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Identification of a Protein That Inhibits the Phosphorylated Form of Nitrate Reductase from Spinach (*Spinacia oleracea*) Leaves[1]

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The low-activity, phosphorylated form of nitrate reductase (NR) became activated during purification from spinach (*Spinacia oleracea*) leaves harvested in the dark. This activation resulted from its separation from an approximately 110-kD nitrate reductase inhibitor protein (NIP). Readdition of NIP inactivated the purified phosphorylated NR, but not the active dephosphorylated form of NR, indicating that the inactivation of NR requires its interaction with NIP as well as phosphorylation. Consistent with this hypothesis, NR that had been inactivated in vitro in the presence of NR kinase, ATP-Mg, and NIP could be reactivated either by dephosphorylation with protein phosphatase 2A or by dissociation of NIP from NR.

Nitrate from the soil is reduced to ammonium in the leaves of higher plants by the activities of cytoplasmic NR (EC 1.6.6.1.) and chloroplastic NiR (Wray and Fido, 1990). Both enzymes are subject to multivalent control at the level of both gene expression and posttranslational modification in response to environmental factors. Illumination and applied nitrate are both necessary to attain maximal rates of transcription and de novo protein synthesis of inducible NR and NiR in leaves (Melzer et al., 1989; Cheng et al., 1992; Mohr et al., 1992). In some plants there is additional control of NR and NiR gene expression by circadian and diurnal rhythms (reviewed by Lillo, 1994). The diurnal regulation may be mediated by variations in the intracellular concentration of reduced N metabolite(s), such as Gln (Deng et al., 1991; Shiraishi et al., 1992). It is thought that this complex regulation of gene expression is required to ensure that energy is not channeled into nitrate assimilation under unfavorable physiological conditions. The relative importance of each type of control, however, is unclear because plants containing a NR gene that expresses mRNA constitutively develop normally (Vincentz and Caboche, 1991).

Posttranslational control of nitrate assimilation is closely linked with photosynthetic activity (Kaiser and Förster, 1989) and control of carbon metabolism. Under aerobic conditions, NR is rapidly inactivated by phosphorylation when the rate of photosynthesis is reduced (Kaiser and Brendle-Behnisch, 1991; Kaiser and Spill, 1991; Huber et al., 1992a, 1992b; MacKintosh, 1992). Perhaps the role of this inactivation of NR is to prevent the accumulation of toxic nitrite that would otherwise occur if NiR activity were reduced in the dark due to decreased supply of reduced Fd from the photosynthetic electron transport system. Reactivation of NR (for example, upon illumination of leaves in the dark) involves dephosphorylation by a type 1 or 2A protein (Ser/Thr) phosphatase, which is blocked by okadaic acid and microcystin (MacKintosh et al., 1990; Huber et al., 1992a; MacKintosh, 1992). NR can also be activated by feeding leaves with Man, which causes accumulation of Man-6-P, depleting cytoplasmic Pi and nucleoside triphosphates (Loughman et al., 1989). Together, these findings have led to a working model that proposes that NR is controlled by a chloroplast-derived signal (metabolites or a special messenger molecule) that reflects the rate of photosynthesis and alters the activities of NR kinase and/or phosphatase (MacKintosh and MacKintosh, 1993).

In this paper we report that the posttranslational control of NR activity is not simply mediated by reversible phosphorylation and that another protein, termed NIP, is required to inhibit the phosphorylated form of NR.

MATERIALS AND METHODS

Plants

Spinach seeds (*Spinacia oleracea* L. cv Polka F1, a gift from Werner M. Kaiser, University of Würzburg, or var Medania, from Nuttings and Son Ltd.) were planted in a mixture of Levington's compost and horticultural vermiculite. Plants were maintained in a growth cabinet (Vindon

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Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiophosphate); NIP, nitrate reductase inhibitor protein; NiR, nitrite reductase; NR, nitrate reductase; NRI, nitrate reductase inhibitor; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.

Scientific Ltd., Oldham, UK) for approximately 5 weeks on a cycle of 11 h of light (approximately 350 μmol m^{-2} s^{-1}; tungsten and fluorescent lamps) at 22°C, followed by 13 h of dark at 17°C, or in a greenhouse during winter with supplementary lighting. Plants were irrigated twice daily with a general feed (Chempak Formula 3, which contains ammonium and nitrate; Chempak Products, Hoddesdon, UK), then provided water only for the 3 d before harvesting (allowing the soil ammonium to be oxidized to the NR inducer, nitrate). No additional nitrate was required to achieve maximal NR activities at the time of harvest. Air humidity was approximately 75%. Leaves were harvested after 2 h in the normal light period (termed "light leaves") or after 2 h in the normal light period followed by 2 h in darkness ("dark leaves"). Under those conditions there was no obvious difference in total NR activity in the light and dark extracts. In some cases, excised light leaves were fed through the petiole with 50 mM Man in the light for 2 h ("Man leaves") or with 10 μM microcystin-LR in the light for 2 h ("microcystin leaves"). Routinely, leaves were frozen immediately in liquid nitrogen and stored at −80°C. There was no obvious difference in NR activities extracted from frozen or fresh leaves, but frozen leaves were easier to powder and extract.

Preparation of Extracts and Ammonium Sulfate Fractionation

Between 100 and 2,000 g of frozen leaves, with petioles removed, were powdered in a Waring blender for 30 s. One volume of buffer A (50 mM Hepes-NaOH [pH 7.5], 5% [v/v] glycerol, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine [plus 10 μM FAD for NR extractions]) was added and the mixture was homogenized for an additional 30 s. Homogenates were centrifuged at 18,000*g* for 30 min and the supernatants were filtered through glass wool to give the extracts, termed light, dark, Man, or microcystin depending on the pretreatment of leaves (see above).

Powdered $(NH_4)_2SO_4$ was added gradually to extracts to give the required saturation and the solution was stirred for 20 min and centrifuged at 18,000*g* for 20 min. The pH was maintained at approximately pH 7.5 and the temperature was kept below 4°C. Pellets were dissolved in a minimum volume of buffer A, desalted on Sephadex G-25 (medium), and used immediately or frozen in liquid nitrogen. For subsequent Q-Sepharose chromatography, fractions were desalted by dialysis against 2 × 2 L buffer A for 4 h. Samples were clarified, if necessary, by centrifugation or by filtration (0.45-μm filter).

Some extracts and $(NH_4)_2SO_4$ fractions were prepared in the presence of protein phosphatase inhibitors, using extraction buffer A containing 25 mM NaF and 1 mM NaPPi (general protein phosphatase inhibitors), and 0.5 μM microcystin-LR plus 10 μM cantharidin (specific inhibitors of PP1 and PP2A) and, for desalting the $(NH_4)_2SO_4$ fractions, buffer A containing 0.5 μM microcystin-LR. These additions were sufficient to ensure complete inhibition of PP1 and PP2A throughout the experiments (data not shown).

Partial Purification of NR and Purification of NR to Homogeneity

NR was partially purified (approximately 250- to 400-fold from the extract) by binding to either Blue-Sepharose or 5'AMP-Sepharose and was eluted with 100 μM NADH (Wray and Fido, 1990). NR was purified to homogeneity from 500 to 1000 g of spinach leaves using a procedure based on that of Fido (1991). Briefly, a 0 to 45% saturated $(NH_4)_2SO_4$ fraction from extracts prepared in buffer A (containing 10 μM FAD) was desalted on G-25 Sephadex (medium) (18 × 3 cm), diluted 3-fold in buffer A (containing 10 μM FAD), and loaded onto a Blue-Sepharose column (7.5 × 2.5 cm), which was washed until the A_{280} of the eluate was zero. The column was inverted and enzyme was eluted with 100 μM NADH in buffer A (containing 10 μM FAD) and loaded onto a 5-mL MAC74 (Fido, 1991) immunoaffinity column (1 mg IgG/mL cyanogen bromide-Sepharose). The MAC74 column was washed in buffer A containing 10 μM FAD plus 150 mM NaCl, and enzyme was eluted in buffer A containing 10 μM FAD plus 1 M KNO_3. Enzyme was concentrated in Amicon 30 (Beverly, MA) concentrators, dialyzed into buffer A containing 10 μM FAD plus 53% (v/v) glycerol, and stored at −20°C. The pure NR had a specific activity of 46 μmol min^{-1} mg^{-1} protein and recovery from the extract was 16%.

Definition of Activity State of NR

Phosphorylated NR in dark or microcystin extracts is more sensitive to inhibition by Mg^{2+} ions when assayed in Hepes, pH 7.5, than is the dephosphorylated enzyme in light or Man extracts (Kaiser and Brendle-Behnisch, 1991; Kaiser and Spill, 1991; Huber et al., 1992a; MacKintosh, 1992). The activity state of NR is defined as its activity in the presence of 10 mM $MgCl_2$ divided by its activity in the absence of $MgCl_2$, multiplied by 100%. In our hands, the activity state of NR in dark extracts has been consistently observed to be between 15 and 50% and that of NR in Man extracts between 85 and 110%.

NR Inactivation and NR Assays

Components were preincubated at 30°C in a total volume of 250 μL of 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT (buffer B). In some experiments, ATP or ATPγS was added to 2 mM. NR activity assays were initiated by the addition of 250 μL of buffer B (or buffer B in which the $MgCl_2$ was replaced by 12 mM EDTA) containing 2 mM KNO_3 plus 200 μM NADH. After 2 to 5 min, reactions were stopped with 50 μL of 0.5 M zinc acetate. Sulfanilamide (150 μL of 1% [w/v] in 3 M HCl) and 150 μL of 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride were added, mixtures were centrifuged at 15,000*g* for 2 min, and nitrite was determined by measuring A_{540}.

Unlike the low-activity-state NR from squash leaves (Lillo and Ruoff, 1992), the low-activity-state spinach enzyme showed no sigmoidal nor hysteretic kinetics. All time courses were linear (data not shown).

Type 1 and 2A Protein (Ser/Thr) Phosphatases

The catalytic subunit of PP2A from bovine cardiac muscle was purified (specific activity 12,000 milliunits mg^{-1}) and provided by Bob MacKintosh (MRC Protein Phosphorylation Unit, Dundee, UK). Skeletal muscle PP1 was purified (specific activity 40,000 milliunits mg^{-1}), and PP1 and PP2A were assayed using 10 μM ^{32}P-labeled glycogen phosphorylase as substrate, as described by MacKintosh et al. (1990). One unit is the amount that catalyzed the dephosphorylation of 1 μmol substrate/min.

RESULTS

The Low-Activity (Phosphorylated) Form of NR Is Activated during Purification, Even in the Presence of Protein Phosphatase Inhibitors

The low-activity (phosphorylated) form of NR from a dark extract of spinach (defined in "Materials and Methods") became activated during purification, even in the presence of protein phosphatase inhibitors (Table I). Activation occurred after chromatography of dark extracts on Blue-Sepharose, 5'-AMP-Sepharose, a MAC74 (immunoaffinity) column, or Superose 12 gel or when NR was precipitated from dark extracts with 30% saturated $(NH_4)_2SO_4$ and the redissolved precipitate was desalted on G-25 Sephadex (Table I). However, little activation occurred if a dark extract was fractionated from 0 to 60% saturated $(NH_4)_2SO_4$ and desalted on G-25 Sephadex (MacKintosh, 1992; Table I).

Identification of a NIP

One possible explanation of the effect of $(NH_4)_2SO_4$ fractionation shown in Table I was that an NR-activating factor, such as NR phosphatase, was concentrated along with NR in the 0 to 30% $(NH_4)_2SO_4$ fraction. However, it seemed unlikely that dephosphorylation was taking place because all procedures were carried out in the presence of a mixture of protein phosphatase inhibitors (see "Materials and Methods") sufficient to completely inhibit all of the type 1 and type 2A (microcystin-sensitive) protein phosphatase activity in the fractions (not shown).

Table I. *Activity state of NR purified by various methods from dark extracts prepared in the presence of protein phosphatase inhibitors*

Portions of dark extracts were taken individually (not sequentially) through the purification steps indicated. Procedures for purifications and the definition of NR activity state are given in "Materials and Methods." Results are means ± SE (*n* determinations).

Purification	Activity State of NR (*n*)
	%
Extract (fresh)	27.2 ± 11.4 (5)
Extract on ice for 8 h	27.0 ± 8.1 (5)
NR partially purified from extract by:	
Blue Sepharose chromatography	96.0 ± 9.4 (3)
MAC74 chromatography	87.0 ± 7.8 (3)
5'-AMP chromatography	95.0 ± 2.8 (3)
Superose 12 gel filtration	84.7 ± 10.9 (3)
Desalted 0 to 30% saturated $(NH_4)_2SO_4$ fraction	94.3 ± 6.1 (3)
Desalted 0 to 60% saturated $(NH_4)_2SO_4$ fraction	37.7 ± 3.3 (3)

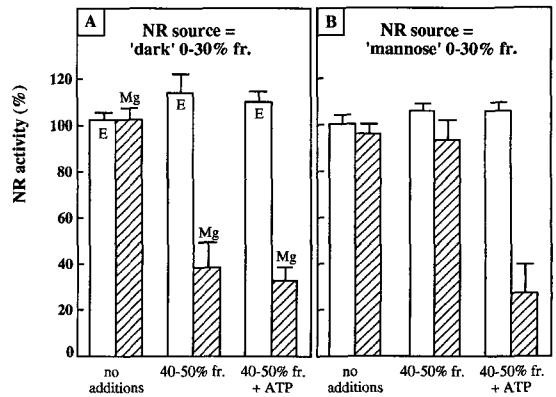

Figure 1. Inactivation [by a 40–50% $(NH_4)_2SO_4$ fraction] of NR in a 0 to 30% $(NH_4)_2SO_4$ fraction prepared in the presence of protein phosphatase inhibitors from a dark extract (A) and a Man extract (B). A, NR in a desalted 0 to 30% $(NH_4)_2SO_4$ fraction (20 μL, 7 mg of protein), prepared in the presence of protein phosphatase inhibitors from a dark extract, was incubated for 20 min with no additions or with a desalted 40 to 50% fraction (100 μL, 5.5 mg of protein) or the 40 to 50% fraction plus 2 mM ATP. NR activity was assayed for 5 min in the presence of EDTA (E, open bars) or $MgCl_2$ (Mg, hatched bars). The 100% value was 7.6 nmol min^{-1} mL^{-1}. B, As for A, except that the source of NR was a 0 to 30% $(NH_4)_2SO_4$ fraction (20 μL, 6 mg of protein) prepared in the presence of protein phosphatase inhibitors from a Man extract. The 100% value was 7.9 nmol min^{-1} mL^{-1}. Preincubation and assay conditions are as described in "Materials and Methods." Inactivation is defined as an increase in the sensitivity of NR to inhibition by Mg^{2+} (see "Materials and Methods"). ATP had little or no effect in the absence of the 40 to 50% fraction (not shown). Results are means ± SE for four determinations and are typical of results seen in several different experiments. fr., Fraction.

An alternative possibility was that an additional factor was required to keep phosphorylated NR inactive, this additional factor being separated from NR in the 0 to 30% $(NH_4)_2SO_4$ fraction but not separated from NR in the 0 to 60% $(NH_4)_2SO_4$ fraction. Consistent with this possibility, a 40 to 50% $(NH_4)_2SO_4$ fraction was able to inactivate the NR in the 0 to 30% fraction prepared from a dark extract in the presence of protein phosphatase inhibitors (Fig. 1A). ATP was not required for this inactivation (Fig. 1A). In contrast, the inactivating effect of the 40 to 50% fraction on NR in a 0 to 30% fraction prepared from a Man extract (i.e. from an extract containing high-activity-state, dephosphorylated NR) was almost totally dependent on ATP-Mg (Fig. 1B).

The inactivating activity in the 40 to 50% fraction was further fractionated by Q-Sepharose anion-exchange chromatography. A single peak of activity was found (at approximately 0.38 M NaCl, at the trailing end of the major protein peak), which caused ATP-independent inactivation of the activated NR in a 0 to 30% fraction that had been prepared from dark extracts in the presence of protein phosphatase inhibitors (Fig. 2A).

The inactivating factor was destroyed by digestion with trypsin and by boiling for 10 min, consistent with the factor

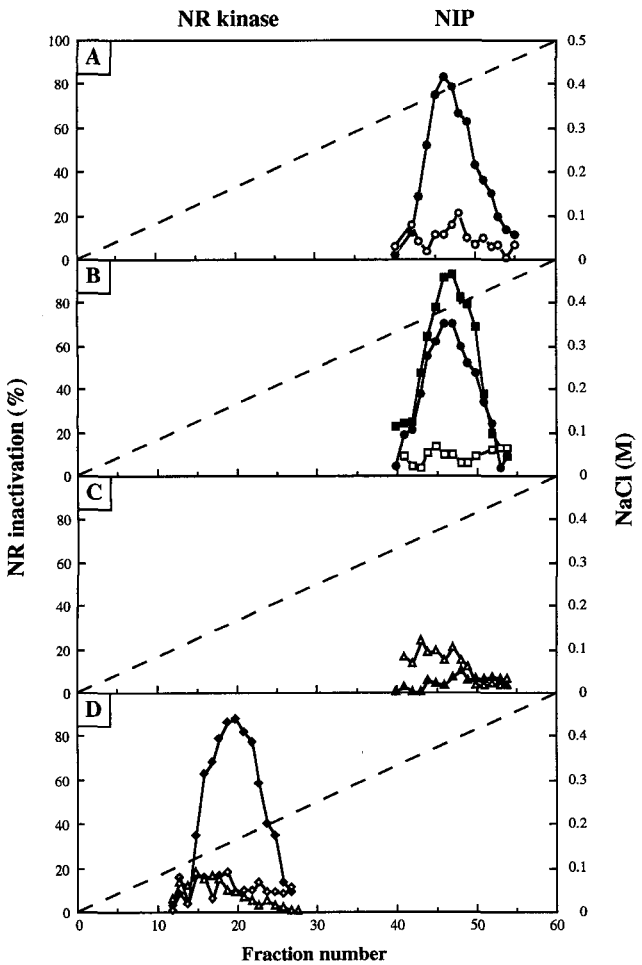

Figure 2. Separation of the inactivating activities of NIP and NR kinase by Q-Sepharose chromatography. A desalted 40 to 50% $(NH_4)_2SO_4$ fraction (250 mg) prepared in the presence of protein phosphatase inhibitors from 2 kg of dark spinach leaves was chromatographed on Q-Sepharose. The column was washed in buffer B until the A_{280} had returned to baseline, and was developed with a linear gradient (broken line) of 0 to 500 mM NaCl in buffer B over 60 min at 3 mL min^{-1}. Fractions (3 mL) were desalted by microdialysis (BRL) and assayed for inactivation of NR as described below. In each case, 10 to 15 nmol min^{-1} mL^{-1} NR with an initial activity state of approximately 100% was used as substrate. A, Aliquots (100 μL) of fractions were assayed for ATP-independent inactivation of the NR in a dark 0 to 30% $(NH_4)_2SO_4$ fraction that had been prepared in the presence of protein phosphatase inhibitors. NR assays were performed in the presence of either 10 mM $MgCl_2$ (●) or EDTA (○). See "Materials and Methods" for incubation and assay conditions. B, Fractions were assayed for inactivation of the NR in a dark 0 to 30% fraction that had been preincubated with 40 milliunits mL^{-1} cardiac PP2A for 20 min at 30°C (followed by addition of 5 μM microcystin to block PP2A activity) (□). ■, The same assays except that 2 mM ATP was included during inactivation. ●, Results of preincubation of column fractions with PP2A (followed by microcystin) prior to inactivation assays using the 0 to 30% fraction (which was not treated with PP2A). NR assays were all carried out in the presence of 10 mM $MgCl_2$. C, Fractions were assayed for inactivation of dephosphorylated NR that had been purified to homogeneity from Man leaves. Incubations were performed in the presence (▲) or absence (△) of ATP. NR assays were all carried out in the presence of 10 mM $MgCl_2$. D, NR kinase was assayed by the inactivation of pure NR in the presence of ATP and NIP (100 μL of pooled fractions 45–48) (◆), ATP alone (△), or NIP alone (◇). NR assays were all carried out in the presence of 10 mM $MgCl_2$.

being a protein. This protein was henceforth termed NIP. NIP had a molecular mass of approximately 110 kD by gel filtration on Sephacryl S300 Superfine or Superose 12 (data not shown). The activity of NIP was stable if fractions were stored at 0°C for 3 weeks, or after freezing in liquid N_2 and rapid thawing at 30°C.

Phosphorylation of NR Is Required for Conversion to the Low-Activity State by NIP

Preincubation of NR in the 0 to 30% $(NH_4)_2SO_4$ fraction from a dark extract with PP2A did not affect its activity (data not shown) but prevented inhibition by NIP unless ATP-Mg was included in the incubation (Fig. 2B). In contrast, pretreatment of the NIP with PP2A had no effect on its inhibitory activity (Fig. 2B). These findings suggested that NIP was able to inactivate phosphorylated but not dephosphorylated NR. Furthermore, since dephosphorylated NR could be inactivated by NIP in the presence of ATP-Mg (Fig. 2B), these results suggested that NR kinase must be present in the 0 to 30% fraction. If these ideas were correct, it would be expected that NIP would be unable to inactivate pure, kinase-free, dephosphorylated NR, even in the presence of ATP. This prediction was confirmed experimentally, as shown in Figure 2C.

Identification of NR Kinase

NR kinase activity could now be measured by the ATP-Mg-dependent inactivation of pure dephosphorylated NR in the presence of NIP. Preliminary experiments suggested that NR kinase activity was present in 0 to 30%, 30 to 40%, and 40 to 50% $(NH_4)_2SO_4$ fractions prepared from a spinach extract, and these fractions were further chromatographed on Q-Sepharose. In each case, inactivating NR kinase activity was eluted at approximately 0.16 M NaCl, at the leading edge of the major protein peak. The highest NR kinase activity was found after Q-Sepharose chromatography of the 40 to 50% $(NH_4)_2SO_4$ (Fig. 2D), and this preparation was used for further studies because it was free of NR. [When present in other $(NH_4)_2SO_4$ fractions, NR was eluted from the column at 0.23 M NaCl, between NR kinase and NIP.] No inactivation occurred in the absence of ATP (Fig. 2D) or in the absence of NIP (Fig. 2D). No inactivation was seen when GTP, UTP, CTP, and non-hydrolyzable ATP analogs were used instead of ATP in NR kinase assays (data not shown).

NR Kinase Behaves Enzymatically and NIP Behaves Stoichiometrically in Inactivation of NR

The inactivating effect of NR kinase was time dependent, and the initial rate of inactivation of NR increased with increasing amounts of NR kinase present in a preincubation (Fig. 3A). These results are consistent with NR kinase behaving enzymatically to inactivate NR in the presence of NIP and ATP-Mg.

In contrast to NR kinase, the inactivation of NR increased as the amount of NIP present in the preincubation was

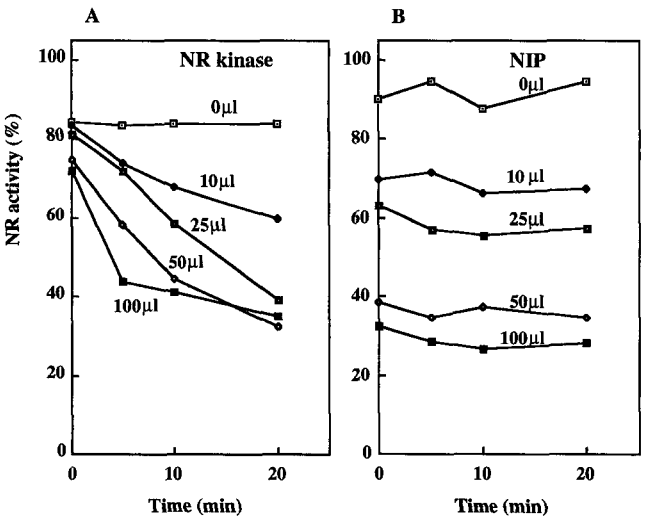

Figure 3. A, Fixed amounts of dephosphorylated NR (partially purified on Blue-Sepharose) plus NIP (100 μL of a desalted Q-Sepharose fraction eluted at 0.38 M NaCl; as in Fig. 2) were present throughout 20-min preincubations carried out at 30°C in buffer B containing 2 mM ATP. Various amounts of NR kinase (from 0–100 μL of a desalted Q-Sepharose fraction eluted at 0.17 M NaCl; see Fig. 2) were added to the preincubations for the times indicated. At the end of the preincubation, 2-min NR assays were carried out in the presence of 10 mM $MgCl_2$, as described in "Materials and Methods." B, An experiment carried out in parallel with A and using the same materials, except that fixed amounts of NR plus NR kinase (100 μL) were present throughout and various amounts of the NIP (from 0–100 μL) were added to the preincubations for the times indicated. The NR activity in the absence of NR kinase and NIP was 10.6 nmol min^{-1} mL^{-1} (100%). Values represent single assays. Similar results from two other experiments were seen by the editor.

increased (Fig. 3B). However, a given amount of NIP caused the same inactivation whether it was present throughout the preincubation or added immediately before the NR assay (Fig. 3B). This finding suggested that NIP was not acting as an enzyme, nor as a substrate for an enzyme, during the incubation, and was consistent with the possibility that NIP was exerting its effect by binding to phosphorylated NR.

The Inactivation of NR by NR Kinase and NIP Is Reversed by Incubation with PP2A or by Repurification of NR

NR that had been inactivated in vitro (by NR kinase and NIP) was reactivated by incubation with PP2A (Table II). Reactivation by PP2A was blocked by microcystin (Table II). PP1 had no effect (Table II). In contrast, NR that was inactivated in the presence of ATPγS was resistant to reactivation by PP2A (Table II), consistent with the well-known inability of protein phosphatases to remove thiophosphate from proteins.

Both phosphorylated and thiophosphorylated enzymes became reactivated after purification on Blue-Sepharose. This chromatography increased the activation state of the phosphorylated enzyme from 32 to 102%, and the thiophosphorylated enzyme from 21 to 89%. The activation states of the phosphorylated and thiophosphorylated enzymes were also increased, to 104 and 100%, respectively, after gel filtration on Superose 12.

DISCUSSION

An Updated Model for Regulation of NR Activity in Spinach Leaves

A revised model is presented in Figure 4 that incorporates roles for both NIP and reversible phosphorylation in effecting rapid changes in NR activity in response to changes in photosynthetic rates in spinach leaves. NIP was identified here as a factor that restored the low-activity state to NR purified in the presence of protein phosphatase inhibitors from dark extracts. Consistent with the data presented in this paper, the new model postulates that NIP inactivates NR, but only after the enzyme has been phosphorylated (Fig. 4).

During experiments to purify NR kinase, Spill and Kaiser (1994) recently concluded that the inactivation of NR was a multicomponent process. They found that two proteins, termed p67 and p100, were required for inactivation of NR. Of the two proteins, fractions containing p67 had histone kinase activity, whereas those containing p100 did not, and p67 was therefore proposed to be NR kinase. Judging by their elution positions from Q-Sepha-

Table II. *Reactivation of NR by protein phosphatase*

NR was inactivated in a 10-min preincubation in the presence of NIP, NR kinase (as for Fig. 3), and nucleotide as indicated (see "Materials and Methods"). A further 20-min incubation was then carried out with (a) no additions, (b) 40 milliunits mL^{-1} PP1, (c) 40 milliunits mL^{-1} PP2A, or (d) 40 milliunits mL^{-1} PP2A plus 2 μM microcystin-LR (MC). NR assays (5 min) were carried out in the presence of 10 mM $MgCl_2$ and 2 μM microcystin. Results are means ± SE for three experiments. Similar reactivation results were seen in another three experiments that used a 0 to 30% $(NH_4)_2SO_4$ fraction as source of both NR and kinase for the initial inactivation.

Nucleotide	NR Activity				
	At 10 min	At 30 min			
		No PP	PP1	PP2A	PP2A + MC
			%		
None	102 ± 1.6	100 ± 2.9	103 ± 4.8	98 ± 1.7	99 ± 1.9
ATP	36 ± 6.5	35 ± 2.6	36 ± 3.7	92 ± 6.0	38 ± 3.3
ATPγS	23 ± 4.3	24 ± 2.5	20 ± 4.9	17 ± 3.4	20 ± 2.9

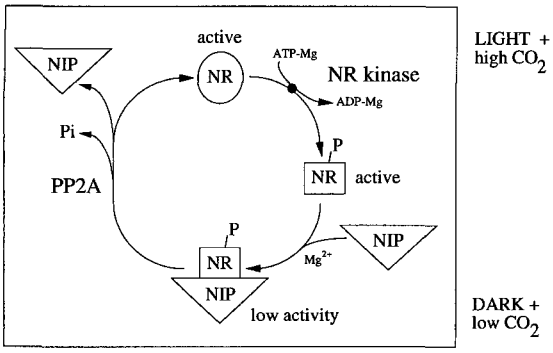

Figure 4. A revised speculative mechanistic model for the reversible control of NR activity in spinach leaves. Conversion of NR to the low-activity form required both phosphorylation by NR kinase and interaction with NIP. Mammalian PP2A dephosphorylated and activated NR in vitro (this study), and the endogenous spinach NR phosphatase is a microcystin-sensitive enzyme that may be PP2A (see introduction). Other details such as the order in which events occur and a possible role for Mg^{2+} in promoting the interaction between phosphorylated NR and NIP are speculative.

rose columns (compare Fig. 2 and Spill and Kaiser, 1994) and their molecular masses, it is almost certain that p100 is identical to NIP and p67 is the NR kinase used in this study. However, the proposal made here, that NIP acts stoichiometrically to inactivate phosphorylated NR, is in apparent contradiction with the results of Spill and Kaiser (1994), who found that preincubation of NR with kinase plus ATP, followed by removal of ATP and incubation with p100, did not lead to NR inactivation. We believe that the failure of Spill and Kaiser to detect inactivation under these conditions is explained by the presence of PP2A in these fractions. We have confirmed their observations but found that the NR was inactivated by p100 (NIP) if microcystin was included (to inhibit the 0.13 milliunit mL^{-1} PP2A that was present when we followed their protocol) or when ATPγS was used instead of ATP.

Figure 4 depicts the inactivation of NR as a two-step process, in which phosphorylation of NR is a prerequisite for interaction of NIP with NR. In vivo, however, it is possible that NIP is always bound to NR and that phosphorylation of NR is the sole trigger required for conversion to the low-activity state of the enzyme. We have not observed any differences in the amount of NIP activity present in leaves harvested after various treatments (data not shown). In the direction of activation of NR, the model predicts that in vitro there should be at least two ways in which NR could be reactivated. Either NR could be dephosphorylated and/or NIP could be dissociated from NR, by purification of NR. These predictions were borne out experimentally (Table II and "Results").

One of the partial activities catalyzed by NR, namely reduction of nitrate by methyl viologen, was inhibited in the low-activity-state enzyme, whereas the ability of NR to reduce Cyt *c* was unaffected (C. Lillo, unpublished data), which suggests that the N-terminal nitrate-reducing domain (Wray and Fido, 1990) of NR may be the site of phosphorylation and/or interaction with NIP.

The Identity and Physiological Role of NIP

There are many reports in the literature of NR-inactivating factors found in plant extracts (e.g. Travis et al., 1969; Wallace and Shannon, 1981; Solomonson et al., 1984; Maki et al., 1987). At least one of these factors was found to be a protease that was inhibited by PMSF (Wallace and Shannon, 1981). NIP is clearly not a protease because its inactivating effect was reversed either by dephosphorylation by PP2A or by dissociation of NR from NIP.

A 115-kD NRI protein, purified from a 40 to 70% $(NH_4)_2SO_4$ fraction of spinach leaf extracts, was found to bind stoichiometrically and reversibly to NR (Yamagishi et al., 1988; Yoshimura et al., 1992). Although the phosphorylation state of the NR used in these studies is unknown, the enzyme was isolated using phosphate buffer, which is well known to inhibit protein phosphatase activity. Therefore, if the NR had been in a phosphorylated state when the initial extracts were made, it is quite possible that NRI was actually interacting with phosphorylated NR, a property that would be consistent with the effect of NIP on NR that is reported here. Furthermore, the NRI effect was reversed by EDTA (Yamagishi et al., 1988), which is also true of NIP. NRI was reported to be a glycoprotein that binds Con A and to be a dimer of subunits linked by disulfide bridges (Yamagishi et al., 1992). However, NIP does not appear to bind to Con A-Sepharose (F. Douglas and C. MacKintosh, unpublished data). Yoshimura et al. (1992) reported that the interaction between NRI and NR was stable to gel filtration, and that NR that had been inactivated by NRI had a higher molecular mass than the active enzyme. However, we found that low-activity-state NR became activated and dissociated from NIP during gel filtration on Superose 12 in buffers containing either $MgCl_2$ or EDTA (Table I and "Results"). Further purification and cross-linking studies will be required to determine whether NRI and NIP are related proteins.

The cooperative effect of phosphorylation and interaction with NIP in converting NR to a low-activity state introduces another level of complexity to the control of an already highly regulated enzyme. At this stage we do not have a good understanding of why this dual regulatory system may be important physiologically. A detailed analysis of the in vivo phosphorylation state, activation state of NR, and interaction of NR and NIP under different physiological and environmental conditions should give clues about the relative contributions of NIP and phosphorylation to the control of NR.

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