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Modifications of the activity of nitrate reductase from cucumber roots

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Abstract

The regulatory properties of NADH-dependent nitrate reductase (NR) in desalted root extracts from hydroponically grown cucumber (*Cucumis sativus* L.) seedlings were examined. The lowest activity of NR was detected in extracts incubated with Mg^{2+} and ATP. An inhibitory effect of Mg-ATP was cancelled in the presence of staurosporine (the protein kinase inhibitor) and completely reversed after addition of ethylenediaminetetraacetate (EDTA) as well as AMP into reaction mixture. Reactivation of enzyme due to AMP presence, contrary to the chelator-dependent NR activation, was sensitive to microcystin LR (the protein phosphatase inhibitor). Above results indicated that the nitrate reductase in cucumber roots was regulated through reversible phosphorylation of enzyme protein. A drop in the activity of NR was also observed after incubation of enzyme at low pH. At low pH, the presence of ATP alone in the incubation medium was sufficient to inactivate NR, indicating that H^+ can substitute the Mg^{2+} in formation of an inactive complex of enzyme. ATP-dependent inactivation of NR at low pH was prevented by staurosporine and reversed by AMP. However, AMP action was not altered by microcystin LR suggesting that in low pH the nucleotide induced reactivation of NR is not limited to the protein phosphorylation.

Additional key words: AMP, ATP, *Cucumis sativus*, magnesium, phosphorylation, regulation of nitrate reductase activity.

Introduction

The first reaction of nitrate assimilation pathway in plants catalyzed by the nitrate reductase (EC 1.6.6.1) is a rate-limiting step in acquisition of the nitrogen in higher plant (Campbell 1999, Sauer and Frébort 2003, Cruz *et al.* 2004). Thus the nitrate reductase is subjected to the tight, metabolic control comprise the transcriptional and posttranscriptional levels (Hoff *et al.* 1994, Campbell 1999). Transcriptional control involves the changes in *Nia* gene expression, whereas a posttranslational modulation of NR activity is due to the modifications of enzyme protein (Kaiser and Huber 1994a). The post-translational modification of NR occurs in response to the changes of different environmental factors and its effect, manifested as a decrease or increase in enzyme activity, is clearly visible already after few minutes. Increasing evidences indicated that in a green tissue a crucial role in a regulation of enzyme activity plays the reversible phosphorylation of NR protein. In many plant species, phosphorylation of conserved serine residue (ser 543 in spinach) in H1 region of NR by the serine-threonine protein kinase is followed by association of the phosphorylated enzyme with the 14-3-3-type inhibitor protein (IP) (Bachmann *et al.* 1996a,b). This association can occur only in the presence of divalent cations (Mg^{2+}) and converts NR into an inactive form (Bachmann *et al.* 1996c, Athwal *et al.* 1998a). Reactivation of nitrate reductase requires dephosphorylation of the enzyme protein which is catalyzed by okadaic acid-sensitive phosphatatse 2A and followed with a subsequent dissociation of 14-3-3 protein from an inactive complex (Huber and Huber 1995). This fast and direct protein modification, which allows the rapid adaptation of plants to changes in environmental conditions such as irradiance, carbon dioxide and oxygen availability, or low temperature, has been well described for NR in green tissues (Kaiser *at al.* 1999, 2000, Yaneva *et al.* 2002). In this study we present data demonstrating that also in the root tissues operates similar posttrancriptional modification of NR protein altering its activity. The role of the protein kinases and phosphatases in regulation of the root NR

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Abbreviations: NR - nitrate reductase; BSA - bovine serum albumin; DTT - dithiotreitol; DMSO - dimethylsulfoxide; EDTA - ethylenediaminetetraacetate; Hepes - hydroxyethylpiperazine ethanesulfonic acid; PMSF - phenylmethylsulphonyl fluoride; PVPP- polyvinylpolypyrrolidone.
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was examined using the specific inhibitors and excised roots as well as the partially purified extract from cucumber roots. Evidence is presented that NR activity in root tissues was sensitive to magnesium ions and that effect could be mimicked by low pH.

Materials and methods

Cucumber (*Cucumis sativus* L. cv. Wisconsin) plants were grown hydroponically for 6 d in N-free solution and then for 1 d in 1/4 strength Hoagland solution under irradiance of 150 μmol m^{-2} s^{-1}, 16-h photoperiod and day/night temperature 25/22 °C. After 6 h of the light period the roots of 7-d-old seedlings were cut, washed briefly with distilled water and frozen in the liquid nitrogen. Frozen roots (1 g) were ground in a chilled mortar with the extraction buffer (1.5 cm^3) containing 50 mM Hepes - KOH (pH 7.5), 1 mM dithiotreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 % bovine serum albumine (BSA) and 1 mM polyvinylpoly-pyrrolidone (PVPP). After filtration of extract through 4 layers of cheesecloth and centrifugation for 15 min at 4 °C and 15 000 *g*, the supernatant was desalted on *Sephadex G-25 Fine* equilibrated with extraction buffer and used as an enzyme source.

Nitrate reductase activity was determined in the presence (actual enzyme activity) or absence (maximal enzyme activity) of $MgCl_2$, according to Kaiser and Huber (1997) with some modifications. The reaction medium contained 50 mM Hepes-KOH (pH 7.5 or 6.0, as indicated in text), 5 mM $MgCl_2$ or 5 mM ethylene-diaminetetraacetate (EDTA), 10 mM KNO_3 and desalted root extract. The reaction was started with 0.2 mM NADH and, after 5 min incubation in 27 °C, the reaction was stopped with 0.066 cm^3 of 1 M zinc acetate. The mixture was centrifuged and the amount of nitrite was determined colorimetrically with sulphanilamide and N-(1-naphthyl)ethylenediamine (Hageman and Reed 1980) and read at 540 nm on spectrophotometr *Beckman DU640*.

Effect of divalent cations (Mg^{2+}) and adenine nucleotides (ATP and AMP) on the activity of nitrate reductase was measured after addition them into the reaction medium. The mixture was preincubated without substrates (KNO_3 and NADH) at time indicated in figures. After preincubation of samples, KNO_3 and NADH were added and the nitrate reductase activity was measured as described above. In some experiments the specific inhibitors of protein kinases and phosphatases (staurosporine or microcystin LR) were included into reaction medium in the concentrations indicated in Results. Staurosporine, the serine-threonine protein kinases inhibitor, was dissolved in 50 % DMSO whereas microcystin LR (an inhibitor of protein phosphatases of type 1 and 2A) was dissolved in distilled water.

Results

Effect of Mg^{2+} ions and pH of reaction medium on the nitrate reductase activity: Desalted supernatant obtained from the cucumber roots (pH 7.5 and 6.0) was incubated 15 min alone (control) and with 5 mM $MgCl_2$ or 5 mM EDTA. NR activity determined at pH 6.0 was significantly lower than at pH 7.5 (Table 1). The presence of EDTA elevated the NR activity at the both pH, although the activity determined at pH 6.0 was still lower than at pH 7.5. The addition of Mg^{2+} into the samples incubated at pH 6.0 had no effect on the NR activity, whereas at pH 7.5 Mg^{2+} distinctly decreased the enzyme activity (Table 1).

A decrease in NR activity caused by Mg^{2+} at pH 7.5 was completely reversed by the addition of EDTA into samples and the enzyme activity returned to its initial level (Fig. 1*A*). No reactivation of NR caused by EDTA was observed at pH 6.0. What is more, after addition of EDTA to the enzyme solution incubated earlier with Mg^{2+} the further drop of NR activity was observed (Fig. 1*B*).

Effect of adenine nucleotides and inhibitors of protein kinases and phosphatases on the inhibition and reactivation of NR: ATP effect on NR activity was determined in the presence of Mg^{2+} ions (actual activity) or in the presence of EDTA (maximal activity) at the pH 7.5 or pH 6.0. The incubation of the desalted extract with ATP at pH 7.5 did not change the maximal NR activity (Fig. 2*A*), whereas in samples containing ATP and Mg^{2+}

Table 1. Effect of pH, Mg^{2+} and EDTA on the NR measured in supernatant obtained from roots of 7-d-old cucumber seedlings. Supernatant was desalted and incubated for 15 min in the presence of 5 mM EDTA or 5 mM $MgCl_2$ at pH 7.5 or 6.0. After incubation 0.2 cm^3 of mixture containing 50 mM Hepes-KOH pH 7.5 or 6.0 KNO_3 and NADH was added into the reaction medium and incubated for 5 min at 27 °C. The NR activity measured in the reaction medium with pH 7.5 incubated without EDTA and $MgCl_2$ was 0.26 μmol(NO_2) g^{-1}(f.m.) h^{-1} (100 %). Experiment was repeated 3 times with the same results.

	NR activity [%] pH 7.5	pH 6.0
Desalted supernatant	100	58
+ 5 mM EDTA	127	84
+ 5 mM $MgCl_2$	67	50

the enzyme activity radically dropped (Fig. 2*B*). Inactivation of nitrate reductase caused by ATP in the presence of Mg^{2+} was very rapid and was observed after 15 min of incubation. Inclusion of staurosporine into reaction medium with Mg^{2+} and ATP at pH 7.5 completely abolished an inhibitory action of ATP on the nitrate reductase activity (Fig. 2*B*). In the samples incubated at pH 6.0 an actual as well as maximal nitrate reductase activity were sensitive to the ATP inhibition (Fig. 2*C*,*D*). Decrease of an actual enzyme activity at pH 6.0 was stronger than at pH 7.5. Staurosporine was also effective in diminishing inhibitory action of ATP at pH 6.0, nevertheless, the neither maximal nor actual activities of NR reached their initial levels (Fig. 2*C*,*D*).

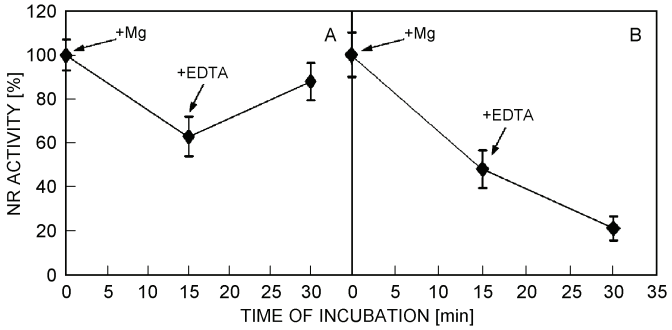

Fig. 1. Effect of EDTA and pH on the reversibility of Mg^{2+} action on the NR activity. Desalted extract obtained from cucumber roots was incubated for 15 min in the presence of 5 mM $MgCl_2$ at pH 7.5 (*A*) or 6.0 (*B*). Then 5 mM EDTA was added and mixture was incubated for the another 15 min. The initial enzyme activity (time 0, determined without incubation) measured without EDTA in the reaction medium was used as 100 % (for *A* and *B* it was 0.63 ± 0.03 and 0.14 ± 0.014 μmol(NO_2) g^{-1}(f.m.) h^{-1}, respectively). Means of 5 replications, *error bars* represent standard deviations (SD).

Above results suggested that protein phosphorylation is involved in the ATP-dependent inhibition of NR. Experiments with microcystin LR, the protein phosphatase inhibitor confirmed that (Fig. 3*B*). Since EDTA reversed Mg^{2+}-ATP inhibition of NR only at pH 7.5, the experiments were done just in this pH. Microcystin was included into reaction medium together with EDTA, after 15-min incubation of supernatant with Mg^{2+} (Fig. 3*A*) or with Mg^{2+} and ATP (Fig. 3*B*). An addition of microcystin into reaction medium had no effect on the EDTA-dependent reactivation of NR (Fig. 3).

Alteration of NR activity caused by ATP was effectively reversed also after AMP application into reaction mixture (Fig. 4), independently if ATP-repression was provided with Mg^{2+} (Fig. 4*A*,*B*) or without divalent cations (+EDTA, Fig. 4*C*). AMP was less effective at pH 7.5 than at pH 6.0. In pH 7.5 microcystin LR incorporated into reaction mixture clearly diminished the stimulation of NR by AMP (Fig. 4*A*) whereas at pH 6.0 the effect of phosphatase inhibitor on the AMP-depended reactivation of NR was subjected upon conditions of previous inactivation of the enzyme. If NR was inhibited by Mg^{2+} and ATP, its AMP-reactivation was almost completely eliminated in the presence of microcystin (Fig. 4*B*). While the inactivation of enzyme was provided in the presence of ATP and EDTA, microcystin only partially abolished the AMP-dependent reactivation of NR activity (Fig. 4*C*).

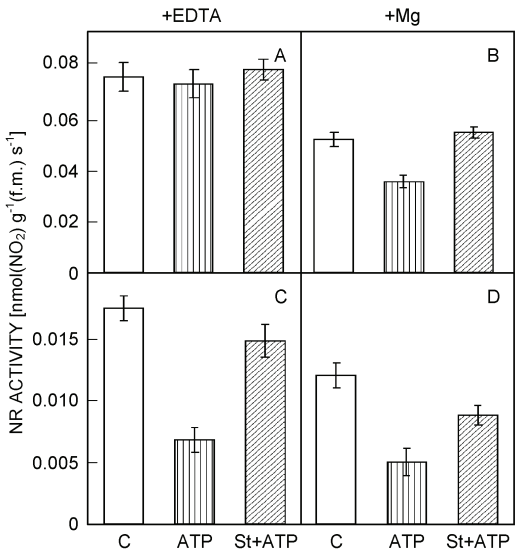

Fig. 2. Effect of staurosporine on ATP-dependent changes of NR activity. Desalted supernatant was incubated for 10 min in the presence of 5 mM EDTA or 5 mM $MgCl_2$ without or with 2 μM staurosporine (St). Then 2 mM ATP was added and samples were incubated for the following 15 min. The pH of the reaction medium was 7.5 (*A* and *B*) and 6.0 (*C* and *D*). Actual (+Mg^{2+}) and maximal (+EDTA) NR activities were measured as described in Materials and methods. Means from 6 replications, *error bars* represent SD.

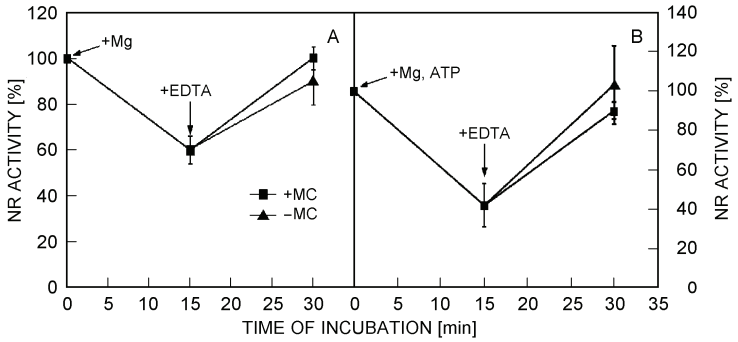

Fig. 3. Effect of protein phosphatase inhibitor on the EDTA dependent reactivation of NR. Desalted extract obtained from cucumber roots was incubated in the presence of 5 mM $MgCl_2$ (*A*) or in the presence of 5 mM $MgCl_2$ and 2 mM ATP (*B*) at pH 7.5. After 15 min 5 mM EDTA without (*triangles*) or with 1 μM microcystin LR (*squares*) were added into the reaction medium. NR activity measured before addition of Mg^{2+} and ATP (time 0) was used as 100 % (0.69 ± 0.04 μmol(NO_2) g^{-1}(f.m.) h^{-1}). Means of 6 replications, *error bars* represent SD.

Discussion

The highest activity of NR was measured after incubation of desalted supernatant from cucumber roots in the presence of 5 mM EDTA (Table 1). Those results have demonstrated that, similarly to the leaf enzyme, the activity of NR from roots has been affected by Mg^{2+} ions. Sensitivity of the root nitrate reductase to Mg^{2+} ions (Fig. 1) also resembled the properties of enzyme originating from photosynthetic tissues (Huber *et al.* 1992, Kaiser and Huber 1994b, Kojima *et al.* 1995). In the plant leaves high sensitivity of NR to Mg^{2+} is due to the higher amount of phosphorylated enzyme protein. It is well known that phosphorylated NR through Mg^{2+} ions can form an inactive complex with 14-3-3 protein (NR-IP) (Kaiser and Huber 1994b, Kaiser *et al.* 2002). The activity of NR was also altered after incubation with both Mg^{2+} and ATP (Fig. 2*B*, 3*B*). A drop of the activity in this combination was distinctly higher than in the presence of Mg^{2+} alone (compare Fig. 3*A* and *B*). Since the effect of ATP was fully canceled by staurosporine (Fig. 2*B*), the specific inhibitor of the serine-threonine protein kinases (Radloff and Gercken 1996), it is evident that during incubation of extract from roots with ATP, the level of phosphorylated NR protein was elevated, altering the activity of enzyme. Involvement of reversible phosphorylation in the regulation of the nitrate reductase activity in cucumber roots was confirmed by facts that ATP effect on NR activity was easily reversed by AMP (Fig. 4) and that this nucleotide-dependent enzyme reactivation was totally repressed in the presence of protein phosphatases inhibitor (microcystin LR). The direct effect of AMP on protein phosphatase activity and its participation in the activation of NR in spinach leaves

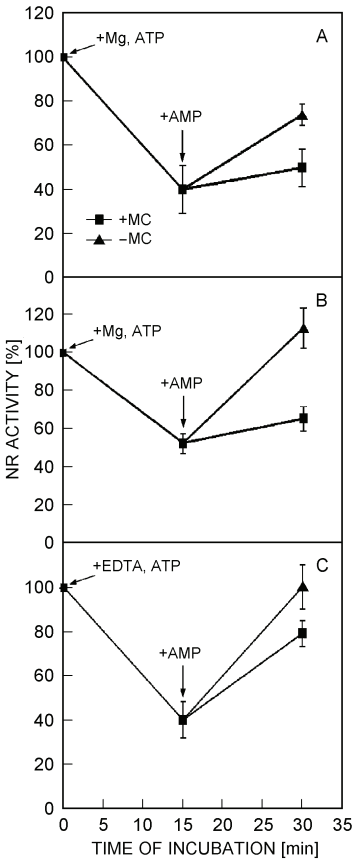

Fig. 4. Effect of microcystin LR, the protein phosphatase inhibitor, on the AMP dependent reactivation of NR at pH 7.5 (*A*) and pH 6.0 (*B* and *C*). Desalted extract obtained from cucumber roots was incubated in the presence 5 mM $MgCl_2$ and 2 mM ATP (*A* and *B*) or in the presence of 5 mM EDTA and 2 mM ATP (*C*) at pH 6.0. After 15 min of incubation 5 mM AMP was added into the reaction medium. Part of samples, besides AMP, contained also 1 μM of microcystin LR (*squares*). The activity of NR measured immediately before addition of both Mg^{2+} and ATP (time 0) was used as 100 % (0.21 μmol(NO_2) g^{-1}(f.m.) h^{-1}). Means of 6 replications, *error bars* represent SD.

was well documented by Huber and Kaiser (1996). Stimulatory effect of AMP on NR activity was also shown by Athwal *et al*. (1998b and 2000). However, Athwal *et al*. suggested that the AMP-dependent enzyme stimulation resulted from the nucleotide interaction with 14-3-3 protein and subsequent dissociation of inactive complex. Our results suggest that AMP-induced reactivation of root NR is rather due to the direct reaction of AMP with protein phosphatases than with an inhibitor protein.

Inhibitory action of ATP on the nitrate reductase activity was also cancelled by EDTA (Fig. 3*B*). Contrary to the AMP action, the chelator-dependent reactivation of enzyme was insensitive to the phosphatase inhibitors suggesting that this increase of NR activity did not result from a dephosphorylation of the enzyme protein and was probably due to the direct effect of EDTA on the enzyme-inhibitor complex. That is, EDTA chelating the Mg^{2+} ions could disrupt inactive complexes of NR with 14-3-3 protein increasing the enzyme activity up to its initial level. Similar results were obtained by Kaiser and Spill (1991) and Glaab and Kaiser (1996) for the nitrate reductase isolated from spinach leaves.

A drop in the activity of NR, comparable with that obtained with Mg^{2+}, was observed after incubation of enzyme at pH 6.0 (Table 1). What is more, at pH 6.0, a subsequent addition of Mg^{2+} ions into reaction medium did not significantly decrease the activity of NR. Athwal *et al*. (1998a) proved for the NR from leaves that the high concentration of protons in medium (low pH) can induce the conformational changes of 14-3-3 protein resulting in the extension of its hydrophobic surface and this enabled its covalent binding with the enzyme. Our results imply that the similar regulatory mechanism of the NR activity can function also in the root tissues. At pH 6.0 the presence of ATP itself in the incubation mixture (Figs. 2*C*, 4*C*) was sufficient to inactivate NR. Since an addition of staurosporine prevented this inactivation (Fig. 2*C*) we assume that alteration of NR caused by ATP resulted from the phosphorylation of enzyme protein and subsequent formation of an inactive complex with 14-3-3 protein. Those results confirm the suggestion of Atwal *et al.* (1998a) that protons can substitute the magnesium ions in the association of phosphorylated NR with 14-3-3 protein. Results obtained with EDTA at pH 6.0 (drop in NR activity) confirmed this supposition. Inhibitory effect of H^{+}-ATP (Fig. 4*C*) similarly as Mg^{2+}-ATP (Fig. 4*B*) was totally reversed by AMP. The reactivation of NR due to AMP was drastically decreased by microcystin LC only when the enzyme was repressed by Mg^{2+}-ATP. If ATP alone altered NR (trials with EDTA and ATP) action of AMP was only slightly affected by inhibitor of phosphatase. Those data suggest that at low pH the reactivation of enzyme induced by AMP is more complicated and is not limited to the protein dephosphorylation.

To sum up, we documented that in root tissues can operate a reversible phosphorylation of the NR protein and its mechanism is similar as in the leaves. Since in green tissues the posttranslational regulation is primarily responsible for NR adaptation to many environmental factors (Huber *et al*. 1992, Kojima *et al*. 1995, Redinbaugh *et al*. 1996) it is possible that the reversible phosphorylation of the nitrate reductase protein operating in roots is also involved in such adaptations.

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