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14-3-3 proteins associate with the regulatory phosphorylation site of spinach leaf nitrate reductase in an isoform-specific manner and reduce dephosphorylation of Ser-543 by endogenous protein phosphatases

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Abstract Three lines of evidence indicate that the 14-3-3 proteins that inactivate the phosphorylated form of spinach leaf NADH:nitrate reductase (NR) bind to the enzyme at the regulatory phosphorylation site (Ser-543). First, a phosphorylated synthetic peptide based on the regulatory site can prevent and also reverse the inactivation of phospho-NR caused by 14-3-3 proteins. Second, sequence-specific and phosphorylation-dependent binding of the aforementioned synthetic peptide to the 14-3-3 proteins was demonstrated in vitro. Third, 14-3-3 proteins were required for the ATP-dependent phosphorylation of NR (as assessed by activity measurements) in the presence of NR-kinase and leaf protein phosphatases. Lastly, we demonstrate specificity of recombinant *Arabidopsis* 14-3-3 isoforms in the interaction with phospho-NR: $\omega > \chi > \upsilon \gg \phi, \psi$.

Key words: Binding site; 14-3-3 Protein; Inhibitor protein; Isoform specificity; Nitrate reductase; Protein phosphatase; Regulatory phosphorylation site

1. Introduction

The first step in nitrate assimilation is the reduction of nitrate to nitrite and is catalyzed by NADH:nitrate reductase (NR; EC 1.6.6.1), which is generally regarded to be a highly regulated step. Control of NR activity involves both regulation of the steady-state level of enzyme protein [1–3] as well as reversible protein phosphorylation [4–6]. The regulation of NR by seryl phosphorylation is a two-stage process: first, Ser-543 on spinach NR is phosphorylated by Ca^{2+}-dependent NR-kinase (NRk) and then phospho-NR interacts with an inhibitor protein (IP) in the presence of divalent cations to yield an inactive complex [7–9]. The IP that inactivates phospho-NR has recently been identified as a mixture of 14-3-3 proteins [10,11]. Because 14-3-3 proteins are known to be involved in protein-protein interactions and kinase-related events in signal transduction pathways, it now seems likely that one or more of the 14-3-3 proteins do bind directly to phospho-NR. Moreover, 14-3-3 proteins have been shown to be sequence-specific phosphoserine-binding proteins, interacting with the motif: R-S-X-phosphoS-X-P [12]. The suggested 14-3-3 binding motif is a good match for the regulatory phosphorylation site of spinach NR: R-T-A-phosphoS_{543}-T-P [13,14]. Thus, the most likely possibility is that the 14-3-3 protein(s) binds directly to the regulatory phosphorylation site of NR as is thought to occur with other proteins that interact with 14-3-3s [12].

However, there is still no experimental evidence that 14-3-3s do indeed bind to phospho-NR, and if so, what portion of the NR protein is involved in the interaction. It is also unclear whether the phosphorylation state of Ser-543 affects the interaction. Consequently, the overall objective of the present study was to determine whether 14-3-3 proteins interact with the regulatory phosphorylation site (Ser-543) of spinach NR. The experimental approach was to use a synthetic peptide (designated NR6) based on the sequences surrounding the regulatory phosphorylation site (residues 535 to 552 of spinach NR). The NR6 synthetic peptide has been shown to be readily phosphorylated by NRk and to compete with native NR for phosphorylation and inactivation [14]. In the present study, we show that spinach leaf 14-3-3 proteins bind in a sequence and phosphorylation-dependent manner to phospho-NR6 (pNR6), that pNR6 can disrupt the native phospho-NR:14-3-3 complex, and that binding of 14-3-3 proteins to phospho-NR blocks dephosphorylation of Ser-543 by endogenous protein phosphatases. Lastly, we present evidence that there may be 14-3-3 isoform specificity involved in regulation of NR.

2. Materials and methods

2.1. Materials

Spinach (*Spinacia oleracea* L. cv. Bloomsdale) was grown in a greenhouse and leaves were harvested directly into liquid nitrogen as described [9]. [γ-^{32}P]ATP (111 TBq/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA); microcystin-LR was obtained from Calbiochem (La Jolla, CA, USA), and all other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of spinach leaf 14-3-3 proteins

The IP, which is a mixture of 14-3-3 proteins, was purified by sequential chromatography of spinach leaf proteins on DEAE-Sepharose, Blue Sepharose, and Resource Q (Pharmacia), and in some experiments by native gel electrophoresis, as previously described [9].

2.3. Expression and purification of GF14 proteins

Five *Arabidopsis* GF14 proteins [15,16] were expressed and purified as previously described [17]. The coding sequences of GF14 proteins were amplified by PCR using synthetic oligodeoxynucleotides and subcloned into the *Nde*I-*Bam*HI site of pET15b. All 14-3-3 clones are in frame determined by sequencing. The pET15b vector provided the expressed GF14 proteins with a His tract at their amino-termini, allowing for purification by Ni^{2+}-charged immobilized metal affinity

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Abbreviations: IP, inhibitor protein; NR, NADH:nitrate reductase; NRk, NR kinase; NR-PP, NR-protein phosphatase.

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chromatography (IMAC) according to the manufacturer's protocol (Novagen). The His tract was removed by human thrombin cleavage, followed by dialysis and IMAC. The purified proteins were dialyzed against a buffer containing 20 mM HEPES (pH 7.5), 40 mM KCl, 0.1 M EDTA, 10% (v/v) glycerol, and 5 mM 2-mercaptoethanol, and the protein concentration was determined using the Bradford microassay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as standard.

2.4. Extraction and assay of NR in leaf extracts

In order to obtain the native phospho-NR/14-3-3 complex (as used in the experiment presented in Fig. 2), leaves were harvested from spinach plants that had been placed in darkness for 1 h. Frozen leaf tissue was then homogenized (1 g fresh weight/2 ml) in extraction buffer containing 50 mM MOPS-NaOH (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 1 mM aminocaproic acid, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μM microcystin-LR. The extract was centrifuged at 14000×*g* for 1 min, and the supernatant was desalted by centrifugal filtration on Sephadex G-25. NADH:NR activity was assayed in the presence and absence of 5 mM Mg^{2+}; reactions, initiated by addition of extract, were run at 25°C and terminated after 4 min by addition of zinc acetate [5]. Activation state of NR is defined as the ($+Mg^{2+}$) NR activity expressed as a percentage of the ($-Mg^{2+}$) NR activity, and in the presence of 14-3-3 proteins, reflects the phosphorylation status of the regulatory site [5,14].

2.5. Partial purification of NR, NRk and NR-phosphatase

Native dephosphorylated NR was obtained from leaves harvested from spinach plants in the light. Partial purification of dephospho-NR was performed as described in detail [9]. Briefly, the leaf extract was fractionated with polyethylene glycol(PEG)-8000, and the fraction that precipitated between 5 and 20% PEG was subjected to anion exchange chromatography using a DEAE-Sepharose column on an FPLC (Pharmacia) system. NR co-elutes in the salt gradient along with NRk and NR-protein phosphatase(s) (NR-PP), but is well resolved from IP, now known to be a mixture of 14-3-3 proteins. In the experiment presented in Fig. 3, the fraction containing NR, NRk and NR-PP was preincubated with ATP and other additions as indicated in the text prior to assay in the presence of 14-3-3s using the $+Mg^{2+}$ and $-Mg^{2+}$ assays.

2.6. Purification and phosphorylation of synthetic peptides

The synthetic peptide NR6 (sequence: GPTLKRTASTPFJNTTSK; J = norleucine), based on the regulatory phosphorylation site of spinach NR, was purchased from Chiron Mimotopes Peptide Systems (San Diego, CA) and synthetic peptide SP2 (sequence: GRJRRISSVEJJDKK), based on the regulatory phosphorylation site of sucrose-phosphate synthase was synthesized on a Milligen/Biosearch 9600 instrument as previously described [18]. Peptides were purified by chromatography using a linear acetonitrile gradient on an Ultremex 5 C18 reversed-phase preparative HPLC column as previously described [18]. Purified peptides were lyophilized, resuspended in Milli-Q water (Millipore, Bedford, MA), and dried again to remove residual TFA. Peptides (1 mM) were phosphorylated (non-radiolabeled) in vitro in a reaction mixture containing 50 mM MOPS-NaOH (pH 7.5), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2.5 mM DTT, 2 mM ATP and partially purified protein kinase (the peak I enzyme [19]). Phosphorylation mixtures were allowed to run overnight at 2 °C. The mixtures were then rechromatographed on the reversed-phase preparative HPLC column, and the phosphopeptide peak was collected and processed as described above. [^{32}P]phosphopeptides were prepared as described above except that [γ-^{32}P]ATP (60 cpm/pmol) was used and the reaction mixtures were not rechromatographed by reversed-phase HPLC.

2.7. Peptide binding assay

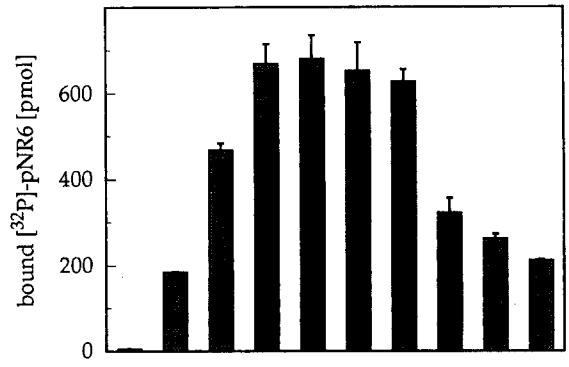
Spinach leaf 14-3-3 proteins, or recombinant *Arabidopsis* GF14 isoforms, were mixed with 20 to 80 μM [^{32}P]pNR6 (60 cpm/pmol) in a buffer containing 50 mM MOPS-NaOH (pH 7.5), 10 mM $MgCl_2$, and 2.5 mM DTT at 0°C for 1 min with other additions as specified in the text. Binding of the ^{32}P-phosphopeptide was determined by centrifugal filtration of the mixture (0.1 ml) through a 1.5 ml column of Sephadex G-25 fine in a table top centrifuge (approx. 350×*g*, 1 min). Uncomplexed ^{32}P-peptide is retained in the column and bound peptide is contained in the void volume. It was verified that ATP does not bind to 14-3-3 proteins (data not shown). Consequently, any residual [γ-^{32}P]ATP in the ^{32}P-phosphopeptide preparation did not contribute to, or interfere with, binding of ^{32}P-phosphopeptides to the 14-3-3 proteins.

3. Results

3.1. Evidence for direct binding of 14-3-3 proteins to pNR6

Muslin et al. [12] recently provided a unifying concept concerning the nature and specificity of 14-3-3 binding to target proteins, by demonstrating that 14-3-3s bind to proteins and peptides containing the motif: R-S-X-phosphoS-X-P. Thus, it was of interest to determine whether spinach IP, which is a mixture of 14-3-3s, would bind to the phosphorylated synthetic peptide pNR6, which is based on the regulatory phosphorylation site of NR. For these experiments, we used a centrifugal filtration technique using Sephadex G-25 as the matrix to separate rapidly bound from free peptide. As shown in Fig. 1, [^{32}P]pNR6 was obtained in the void volume only when 14-3-3 proteins were added indicating formation of a high molecular weight 14-3-3/[^{32}P]pNR6 complex. The amount of [^{32}P]pNR6 bound was a function of 14-3-3 protein concentration under the conditions tested, as would be expected (Fig. 1).

Several lines of evidence indicated that the interaction between the 14-3-3 protein(s) and pNR6 was rather specific in nature. First, addition of salt (up to 0.2 M NaCl) did not reduce peptide binding indicating that the interaction was not a non-specific electrostatic effect. Second, the binding was both sequence- and phosphorylation-dependent. Sequence specificity of the interaction was examined by testing other peptides for their ability to compete with [^{32}P]pNR6 for binding to the 14-3-3s. As expected, addition of increasing con-



IP	[μM]	0	7.5	20	35	35	35	35	35	35	35
SP2	[μM]	-	-	-	-	80	-	-	-	-	-
pSP2	[μM]	-	-	-	-	-	80	-	-	-	-
NR6	[μM]	-	-	-	-	-	-	80	-	-	-
pNR6	[μM]	-	-	-	-	-	-	-	20	40	80

Fig. 1. Competitive binding of synthetic peptides to 14-3-3 proteins. Spinach leaf 14-3-3s (35 μM) were mixed with 40 μM [^{32}P]pNR6 (60 cpm/pmol) in the presence and absence of other phosphorylated (nonradioactive) or unphosphorylated peptides. The control contained no additional competing peptide. The sequences of the synthetic peptides are as follows (J is norleucine): NR6, GPTLKRTASTPFJNTTSK; pNR6, GPTLKRTA**phosphoS**TPFJNTTSK; SP2, GRJRRISSVEJJDKK; pSP2, GRJRRI**SphosphoS**VEJJDKK; Values are means of three determinations ± standard error.

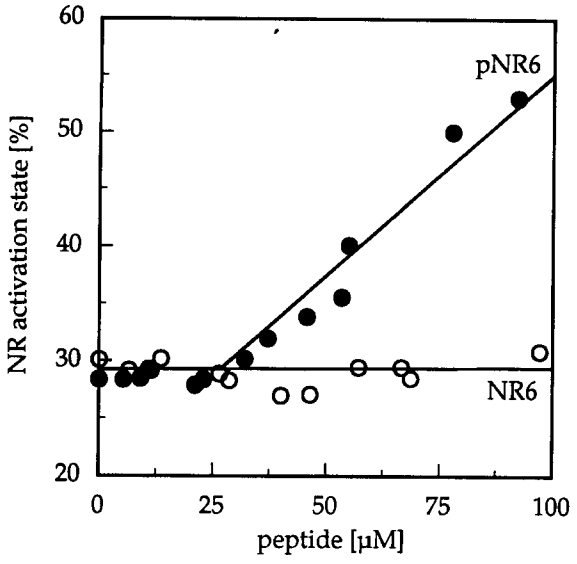


Fig. 2. The pNR6 synthetic peptide disrupts the native phospho-NR/14-3-3 interaction. Desalted extracts, containing inactive NR extracted from dark leaves in the presence of protease and phosphatase inhibitors, were preincubated for 4 min at 0°C with the indicated concentration of pNR6 or unphosphorylated NR6 synthetic peptide. Activation state of NR was then assessed as described in Section 2.4.

centrations of non-radioactive pNR6 effectively competed and could displace bound ^{32}P-phosphopeptide (Fig. 1). Unphosphorylated NR6 had no effect, indicating that binding was strictly dependent upon phosphorylation of the serine residue equivalent to Ser-543. However, other phosphoserine-containing peptides such as pSP2, which is based on the regulatory phosphorylation site of SPS and does not match the proposed 14-3-3 binding motif, did not compete with [^{32}P]pNR6 for binding. Likewise, unphosphorylated SP2 had no effect. Thus, the in vitro binding was sequence specific.

3.2. pNR6 can disrupt the inactive pNR/14-3-3 complex

If 14-3-3 proteins bind reversibly to the regulatory phosphorylation site on native NR, then addition of pNR6 should reverse the 14-3-3/phospho-NR interaction that inhibits enzymatic activity. As shown in Fig. 2, addition of increasing concentrations of pNR6 to a desalted dark leaf extract resulted in a significant increase in NR activation state. Addition of unphosphorylated NR6 had no effect, demonstrating a strict dependence on phosphorylation of the peptide. These results suggest that the phosphopeptide specifically promotes dissociation of preformed 14-3-3/phospho-NR complexes, thereby activating NR. Interestingly, pNR6 was only effective at concentrations greater than 25 μM (Fig. 2). This may indicate that 14-3-3 proteins are more abundant than NR protein and that uncomplexed 14-3-3s must bind pNR6 before the 14-3-3:phospho-NR complex is disrupted.

3.3. 14-3-3 proteins block the dephosphorylation of Ser-543 in native NR

If 14-3-3s interact directly with the phosphoserine residue of the binding motif, then it would be expected that the presence of 14-3-3s would reduce the rate of dephosphorylation of the phosphoserine. This has been demonstrated for the protein phosphatase type 1-catalyzed dephosphorylation of Raf-1 [12]. The results presented in Fig. 3 are also consistent with this postulate. The experiment presented in Fig. 3 involved two stages. In the first, a mixture of partially purified dephospho-NR, NRk and NR-PP (but devoid of 14-3-3s) was incubated with Mg·ATP, Ca^{2+}, and other additions as indicated, and in the second stage, an aliquot was removed at the times indicated, supplemented with 14-3-3s, and the activation state of NR was immediately assessed. In the control incubation (no further additions), NR was not inactivated (Fig. 3) by preincubation with ATP, indicating that phosphatase activity exceeded kinase activity and thus phospho-NR, needed for binding of the 14-3-3 protein, did not accumulate. However, addition of microcystin-LR to the first stage incubation inhibited NR-PP, and thereby allowed phospho-NR to accumulate and the inactive complex to form when 14-3-3s were added just prior to assay. The most important result is that the same effect was obtained when 14-3-3 proteins (i.e. the IP preparation) were added to the first stage incubation (Fig. 3) suggesting that the 14-3-3 protein(s) bind to the regulatory phosphorylation site and restrict accessibility of the phosphoserine to the protein phosphatase(s).

3.4. Evidence for isoform specificity

In both plants and animals, multiple genes encode the different members of the 14-3-3 family. In the best characterized plant system, five different members of the *Arabidopsis* family have been cloned [16]. The characterized *Arabidopsis* isoforms, designated GF14 ω, ψ, χ, φ, and υ, were produced as recombinant proteins and tested individually for their ability to inactivate phospho-NR and also to bind [^{32}P]pNR6. As shown in Fig. 4, the ω isoform was most effective in inactivation of phospho-NR, followed by χ and υ. The φ and ψ isoforms had no effect at the concentrations tested. The isoform specificity

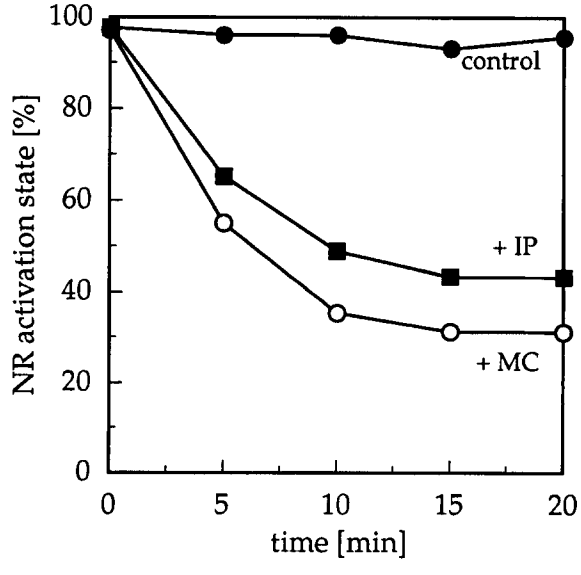


Fig. 3. 14-3-3 proteins inhibit the dephosphorylation of Ser-543 of native phospho-NR. A partially purified mixture of dephospho-NR, NRk and NR-PP, obtained from anion exchange chromatography, was preincubated with 2 mM ATP and no additions ('control') or 10 μM 14-3-3 proteins ('+IP') or 2 μM microcystin-LR ('+MC'). The mixtures were preincubated at 25°C and at the times indicated, aliquots were withdrawn, supplemented with 10 μM 14-3-3 proteins and NR activation state was measured immediately as described in Section 2.4.

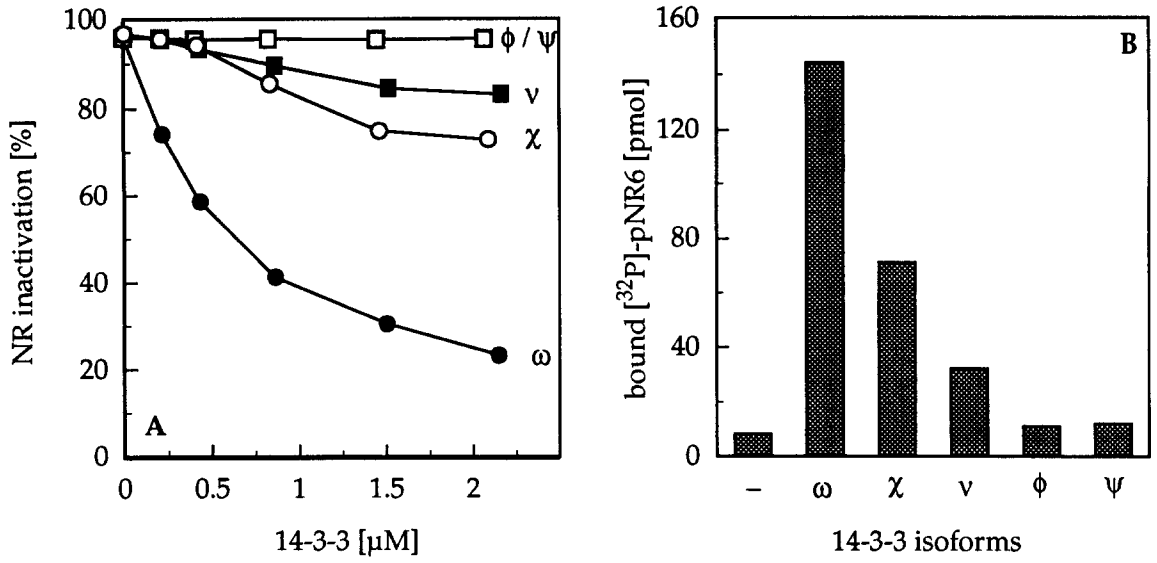


Fig. 4. Isoform specificity for *Arabidopsis* 14-3-3 proteins in the inactivation of native phospho-NR and binding to [^{32}P]pNR6. (A) A partially purified mixture of dephospho-NR and NRk was preincubated with 2 mM ATP, 2.5 μM microcystin-LR, and increasing concentrations of the indicated 14-3-3 isoform. After preincubation at 25°C for 15 min, the activation state of NR was measured as described in Section 2.4. (B) Recombinant *Arabidopsis* 14-3-3 proteins (2 μM) were mixed with 40 μM [^{32}P]pNR6 at 0°C for 1 min and binding was measured by centrifugal filtration as described in Section 2.7.

may be explained by their differential ability to bind to the phosphorylated form of the regulatory site peptide pNR6 (Fig. 4B). Apparently, the ability to bind the synthetic peptide pNR6 in vitro was correlated with the ability to bind to native phospho-NR to produce the inactive enzyme complex. The isoform specificity was surprising, because in a previous study, Muslin et al. [12] were unable to demonstrate specificity among mammalian ζ, η, β and τ isoforms in terms of binding to a Ser-259 phosphorylated peptide derived from Raf-1. It is interesting that plant isoforms appear to differ in their binding properties in vitro.

4. Discussion

Identification of the inhibitor protein, required to inactivate phospho-NR, as 14-3-3 protein(s) [10,11] strengthened the original suggestion that the inhibitor functions by binding to phospho-NR. However, direct evidence for a physical association between the two has been lacking and the site(s) of interaction is also not known. Based on the suggestion by Muslin et al. [12] that 14-3-3s bind to phosphoserine in the motif: R-S-X-phosphoS-X-P, we set out to determine whether spinach leaf 14-3-3s bind to the regulatory phosphorylation site of NR. Three lines of evidence indicate that this is the case. First, spinach 14-3-3s bind to [^{32}P]pNR6, a synthetic peptide derived from the regulatory phosphorylation site of spinach NR. Second, pNR6 disrupted the preformed association between 14-3-3/phospho-NR. Third, 14-3-3s blocked dephosphorylation of the regulatory phosphorylation site on NR, as would be expected if the 14-3-3 protein(s) bound directly to the Ser-543. Similar results to those listed above were obtained by Muslin et al. [12] for the interaction of Raf-1 with 14-3-3s. Our results lend strong support to the notion that 14-3-3s bind to a distinct phosphoserine-containing motif.

We suggest that binding of 14-3-3(s) to the R-T-X-phosphoS$_{543}$-X-P binding motif in the hinge 1 region of NR may explain why the rate of enzyme activation (dephosphorylation) in vivo is much slower than the rate of inactivation (phosphorylation) in vivo [20]. It may be a general feature of 14-3-3-regulated enzymes that dephosphorylation is necessarily slower than the rate of activation. If true, this may have important physiological consequences and implications for the control of 14-3-3-regulated enzymes in vivo.

Our results are also significant because recombinant *Arabidopsis* 14-3-3 isoforms appear to differ in their ability to bind to, and inactivate, phospho-NR. It is not at all clear at the present time how restricted in function(s) each 14-3-3 isoform may be. Of particular relevance to the present study, it has recently been shown that at least four of the five cloned *Arabidopsis* isoforms are able to complement the yeast double mutant *bmh1-bmh2*, which alone is lethal [21]. However, the *Aradidopsis* isoforms appear not to be equivalent in regulation of spinach NR, at least in our heterologous in vitro system. Isoform specificity has been observed in at least some other systems. For example, mammalian 14-3-3 proteins associate with A20, a zinc finger protein, with considerable specificity in vitro [22]. It will be interesting to try to determine the basis for the isoform specificity in systems where it exists. In the case of NR (and binding to pNR6), it may be that substitution of a Thr residue at P-2 (a conservative substitution for the more common Ser residue) in the proposed binding motif may reduce binding of some (φ, ψ) but not other (ω, χ, υ) *Arabidopsis* isoforms. Future studies will examine this possibility.

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NC Agricultural Research Service and does not imply its approval to the exclusion of other products that might also be suitable.

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