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Modulation of nitrate reductase in vivo and in vitro: Effects of phosphoprotein phosphatase inhibitors, free Mg^{2+} and 5′-AMP

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Abstract. Nitrate reductase in spinach (*Spinacia oleracea* L.) leaves was rapidly inactivated in the dark and reactivated by light, whereas in pea (*Pisum sativum* L., roots, hyperoxic conditions caused inactivation, and anoxia caused reactivation. Reactivation in vivo, both in leaves and roots, was prohibited by high concentrations (10–30 μM) of the serine/threonine-protein phosphatase inhibitors okadaic acid or calyculin, consistent with the notion that protein dephosphorylation catalyzed by type-1 or type-2A phosphatases was the mechanism for the reactivation of NADH-nitrate reductase (NR). Following inactivation of leaf NR in vivo, spontaneous reactivation in vitro (in desalted extracts) was slow, but was drastically accelerated by removal of Mg^{2+} with excess ethylenediaminetetraacetic acid (EDTA), or by desalting in a buffer devoid of Mg^{2+}. Subsequent addition of either Mg^{2+}, Mn^{2+} or Ca^{2+} inhibited the activation of NR in vitro. Reactivation of NR (at pH 7.5) in vitro in the presence of Mg^{2+} was also accelerated by millimolar concentrations of AMP or other nucleoside monophosphates. The EDTA-mediated reactivation in desalted crude extracts was completely prevented by protein-phosphatase inhibitors whereas the AMP-mediated reaction was largely unaffected by these toxins. The Mg^{2+}-response profile of the AMP-accelerated reactivation suggested that okadaic acid, calyculin and microcystin-LR were rather ineffective inhibitors in the presence of divalent cations. However, with partially purified enzyme preparations (5–15% polyethyleneglycol fraction) the AMP-mediated reactivation was also inhibited (65–80%) by microcystin-LR. Thus, the dephosphorylation (activation) of NR in vitro is inhibited by divalent cations, and protein phosphatases of the PP1 or PP2A type are involved in both the EDTA and AMP-stimulated reactions. Evidence was also obtained that divalent cations may regulate NR-protein phosphatase activity in vivo. When spinach leaf slices were incubated in Mg^{2+}-and Ca^{2+}-free buffer solutions in the dark, extracted NR was inactive. After addition of the Ca^{2+}/Mg^{2+}-ionophore A 23187 plus EDTA to the leaf slices, NR was activated in the dark. It was again inactivated upon addition of divalent cations (Mg^{2+} or Ca^{2+}). It is tentatively suggested that Mg^{2+} fulfills several roles in the regulatory system of NR: it is required for active NR-protein kinase, it inactivates the protein phosphatase and is, at the same time, necessary to keep phospho-NR in the inactive state. The EDTA- and AMP-mediated reactivation of NR in vitro had different pH optima, suggesting that two different protein phosphatases may be involved. At pH 6.5, the activation of NR was relatively slow and the addition or removal of Mg^{2+} had no effect. However, 5′-AMP was a potent activator of the reaction with an apparent K_m of 0.5 mM. There was also considerable specificity for 5′-AMP relative to 3′- or 2′-AMP or other nucleoside monophoposphates. We conclude that, depending upon conditions, the signals triggering NR modulation in vivo could be either metabolic (e.g. 5′-AMP) or physical (e.g. cytosolic $[Mg^{2+}]$) in nature.

Key words: AMP – Cation (divalent) – Nitrate reductase – *Pisum* – Protein phosphatase – *Spinacia*

Introduction

It has long been recognized that assimilatory nitrate reductase (NR; EC 1.6.6.1) is regulated at the level of protein synthesis and degradation, and also by enzyme modulation (Solomonson and Barber 1990). In recent years, evidence has accumulated that NR is modulated by reversible protein phosphorylation/dephosphorylation, both in leaves and roots (Kaiser and Spill 1991; Kaiser et al.1992; Huber et al. 1992; Glaab and Kaiser 1993; MacKintosh 1992; Huber et al. 1993; MacKintosh

This paper is dedicated to Prof. O.H. Volk on the occasion of his 90th birthday

Abbreviations: DTT = dithiothreitol; Mops = 3-(N-morpholino)-propanesulfonic acid; NR = NADH-nitrate reductase; NRA = nitrate-reductase activity; PP = protein phosphatase

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and MacKintosh 1993). Meanwhile, some of the auxiliary proteins catalyzing these steps have been partially purified (Spill and Kaiser 1993). It is, however, still not clear how the activities of the protein kinases and phosphatases are themselves regulated. There is some evidence that NR kinase is affected by sugar phosphates (Huber et al. 1993), much like sucrose-phosphate-synthase kinase (Weiner et al. 1992), but no other metabolic effectors have been identified to date. Some information about the nature of the participating protein phosphatases (PPs) has been derived from inhibitor studies, using toxins from marine sponges such as microcystin-LR, okadaic acid and calyculin, which inhibit, with different affinities, type-2A and type-1 protein phosphatases (Bialojan and Taki 1988; Hardie 1993). The activation/dephosphorylation of NR in vivo was completely prevented by pretreating leaves with okadaic acid (Huber et al. 1992), and activation of NR in pea roots was also prevented (Glaab and Kaiser 1993), indicating involvement of a type-1 or type-2A PP. In in-vitro studies, only PP 2A could dephosphorylate and activate NR (MacKintosh 1992), suggesting a specific role for PP 2A and not PP 1. Indications have been obtained that inactivated (i.e. phosphorylated) NR in crude extracts is only slowly activated in buffers containing Mg^{2+} or Ca^{2+} when extracts are preincubated at 25°C before assay.

In animal cells, the central enzyme of a protein-kinase cascade is a 5′-AMP-stimulated protein kinase (MacKintosh and MacKintosh 1993, and literature cited therein). In plants, the situation appears to be different; 5′-AMP has been shown to accelerate reactivation (dephosphorylation) of NR in extracts from leaves or roots (Kaiser and Spill 1991; Kaiser et al. 1992; Glaab and Kaiser 1993). Thus in plants, AMP might activate a PP instead of a kinase. Activity of phospho-NR was also increased by chelation, or by physical removal of divalent cations (Kaiser and Spill 1991; Huber et al. 1992). The required AMP concentrations were considered as too high to be physiologically relevant, and it is as yet unknown to what extent variations of Mg^{2+} activities in the cytosol exist which might be involved in the regulation of NR (or of the participating kinases and phosphatases), and of other enzymes belonging to the family of proteins modulated by protein phosphorylation/dephosphorylation.

In the present study we examined the effect of various PP inhibitors on the reactivation of NR in vivo and in vitro, in order to find out to what extent specific or non-specific PPs were involved. The specificity for AMP was investigated and the role of cytosolic free Mg^{2+} for the reactivation of NR and on the efficiency of the phosphatase inhibitors was also examined. The results obtained indicate the potential for involvement of both metabolic (5′-AMP) and physical (Mg^{2+}) factors in the regulation of NR in vivo.

Materials and methods

Plant material. For experiments carried out in Würzburg, spinach (*Spinacia oleracea* L. cv. Polka F_1) was obtained from a local seed merchant, and was grown in a greenhouse under additional illumination (HQi, 400 W; Schreder, Winterbach, Germany[1]), at a variable total photon flux density of 250–400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR), and a mean daylength of 11 h. Air humidity varied from 60 to 80%, and day/night temperatures were from 20/26°C to 16/22°C. Similar growth conditions were used in Raleigh, N.C., USA, but the spinach cultivar was 'Bloomsdale'. Peas (*Pisum sativum* L. cv. 'Kleine Rheinländerin') were grown hydroponically under slightly different conditions, as described recently (Glaab and Kaiser 1993).

Protein-phosphatase inhibitors. Okadaic acid and calyculin were obtained from Moana Bioproducts of Hawai Inc. (Honolulu, USA). Microcystin-LR was from Calbiochem, La Jolla, Cal., USA. Stock solutions were prepared from all inhibitors in dimethyl sulfoxide, exceeding the desired maximum final concentration by a factor of 100.

Preparation of tissue extracts. Plants were harvested usually after 2 h in the light phase. Leaves were rinsed briefly with distilled water, blotted dry, weighed and frozen and ground in liquid nitrogen. The tissue powder was extracted directly or kept at −80°C until further use. If not mentioned otherwise, about 1 g of frozen tissue powder was ground in 2 ml of buffer, containing 50–100 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 10 μM FAD, 10 μM leupeptin. Subsequently, the slurry was centrifuged (16 000·g, 10 min, 4°C), and the clear supernatant was desalted on Sephadex G-25 equilibrated with the extraction buffer. Pea roots were extracted in a similar way, with slight modifications described recently (Glaab and Kaiser 1993).

In-vivo modulation of NR. For dark-light modulation of NR in vivo, freshly harvested leaves were placed with their petioles in water, and were exposed to light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) at 22°C, or to darkness for the times indicated in the legends of figures and tables. Leaves were quenched in liquid nitrogen, and crude extracts were prepared as described above.

In-vitro modulation of NR. 14 Desalted leaf extracts were incubated at 22°C, with effectors added as indicated in legends of figures and tables. At the times indicated, aliquots (50–100 μl) of the incubation mixture were injected into 900–950 μl of the reaction medium.

Determination of nitrate-reductase activity NRA. Enzyme activity was measured in a medium containing the buffer described above, and in addition 1 mM KNO_3 and 0.2 mM NADH. Nitrate reduction was allowed to proceed for 2–3 min, and was terminated by addition of 125 μl of zinc acetate (0.5 M). Formed nitrite was determined colorimetrically (Hagemann and Reed 1980). The NRA is usually expressed in $\mu mol \cdot (g\ FW)^{-1} \cdot h^{-1}$, but data can be recalculated to the soluble protein content, which was 13 ± 1 $mg \cdot (g\ FW)^{-1}$ for leaves, and 2.7 ± 0.6 $mg \cdot (g\ FW)^{-1}$ ($n = 6$) for roots.

Reproducibility of data. Most experiments were done several times in two locations (Raleigh, USA, and Würzburg, Germany). In general, typical results from one experiment with at least two independent preparations are presented.

Results and discussion

Inhibitor effects on NR reactivation in vivo. Nitrate reductase in spinach leaves was inhibited in the dark, and was rapidly reactivated upon illumination (Huber et al. 1992;

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Table 1. Effect of okadaic acid (Oka) or calyculin (Caly) on the reactivation of NR in intact spinach leaves or in pea root segments. Leaf NR was inactivated by a 90-min dark treatment, and reactivated by a 60-min illumination period (300 μmol · m^{-2} · s^{-1}). Root NR was inactivated by keeping the roots (root tip segements of 3 cm length) for 30 min in a nutrient solution flushed with oxygen, and was reactivated by flushing the root segements with nitrogen for another 90 min. Means from two separate determinations. The NRA is given as μmol NO_2^- · (g FW)$^{-1}$ · h^{-1}

Spinach leaves	90 min dark ⇒ 60 min light	
Control	6.5	16.5
+ Oka 3 μM	6.2	8.9
+ Oka 10 μM	4.5	7.2
+ Oka 30 μM	4.2	3.6
+ Caly 1 μM	6.2	10.1
+ Caly 3 μM	6.0	8.0
Pea roots	**30 min O_2 ⇒ 90 min N_2**	
Control	0.7	2.2
+ Oka 10 μM	0.7	1.4
+ Caly 5 μ M	0.6	1.3

Table 2. Effect of various nucleoside monophosphates on the rate of NR activation in vitro at pH 6.5. Crude leaf extracts were prepared from darkened spinach leaves and desalted into a buffer containing 50 mM Mops-NaOH (pH 6.5), 10 mM $MgCl_2$, 0.1 mM EDTA and 2 mM DTT. The extracts were supplemented with 0.5 mM of the indicated nucleoside monophosphates. The NRA was assayed (+Mg^{2+} assay) as described in *Materials and methods*. The increase in activity as a result of a 10-min preincubation at 25°C is recorded below

Additions	Increase in NRA (μmol · (g FW)$^{-1}$ · h ^{1})
None (control)	0.7
5′-AMP	3.0
3′-AMP	1.5
2′-AMP	1.3
IMP	2.1
GMP	1.9
UMP	1.4
CMP	1.3

Kaiser et al. 1992; MacKintosh 1992; Riens and Heldt 1992). In roots, inactivation was achieved by hyperoxic conditions, and reactivation by anoxia (Glaab and Kaiser 1993). Table 1 summarizes some of these observations, and demonstrates in addition that in all these cases, reactivation in vivo was prevented by okadaic acid or calyculin. It has to be mentioned, however, that the required inhibitor concentrations (10–30 μM) were rather high.

Inhibitor effects in vitro. The enzyme from leaves which had been kept in the dark for 1 h was usually inhibited by Mg^{2+} (in the assay) from 50 to 90% of its maximum activity. In crude, desalted leaf extracts at room temperature (22° C), NR was slowly reactivated when the incubation buffer contained 10 mM Mg^{2+} (Fig. 1). Reactivation was accelerated either by removing divalent cations by excess EDTA (15 mM), or by addition of AMP (Fig. 1) or similar nucleoside monophosphates (Table 2).

The results indicated that the dephosphorylation/activation of NR in vitro was inhibited by Mg^{2+}. This was verified, and as shown in Fig. 2, inhibition of NR activation was observed with either Mg^{2+}, Mn^{2+} or Ca^{2+}; Mg^{2+} and Mn^{2+} were equally effective (concentration for 50% inhibition, IC_{50} = 0.6 mM), whereas Ca^{2+} was slightly less effective (IC_{50} = 1.7 m M) as inhibitor. Because bivalent cations also inhibit phospho-NR enzymatic activity (Kaiser and Brendle-Behnisch 1991), it is reasonable to question whether interaction of bivalent cations with a single site on the NR protein itself might be responsible for both effects, namely for inhibition of phospho-NR activity and the inhibition of the activation/dephosphorylation reaction. It appears that two sites may be involved because, in the former case, all three bivalent cations are equally effective and the IC_{50} values are lower (0.2–0.3 mM) than those required for the latter effect (compare Kaiser and Brendle-Behnisch 1991). Thus, we tentatively suggest that inhibition of NR activation in vitro may reflect an interaction of the bivalent cations with the PP itself.

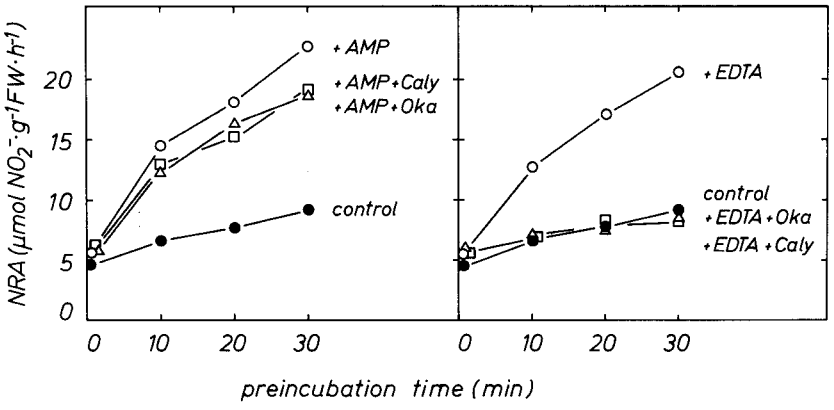

Fig. 1. Effect of okadaic acid (*Oka*; 10 μM) or calyculin (*Caly*; 1 μM) on the reactivation of NR in vitro, mediated by AMP (5 mM), or EDTA (10 mM). Crude extracts were prepared from spinach leaves harvested after 2 h in the dark, in buffer containing 20 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 10 μM FAD. The cleared extract was desalted on Sephadex G25, equilibrated with the same buffer, but containing only 10 mM $MgCl_2$ and no $CaCl_2$. Aliquots (500 μl) were preincubated at 22°C, in the presence or absence of EDTA, AMP and toxins, as indicated in the figure. At the times indicated, aliquots (100 μl) were removed and injected into 900 μl of the standard reaction mixture for nitrate reduction. Run time was 3 min

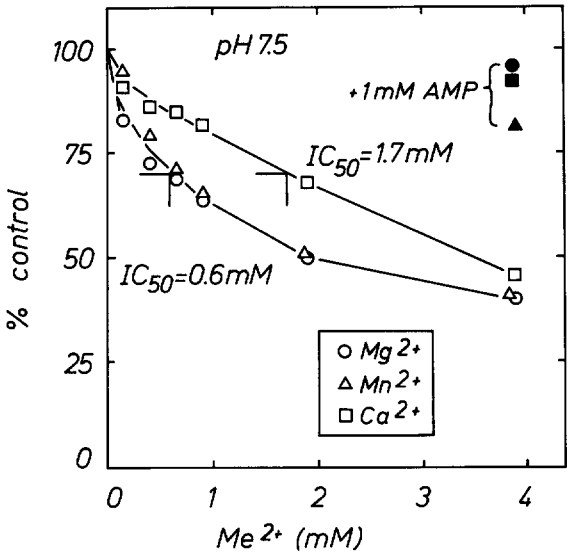

Fig. 2. Inhibition of the rate of NR activation in vitro by bivalent cations in the absence (*open symbols*) or presence (*closed symbols*) of AMP (1 mM). Extracts were prepared from darkened spinach leaves and desalted into buffer containing 50 mM 3-(N-morpholino)propanesulfonic acid (Mops)-NaOH (pH 7.5), 0.1 mM EDTA, and 2.5 mM DTT. Extracts were supplemented with increasing concentrations of Mg^{2+}, Mn^{2+}, and Ca^{2+}; the concentration of free Mg^{2+} in the preincubation mixture was calculated assuming complete chelation of the EDTA present. As indicated, 1 mM AMP was also present. The mixtures (100 µl) were preincubated at 25°C for 10 min prior to initiation of the assay by addition of 900 µl for the standard reaction mixture (5 mM Mg^{2+})

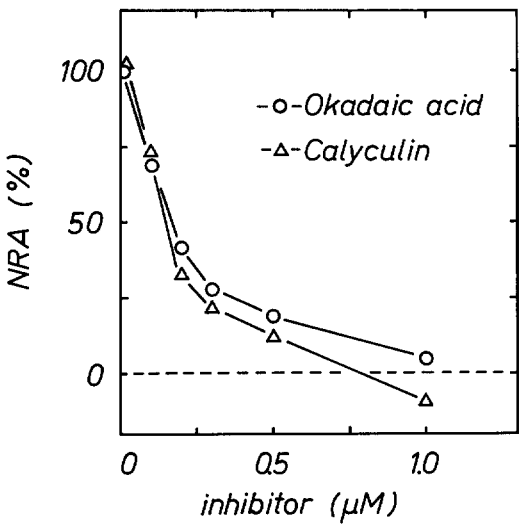

Fig. 3. Inhibition of the EDTA-mediated reactivation of NR in vitro, by different concentrations of okadaic acid or calyculin. Extracts were prepared from spinach leaves which had been kept in the dark for 2 h, as described before. Preincubation time was 20 min; run time for the NR assay was 3 min. The EDTA concentration was 15 mM, on a background of 10 mM $MgCl_2$, 100% = NRA after preincubation with EDTA, but without inhibitor

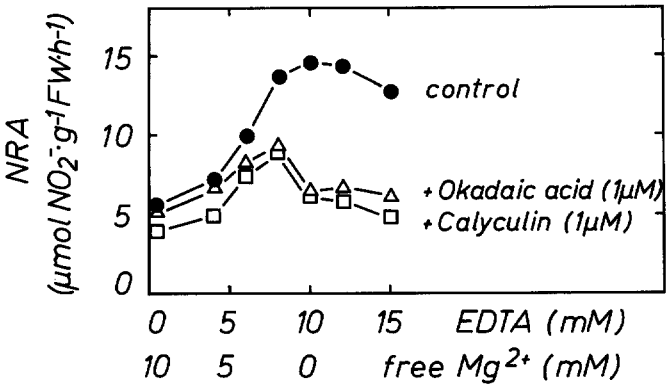

Fig. 4. A typical representation of the in-vitro reactivation of NR in response to the EDTA concentration (or the corresponding free-Mg^{2+} concentration), as affected by okadaic acid or calyculin. A crude, desalted extract was prepared from spinach leaves harvested after 2 h in the dark (see Fig. 1). The extract was preincubated after addition of EDTA (on a background of 10 mM Mg^{2+}), and NR was essayed as described before. Since the logarithmic stability constant of the Mg-EDTA complex at pH 8 is 6.4, the calculated free-Mg^{2+} concentration is, for practical purposes, equal to the difference between the total Mg^{2+} concentration and the total EDTA concentration

Another difference which distinguishes the effect of bivalent cations on the two processes concerns the effect of AMP. A high concentration of AMP could prevent the bivalent-cation-dependent inhibition of NR activation (Fig. 1), whereas AMP had relatively little direct effect on the inhibition of phospho-NR enzymatic activity by Mg^{2+} (data not shown).

The slow spontaneous reactivation of NR and the AMP-accelerated reactivation were insensitive to okadaic acid or calyculin, but the EDTA-accelerated reactivation was inhibited by both toxins (Fig. 1). Figure 3 shows that, in vitro, required inhibitor concentrations were at least one order of magnitude lower than in vivo (compare Table 1). As calyculin and okadaic acid were of equal effectiveness, it is concluded that reactivation was catalyzed by a type-2A phosphatase, and not by a type-1 phosphatase, which is significantly more sensitive to calyculin than to okadaic acid (Hardie 1993). This conclusion is consistent with the direct evidence obtained by MacKintosh (1992) that phospho-NR could be activated in vitro only with the catalytic subunit of PP 2A, not PP 1. It has been shown above that the AMP-accelerated reactivation in the desalted leaf extracts was almost insensitive to the toxins. Thus, it might be concluded that this reaction was catalyzed by a different, perhaps unspecific phosphatase. In Fig. 4, the AMP-accelerated reactivation was measured at different Mg^{2+} activities, adjusted by increasing EDTA concentrations on a constant background of 10 mM Mg^{2+}, in the presence and absence of okadaic acid or calyculin. It is obvious that the inhibition by the toxins was virtually absent at high Mg^{2+} concentrations (as in Fig. 1), but that some inhibition occurred at intermediate Mg^{2+} concentrations. Thus, the inhibitor action by itself appeared to be magnesium-sensitive.

All of the in-vitro inhibition studies were conducted with desalted, crude leaf extracts. We investigated the ap-

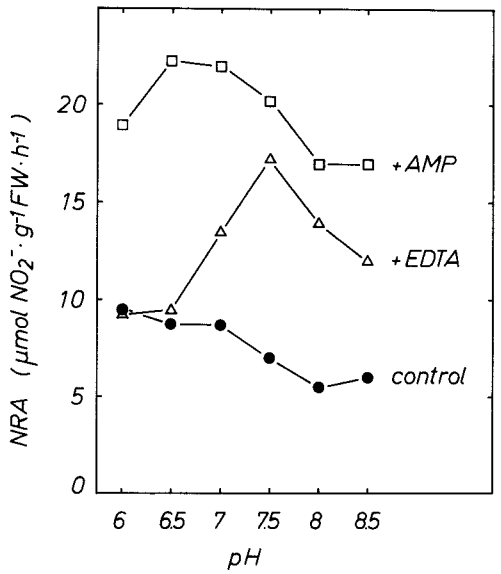

Fig. 5. pH-response curve of the reactivation of spinach leaf NR, by preincubation in the presence or absence of AMP (5 mM) or EDTA (10 mM). The extraction buffers all contained 10 mM $MgCl_2$. 2-(N-Morpholino)ethanesulfonic acid (Mes) was used for pH 6 and 6.5, Hepes for pH 7 and 7.5, and 4-(2-hydroxyethyl)-1-piperazine-propanesulfonic acid (Epps) for pH 8 and 8.5. All buffers were 50 mM, and pH was adjusted with KOH. The desalting columns and the preincubation buffers contained 5 mM $MgCl_2$. Preincubation time was 10 min; run time for the NR assay was 2 min. It is important to note that all extracts, after preincubation at various pH values, were essayed at pH 7.6, in 50 mM Hepes-KOH containing 10 mM $MgCl_2$

parent interaction between Mg^{2+} and microcystin-LR using partially purified enzyme preparations. Fractionation of a dark leaf extract with polyethyleneglycol (5–15% fraction) yields a preparation which contains both phospho-NR and the requisite PP(s) required for activation/dephosphorylation. The activation of phospho-NR in the partially purified, concentrated preparations was also inhibited by 10 mM Mg^{2+}, and the rate of activation could be increased by addition of excess EDTA (15 mM) or AMP (5 mM) (Table 2, line 1). Addition of 1 μM microcystin-LR to the same desalted enzyme preparation (Table 2, line 2) resulted in complete inhibition of the control (containing Mg^{2+}) and the EDTA-stimulated reaction. The AMP-stimulated reaction was also strongly inhibited (65%), which contrasts sharply with the lack of inhibition seen in crude extracts. Almost identical results were obtained when microcystin-LR was added to the enzyme preparation *before* desalting (Table 2, line 3). The persistent inhibition is consistent with the notion (MacKintosh 1992) that microcystin-LR binds tightly to the catalytic subunit of the PP and is not readily dissociated. There are two important points to come from the results presented in Table 2. First, it is clear that binding of microcystin-LR to the PP(s) responsible for activation of NR is not prevented by Mg^{2+}. Second, the AMP-stimulated activation of NR in vitro is, at least in part, catalyzed by a type-1 or type-2A PP. Thus, the observed differential sensitivity to okadaic acid of the AMP- and the EDTA-accelerated reactivation seen with crude extracts but not with partially purified preparations does not unequivocally indicate participation of an alkaline phosphatase or different PPs. It is clear that in both cases, type-1 or type-2A PPs are involved.

The pH-response profile of the AMP-mediated reactivation of NR had a broad optimum in the slightly acidic to neutral pH range, whereas the EDTA-accelerated reactivation had an optimum at pH 7.5 (Fig. 5). This is one hint that, in fact, two different PPs participated in NR reactivation in vitro.

The lack of EDTA-stimulation of the rate of NR activation (+10 mM Mg^{2+}) at pH values below pH 7 could be the result of reduced chelation capacity of EDTA at slightly acidic values. In order to test this possibility, leaf extracts were desalted into solution buffered at pH 6.5 or 7.5, both lacking Mg^{2+}. In the absence of Mg^{2+}, the rate of NR activation in vitro at pH 6.5 was much lower than at pH 7.5, and addition of Mg^{2+} (10 mM) caused inhibition only at the higher pH (data not shown). The lack of Mg^{2+} inhibition of the rate of NR activation at pH 6.5 confirms that the lack of EDTA stimulation at acidic pH values (Fig. 5) is not caused by ineffective chelation. Occasionally, at pH 6.5 ($-Mg^{2+}$), a slow spontaneous rectivation occurred similar to that shown in Fig. 1. This reactivation was insensitive to okadaic acid (not shown). Thus, a minor part of the reactivation was probably due to the action of unspecific phosphatases.

The rate of NR activation at pH 6.5 (in the absence of Mg^{2+}) was also stimulated by AMP (0.5 mM; data not shown). Thus, AMP stimulation cannot be attributed, even in part, to chelation of Mg^{2+}. Consequently, we further investigated the specificity of the AMP effect at pH 6.5. As shown in Table 3, 5′-AMP (at 0.6 mM) was more effective than either 3′- or 2′-AMP. Furthermore, in comparison of various 5′-nucleoside monophosphates, purines were more effective than pyrimidines, and 5′-AMP produced the largest stimulation of the compounds tested.

The saturation kinetics of the various AMP isomers were also investigated. As shown in Fig. 6, stimulation by 5′-AMP occurred at relatively low concentrations, and

Table 3. Effect of microcystin-LR on the EDTA- and AMP-stimulated activation of NR in vitro in partially purified preparations. An extract from dark spinach leaves (10 g per 20 ml) was fractionated with polyethyleneglycol (5–15%). The final pellet was resuspended in one-fifth of the original volume of a buffer containing 50 mM Mops (pH 7.5), 10 mM $MgCl_2$ and 1 mM DTT. An aliquot, desalted into the same buffer served as the control (line 1). Another aliquot was supplemented with 1 μM microcystin-LR (MC-LR), incubated at 0°C for 30 min, and then desalted into the above buffer (line 3). The NR activation in vitro was measured in the three enzyme preparations without other additions, or in the presence of excess (15 mM) EDTA or 5 mM AMP

Enzyme treatment	NR activation (percent above initial value)		
	Control	+EDTA	+AMP
Desalted, no addition	25	34	100
Desalt ⇒ add MC-LR	0	0	35
Add MC-LR ⇒ desalt	1	1	35

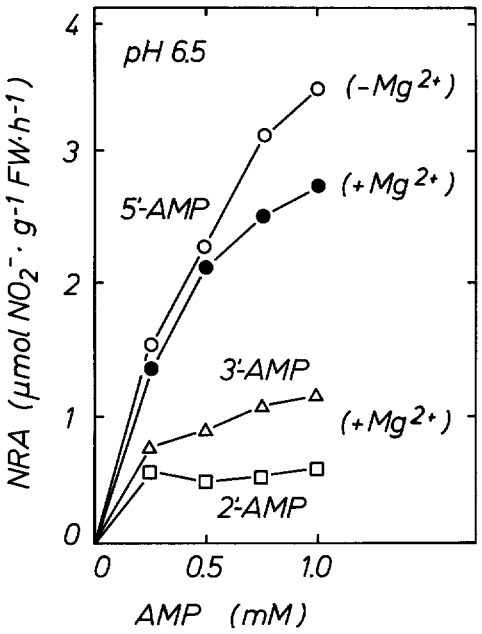

Fig. 6. Saturation kinetics for the stimulation of NR activation in vitro by various isomers of AMP. A crude extract was prepared from darkened spinach leaves and desalted into buffer containing 50 mM Mops-NaOH (pH 6.5), 0.1 mM EDTA and 2 mM DTT. Part of the desalted extract was supplemented with 5 mM $MgCl_2$. As indicated, increasing concentrations of 5'-, 3'-, or 2'-AMP were added to the extracts and the NRA was assayed ($+Mg^{2+}$) as described in *Materials and methods* at time zero and after a 15-min preincubation at 25°C. The increase in NR activity is plotted

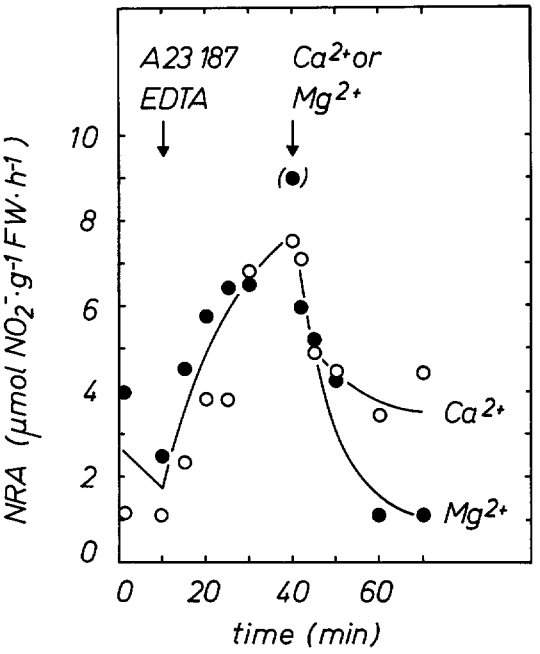

Fig. 7. Representative documentation of the effect of the Ca^{2+}/Mg^{2+}-ionophore A 23187 and EDTA or Ca^{2+} or Mg^{2+} on NRA in dark-incubated slices of spinach leaf in buffer solution. For preparation of leaf slices, attached leaves were kept in the dark for 1 h. Leaf discs (11 mm in diameter) were punched out from four different leaves, and pooled to give one sample with a fresh weight of about 170 mg. The discs were cut with a new razor blade into slices about 1 mm broad, which were suspended in 2 ml of buffer solution (50 mM Hepes-KOH, pH 8.0; 1 mM $CaSO_4$). The slices were gently vacuum-infiltrated and the medium was replaced by another 2 ml of the same buffer solution, in order to remove oxalic acid which trapped divalent cations. Incubation was carried out in a temperature-controlled sample holder at 22°C, in the above-described buffer. A 23187 (final concentration 100 µM), EDTA (10 mM), $CaCl_2$ (30 mM) or $MgCl_2$ (30 mM) were added as indicated in the figure. At each time point, the respective samples were removed, the medium was sucked off, and slices were quenched by addition of liquid nitrogen. Subsequently, the frozen slices were ground and extracted by addition of buffer (2 ml·(g FW)$^{-1}$; 50 mM Hepes-KOH, pH 7.6; 10 mM $MgCl_2$). After centrifugation (5 min, 16000·g), 150 µl of the greenish supernatant was used directly (without desalting) for measuring NRA in a standard assay (run time 3 min). Two separate samples were used for each time point

was essentially unaffected by Mg^{2+}. As expected, the 3'- and 2'-isomers were substantially less effective. Woolf replots of the data in Fig. 6 indicated that the apparent K_m for 5'-AMP was about 0.5 mM, both in the presence and absence of Mg^{2+} (data not shown). The specificity and relatively low apparent K_m (5'-AMP) suggests that fluctuations in cytosolic [AMP] could play an important role in modulating the rate of NR activation, at least under some conditions.

Role of cytosolic free Mg in NR modulation. From the above in-vitro data the impression prevails that cytosolic free Mg^{2+} plays a decisive role in NR regulation. The participating PPs were progressively inhibited if the free [Mg^{2+}] exceeded 0.5 mM, whereas ATP-dependent NR inactivation by the tentative protein kinase depends on free Mg^{2+} (Kaiser and Spill 1991; Spill and Kaiser 1993). Thus, any change in cytosolic free Mg^{2+} should directly affect the activities of the kinase and phosphatase, and thereby the phosphorylation status of NR. However, to our knowledge no data are yet available on the size and on variations of cytosolic Mg^{2+} activities in higher-plant cells. In order to gain some first insight into a possible role of Mg^{2+} in vivo, experiments were carried out with leaf slices in buffer solutions. In contrast to mesophyll protoplasts, leaf slices can be prepared easily from any leaf material, need no osmotic support, and are yet almost as accessible to added solutes as are protoplasts. When leaf slices were incubated for 1 h in aerated buffer solution, the dark-extracted NR was inactive, as with whole leaves. After addition of EDTA plus the Ca/Mg-ionophore A 23187, NRA increased in the dark. It was again inactivated after addition of excess Mg^{2+} or Ca^{2+} (Fig. 7).

Concluding remarks. The results of the present study establish the *potential* for modulation of the in-vivo rate of NR dephosphorylation/activation by the cytosolic concentration of (i) Mg^{2+} and (ii) 5'-AMP. With respect to the former case, it is clear that the cytosol does contain free Mg^{2+} but it is not known whether the concentration of Mg^{2+} changes. However, experimental manipulation using the ionophore A 23187 clearly altered intracellular Mg^{2+}, and NRA changed in response as would be expected if cytosolic Mg^{2+} inhibits NR-PP in situ. It is conceivable that even highly localized changes in cytoso-

lic [Mg^{2+}], which may be difficult to detect, could be involved in the regulation of NR activation in vivo.

With respect to the role of AMP, the specificity for 5′-AMP at concentrations presumed to be within the physiological range suggest that increases in AMP could stimulate NR-PP and thereby activate NR in vivo. As discussed by Kaiser et al. (1992), changes in AMP are more likely to occur with changes in oxygen availability rather than with light/dark signals, where [AMP] appears to be constant. Conceivably, the increase in cellular AMP when darkened leaves or roots are subjected to anoxia (Kaiser et al. 1992; Glaab and Kaiser 1993) could contribute to the activation of NR that is observed. Anoxic utilization of nitrate by NR is thought to be important for anaerobic germination and the flooding tolerance of some species (Reggiani et al. 1993, and refs. therein). Consequently, regulation of NR-phosphatase by 5′-AMP may be of physiological significance.

In spite of the above-suggested role of cytosolic free Mg^{2+} and AMP in the modulation of NR and of the participating protein kinase and PP, it seems clear that the search for other metabolic and/or physical triggers has to continue. Also, data obtained with crude extracts are presently re-examined with the more-purified systems obtained recently (Spill and Kaiser 1993).

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