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PHOTOSYNTHESIS AND THE INDUCTION OF NITRATE REDUCTASE IN PLANTS

D. Kaplan, N. Roth-Bejerano and S.H. Lips

Department of Biology,
Ben-Gurion University of the Negev
Beer Sheva, Israel

Summary

Nitrate induces nitrate reductase (NR) in darkness only upon addition of suitable sources of reducing equivalents which permit the formation of nitrite; nitrite itself allows induction of NR in darkness without supplementation of exogenous reducing cofactors. Inhibition of constitutive NR(tungstate) prevents induction of the enzyme by nitrate but not by nitrite. Since nitrate induces NR only under conditions allowing production of nitrite, while the inductive capacity of nitrite is free of this constraint - NR may be considered a product rather than a substrate inducible enzyme. The relative rapid effect of nitrite on the activity of NR detectable in crude extracts raises also the possibility that nitrite activates an inactive form of the enzyme without necessarily triggering its de novo synthesis.

1. Introduction

The ability of nitrate to induce nitrate reductase (NR) in higher plants has been widely accepted[1]. Nitrate alone, however, is not always sufficient to induce NR 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 NR induction 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 NR in the dark[5]. Nitrate, therefore, promotes NR induction 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, PGA or glycolate; this exogenous 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 an inducer or activator of NR should be considered.

2. Materials and Methods

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 25°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 of nitrate reductase (NR) was obtained placing detached leaves in Petri dishes (10 cm dia.) 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 of 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.

After induction, the leaves were thoroughly rinsed and immediately homogenized in buffer Tris-HCl 0.1 M containing 3×10^{-5} M EDTA. Homogenization was done using 2 ml medium/gm tissue, with an ice cold mortar and pestle. The homogenate was filtered through 2 layers of gauze and the filtrate used in enzyme assays.

Nitrate reductase (EC 1.6.6.2) was assayed according to Hewitt and Nicholas[9]. Nitrite content of assay mixture was determined before and after 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 hours 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 Figs. 4 and 5.

3. Results

Nitrite as an Inducer of NR. Nitrite is a more efficient inducer of NR than nitrate in barley leaves (fig. 1). NR production in the tissue in response to nitrite is remarkably large in etiolated barley leaves. Both in dark and light-grown leaves, more NR is induced after 24 hours by 5 mM KNO_2 than by 50 mM KNO_3.

The induction of NR by nitrite is very rapid initially, as in the case of bean seed cotyledons previously reported[8]. This rapid initial induction (first 2-3 hours) 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 hours,

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

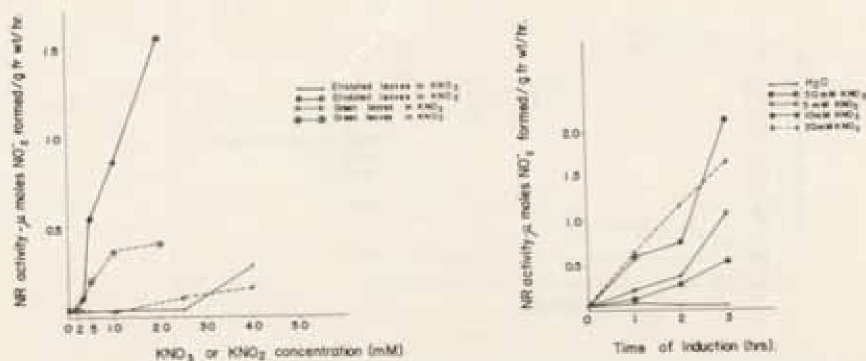

Fig. 1. (Left) Induction of NR in the presence of KNO_3 or KNO_2. Barley leaves of plants grown in the dark (etiolated leaves) or in the light (green leaves) were induced in darkness for 24 hours.

Fig. 2. (Right) 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 figure.

level of NR after only 3 hours than 50 mM KNO_3 (fig. 2). Etiolated barley leaves are capable of an intense and rapid induction of NR in the presence of nitrite, while nitrate induces very little enzyme (Table 1).

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

Induction medium	NR activity; mμmoles NO_2^- formed/gm fr wt/hr		
	1 hr	2 hrs	3 hrs
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

Effect of Glycolate and NADH on NR Induction.

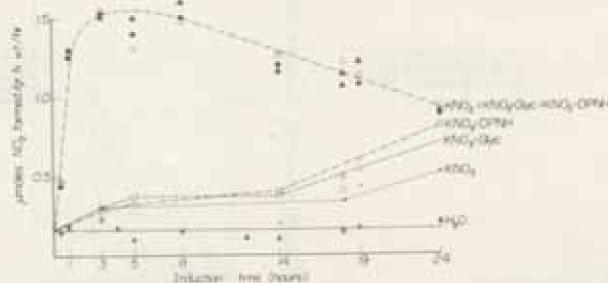

Fig. 3. Kinetics of nitrate reductase induction in different media

The slow induction of NR 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 NR induction. The addition of electron donnors is apparently unnecessary to obtain high rates of NR 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. Induction of NR in presence of tungstate results apparently in the synthesis of an inactive protein[11]. Haymer et al. suggested that W replaces Mo at the active site of the enzyme as a result of which an inactive NR protein is obtained. Figure 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 hours (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 NR 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 increase in NR activity will be observed. Addition of molibdate after 4 hours 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 molibdate indicates, therefore, activation

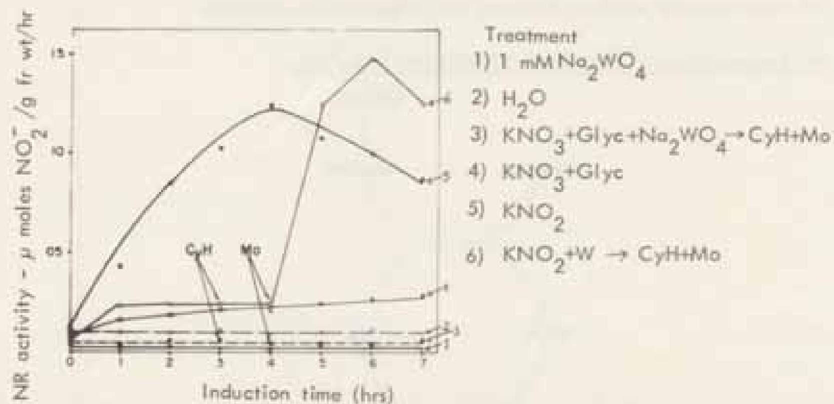

Fig. 4. Induction of nitrate reductase in etiolated barley leaves in the dark. Seedlings were grown in 0.5 mM $CaSO_4$ and some of them (1,3,6) were given in addition 1 mM Na_2WO_4 through the roots 24 hours prior to induction.

of the protein synthesized before cycloheximide addition[11].

Inhibition of Constitutive Nitrate Reductase in the Light. Figure 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 NR is inactive due to the exchange of its Mo by W, nitrate will not induce the synthesis of the NR protein while nitrite does. Activity of constitutive NR is essential, therefore,

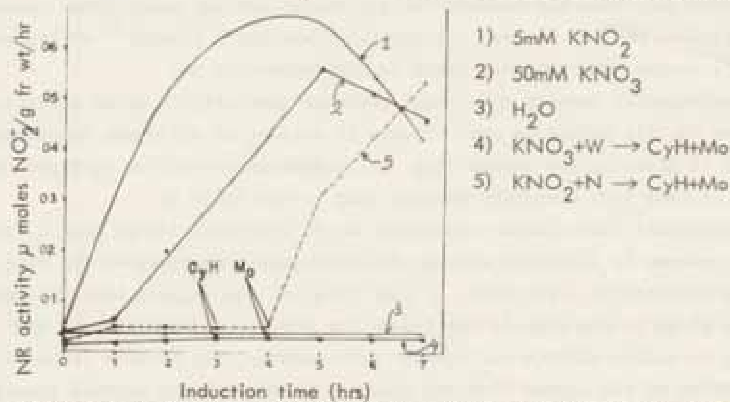

Fig. 5. Induction of nitrate reductase in green barley leaves in the light. Seedlings were grown in 0.5 mM Ca_2SO_4 and some of them (4,5) were given in addition 1 mM Na_2WO_4 through the roots 24 hours prior to induction.

for induction by nitrate but not for induction by nitrite.

The Interaction of Factors Affecting NR Induction.

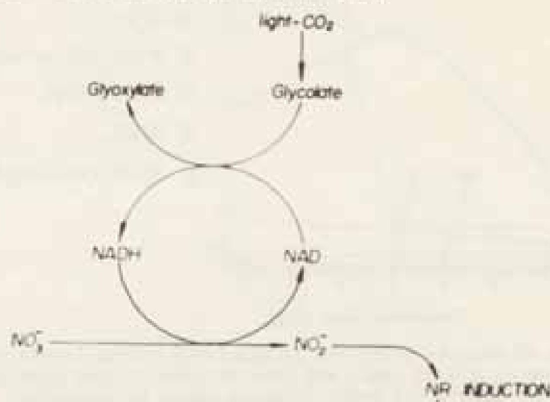

Fig. 6. Interrelationship of several factors affecting induction of nitrate reductase in the presence of nitrate or nitrite.

4. 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 previously shown[5] that addition of glycolate, glucose or PGA to nitrate induction medium, in the dark, will enhance induction of NR. These compounds are oxidized by the tissue and may yield NADH, required to reduce nitrate. One may wonder, consequently, whether a product[12] of the reduction of nitrate rather than nitrate itself is the inducer of NR.

The experimental results shown here indicate that nitrite is an effective inducer of NR. 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 hypothesis that nitrate reductase may be a product rather than a substrate inducible enzyme is supported also by the differences observed on the rate of NR synthesis affected by a glycolate or NADH (Fig. 3). We suggest that the rate of induction of NR 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 NR, at a rate unaffected by glycolate or NADH. This is, indeed, the case, as shown in Figure 3.

Another way to test the inductive capacities of nitrate and nitrite is to

determine whether induction of NR is possible when most of the constitutive enzyme is inhibited. The basic assumption in this experiment is that nitrate may serve as a NR inducer only when converted to nitrite. If the constitutive NR 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 Figs. 4 and 5 show that only nitrite-induced NR is unaffected by the inhibition of the constitutive enzyme, supporting the idea that nitrite is the inducer of the system.

Figure 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 constitutive enzyme is not limited (e.g., 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. NR induction in the dark is possible, therefore, only when nitrite is supplied or nitrate is supplemented by suitable electron donnors (Fig. 3).

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

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