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Nitrate Reductase as a Product-Inducible Enzyme

Drora KAPLAN, Nurit ROTH-BEJERANO, and Herman LIPS
Ben Gurion University of the Negev Department of Biology, Beer Sheva

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Nitrite is a very effective inducer of nitrate reductase. Nitrate induces the enzyme in darkness only upon addition of suitable sources of reducing equivalent which permit the formation of nitrite; nitrite itself brings about induction of the enzyme in darkness without supplementation of exogenous reducing cofactors. Inhibition of constitutive nitrate reductase by tungstate prevents induction of the enzyme by nitrate but not by nitrite. Since nitrate induces the enzyme only under conditions allowing production of nitrite while the induction capacity of nitrite is free of this constraint, nitrate reductase be considered a product-inducible rather than a substrate-inducible enzyme.

The ability of nitrate to induce nitrate reductase in higher plants has been widely accepted [1]. Nitrate alone, however, is not always sufficient to induce the enzyme in higher plants. Light [2] and CO_2 [3] are also necessary, suggesting that photosynthesis furnishes some essential component of the induction process triggered by nitrate. We have previously shown [4] that induction of nitrate reductase may be obtained by spraying suitable amounts of plant hormones on the leaves of tobacco plants in the dark. It has been shown also that adding certain respiratory substrates to nitrate in etiolated barley leaves allows induction of the enzyme in the dark [5]. Nitrate, therefore, promotes induction of nitrate reductase only under conditions which permit formation of nitrite. The reduction of nitrate requires photosynthesis of a carbon compound which yields, upon oxidation, the electrons necessary for the reduction. We have suggested [6] that glycolate is the photosynthetic compound required for nitrate reduction because the enzyme system which oxidizes glycolate and the enzyme-reducing nitrate are located in microbodies. Glycolate dehydrogenase activity has been detected in plants actively reducing nitrate [7].

Nitrate reduction takes place in the dark when the leaves are supplemented with suitable respiratory substrates [5] such as glucose, phosphoglyceric acid or glycolate; this exogeneous supply substitutes photosynthesis as a source of energy-yielding substrates for the reduction of nitrate and the generation of nitrite.

Since reduction of nitrate to nitrite takes place in both cases (photosynthesis or supplementation of suitable respiratory substrates), the possible involvement of nitrite as the inducer of nitrate reductase should be considered.

Enzyme. Nitrate Reductase [NAD(P)H] (EC 1.6.6.2).

MATERIALS AND METHODS

Plant Material

Etiolated barley (*Hordeum vulgare* L., var. *Esperanza*) leaves were obtained from seedlings germinated and grown in the dark for 7–9 days. The plants were grown in 0.5 mM $CaSO_4$ at 24 °C. Other barley seeds were germinated and grown in the light for the same period of time (7–9 days) in 0.5 mM $CaSO_4$. These plants constituted the source of "green leaves" used in some of the experiments described.

Induction Procedure

Induction of nitrate reductase was obtained by placing detached leaves in petri dishes (10 cm diameter) lined with filter paper impregnated with induction medium. The medium for induction consisted of KNO_3 or KNO_2 at concentrations indicated in every experiment, 0.2 ml 0.1% Tween and 10 µg chloramphenicol. The total volume of the induction medium was 10 ml. Respiratory substrates were added to the medium as indicated in text.

Homogenization

After induction, the leaves were thoroughly rinsed and immediately homogenized in 0.1 M Tris-HCl

buffer containing 30 μM EDTA. Homogenization was done using 2 ml medium/g tissue, with an ice-cold mortar and pestle. The homogenate was filtered through 2 layers of gauze and the filtrate used in enzyme assays.

Enzyme Assay

Nitrate reductase was assayed according to Hewitt and Nicholas [9]. Nitrite content of assay mixture was determined before and after the reaction period; enzyme activity was expressed as the increase of nitrite concentration.

Nitrate was determined as described by Sloan and Sublett [10], the procedure followed was based on the non-enzymatic reduction of nitrate to nitrite.

Application of Tungstate and Cycloheximide

Na_2WO_4 1 mM was given through the root system of the seedlings 24 h prior to leaf detachment and induction in petri dishes. Cycloheximide was added to the induction medium at a concentration of 100 μg/ml at times indicated in Fig. 4 and 5.

RESULTS

Nitrite as an Inducer of Nitrate Reductase

Nitrite is a more efficient inducer of nitrate reductase than nitrate on barley leaves (Fig. 1). Production of the enzyme in the tissue in response to nitrite is remarkably large in etiolated barley leaves. Both in dark and light-grown leaves, more nitrate reductase is induced after 24 h by 5 mM KNO_2 than by 50 mM KNO_3.

The induction of nitrate reductase by nitrite is very rapid initially, as in the case of bean seed cotyledons previously reported [8]. This rapid initial induction (first 2–3 h) is followed by a slowly decreasing level of enzyme activity. The initial rate of induction, therefore, may be the best indicator of the inductive capability of nitrate and nitrite. As in long-term induction experiments (24 h, Fig. 1), nitrite is more effective than nitrate during the first hours of induction. In light-grown barley leaves induced in light, 2 mM KNO_2 induce twice the level of the enzyme after only 3 h than 50 mM KNO_3 (Fig. 2). Etiolated barley leaves are capable of an intense and rapid induction of the enzyme in the presence of nitrite, while nitrate induces very little enzyme (Table 1).

Characteristics of the Enzyme Induced by Nitrite

Table 2 summarizes the dependence of the enzymes induced by nitrate and by nitrite on substrate and reducing cofactors. Nitrite-induced nitrate reductase depends on nitrate as the source of nitrite in the assay

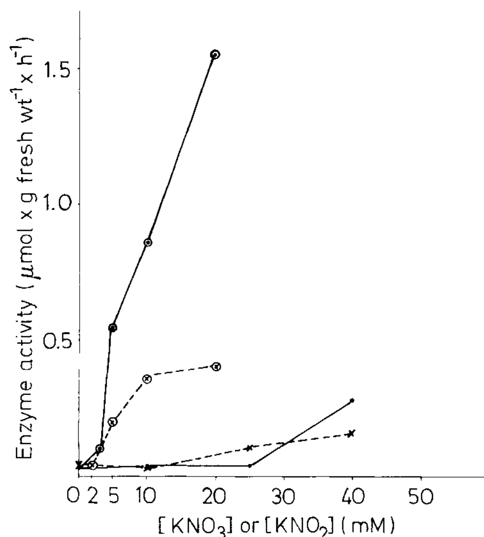

Fig. 1. *Induction of nitrate reductase in the presence of KNO_3 or KNO_2.* Barley leaves of plants grown in the dark (etiolated leaves) or in the light (green leaves) were induced in darkness for 24 h. Etiolated leaves in (●——●) KNO_3; (⊙——⊙) KNO_2; green leaves in (×----×) KNO_3; (⊗----⊗) KNO_2

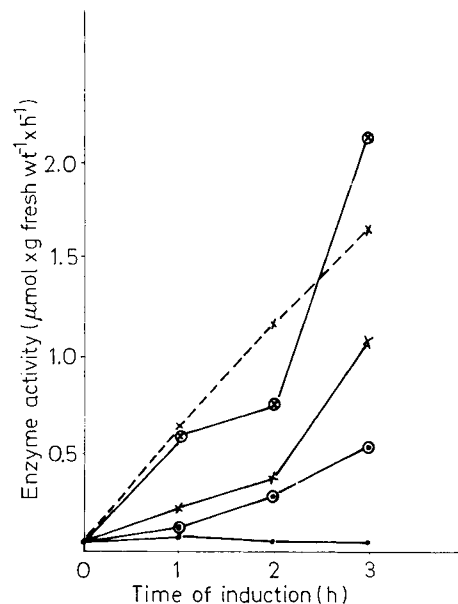

Fig. 2. *Short-term induction of nitrate reductase in the presence of KNO_3 or KNO_2.* Leaves of barley plants grown in the light were placed in induction media in the light for the periods of time indicated in the figure. (●) H_2O; (⊙——⊙) 50 mM KNO_3; (×——×) 5 mM KNO_2; (⊗——⊗) 10 mM KNO_2; (×----×) 20 mM KNO_2. Enzyme activity is expressed in terms of NO_2^-

Table 1. *Short-term induction of nitrate reductase*
Leaves of etiolated barley plants (grown in the dark) were placed in induction medium in the dark for periods of time indicated. Activity is expressed in terms of NO_2^- formed

Induction medium	Nitrate-reductase activity after		
	1 h	2 h	3 h
	nmol × g fresh wt^{-1} × h^{-1}		
H_2O	84	56	56
KNO_3 10 mM	56	112	112
KNO_3 25 mM	56	112	168
KNO_3 50 mM	28	28	112
KNO_2 2 mM	256	512	712
KNO_2 5 mM	312	940	1000
KNO_2 10 mM	350	1220	1500
KNO_2 20 mM	228	1284	1000

Table 2. *Cofactor requirements of nitrate reductase induced by KNO_3 or KNO_2 in etiolated barley leaves*
Induction period, 24 h in the dark

Cofactors added to assay			Activity after induction in	
KNO_3	NADH	NADPH	50 mM KNO_3	10 mM KNO_2
			nmol × g fresh wt^{-1} × h^{-1}	
+	+	−	371	712
−	+	−	312	140
+	−	−	42	183
+	−	+	107	192

mixture; NADH rather than NADPH, is required in both preparations as the donor of electrons.

The apparent independence of nitrate-induced nitrate reductase from added nitrate in the assay mixture is due to the presence of the substrate (nitrate) in the crude enzyme extract. The content of nitrate in the tissue (Table 3) after 24 h induction in nitrate is sufficient to support at least 15 min of nitrate reductase activity *in vitro*. This, apparently, is not the case with the nitrate content of tissue incubated in 10 mM KNO_2.

The lower rate of induction of the enzyme by KNO_3 as compared to that of KNO_2 cannot be ascribed to a limited uptake of nitrate by the leaves (Table 3). Oxidation of nitrite to nitrate cannot be, consequently, the reason for the greater efficiency of nitrite as an inducer of nitrate reductase.

Another characteristic of the inductive system is the production of an inactive protein in the presence

Table 3. *Nitrate and nitrite content of etiolated barley leaves after 24 h induction in the dark*
Induction media contained KNO_3 or KNO_2

Induction medium	NO_3^-	NO_2^-
	nmol/g fresh wt	
H_2O	traces	0
KNO_3 10 mM	117	21
KNO_3 25 mM	322	32
KNO_3 50 mM	1264	57
KNO_2 2 mM	147	71
KNO_2 5 mM	176	343
KNO_2 10 mM	235	628
KNO_2 20 mM	294	1457

of the inducer and tungstate [11]. Haymer *et al.* suggested that W replace Mo at the active site of the enzyme as a result of which an inactive protein is obtained. In barley leaves, tungstate inhibits synthesis of active nitrate reductase after induction of nitrate or nitrite (Fig. 4). These observations support the assumption that the same enzyme is synthesized in the presence of both nitrate or nitrite.

Effect of Glycolate and NADH on Induction of Nitrate Reductase

The slow induction of nitrate reductase in etiolated barley leaves by nitrate in the dark may be significantly increased by addition of glycolate or NADH to the induction medium (Fig. 3). Addition of the same compounds to nitrite under identical experimental conditions, has no stimulatory effect on the rate of induction. The addition of electron donors is apparently unnecessary to obtain high rates of induction in the dark if nitrite is the inducer but the same compounds stimulate very effectively the slow induction of the enzyme in the presence of nitrate.

Inhibition of Constitutive Nitrate Reductase in the Dark

Fig. 4 shows the results of a series of treatments designed to test the requirement of the activity of the constitutive nitrate reductase on the nitrate-mediated induction of the enzyme. The constitutive level of nitrate reductase, present in tissue not exposed to any inducer (treatment 2 in Fig. 4), may be inhibited by adding 1 mM Na_2WO_4 to the nutrient medium of the seedlings during 24 h (treatment 1 in Fig. 4). Addition of nitrate (treatment 4, Fig. 4) and nitrite (treatment 5, Fig. 4) to leaves without tungstate brings about induction of nitrate reductase although at very different rates. If the addition of nitrate or nitrite is done to tungstate-pretreated leaves (treatments 3 and 6, Fig. 4) no in-

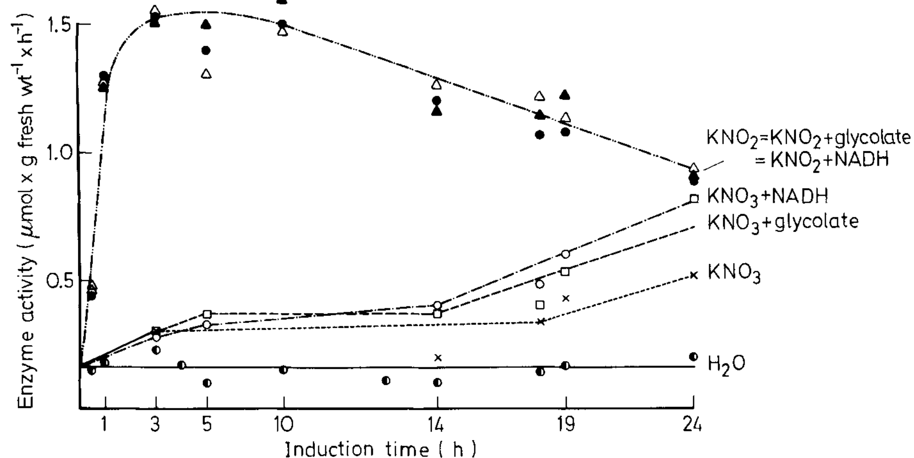

Fig. 3. *Kinetics of nitrate reductase induction in different media.* (◐) H_2O; (×) 50 mM KNO_3; (□) 50 mM KNO_3 + 50 mM glycolate; (●) 50 mM KNO_3 + 0.8 mg NADH; (○) 10 mM KNO_2; (△) 10 mM KNO_2 + 50 mM glycolate; (▲) 10 mM KNO_2 + 0.8 mg NADH

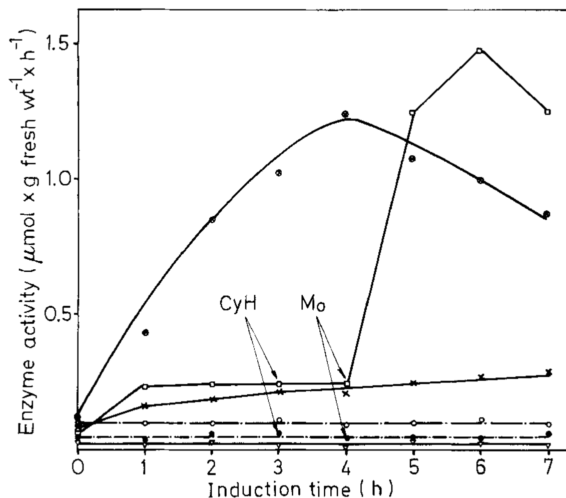

Fig. 4. *Induction of nitrate reductase in etiolated barley leaves in the dark.* Seedlings were grown in 0.5 mM $CaSO_4$ and some of them (▽, ●, □) were given in addition 1 mM Na_2WO_4 through the roots 24 h prior to induction. 1 mM Na_2WO_4 (▽); H_2O (○); 50 mM KNO_3 + 50 mM glycolate + 1 mM Na_2WO_4 transferred as indicated in figure to 100 μg/ml cycloheximide (CyH) and 10 mM Na_2MoO_4 (●); 5 mM KNO_2 + 1 mM Na_2WO_4 transferred as indicated in figure to 100 μg/ml cycloheximide and 10 mM Na_2MoO_4 (□); 50 mM KNO_3 + 50 mM glycolate (×); 5 mM KNO_2 (⊗)

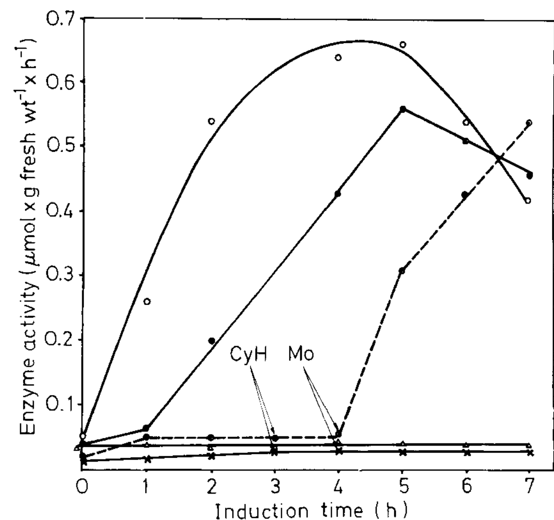

Fig. 5. *Induction of nitrate reductase in green barley leaves in the light.* Seedlings were grown in 0.5 mM Ca_2SO_4 and some of them (×, ⊙) were given in addition 1 mM Na_2WO_4 through the roots 24 h prior to induction. (○) 5 mM KNO_2; (⊗) 50 mM KNO_3; (△) H_2O; (×) 50 mM KNO_3 + 1 mM Na_2WO_4 transferred as indicated in figure to 100 μg/ml cycloheximide (CyH) and 10 mM Na_2MoO_4; (⊙) 5 mM KNO_2 + 1 mM Na_2WO_4 transferred as indicated in figure to 100 μg/ml cycloheximide and 10 mM Na_2MoO_4

crease in enzyme activity will be observed. Addition of molybdate after 4 h of induction will allow rapid activation of the protein synthesized in response to nitrite but no such activation was observed when nitrate was used as the inducer. Cycloheximide was added to prevent further synthesis of protein the addition of molybdate indicated, therefore, activation of the protein synthesized before cycloheximide addition [11].

Inhibition of Constitutive Nitrate Reductase in the Light

Fig. 5 shows the effect of nitrate and nitrite on the induction of nitrate reductase in green leaves, induced in light, after prior inhibition of the constitutive enzyme with tungstate. In this case too, it seems evident that when the constitutive nitrate reductase is inactive due to the exchange of its Mo by W, nitrate will not induce the synthesis of the enzyme protein

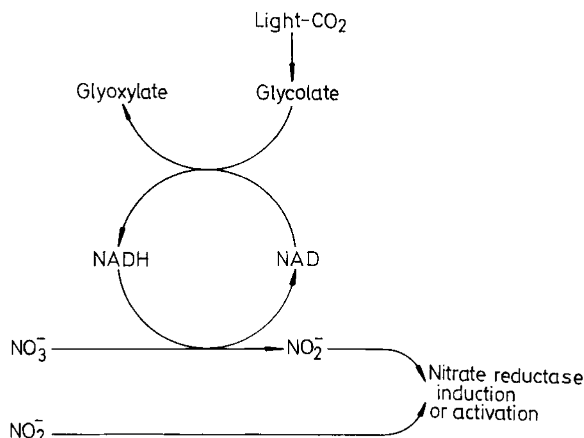

Fig. 6. *Interrelationship of several factors affecting induction of nitrate reductase in the presence of nitrate or nitrite*

while nitrite does. Activity of constitutive nitrate reductase is essential, therefore, for induction by nitrate but not for induction by nitrite.

The Interaction of Factors Affecting Induction of Nitrate Reductase

The interrelationships of light, CO_2, glycolate metabolism and the induction process of nitrate reductase as mediated by nitrate or nitrite have been schematically represented in Fig. 6.

DISCUSSION

The synthesis of nitrate reductase in plants adequately supplied with nitrate does not take place to any significant extent if the leaves are kept in the dark during the induction period. We have recently shown [5] that addition of glycolate, glucose or phosphoglyceric acid to nitrate induction medium, in the dark, will enhance induction of the enzyme. These compounds are oxidized by the tissue and may yield NADH, required to reduce nitrate. One may wonder, consequently, whether a product of the reduction of nitrate rather than nitrate itself is the inducer of nitrate reductase.

In view of these considerations and since cases of product-inducible enzymes are well known [12], we decided to study the possible involvement of nitrite in the induction of nitrate reductase.

The experimental results shown here indicate that nitrite is an effective inducer of nitrate reductase. Its advantage over nitrate is evident in all cases considered: etiolated or green barley leaves (Fig. 1), induction in dark or in light (Fig. 2), in short or long term induction periods (Fig. 1 and Table 1).

The enzyme induced by nitrite is capable of producing nitrite from nitrate and requires NADH as a cofactor (Table 4); its activity is inhibited by adding tungstate to the induction medium (Fig. 4). These three characteristics are typical of nitrate reductase and the possibility of nitrite inducing a different nitrate-reducing enzyme becomes remote.

Leaf tissue exposed to nitrite will produce some nitrate. It may be argued that this resulting nitrate, rather than the nitrite originally supplied, is responsible for the induction of nitrate reductase. This explanation should be also discarded in view of the fact that the levels of the enzyme bear no relation to the amount of nitrate in the tissue (Table 3).

The hypothesis that nitrate reductase may be a product-inducible rather than a substrate-inducible enzyme is supported also by the differences observed in the rate of its synthesis when affected by glycolate or NADH (Fig. 3). We suggest that the rate of induction of the enzyme in the dark is limited by the supply or generation of NADH, necessary to reduce nitrate and obtain, subsequently, the nitrite required for the induction of the enzyme. One may expect, therefore, that nitrite itself would cause an immediate and fast production of the enzyme, at a rate unaffected by glycolate or NADH. This is, indeed, the case, as shown in Fig. 3.

Another way to test the inductive capacities of nitrate and nitrite is to determine whether induction of nitrate reductase is possible when most of the constitutive enzyme is inhibited. The basic assumption in this experiment is that nitrate may serve as a inducer of nitrate reductase only when converted to nitrite. If the constitutive enzyme is not operative due to the replacement of Mo by W at its active center, no reduction of nitrate to nitrite will be possible and, under these conditions, nitrate will not trigger the synthesis of inducible nitrate reductase. The inhibition of the constitutive enzyme, on the other hand, will not affect the production of inducible nitrate reductase by nitrite if the enzyme is a product-inducible protein. The results in Fig. 4 and 5 show that only nitrite-induced enzyme is unaffected by the inhibition of the constitutive enzyme, supporting the idea that nitrite is the inducer of the system.

Fig. 6 summarizes the observations made in our and other laboratories concerning the requirements and limitation of the nitrate reductase induction system. The enzyme may be induced by nitrite under all conditions because the synthesis of the enzyme is directly triggered by this compound. Induction by nitrate seems to be indirect and will be possible only when the reduction of nitrate to nitrite by the consti-

tutive enzyme is not limited by the availability of NADH. In leaves of higher plants NADH for the reduction of nitrate is supplied by the oxidation of a photosynthetic carbon compound (presumably glycolate) which rapidly disappears in the dark. Induction in the dark is possible, therefore, only when nitrite is supplied or nitrate is supplemented by suitable electron donors (Fig. 3).

The effect of nitrite in induction of nitrate reductase has been previously observed [13]. Its role in the induction of the enzyme has been presumably disregarded due to the relatively long induction periods usually studied (about 24 h). The initial fast induction of the enzyme in the presence of nitrite does not persist for more than 2 to 3 h in etiolated barley leaves, slowly declining thereafter (Fig. 3). After 24 h the level of nitrate reductase in nitrite is not significantly higher than in nitrate. This decreasing level of nitrate reductase activity after induction by nitrite may be the main reason which led to the general disregard of its inductive capability.

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D. Kaplan, N. Roth-Bejerano, and S. H. Lips, Department of Biology, Ben Gurion University of the Negev, Beer Sheva, Israel