Planta 138, 205–209 (1978)

Planta
© by Springer-Verlag 1978

Nitrite Activation of Nitrate Reductase in Higher Plants

D. Kaplan, A.M. Mayer*, and S.H. Lips
Department of Biology, Ben Gurion University of the Negev, Beer Sheva, Israel

Abstract. Comparative studies of nitrate-activated nitrate reductase (NR-NO_2) and nitrate-induced nitrate reductase (NR-NO_3) (EC 1.6.6.2) indicate that the enzymes differ in structure, heat stability, and pH dependence, but have the same cofactor requirment. NR-NO_2 developes in barley (*Hordeum vulgare* L. var. Dvir) seedlings as NR-NO_3 disappears. A transition from the active to the inactive form of nitrate reductase takes place. Nitrite seems to activate the inactive form of the enzyme.

Key words: *Hordeum* Nitrite – Nitrate reductase.

Introduction

Nitrate reductase is considered a substrate-inducible enzyme (Hewitt, 1975), although the capacity of nitrite to elicit nitrate reductase activity has also been observed (Ingle et al., 1966; Kaplan et al., 1974a). A more detailed study of the effect of nitrite showed that the response to nitrite in bean seed cotyledons is relatively rapid (Kaplan et al., 1974a). This observation was later extended to leaves of several other species of plants (Kaplan et al., 1974b). The expression of the inductive capacity of nitrate in green leaves requires light or the supply of suitable respiratory substrates, such as glycolate or sucrose (Roth-Bejerano and Lips, 1973). In contrast, the capacity of nitrite to elicit nitrate reductase activity is not light-dependent (Lips et al., 1973). Nitrite evoked an immediate and rapid increase of nitrate reductase activity in leaves, which reached its maximal effect 2–3 h after nitrite addition to the plant material (Kaplan et al., 1974b).

* *Address:* Department of Botany, Hebrew University of Jerusalem, Israel

The characteristics of the nitrite effect suggest that an inactive form of nitrate reductase is activated. Inactive nitrate reductase is formed in *Chlorella* in response to cyanide and NADH (Solomonson et al., 1973) and nitrite protects the enzyme from inactivation by cyanide (Solomonson, 1974). Furthermore, environmental conditions such as high oxygen tension or high light intensity also produce the reversible inactivation of nitrate reductase in *Chlorella* (Pistorius et al, 1974), indicating that cyanide inactivation of the enzyme may occur under physiologic conditions. In this paper we report the results of a comparative study of the nitrite-activated enzyme (NR-NO_2) with the enzyme normally formed in the leaves of plants growing in a nitrate-containing nutrient solution (NR-NO_3).

Materials and Methods

Plant Material

Seedlings of barley (*Hordeum vulgare*, L., cv. Dvir) were grown on a layer of vermiculite on a polyethylene net suspended over the nutrient solution. Nutrient solutions used were either 0.5 mM $CaSO_4$ or 50% Hoagland solutions. Pea plants (*Pisum sativum*, L., var. Dan) were grown in vermiculite on 0.5 mM $CaSO_4$.

Homogenization

Leaves were ground in 0.1 M phosphate buffer, pH 7.4 containing 1 mM EDTA, in an ice-cold mortar with a pestle, using 2 ml medium per gram fresh weight of leaves. The resulting homogenate was filtered through 2 layers of gauze and the filtrate centrifuged at 20,000 g for 15 min. The resulting pellet was discarded and the supernatant used as the enzyme extract.

Activation

KNO_2 was added to the crude enzyme extract up to the concentrations indicated in each case, the extract then incubated for 5 min at 30°. The resulting nitrate reductase activity was designated NR-

0032-0935/78/0138/0205/$01.00

NO_2 to distinguish it from the enzyme obtained from plants grown in solutions containing nitrate, which was called NR-NO_3.

Nitrate Reductase Assay

Enzyme activity was determined essentially as described by Hewitt and Nicholas (1964). Assays were performed in test tubes in a water bath at 30°. Each tube contained 2 ml assay mixture, consisting of: 0.2 ml of 0.1 M KNO_3, 0.2 ml of 2.6×10^{-5} M FAD, 0.4 ml of 2 mg/ml NADH, 0.1 ml of 0.1 M phosphate buffer pH 7.4, 0.1 ml of 1% Triton X-100, 0.1 ml extract, and 0.9 ml water. Nitrite determinations were done at 5 and 30 min after the beginning of the reaction; enzyme activity is expressed as the increase in nitrite between 5 and 30 min per g fresh weight of leaves per h.

DEAE-cellulose Ion Exchange Chromatography

The 20,000 g supernatant obtained from a leaf homogenate was adsorbed to a DEAE-cellulose anion exchange column (25 × 2 cm) previously equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA. Protein was eluted from the column using a linear gradient of KCl from 0–0.3 M in the buffer solution used for equilibration and homogenization.

Results

Leaves of barley seedlings grown in the light on 0.5 mM $CaSO_4$ were homogenized as previously described. KNO_2 was added to the resulting extract to different concentrations and the mixture allowed to stand for 5 min at 30° C before enzyme activity was measured (Table 1). The activity of nitrate reductase following addition of nitrite reaches saturation at about 0.5 mM KNO_2. Similar responses to nitrite added were observed when using homogenates of other plant species (Table 2) some of which show somewhat higher saturation levels.

Neither ferricyanide nor ascorbic acid was an effective activator of nitrate reductase in homogenates of plants grown in the absence of nitrate. Response to added nitrite is quite rapid (Table 3), nitrate reductase activation reaching its maximal value 5–10 min after addition of nitrite, but the activity of the enzyme declines with time. This decline in nitrite-activated NR was observed also in vivo as previously reported (Kaplan et al., 1974b).

Heat inactivation of NR-NO_2 was compared to that of the enzyme formed in response to nitrate, NR-NO_3. At the lower temperatures tested both enzymes behaved similarly. However, NR-NO_2 appeared to be less sensitive at the higher temperatures (Fig. 1).

NR-NO_2 shows an absolute dependence on NADH (Table 4) and requires nitrate as substrate. NADPH does not act as an electron donor. These requirements of the nitrite-activated enzyme are similar to those of NR-NO_3.

Table 1. KNO_2 activation of nitrate reductase in homogenates of barley leaves obtained from 9-day-old seedlings grown in the absence of nitrate. KNO_2 was added to the homogenates for 5 min at 30° C prior to nitrate reductase assay

mM KNO_2	Nitrate reductase activity µmol NO_2^- formed/g fr wt/h
0	0.06
0.05	0.25
0.10	0.45
0.25	0.85
0.50	1.93
1.00	2.15

fr wt = fresh weight

Table 2. KNO_2 activation of nitrate reductase in extracts of different nitrate-depleted plant species. Conditions as in Table 1

mM KNO_2	NR activity: µmol NO_2^- formed/g fr wt/h			
	Green barley leaves	Etiolated barley leaves	Cucumber cotyledons	Pea leaves
0	0.08	0.10	0.30	0.01
0.25	0.25	0.53	–	0.31
0.50	2.44	1.09	2.57	0.88
1.00	2.59	1.60	3.74	1.07

fr wt = fresh weight

Table 3. Effect of time of addition of 2 mM KNO_2 to homogenate of barley leaves, at 30° C, prior to nitrate reductase assay

Minutes in KNO_2	NR activity µmol NO_2^- formed/g fr wt/h
5	4.6
10	5.0
15	4.1
20	3.5
25	3.0
30	2.8

fr wt = fresh weight

Plants grown in Hoagland were transfered to 0.5 mM $CaSO_4$ and the decrease of nitrate reductase following nitrate depletion was followed (Table 5). Samples of leaves were homogenized, and KNO_2 was added to the extract to obtain a final concentration of 0.5 mM. The lower the level of NR-NO_3, the larger, was the response to nitrite giving rise to NR-NO_2. Homogenates of leaves obtained from plants grown in an adequate level of nitrate (0 time in $CaSO_4$ in Table 5) showed no response to added nitrite. Optimal activation was observed at 20° C with a rapid decline above 30° C (Table 6) presumably due to enzyme

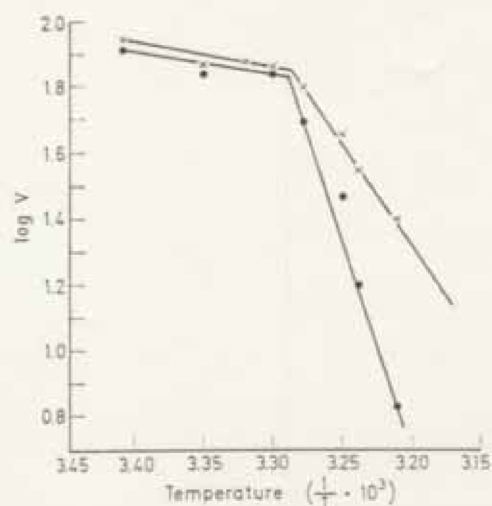

Fig. 1. Heat inactivation of NR-NO_3^- and NR-NO_2^- activities. The extracts were treated for 5 min at the temperature indicated and then assayed for NR activity. NR-NO_3^- ·—·—·; NR-NO_2^- ×—×

Table 4. Cofactor requirements of NR-NO_2^-, after activation of the enzyme in a homogenate of barley leaves grown in the absence of nitrate

NO_2^- activation (0.5 µmol/ml)	KNO_3 (10 µmol/ml)	NADH (0.2 mg/ml)	NADPH (0.2 mg/ml)	NR activity µmol NO_2^- formed/g fr wt/h
−	−	+	−	0.13
−	+	+	−	0.13
−	+	−	+	0
−	+	−	−	0
+	−	+	−	0.17
+	+	+	−	0.66
+	+	−	+	0
−	+	−	−	0

fr wt = fresh weight

Table 5. Barley seedlings grown in 50% Hoagland solutions were transferred to 0.5 mM $CaSO_4$. After periods of time indicated, some of the plants were homogenized and NR activity determined before and after a 5-min activation period with 0.5 mM KNO_2

Hours in $CaSO_4$	NR activity: µmol NO_2^- formed/g fr wt/h		
	Before NO_2^- activation	After NO_2^- activation	Δ NR
0	6.5	6.5	0
23	2.2	2.3	0.1
65	1.5	2.8	0.7
89	0.8	3.0	2.2

fr wt = fresh weight

Table 6. Effect of temperature during activation period of 5 min with 0.5 mM KNO_2, using a homogenate of barley leaves

Temperature during activation (°C)	NR activity µmol NO_2^- formed/g fr wt/h
0	0.45
10	0.65
20	0.69
30	0.65
40	0.32
50	0.29
60	0.16

fr wt = fresh weight

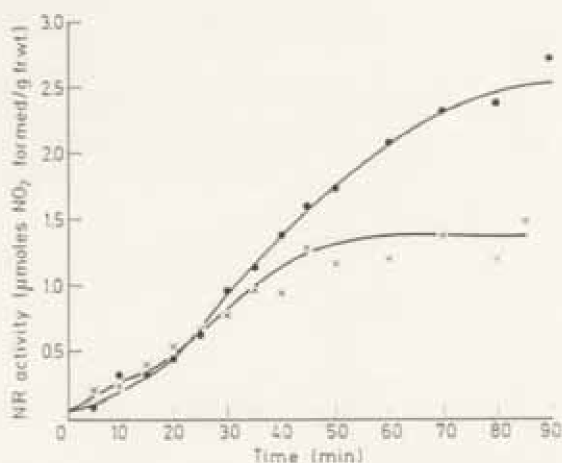

Fig. 2. Kinetics of NR-NO_3^- and NR-NO_2^- activity. NR-NO_3^- ·—·—·; NR-NO_2^- ×—×

inactivation. Even at 0° C activation took plase to the extent of 65% of the maximum observed.

A comparison of the kinetics of the reduction of nitrate by NR-NO_2 and NR-NO_3 was made (Fig. 2). Under normal assay conditions, both activities show a slight initial lag. However, NR-NO_3 is linear with time for a longer period than NR-NO_2. This might indicate greater stability of the NR-NO_3 form of the enzyme. The dependence of the two enzyme activities on pH was examined (Fig. 3). NR-NO_3 had a broader pH maximum and peaked at about pH 7.5, while NR-NO_2 showed a greater response with an optimum at about pH 7.0. NR-NO_2 appeared to be less sensitive to pH changes than NR-NO_3, since even at the extreme pH values no more than 50% inhibition could be observed, while NR-NO_3 is almost completely inactive above pH 8.5 and below pH 6.

Increased NR-NO_2 activity might be an artifact due to nitrite stimulation of NADH oxidase. This

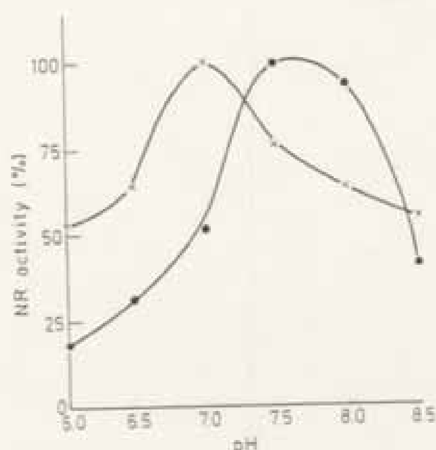

Fig. 3. The NR-NO_3^- and NR-NO_2^- activities as a function of the pH values in the reaction mixture were measured. NR-NO_3^- —·—·—; NR-NO_2^- ×—×

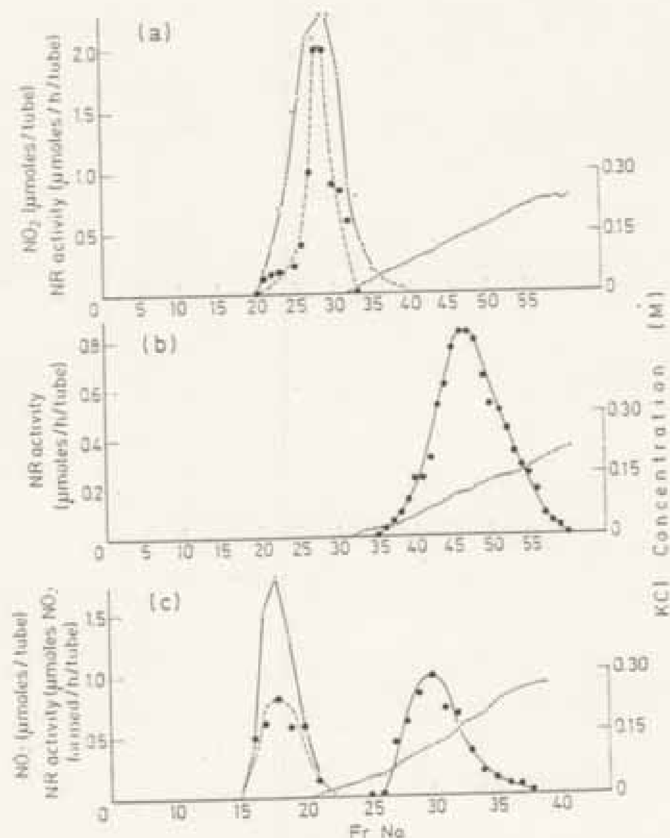

Fig. 4a–c. Elution of: a NR-NO_2^- activity, b NR-NO_3^- activity, and c a mixture of NR-NO_2^- and NR-NO_3^- activites from 3 parallel columus of DEAE cellulose by a *leaner* gradient of potassium chloride. NR-NO_2^- ×—×; NO_2^- ○—○; NR-NO_3^- —·—· KCl...

would result in enhanced color yield due to removal of NADH. However, NADH oxidase was found to be an easily removable, particulate enzyme, whose activity was unaffected by nitrite or nitrate. Furthermore, removal of residual NADH with malate or alcohol dehydrogenase – at the end of the assay and prior to addition of reagents for the estimation of nitrite – did not affect the estimated activity of NR-NO_2.

NR-NO_3 and NR-NO_2 were each adsorbed onto a DEAE-cellulose ion exchange column and then eluted with a linear gradient of KCl. The individual enzymes were eluted at different KCl concentrations as indicated in Figures 4A and 4B. Identical results were obtained when the column was loaded with a mixture of NR-NO_3 and NR-NO_2 (Fig. 4C). Nitrite and NR-NO_2 were eluted off the column together, before addition of KCl to the eluting buffer, while NR-NO_3 was eluted off at about 0.1 M KCl.

Discussion

The rapid response of nitrate-depleted plants to nitrite in vivo (Kaplan et al., 1974a, b) and the independence of this response from factors known to be essential for nitrate induction of nitrate reductase (light, supplementation of respiratory substrates), (Lips et al., 1973) led us to consider the possibility of the activation of a preexisting nitrate reductase by nitrite. An inactive form of nitrate reductase in *Chlorella* was demonstrated by Solomonson et al. (1973).

The results presented in this paper indicate that nitrite elicits nitrate reductase activity in homogenates of uninduced plants grown in the absence of nitrate. The cofactor requirements of the activated nitrate reductase are the same as those of the nitrate-induced enzyme. Temperature and pH sensitivity of NR-NO_2 are different from those characteristic of NR-NO_3. The activity of NR-NO_2 in vitro decays faster than that of NR-NO_3, a situation similar to that described for the nitrite-activated enzyme in vivo (Kaplan et al., 1974b).

Nitrite activation of nitrate reduction was rapid, 92% of the maximum observed being detected 5 min after addition of nitrite to the extract. Maximum activation is obtained between 5 and 10 min after nitrite addition, and this is followed by a rapid decay of activity – 56% of maximum after 30 min activation at 30°.

Nitrite activation of nitrate reductase is observable in extracts from plants with suboptimal levels of nitrate. The lower the values of NR-NO_3, the larger is the capacity of nitrite to activate nitrate reductase. The two nitrate reductase enzymes considered can be separated on a DEAE-cellulose column.

The data presented here indicate that NR-NO_3 and NR-NO_2 are not identical, and the physiologic significance of NR-NO_2 is uncertain since nitrite does not accumulate in plants under normal growth conditions. However, the fact that NR-NO_2 developes as a function of the disappearance of NR-NO_3 may indicate that we are dealing with two interchangeable forms of nitrate reductase: One of them (NR-NO_3) develops in response to nitrate in vivo; the second form (NR-NO_2) is an inactive form that may be activated in vitro or in vivo by nitrite. Our preliminary work with DEAE-cellulose columns indicates that the two forms differ in their protein conformation or structure.

This view may parallel the work of Nason et al. (1971) with the *Neurospora* nitrate reductase, showing that the subunit of the enzyme containing molybdenum is constitutive and only the subunit responsible for NADH oxidation is inducible. The effect of nitrite on nitrate reductase in vitro reported here, suggests that nitrite activates an inactive form of nitrate reductase that is present in plants under suboptimal nitrate nutrition conditions. The nature of the activation process is at present under investigation.

References

Hewitt, E.J.: Assimilatory nitrate-nitrite reduction. Ann. Rev. of Plant Physiol. **26**, 73–100 (1975)

Hewitt, E.J., Nicholas, D.J.D.: Enzymes of inorganic nitrogen metabolism. In: Modern Methods of Plant Analysis. Vol. 7, pp. 67–172. Berlin-Göttingen-Heidelberg-New York: Springer 1964

Ingle, J., Joy, K.W., Hageman, R.H.: The regulation of activity of the enzymes involved in the assimilation of nitrate by higher plants. Biochem. J. **100**, 577–588 (1966)

Kaplan, D., Roth-Bejerano, N., Lips, S.H.: Nitrate reductase as a product inducible enzyme. Europ. J. Biochem. **49**, 393–398 (1974a)

Kaplan, D., Roth-Bejerano, N., Lips, S.H.: Photosynthesis and the induction of nitrate reductase in plants. Proceedings of the 3rd International Congress of Photosynthesis. Rehovot. Israel. Vol. **2**: pp. 1517–1524 (1974b)

Lips, S.H., Kaplan, D., Roth-Bejerano, N.: Studies on the induction of nitrate reductase by nitrite in bean seed cotyledons. Europ. J. Biochem. **37**, 589–592 (1973)

Nason, A., Lee, K.Y., Pan, S.S., Ketchum, P.A., Lambert, A., DeVries, J.: In vitro formation of assimilatory reduced nicotineamide adenine dinucleotide phosphate: nitrate reductase from a Neurospora mutant and a component of molybdenum enzymes. Proc. Natl. Acad. Sci. USA **68**, 3242–3246 (1971)

Pistorius, E.K., Gewitz, H.S., Voss, H., Vennesland, B.: Reversible inactivation of nitrate reductase in Chlorella vulgaris in vivo. Planta **128**, 73–80 (1974)

Roth-Bejerano, N., Lips, S.H.: Induction of nitrate reductase in leaves of barley in the dark. New Phytologist. **72**, 253–257 (1973)

Solomonson, L.P.: Regulation of nitrate reductase activity by NADH and cyanide. Biochim. Biophys. Acta **334**, 297–308 (1974)

Solomonson, L.P., Jetschman, K., Vennesland, B.: Reversible inactivation of the nitrate reductase of Chlorella vulgaris Beijerinck. Biochim. Biophys. Acta **309**, 32–43 (1973)

Received 20 July; accepted 18 November 1977