Deletion of the Nitrate Reductase N-Terminal Domain Still Allows Binding of 14-3-3 Proteins but Affects Their Inhibitory Properties[1]

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Nitrate reductase (NR) is post-translationally regulated by phosphorylation and binding of 14-3-3 proteins. Deletion of 56 amino acids in the amino-terminal domain of NR was previously shown to impair this type of regulation in tobacco (*Nicotiana plumbaginifolia*) (L. Nussaume, M. Vincentez, C. Meyer, J.-P. Boutin, M. Caboche [1995] Plant Cell 7: 611–621), although both full-length NR and deleted NR (ΔNR) appeared to be phosphorylated in darkness (C. Lillo, S. Kazazaic, P. Ruoff, C. Meyer [1997] Plant Physiol 114: 1377–1383). We show here that in the presence of Mg^{2+} and phosphatase inhibitors, NR and endogenous 14-3-3 proteins copurify through affinity chromatography. Assay of NR activity and western blots showed that endogenous 14-3-3 proteins copurified with both NR and ΔNR. Electron transport in the heme-binding domain of ΔNR was inhibited by Mg^{2+}/14-3-3, whereas this was not the case for NR. This may indicate a different way of binding for 14-3-3 in the ΔNR compared with NR. The ΔNR was more labile than NR, in vitro. Lability was ascribed to the molybdopterin binding domain, and apparently an important function of the 56 amino acids is stabilization of this domain.

Nitrate reductase (NR) is regulated post-translationally by phosphorylation/dephosphorylation and binding of inhibitor proteins, members of the so called 14-3-3 family, to the phosphorylated form of NR (Huber et al., 1996; Moorhead et al., 1996; Su et al., 1996). NR is rapidly inactivated in this way in response to, for instance, a sudden lowering of the light intensity (Kaiser and Brendle-Behnisch, 1991; MacKintosh, 1992; Kojima et al., 1995). To detect this type of regulation, NR activity is assayed in the presence of Mg^{2+}, because formation of the NR/14-3-3 complex requires a divalent cation (MacKintosh et al., 1995; Athwal et al., 1998). A deleted NR lacking 56 amino acids in the amino-terminal end (ΔNR) was previously shown not to obey the usual post-translational dark-inactivation in vivo and was not inactivated by ATP in vitro (Nussaume et al., 1995; Lillo et al., 1997). ΔNR was suggested not to be phosphorylated at the regulatory site, and/or not to bind the endogenous 14-3-3 proteins (Nussaume et al., 1995). ΔNR affinity purified from dark-exposed tobacco leaves was, however, clearly inhibited by yeast 14-3-3, and the ΔNR appeared to be phosphorylated and capable of binding yeast 14-3-3 proteins (Lillo et al., 1997). Six isoforms of 14-3-3 proteins have so far been identified in tobacco (Piotrowski and Oecking, 1998). Different 14-3-3 isoforms may have different functions in the cell (Bachmann et al., 1996a; Sehnke and Ferl, 1996), and it is possible that the ΔNR is incapable of binding to the specific 14-3-3 that regulates NR activity. This would explain the fact that only modest inhibition of ΔNR by Mg^{2+} was detected in the crude extracts, although partially purified ΔNR was inhibited by yeast 14-3-3 in the presence of Mg^{2+} (Lillo et al., 1997). However, the work presented here shows that the ΔNR is inhibited by endogenous 14-3-3 proteins. Binding of 14-3-3 proteins to NR has also been demonstrated by immunoprecipitation (Weiner and Kaiser, 1999). We have copurified NR and 14-3-3 proteins by Blue Sepharose affinity purification; and in the presence of phosphatase inhibitors and Mg^{2+}, 14-3-3 proteins were found to co-purify with NR and ΔNR based on activity assays and western blots. Under certain conditions, the stability of NR and ΔNR is different (Nussaume et al., 1995). Lability of ΔNR was further confirmed in the present work, and we suggest that stabilization of NR activity is an important function of the N-terminal domain of NR.

RESULTS

Inhibition of Purified NR and ΔNR by Mg^{2+} and Endogenous 14-3-3

We partially purified NR from different sources in the presence of phosphatase inhibitors and in the presence or absence of Mg^{2+} (Table I). In agreement with previous results, the phosphorylation state is preserved when NR is partially purified in the presence of phosphatase inhibitors (Lillo et al., 1997). This was confirmed by the high Mg^{2+} inhibition always

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Table I. *Inhibition of NR and ΔNR activity by 5 mM free Mg^{2+} in the assay*

Tests were made with crude extracts and after Blue Sepharose affinity purification of NR from spinach, and tobacco C1 and ΔNR from tobacco del7. Extracts were made from plants 30 min after transfer to darkness. After purification NR and ΔNR activity was also tested in the presence of exogenous 14-3-3 (30 nM yeast 14-3-3 protein). Data presented are mean values from *n* preparations as indicated. SE is given.

Plant Source	Extraction Buffer	Inhibition by Mg^{2+}			
		Crude extract	Crude extract (after centrifugation)	Affinity purified (assay without yeast 14-3-3)	Affinity purified (assay with yeast 14-3-3)
		%			
Spinach ($n = 3$)	0.1 M HEPES, 25 mM NaF, 0.1 μM okadaic acid, 10 mM $MgCl_2$	84 ± 5	83 ± 6	66 ± 7	68 ± 5
Spinach ($n = 3$)	0.1 M HEPES, 25 mM NaF, 0.1 μM okadaic acid	86 ± 4	81 ± 4	0 ± 4	80 ± 8
Tobacco C1 ($n = 6$)	0.1 M HEPES, 0.1 μM okadaic acid, 10 mM $MgCl_2$	81 ± 2	77 ± 4	46 ± 4	60 ± 4
Tobacco C1 ($n = 3$)	0.1 M Phosphate, 25 mM NaF, 0.1 μM okadaic acid	53 ± 1	52 ± 8	6 ± 3	68 ± 6
Tobacco del7 ($n = 6$)	0.1 M HEPES, 0.1 μM okadaic acid, 10 mM $MgCl_2$	45 ± 5	24 ± 6	34 ± 3	55 ± 7
Tobacco del7 ($n = 3$)	0.1 M Phosphate, 25 mM NaF, 0.1 μM okadaic acid	48 ± 12	50 ± 13	6 ± 6	68 ± 4

observed when yeast 14-3-3 was added to the assay buffer (Table I). Preparations made in the absence of 10 mM Mg^{2+} in all buffers were clearly different from preparations made in the presence of Mg^{2+}. When NR was affinity purified in the absence of Mg^{2+}, almost no difference was seen between assays with EDTA and assays with Mg^{2+}, i.e. Mg^{2+} added to the assay gave only 0% to 6% inhibition (Table I). Apparently, when prepared in the absence of Mg^{2+}, endogenous 14-3-3 was not retained in the preparation. However, when Mg^{2+} was present during purification, Mg^{2+} inhibition of partially purified NR was 41% to 66%, reflecting that endogenous 14-3-3 was present. This supports the assumption that one effect of Mg^{2+} is indeed to facilitate binding of 14-3-3 to phosphorylated NR, not just to inhibit activity of the NR/14-3-3 complex. Both tobacco NR (from C1 plants) and ΔNR (from del7 plants) purified in the presence of Mg^{2+} were inhibited by Mg^{2+}, showing that endogenous 14-3-3 copurified with NR as well as with ΔNR (Table I). However, less Mg^{2+} inhibition was observed for ΔNR compared with NR, indicating that less endogenous 14-3-3 was present in the ΔNR preparation compared with the NR preparation. Purification of NR and ΔNR in phosphate buffer confirmed that ΔNR and NR were both phosphorylated in dark-exposed tobacco plants. Phosphate is known to inhibit phosphatases, and clearly after purifying tobacco NR and ΔNR in phosphate buffer, strong inhibition by Mg^{2+} in the presence of yeast 14-3-3 was observed. However, Mg^{2+} alone inhibited NR activity by only 6% showing that endogenous 14-3-3 did not copurify with either NR or ΔNR when prepared in this buffer (Table I).

During this work, we observed that Mg^{2+} inhibition of the ΔNR activity decreased significantly after centrifugation of the crude extract. Activity was, therefore, tested immediately after extraction and again after centrifugation also for extracts of other plants. However, the decrease in Mg^{2+} inhibition during centrifugation was observed only for ΔNR and only in HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], not in phosphate buffer. We then wanted to determine whether centrifugation or time was crucial for the decrease in Mg^{2+} inhibition of the ΔNR enzyme. A 1-min centrifugation in a microcentrifuge at 12,000*g* did not lead to more than a 4% decrease in Mg^{2+} inhibition. Furthermore, when part of the extract was left at 0°C, whereas the other part of the extract was centrifuged for 10 min at 30,000*g*, the decrease of Mg^{2+} inhibition was observed in both cases, i.e. time was the critical factor for activation of ΔNR, not removal of debris by centrifugation. These results point to previous suggestions that some factor in the crude extract appears to activate ΔNR, whereas NR is not activated by the same factor (Pigaglio et al., 1999).

Generally, only small differences in activity were seen between assays with and without Mg^{2+} when testing ΔNR in crude extracts made with HEPES

buffer and assayed after centrifugation (Table I; Nussaume et al., 1995; Lejay et al., 1997). To determine whether this difference was due to kinetic characteristics, we tested the concentration of cations necessary for half maximal inhibition of NR and ΔNR partially purified from dark-exposed plants in the presence of phosphatase inhibitors (phosphorylated enzyme). Cations which could be of importance in addition to Mg^{2+} in vivo include the polyamine spermidine, which is known to reach fairly high concentrations in plant tissue (0.3 mM) and is involved in regulation of various processes (Flores and Galston, 1984). Although highly speculative, inhibition by spermidine could represent a possible negative feedback mechanism in NO_3 assimilation by a metabolite rich in nitrogen. The concentrations of Mg^{2+} and spermidine were varied in the range 0 to 5 mM and 0 to 1 mM, respectively, in the presence, as well as absence, of yeast 14-3-3. Mg^{2+} and spermidine did not inhibit NR or ΔNR at these concentrations unless 14-3-3 was present. In the presence of 14-3-3, 1 mM Mg^{2+} or 0.2 mM spermidine gave half maximal inhibition of NR and ΔNR. No difference in IC_{50} values was seen for NR and ΔNR (Table II). The maximal inhibition of NR and ΔNR activity varied between 70% and 80% in these experiments. At higher concentrations of Mg^{2+} (10 mM) and spermidine (2 mM), some inhibition of NR activity was detected, even in the absence of 14-3-3. The same IC_{50} values were found for Mg^{2+} and for spermidine in the presence of endogenous tobacco 14-3-3. However, no difference was found between NR and ΔNR with respect to IC_{50} values.

Immunoblot Analysis

Western blots confirmed that in the presence of phosphatase inhibitors and Mg^{2+}, 14-3-3 proteins copurified with NR. This was found for both spinach and squash (blots not shown), as well as for full-length and deleted tobacco NR (Fig. 1). Lanes 1 and 3 show that 14-3-3 proteins were present in preparations of Blue Sepharose-purified NR and ΔNR when Mg^{2+} was present in all buffers during purification, whereas 14-3-3 proteins were not detectable when Mg^{2+} was omitted (lanes 2 and 4). The NR/14-3-3 complex was eluted from Blue Sepharose with 0.3 M KNO_3 (lanes 1 and 3) or 100 μM NADH (lanes 5 and 6).

Table II. *Concentration of Mg^{2+} or spermidine necessary for half-maximal inhibition (IC_{50}-values) of purified NR and ΔNR activity in the presence of 30 nM yeast 14-3-3*

Three preparations from C1 and del7 plants harvested after 30 min of darkness and prepared in the presence of phosphatase inhibitors were tested. SE is given.

Plant Source	IC_{50}	
	Mg^{2+}	Spermidine
	mM	
Tobacco, C1	0.95 ± 0.25	0.21 ± 0.02
Tobacco, del7	1.25 ± 0.25	0.16 ± 0.03

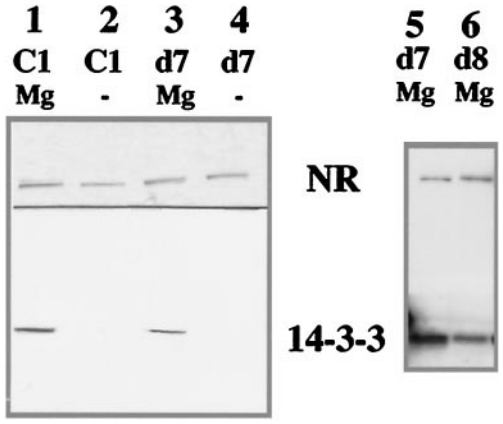

Figure 1. Protein-gel blots showing NR, ΔNR, and 14-3-3 proteins after Blue Sepharose chromatography of extracts from C1, del7 (d7), and del8 (d8) plants. Affinity-purified NR and ΔNR were subjected to SDS-PAGE. NR purified in the presence (lane 1) and absence of Mg^{2+} (lane 2). ΔNR (d7) purified in the presence (lane 3) and absence of Mg^{2+} (lane 4). Lanes 5 and 6 show ΔNR from del7 and del8 purified in the presence of Mg^{2+}, and a different assay for detecting 14-3-3 protein was used (see "Materials and Methods"). Samples tested in lanes 1 through 4 were eluted with KNO_3 from the Blue Sepharose column and concentrated on Centricon 30. Samples tested in lanes 5 and 6 were eluted with NADH from the Blue Sepharose column and concentrated on Centricon 100. Amounts of protein added to wells 1 through 4 were approximately 6 μg and for wells 5 and 6, approximately 2 μg. Following transfer of the proteins to a nitrocellulose membrane, the membrane was cut in two halves. Top, Upper half of the membrane was probed with antiserum raised against squash NR. Bottom, Lower half of the membrane was probed with antiserum raised against spinach 14-3-3 proteins.

For all species tested, Mg^{2+} in the extraction buffer and buffers used during purification was necessary for detection of 14-3-3 protein on the blot. As a control, a crude (spinach) extract was passed through an antibody column, Mac74 (MacKintosh et al., 1995), which removed 90% of NR activity. This extract, low in NR, was then passed through a Blue Sepharose column, and proteins were eluted with 0.3 M KNO_3. Only negligible amounts of 14-3-3 bound to the column. The experiment confirmed that 14-3-3 elution from the Blue Sepharose column is indeed dependent on the presence of NR.

The results shown in Table I and blots shown in Figure 1 strongly support the assumption that endogenous 14-3-3 proteins bind to ΔNR as well as NR. Densitometer measurements of four different preparations of NR and ΔNR on various blots indicated that, to some degree, 14-3-3 bands were weaker for del7 extracts compared with C1 extracts. However, considerable variations among extracts prevented strict conclusions.

Partial Activities

Different partial activities of affinity-purified tobacco NR and ΔNR were tested with dephosphory-

lated enzymes (leaves harvested in the light, no phosphatase inhibitors present in extraction buffers) and phosphorylated enzymes (leaves harvested after 30 min of darkness, phosphatase inhibitors in extraction buffers). As expected, dephosphorylated NR and ΔNR activities were not inhibited by Mg^{2+}. Like the partial activity of phosphorylated spinach NR (Bachmann et al., 1996b), the reduced methylviologen (MV) to NR partial activity of tobacco NR was inhibited by Mg^{2+} when leaves were harvested from darkness. Also the MV to ΔNR activity was inhibited by Mg^{2+} (Table III). However, ΔNR and NR behaved differently, because for ΔNR the NADH to NADH-cytochrome c reductase partial activity (CR) was inhibited by Mg^{2+}. This surprising result was confirmed with four independent preparations of purified ΔNR and NR. Inhibition of NADH to CR by Mg^{2+} was dependent on the phosphorylation state of ΔNR; inhibition was only seen for enzyme prepared from dark-treated leaves (Table III). NADH to ferricyanide-NR partial activity of ΔNR and NR was not inhibited by Mg^{2+} (Table III). The NADH to ferricyanide-NR partial activity involves only the FAD-binding domain, whereas NADH to CR involves also the heme-binding domain (Rouzé and Caboche, 1992). The binding site for 14-3-3 proteins is at the hinge between the heme-binding and Mo-cofactor binding domain (Bachmann et al., 1996b; Moorhead et al., 1996), and it may, therefore, be expected that the heme-binding domain, but not the FAD-binding domain, is influenced by Mg^{2+} in combination with 14-3-3. The surprising result is that the deletion in the N-terminal region of NR is necessary to see any effect of 14-3-3 and Mg^{2+} on the partial NADH to CR activity. The results show that ΔNR and NR have some different characteristics with respect to 14-3-3.

Labile ΔNR Activity

When using NADH as an electron donor and NO_3 as an acceptor, electron transfer includes all three cofactor binding domains of NR (FAD-, heme-, and molybdopterin-binding domains), whereas Cytochrome c (cyt c) as substrate needs only active FAD- and heme-binding domains. The ratio of CR to NR and ΔNR activities varied between preparations, but was always higher for ΔNR. An average of six different preparations after affinity purification gave a ratio of 3 for NR and 7 for ΔNR (Table IV). The number of units of purified NR and ΔNR was clearly related to the number of units in the crude extracts (Table IV), and the yield varied from 34% to 48%. The NR activity was approximately three times higher in crude extracts from C1 plants compared with del7 plants (data derived from Table IV). However, CR activity was similar in preparations from del7 and C1 plants. This shows that del7 plants contained a high proportion of inactive ΔNR, i.e. only the partial CR activity was functional. Alternatively, the ΔNR was immediately inactivated when extracted and assayed. Clearly, ΔNR is more labile than NR in vitro. For instance, $(NH_4)_2SO_4$ precipitation of ΔNR resulted in unstable enzyme (Nussaume et al., 1995). Following Blue Sepharose purification, ΔNR activity was unstable if not immediately desalted. For instance, after incubation of the enzyme at 25°C for 60 min, 80% of the ΔNR activity was lost, whereas NR activity was unchanged under these conditions (Fig. 2). Inactivation of ΔNR was not caused by proteolysis because a western blot was performed with the ΔNR sample at time 0, 60, and 120 min of incubation at 25°C, and showed no changes in ΔNR protein (blot not shown). CR was stable during such experiments, which confirms that inactivation of activity was not due to proteolysis, but rather, caused by inactivation of the Mo-containing domain. NR and ΔNR were eluted with a high salt concentration (0.3 M KNO_3), and after desalting, both NR and ΔNR were stable during 1 h at 25°C (Fig. 2). However, attempts to ascribe the lability of ΔNR to a salt effect were not successful because, although ΔNR was much more stable after desalting of the Blue Sepharose fractions, adding back KNO_3 or other salts (KCl, K_2SO_4) up to 0.3 M did not render the enzyme unstable. Apparently, ΔNR is unstable after the conformation change that probably takes place during elution from Blue Sepharose. However, this behavior is still not understood.

Tobacco NR and ΔNR were routinely eluted with NO_3^- from the Blue Sepharose column because ΔNR activity was hardly detectable after elution with NADH. However, CR was still present in these NADH-eluted preparations. Following NADH elution, the ratio of CR to NR was 2 and the ratio of CR to ΔNR was 20. Further tests of NADH-eluted ΔNR showed that the very terminal activity, i.e. the reduced bromphenol blue to NR that involves the molybdopterin cofactor binding domain only (Rouzé and Caboche, 1992), was also inactivated (data not shown). Reversible inactivation of NR by NADH has previously been reported for Chlorella and wheat and was shown to depend on FAD and formation of superoxide, and involve the molybdopterin-binding domain (Moreno et al., 1972; Aryan and Wallace, 1985). Attempts to regain activity by incubation with

Table III. *Inhibition of partial activities of NR and ΔNR by 5 mM Mg^{2+} in the presence of 30 nM yeast 14-3-3 protein*

Assays were run in triplicate, and SE ≤ 5%.

Plant Source and Light Conditions	Inhibition by Mg^{2+}		
	NADH:CR	NADH:FR	MV:NR
		%	
Tobacco, C1, light	0	11	8
Tobacco, C1, darkness	10	5	73
Tobacco, del7, light	9	12	8
Tobacco, del7, darkness	46	4	71

Table IV. *Total activity in units (nmol NO_2^- formed per NADH consumed or Cyt c reduced per min) in crude extract and affinity purified NR and ΔNR preparations using 10 g of leaf tissue*

There were *n* repeats for each type of extraction buffer and species. CR was measured in three of the preparations in each case. SE is given.

Plant Source (NR/ΔNR)	Extraction Buffer	Crude Extract (NR Units)	Affinity Purified (NR Units)	Affinity Purified (CR Units)
Tobacco, C1 (NR)	0.1 M HEPES, 0.1 μM okadaic acid, 10 mM $MgCl_2$	789 ± 160 (n = 4)	379 ± 92	1,413 ± 232
Tobacco, del7 (ΔNR)	0.1 M HEPES, 0.1 μM okadaic acid, 10 mM $MgCl_2$	254 ± 39 (n = 4)	124 ± 10	1,261 ± 120
Tobacco, C1 (NR)	0.1 M Potassium-phosphate, 25 mM NaF, 0.1 μM okadaic acid	1,330 ± 204 (n = 3)	455 ± 73	1,080 ± 272
Tobacco, del7 (ΔNR)	0.1 M Potassium-phosphate, 25 mM NaF, 0.1 μM okadaic acid	385 ± 39 (n = 4)	155 ± 28	736 ± 53

an oxidizing agent like ferricyanide, 0.5 mM, as described by Aryan and Wallace (1985), increased ΔNR activity slightly. However, ΔNR activity was still very low compared with NO_3^- eluted ΔNR or NR. Since most of the ΔNR protein was still inactive with respect to the terminal activity after treatment with ferricyanide, this argues against (reversible) inactivation by superoxide, but supports the assumption that the molybdopterin cofactor is released or nonfunctional (Nussaume et al., 1995).

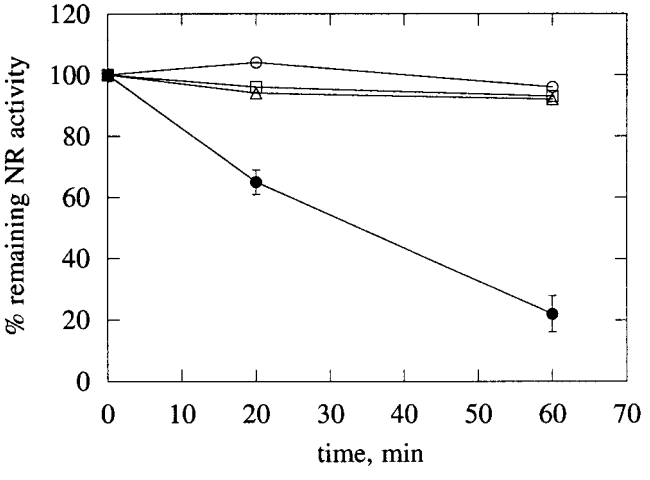

Figure 2. Stability of NR and ΔNR in fractions of freshly prepared affinity-purified enzyme eluted with 0.3 M KNO_3. ΔNR not desalted (●), NR not desalted (○), ΔNR desalted (△), and 0.3 M KNO_3 was added to desalted ΔNR (□). Storage temperature was 25°C. The experiment was repeated three times with different enzyme preparations. Data presented represent (△) NR prepared in phosphate buffer and in the absence of Mg^{2+}, however, the same results were obtained for preparations made in HEPES buffer in the presence or absence of Mg^{2+} and phosphatase inhibitors. SE is indicated when exceeding the size of the symbol.

DISCUSSION

Following purification of phosphorylated NR and ΔNR, IC_{50} values for yeast 14-3-3 (Lillo et al., 1997), Mg^{2+}, and spermidine (present work) were the same for NR and ΔNR. No differences of kinetic constants were found that would explain the modest Mg^{2+}-inhibition (Nussaume et al., 1995; present paper) often found for ΔNR in crude extracts.

Following centrifugation, i.e. 15 min after extraction, Mg^{2+} inhibition of ΔNR in HEPES buffer was only about 25% (Table I); in agreement with values found previously under similar extraction and assay conditions (Nussaume et al., 1995). However, in the present work, we also show that when ΔNR activity was tested in extracts immediately after homogenization of leaves harvested from darkness, stronger Mg^{2+} inhibition was observed (45%), but still not as strong as for NR (81%). The decrease in Mg^{2+} inhibition after extraction was not observed for NR. This implies that modest Mg^{2+} inhibition of ΔNR in crude extracts may be caused by reactions taking place both in the plant and after extraction. When extracts were made in phosphate buffer, less inhibition of NR was observed compared with HEPES buffer; Mg^{2+} inhibition was almost the same for NR and ΔNR (Table I). High concentration of phosphate is likely to promote release of 14-3-3 protein from NR and may therefore result in NR appearing more similar to ΔNR. Phosphate is known to stimulate NR activity by binding to the molybdopterin-binding domain (Solomonson and Barber, 1990) and, possibly, phosphate stabilizes ΔNR and also makes ΔNR and NR more similar in activity assays.

The fact that the ΔNR protein was inactivated by yeast 14-3-3 after purification led us to propose previously a model where a putative activating factor would remain bound to the ΔNR protein in crude extracts and would impede 14-3-3 binding (Pigaglio

et al., 1999). We show here that endogenous 14-3-3 copurified with ΔNR through the purification process, as shown by activity measurements in the presence of Mg^{2+} (Table I), as well as western blots (Fig. 1). Thus, our previous model appears to be no longer valid, as the ΔNR protein seems to bind 14-3-3 proteins to almost the same extent as NR. An explanation for this observation could be that 14-3-3 proteins bind the ΔNR protein to a different site and/or in a different manner. Although no alternative binding site for 14-3-3 proteins has so far been described for NR, other 14-3-3 interacting proteins, like the Raf kinase, present multiple sites of interaction with 14-3-3 (Tzivion et al., 1998). To reconcile our previous model with the present data, we propose that the factor which remains bound to the ΔNR protein is in fact the interacting 14-3-3 protein(s). In this case, 14-3-3 binding would inactivate ΔNR less, either because the 14-3-3 proteins are also bound to another site on ΔNR or because the ΔNR/14-3-3 complex adopts a different conformation due to the removal of the NR N-terminal region. This hypothesis is supported by the fact that 14-3-3 proteins inhibited CR partial activity to a different extent in NR and ΔNR proteins (Table III).

Clearly, the deletion in the N-terminal domain influenced characteristics of the ΔNR/14-3-3 complex because electron transport in the heme-binding domain of ΔNR was inhibited by Mg^{2+}/14-3-3, whereas this was not the case for NR (Table III). A striking effect of the deletion was the influence on stability (Fig. 2; Table IV); NADH to CR activity was retained, but the terminal activity (associated with molybdopterin-binding domain) was inactivated. Generally, when NADH and NO_3^- were used as substrates, del7 plants had only 30% of NR activity, compared to C1 plants. However, when the partial CR activity was tested, activities in C1 and del7 plants were similar (Table IV). This shows that, for the ΔNR, a large proportion of the terminal activity was inactive or less active than for NR. This deactivation took place during extraction or also in the plant. It is therefore likely that an important function of the N-terminal domain is stabilization of the molybdo-pterin-binding domain.

The question that still remains open is the actual activation state of the ΔNR protein in the plant. If regulation of ΔNR is seriously impaired in vivo, accumulation of nitrite in the leaves in darkness would be expected (Riens and Heldt, 1992). Accumulation of nitrite was, however, never observed in del7 plants, since extracts did not have any background level of nitrite. Moreover, as reported by Lejay et al. (1997), almost no stimulation of NO_3^- reduction in the dark was detected in del7 plants compared with control plants, despite of the high activation state of the ΔNR protein. Since accumulation of nitrite could have serious negative effects on plants, redundant strategies may have evolved to assure that this toxic compound is not allowed to accumulate. It has been proposed that the limited availability of reducing power in darkness would prevent or at least decrease any effect of the ΔNR higher-activation state. Indeed, when the del7 and del8 plants were grown in the light in very low CO_2 concentrations, where reducing power is probably not limiting, a higher NO_3 reduction rate was observed (Lejay et al., 1997). Other evidence also supports a possible higher activation state of the ΔNR protein in vivo. It has been proposed that the stability of NR is decreased upon inactivation (Weiner and Kaiser, 1999), and we have previously shown that the ΔNR protein is more stable in darkened plants than the NR protein (Pigaglio et al., 1999).

Taken together, these results indicate that the ΔNR protein is probably less inactivated by 14-3-3 proteins than NR in planta and in vitro, although 14-3-3 proteins seem to remain bound to it, and that the Mg^{2+} inhibition of this protein is decreasing with time after extraction. Thus, we propose that the absence of the NR N-terminal domain affects in some way the enzymatic activities of the ΔNR/14-3-3 complex.

MATERIALS AND METHODS

Plant Material

Plants tested were: squash (*Cucurbita maxima* L. cv Buttercup), spinach (*Spinacea oleracea* L. cv Merkur), *Nicotiana plumbaginifolia* var. Viviani C1 with the full-length tobacco NR (Vincentz and Caboche, 1991), and *N. plumbaginifolia* del7 and del8 with a ΔNR lacking 56 amino acids in the N-terminal domain (Nussaume et al., 1995). del7 and del8 are independent transformants carrying the same transgene, and there was no difference in the level of NR expression between them. NR was constitutively expressed using the cauliflower mosaic virus 35 S promoter in the *Nicotiana*. All the NR activity detected in these transgenic plants is derived from the transgene expression, as 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 were 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, followed by 30 min of darkness. Samples were homogenized with 30 mL of 0.1 M HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 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 (stock solution made up in dimethylsulfoxide, Calbiochem, San Deigo), filtered through cheesecloth, and centrifuged for 10 min at 31,000*g*. In some cases, $MgCl_2$ was omitted, see "Results." The supernatant was mixed with 3 g of Blue Sepharose equilibrated in homogenization buffer (omitting polyvinylpolypyrrolidone), and shaken

for 40 min, then washed with 100 mL of one-half-strength extraction buffer (omitting polyvinylpolypyrrolidone), packed into a syringe, and NR was then eluted with 100 μM NADH or 0.3 M KNO_3 (in case of tobacco) in the one-half-strength extraction buffer. The most active Blue Sepharose fractions were desalted on a Sephadex G-25 PD-10 column (Pharmacia Biotech, Piscataway, NJ) with 25 mM HEPES (pH 7.5), 7 mM Cys, 0.2 mM EDTA, and 0.1 μM okadaic acid, and concentrated in centricon 30 (Millipore, Bedford, MA), or, when mentioned, in centricon 100. For tobacco, NaF strongly inhibited NR activity in the presence of HEPES buffer and had to be avoided. When NaF was included, potassium phosphate buffer was used, otherwise additions were as for HEPES buffer. For efficient binding of tobacco NR to Blue Sepharose, the crude extract was first desalted on Sephadex G-25 columns equilibrated with one-half-strength extraction buffer. Recovery was about 30%, and the specific NADH to NR activity 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 a protein assay (Bio-Rad, Hercules, CA) using γ-globulin as a standard. NR was purified 1,000-fold when eluted with NADH (as for spinach and squash) and about 200-fold when eluted with 0.3 M KNO_3 (tobacco).

Assay of NR

The assay mixture contained 50 mM HEPES-KOH (pH 7.5), 100 μM NADH, and 5 mM KNO_3 with 2 mM EDTA or 5 mM $MgCl_2$. The assay volume was 0.75 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.

To measure MV to NR, NADH was substituted by 100 μM MV and 0.8 mg $Na_2S_2O_4$. To measure NADH to CR, KNO_3 was substituted by 0.5 mg Cyt c. Controls with addition of an anti-NR monoclonal antibody (96[9]25), which inhibits CR activity, assured that the CR measured was indeed linked to NR (Chérel et al., 1990). To measure NADH to ferricyanide reductase, KNO_3 was substituted by 0.5 mM potassium ferricyanide. (for details on assays, see Wray and Fido, 1990). One unit of enzyme activity is defined as the rate of 1 μmol of nitrite produced per NADH reduced or 1 μmol Cyt c reduced in 1 min. All assays were performed at 25°C. NADH to NR, MV to NR, and reduced bromphenol blue to NR were assayed by incubating for 5 min and measuring the nitrite produced as the diazo-compound formed from sulfanilamide (Lillo, 1983). NADH to CR was measured by continuously following the production of reduced Cyt c in a spectrophotometric cell at 550 nm.

Purification of Yeast 14-3-3 Proteins

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

Immunoblot Analysis

Proteins were separated using a discontinuous buffer system SDS-PAGE (12% gels) electrophoresis, according to the method of Laemmli (1970). Protein preparations, 2 to 6 μg, were applied to each well. Proteins were transferred to a nitrocellulose membrane (0.45 μm) using a mini trans-blot electrophoretic cell (Bio-Rad). The transfer buffer used was 25 mM Tris, 192 mM Gly, and 20% (v/v) methanol, pH 8.3. The upper section of the membrane was incubated with rabbit polyclonal antibodies raised against purified squash NR, and the lower section was treated with polyclonal antibodies raised against spinach 14-3-3 in sheep. The immunocomplexes were recognized using protein G conjugated to horseradish peroxidase. The membranes were developed using a color development method (Fig. 1, lanes 1–4; Bio-Rad) and the ECL chemiluminescent detection method (Fig. 1, lanes 5 and 6; Amersham Pharmacia, Uppsala). The 14-3-3 antibodies were kindly provided by Drs. Moorhead and MacKintosh (Department of Biochemistry, University of Dundee, Dundee, UK).

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