Binding to 14-3-3 proteins is not sufficient to inhibit nitrate reductase in spinach leaves

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Abstract To assess the role of 14-3-3 proteins in the magnesium-dependent inhibition of nitrate reductase (NR) we tested the effect of magnesium on NR binding to 14-3-3s by coimmunoprecipitation and gel filtration. The stability of the 14-3-3 complex of NR was, unlike its activity, unaffected by magnesium. We therefore conclude that binding to 14-3-3s per se does not inhibit NR. Magnesium inhibited 14-3-3-bound NR much more strongly than 14-3-3-free NR. 14-3-3s possibly reinforce NR inhibition by magnesium.

Key words: 14-3-3 Protein; Nitrate reductase; Protein phosphorylation; Protein–protein interaction; Enzyme inhibition

1. Introduction

14-3-3 proteins are highly conserved and ubiquitously expressed eukaryotic proteins [1]. 14-3-3s exist as homo- or heterodimers, are generally thought to promote communication between proteins as linkers or molecular frameworks and appear to be involved in a panoply of signalling pathways [2]. The exact functions of 14-3-3s are largely unknown. Many enzymes share a so-called 14-3-3 binding site containing a phosphorylated serine residue [3]. In plants, an increasing number of 14-3-3 ligands have been identified, including the metabolic enzymes nitrate reductase (NR; EC 1.6.6.1) and sucrose-phosphate synthase (SPS; EC 2.4.1.14) [4–8].

Phosphorylation of spinach leaf NR in the hinge 1 region on serine 543 creates a binding site for 14-3-3s and leads to NR inhibition in the presence of divalent cations and 14-3-3s [4,5,9–12]. The mechanism of this inhibition is poorly understood. It is currently believed that 14-3-3 binding to NR requires divalent cations and that such binding alone inhibits NR [10–15]. However, this rather simple explanation might be misleading since it is only based on indirect evidence: (1) a magnesium requirement for the non-transient drop of NR activity upon NR phosphorylation and binding to 14-3-3s [4,7,15–17], and (2) a weak stimulation by magnesium of 14-3-3 binding to short synthetic phosphopeptides [5,17] that have a larger conformational freedom than their corresponding proteins. Notably, a magnesium requirement for the physical association of NR with 14-3-3s (so far examined by blot overlay [7] or the yeast dihybrid system [9]) was not demonstrated. A conserved acidic stretch in the N-terminus of NR has been proposed to be involved in the inactivation of the enzyme [18]. This has led to the speculation that an activating factor is released from NR upon NR phosphorylation and binding to 14-3-3s [19]. Such factor has not been identified and the acidic stretch has been shown to be unnecessary for the 14-3-3 and magnesium-dependent inhibition of the enzyme [16].

Here, we tested the proposed function of 14-3-3s as inhibitors of NR by coimmunoprecipitation and gel filtration, two classical and powerful techniques to examine protein–protein interactions under native conditions. We assessed the stability of the 14-3-3 complex of NR with or without magnesium and asked how this affects NR activity.

2. Materials and methods

2.1. Materials, extraction, fractionation and detection of proteins, NR activity assay

Growth of spinach, protein extraction from leaves and NR activity assays with $MgCl_2$ or EDTA (3 min assays without preincubation of the extract) were carried out as previously [15] except that the following extraction buffer was used: 50 mM MOPS/NaOH, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.5, 1 mM DTT, 0.5% (v/v) Triton X-100 and 0.5 mM freshly prepared PMSF.

Gel electrophoresis, Western transfer and immunodecoration were done according to [20]. The amounts of NR or 14-3-3s were measured as in [21]: immunoprecipitated, partially purified or crude proteins were gel fractionated in parallel to serial dilutions of purified NR or 14-3-3s of known protein contents, blotted and quantified after they were immunodecorated or stained with Coomassie brilliant blue R 250.

2.2. Antibody production and protein purification

Serum antibodies were raised in rabbits (New Zealand Whites). Peptide antigens were prepared by coupling synthetic peptides (DRQYHPAPMSGVVRTP or QPNLDKMGFDIKE, close to N- or C-terminus of spinach leaf NR) to Keyhole limpet hemocyanin [22]. Antisera to full length 14-3-3s were raised against purified 14-3-3s. They were purified from spinach leaves as in [5].

14-3-3s were coprecipitated with NR as in [21]. The efficiency of NR immunoprecipitation was $>90\%$ if crude enzymes from illuminated or darkened leaves or if partially purified and in vitro phosphorylated or dephosphorylated enzymes were used. This was assessed from NR protein that was precipitated and that was left in the supernatant after precipitation. Both NR antibodies crossreacted with a single band at 110 kDa since such band was not decorated in the presence of antigenic peptides.

14-3-3-free NR with NR-kinase and -phosphatase was partially purified with polyethyleneglycol and by anion exchange [23]. Enzymes were gel filtrated at 4–6°C on a Sephacryl S300 HR 16/60 column (Pharmacia) with buffers as indicated (Fig. 2). 1.0-ml fractions were collected at 0.20 ml/min.

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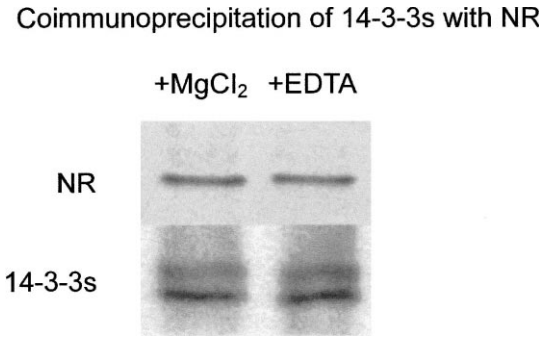

Fig. 1. The 14-3-3 complex of NR is stable without magnesium. After leaves were darkened for 30 min, protein extracts were prepared, desalted over a Sephadex G25 column into extraction buffer without Triton and NaCl and brought to 100 nM okadaic acid. NR was then precipitated after the extracts were incubated with antibodies to the N-terminus at 23°C for 30 min in the absence or presence of free magnesium (with or without 10 mM EDTA). The precipitates were fractionated on 12% Laemmli gels and NR or 14-3-3s were immunodecorated following their transfer onto a Immobilon P membrane. Results show the 110-kDa NR monomer and the 30/32 kDa 14-3-3 doublet that was pulled down together with NR. The 14-3-3 binding stoichiometry of NR was 1.7. The antibodies failed to precipitate NR or 14-3-3s in the presence of antigenic peptide.

3. Results and discussion

3.1. Binding to 14-3-3s does not inhibit NR unless magnesium is present

NR is well known to be rapidly inactivated by phosphorylation and binding to 14-3-3s in leaves upon a light/dark transition [14]. To test whether NR binding to 14-3-3s requires divalent cations and is disrupted in the presence of excessive EDTA [10–15], we prepared crude extracts from darkened leaves. We then checked the stability of the 14-3-3 complex of NR by coimmunoprecipitation in the presence or absence of magnesium. The absence of magnesium did not reduce the amount of 14-3-3s that were pulled down together with NR (Fig. 1). So, the stability of the 14-3-3 complex of NR was, unlike its activity (Table 1) [10–15], unaffected by magnesium. This was confirmed by gel filtration (Fig. 2). Magnesium had only little, if any, influence on the elution of NR (Fig. 2A) or 14-3-3s (Fig. 2B) from a sizing column that was loaded with a mix of partially purified and phosphorylated NR. 14-3-3s eluted in two major peaks. The narrower second peak coeluted with purified 14-3-3s (not shown). Hence, the broader first peak were probably ligand-bound 14-3-3s and the second peak ligand-free 14-3-3s. Since we used only partially purified NR, the 14-3-3s in the first peak were probably bound to ligands in addition to NR. To assess how much of the 14-3-3s in the first peak were bound to NR, we removed NR from partially purified enzymes by immunoprecipitation before they

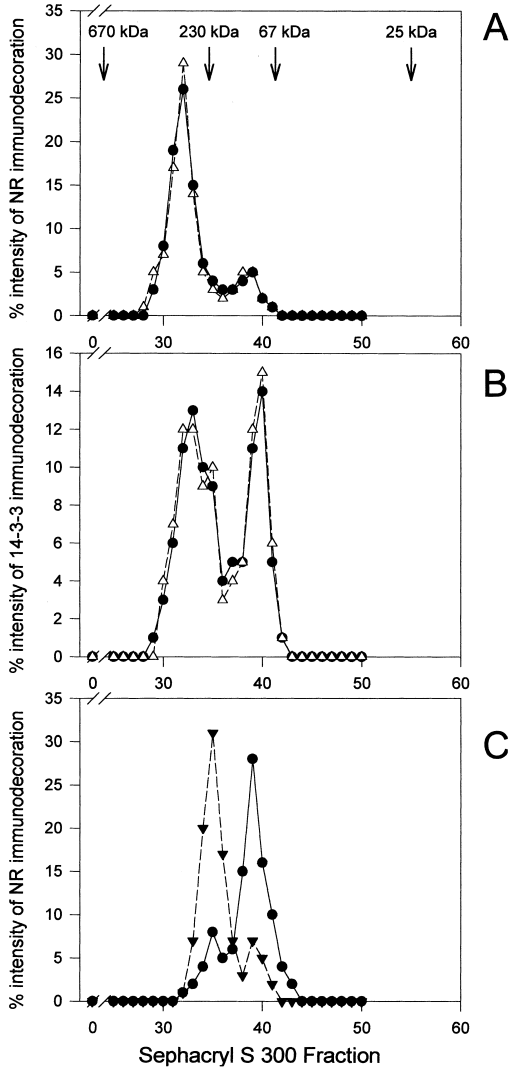

Fig. 2. Magnesium hardly influences gel filtration of the 14-3-3 complex of NR. NR was partially purified, desalted, and incubated at room temperature for 30 min to: (1) dephosphorylate NR or (2) phosphorylate NR after addition of 5 nM okadaic acid and 20 μM ATP. The phosphorylated NR was then mixed with purified 14-3-3s (7 μg per μg NR) and gel filtrated without (△) or with (●) free magnesium (extraction buffer minus Triton with or without 10 mM EDTA, respectively). Aliquots were taken from the eluted fractions to measure by densitometry the intensity of the immunodecoration of NR (A) or 14-3-3s (B) following protein fractionation on 10% Laemmli gels and Western blotting. (C) Phosphorylated (●) or dephosphorylated NR (▼) was gel filtrated without EDTA and analyzed for NR as above. Recovery of loaded NR (protein and activity with or without magnesium) or 14-3-3s after gel filtration was between 88 and 96%. Gel filtration was calibrated with thyroglobin, catalase, albumin and chymotrypsinogen A (670, 230, 67 and 25 kDa).

Table 1
Magnesium inhibition of NR remains after immunoprecipitation and EDTA treatment

Conditions	NR activity (μmol h^{-1} g^{-1} FW)	
	Magnesium assay	EDTA assay
Crude extract	4.6 ± 0.3	24 ± 3
After immunoprecipitation with magnesium	4.4 ± 0.4	21 ± 2
After immunoprecipitation with EDTA	4.6 ± 0.4	20 ± 3

Data are based on the experiment described in Fig. 1. NR activity was assayed with 10 mM $MgCl_2$ or 15 mM EDTA: (1) in the crude extract before immunoprecipitation; and (2) after immunoprecipitation in resuspended immunoprecipitates. Data are means ± S.E. from three independent experiments.

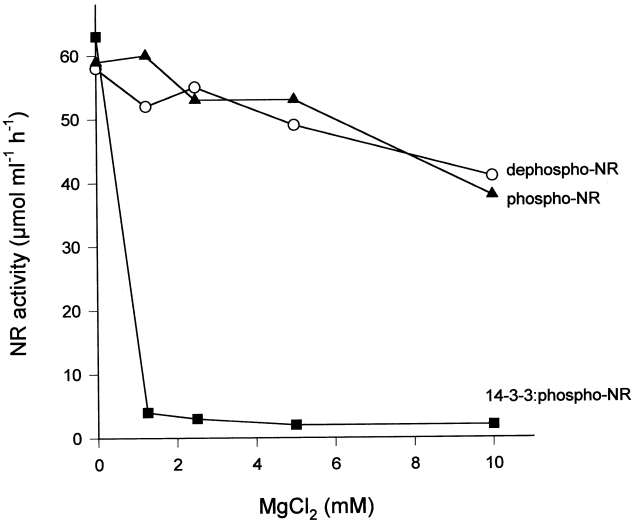

Fig. 3. The degree of magnesium inhibition varies among NR forms. NR was immunoprecipitated from dephosphorylated or phosphorylated NR (Fig. 2) and from phosphorylated NR after addition of purified 14-3-3s (7 μg per μg NR). NR activity was then assayed as a function of $MgCl_2$ after precipitates were resuspended. Data points are means of duplicate assays that varied less than 7% and are typical of three independent experiments.

were mixed with 14-3-3s and gel filtrated as described in Fig. 2. In this control experiment (not shown), the second 14-3-3 peak was about 30% larger than in Fig. 2B at the expense of the first peak. This suggests that at least a minor portion of the 14-3-3s in the first peak (Fig. 2A) was bound to NR during gel filtration in both, the presence or absence of free magnesium.

As one would expect for 14-3-3-free NR, NR eluted later if loaded without 14-3-3s (Fig. 2C). Interestingly, without the 14-3-3s, dephosphorylated NR eluted earlier than phosphorylated NR (Fig. 2C). It is possible that dimeric NR had fallen apart into monomers during the sizing step upon phosphorylation in the absence of 14-3-3s. A possible function of 14-3-3s might therefore be to stabilize native NR upon phosphorylation which is reminiscent to the generally proposed function of 14-3-3s as molecular frameworks [2].

3.2. *Magnesium inhibits all NR forms, in particular the 14-3-3 complex*

As can be inferred from the stability of the 14-3-3 complex of NR, binding to 14-3-3s per se cannot explain NR inhibition. Alternatively, it is conceivable that magnesium inhibits NR after NR has bound to 14-3-3s. To test this, we purified serine 543 phospho- or dephospho-NR and the 14-3-3 complex. We then determined the inhibition by magnesium of each NR form. As shown in Fig. 3, magnesium inhibits the 14-3-3 complex most strongly. The complex was almost completely inhibited by 10 mM magnesium that is used in our standard assay conditions to measure the activation state of NR [15]. Magnesium inhibited 14-3-3-free NR only weakly (Fig. 3). Interestingly, the extent of this inhibition varies among plant species; e.g. magnesium inhibits 14-3-3-free NR from ricinus leaves much more strongly than the spinach enzyme for yet unknown reasons [24].

4. Concluding remarks

In short, we provide compelling evidence that spinach leaf NR is inhibited by magnesium plus 14-3-3s because magnesium strongly inhibits the 14-3-3 complex of NR and not, as presently claimed [10–15], because of the binding of 14-3-3s to NR. 14-3-3s obviously reinforce NR inhibition by magnesium. It is currently unknown which part of the 14-3-3 complex of NR binds to magnesium. Recent evidence indicates that magnesium binds to 14-3-3s [25]. It is also possible that magnesium binds to both, NR and 14-3-3s upon formation of the complex. Perhaps, upon binding to NR, the 14-3-3s bring magnesium closer to NR (Fig. 4, right). Alternatively, binding to magnesium might let the complex twist into a catalytically inactive form (Fig. 4, left). If any activating factor is involved in NR inhibition [19], such factor should be released upon binding of the complex to magnesium.

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References

[1] Aitken, A., Collinge, D.B., van Heusden, B.P.H., Isobe, T., Roseboom, P.H., Rosenfeld, G. and Soll, J. (1992) Trends Biochem. Sci. 17, 498–501.
[2] Xiao, B., Smerdon, S.J., Jones, D.H., Dodson, G.G., Soneji, Y., Aitken, A. and Gamblin, S.J. (1995) Nature 376, 188–191.
[3] Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Cell 84, 889–897.
[4] Moorhead, G., Douglas, P., Morrice, N., Scarabel, M., Aitken, A. and MacKintosh, C. (1996) Curr. Biol. 6, 1104–1113.
[5] Bachmann, M., Huber, J.L., Liao, P.C., Gage, D.A. and Huber, S.C. (1996) FEBS Lett. 387, 127–131.
[6] Toroser, D., Athwal, G.S. and Huber, S.C. (1998) FEBS Lett. 435, 110–114.

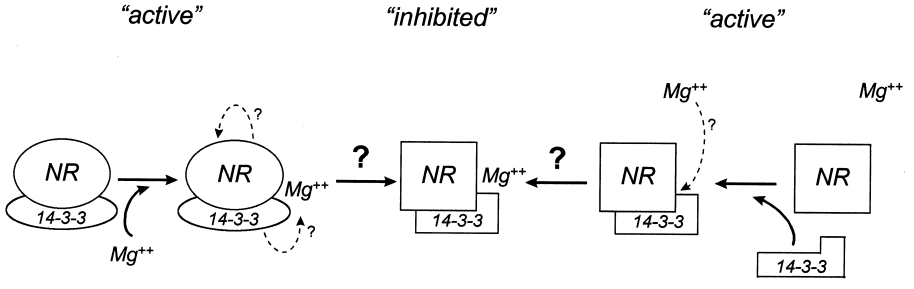

Fig. 4. Hypothetical model about two alternatives how the 14-3-3 complex of NR is inhibited by magnesium. 14-3-3s allow magnesium to bind more strongly to NR (right) or magnesium promotes the formation of an inactive complex (left).

[7] Moorhead, G., Douglas, P., Cotelle, V., Harthill, J., Morrice, N., Meek, S., Deiting, U., Stitt, M., Scarabel, M., Aitken, A. and MacKintosh, C. (1999) Plant J. 18, 1–12.
[8] Chung, H.J., Sehnke, C. and Ferl, R.J. (1999) Trends Plant Sci. 9, 367–371.
[9] Kanamaru, K., Wang, R., Su, W. and Crawford, N.M. (1999) J. Biol. Chem. 274, 4160–4165.
[10] MacKintosh, C., Douglas, P. and Lillo, C. (1995) Plant Physiol. 107, 451–457.
[11] Glaab, J. and Kaiser, W.M. (1995) Planta 195, 514–518.
[12] Bachmann, M., McMichael Jr., R.W., Huber, J.L., Kaiser, W.M. and Huber, S.C. (1995) Plant Physiol. 108, 1083–1091.
[13] Bachmann, M., Shiraishi, N., Campbell, W.H., Yoo, B.-C., Harmon, A.C. and Huber, S.C. (1996) Plant Cell 8, 505–517.
[14] Kaiser, W.M., Weiner, H. and Huber, S.C. (1999) Physiol. Plant. 105, 385–390.
[15] Kaiser, W.M. and Huber, S.C. (1997) J. Exp. Bot. 48, 1367–1374.
[16] Lillo, C., Kazazaic, S., Ruoff, P. and Meyer, C. (1997) Plant Physiol. 114, 1377–1383.
[17] Athwal, G.S., Huber, J.L. and Huber, S.C. (1998) Plant Physiol. 118, 1041–1048.
[18] Nussaume, L., Vincentz, M., Meyer, C., Boutin, J.P. and Caboche, M. (1995) Plant Cell 7, 611–621.
[19] Pigaglio, E., Durand, N. and Meyer, C. (1999) Plant Physiol. 119, 219–229.
[20] Weiner, H. (1995) Plant Physiol. 108, 219–225.
[21] Weiner, H. and Kaiser, W.M. (1999) FEBS Lett. 455, 75–78.
[22] Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.
[23] Bachmann, M., Huber, J.L., Athwal, G.S., Wu, K., Ferl, R.J. and Huber, S.C. (1996b) FEBS Lett. 398, 26–30.
[24] Kandlbinder, A., Weiner, H. and Kaiser, W.M. (2000) J. Exp. Bot. 51, 1099–1105.
[25] Athwal, G.S., Lombardo, C.R., Huber, J.L., Masters, S.C., Fu, H. and Huber, S.C. (2000) Plant Cell Physiol. 41 (4), 523–533.