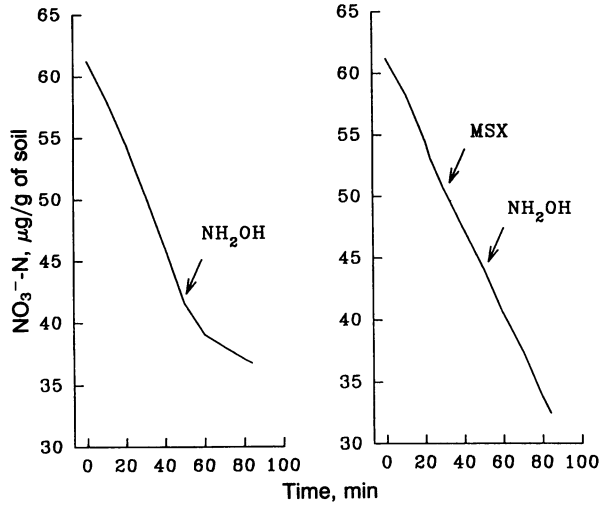

FIG. 1. Effect of MSX on the inhibition of ANR activity in Harps soil by hydroxylamine (NH_2OH). Slurries containing 10 g (dry weight) of soil, 30 ml of water, 0.6 mg of KNO_3-N, 5 mg of glucose-C, and 14 mg of K_2SO_4 were incubated at 30°C and treated with 0.5 mg of NH_2OH and 0_2 or 10 μmol of MSX at the times indicated.

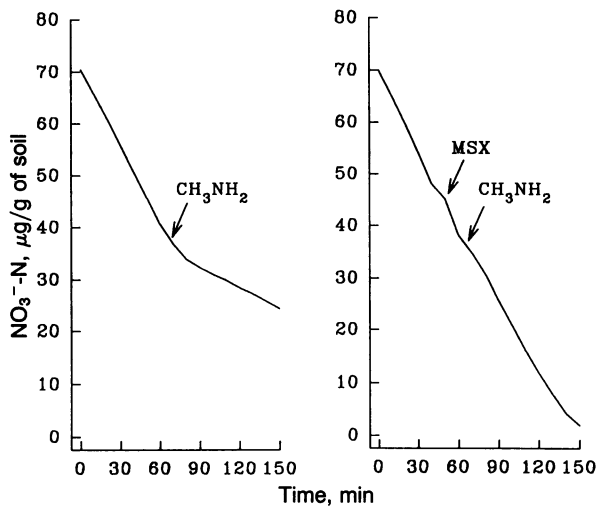

FIG. 2. Effect of MSX on inhibition of ANR activity in Harps soil by methylamine (CH_3NH_2). Slurries containing 10 g (dry weight) of soil, 30 ml of water, 0.6 mg of KNO_3-N, 5 mg of glucose-C, and 14 mg of K_2SO_4 were incubated at 30°C and treated with 20 mg of CH_3NH_2 and 0_2 or 10 μmol of MSX at the times indicated.

al. (17) studied the effects of 20 amino acids on ANR activity in a cyanobacterium (*Anacystis nidulans*) and the ability of these amino acids to act as amino donors to 2-ketoglutarate by transaminase activity in cell-free extracts of *A. nidulans*. They found that the ability of 16 of these amino acids to inhibit ANR activity in *A. nidulans* was highly correlated ($r = 0.96$) with their ability to act as amino group donors to 2-ketoglutarate, the exceptions being glutamine, asparagine, methionine, and glycine. They concluded that the ability of these 16 amino acids to inhibit ANR activity was related to their ability to deplete 2-ketoglutarate in the cytosome and not to the amino acids *per se*.

Much of the work concerning regulation of ANR activity in bacteria has involved studies using photosynthetic cyanobacteria, and caution is clearly required when comparing the physiology of nonphotosynthetic and photosynthetic bacteria. For example, it is well established that the reduction of nitrate in cyanobacteria is closely linked to photosynthetic activity and the availability of CO_2 for such activity (12, 18–21). It has been proposed that the influence of CO_2 availability on ANR activity in cyanobacteria is due to a CO_2-derivative (2-ketoglutarate) having a positive influence on ANR activity in microorganisms (17).

Evidence that 2-ketoglutarate may be a positive effector of ANR activity (17) and that L-glutamine may be a negative effector (10) suggests that the short-term regulation of ANR activity in bacteria may be controlled by the ratio of 2-ketoglutarate to L-glutamine. Such control would have an obvious parallel with the well-established mechanism for the short-term control of glutamine synthetase activity in nonphotosynthetic bacteria by a regulatory cascade influenced by the ratio of 2-ketoglutarate to L-glutamine (22). Recent work demonstrated the existence of certain elements of a similar cascade controlling aspects of nitrogen assimilation in photosynthetic bacteria (23, 24).

In summary, the experiments reported support the conclusion that the inhibitory effect of NH_4^+ on ANR activity in soil is not due to NH_4^+ *per se* but to L-glutamine formed by microbial assimilation of NH_4^+.

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1. National Research Council (1978) *Nitrates: An Environmental Assessment* (Natl. Acad. Press, Washington).
2. Royal Society Study Group (1983) *The Nitrogen Cycle of the United Kingdom* (Royal Soc., London).
3. Jones, J. M. & Richards, B. N. (1977) *Soil Biol. Biochem.* **9**, 383–392.
4. Tiedje, J. M., Sorensen, J. & Chang, L. (1981) *Ecol. Bull.* **33**, 331–342.
5. Rice, C. W. & Tiedje, J. M. (1989) *Soil Biol. Biochem.* **21**, 597–602.
6. Sias, S. R. & Ingraham, J. L. (1979) *Arch. Microbiol.* **122**, 263–270.
7. Tiedje, J. M. (1988) in *Environmental Microbiology of Anaerobes*, ed. Zehnder, A. J. B. (Wiley, New York), pp. 179–244.
8. Jansson, S. L. (1958) *Lantbrukshoegsk. Ann.* **24**, 101–361.
9. Recous, S. & Mary, B. (1990) *Soil Biol. Biochem.* **22**, 913–922.
10. McCarty, G. W. & Bremner, J. M. (1991) *Proc. Natl. Acad. Sci. USA* **89**, 453–456.
11. Brenchley, J. E. (1973) *J. Bacteriol.* **114**, 666–673.
12. Flores, E., Guerrero, M. G. & Losada, M. (1980) *Arch. Microbiol.* **128**, 137–144.
13. Tachiki, T., Suzuki, H., Wakisaka, S., Yano, T. & Tochikura, T. (1986) *J. Gen. Appl. Microbiol.* **32**, 545–548.
14. Tate, S. S. & Meister, A. (1973) in *The Enzymes of Glutamine Metabolism*, eds. Prusiner, S. & Stadtman, E. R. (Academic, New York), pp. 77–127.
15. Moore, R. A. & Gordon, J. K. (1984) *Arch. Microbiol.* **140**, 193–197.
16. Yoch, D. C., Zhang, Z. & Claybrook, D. L. (1983) *Arch. Microbiol.* **134**, 45–48.
17. Romero, J. M., Flores, E. & Guerrero, M. G. (1985) *Arch. Microbiol.* **142**, 1–5.
18. Flores, E., Ramos, J. L., Herrero, A. & Guerrero, M. G. (1983) in *Photosynthetic Prokaryotes: Cell Differentiation and Function*, eds. Papageorgiou, G. C. & Packer, L. (Elsevier, New York), pp. 363–387.
19. Lara, C., Romero, J. M., Flores, E., Guerrero, M. G. & Losada, M. (1984) in *Advances in Photosynthesis Research*, Vol. II, ed. Sybesma, C. (Nijhoff, Boston), pp. 7-715–7-718.
20. Romero, J. M., Coronil, T., Lara, C. & Guerrero, M. G. (1987) *Arch. Biochem. Biophys.* **256**, 578–584.
21. Lara, C., Romero, J. M., Coronil, T. & Guerrero, M. G. (1987) in *Inorganic Nitrogen Metabolism*, eds. Ullrich, W. R., Aparicio, P. J., Syrett, P. J. & Castillo, F. (Springer, New York), pp. 45–52.
22. 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.
23. Vega-Palas, M. A., Madueno, F., Herrero, A. & Flores, E. (1990) *J. Bacteriol.* **172**, 643–647.
24. Tsinoremas, N. F., Castets, A. M., Harrison, M. A., Allen, J. F. & Tandeau de Marsac, N. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4565–4569.