Biochimica et Biophysica Acta, 350 (1974) 162–170

BBA 67185

INHIBITOR OF NITRATE REDUCTASE IN THE ROOTS OF RICE SEEDLINGS AND ITS EFFECT ON THE ENZYME ACTIVITY IN THE PRESENCE OF NADH

S. S. KADAM, A. P. GANDHI, S. K. SAWHNEY and M. S. NAIK
Biochemistry Division, Indian Agricultural Research Institute, New Delhi – 110012 (India)
(Received November 23rd, 1973)

SUMMARY

Roots of rice seedlings contain a substance which inhibits the activity of nitrate reductase from leaves when NADH or $FMNH_2$ is used as an electron donor. The activity of reduced benzyl viologen:nitrate reductase is, however, not affected. The inhibitor is non-dialysable, heat labile and inactive at 0 °C. It has no effect on nitrite reductase. NADH, a positive effector of nitrate reductase, when added along with the inhibitor, counteracts the effect of the latter. Similar results are obtained with the partially purified enzyme. It is suggested that the inhibitor might act at the NADH site of the nitrate reductase enzyme complex.

INTRODUCTION

Nitrate reductase (nitrate oxidoreductase (NADH), EC 1.6.6.1), which catalyses the first step in the assimilation of nitrate is ubiquitous in almost all organs of higher plants [1], but its activity in the roots is generally found to be very low [2, 3]. The failure to detect high levels of enzyme activity in roots has been attributed to the lack of knowledge about the optimum conditions for its extraction and assay [4, 5]. The possibility of the presence of a nitrate reductase inhibitor of a phenolic nature, in the roots of apple trees has also been indicated [6, 7]. The inhibitor could be readily eliminated from root extracts by dialysis against a buffer containing polyvinylpyrrolidone. In this paper we report the presence of a non-dialysable and heat labile inhibitor in roots of rice seedlings. It has recently been shown that rice leaf nitrate reductase is specifically activated by preincubation with NADH [8]. We now report the combined effect of NADH and the inhibitor on the activity of leaf nitrate reductase.

EXPERIMENTAL PROCEDURE

Extraction of inhibitor

Seedlings of rice (*Oryza sativa* L) variety improved Sabarmati were grown in distilled water for 15 days under normal light [9]. They were then transferred to a

Hoagland solution containing 15 mM NO_3^- for the induction of the enzymes. The extracts were prepared both from leaves and roots by grinding them separately at 0–4 °C in a chilled pestle and mortar using 0.1 M phosphate buffer, pH 7.5 containing 10^{-3} M cysteine with a 1:6 ratio of the tissue to the extraction medium. The same buffer was used for the extraction of the inhibitor from the roots. Nitrate reductase from the roots was, however, extracted by using the modified medium of Miflin [5], which contains in the phosphate buffer, EDTA, glutathione, $MgCl_2$ and sucrose. The homogenates in each case were centrifuged at 10 000 × *g* for 20 min and the supernatant solutions were used as sources of the enzyme and the inhibitor. These were dialysed for 24 h at 0 °C with frequent changes of 0.01 M phosphate buffer, pH 7.0, in order to remove metabolites and coenzymes.

Assay of enzymes

After preincubation of the dialysed leaf extract with the dialysed root extract, nitrate reductase and nitrite reductase activities were assayed using NADH and reduced methyl viologen as electron donors, respectively, as described earlier [9]. For the assay of nitrate reductase chemically reduced benzyl viologen and FMN were also used. The reaction mixture in both the cases contained in 2 ml the following, in μmoles: phosphate buffer, pH 7.5, 85; KNO_3, 20; enzyme, 0.2 ml; root extract, 0.3 ml; reduced benzyl viologen/FMN, 1. A thin layer of liquid paraffin was placed on the surface of the reaction mixture to prevent the oxidation of reduced electron donors, which were reduced by the gentle addition of 0.75 μmoles of freshly prepared solution of $Na_2S_2O_4$. The mixture was incubated at 30 °C and the reaction was stopped in both the cases by vigorous shaking. In suitable aliquots, the quantity of nitrite was determined as previously described [9].

Reagents

Chemicals were obtained as follows: NADH from Patel Chest Institute, New Delhi, benzyl viologen and methyl viologen from British Drug Houses, Poole, England and FMN from Sigma Chemical Company, St. Louis, Mo.

RESULTS

Inhibitory effect of root extract

Inhibition of leaf nitrate reductase by preincubation with extracts of roots obtained from rice seedlings is shown in Table I. Nitrate reductase is known to be an unstable enzyme [10] and hence during a 1 h preincubation at 25 °C about 46% of the activity was lost. The process of inactivation was considerably accelerated by preincubation with root extract. Uninduced root extract, obtained from seedlings grown without nitrate, was also equally effective. The extent of inhibition increased with increasing quantities of root extract from 0.1 to 0.5 ml (Table II).

Temperature dependence

Preincubation of leaf nitrate reductase with root extract at 0 °C considerably retarded the rate of inactivation (Table III). At this temperature, no increase in the extent of inhibition was noticed between 40- and 60-min incubation. The slight inhibition observed could have occurred during incubation for the assay of the enzyme

TABLE I

INHIBITION OF LEAF NITRATE REDUCTASE BY ROOT EXTRACTS

Dialysed root extracts were prepared from seedlings grown with (induced) or without (uninduced) nitrate. Dialysed leaf extracts containing nitrate reductase were preincubated at 25 °C with or without root extracts for different time intervals and then the activity of the enzyme was assayed by using 0.68 μmole NADH as the electron donor, as described in the text.

Conditions of preincubation Preincubation period with or without root extracts (min)	Nitrate reductase (nmoles NO^-_2 formed/0.2 ml per 30 min)			
	0	20	40	60
Leaf extract	112	86	75	60
Leaf extract + root extract (induced)	97	66	38	20
Leaf extract + root extract (uninduced)	89	64	40	20
Root extract (induced)	10	Nil	Nil	Nil
Root extract (uninduced)	4	Nil	Nil	Nil

TABLE II

INHIBITORY EFFECT OF DIFFERENT CONCENTRATIONS OF ROOT EXTRACT ON LEAF NITRATE REDUCTASE

Dialysed leaf extract (0.2 ml) was preincubated with different quantities of dialysed root extract at 25 °C for 20 min and the nitrate reductase was assayed by using NADH as the electron donor as in Table I

Quantity of root extract added (ml)	Nitrate reductase activity
Nil (no preincubation)	180
Nil (20-min preincubation)	84
0.1	21
0.3	17
0.5	11
0.7	11
1.0	10

TABLE III

EFFECT OF PREINCUBATION TEMPERATURE ON THE INACTIVATION OF LEAF NITRATE REDUCTASE BY ROOT EXTRACTS

Preincubation of the leaf extract with root extract was done at 0 °C and 25 °C. Nitrate reductase was assayed as in Table I.

Preincubation temperature	Nitrate reductase activity	
	0 °C	25 °C
40 min-leaf extract	98	76
40 min-leaf extract + root extract	68	37
60 min-leaf extract	98	60
60 min-leaf extract + root extract	68	20

TABLE IV

EFFECT OF HEAT TREATMENT ON THE INHIBITORY FACTOR

The root extract was treated at 0, 60 and 100 °C for different periods. Leaf extract was then incubated with these extracts at 25 °C for 40 min. Nitrate reductase activity was then assayed as in Table I. In the control the leaf extract was preincubated without the root extract. Inhibition (%) of enzyme activities is also calculated.

Heat treatment of leaf extract,		Nitrate reductase activity	Inhibition (%)	Loss of inhibitory effect (%)
Temperature	Period (min)			
Control		84	—	—
0	300	48	43	—
60	30	54	36	16
60	60	54	36	16
60	90	56	33	23
60	120	56	33	23
60	180	60	29	33
60	240	60	29	33
60	300	69	18	58
100	10	85	Nil	100

at 30 °C. In Table I also about 20% inhibition was observed when the enzyme and the inhibitor were not preincubated before starting the assay of the enzyme. It is thus conceivable that the inhibitor is not active at 0 °C.

Properties of the inhibitor

The root extract did not lose its inhibitory effect even after prolonged dialysis against 0.01 M phosphate buffer, pH 7.5. It was completely inactivated when kept in boiling water for 10 min. However, it was fairly stable at 60 °C and lost only about 60% of its activity in 5 h at that temperature (Table IV).

The inhibitory factor was effective when either NADH or $FMNH_2$ were used as electron donors for the assay of nitrate reductase (Table V). The extent of inhibition was about 50% in both the cases. However, when benzyl viologen reduced by sodium

TABLE V

EFFECT OF INHIBITOR WHEN DIFFERENT ELECTRON DONORS FOR NITRATE REDUCTASE ASSAY ARE USED

Activities of nitrate reductase were assayed in leaf and root extracts separately by using NADH, $FMNH_2$ and reduced benzyl viologen as electron donors. Leaf extract was also preincubated with the root extract at 25 °C and the enzyme activities were assayed as given in the text.

Electron donor	Nitrate reductase activity					
	Leaf*	Root*	Leaf + Root*	Leaf**	Root**	Leaf + Root**
NADH	256	32	136	168	12	84
$FMNH_2$	56	Nil	28	32	Nil	18
Reduced benzyl viologen	126	Nil	118	142	Nil	136

* Period of preincubation is 40 min.
** Period of preincubation is 60 min.

dithionite was used, the inhibitor was not effective. Although cysteine was added to the buffer used for the extraction of the enzyme, it must have been removed during dialysis of the extract. Hence the effect of preincubation with cysteine as well as nitrate on the activity of the enzyme in the presence of the root inhibitor was studied.

The results in Table VI show that pretreatment of leaf nitrate reductase with

TABLE VI

EFFECT OF PREINCUBATION WITH CYSTEINE AND NITRATE ON THE INHIBITOR

The leaf extracts were preincubated with equal quantities of 10^{-3} M cysteine or 0.067 M KNO_3 at 25 °C for 40 min, along with the root extract. Nitrate reductase was assayed as in Table I. Activities are expressed as a percentage of the activity in the control, in which the leaf extract alone was preincubated without any addition.

Preincubation treatment	Nitrate reductase activity
Leaf extract alone	100
Leaf extract + cysteine	100
Leaf extract + cysteine + root extract	39
Leaf extract + nitrate + root extract	32
Leaf extract + root extract	39

cysteine or nitrate did not offer any protection from inactivation by the root extract. Similarly addition of bovine serum albumin or casein to the preincubation mixture did not prevent the inhibition of nitrate reductase by the root extract.

Effect on nitrite reductase

The results in Table VII show that the root extract did not inhibit leaf nitrite reductase which was assayed by using dithionite-reduced methyl viologen as an electron donor. At the same time nitrate reductase (NADH) was inhibited by about 50%.

TABLE VII

EFFECT OF INHIBITOR ON NITRITE REDUCTASE

Leaf extract was preincubated with root extract at 25 °C for 40 min. Nitrate reductase was assayed using NADH as the electron donor as given in Table I. Nitrite reductase was assayed with methyl viologen reduced by sodium dithionite [9] as electron donor.

Assay conditions	Nitrate reductase (nmoles NO_2^- formed/ 0.2 ml per 30 min)	Nitrite reductase (nmoles NO_2^- consumed/ 0.2 ml per 30 min)
Leaf extract alone	62	920
Root extract alone	10	330
Leaf extract + root extract	36	1240

Effect of light

Light plays an important role in the synthesis of nitrate reductase in the leaves of rice seedlings [9]. The results in Table VIII show that no inhibitory substance is detected in the shoots of green or etiolated seedlings. However, it is invariably present in the roots of both green and etiolated plants grown with or without nitrate.

TABLE VIII

DETECTION OF INHIBITOR IN LEAVES AND ROOTS OF GREEN AND ETIOLATED SEEDLINGS

Dialysed extracts of leaves and roots of normal green and completely etiolated seedlings were prepared as given in the text. Leaf extracts containing nitrate reductase were then preincubated at 25 °C with leaf extracts which lacked nitrate reductase or with different root extracts. Finally the nitrate reductase activity was assayed as described earlier. Activities are expressed as a percentage of the activity in the control, in which the leaf extract alone was preincubated without any addition.

Conditions of growth of plant from which inhibitor is extracted (Period of preincubation (min):	Additions to preincubation mixture	Nitrate reductase	
		20	40
Continuous light	Nil	100	100
Continuous light	Leaf extract (uninduced)	92	100
Continuous light	root extract (induced)	50	43
Continuous light	root extract (uninduced)	47	43
Etiolated	leaf extract	98	96
Etiolated	root extract	52	39

TABLE IX

EFFECT OF NADH ON THE ACTIVITY OF THE INHIBITORY FACTOR

Leaf extract (0.2 ml) containing nitrate reductase was preincubated with 0.1 ml solution containing 0.68 μmole NADH for different time intervals as indicated in the Table. The subsequent period of preincubation with the root extract was adjusted in such a way so as to give a total 40-min preincubation. Nitrate reductase activities are compared with the treatment in which NADH pretreatment was omitted. In the second treatment, the leaf extract was preincubated with the root extract for 40 min with or without NADH.

Time of preincubation of leaf extract (min)		Nitrate reductase activity	% Control
	With root extract		
Without NADH			
40	Nil (control)	286	100
40*	40	72	25
5	35	96	34
10	30	120	42
15	25	144	50
20	20	160	56
30	10	172	60
With NADH			
40	Nil	608	213
40*	40	366	128
5	35	372	130
10	30	372	130
15	25	428	150
20	20	464	162
30	10	508	178

* The second treatment was used (see legend).

Effect of preincubation in the presence of NADH

As reported earlier [8], the activity of nitrate reductase was stimulated when the leaf extract was preincubated with NADH alone for 40 min (Table IX). When it was preincubated with NADH and root extract together the activation of the enzyme was only about 28% as compared to the control. Thus the inhibitor in the root counteracted the stimulatory effect of NADH. However, the activity observed in this treatment was about five-fold more than that found when the enzyme was preincubated with the root extract alone in the absence of NADH. Similarly as the period of contact with NADH was increased from 5 to 30 min and that of root extract was correspondingly decreased, so as to have a total preincubation period of 40 min, the activity of nitrate reductase was considerably stimulated. The activity of the enzyme preincubated with the root inhibitor in the presence of NADH was invariably 3–4-fold more than that in the corresponding treatment in the absence of NADH.

In the next experiment (Table X), increasing concentrations of NADH were added to the leaf and root extracts during preincubation. Even as little as 68 nmoles of NADH doubled the activity of nitrate reductase. The maximum stimulation was observed with a 0.15 ml solution containing 1100 nmoles NADH.

TABLE X

EFFECT OF NADH CONCENTRATION ON THE INHIBITOR

Leaf extract (0.2 ml) containing nitrate reductase was preincubated with 0.1 ml root extract for 40 min at 25 °C with different quantities of solution containing 6.8 μmole NADH/ml as indicated in the Table. Final volume of the preincubation mixture was 0.5 ml. Subsequently while assaying the activity of nitrate reductase, the quantity of NADH added was adjusted in such a way that a final quantity of 0.2 ml NADH was supplied.

Concentration of NADH added (ml)		Nitrate reductase activity
Preincubation mixture	Assay mixture	
Nil	0.2	88
0.01	0.19	200
0.05	0.15	340
0.1	0.1	420
0.15	0.05	464
0.2	Nil	340

Experiments with partially purified extracts

For the experiments described above dialysed leaf and root extracts were used, in order to avoid interference, if there was any, from metabolites and coenzymes. The results were finally confirmed with partially purified extracts as follows.

Nitrate reductase from leaves was partially purified as described by Eaglesham and Hewitt [11] by fractionation with 45% saturated ammonium sulphate and a Sephadex G-25 column. Since the enzyme is unstable, it was considerably inactivated during the process of purification. Thus no significant increase in the specific activity was observed. However, about 75% of the total protein in the crude extract was eliminated in the fraction collected from the Sephadex G-25 column. The rest of the fractions showed negligible nitrate reductase activity. The root extract was given heat treatment for 1 h at 60 °C. As shown above (Table IV), the inhibitory factor was not

TABLE XI

EFFECT OF INHIBITOR ON PARTIALLY PURIFIED NITRATE REDUCTASE

Leaf nitrate reductase was partially purified as explained in the text. The root extract was given heat treatment at 60 °C for 1 h. The enzyme was preincubated with or without the inhibitor at 25 °C for 1 h. NADH was included at 0.4 mM concentration where indicated. Nitrate reductase activity was assayed as given in Table I.

Treatment	Nitrate reductase (nmoles NO_2^-/mg protein per 20 min)
Enzyme alone	12.3
Enzyme + root extract	4.6
Enzyme + root extract + NADH	31.5

inactivated by this treatment. The partially purified nitrate reductase and the heat-treated root extract were used and the results are given in Table XI.

The enzyme activity was about 60% inhibited by preincubation with the inhibitor. When NADH was added along with the root extract the activity was stimulated almost seven-fold and was even higher than in the original enzyme kept at 25 °C for 1 h. Thus the inhibitory and stimulatory effect of the root factor(s) and of NADH, respectively, were confirmed also with partially purified enzyme.

DISCUSSION

The pathway of electron transfer within the nitrate reductase enzyme complex as suggested by Schrader et al. [10] envisages a transfer of electrons from NADH to a flavin moiety and then to molybdenum which ultimately reduces nitrate attached at the active site of the enzyme. In this scheme, the non-physiological electron donor, reduced benzyl viologen is expected to reduce molybdenum directly, thus bypassing the site of the enzyme which accepts electrons from NADH or $FMNH_2$. Our results show that the inhibitory factor in the roots had no effect on the enzyme activity when reduced benzyl viologen was used as the reductant. The inhibitor is thus probably acting at a site where NADH is donating electrons to nitrate reductase. Preincubation of the enzyme with NADH alone stimulated the enzyme but the extent of stimulation was considerably diminished by adding the inhibitor along with NADH. Thus NADH and the inhibitor showed exactly opposite effects on the enzyme. When the two were added together the activity observed was the resultant of the two opposing effectors. It has been earlier reported that the inhibition of nitrate reductase from spinach by parachloromercuribenzoate, a specific –SH inhibitor, is reversed by pretreatment with NADH [12].

The mechanism by which NADH acts as a positive effector of nitrate reductase is not clear [8]. It is possible that the site of the enzyme complex which accepts electrons from NADH, may be specifically activated by NADH. Numerous reports have indicated the involvement of –SH groups in nitrate reductase at the site accepting electrons from NADH [10]. It is, however, interesting to note that while preincubation with NADH protected the enzyme from the root inhibitor, cysteine and nitrate were without any effect.

The inhibitor was found to be non-dialysable, heat labile and inactive at 0 °C,

thus indicating a possible involvement of an enzymatic reaction. Since inclusion of bovine serum albumin or casein in the incubation mixture did not offer any protection to the inactivation of nitrate reductase, it is unlikely that the inhibitor is a general proteolytic enzyme. It showed a specific action on nitrate reductase (NADH) but nitrite reductase was not affected at all.

Physiological significance

In our study, the effect of the root factor has been studied on the enzyme from the leaves. Since this or a similar factor could not be detected in leaves, the physiological significance, if any, of the presence of this factor in the roots has to be sought in the possible control of nitrate assimilation in the roots. The rate of nitrate assimilation is very high in the green tissues of the plants, since photosynthetic reactions play an important role in the generation of the reducing power as well as in the synthesis of the enzyme [9]. An abundant and continuous supply of nitrate is, therefore, necessary to these tissues, in the absence of which, induction of the enzymes will be blocked. A mechanism which controls the quantity of nitrate reduced in the roots, so that the shoots are not deprived of this nutrient, is, therefore, of vital importance for the growth of the plants [13]. It has been pointed out that a sufficient supply of carbohydrates must be present in the roots for reduction as well as retention of nitrate and that NADH availability is a limiting factor for in vivo nitrate reduction [14]. Hence the inhibitory factor present in the roots, can possibly control the supply of nitrate to the shoots and only when sufficient NADH is available in the roots, can this inhibition be reversed. A control mechanism for maintaining the proper nitrate level could thus function by the action of the inhibitor and NADH as negative and positive effectors of the enzyme, respectively.

REFERENCES

1 Beevers, L. and Hageman, R. H. (1969) Annu. Rev. Plant Physiol. 20, 495–522
2 Vaidyanathan, C. S. and Street, H. E. (1959) Nature 184, 531–533
3 Bowerman, A. and Goodman, P. J. (1971) Annu. Bot. 35, 353–366
4 Sanderson, G. W. and Cocking, E. C. (1964) Plant Physiol. 39, 416–422
5 Miflin, B. J. (1967) Nature 214, 1133–1134
6 Grasmanis, V. O. and Nicholas, D. J. D. (1967) Phytochemistry 6, 217–218
7 Klepper, L. and Hageman, R. H. (1969) Plant Physiol. 44, 110–114
8 Gandhi, A. P., Sawhney, S. K. and Naik, M. S. (1973) Biochem. Biophys. Res. Commun., 55, 291–296
9 Sawhney, S. K. and Naik, M. S. (1972) Biochem. J. 130, 475–486
10 Schrader, L. E., Ritenour, G. L., Eilrich, G. L. and Hageman, R. H. (1968) Plant Physiol. 43, 930–940
11 Eaglesham, A. R. J. and Hewitt, E. J. (1971) FEBS Lett. 16, 315–318
12 Relimpio, A. Ma., Aparico, P. J., Paneque, A. and Losada, M. (1971) FEBS Lett. 17, 226–230
13 Wallace, W. and Pate, J. S. (1967) Annu. Bot. 31, 213–228
14 Minotti, P. L. and Jackson, W. A. (1970) Planta (Berl) 95, 36–44