RELATIONSHIPS BETWEEN NITRATE UPTAKE AND NITRATE REDUCTASE ACTIVITY IN *CUCUMIS SATIVUS* L., ROOTS

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ABSTRACT

Anti-NR IgG fragments obtained after papain digestion of polyclonal antibodies gave the positive immunological reaction with both, a soluble and plasma membrane-bound nitrate reductase. Anti-NR antibody as well as IgG fragments almost totally inhibited the nitrate reductase activity in cytosol proving a crossreaction of antibody with the catalytic site of a soluble NR. Anti-NR IgG fragments, but not undigested polyclonal antibodies affected the activity of the nitrate reductase associated with plasma membranes. Discrepancy in the action of intact antibodies and fragments obtained after they digestion were interpreted as a consequence of same differences in the ability of those molecules to the penetration through the membrane.

Undigested anti-NR antibody have no effect on the nitrate uptake by intact plants, as well as by the right-side plasma membrane vesicles. On the other hand, IgG fragments of polyclonal antibodies abolished almost totally the nitrate uptake in the case of intact seedlings, but have only slight effect on the NO_3^- uptake in plasma membranes. On the basis of above findings, some relations between nitrate uptake and its assimilation inside the cell are suggested. Since IgG fragments only slightly changed the NO_3^- absorption in vesicles whereas the activity of plasmalemma associated nitrate reductase was strongly repressed, we concluded that the PM-NR is not structurally involved in the nitrate transport through the membrane.

KEY WORDS: nitrate reductase, nitrate transport, polyclonal antibody, anti-NR IgG fragments.

INTRODUCTION

On the basis of some similarities in the common features between NR and nitrate uptake, such as the presence of a constitutive and a nitrate-inductive activities, Butz and Jackson (1977) suggested that the nitrate reductase (NR, EC 1.6.6.1) may acts as both, a nitrate-transporting and a nitrate-reducing protein. Although today it is obvious that the main place of the nitrate reduction is cytosol, many observations were done indicating that besides the bulk cytosolic nitrate reductase, a small part of NR activity is specific for the plasmalemma of plant cells (Ward et al 1988, Tischner et al 1989, Stohr et al 1993, Kłobus et al. 1994, Mayerhoff et al. 1994, Marco et al. 1994). It was proved for algae (Stohr et al. 1993) and for higher plant cells (Kłobus et al. 1994) that the plasmalemma associated nitrate reductase is an integral membrane protein and differs from the cytosolic form of enzyme in its hydrophobicity, molecular mass and partial activities. Plasmalemma localization of the nitrate reductase recollected the hypothesis of Butz and Jackson, and many experiments were done to reviewed they concept. Results obtained in dif-

ferent laboratories were inconsistent. The same rate of the nitrate uptake by wild barley seedlings and by mutants lacking NR-coding gene (Warner and Huffaker 1989) suggested that the nitrate transport is independent from expression of NR protein. On the other hand, results of Ward et al. (1988) seemed to confirm the contribution of plasma membrane associated NR in the anion uptake. At the presence of an anti-nitrate reductase IgG fragments they observed the inhibition of PM-NR activity, as well as the fall of the nitrate uptake in barley roots. Similar results were obtained with *Chlorella* cells (Tischner et al. 1989). Uptake experiments of Ward et al. (1988) were provided with intact barley seedlings. This complex experimental system possesses many potential sites for a specific and unspecific reaction with fragments of polyclonal IgG. Thus, it can not be excluded that diminished nitrate uptake observed after anti-NR IgG treatment of barley seedlings was due not only to the specific reaction with plasma membrane-bound nitrate reductase. We assumed that a much simpler system would be more suitable to elucidate the contribution of membrane associated nitrate reductase form in the nitrate transport. For this reason, the effect of anti-NR immunoglobulin G fragments on the nitrate uptake in plasma membrane vesicles was determined. This effect was compared with the results obtained for whole cucumber seedlings. Changes in H^+ ATPase activity and H^+ transport followed an antibody fragments treatment of vesicles were also estimated.

Abbreviations:

NRs – soluble nitrate reductase,
PM-NR – plasma membrane-associated nitrate reductase,
anti-NR IgG – anti-nitrate reductase immunoglobuline,

MATERIAL AND METHODS

Plant material

Cucumber (*Cucumis sativus* L., var. Wisconsin) seeds germinated in darkness were transferred for three days into nitrate free solution and than for one day to the nitrate containing nutrient solution, according to Kłobus (1990). Cucumber seedlings grown in a light/dark rhythm of 16h/8h and 25°C/22°C.

Antiserum preparation

Antiserum to *Cucumis sativus* NR was prepared as described by Marciniak et al. 1995. Purification of IgG and its hydrolysis with papain were done according to Lifter and Choi (1978). Separation of cleaved anti NR IgG fragments from papain was made on a Sephadex G-150. Chromatography on a DEAE-Sephacyl column was used for purification and separation of Fab from Fc fragments (Jeanjean et al. 1984). Fab fragments were pooled, dialyzed and concentrated prior to the enzyme and uptake experiments. Similar procedures were used for digestion and purification of IgG fragments from preimmune serum.

Dot-blot assays

Immunoblotting was carried out as described by Marciniak et al. (1995). Instead of soluble NR the plasma membrane suspension was used and anti-NR IgG fragments replaced polyclonal anti-NR antibody.

Nitrate uptake in the cucumber seedlings

Seven-day old NO_3^- induced cucumber seedlings were equilibrated 30 min. with 1 mM KNO_3 and 1 mM MES, pH 6.2 (uptake solution). Then, a group of three seedlings were transferred to 5 cm³ of the fresh uptake solution (control) and to the uptake solution containing preimmune serum, polyclonal anti-NR antibodies or corresponding IgG fragments (500 µg protein). Nitrate uptake by plants was calculated from NO_3^- depletion in the uptake solution after 15 min. in the room temperature.

Nitrate transport in the plasma membrane vesicles

Right-side out oriented plasma membranes isolated from cucumber root microsomes by phase partitioning as described previously (Kłobus 1995), were diluted in 5 mM K-phosphate (pH 8.0), 330 mM sorbitol, 1 mM DTT, 0.1 mM EDTA and 100 mM KCl to the protein concentration about 2 mg·cm⁻³. Before uptake experiments, preimmune serum, polyclonal anti-NR antibodies or corresponding IgG fragments, in the ratio of protein 1:1 were added to the suspension of PM vesicles and the mixture was equilibrated 30 min. at 0-4°C. For a transport assay, 100 µl of mixture was mixed vigorously with 900 µl of the uptake solution containing 5 mM K-phosphate buffer (pH 6.0), 330 mM sorbitol, 10 mM KCl and 50 µM KNO_3 . Part of samples was immediately filtered under vacuum on 0.45 mm Millipore filters and the initial nitrate concentration in solution was determined. The final nitrate concentration was determined after vacuum filtration of uptake solutions obtained from the samples incubated 10 min. at the 25°C. Nitrate uptake by vesicles was calculated from NO_3^- depletion in the uptake solution.

Determination of the NO_3^- concentration

NO_3^- level was determined spectrophotometrically after high-performance liquid chromatography of solutions, according to Thayer and Huffaker (1980).

H^+ – transport in inside-out PM vesicles

Prior to the H^+ -transport determination, approximately 100 µg of the inside-out vesicles obtained as described previously (Kłobus and Buczek 1995) were diluted in 5 mM BTP-MES (pH 7.5), 330 mM sorbitol, 1 mM DTT, 0.1 mM EDTA, 100 µg protein of preimmune serum, polyclonal anti-NR antibodies or corresponding IgG fragments, and incubated 30 min. at 0-4°C. Then 100 µl of mixture was added to the 900 µl of 5 mM BTP-MES (pH 7.5), 330 mM sorbitol, 0.1% BSA, 3.75 mM ATP, 3.75 mM MgSO_4 , 100 mM KCl and 10 µM acridine orange. The reaction was started by the addition of ATP. Changes in A_{495} were determined spectrophotometrically at 25°C, every 30 sec.

Enzyme assays

The enzyme activities were determined in the soluble (after 100 000 g) or the plasma membrane fractions obtained from cucumber roots as described previously (Kłobus and Buczek 1995). Preimmune serum, polyclonal anti-NR antibodies or corresponding IgG fragments were added to the cell fractions and the relationship between an antibody protein and a soluble protein or a membrane protein was 1:1. After 30 min. in 0-4°C the mixtures were used to the determination of the nitrate reductase (Aslam et al. 1987) or H^+ ATPase (Gallagher and Leonard 1982) activities in the presence of 0.1% Triton X-100.

Protein was quantified by the method of Bradford (1976) with BSA as the standard.

All presented data are the means of at least three replicates and standard deviations of the means were always less than 10% (in uptake experiments) or 5% (in H^+ transport experiments) of the data value.

RESULTS

A positive immunological reaction between IgG fragments obtained after purification of papain digested anti -NR antibodies on DEAE-Sephacyl, with the soluble NR, as well as with the plasma membranes were observed (Fig. 1, line 1). The preimmune serum IgG fragments reacted with no fraction (Fig. 1, line 2).

The crossreaction of both, the intact anti-NR serum and the corresponding IgG fragments, with soluble nitrate reductase was evidenced also by an inactivation of enzyme (Table 1). The activity of NR in plasma membrane fraction pretreated 30 min. with the intact antibodies was not changed. Incubation of plasma membranes with anti-NR IgG fragments gave 60% inhibition of the nitrate reductase activity (Table 1). No changes in H^+ -ATPase, a plasma membrane marker, nor ATP-dependent H^+ transport were observed in consequence of PM incubation with a preimmune serum, intact anti-NR, nor anti-NR IgG fragments.

Nitrate uptake by 7-days old cucumber seedlings showed very high sensitivity to the cleaved anti-NR antibodies (Table 2), but not to the intact anti-NR. The effect of antibodies observed in uptake experiments made with the right-side out plasma membrane vesicles isolated from cucumber roots was not as distinguish, as in the case of intact seedlings. NO_3^- uptake into vesicles preincubated with undigested anti-NR antibodies was unchanged, whereas incubation of PM with corresponding IgG fragments slightly diminished the uptake rate. To estimate, if the effect of anti-NR IgG fragments on the nitrate absorption is specific, we determined also they influence on the proton transport into plasma membrane vesicles. No

Fig. 1. Immunoreaction of IgG anti-NR fragments with a soluble (A) and plasma membrane (B) fraction.

A soluble fraction (A) was obtained after centrifugation of cucumber root homogenate at $100\,000 \times g$. Plasma membranes (B) were collected from the upper phase 6.5% two-phase system, as described Klobus et al. (1994). Fractions ($3 \mu\text{g}$ of protein) were dot-blotted onto nitrocellulose and probed with IgG fragments of anti-NR (line 1) or IgG fragments obtained from preimmune serum (line 2).

TABLE 1. The effect of anti-NR IgG fragments on the nitrate reductase activity in the soluble and plasma membrane fraction of cucumber root cells

treatment	soluble NR	PM-NR	H ⁺ -ATPase
	nmol NO ₂ ⁻ $\times$ mg ⁻¹ protein $\times$ h ⁻¹		$\mu\text{mol P}_i \times \text{mg}^{-1}$ protein $\times$ h ⁻¹
none	114.30	125.38	114.45
preimmune IgG fragments	109.87	115.98	104.32
intact anti-NR IgG	14.52	107.91	108.97
anti-NR IgG fragments	13.89	54.12	111.06

A soluble and plasma membrane fractions were mixed with preimmune or anti-NR IgG fragments (protein relationship was 1:1). After 30 min. at 4°C mixtures were used to the determination of the nitrate reductase or H⁺ATPase activities.

TABLE 2. The effect of anti-NR IgG fragments on the nitrate uptake in the cucumber seedlings and plasma membrane vesicles isolated from the root cells.

treatment	NO ₃ ⁻ uptake rate	
	Intact cucumber seedlings ($\mu\text{mol NO}_3^- \times \text{g}^{-1}$ fr.wt. $\times$ h ⁻¹)	right-side out PM vesicles (nmol NO ₃ ⁻ $\times$ mg ⁻¹ protein)
none	1.817 $\pm$ 0.153 (100%)	15.781 $\pm$ 1.32 (100%)
preimmune IgG fragments	1.915 $\pm$ 0.176 (105%)	15.476 $\pm$ 1.41 (98%)
intact anti-NR IgG	1.964 $\pm$ 0.164 (108%)	15.477 $\pm$ 1.38 (99%)
anti-NR IgG fragments	0.106 $\pm$ 0.011 (5%)	11.532 $\pm$ 1.02 (73%)

After equilibration with 1 mM KNO₃ and 1 mM MES (pH 6.2) a group of three nitrate induced cucumber seedlings were put in 5 cm³ of the uptake solution (control) and in the uptake solution containing antibodies (500 μg protein), as indicated in the table. Nitrate uptake by plants was calculated from NO₃⁻ depletion in the uptake solution after 15 min. in the room temperature. In experiments with the right-side out plasma membrane vesicles, pre-immune serum, intact anti-NR antibody or corresponding IgG fragments in the ratio of protein 1:1 were added to the suspension of PM and the mixture was equilibrated 30 min. at 0-4°C. For transport assays, 100 μl of mixture was mixed vigorously with 900 μl of the uptake solution containing 5 mM K-phosphate buffer (pH 6.0), 330 mM sorbitol, 10 mM KCl and 50 μM KNO₃. Nitrate uptake was calculated from NO₃⁻ depletion in the uptake solution.

changes in H⁺ flux in vesicles treated with an intact or digested anti-NR antibodies were observed (Table 3).

DISCUSSION

As we reported previously (Klobus et. al. 1994) intact polyclonal antibodies raised against the soluble nitrate reductase exhibited a high immunological specificity to denatured and native form of plasma membrane proteins. Anti-NR IgG fragments obtained after papain digestion of the polyclonal antibodies gave also a positive immunological reaction, not only with a soluble NR, but also with plasma membranes (Fig. 1).

This confirms that purification of IgG fragments by ion exchange chromatography on a DEAE-Sephacyl column resulted in the separation of Fab fragments of anti-NR, bearing the antigen specific sites. Almost total inhibition of the NR activity in cytosol generated by the intact anti-NR and corresponding Fab fragments proved that antibodies crossreacted with the catalytic site of a soluble NR. The lack of the inhibitory effect of intact anti-NR on the activity of PM-associated nitrate reductase we interpreted previously (Klobus et al. 1994) as a consequence of the fact, that epitopes recognized were not in or near the catalytic center of this form of enzyme. However, presently we estimated that Fab fragments of anti-NR antibodies repressed the activity of PM-NR (Table

TABLE 3. The effect of anti-NR on H⁺ transport into plasma membrane vesicles isolated from cucumber roots

treatment	H ⁺ transport ($\Delta A_{495} \times \text{mg}^{-1} \text{ protein} \times \text{min}^{-1}$)
none	0.126 $\pm$ 0.004
preimmune IgG fragments	0.127 $\pm$ 0.004
intact anti-NR IgG	0.129 $\pm$ 0.003
anti-NR IgG fragments	0.126 $\pm$ 0.004

The proton transport was measured as a decrease in the acridine orange absorbance (A_{495}) with inside-out plasma membrane vesicles after they treatment with preimmune serum, intact anti-NR antibody and anti-NR IgG fragments, as described in Material and Methods.

1). In the light of these findings, our previous suggestion must be amplified. Presumably, the discrepancies in an inhibitory effect of polyclonal anti-NR antibodies and corresponding Fab fragments on the PM-associated nitrate reductase were due to the differences in its penetration of membrane. The cleaved IgG fragments are much smaller (50 kDa) than intact anti-NR molecules (150kDa) and it seems possible that they can easier reach the active site of the enzyme protein, which is build up in the membrane. Earlier estimations that the nitrate reductase associated with plasma membrane is a hydrophobic, integral membrane protein (Stohr et al. 1993, Kłobus et al. 1994) support this suggestion. Moreover, different sensitivity of the soluble NR and PM-NR to anti-NR Fab fragments (90% inhibition of NRs compare with 60% inhibition of PM-NR) may indicate some deviation in an amino acid sequence between those two forms of enzyme. Such observations are consistent with the view of Campbell and Kinghorn (1990) that NR from different sources, sharing functional and structural similarities, can be highly divergent in an amino acid composition.

Similarly to barley (Ward et al. 1988), a treatment of intact cucumber seedlings with anti-NR IgG fragments abolished the nitrate uptake, whereas undigested anti-NR antibodies have no effect. Differences in the effect of entire antibody and Fab fragments obtained after digestion can be a consequence of the diversity in they molecular masses. It was postulated by Ward et al. (1889) that the intact IgG are to large to penetrate the cell wall and to bind to NO₃⁻ transport site on the plasmalemma. However, the intact antibodies were also ineffective in uptake experiments provided with plasma membrane vesicles (Table 2). These results excluded the cell wall as a possible barrier for uncleaved antibody action. Moreover, in the literature there are few examples about a good infiltration of intact antibodies through the cell wall (Jeanjean et al. 1981, Brass et al. 1981). The above findings suggest that the plasma membrane, not the cell wall, impeded the antibody entrance to the cell, and that the site of antibody action is localized inside of the cell.

Only slight effect of the Fab fragments on nitrate absorption measured with plasma membranes and almost total uptake repression obtained in experiments with intact cucumber seedlings implicate some connections between the nitrate uptake and a soluble NR activity. Although, Warner and Huffaker (1989), on the basis of the same rate of nitrate uptake by wild barley seedlings and by mutants lacking the NR-coding gene, concluded that the nitrate transport is independent from the expression of NR protein. In our opinion, these data did not exclude the contribution of NRs activity in regulation of the nitrate transport, since NR-deficient mutants can still produce 5-10% of the nitrate reductase and they are not reduced in the nitrogen metabolism efficiency (Charel et al. 1990).

It was shown (Table 1 and 2) that the decrease in nitrate absorption, contrary to the PM-NR activity, resulted from IgG fragments action was relatively small. On this basis, we concluded that the PM-NR is not structurally involved in nitrate transport through the membrane. Another functional linkage between these two activities should exist. As recently shown for *Nicotiana tabacum*, the plasma membrane-bound nitrate reductase might serve as a regulator between carbon and nitrogen metabolism. (Stohr and Ullrich 1997). Earlier findings of Stohr et al. (1995) suggested that in *Chlorella* cells PM-NR is probably involved in signal transduction of blue-light regulation of NO₃⁻ uptake.

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POBIERANIE AZOTANÓW I AKTYWNOŚĆ REDUKTAZY AZOTANOWEJ W KORZENIACH *CUCUMIS SATIVUS* L.

STRESZCZENIE

Fragmenty IgG uzyskane w wyniku proteolizy poliklonalnych przeciwciał przeciwko reduktazie azotanowej dawały pozytywną reakcję immunologiczną z rozpuszczalną jak i plazmolemmową formą enzymu. Zarówno poliklonalne przeciwciała anti-NR jak i ich fragmenty niemal całkowicie hamowały aktywność cytosolowej reduktazy azotanowej wskazując, iż miejscem reakcji przeciwciało-enzym jest centrum katalityczne reduktazy. Inkubacja błon plazmatycznych z fragmentami IgG spowodowała obniżenie aktywności NR związanej z plazmolemmą, podczas gdy poliklonalne przeciwciała anti-NR były nie efektywne. Powyższe rozbieżności mogą wynikać z różnej penetracji błony przez całe przeciwciała i ich fragmenty, różniących się znacznie wielkością.

Poliklonalne przeciwciała anti-NR nie zmieniały pobierania azotanów przez całe siewki jak i przez wyizolowane pęcherzyki plazmolemmy o właściwej orientacji. Absorpcja NO_3^- przez siewki ogórka była natomiast niemal całkowicie hamowana w obecności anti-reduktazowych fragmentów IgG. Z drugiej strony, fragmenty poliklonalnych przeciwciał anti-NR obniżały tylko nieznacznie transport azotanów do pęcherzyków plazmolemmowych. W kontekście powyższych wyników dyskutowany jest udział rozpuszczalnej i plazmolemmowej formy reduktazy azotanowej w pobieraniu azotanów.

SŁOWA KLUCZOWE: reduktaza azotanowa, transport azotanów, przeciwciała poliklonalne, fragmenty IgG anti-NR.