14-3-3 proteins control proteolysis of nitrate reductase in spinach leaves

Hendrik Weiner*, Werner M. Kaiser

Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl Botanik I, Molekulare Pflanzenphysiologie und Biophysik, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany

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Abstract To test a possible role of 14-3-3 proteins in the degradation of nitrate reductase (NR) in leaves, we monitored 14-3-3s bound to NR in leaf extracts. The amount of 14-3-3s that coimmunoprecipitated with serine 543 phospo-NR (p-NR) increased upon a light/dark transition. This was accompanied by a similar increase in the protein turnover rate of NR in leaves. Purified NR was degraded in extracts from darkened but not from illuminated leaves. Removal of 14-3-3s from such extracts prevented NR degradation. We conclude that the availability of 14-3-3s for p-NR regulates the stability of NR.

Key words: 14-3-3 protein; Nitrate reductase; Protein turnover rate; Protein:protein interaction; Protein phosphorylation

1. Introduction

14-3-3 proteins are highly conserved and ubiquitously expressed eukaryotic proteins [1]. They generally promote communication between other proteins and appear to be involved in a myriad of signalling pathways [2]. However, the exact functions of 14-3-3s are still poorly understood. Many enzymes share a so-called 14-3-3 binding motif containing a phosphorylated serine residue [3]. In plants, an increasing number of 14-3-3 binding enzymes have been identified, including 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 on serine 543 creates a binding site for 14-3-3s and, after binding, NR is inactivated [9]. Phosphorylation and inactivation of NR in leaves upon darkening is usually accompanied by an enhanced propensity of NR to be degraded [10–12]. It has been speculated that such degradation of NR is associated with the increase of the phosphorylation state of NR because p-NR may be the degraded NR form [12] or a down-regulator of NR synthesis [6]. Although attractive, this seems to be overly simple as it does not explain why NR disappears almost completely in leaves during extended dark periods after the phosphorylation state of NR has increased only 2-fold or less upon darkening [12,13]. A possible role of 14-3-3s in this context has been overlooked so far and is described here.

2. Materials and methods

2.1. Materials, extraction, fractionation and detection of proteins

Growth of spinach and protein extraction from leaves were carried out as described previously [5] except that the following extraction buffer was used: 50 mM MOPS/NaOH pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.5% (v/v) Triton X-100, 0.5 mM freshly prepared PMSF. Gel electrophoresis, Western transfer and immunodecoration were done according to [14].

2.2. Production of antibodies, purification and quantification of proteins

Serum antibodies were raised in rabbits (New Zealand Whites). Peptide antigens were prepared by coupling synthetic peptides to keyhole limpet hemocyanin [14]. The following peptides were used: DRQYHPAPMSGVVRTPC (NR), LRERGHFSPSRTFVEEV (SPS) and ISKDYMESKSAAEL (phosphoenolpyruvate carboxylase, PepC). Antisera against full length 14-3-3s were raised using purified 14-3-3s as antigen. These 14-3-3s were obtained from spinach leaves via column purification as described in [5]. For precipitation, coprecipitation and depletion (Sections 3.1–3.3), serum antibodies were purified with protein A-Sepharose beads and coupled to such beads with dimethylpimelimidate [15] before they were added to leaf protein extracts (1 g of fresh leaves per 3 ml extraction buffer, precleared with protein A beads). The resulting suspension was rotated on a multiaxle rotating mixer at 4°C for about 40 min before immune complexes were collected for subsequent experiments or, without additional washes, for fractionation on Laemmli gels and further analysis (see Section 3). The efficiency of immunoprecipitation of 14-3-3s, SPS, NR and PepC from leaf extracts was $>90\%$.

To determine the amounts of individual proteins and their abundances, crude leaf proteins or immunoprecipitated proteins were gel-fractionated in parallel to serial dilutions of purified protein preparations of known protein contents, blotted and quantified after they were immunodecorated. Purified proteins were obtained as follows: individual proteins were immunopurified, fractionated on Laemmli gels and eluted (Bio-Rad Model 422 electroeluter). The protein recovery from gels after elution was 50–70% or 30–50% for 14-3-3s or NR, respectively, as evidenced by staining with Coomassie brilliant blue R 250 of the eluted proteins. The composition and concentration of amino acids of the eluted protein and of leaf total protein were determined on an Applied Biosystems 420 A/H amino acid analyzer equipped with an automated hydrolysis unit. The chemical reactions were carried out according to the manufacturer's recommendation.

Immunopurified serine 543 p-NR or the corresponding dephospho form of NR were obtained from partial purified and 14-3-3-free NR preparations containing NR kinase and NR phosphatase [5]. Following in vitro phosphorylation or dephosphorylation of serine 543 [5], the corresponding NR forms were immunopurified (see above) and subsequently used as described in Section 3.3. In vitro phosphorylation was carried out in the presence of 0.10 μM okadaic acid.

2.3. Protein synthesis measurements

Leaves were fed [^{35}S]methionine and treated as described in Section 3.2. Apparent rates of NR, SPS and PepC synthesis were obtained during the time course of labelling from the initial rates of incorporation of [^{35}S]methionine into each protein, and from the increase in the specific label of cellular-free methionine (i.e. non-protein-bound methionine that was 35 nmol methionine per g fresh weight under illumination or darkness). Apparent rates of protein synthesis are given as 'amount of freshly synthesized protein in % of its steady-state total protein level per hour'. Such rates are based on (1) the assumption that free methionine is metabolically active and together with the fed label equally distributed throughout intra- and extracellular leaf compartments, (2) published methionine contents of NR, SPS and PepC, and (3) abundances of 0.07, 0.08 and 0.5% of the total protein for NR, SPS and PepC, respectively (H. Weiner, unpublished).

*Corresponding author. Fax: (49) (931) 888 6158.
E-mail: weiner@botanik.uni-wuerzburg.de

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3. Results and discussion

3.1. 14-3-3s bind strongly to p-NR in darkened but not in illuminated leaves

To analyze binding of 14-3-3s to NR we measured the amounts of 14-3-3s bound to NR via coimmunoprecipitation after NR was phosphorylated in leaves. As shown in Fig. 1, a 14-3-3 doublet was pulled down together with NR from 'dark' extracts. The molar ratio of 14-3-3 to NR was almost 2 in such precipitates (Table 1). In contrast, we could detect very little, if any, coprecipitated 14-3-3s if we used 'light' extracts. The difference in the coprecipitable amounts of 14-3-3s was more than 20-fold (Table 1). This difference was unexpected because the overall amounts of 14-3-3s in leaves did not change (not shown) and because the phosphorylation state of NR on serine 543 increased as usual only 2-fold or less upon a light/dark transition ([12,13], H. Weiner and W.M. Kaiser, unpublished). So, although phosphorylation of NR on serine 543 is essential [5–9], it does not seem to be sufficient for binding of 14-3-3s to NR in leaves. The molecular basis for the light/dark changes in binding of 14-3-3s to p-NR is presently unknown. It may be the result of a mechanism that influences the availability of 14-3-3s for their ligands and that possibly involves phosphorylation of 14-3-3s [1,16] or phosphorylation of p-NR [17]. Importantly, 14-3-3s are only 4–5 times more abundant than NR in leaves (0.3% and 0.07%, respectively, of the total leaf protein (Section 2.2)). 14-3-3s might therefore be limiting for the formation of the 14-3-3:p-NR complex in leaves, given that 14-3-3s bind to a multitude of cellular phosphoproteins [1–9] that might have to compete with p-NR for binding to 14-3-3s.

PepC does not contain a 14-3-3 binding motif according to Muslin et al. [3] and, therefore, as would be expected, 14-3-3s did not appear in PepC immunoprecipitates (Fig. 1). In contrast to NR, the amount of 14-3-3s that bound to SPS was the same whether 'dark' or 'light' extracts were used. Further, unlike NR, only one 14-3-3 band was pulled down together with SPS. This 14-3-3 band comigrated in a Laemmli gel with the lower band of the 14-3-3 doublet of NR immunoprecipitates. Recently, Moorhead et al. also found such a 30 kDa 14-3-3 band in SPS immunoprecipitates using protein extracts from potato tubers [8]. As shown next, a change in the amount of bound 14-3-3s seems to play a role for the stability of its ligand.

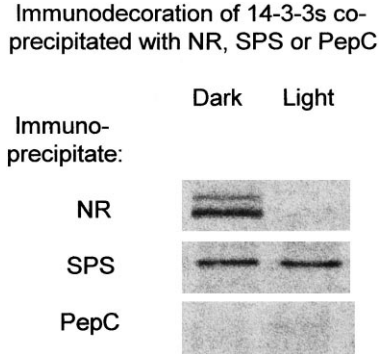

Fig. 1. Much more 14-3-3s coimmunoprecipitate with NR from 'dark' than from 'light' extracts. After leaves were illuminated or darkened for 30 min, extracts were prepared from which SPS, NR and PepC were immunoprecipitated. The precipitates were fractionated on 12% Laemmli gels and 14-3-3s were immunodecorated following western blotting. 80–90% of 14-3-3s remained in the extracts after immunoprecipitation of NR or SPS, and more than 95% 14-3-3s remained after precipitating PepC. The sizes of the 14-3-3 bands were 30 and 32 kDa in NR precipitates and 30 kDa in SPS precipitates (determined with Bio-Rad's low range protein standards for SDS-PAGE). Results are typical for three independent experiments.

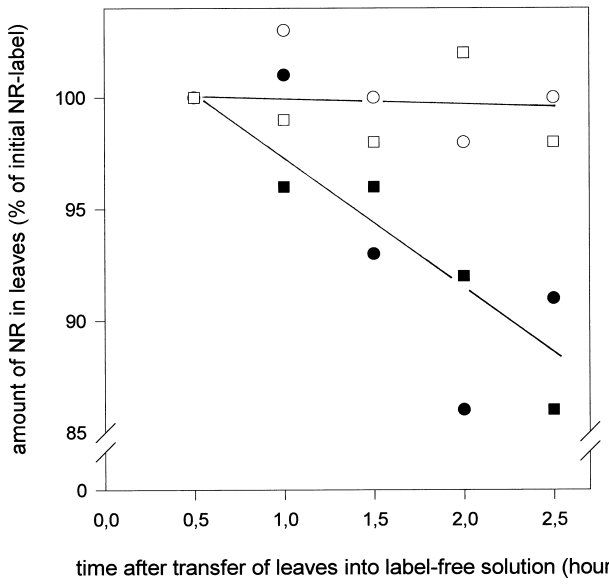

Fig. 2. The stability of NR drops upon darkening. Leaves were cut at the petiole, transferred into water, illuminated and fed carrier-free [^{35}S]methionine (0.25 mCi/ml) through the transpiration stream. After 30 min leaves were transferred into label-free water for an additional 30 min. Subsequently, half of the leaves were kept under illumination and half were darkened. During the next 2 h leaf samples were taken as indicated for protein extraction and immunoprecipitation. Following fractionation on 7% Laemmli gels, the immunoprecipitated NR subunit was cut out and the band was dissolved and counted. Initial label of NR (100% on *y*-axis) corresponds to about 800 cpm per such band obtained from the first leaf samples during the time course. Data points are means of duplicate immunoprecipitations which varied less than 4%. Results are from two independent experiments (closed symbols for darkened leaves, □,■ and ○, ●)

3.2. NR becomes unstable in leaves upon a light/dark transition

To test whether the loss of NR in leaves during a dark period results from changes in NR synthesis or turnover, we labelled leaf proteins metabolically and analyzed their stability. For comparison to NR, we examined SPS and PepC that have, unlike NR, a rather constant steady-state protein level in the dark period [18,19]. As shown in Fig. 2, NR was rather stable in illuminated leaves but was unstable if leaves were

Table 1
14-3-3 binding stoichiometry of NR

Leaf extract (noon)	Amount of precipitated protein (ng)		mol 14-3-3 per mol NR
	NR	14-3-3s	
Dark (30 min)	210 ± 14	104 ± 9	1.8 ± 0.3
Light	220 ± 20	< 5	< 0.1

NR and NR-bound 14-3-3s were precipitated with NR antibodies from extracts from darkened or illuminated leaves (Section 2.2). The amount of NR or 14-3-3s in the precipitates were quantified by immunoblotting using standard preparations of NR or 14-3-3s (Section 2.2). The molar ratio of 14-3-3s to NR is based on published molecular weights. Data shown are from three independent experiments.

darkened. SPS and PepC were also stable in illuminated leaves but, unlike NR, remained stable upon darkening (data for SPS and PepC not shown). Because of the large increase in 14-3-3 binding to NR (Table 1) and because different bands of 14-3-3s bound to NR and SPS (Fig. 1), it may be possible that certain 14-3-3 isoforms that bind to NR but not to SPS or PepC, enhance the turnover rate of their ligands. Taken together, it seems that binding to 14-3-3s rather than phosphorylation per se ([12], see Section 1) is critical for NR degradation.

The apparent rates of synthesis of NR, PepC and SPS were all about 2–3-fold higher in illuminated than in darkened leaves but overall, these rates were rather low (Fig. 3). The latter was expected because with the exception of NR in darkened leaves, the steady-state protein levels did not change and their turnover rates were low (see above). So, the observed light/dark decrease in NR synthesis was small compared to the corresponding increase in the rate of NR turnover (Fig. 2) and therefore, did not contribute largely to the decrease of the steady state protein level of NR in the dark period [10–12].

The regulation of NR by 14-3-3 is best understood in spinach leaves. Such regulation may not be representative for other species, at least partly. For example, *Ricinus communis* leaf NR contains the 14-3-3 binding motif but is obviously not regulated by 14-3-3s because, unlike the spinach enzyme, ricinus NR (a) has an extremely low activation state in both, illuminated or darkened leaves, (b) retains this low activation state after partial purification in 14-3-3-free preparations and (c) does not disappear in darkened leaves (A. Kandlbinder, H. Weiner and W.M. Kaiser, unpublished).

3.3. 14-3-3s are necessary for proteolysis of purified NR

Next, we tested the stability of purified NR forms in crude extracts because NR is well known to be unstable in leaf extracts and because binding of 14-3-3s to NR (Fig. 1, Table 1) was accompanied by an enhanced turnover rate of NR in leaves (Fig. 2). As shown in Fig. 4, p-NR but not the corresponding dephospho form of NR disappeared in crude extracts. This loss of NR depended on 'dark' extracts, was prevented after 14-3-3s were removed and was the result of proteolysis, as it was blocked by 0.5 mM PMSF (the latter not shown). Thus, it seems that an essential factor for NR degradation is absent or inactive in 'light' extracts.

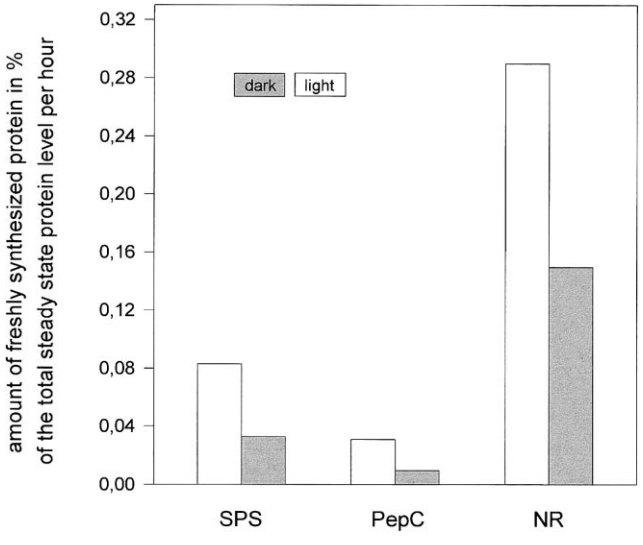

Fig. 3. Protein synthesis. Leaves were darkened or illuminated and fed [^{35}S]methionine as described for Fig. 2. Leaf samples were taken every 30 min during the first 2 h after label was added to monitor the uptake of label into cellular methionine pools and into individual proteins following immunoprecipitation and fractionation on Laemmli gels. Initial rates of protein synthesis were obtained from such time course analysis (Section 2.2). Data from one experiment are shown which was repeated one time for SPS or two times for NR and PepC yielding equivalent results.

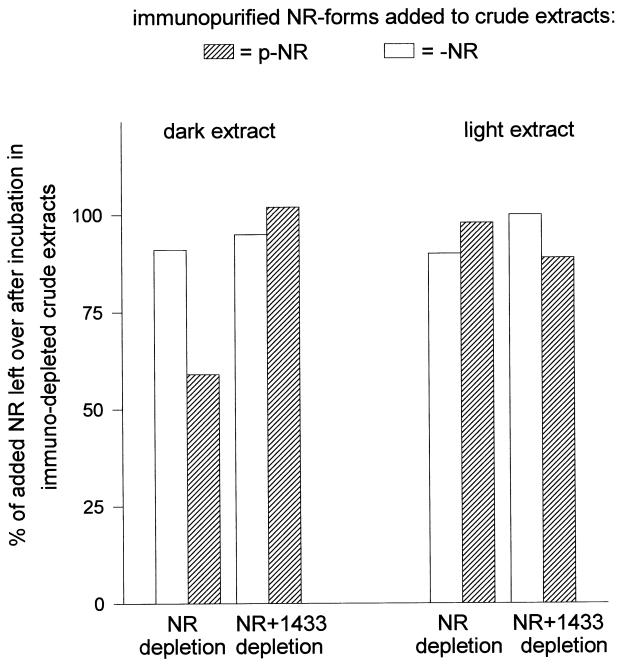

Fig. 4. 14-3-3s promote proteolysis of NR in extracts from darkened leaves. Protein extracts were prepared without PMSF from illuminated or darkened leaves. These extracts were desalted over Sephadex G25, immunodepleted of NR or of NR+14-3-3s as indicated and supplemented with 0.10 μM okadaic acid to prevent dephosphorylation of NR before immunopurified serine 543 phospho- or dephospho-NR (14-3-3-free, see Section 2.2) was added and before the resulting mixtures were incubated at 25°C. Reactions were terminated 45 min later with TCA and the NR subunit was immunodecorated and quantified following Laemmli gel fractionation and Western blotting. Immunodetection of NR was carried out with NR antibodies directed against the N-terminus (see Section 2.2) or against the C-terminus of NR (QPNLDKMGFDIKE). In both cases only one NR band (110 kDa) was identified. Data shown are means of duplicate assays which varied less than 5% and which are typical for three or two independent experiments with extracts from darkened or from illuminated leaves, respectively, using different preparations of p-NR or -NR in each experiment.

We did not detect ubiquinated NR forms using anti-ubiquitin antibodies on Westerns containing immunoprecipitated NR from crude leaf extracts or from partially purified NR preparations from illuminated or darkened leaves, not even after feeding leaves with derivatives of calpain inhibitor 1, very potent and widely used proteasome inhibitors [20,21]. So, NR seems to be degraded, at least in vitro, by an ubiquitin/proteasome-independent mechanism.

4. Concluding remarks

14-3-3s exist as homo- or heterodimers and are generally thought to function as linkers between different or between the same polypeptides [2,22–25]. Assuming that native NR is a homodimer [10] and highly phosphorylated in darkened leaves

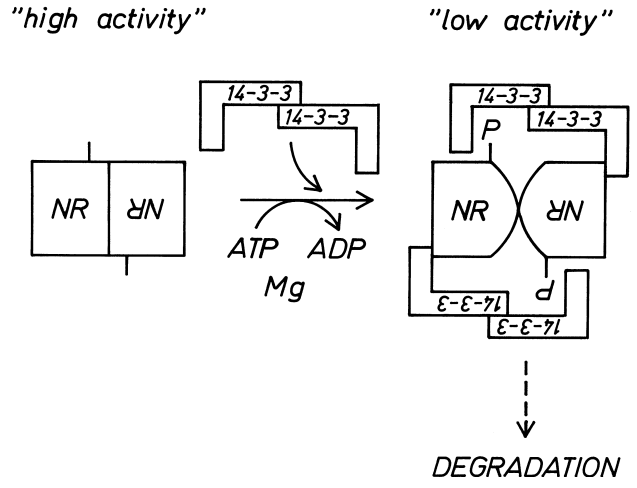

Fig. 5. Hypothetical model showing how 14-3-3s function as stabilizers to prevent dissociation of the NR dimer upon phosphorylation of serine 543. Formation of such a complex leads to inactivation and, subsequently, to proteolysis of NR.

[12], our results imply that two 14-3-3 dimers bind to native p-NR. It is possible that one 14-3-3 dimer interacts with different NR subunits to stabilize native NR following phosphorylation because (a) one 14-3-3 dimer has two potential binding sites [22–25], (b) the N-terminus of NR seems to be involved in binding to 14-3-3s [11,17] in addition to the 14-3-3 binding motif around serine 543, and (c) phosphorylation of partially purified and 14-3-3-free NR promotes dissociation of native NR (H. Weiner, unpublished). Fig. 5 highlights three possible functions of 14-3-3s: they (I) contribute to inactivation, (II) prevent dissociation and (III) trigger proteolysis of NR.

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