Identification of a regulatory phosphorylation site in the hinge 1 region of nitrate reductase from spinach (*Spinacea oleracea*) leaves

Pauline Douglas*, Nick Morrice, Carol MacKintosh

Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee, DD1 4HN, Scotland, UK

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Abstract Purified nitrate reductase (NR) from spinach leaves was phosphorylated in vitro by NR-inactivating kinase on Ser-543 which is located in the hinge 1 region between the molybdenum-cofactor and haem-binding domains. Phosphorylation of Ser-543 allowed NR to be inhibited by the inhibitor, NIP. Degraded NR preparations in which a proportion of the subunits had lost 45 amino acids from the N-terminus during purification could be phosphorylated by NR kinase on Ser-543, but could not subsequently be fully inhibited by NIP, suggesting a role for the N-terminal tail of NR in NIP binding.

Key words: Nitrate reductase; Phosphorylation site; *Spinacea oleracea*; Phosphoserine; Inactivation

1. Introduction

Nitrate in plant cells is reduced to ammonia by the concerted actions of nitrate reductase (NR) in the cytoplasm and nitrite reductase (NiR) in the plastid [1,2]. Ammonia is then converted to an organic form by the action of glutamine synthase (GS) [3] Nitrate assimilation is highly regulated. The activities of NR, NiR and GS change in response to external nitrogen supplies (nitrate, ammonia), environmental conditions (light, CO_2 levels), and intra- and intercellular messengers and metabolites (carbon and nitrogen metabolites, cytokinins) [1,3,4,5]. These enzyme activity changes are mediated by a variety of mechanisms; at the level of mRNA synthesis, protein production and degradation, and by rapid post-translational changes in enzyme activity [5–8]. It is logical to propose that these controls give the flexibility required to ensure that nitrate assimilation is coordinated with carbon metabolism at different stages of growth, while avoiding overaccumulation of potentially toxic intermediates such as nitrite. The relative contribution of each type of control to plant metabolism and growth is starting to be assessed quantitatively by examining the physiological consequences of deregulating these mechanisms in transgenic and mutant plants [9,10].

Under aerobic conditions, NR in leaves is inactivated within minutes when photosynthetic rates are reduced, for example, in the dark [11–13]. Perhaps the role of this rapid inactivation of NR is to ensure that nitrite does not accumulate in the dark when levels of reduced ferredoxin (the NiR cofactor) may become low enough to restrict nitrite reduction in the plastid. The mechanism of the NR inactivation is an unusual two-step process in which phosphorylation of NR does not inactivate the enzyme directly but allows it to interact with an inhibitory protein [14–16], termed NIP [15]. NIP inactivates phosphorylated NR in the presence of Mg^{2+} or Ca^{2+} ions, but has little effect on dephosphorylated NR [15,16]. The low-activity form of NR from spinach leaves harvested in the dark (a complex of phosphorylated NR and NIP) can be reactivated in vitro by dissociation of the two proteins during purification or by dephosphorylation of NR [15]. Therefore, it should be possible to deregulate this post-translational control in transgenic plants either by mutating the regulatory phosphorylation site(s), or by mutating NR or NIP so that these two proteins are no longer able to interact with each other.

Plant NRs are homodimers of approximately 110 kDa subunits that each have three functional domains (Fig. 1). Electrons from the reductant NAD(P)H feed into the C-terminal FAD domain and are shuttled through the central haem domain to the molybdate-pterin (MoCo) in the N-terminal domain where nitrate is reduced [1,17]. In this paper, we show that in vitro phosphorylation of Ser-543, which lies in the hinge 1 region between the haem and MoCo domains of NR (Fig. 1), allows subsequent inhibition of the enzyme by NIP. However, in some of our NR preparations a proportion of the subunits had become proteolysed, with the loss of 45 amino acids from the N-terminus during purification. While the proteolysed preparations could be phosphorylated normally by NR kinase they could not be fully inactivated by NIP, suggesting a role for the N-terminal tail of NR in NIP binding.

2. Materials and methods

2.1. Materials

Spinach was grown, leaves were harvested and frozen as described [15]. [γ-^{32}P]ATP was from Amersham International (Amersham, UK), ProBlott from Applied Biosystems and Sequelon arylamine PVDF membrane (GEN920033) from Millipore Ltd (Watford, UK).

2.2. Purification of NR

NR was purified to homogeneity from 500 to 1000 g of spinach leaves by Blue-Sepharose chromatography and MAC74 immunoaffinity purification as described [15, 17]. Enzyme was concentrated in Amicon 30 (Beverly, MA) concentrators, dialysed into buffer A (50 mM HEPES-NaOH, pH7.5, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 10 μM FAD) containing 53% (v/v) glycerol, and stored at −20°C. The pure NR had a specific activity of between 30 and 50 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein for three different preparations.

2.3. Partial purification of NR kinase

Frozen spinach leaves (500 g) were homogenised in a Waring Blender with 1.5 vol of buffer B (50 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF), centrifuged at 10,000 × *g* for 30 min and the supernatant, termed extract, filtered through two layers of glass wool. The extract was made 18% (w/v) in polyethylene glycol (PEG) by addition of a solution of 50% (w/v) PEG-8000 (Sigma) in buffer B and

*Corresponding author. Fax: (44) (1382) 223778.
E-mail: pdouglas@bad.dundee.ac.uk

Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; NIP, nitrate-reductase inhibitor protein.

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the mixture was incubated with gentle stirring at 4°C for 30 min followed by centrifugation at 4500 × *g* for 15 min. The pellet was resuspended by gentle Dounce homogenisation in a minimal volume of buffer B and loaded onto a 100 ml column of Q-Sepharose equilibrated in buffer B. After washing with buffer B, NR kinase was eluted in buffer B containing 0.3 M NaCl (~250 ml). Protein was precipitated in 20% (w/v) PEG, centrifuged and resuspended as before. The solution was clarified by centrifugation, loaded onto a 10 × 1.6 cm column of Hi-load Q (Pharmacia) equilibrated in buffer B and the column washed in buffer B until the A_{280} had returned to baseline. The column was developed with a linear gradient of 0–500 mM NaCl in buffer B over 70 min at 3 ml · min^{-1}. Fractions (3 ml) were desalted individually by microdialysis (BRL Microdialyser). NR kinase activity was identified (at ~0.16 M NaCl, see Fig. 2D in [15]) by its ability to inactivate purified NR in the presence of ATP and NIP as described in [15].

2.4. Partial purification of NIP and NIP assays

A 40–70% saturated $(NH_4)_2SO_4$ fraction of spinach leaf extract was dialysed into buffer B. The pH was reduced to 5.5 by dropwise addition of 5 N acetic acid, stirred gently for 10 min and precipitated protein removed by centrifugation. The supernatant was adjusted to pH7.5 by addition of ammonium hydroxide, clarified by centrifugation and loaded onto a 20 ml column of Q-Sepharose equilibrated in buffer B. After washing with buffer B containing 0.2 M NaCl, NIP was eluted in buffer B containing 0.45 M NaCl (~30 ml), dialysed in buffer B and chromatographed on Hi-load Q as described above for NR kinase.

NIP activity was identified (at ~0.38 M NaCl, see Fig. 2 in [15]) by its ability to inhibit NR in the presence of ATP and NR kinase [15]. Unless otherwise stated, preincubations contained 5 μg of a desalted 0–30% saturated $(NH_4)_2SO_4$ fraction of a spinach leaf extract (this fraction was the source of both NR and NR kinase), 2 mM ATP and 15 μl NIP fraction in a total volume of 50 μl in buffer B. NR assays were initiated by addition of 50 μl of buffer B containing 2 mM KNO_3 plus NADH. Nitrite was measured as in [15].

2.5. Phosphorylation of NR by partially purified NR kinase

Incubations (500 μl) contained 10 μg NR, 200 μl of NR kinase (peak fraction from Hi-load Q column, see section 2.4.), 1 μM microcystin-LR (to inhibit protein phosphatases [18]) and 0.1 mM [γ-^{32}P]ATP (5,000 cpm/pmol by Cerenkov counting) in buffer B. The reactions were initiated by addition of ATP and were stopped by heating to 100°C in SDS sample buffer. Samples were electrophoresed on a 7.5% polyacrylamide gel in the presence of SDS according to Laemmli [19].

2.6. Digestion of ^{32}P-labelled NR with alkylated trypsin and separation of phosphopeptides

After autoradiography of the gel, the radiolabelled band corresponding to NR was excised and counted by Cerenkov counting. Gel slices were washed in Milli-Q water (5 × 1 ml) for 1 h and brought to near dryness by rotary evaporation. The gel slices were suspended in 250 μl buffer C (50 mM Tris-HCl pH8, 0.01% alkylated Triton X-100) containing 3 μg of alkylated trypsin (Boehringer) and incubated at 30°C for 20 h. The supernatant was removed and a further 250 μl buffer C without trypsin was added for 1 h. The pellet and recombined supernatants were analysed by Cerenkov counting and recovery of ^{32}P from the gel slice was determined. β-Mercaptoethanol (2 μl) was added and the solution agitated for 1 h. 4-Vinyl pyridine (4 μl) was added (to alkylate cysteines) and the solution left for a further 2 h, with gentle vortex mixing every 15 min. The digest was centrifuged at 13,000 × *g* to pellet the 4-vinyl pyridine and the supernatant was applied to a 150 × 2.1 mm Vydac C_{18} column equilibrated in 0.1% trifluroacetic acid (TFA) attached to a Applied Biosystems 140B HPLC system. The column was developed with a linear acetonitrile gradient in 0.08% TFA with an increase in acetonitrile concentration of 0.5% per min. Absorbance at 214 nm was recorded with an on-line monitor. The flow rate was 0.2 ml min^{-1} and fractions of approximately 100 μl were collected manually and Cerenkov counted to identify phosphopeptides. If phosphopeptides were not pure, samples were rechromatographed on C_{18} as described above except that a gradient of 0.25% increase in acetonitrile concentration per min was used.

2.7. Sequence analysis of tryptic peptides

Phosphopeptides (~5–10 picomoles) were sequenced directly on an Applied Biosystems 476A protein sequencer. In order to identify phosphorylation sites, phosphopeptides were also covalently attached to a Sequelon arylamine PVDF membrane, subjected to Edman degradation in an Applied Biosystems 470A/120A sequencer, and the ^{32}P-radioactivity released after each cycle of Edman degradation was extracted and counted as described in [20].

3. Results

3.1. NR is phosphorylated on Ser-543 by NR kinase in vitro

NR kinase was partially purified and assayed by its ability to inactivate NR in the presence of MgATP and NIP [15, 16]. Purified NR was phosphorylated with NR kinase and Mg[γ-^{32}P]ATP and run on SDS-PAGE. After the radiolabelled NR band was excised, digestion of the gel slice with alkylated trypsin released between 73 and 89% of the radioactivity in three separate experiments using three preparations of NR and two preparations of NR kinase.

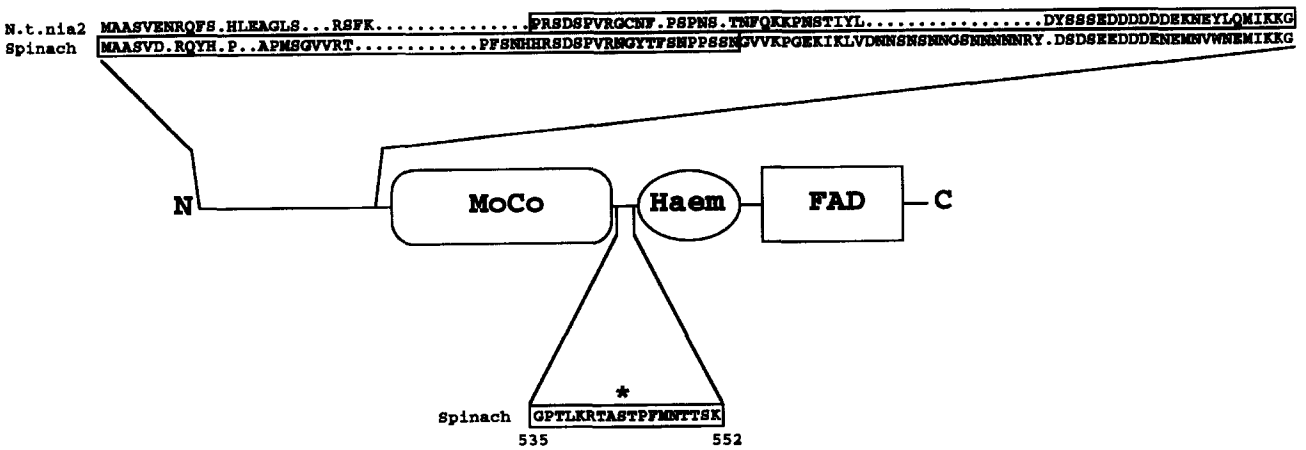

Fig. 1. Regulatory sites on higher plant nitrate reductase (NR). A schematic diagram of one subunit of the NR homodimer indicating the functional domains and linking hinges. FAD, flavin domain; haem, haem-Fe domain; MoCo, molybdenum cofactor domain; C, C terminus of NR; N, N terminal tail of NR. The sequence expanded from hinge 1 shows the regulatory phosphorylation site in spinach NR that was identified here, with an asterisk indicating the phosphorylated serine residue, Ser-543. The sequences expanded from the N-terminal tail are tobacco NR in which the boxed region shows the 56 amino acids deleted in the DNR construct of Nussaume et al. [27] (upper sequence), and spinach NR in which the boxed region shows the sequence that is sometimes lost by proteolysis during purification of NR (lower sequence). The sequence alignments and gaps (dots) were introduced in Nussaume et al. [27] to maximise homologies with the N-termini of other NRs and are retained here to ease comparisons.

In the first experiment, chromatography of the tryptic digests on a C_{18} column at pH 1.9 revealed one major peak of ^{32}P-radioactivity which was eluted at 16.4% acetonitrile (equivalent to peak 1 on Fig. 2). The radioactive peak coincided with the shoulder of a peak of absorbance at 214 nm. Therefore, the peak fractions of ^{32}P-radioactivity were rechromatographed on the C_{18} column using a shallower gradient to give pure phosphopeptide (section 2). The sequence of this peptide was TASTPFMNTTSK (Fig. 3), identical to residues 541 to 552 of the amino acid sequence derived from the cDNA of spinach NR [21]. Phosphoamino acid analysis showed that only phosphoserine was present in this peptide (data not shown). In order to identify which of the two possible serines was phosphorylated, solid phase sequencing (section 2; [20]) was carried out. A burst of ^{32}P release at the third cycle of Edman degradation showed that only the first serine in this sequence, Ser-543, was phosphorylated (Fig. 3).

In two further phosphorylation experiments, again the major phosphopeptide was eluted from the C_{18} column at 16.4% acetonitrile. However, three additional phosphopeptides, eluted at 18.3%, 22.2% and 30.0% acetonitrile, were also identified (peaks 2, 3 and 4, respectively on Fig. 2). The phosphopeptide which was eluted at 18.3% acetonitrile was found to be identical to that of the peptide at 16.4% acetonitrile (Fig. 3), except that Met-547 had become oxidised to methionine-sulphone. Methionine oxidation presumably explains the shift in HPLC elution properties of this peptide. The abundance of the phosphopeptide at 22.2% acetonitrile was too low to sequence. The amino acid sequence of the phosphopeptide at 30.0% acetonitrile was found to be PVSFTAASLAPEFQNAIP, identical

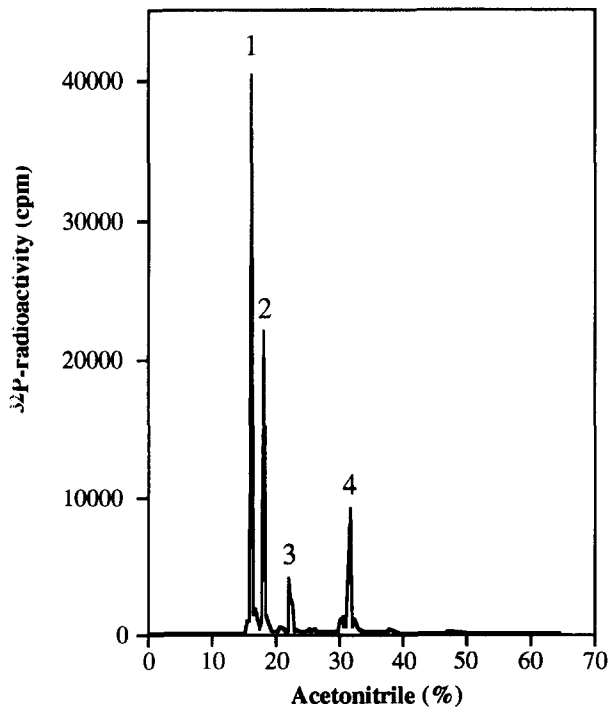

Fig. 2. Chromatography on C_{18} of tryptic phosphopeptides from NR phosphorylated by NR kinase. ^{32}P-labelled NR was digested with trypsin and the digest chromatographed on a C_{18} column (section 2). In the first phosphorylation experiment (see section 3) only peak 1 of ^{32}P-radioactivity, eluted at 16.4% acetonitrile, was seen. The profile of four phosphorylated peaks shown here was seen in two further experiments.

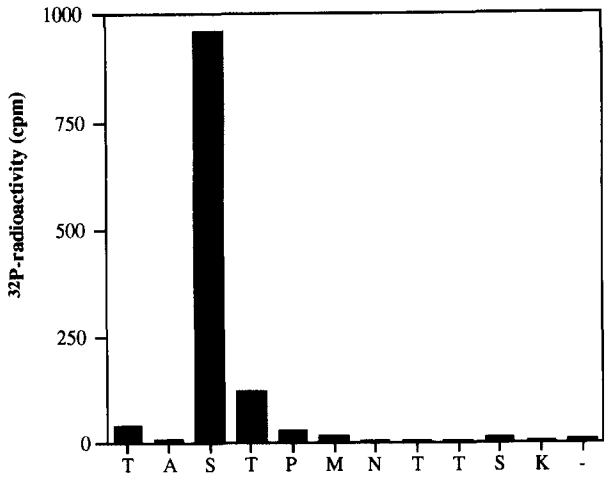

Fig. 3. Identification of Ser-543 as the serine residue in the hinge 1 region of nitrate reductase that was phosphorylated by NR-inactivating kinase. Phosphopeptide (~5–10 pmol) eluted from the C_{18} column at 16.4% acetonitrile (equivalent to peak 1, Fig. 2) was coupled covalently to an arylamine membrane and sequenced on an Applied Biosystems 470A/120A sequencer. The figure shows ^{32}P-radioactivity released after each cycle of Edman degradation and the amino acid sequence of the peptide using the single letter code for amino acids which was determined on an Applied Biosystems 476A protein sequencer.

to residues Pro-531 to Pro-548 of the P subunit of glycine dehydrogenase from pea (*Pisum sativum*) [22]. Glycine dehydrogenase has approximately the same molecular mass (114 kDa for the pea enzyme, [22]) as the NR subunit and was probably introduced as a contaminant of the NR kinase preparation.

3.2. *Inactivation of phosphorylated NR by NIP*

In common with others [23,24] we have found that the 115 kDa NR subunit sometimes becomes proteolysed during purification to give a second NR band with an apparent molecular mass of 109 kDa on SDS-PAGE (Fig. 4A). The two bands of a proteolysed NR preparation were electrophoretically transferred from an SDS-gel onto ProBlott and sequenced. The N-terminal sequence of the lower band was found to be GVVKPGEKIKLVDNN, which identified this band as NR with 45 amino acids removed from the N-terminus (Fig. 1). In agreement with previous findings that the terminal methionine of NR is blocked [25], no full-length NR sequence was identified from the upper band. Sequences beginning HHRSDSPVR (NR minus 24 amino acids from the N-terminus; Fig. 1) and GVVKPGEKI were present in trace amounts.

As reported previously [15,16], both phosphorylation of NR and binding of NIP were required to inactivate NR (Fig. 4B and data not shown). However, we noticed that more degraded preparations of NR were less sensitive to NIP inhibition after phosphorylation than intact NR preparations. For example, Fig. 4A shows a time course of NR phosphorylation of a partially degraded preparation of NR. The lower NR band (NR minus the N-terminal 45 amino acids) was phosphorylated at the same rate as the upper band of intact NR (Fig. 4A). Both upper and lower bands were phosphorylated on Ser-543 (data not shown). However, at maximal phosphorylation, this preparation could only be 60% inhibited by NIP (Fig. 4B). Another preparation in which approximately 60% of the NR subunits

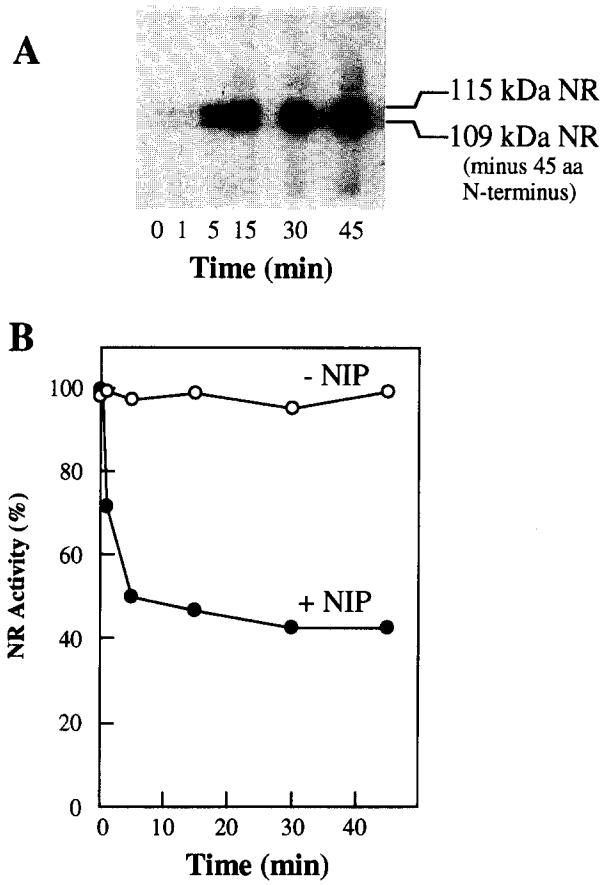

Fig. 4. The effect of phosphorylation on the inhibition of a proteolysed NR preparation by NIP. (A) NR was phosphorylated in incubations (500 μl) containing 10 μg NR, 200 μl of NR kinase (peak fraction from Hi-load Q column, see section 2.4.), 1 μM microcystin-LR (to inhibit protein phosphatases [18]) and 0.1 mM [γ-^{32}P]ATP (~5,000,000 cpm/nmol by Cerenkov counting) in buffer B. The reactions were initiated by addition of ATP. Aliquots (50 μl) were removed at the times indicated, trichloroacetic acid (TCA) added to a final concentration of 15% (w/v) and precipitated protein collected by centrifugation for 3 min at 13,000 × g. Pellets were washed three times in 15% (w/v) TCA and counted by Cerenkov, washed in ether to remove TCA, subjected to electrophoresis on 7% SDS-polyacrylamide gels, stained with Coomassie Brilliant blue and autoradiographed (autoradiograph shown). (B) Aliquots (2.5 μl) of the incubation from (A) were assayed for NR activity in the presence of Mg^{2+} and in the presence (●) or absence (○) of NIP (20 μg from a desalted NIP fraction eluted from the Hi-Load Q-column) as described in [15] and section 2.

were proteolysed, could only be inhibited 35% by NIP after maximal phosphorylation (not shown). The concentration of NIP was not limiting in these experiments, but was tenfold higher than the concentration which caused >95% inhibition of phosphorylated NR in a rapidly prepared and desalted 0–30% saturated $(NH_4)_2SO_4$ fraction of a spinach leaf extract.

4. Discussion

Here we report that the in vitro phosphorylation of NR on a single serine residue (Ser-543) is sufficient to allow inactivation of intact NR by NIP binding. Ser-543 is conserved in all the sequences of higher plant NRs that have been published [26], suggesting that this regulatory mechanism may occur in many plant species. Ser-543 is not conserved in NR from Chlorella or fungi [26].

In an earlier study, Huber et al. [12] phosphorylated spinach NR in vivo by feeding leaves with ^{32}P in the light and the dark. The NR was then immunoprecipitated from extracts and digested with trypsin. The seryl phosphorylation status of two tryptic NR peptides, analysed by thin layer chromatography (TLC), was found to correlate closely with changes in NR activity [12]. It was suggested, therefore, that NR was regulated by multisite phosphorylation in vivo. This may be the case. For example, there may be other NR kinases that have not yet been identified in vitro and that phosphorylate other serine residues in NR. However, an alternative explanation for the in vivo data [12] is that both phosphorylated spots that were identified by TLC represented the same phosphopeptide (Thr-541–Lys-552) that has been identified in this study, but containing Met-547 in different oxidation states (see section 3).

We also observed that NR that had been partially proteolysed at the N-terminus could be phosphorylated at the same rate as intact NR, but could not subsequently be completely inhibited by NIP, even when more than saturating concentrations of NIP were used (Fig. 4). This N-terminal proteolysis was probably an artifact of NR extraction and purification. Nevertheless, these findings suggest that both the N-terminal tail and the phosphorylation site in the hinge 1 region take part in the NR inactivation mechanism. For example, the N-terminal tail may contain the site which interacts directly with NIP upon phosphorylation of NR. Alternatively, the N-terminal tail may be a mobile region that changes its conformation after phosphorylation in the hinge 1 region to reveal a NIP binding site in another part of the protein. Strong evidence that the N-terminal tail is required for inactivation was provided by Nussaume et al. [27] who found that a deletion of 56 amino acids close to the N-terminus of NR from tobacco (Fig. 1) abolished the ability of the enzyme to be inactivated in the presence of ATP. Furthermore, in transgenic plants expressing this N-terminal deletion construct, inactivation of NR in the dark was abolished [27]. The MoCo domain (which lies between the N-terminal tail and the Ser-543) may also play a part in the inactivation mechanism. A mutation in the MoCo domain drastically impaired phosphorylation of NR in Arabidopsis [28] and the methyl viologen-reducing activity of the MoCo domain has been shown to be inhibited by phosphorylation and NIP binding (Cathrine Lillo, personal communication).

The results in this paper extend our previous model (Fig. 4 in [15]) by showing that inactivation of NR requires (a) phosphorylation at Ser-543, (b) interaction with NIP, (c) the presence of Ca^{2+} or Mg^{2+} ions and (d) intact NR that has not been proteolysed at the N-terminus. If any of these criteria are not met, NR cannot be inactivated fully in vitro. It follows that reactivation of NR could be due to (a) dephosphorylation of Ser-543, (b) release of NIP (for example by separation during purification or by chelating Mg^{2+}), and/or (c) N-terminal proteolysis of NR. This model highlights the importance of distinguishing between these various mechanisms when carrying out in vitro activation/inactivation studies; for example, when testing metabolites as potential regulators of NR kinase and NR phosphatase. Some studies have treated NR activation as synonymous with NR phosphatase activity [29]. However, it is now clear that the only certain way to define NR dephosphorylation is to demonstrate inhibition by a specific inhibitor of type 2A

protein phosphatases (such as microcystin and okadaic acid) and to demonstrate release of $^{32}P_i$ from radiolabelled NR.

It is clearly now critical to establish whether phosphorylation of Ser-543 underlies the acute inhibition of NR by NIP in vivo in response to factors that reduce photosynthetic rates, and to test the physiological effects of mutating Ser-543 of NR in transgenic plants. The identification of the inactivating phosphorylation site and the elution positions of the tryptic phosphopeptides during C_{18} chromatography reported in this paper will greatly facilitate these investigations.

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