Pergamon

PII: S0031-9422(96)00536-5

Phytochemistry, Vol. 44, No. 1, pp. 11–16, 1997
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0031-9422/97 $17.00 + 0.00

INVOLVEMENT OF MAGNESIUM AND ATP IN THE REGULATION OF NITRATE REDUCTASE ACTIVITY IN *SINAPIS ALBA*

RAUL HERRERA* and CHRISTOPHER B. JOHNSON†

Department of Botany, Plant Science Laboratories, The University of Reading, Whiteknights, P.O. Box 221, Reading RG6 6AS, U.K.

(*Received* 8 *July* 1996)

Key Word Index—*Sinapis alba*; Brassicaceae; mustard; nitrate reductase; enzyme modulation; phosphorylation; light.

Abstract—The activity of NADH: nitrate reductase (NR, EC 1.6.6.1) was extracted from mustard (*Sinapis alba*) cotyledons and leaves. When seedlings/plants were grown following light/dark cycles in the presence of nitrate, an increase in the activity of NR was observed during light periods compared to dark periods. An inhibition in NR activity was observed after treatment with Mg^{2+} and ATP in extracts from both cotyledons and leaves prepared from tissue harvested during light periods. No apparent inhibition was observed in tissue extracts harvested during dark periods. Two isoforms of mustard NR (NR_1 and NR_2) were separated by the use of anion exchange chromatography. The results indicate that NR_2 (the more negatively charged isoform) corresponds to a light-inducible form, which was selectively and strongly inhibited by Mg^{2+}/ATP. The results presented here indicate that the modulation of NR activity by light can be attributed, at least in part, to a protein reversible phosphorylation/dephosphorylation mechanism on NR_2. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

A drop in the activity of nitrate reductase (NR, EC 1.6.6.1) is usually observed in plants when transferred from light to darkness. The activity rises when plants are transferred back to light. The response could take between minutes and hours, but no decisive evidence exists so far clarifying the mechanism(s) participating in such regulation, although there is evidence that the fluctuations observed in the activity of NR could be an effect of regulation at all levels of gene expression: transcriptional, translational and post-translational [1–3].

In general, the activity of an enzyme could be modulated post-transcriptionally by a series of mechanisms which include glycosylation, acetylation, ADP-ribosylation and phosphorylation. Modulation of enzyme activity by phosphorylation has been reported extensively in recent years; for example, the stromal enzymes pyruvate Pi dikinase and RuBisCO have been reported to be phosphorylated *in vivo* [4, 5]. Similarly, the cytosolic enzymes phosphoenolpyruvate carboxylase [6] and sucrose phosphate synthase are reversibly phosphorylated [7].

Phosphorylation and dephosphorylation have been postulated as providing a regulatory mechanism of NR activity for different species, such as pea [8], squash [9] and spinach [10]. Huber *et al.* [11] have reported that the presence of magnesium and ATP results in an inhibition of NR activity, comparable to the NR activity obtained from dark-treated spinach leaves. This post-translational mechanism appears to have an important role in regulating the activity of the enzyme in light/dark transitions. The most dramatic example is a five- to 10-fold drop in NR activity in spinach within minutes following dark treatment [11, 12], in which the presence of magnesium is essential. Furthermore, Huber *et al.* [11] were able to phosphorylate serine groups *in vivo* in NR with inorganic [^{32}P]phosphate, allowing inactivation of the enzyme activity. A phosphatase protein has been identified as being responsible for the removal of the phosphate group, with the consequent activation of the enzyme activity [10]. Recently, a protein inhibitor of NR activity has been isolated which interacts with the phosphorylated form of NR in spinach [13, 14] and a post-translational regulatory site on the NR protein has been identified in both spinach [15] and *Arabidopsis* [16].

It has been suggested that the regulation of NR activity involves a link between the carbon and the nitrogen pathways [17–19]. A reduction in NR activity has been observed when leaves are exposed to low levels of CO_2, and the effect was reversed after transferring the leaves to air [12]. The NR activity extracted from leaves supplied with air had previously been demonstrated to be highly sensitive to inhibition

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*Present address: Departamento de Ciencias Biológicas, Facultad de Recursos Naturales, Universidad de Talca, 2 Norte 685, Chile.

†Author to whom correspondence should be addressed.

by trinucleotides [20]. In addition, the enzyme extracted from dark-treated spinach leaves was shown to be more sensitive to inhibition by $MgCl_2$ [3]. The same authors that had previously demonstrated the phosphorylation of the spinach enzyme have suggested that a Mg^{2+} dependent phosphorylation/dephosphorylation reaction could represent the mechanism involved in the regulation of NR activity by light [10–12]. The cation Ca^{2+} has also been found to inhibit the NR activity in squash [21] and barley [22].

Mustard (*Sinapis alba* L.) NR is a NADH-linked enzyme which is regulated by nitrate and light [23]. The appearance of two NR isoenzymes in cotyledons has been reported in response to nitrate and light (20). The appearance of these two NR activities can be modulated via phytochrome and has been claimed to be exclusively due to *de novo* synthesis of proteins (24).

The purpose of the present study was to determine the effect of Mg^{2+}/ATP on the NR activity in mustard leaves and cotyledons grown under day/night cycles, and the effect of Mg^{2+}/ATP on the two isoenzymes.

RESULTS

Effect of Mg^{2+}*/ATP on NR activity*

The removal of low molecular weight compounds from the protein extracts was considered essential due to the presence of cations or anions which could interfere with the enzymatic assay and the inhibitory studies. In fact, when the NR activity assays in crude and desalted extracts were compared, an increase of approximately 40% in the activity was observed after desalting (data not shown). When desalted extracts from *S. alba* tissues were assayed in the presence of Mg^{2+}/ATP, different inhibition rates were observed (Table 1). Light extracts from both mature and young seedlings were more sensitive to inhibition with Mg^{2+}/ATP than were dark extracts. NR activity from light treated plants was inhibited by 33–39%, compared with dark-treated material where inhibition values of 9% and 7% for mature plants and cotyledons, respectively, were observed. It may be noted that the activity of light treated tissues incubated with Mg^{2+}/ATP is comparable to the activity of dark-treated tissues without treatment.

Table 1. Effect of Mg^{2+}/ATP on NR activity*

	Nitrate reductase activity (pKat/g fresh wt)	
	Mature plant	Young seedling
Light		
Control	258±14 (100)	65.9±5 (100)
Mg^{2+}/ATP	158±12 (61)	44.4±3 (67)
Dark		
Control	153±19 (100)	41.7±3 (100)
Mg^{2+}/ATP	139±11 (90)	38.9±3 (93)

*The desalted extracts were preincubated in the presence of $MgCl_2$ (5 mM) and ATP (1 mM) for 15 min at 25°. The enzymatic assay was performed as described in the Experimental. The percentage of the control activity is expressed in parentheses.

Effect of Mg^{2+}*/ATP on the two NR isoforms*

Schuster *et al.* [23] reported the appearance of multiple forms of NR in 90-hr old mustard cotyledons. They found two isoenzymes in the presence of NO_3^- after light treatment, but only one in the dark. We have found both isoforms in 60-hr old cotyledons and mature plants following a 12 hr light/12 hr dark cycle. These isoforms were found in both light- and dark-treated mustard plants (Figs 1 and 2). The first isoform (NR_1) was eluted from an anion exchange column after addition of approximately 245 mM Cl^-, and the second isoform (NR_2) at a higher concentration of Cl^- (320 mM). Based on the results obtained (Figs 1 and 2), it could be predicted that the two NR isoforms (NR_1 and NR_2) have different charge properties, with NR_2 apparently more negatively charged than NR_1. In general, the activity of NR_1 was higher than the activity of NR_2. In dark conditions a general decrease in NR activity was observed.

Protein fractions previously separated through the Mono Q column and containing NR activity were preincubated with magnesium (5 mM) and ATP (1 mM) for 15 min at 25° with gentle shaking, and subsequently assayed for NR activity. The results indicate that NR_2 separated from light-treated mature plants was inhibited extensively by Mg^{2+}/ATP (Fig. 1(B)). When the same fractions were preincubated in buffer but in the absence of Mg^{2+}/ATP, no inhibition in the NR activity was observed (Fig. 1(A)). No inhibition of NR_2 activity was observed in the Mono-Q fractions obtained from dark-treated mature plant leaves (Fig. 2), although the activity of NR_2 was lower than that of NR_1.

A quantitative analysis was carried out by measuring the area of each peak and estimating the proportion of NR activity of each isoform. The total activity recovered from the column was used as reference. The results showed that NR_2 extracted for light-treated plants and cotyledons was inhibited by Mg^{2+}/ATP treatment (Table 2), while no apparent inhibition was observed on NR_1. In contrast, neither of the two NR isoforms extracted from dark-harvested plants suffered significant inhibition by Mg^{2+}/ATP.

A similar behaviour was observed in young seedlings. In light-treated cotyledons, NR_2 was inhibited by Mg^{2+}/ATP treatment (Table 2). As with mature plants, no inhibitory effect of Mg^{2+}/ATP was observed on NR activity extracted from dark-treated seedlings or on NR_1 even in light-treated plants. In short, a similar response to inhibition by Mg^{2+}/ATP of NR_1 and NR_2 isoforms was observed for young seedlings and mature plants.

Two explanations for the apparent selective inhibition are possible: either the inhibition is specific for

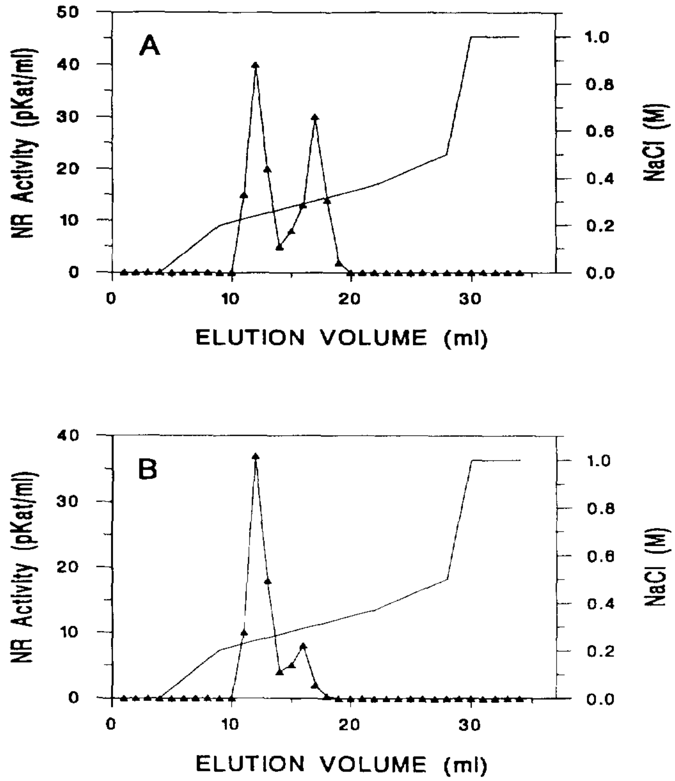

Fig. 1. Effect of Mg^{2+}/ATP on NR fractions from light-treated plant extracts. Leaf extract of light-treated mustard plants was applied to a Mono Q column. Each fraction collected was preincubated for 15 min at 25° in the presence of extraction buffer (A) or with 5 mM $MgCl_2$ and 1 mM ATP (B).

NR_2, or the molecule responsible for the inhibition eluted together with the NR_2 fractions after the Mono Q separation, but not with the NR_1 fraction. In order to clarify this point, desalted extracts were preincubated with Mg^{2+}/ATP and NR activity was assayed in each fraction obtained after the Mono Q separation (Fig. 3(A)). The results indicate that NR_2 from light-treated plants was selectively inhibited by Mg^{2+}/ATP. It can be concluded, therefore, that the inhibition of NR_2 is due to its sensitivity to inhibition by Mg^{2+}/ATP. On the other hand, in the dark extract no inhibition of the NR_2 isoenzyme activity was observed (Fig. 3(B)). These results suggest that light plays a fundamental role in the regulation of the NR activity and, as far as the phosphorylation mechanism is concerned, this regulation should be addressed to NR_2.

DISCUSSION

Modulation of NR activity

Sephadex G-25 columns were used to remove low molecular weight compounds. It could be observed that the activity of light- or dark-treated extracts increased after desalting. Apparently, the enzyme is inhibited by an endogenous low molecular weight molecule. Interestingly, the enzyme from spinach CO_2-treated leaves remains inhibited even after low molecular weight compounds are removed by the same procedure [25]. Low molecular weight molecules acting as enzyme modulators could be involved in the rapid response of NR activity to a light/dark transition. For example, Riens and Heldt [3] reported the inactivation of NR with a half-life of 2 min after sudden darkening.

The work reported here supports the role of phosphorylation of the enzyme as a possible mechanism of regulation of its activity. Mustard NR of light-treated mature plants was shown to be strongly inhibited by the presence of Mg^{2+}/ATP. Huber *et al.* [11], working with spinach, had found the same behaviour in the presence of Mg^{2+} and ATP; however, they also found that the enzyme extracted from material which had been kept in the dark was more sensitive to divalent cation (Mg^{2+}) inhibition. In general, NR from both mustard and spinach extracted from light-treated material is inhibited by Mg^{2+} and ATP.

Separation of two NR isoforms in mustard

The separation of two isoforms in mustard agreed with the previous results reported by Schuster *et al.* [23]. We found both isoenzymes in light- and dark-treated extracts, and the regulation of the NR activity resulting from the day/night transition seems to be primarily over NR_2. The activity of NR_2 in both mature

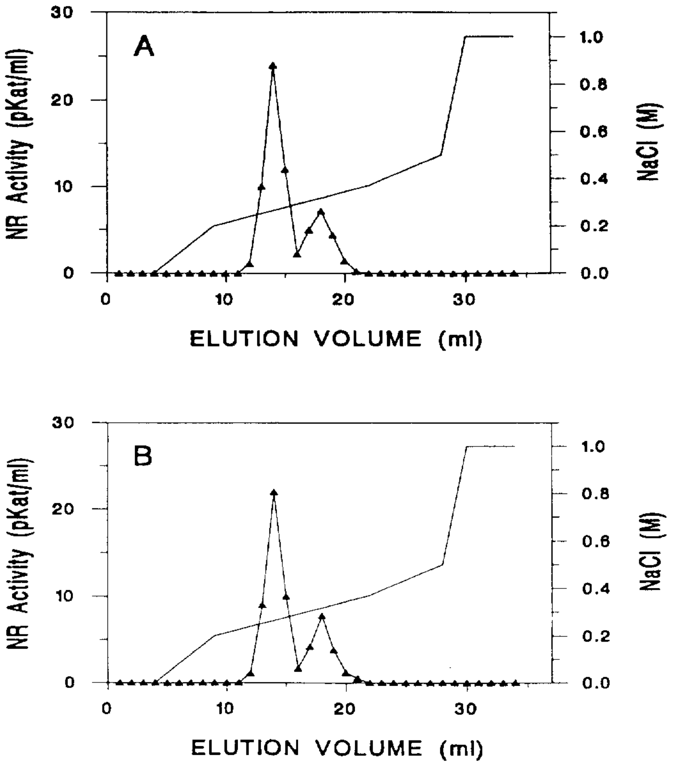

Fig. 2. Effect of Mg^{2+}/ATP on NR fractions from dark-treated plant extracts. Activity profile of leaf extract from dark-treated mustard plants applied on a Mono Q column. Each fraction collected was preincubated for 15 min at 25° in the presence of extraction buffer (A) or with 5 mM $MgCl_2$ and 1 mM ATP (B).

plants and young seedlings was affected by Mg^{2+}/ATP, being inhibited in both cases to the same extent. Not surprisingly, the effect of Mg^{2+}/ATP was on NR_2 because this isoform is the predominant form induced by light [21]. The inhibition by Mg^{2+}/ATP was observed in both plants and young seedlings in a light regime. No response could be observed for the activity extracted after dark periods.

Table 2. Quantitative analysis of NR inhibition by Mg^{2+}/ATP

	Nitrate reductase activity (pKat ml^{-1})	
	Without Mg^{2+}/ATP	With Mg^{2+}/ATP
Plants		
Light	NR_1 5.88 (52)	5.65 (50)
	NR_2 5.43 (48)	2.03 (18)
Dark	NR_1 3.68 (71)	3.63 (68)
	NR_2 1.51 (29)	1.51 (29)
Cotyledons		
Light	NR_1 3.01 (58)	3.12 (60)
	NR_2 2.18 (42)	0.57 (11)
Dark	NR_1 1.42 (63)	1.35 (60)
	NR_2 0.83 (37)	0.83 (37)

*The area of each peak eluted from the Mono Q column was measured to establish the isoform proportion. The proportional activity of each isoform was established by comparison with the total NR activity which correspond to the total activity collected from the column. Figures in brackets correspond to the percentage of activity of each peak.

To establish that the inhibition by Mg^{2+}/ATP on the separated NR_2 was in fact the phenomenon observed, two approaches were used. First, the preincubation of each separated fraction with Mg^{2+}/ATP; and, secondly, the preincubation of the extract with Mg^{2+}/ATP prior the separation of the isoforms through the Mono Q column. In both cases, only the second isoform suffered the inhibition of the activity in light-treated material. On the other hand, NR_2 was insensitive to inhibition when the extract was prepared from dark-acclimatized material. Thus it appears that NR_2 has been activated in the light in a manner that can be reversed by the treatment with Mg^{2+}/ATP. The reversibility of the inactivation process has been confirmed previously after the addition of AMP and the reactivation of the inhibited enzyme in pea [26] and spinach [27]. However, such reversibility in *Sinapis* NR was not obvious (data not shown).

The data presented here indicate that phosphorylation/dephosphorylation could play a role *in vivo* in the activation and inhibition of mustard NR. Light induces the activation of NR activity, and during dark periods phosphorylation of the enzyme could take place, inhibiting its activity. Furthermore, this phosphorylation must be selectively on the second isoenzyme (NR_2). This mechanism could explain the reduction in the NR

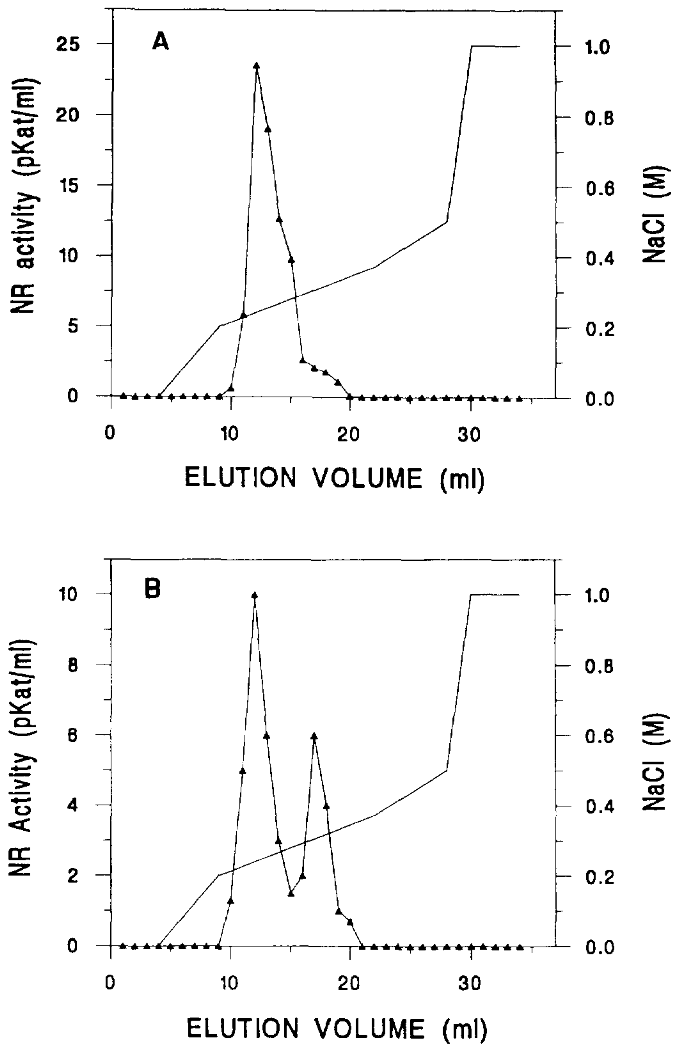

Fig. 3. Effect of Mg^{2+}/ATP on NR activity from light- and dark-treated cotyledon extracts. The enzyme from light-treated cotyledons (A) and dark-treated cotyledons (B) was extracted, desalted, and preincubated with $MgCl_2$ (5 mM) and ATP (1 mM) for 15 min at 25°. After incubation, the isoforms were separated using a Mono Q column. The enzymatic assay was performed directly on the fractions collected.

activity in plant leaves observed during dark periods. However, further experiments need to be done in order to establish the precise mechanism involved in the inhibition by Mg^{2+}/ATP. In spinach, several protein kinases have been isolated that phosphorylate NR [28]. Furthermore, a protein inhibitor has been recognized which reacts specifically with a phosphorylated NR [29–30]. Similarly, in both spinach [11] and mustard two populations of NR with different sensitivities to divalent cations have been found to be present.

EXPERIMENTAL

Plant material and growth conditions. Seeds (*S. alba* L.) were obtained from Suttons Seeds, Torquay, Devon, U.K., and germinated on 1.5% agar in growth chambers. They were allowed to grow in the presence of 40 mM KNO_3 following a 12 hr day/night cycle for $2\frac{1}{2}$ days at 2°. White light was supplied by the use of a bank of warm-white fluorescent tubes. The enzyme was extracted from cotyledons collected after 3 hr of light period (light-treated) or after 3 hr of dark period (dark-treated).

Mature plants were grown on compost in the greenhouse following the natural day/night cycle (January–February) for 1 month before enzyme extraction. The material was watered every day with a 40 mM KNO_3 solution. The enzyme was extracted from mature plants 3 hr after sunset (dark-treated) or sunrise (light-treated). At least three replicates per treatment were performed.

Protein extraction. The tissue (0.5 g) was frozen in liquid nitrogen and ground using a mortar and pestle in the presence of extraction buffer (1 ml), consisting of 100 mM MOPS-KOH (pH 7.5), 0.1 mM EDTA and 830 mg ml^{-1} FAD. The homogenate was centrifuged at 14 000 *g* for 20 min. The supernatant obtained was filtered by using a Millipore filter (0.45 μm) and then desalted on a Sephadex G-25 column (PD-10 Pharmacia) pre-equilibrated with extraction buffer minus EDTA.

NR enzymatic assay. The incubation with Mg^{2+}/ATP was performed in extracts previously desalted on Sephadex G-25 columns. When appropriate, the extract was preincubated for 15 min at 25° in the presence of either $MgCl_2$ (5 mM) and ATP (1 mM) or the extraction buffer (control) before the assay of NR activity. The enzymatic assay was carried out by mixing 180 μl substrate (0.5 mM NADH, 1.4 mM KNO_3) and 100 μl of protein extract. The mixture was incubated for 30 min at 37°. The reaction was stopped by the addition of 500 μl sulphanilic acid (1% w/v in HCl) and 500 μl *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.1% w/v in H_2O) and the reaction product was measured at 540 nm in a spectrophotometer [28]. A calibration curve with nitrite was prepared to transform the absorbance into concentration. All determinations were made in triplicate and the results expressed as the means±S.E. NR activity was expressed in Katals (moles of NO_2^- produced per second).

Anion exchange chromatography. A Mono Q 5/5 HR column (0.5 cm × 5.0 cm) attached to a Pharmacia FPLC (LCC-500) system was used. The column was pre-equilibrated with 20 mM potassium phosphate buffer (pH 6.6). An overloading of the column was made, each time applying samples of 2 ml and performing a short wash with phosphate buffer between the applications. The elution was made using increasing concentrations of phosphate buffer containing 1 M NaCl at a flow rate of 1 ml min^{-1}, and fractions of 1 ml were collected and assayed. In most of the runs, the activity obtained after separation of the isoforms was high enough for further inactivation assays to be performed.

Acknowledgement—This work was supported by a Beca Presidente de la República Fellowship from the Government of Chile to R. H.

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