Plant Physiol. (1997) 114: 1377–1383

Characterization of Nitrate Reductase from Light- and Dark-Exposed Leaves[1]

Comparison of Different Species and Effects of 14-3-3 Inhibitor Proteins

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Nitrate reductase (NR) was extracted and partially purified from leaves of squash (*Cucurbita maxima*), spinach (*Spinacia oleracea*), and three transgenic *Nicotiana plumbaginifolia* leaves in the presence of phosphatase inhibitors to preserve its phosphorylation state. Purified squash NR showed activation by substrates (hysteresis) when prepared from leaves in the light as well as in darkness. A 14-3-3 protein known to inhibit phosphorylated spinach NR in the presence of Mg^{2+} decreased by 70 to 85% the activity of purified NR from dark-exposed leaves, whereas NR from light-exposed leaves decreased by 10 to 25%. Apparent lack of posttranslational NR regulation in a transgenic *N. plumbaginifolia* expressing an NR construct with an N-terminal deletion (ΔNR) may be explained by more easy dissociation of 14-3-3 proteins from ΔNR. Partially purified ΔNR was, however, inhibited by 14-3-3 protein, and the binding constant of 14-3-3 protein (4×10^8 M^{-1}) and the NR-inhibiting protein concentration that results in a 50% reduction of free NR (2.5 nM) were the same for NR and ΔNR. Regulation of NR activity by phosphorylation and binding of 14-3-3 protein was a general feature for all plants tested, whereas activation by substrates as a possible regulation mechanism was verified only for squash.

Evidence for the involvement of phosphorylation for inactivation of NR (EC 1.6.6.1) is reported for several species, including spinach (*Spinacia oleracea*) (Kaiser and Spill, 1991) Arabidopsis (Su et al., 1996), *Nicotiana plumbaginifolia* (Nussaume et al., 1995), *Brassica campestris* (Kojima et al., 1995), maize (*Zea mays*) (Merlo et al., 1995), and barley (*Hordeum vulgare*) (Lillo et al., 1996a). Generally, the phosphorylated form of NR is thought to predominate over the dephosphorylated NR in darkness and vice versa. Phosphorylation of NR at a regulatory conserved Ser residue and association of an inhibiting protein belonging to the 14-3-3 family as a mechanism of down-regulating NRA is well established for spinach (Douglas et al., 1995; Bachmann et al., 1996; Moorhead et al., 1996). The involvement of 14-3-3 proteins in the inhibition of NR from species other than spinach is verified in the present report.

In addition to phosphorylation/dephosphorylation, another posttranslational regulation mechanism for NR has been suggested, based on the observation that squash NR is slowly activated by its substrate, NADH (hysteresis) (Lillo and Ruoff, 1992). To elucidate a possible connection between the two mechanisms, we decided to characterize and compare biochemical properties of NR from light- and dark-exposed leaves of different species. Our study included spinach, which is the best-characterized species regarding phosphorylation/dephosphorylation of NR, and squash (*Cucurbita maxima*), which is best characterized regarding hysteretic properties of NR. Additionally, transgenic *N. plumbaginifolia* was included in the present investigation because it was previously shown that an N-terminal deletion of 56 amino acids abolished the phosphorylation regulation of NR when tested in crude extracts (Nussaume et al., 1995). This means that NRA in crude extracts from dark-treated leaves was no longer inhibited by Mg^{2+}. Also, in contrast to other plants, incubation with MgATP no longer had any inhibitory effect on NRA in crude extracts.

The *N. plumbaginifolia* genotypes included in this study constitutively expressed NR by linkage of the NR-coding sequence to the cauliflower mosaic virus 35S promoter (Vincentz and Caboche, 1991). The *N. plumbaginifolia* C1 line contained a full-length tobacco NR-coding sequence under the control of the 35S promoter. With the C1 line, NRA in crude extracts made from leaves in darkness was strongly inhibited by Mg^{2+}, whereas NR extracted from leaves in the light was only weakly inhibited by Mg^{2+}, which implies that the NR had the common posttranslational regulation by phosphorylation. On the other hand, the activity of ΔNR from del 7 or del 8 transgenic plants in crude extracts showed almost no difference when extracted from leaves subjected to light/dark shifts. This may be caused by an altered phosphorylation response or altered NIP binding by ΔNR (Douglas et al., 1995). However, in this paper we show that after partial purification the de-

[1] This work was financially supported by a grant from the Nansen Foundation to C.L.

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Abbreviations: NIP, reductase-inhibiting protein; NR, reductase; ΔNR, deleted NR; NRA, nitrate reductase activity.

leted NR did show inhibition by 14-3-3 protein like the full-length NR.

MATERIALS AND METHODS

Plants tested were squash (*Cucurbita maxima* L. cv Buttercup), spinach (*Spinacia oleracea* L. cv Merkur), transgenic *Nicotiana plumbaginifolia* (var Viviani) C1 with the full-length tobacco NR (Vincentz and Caboche, 1991), and transgenic *N. plumbaginifolia* del 7 and del 8 with a deleted NR lacking 56 amino acids in the N-terminal domain (Nussaume et al., 1995). del 7 and del 8 are independent transformants carrying the same transgene, and there was no difference in the level of NR expression between them. NR was constitutively expressed by the cauliflower mosaic virus 35S promoter in the transgenic *N. plumbaginifolia* genotypes. All of the NRA detected in these transgenic plants is derived from the transgene expression, since the endogenous NR gene is inactivated by a retrotransposon insertion. Plants were grown at 20°C with a 12-h photoperiod at 80 μmol m^{-2} s^{-1} and watered with Hoagland solution containing 15 mM KNO_3 three times a week and always the day before harvesting (Lillo, 1994).

Extraction and Purification of NR

Leaves (10 g) were harvested in the morning 2 h after the onset of light and after an additional 30 min of darkness. Samples were homogenized with 30 mL of 0.1 M Hepes-KOH (pH 7.5), 3% (w/v) polyvinylpolypyrrolidone, 1 mM EDTA, 7 mM Cys, 7.5 μM leupeptin, 0.1 mM PMSF, 1 μM FAD, 1 μM Na_2MoO_4, 25 mM NaF, and 0.1 μM okadaic acid, filtered through cheesecloth, and centrifuged for 10 min at 31,000*g*. The supernatant was mixed with 3 g of Blue Sepharose and shaken for 40 min, washed with 100 mL of half-strength extraction buffer (omitting polyvinylpolypyrrolidone), and packed into a syringe, and NR was then eluted with 100 μM NADH in the half-strength extraction buffer. The most active Blue Sepharose fractions were desalted on a Sephadex G-25 PD-10 (Pharmacia) column with 25 mM Hepes (pH 7.5), 7 mM Cys, 0.2 mM EDTA, and 0.1 μM okadaic acid and used immediately in experiments. For *N. plumbaginifolia* the procedure needed slight modifications because the general phosphatase inhibitors strongly inhibited NRA in the presence of Hepes buffer. Potassium phosphate buffer was therefore used instead of Hepes. For efficient binding of *N. plumbaginifolia* NR to Blue Sepharose, the crude extract was first desalted on Sephadex G-25 columns equilibrated with half-strength extraction buffer. There was a difference for NR from C1 and del 7/8 regarding elution of enzyme activity from the Blue Sepharose column; only NR from C1 was successfully eluted with NADH, not the del 7/8 NR. For a better comparison, all three *N. plumbaginifolia* NRs were therefore eluted with 0.3 M KNO_3 as described by Mendel (1980). Recovery was about 30%, and the specific NADH:NRA was 10 μmol NO_2^- min^{-1} mg^{-1} protein for spinach and squash, and 2 μmol NO_2^- min^{-1} mg^{-1} protein for tobacco NR and ΔNR. Protein was measured with the protein assay using γ-globulin as a standard (Bio-Rad). NR was purified 1000-fold when eluted with NADH (as for spinach and squash) and about 200-fold when eluted with 0.3 M KNO_3 (*N. plumbaginifolia*). Squash and spinach NR was also purified on a 10-fold larger scale, and a second purification step with monoclonal antibodies (Mac 74) was included (MacKintosh et al., 1995). Effects of 14-3-3 proteins on NRA were confirmed with these preparations.

In experiments presented in Tables I and II the extraction buffer used was 50 mM Hepes-KOH (pH 7.5), 3% (w/v) polyvinylpolypyrrolidone, 1 mM EDTA, 7 mM Cys, 7.5 μM leupeptin, 0.1 mM PMSF, 1 μM FAD, 1 μM Na_2MoO_4, 25 mM NaF, 1 μM microcystin-LR, and 8 mL of extraction buffer for 2 g of leaves. The extracts were tested without desalting, and 75 μL of extract was used for a 0.75-mL assay volume.

Assay of NR

The assay mixture contained 50 mM Hepes-KOH (pH 7.5), 100 μM NADH, and 5 mM KNO_3 with or without 5 mM $MgCl_2$. The assay volume was 0.75 or 0.5 mL. Activity was measured in crude extracts by determining NO_2^- formation and in (partially) purified NR preparation also by continuously following the decrease of NADH at 340 nm (Lillo and Ruoff, 1992). NADH oxidation in the absence of KNO_3 was negligible.

Purification of Yeast 14-3-3 Proteins

The 6-His-tagged BMH1 and BMH2 14-3-3 isoforms from *Saccharomyces cerevisiae* were expressed in *Escherichia coli* DH5α from the trc promotor in plasmid pTrcHisA and purified to homogeneity (Moorhead et al., 1996).

Determination of Binding Constant between NR and 14-3-3 (NIP)

The reversible binding between NIP and NR (in the presence of Mg^{2+} ions) causes a decrease in NRA until equilibrium is established and NRA levels approach constant values.

$$\mathrm{NR} + \mathrm{NIP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{NR}\cdot\cdot\mathrm{NIP} \qquad \text{(Reaction 1)}$$

In the presence of excess NIP, reaction 1 becomes pseudo-first order in NR

$$\mathrm{NR\ (free)} \underset{k_{-1}}{\overset{k_1[\mathrm{NIP}]}{\rightleftharpoons}} \mathrm{NR\ (NIP\ bound)} \qquad \text{(Reaction 1')}$$

with the kinetics

$$\log\left(\frac{[\mathrm{NR}] - [\mathrm{NR}]_\infty}{[\mathrm{NR}]_o - [\mathrm{NR}]_\infty}\right) = -(k_1[\mathrm{NIP}] + k_{-1})t \qquad \text{(Eq. 1)}$$

where $[NR]_o$ and $[NR]_\infty$ are initial and equilibrium concentrations of NR, respectively, and t is time.

Because the activity of NIP-bound NR is considerably

lower than the activity of free NR, the concentration of NR ([NR]) can be expressed as NRA, as long as substrate concentrations remain saturating, i.e. we can write [NR] = constant × NRA. Equation 1 can now be rewritten in the following form:

$$\log\left[\frac{NRA(t) - NRA_\infty}{NRA_0 - NRA_\infty}\right] = -(k_1[NIP] + k_{-1})t \quad \text{(Eq. 2)}$$

The slope $-(k_1[NIP] + k_{-1})$ has been determined by Guggenheim's method (Purich, 1980). In this method $\log(NRA_i - NRA_{i+\Delta})$ is calculated at constant time intervals Δt (here 100 s) and plotted versus time. The slope in the Guggenheim plot is $-(k_1[NIP] + k_{-1})$. By finally plotting the slope against different initial NIP concentrations, a straight line is observed, with k_1 as the slope and k_{-1} as the ordinate intersect. The binding constant $K_{NR\text{-}NIP}$ between NR and NIP (reaction 1) is calculated as the ratio between rate constants k_1 and k_{-1}, i.e.

$$K_{NR\text{-}NIP} = \frac{[NR{\cdot\cdot}NIP]}{[NR][NIP]} = \frac{k_1}{k_{-1}} \quad \text{(Eq. 3)}$$

RESULTS

Factors Affecting NRA in Crude Extracts

NRA in crude extracts prepared from plants in darkness is generally inhibited 50 to 80% by 5 mM Mg^{2+} (Kaiser and Brendle-Behnisch, 1991; MacKintosh, 1992; Lillo, 1994), except for the *N. plumbaginifolia* del 7/8, which shows much less inhibition by Mg^{2+} (Table I; Nussaume et al., 1995). However, Mg^{2+} inhibition was largely abolished within 2 h when extracts were stored at 4°C unless phosphatase inhibitors were included in the extraction buffer. This was shown for squash, spinach, and tobacco NR (Table I). Similar results were observed when leaves were extracted in phosphate buffer (data not shown) rather than Hepes. Desensitization toward Mg^{2+} can be caused by dephosphorylation of NR at a conserved Ser residue located in the hinge 1 region between the heme-binding domain and the Mo-binding domain. Additionally, proteolytic activity may result in a truncated NR that does not bind NIP (Douglas et al., 1995). Since phosphatase inhibitors were crucial to preserve Mg^{2+} inhibition, activation of NR during storage of the crude extracts was likely to be caused by phosphatases. The results also suggested that it is possible to preserve the phosphorylation state of NR and binding ability of NIP when NR is quickly purified (in less than 2 h) in the presence of phosphatase and protease inhibitors, as described in "Materials and Methods," and performed in experiments presented later.

Extracts from various plants showed increased NRA during assay time when made from dark-exposed leaves but not from leaves in the light (Table II). NRA from *N. plumbaginifolia* del 7 increased less than NR from *N. plumbaginifolia* C1 and other plants tested, in agreement with the apparent lack of posttranslational inactivation/activation of ΔNR. Dephosphorylation of phosphoNR is known to lead to increased NRA (MacKintosh, 1992). However, increased NRA during the assay was not inhibited by 25 mM NaF, 1 mM $Na_2H_2P_2O_7$, and 1 μM microcystin-LR added to the assay buffer (same inhibitors that were shown to prevent activation during storage of extracts), and activation is therefore not explained by phosphatase activities. Activation of NRA due to protease activity is also possible (Douglas et al., 1995). However, activation was much less in the presence of Mg^{2+}, implying that activity of the protease involved would be inhibited by Mg^{2+}. One explanation is that the cause of activation is a (slow) dissociation of NIP from NR in the absence of Mg^{2+}. Less activation of ΔNR may be the result of ΔNR being devoid of NIP already in situ or a rapid dissociation of NIP from ΔNR during extraction; hence further activation would not take place. In other words, NIP may be more readily dissociated from ΔNR compared with the full-length NR. NR activation during assay could also be due to activation by substrates as previously shown for squash and maize NR (Lillo and Ruoff, 1992). However, examination of partially purified squash NR showed that there was no difference between NR extracted from leaves in the dark and light with respect to substrate activation (Table II). Activation in crude extracts was observed only for NR from dark-treated leaves and is therefore most likely caused by slow dissociation of 14-3-3 proteins from NR for all species. Attempts to induce hysteresis in partially purified spinach NR by conditions that would rapidly induce hysteresis in squash, e.g. exclusion of Cys from the desalting buffer or storage of NR at

Table I. *Activation of NR during storage in the extraction buffer*

Extracts were made from dark-treated leaves with or without phosphatase inhibitors (25 mM NaF, 1 mM $Na_2H_2P_2O_7$, and 1 μM microcystin-LR) in the extraction buffer. NRA was tested in the presence (+) and absence (−) of 5 mM $MgCl_2$ immediately after homogenization (time 0) and after storage of the extract for 2 h at 4°C. Activity at time 0 tested in the absence of Mg^{2+} is set to 100%. For the different species 100% equals (in μmol NO_2^- g^{-1} fresh weight h^{-1}): spinach, 6.6; tobacco C1, 4.2; tobacco del 7, 2.4; and squash, 10.2. Data are means of experiments repeated twice, and results were within the 10% range.

Source of NR	No Inhibitors				Plus Phosphatase Inhibitors			
	Time 0		Time 2 h		Time 0		Time 2 h	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
	%							
Spinach	100	45	180	165	100	30	95	40
Tobacco, C1	100	48	126	89	100	38	108	44
Tobacco, del 7	100	88	100	86	100	76	84	65
Squash	100	46	120	100	100	36	110	60

Table II. *Increase of NRA during assay of crude extracts or partially purified NR of different species*

Data presented are from assays performed in the absence of Mg^{2+}. In crude extracts NRA was determined by measuring nitrite formation after 2.5, 5, and 10 min of incubation. For partially purified preparations, NRA was measured by recording the NADH consumption continuously. Maximal activity (during the 5- to 10-min span) was compared with initial activity (0–2.5 min), and the difference is given as the percentage increase. Data are typical of several experiments.

Species Tested	Crude Extract		Partially Purified	
	Light	Darkness	Light	Darkness
		%		
Spinach	0	81	0	0
Tobacco, C1	4	67	0	0
Tobacco, del 7	4	24	0	0
Squash	2	104	100	100

high pH (pH 8.5), did not succeed; the spinach NR always showed linear rates of product formation.

Kinetic Parameters

Characterization of partially purified NR from squash and tobacco showed that K_m values for nitrate (50–60 μM) and NADH (1.5–2.0 μM) did not vary significantly between light- and dark-treated plants. This is in agreement with results with crude spinach extracts with high- or low-activity forms of NR (Kaiser and Brendle-Behnisch, 1991; Kaiser and Spill, 1991). Furthermore, there was no significant difference between the tobacco NR and ΔNR with respect to K_m values. Yeast 14-3-3 proteins (10 and 20 nM) inhibited squash NR in a noncompetitive manner with respect to nitrate, i.e. lowering V_{max} but not changing the K_m (data not shown). For tobacco NR and ΔNR, 14-3-3 proteins were tested with respect to NADH and nitrate and was found to be a noncompetitive inhibitor regarding both substrates (data not shown).

Influence of Temperature

The thermosensitivities of ammonium-sulfate-precipitated tobacco NR and ΔNR were previously shown to be different (Nussaume et al., 1995). We therefore tested whether another temperature effect, i.e. activation energy, would differ between NR and ΔNR or NR from light- and dark-treated plants. When measured in the crude extract of *N. plumbaginifolia* del 7 and del 8, activation energy for the NR reaction was only half that of *N. plumbaginifolia* C1 (Table III) and thereby confirmed differences between NR and ΔNR with respect to temperature (Nussaume et al., 1995). Differences in activation energies are reflected in different Q_{10} (reaction rate at a temperature T + 10°C divided by reaction rate at T) values: 1.3 in crude extracts of *N. plumbaginifolia* del 7 and del 8 and 1.6 for *N. plumbaginifolia* C1 and other species (calculated for the 3–25°C temperature range). However, activation energies for partially purified tobacco NR and ΔNR were the same. Activation energy was approximately 32 kJ mol^{-1} for partially purified NR of all species tested (Table III), and no difference was found between NR from light- and dark-treated plants. Nitrite formation was linear in the assay for at least 30 min in a 3 to 25°C temperature range for both crude extracts and partially purified NR. The low stability of ΔNR (Nussaume et al., 1995) is therefore prominent only after certain treatments of the enzyme, e.g. ammonium sulfate precipitation.

Influence of pH

Previously, we showed that the pH optimum for NR in crude extracts from light-exposed squash leaves is 0.2 pH units higher than for dark-exposed leaves (Lillo, 1994). This was found also for partially purified squash NR because phosphorylated NR has a slightly lower pH optimum than dephosphorylated NR in the case of squash NR (Table IV). However, this was different for spinach and tobacco because both crude extracts (data not shown) and partially purified NR (Table IV) from leaves in light and in darkness showed the same pH optimum. Generally, 5 mM Mg^{2+} in the assay buffer resulted in slightly increased NRA at pH 6.5, almost no effect at approximately pH 7.5, and then inhibition of NRA at higher pH (Fig. 1). This resulted in a shift in pH optimum toward a lower pH value in the presence of Mg^{2+} (Table IV). This behavior was found for all species tested and for enzyme extracted from dark- as well as light-treated leaves. The pH curves determined are illustrated in Figure 1 for squash NR from dark leaves. A slight difference in pH optimum between tobacco NR and ΔNR was found, which became more pronounced in the

Table III. *Activation energy of the NR reaction in the crude extract and for partially purified NR from different species*

NRA was tested at 3, 10, 17, and 25°C by measuring nitrite formation during the first 3 min of assay as well as during the 3- to 10-min time span (same activation energies were obtained in both cases). Activation energies were determined by simple Arrhenius plots. The results are based on different preparations (*n*) as indicated in each case. Results are means ± SE.

Species Tested	Crude Extract	Partially Purified	
	Light	Light	Dark
		kJ mol^{-1}	
Spinach	36 (1)	30 (1)	36 (1)
Tobacco, C1	36 ± 2 (4)	36 (2)	32 ± 2 (3)
Tobacco, del 7	18 ± 2 (4)	30 (1)	32 (2)
Tobacco, del 8	14 (2)		34 (1)
Squash	30 (1)	30 ± 2 (3)	28 (1)

Table IV. *pH optimum for partially purified NR from leaves of different species harvested in the light or after 30 min of darkness*

Ten different 50 mM Hepes-KOH buffers were used in the pH range 6.5 to 8.75 to determine the pH optimum. Activities were determined by measuring nitrite formation during 3 min of incubation. Each pH optimum given is based on data from at least three different preparations of NR.

Species Tested	Light		Dark	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
Spinach	8.0	7.5	8.0	7.5
Tobacco, C1	7.3	6.8	7.3	6.8
Tobacco, del 7	7.4	7.2	7.4	7.2
Squash	7.5	7.3	7.3	7.1

presence of Mg^{2+} because del 7 NR was less sensitive for Mg^{2+} effects on pH (Table IV). Addition of 14-3-3 proteins shifted the pH optimum toward more alkaline values (Fig. 1). This was found for squash, spinach, and tobacco. However, these pH curves were obtained by assaying the nitrite production during 5 min of assay. Further analysis revealed that equilibrium between NR and 14-3-3 was not reached within the assay time at all pH values tested. 14-3-3 proteins inhibited NR more rapidly at low pH compared with high pH. When NRA was assayed by continuously following NADH consumption at 340 nm, it was easy to observe that equilibration of 15 nM 14-3-3 protein and NR needed more than 10 min at pH 8 and higher but less than 5 min at low pH values (data not shown).

Effects of 14-3-3 Proteins on NR from Light- and Dark-Treated Leaves

14-3-3 proteins inhibited partially purified NR from all species tested, and the inhibition was stronger when NR was prepared from dark-exposed plants as expected (Table V). Surprisingly, the tobacco ΔNR was inhibited by 14-3-3 proteins. When ΔNR was purified in the presence of phosphatase inhibitors, NRA was inhibited 70% by 14-3-3 proteins. However, when phosphatase inhibitors were excluded, 14-3-3 proteins inhibited ΔNR by only 18%, showing that inhibition by 14-3-3 proteins is indeed dependent on the phosphorylation state of ΔNR as for the full-length NR.

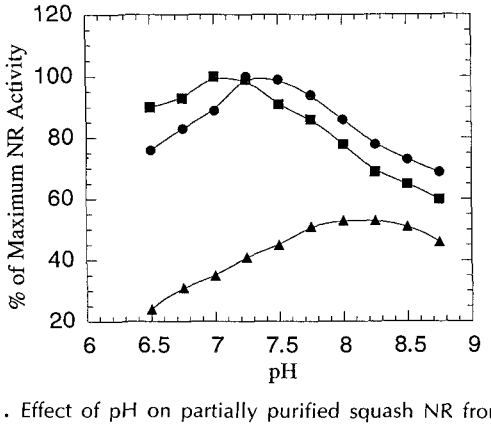

Figure 1. Effect of pH on partially purified squash NR from dark-exposed leaves. Activity was determined by measuring nitrite formed during the 5-min assay. Activity occurred in the absence of free Mg^{2+} (●), in the presence of 5 mM Mg^{2+} (■), and in the presence of 5 mM Mg^{2+} and 15 nM 14-3-3 protein (▲).

Kinetics of NR-NIP Binding and Binding Constant

As mentioned before, the binding between NR and NIP is not an instantaneous process but requires time until equilibrium is established. Depending on the concentration of NIP, the time scale varies from a few seconds to 5 to 10 min. Because the NR·NIP complex has a considerably lower NRA than free NR, a decrease in NRA is observed during the reaction between NR and NIP. Figure 2A shows the exponential decrease in NRA in the presence of different NIP concentrations. Controls without any NIP showed that NRA was constant during the assay time. From Guggenheim plots (see "Materials and Methods") the slope $-(k_1[NIP] + k_{-1})$ has been determined for the different NIP concentrations of Figure 2A. Figure 2B shows the numerical values of these slopes as a function of NIP concentration with the determination of $k_1 = 3.2 \times 10^5\ s^{-1}\ M^{-1}$ and $k_{-1} = 7.9 \times 10^{-4}\ s^{-1}$. The determined binding constant, $K_{NR\text{-}NIP}$, is $4 \times 10^8\ M^{-1}$ (Eq. 3). The NIP concentration that results in a 50% reduction of free NR compared with the initial NR concentration before binding is calculated as $1/K_{NR\text{-}NIP} = 2.5 \times 10^{-9}$ M. Experiments were repeated twice. The same experiments were performed with the ΔNR of *N. plumbaginifolia* del 7 and del 8. No significant differences in NR-NIP binding were found between the full-length tobacco NR and the ΔNR. Time-dependent inactivation of NR at relatively low concentrations of NIP, as described in Figure 2A, was also observed for spinach and

Table V. *Effects of 30 nM 14-3-3 protein on activity of partially purified NR from different species*

Leaves were harvested in the light or darkness. NRA was determined by following NADH consumption and confirmed by measurement of nitrite formation. Dark values are means of three different preparations. Light values are means of one or two preparations. Differences between preparations were generally <10%.

Species Tested	Inhibition by 14-3-3	
	Light	Dark
	%	
Spinach	20	86
Tobacco, C1	24	74
Tobacco, del 7	10	70
Squash	10	82

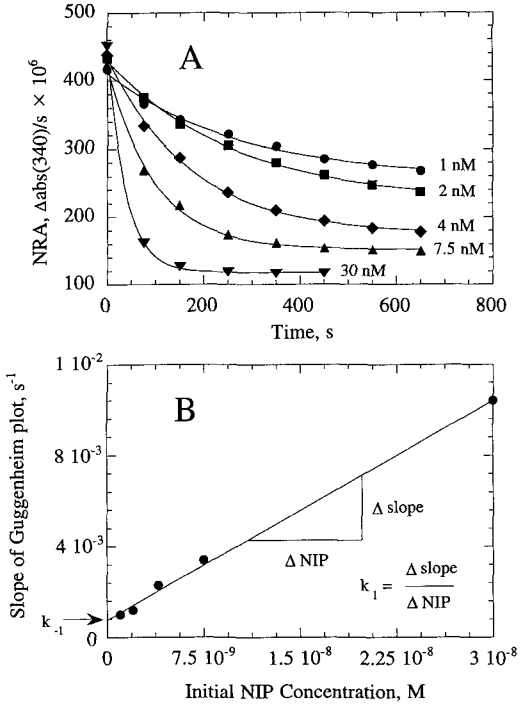

Figure 2. A, Exponential decrease of activity of purified *N. plumbaginifolia* C1 NR in the presence of 1, 2, 4, 7.5, and 30 nM NIP (yeast 14-3-3 protein). At time 0, NIP was added to the assay mixture and the slope of the decrease in NADH absorption (at 340 nm) was measured. Solid lines represent fits of the exponential data to reversible first-order kinetics, i.e. to exponential functions of the form $A + B \times \exp(-Ct)$, where A, B, and C are constants and t is time. The initial reaction rate, Δabs_{340} s^{-1}, corresponds to 4.0 nmol NADH mL^{-1} min^{-1}. B, Calculated slopes $-(k_1[NIP] + k_{-1})$ from linear Guggenheim plots for the NIP concentrations shown in A. The linear regression line has the form 7.9×10^{-4} s^{-1} + 3.2×10^5 s^{-1} M^{-1} × [NIP] with a correlation coefficient of 0.9987.

squash NR. The NIP concentration that results in a 50% reduction of free NR compared with the initial NR concentration before binding was not determined exactly for squash and spinach but was clearly found to be <7 nM (data not shown).

DISCUSSION

Increased NRA during storage of extracts or during assays can be caused by dissociation of inhibiting 14-3-3 proteins, dephosphorylation of phosphoNR, or activation by the substrates NADH and nitrate. The mode of activation depends on the species and incubation conditions. Substrate activation was evident for squash NR and was previously also shown for maize NR (Lillo and Ruoff, 1992). However, tobacco and spinach NR did not show substrate activation. The hypothesis that slow activation by substrates (hysteresis) might be connected with the phosphorylated form of the enzyme extracted from plants in darkness (Huber and Huber, 1995; Lillo et al., 1996b) was not verified. On the other hand, hysteresis was observed for NR purified from squash leaves in the light as well as darkness (Table II) and may therefore be an additional way of regulating NR independently of the phosphorylation state of the enzyme. It remains to be revealed what differences on the molecular level induce substrate activation of squash and maize NR but not spinach and tobacco NR.

Activation during assay of NR in crude extracts can be explained by dissociation of NIP from phosphoNR, whereas NR activation during storage of crude extracts appears to be mainly the result of internal phosphatases in the extracts converting phosphoNR into dephosphoNR (Tables I and II), which is not inhibited by NIP. Under the conditions of the assay, the crude extract is diluted 10 times and the concentrations of 14-3-3 proteins are such that the NR··14-3-3 complex (slowly) dissociates to a lower [NR··14-3-3]/[NR] ratio. The time scale for the 14-3-3 and NR equilibrium as described in Figure 2A agrees with the time scale observed in assays (Table II). Furthermore, since Mg^{2+} is necessary for the binding of 14-3-3 to NR, activation was especially prominent in the absence of free Mg^{2+} as expected (Table II).

NR from light- and dark-treated leaves generally showed identical biochemical characteristics with respect to K_m for NADH and nitrate, activation energy, and pH optimum (Tables III and IV). An exception is the squash NR, which showed a slightly lower pH optimum when isolated from dark-treated leaves.

At relatively high concentrations of NIP (30 nM), almost 90% of complete inactivation was achieved within 75 s (Fig. 2A). At even higher NIP concentrations, time dependence was hardly observable. However, at lower NIP concentrations, several minutes were needed to obtain equilibrium between NIP and NR (Fig. 2A). This time dependence was not observed in previously published results (MacKintosh et al., 1995). However, in the experiments published previously, NIP was varied only 10-fold and the preparation of NIP used was only partially purified, and also, a partially purified kinase was added to the assay system. Therefore, in previous experiments several other components would be present that might bind NIP or influence the equilibrium between NIP and NR. That means that one might easily be using NIP concentrations in the higher range (higher than 30 nm) and at such NIP concentrations that the inactivation process is very rapid, as shown in Figure 2A.

The potency of a mixture of spinach NIP was shown to be very similar to that of yeast 14-3-3 proteins (Moorhead et al., 1996), and it is likely that the tobacco NIP is also as potent as yeast 14-3-3 proteins. However, it should be mentioned that the observation that 14-3-3 proteins inactivate deleted tobacco NR is based on experiments using yeast 14-3-3 proteins and may be different using tobacco 14-3-3 proteins.

Inhibition by 14-3-3 proteins is a general feature of phosphoNR for all species tested. Surprisingly, the ΔNR was also inhibited by 14-3-3 after partial purification and opens up the possibility that ΔNR may be regulated by phosphorylation/dephosphorylation like NR. However, the apparent lack of posttranslational regulation of ΔNR when tested immediately after homogenization (crude extracts) points

to differences in dissociation of the NR··NIP and ΔNR··NIP complex due to some components in the plant and may indicate that ΔNR is devoid of NIP in situ. Reactivation of (Δ)NRA is the subject of further studies.

ACKNOWLEDGMENTS

The authors acknowledge the generous gift of yeast 14-3-3 protein from Drs. C. MacKintosh and G. Moorhead (Department of Biochemistry, University of Dundee, UK).

Received February 21, 1997; accepted May 3, 1997.

LITERATURE CITED

Bachmann M, Huber JL, Liao P-C, Gage DA, Huber SC (1996) The inhibitor protein of phosphorylated nitrate reductase from spinach (*Spinacea oleracea*) leaves is a 14-3-3 protein. FEBS Lett **387:** 127–131

Douglas P, Morrice N, MacKintosh C (1995) Identification of a regulatory phosphorylation site in the hinge 1 region of nitrate reductase from spinach (*Spinacea oleracea*) leaves. FEBS Lett **377:** 113–117

Huber SC, Huber JL (1995) Metabolic activators of spinach leaf nitrate reductase: effects on enzymatic activity and dephosphorylation by endogenous phosphatases. Planta **196:** 180–189

Kaiser WM, Brendle-Behnisch E (1991) Rapid modulation of spinach leaf nitrate reductase activity by photosynthesis. I. Modulation *in vivo* by CO_2 availability. Plant Physiol **96:** 363–367

Kaiser WM, Spill D (1991) Rapid modulation of spinach leaf nitrate reductase by photosynthesis II. *In vitro* modulation by ATP and AMP. Plant Physiol **96:** 368–375

Kojima M, Wu SJ, Fukui H, Sugimoto T, Nanmori T, Oji Y (1995) Phosphorylation/dephosphorylation of Komatsuna (*Brassica campestris*) leaf nitrate reductase in vivo and in vitro in response to environmental light conditions: effects of protein kinase and protein phosphatase inhibitors. Physiol Plant **93:** 139–145

Lillo C (1994) Light/dark regulation of higher plant nitrate reductase related to hysteresis and calcium/magnesium inhibition. Physiol Plant **91:** 295–299

Lillo C, Ruoff P (1992) Hysteretic behavior of nitrate reductase. Evidence of an allosteric binding site for reduced pyridine nucleotides. J Biol Chem **19:** 13456–13459

Lillo C, Smith LH, Nimmo HG, Wilkins MB (1996a) Regulation of nitrate reductase and phosphoenolpyruvate carboxylase activities in barley leaf protoplasts. Planta **200:** 181–185

Lillo C, Smith LH, Nimmo HG, Wilkins MB (1996b) Rhythms in magnesium ion inhibition and hysteretic properties of nitrate reductase in CAM plant *Bryophyllum fedtschenkoi*. Physiol Plant **98:** 140–146

MacKintosh C (1992) Regulation of spinach leaf nitrate reductase by reversible phosphorylation. Biochim Biophys Acta **1137:** 121–126

MacKintosh C, Douglas P, Lillo C (1995) Identification of a protein that inhibits the phosphorylated form of nitrate reductase from spinach (*Spinacea oleracea*) leaves. Plant Physiol **107:** 451–457

Mendel RF (1980) Comparative affinity chromatography of nitrate reductase from wild-type and molybdenum cofactor-defective cell cultures of *Nicotiana tabacum*. Biochem Physiol Pflanz **175:** 216–227

Merlo L, Ferretti M, Passera C, Ghisi R (1995) Light-modulation of nitrate reductase activity in leaves and roots of maize. Physiol Plant **94:** 305–311

Moorhead G, Douglas P, Morrice N, Scarabel M, Aitken A, MacKintosh C (1996) Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusicoccin. Curr Biol **6:** 1104–1113

Nussaume L, Vincentz M, Meyer C, Boutin J-P, Caboche M (1995) Post-transcriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. Plant Cell **7:** 611–621

Purich D (1980) Enzyme kinetics and mechanism, part B. Methods Enzymol **64:** 202–203

Su W, Huber SC, Crawford NM (1996) Identification in vitro of a post- translational regulatory site in the hinge 1 region of Arabidopsis nitrate reductase. Plant Cell **8:** 519–527

Vincentz M, Caboche M (1991) Constitutive expression of nitrate reductase allows normal growth. EMBO J **10:** 1027–1035