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Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers

Werner M. Kaiser[1,3] and Steven C. Huber[2]

[1] *Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für Molekulare Pflanzenphysiologie und Biophysik, Julius-von-Sachs-Platz 2, D-97082 Würzburg, Germany*

[2] *USDA Plant Science Research and Departments of Crop Science and Botany, NC State University, Raleigh, NC 27695-7631, USA*

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Abstract

Assimilatory nitrate reductase (NR) of higher plants is a most interesting enzyme, both from its central function in plant primary metabolism and from the complex regulation of its expression and control of catalytic activity and degradation. Here, present knowledge about the mechanism of post-translational regulation of NR is summarized and the properties of the regulatory enzymes involved (protein kinases, protein phosphatases and 14-3-3-binding proteins) are described. It is shown that light and oxygen availability are the major external triggers for the rapid and reversible modulation of NR activity, and that sugars and/or sugar phosphates are the internal signals which regulate the protein kinase(s) and phosphatase. It is also demonstrated that stress factors like nitrate deficiency and salinity have remarkably little direct influence on the NR activation state. Further, changes in NR activity measured *in vitro* are not always associated with changes in nitrate reduction rates *in vivo*, suggesting that NR can be under strong substrate limitation. The degradation and half-life of the NR protein also appear to be affected by NR phosphorylation and 14-3-3 binding, as NR activation always correlates positively with its stability. However, it is not known whether the molecular form of NR *in vivo* affects its susceptibility to proteolytic degradation, or whether factors that affect the NR activation state also independently affect the activity or induction of the NR protease(s). A second and potentially important function of NR, the production of nitric oxide (NO) from nitrite is briefly described, but it remains to be determined whether NR produces NO for pathogen/stress signalling *in vivo*.

Key words: Nitrate reductase, nitrite, nitric oxide, regulation, protein phosphorylation, 14-3-3-binding, signalling, sugars.

Introduction

Nitrate is the major N-source for plants and, in addition, it is an important signalling molecule that influences plant growth and differentiation. Accordingly, for many decades plant scientists have been engaged in elucidating the mechanism and regulation of nitrate uptake, transport and reduction in plants. Nitrate itself appears to be a signal (Scheible *et al*., 1997*a*) that can directly affect the expression of genes related to nitrate uptake, transport, assimilation, and related carbon metabolism (Scheible *et al*., 1997*b*). Indirectly, nitrate may also affect plant–pathogen interactions via the production of nitric oxide (NO), which is catalysed by nitrate reductase (NR) under certain conditions. This article summarizes data collected over the last ten years on the function and post-translational regulation of higher plant nitrate reductase (NR). The mechanism of the post-translational modulation of NR is summarized briefly followed by a description of the physiological relevance of that regulation and the environmental (stress) factors involved in the control of NR. For more details on the structure, function and transcriptional regulation of NR the reader may refer to an excellent recent summary (Campbell, 1999).

[3] To whom correspondence should be addressed. Fax: +49 931 888 6125. E-mail: kaiser@botanik.uni-wuerzburg.de

How is nitrate reductase (NR) activity controlled?

The NR protein represents a short, soluble electron transport chain localized in the cytosol, which catalyses the transfer of two electrons from NAD(P)H to nitrate (+5), which is reduced to nitrite (+3), and further reduced to ammonium (−3) in the plastids. A second, less well understood function of NR is the reduction of nitrite to nitric and nitrous oxides (see below). Both, nitrite and ammonia (and also NO), are toxic at higher concentrations. Indeed, under normal conditions nitrite concentrations in leaves are rarely above 15 nmol g^{-1} FW. Ammonium concentrations are usually orders of magnitude higher, and may exceed total leaf concentrations of 10 µmol g^{-1} FW (Lang and Kaiser, 1994). Thus, production of nitrite appears under rigid control. This is achieved by the control of (i) NR expression; (ii) NR catalytic activity; and (iii) NR protein degradation.

NR is inducible, depending on the availability of nitrate and light (Galangau *et al*., 1988; Vincentz *et al*., 1993; for a review see Campbell, 1999), but this aspect will not be considered here. Instead, the study focuses on the control of catalytic activity and of NR protein degradation.

In the last decade it became clear that the activity of the existing NR enzyme is rapidly and reversibly modulated. One very simple but important prerequisite for observing this rapid modulation was to measure NR activity in the presence of millimolar concentrations of divalent cations instead of using phosphate buffers which had been frequently applied. Phosphate buffers give high NR activity, but bind divalent cations, which play an important role in NR modulation (see below). Thus, in the absence of divalent cations (and in the presence of EDTA), post-translational modulation of NR activity can barely be detected. For example, in crude leaf extracts prepared with 10 mM Mg^{2+}, NR activity changed within minutes when leaves were exposed to a dark/light transient. When the same extracts were measured in the presence of excess EDTA, very little change in NR activity was observed. NR was also inactivated *in vitro* by preincubation with ATP, suggesting that the enzyme was phosphorylated. Subsequent investigations with ^{32}P-labelling techniques showed that NR was indeed inactivated by phosphorylation on a serine residue (ser 543 in spinach), which is located in the hinge-1 region of the molecule. However, during early attempts to purify an NR kinase it became clear that phosphorylation per se was not sufficient, but an additional protein was required to inactivate NR. That additional protein factor was initially termed 'inhibitor protein', or 'IP' or NIP (for nitrate reductase inhibitor protein). It was later identified to belong to the large group of 14-3-3-proteins, which are binding proteins with multiple functions in all eukaryotes (for reviews see Kaiser and Huber, 1994*a*; Kaiser *et al*., 1999).

Together, all these findings led to the scheme for NR modulation depicted in Fig. 1. According to that, NR exists basically in three states: free NR (active), phosphorylated NR (pNR; active), and pNR: 14-3-3 complex (inactive). The ratio of these three NR-forms is variable, depending on external conditions. The percentage of active NR (NR + pNR) measured in the presence of excess Mg^{2+} to the total NR activity measured in the presence of excess EDTA was termed the 'activation state'. For example, if one half of the existing NR would be phosphorylated and bound to 14-3-3s in the presence of millimolar concentrations of Mg^{2+}, the activation state (based on activity measurements) would be about 50%. It is important that phosphorylation plus 14-3-3-binding does not change the substrate affinities of NR in a crude extract (Kaiser and Spill, 1991). Apparently, the pNR–14-3-3 complex is simply switched off, whereas

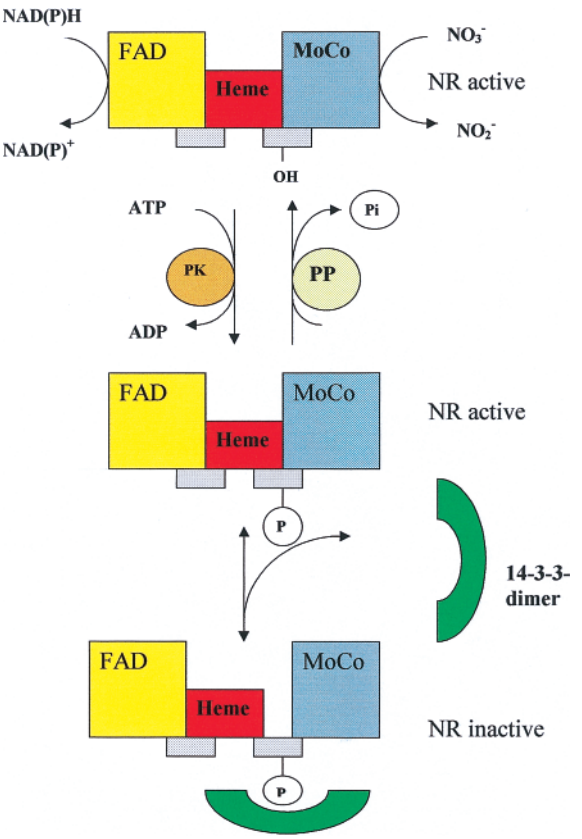

Fig. 1. Model for the post-translational modulation of NR. The enzyme consists of three different functional and structural domains (labelled in different colours), which are connected by two hinge regions (in grey). The serine-543 phosphorylation site is located in hinge-1, which connects the heme and the MoCo domain. The phosphorylated ser-motif is recognized by a 14-3-3 dimer which binds and, in presence of divalent cations converts NR into a completely inactive complex, which cannot transfer electrons from NAD(P)H to nitrate. This is schematically indicated by the 'gap' between the *heme* and the MoCo domain within the complex. Further explanations in the text.

the remaining free NR in the extract works normally. Formation of the pNR–14-3-3 complex may cause a change in NR conformation that interrupts electron transport between the heme and the MoCo-domain, as indicated schematically in Fig. 1.

Divalent cations fulfil several purposes in that model: (i) the protein kinase itself is a Ca-dependent enzyme; (ii) the substrate for the kinase is Mg-ATP; (iii) divalent cations are required to bring the pNR–14-3-3 complex into an inactive form. It is still not totally clear, whether divalent cations are required for 14-3-3 binding to pNR, or whether they are needed for the complex to switch from an active into an inactive form or both (Weiner and Kaiser, 2000). It is, however, absolutely clear that after complete chelation of divalent cations subsequent to NR phosphorylation, NR remains active, irrespective of its phosphorylation state.

Next, the properties of the regulatory enzymes and proteins that are responsible for modulation of NR activity by post-translational modification are briefly summarized.

CDPKs and SNF1-related protein kinases phosphorylate NR

Spinach leaf extracts contain several major peaks of protein kinase activity that were originally designated PK_I, PK_{II} and PK_{III} (in order of elution during FPLC ion exchange chromatography) and can phosphorylate NR, or a synthetic peptide based on the Ser-543 sequence. PK_I and PK_{II} are calcium-dependent kinases and are known to be CDPKs (Bachmann *et al*., 1996*a*; Douglas *et al*., 1998), whereas the calcium-independent PK_{III} has been shown to be an SNF1-related protein kinase (SnRK1; Sugden *et al*., 1999). Like other SNF1-related protein kinases, the PK_{III} (or 'HRK-C' in Sugden *et al*., 1999) is itself potentially regulated by phosphorylation. There is also evidence that the phosphorylation/inactivation of NR catalysed by these enzymes may be inhibited by metabolites (Bachmann *et al*., 1995). The extent to which metabolites may regulate NR phosphorylation *in situ* is discussed further below. Another remaining question is the extent to which the three kinases mentioned above might individually contribute to NR phosphorylation. That calcium-dependent protein kinases play a significant role is suggested by the observation that treatment of darkened leaf discs with EGTA + A23187 (divalent cation ionophore) resulted in NR activation, consistent with a role for CDPKs in NR phosphorylation (Kaiser and Huber, 1994*b*).

pNR is dephosphorylated by a type 2A protein phosphatase

Phospho-NR is dephosphorylated and activated by a type 2A protein phosphatase (MacKintosh, 1992), and thus, light activation *in situ* can be blocked by feeding inhibitors such as okadaic acid. The type 2A protein phosphatases in plants (Deruère *et al*., 1999) and animals (Shenolikar, 1994) are heteromeric proteins, usually composed of a catalytic C subunit and two regulatory subunits, A and B. It is thought that pNR and the phosphorylated form of sucrose-phosphate synthase are dephosphorylated by the same 2A phosphatase holoenzyme. Little is known about the structure and composition of the plant proteins, but early results suggested that the activity of the enzyme towards pNR was regulated by metabolites *in vitro*. Specifically, inorganic phosphate was an inhibitor, and sensitivity to Pi-inhibition was affected by light/dark treatment of leaves prior to extraction (Weiner *et al*., 1993).

14-3-3 inhibitor proteins

The only site on the native NR molecule that has been shown to interact with 14-3-3s is the sequence surrounding the regulatory phosphorylation site at Ser-543 (spinach numbering) in hinge-1 (Bachmann *et al*., 1996*a*; Su *et al*., 1996). The amino acid sequence surrounding Ser-543 corresponds closely with the original binding motif reported earlier (Muslin *et al*., 1996): RSXpSXP, where X is (in principal) any amino acid and pS is phosphoserine. It is clear that the 14-3-3s bind directly to the phosphoserine (and immediately adjacent residues). One line of evidence in support of this notion is that phosphorylation-dependent binding of 14-3-3s to synthetic peptides can be readily demonstrated *in vitro*. The 14-3-3s bind to certain phosphopeptides in solution (Bachmann *et al*., 1996*b*) and also to certain phosphopeptides immobilized on the surface of a gold chip (Athwal *et al*., 2000). Divalent cations promote binding to phosphopeptides (Athwal *et al*., 2000) which may provide at least part of the explanation for the earlier observation that millimolar concentrations of Mg^{2+} are required to maintain the inactive form of NR (now known to be a pNR–14-3-3 complex; see above). This is consistent with the observation that copurification of 14-3-3s with tobacco pNR can only be observed if Mg^{2+} is present (Provan *et al*., 2000). Similarly, binding of 14-3-3s to the phosphorylated form of the plasma membrane H^+-ATPase was dependent on Mg^{2+} (Fullone *et al*., 1998). Thus, binding of 14-3-3s to target proteins may generally show stimulation or dependence on divalent cations, and it is possible that this is of physiological significance *in vivo*.

Recently, immunological techniques have been developed with antibodies that specifically recognize pNR and dephospho-NR (H Weiner and WM Kaiser, unpublished results), and the amount of 14-3-3s bound to pNR was also determined by co-immunoprecipitation with anti-NR antibodies (Weiner and Kaiser, 1999, 2000).

Two 14-3-3 molecules were pulled down together with one NR dimer. Cells contain many targets for 14-3-3 proteins, and although the total amount of 14-3-3 proteins exceeds the amount of total NR protein by a factor of 4–5, practically all pNR is usually complexed by 14-3-3s due to the high binding affinity of 14-3-3s to pNR (Weiner and Kaiser, 1999). Interestingly, substantial co-immunoprecipitation of 14-3-3s with pNR (extracted from darkened spinach leaves) was observed in the complete absence of Mg^{2+} (Weiner and Kaiser, 2000). This apparent contradiction with the examples cited above where binding is dependent on Mg^{2+} might suggest that an additional binding site for 14-3-3s occurs on NR. However, even if a second site exists, it is clear that interaction at the 'alternate site' alone is not sufficient for inhibition of enzymatic activity.

Environmental (stress)-factors that trigger the modulation of NR

Response of NR in leaves to photosynthesis

It was mentioned above that NR in leaves is active in the light, and less active in the dark. A typical activation state in the light would be 70–90%, which is reduced in the dark to 10–30%. Surprisingly, although there still exists a considerable portion of active NR in extracts from darkened leaves, nitrate reduction of green leaves in the dark is almost absent (Kaiser *et al*., 2000). The reason is not yet known, but an additional limitation by as yet unknown factors may exist, for example, by the substrate NADH. However, it seems extremely difficult to measure cytosolic NADH levels in plant cells. Indirect estimations have suggested that it may be very low, at least far below the K_m of NR for NADH (4–8 μM, Kaiser *et al*., 1999; Campbell, 1999).

Light is not a direct signal to activate NR. Even in continuous strong light, NR becomes inactive when CO_2 is absent. Thus, photosynthesis is required for NR activation. Most probably, assimilates exported out of the chloroplast function as signals. Indeed, NR can be activated in the dark by feeding sugars to the leaves. Moreover, dark inactivation of NR in a starchless mutant of *N. sylvestris*, which accumulates high levels of sugar-Ps as a result of the block in starch synthesis, is severely attenuated relative to the wild type (Bachmann *et al*., 1995). Lastly, excision of leaves in the light causes build-up of photosynthates in the leaf and 'hyperactivation' of NR in the light (Huber *et al*., 1992*a*, *b*). All of these responses indicate that NR activation state is sensitive to metabolites and may be explained by the observation that *in vitro*, the NR protein kinase is inhibited by physiological concentrations of hexose monophosphates (Kaiser *et al*., 1999). This response to photosynthates reflects the need to co-ordinate carbon and nitrogen assimilation. It makes nitrate reduction sensitive to stomatal resistance. Thus, when plants close stomata under drought in order to preserve water, not only photosynthesis drops but NR becomes less active.

Response of leaf NR activation to nitrate supply

Variations in nitrate supply usually do not change the activation state of NR. Nitrate-deficient plants, having low contents of NR protein and low overall NR activity in their leaves, may still have about the same NR activation state (high in the light, low in the dark) as nitrate-sufficient plants. Only in rare cases where NR contents were extremely low due to persistent nitrate deficiency (Man *et al*., 1999) or mutation of the *Nia* genes (Scheible *et al*., 1997*b*), was the regulation apparently affected. In both cases, NR was much less dark-inactivated (Man *et al*., 1999). Again, the reason for that is not yet known. Normally, in tobacco plants with high nitrate supply, synthesis of NR mRNA in leaves may start in the late night phase to reach a maximum around midday, whereas NR protein (activity) increases in the first half of the day and remains constant or decreases slightly from midday until darkness. Interestingly, when nitrate supply to barley plants stopped and the internal nitrate concentrations became extremely low, NR protein and activity decreased very strongly during the day, indicating rapid NR degradation (Man *et al*., 1999). In that situation NR was still activated in the dark, whereas normally NR degradation (e.g. in the dark) is preceded by inactivation (see below).

Modulation of NR in roots: response to anoxia

Roots have no need to change their nitrate or nitrite reduction rate as rapidly as leaves. Assimilate supply from the shoot may vary only slowly within hours or days, as does the nitrate supply. However, there is one natural situation where root NR is also rapidly modulated: root oxygen supply may become critical for respiration when soils are flooded, due to diffusional limitations in wet soils. It has long been known that NR is more active in anoxic plant tissues than in air, but the reason for this is still unclear. It has been shown that under sudden anoxia, in darkened leaves, but also in roots, NR was activated within minutes (Glaab and Kaiser, 1993; Botrel *et al*., 1996; Botrel and Kaiser, 1997). It is known that the cytosol of anoxic cells is acidified from the normal pH 7.2 down to pH 6.5. Artificial acidification of darkened leaf discs or of root pieces also activated NR (Kaiser and Brendle-Behnisch, 1995), and therefore it was concluded that the activation of NR by anoxia was probably mediated by cytosolic acidification. However, other explanations may seem possible. The anoxia- or acidification-dependent NR activation was inhibited by PP2A inhibitors, indicating that the activation

was due to dephosphorylation (Glaab and Kaiser, 1993; Kaiser and Brendle-Behnisch, 1995).

During anoxia, the reduction of externally added nitrite is very low or absent (Botrel *et al*., 1996), because the oxidative pentose phosphate cycle is not operating due to lack of substrate. But together with the activation of NR, anoxia always leads to a strong accumulation of nitrite and to nitrite excretion by the roots. Surprisingly, in anoxic leaf tissues, the reduction of nitrate and the accumulation of nitrite are still much lower than should be expected from the high NR activity determined in the extracts. Again, that indicates that in roots, as in leaves, nitrate reduction rates are not only dictated by the NR activation state, but also by other factors such as substrate (NADH?) availability.

Whether the high nitrite production by anoxic tissues fulfils a physiological purpose, is not yet known. Production of the weak acid nitrite and excretion of undissociated nitric acid may help to stabilize cytosolic pH. However, preliminary measurements of cytosolic pH gave no clear evidence for that. Alternatively, nitrate reduction may decrease the rate of alcoholic or lactic acid fermentation. Lactic acid especially may become toxic, as it is not as easily excreted as alcohol or nitric acid.

As in leaves, sugar feeding also activates NR in normoxic roots (Botrel and Kaiser, 1997). It was concluded that assimilate translocation from shoot to root will also affect the nitrate reduction rate of roots, but this is probably a slow response.

Salt stress

It has been shown that in maize seedlings under mild salt stress, the activation state of NR is hardly changed, both in light and dark (Abd-El Baki *et al*., 2000). In leaves, NR activity decreased whereas in roots in increased with increasing salinity. Partly, this was due to changes in leaf nitrate contents. In leaves from salt-stressed seedlings, nitrate concentrations decreased to very low levels whereas chloride increased. The diurnal pattern of NR-mRNA levels in leaves also changed slightly. In control plants, NR-mRNA had a distinct maximum in the morning, and a second, less expressed maximum in the late night hours. This latter maximum disappeared with salinity, and accordingly the increase in NR protein and activity in the early light phase was slowed down. Thus, salt stress appeared to affect NR expression rather than post-translational NR modulation.

NR phosphorylation and 14-3-3-binding are also involved in the control of NR degradation

As mentioned above, NR has a rather short half-life of several hours. Thus, the existing amount of NR protein depends not only on the rate of synthesis, but also on the rate of degradation. NR synthesis is high in the light and low in the dark (Weiner and Kaiser, 1999). There are a number of artificial treatments which all activate NR, such as anoxia or tissue acidification, or the permeating analogue of 5′-AMP, AICAR (5-aminoimidazole-4-carboxyamide ribonucleoside), or uncouplers of respiration like CCCP or DNP. Indeed, NR protein in darkened leaves appeared more stable whenever it was activated by any of these treatments (for review see Kaiser *et al*., 1999, also compare Fig. 2), suggesting that the inactive form of NR (bound to 14-3-3s) would be more easily degraded than the active form. With ^{35}S-methionine feeding it was shown that NR degradation was much more rapid in the dark than in the light. Interestingly, purified NR added to extracts from darkened leaves, where endogenous NR had been removed by immunoprecipitation, was also degraded and that degradation *in vitro* was accelerated when NR was phosphorylated and 14-3-3s were added (Weiner and Kaiser, 1999). Conclusively the pNR–14-3-3 complex appeared a better substrate for specific proteolysis than free NR.

However, these conclusions may be questioned according to recent data (Cotelle *et al*., 2000). In sugar-starved *Arabidopsis* suspension cells, NR and many other proteins were rapidly degraded, pointing to the important role of sugars and of sugar sensing for cellular signalling. Overlay experiments showed that in sugar-starved cells, 14-3-3 binding to various targets was lost. Sugar feeding reversed both the loss of 14-3-3 binding and proteolytic degradation of proteins. These experiments are in sharp contrast to the above-mentioned observations and conclusions; Cotelle *et al*. suggest that sugar starvation and 14-3-3 release may be a signal initiating proteolysis (Cotelle *et al*., 2000): whereas it is suggested here that 14-3-3 binding initiates degradation. The question then was whether, in the above-described treatments that activate and stabilize NR, sugars would disappear (= sugar-starved) or remain high (= sugar-sufficient).

In darkened aerobic leaf tissues, where NR was degraded, there was indeed a rapid consumption mainly of sucrose, but also of hexoses, consistent with the above role of sugar signalling. Under anoxia, where NR was stabilized and active, sucrose levels decreased, yet at the same time hexose levels increased drastically (Fig. 2).

In AICAR-treated tissues, where NR was also stabilized, sucrose was consumed more slowly than in controls, and hexoses also increased (Fig. 2). A similar situation was observed for uncoupler-treated tissues (not shown). Thus, whenever NR was stabilized, cells were sugar-sufficient, eventually changing from high-sucrose cells to high-hexose cells, as shown in Fig. 2. Figure 3 summarizes such data: across treatments there was a positive correlation between sugar content and maximum NR activity (+EDTA) after 28 h of darkness. These observations are consistent with the findings by Cotelle *et al*.

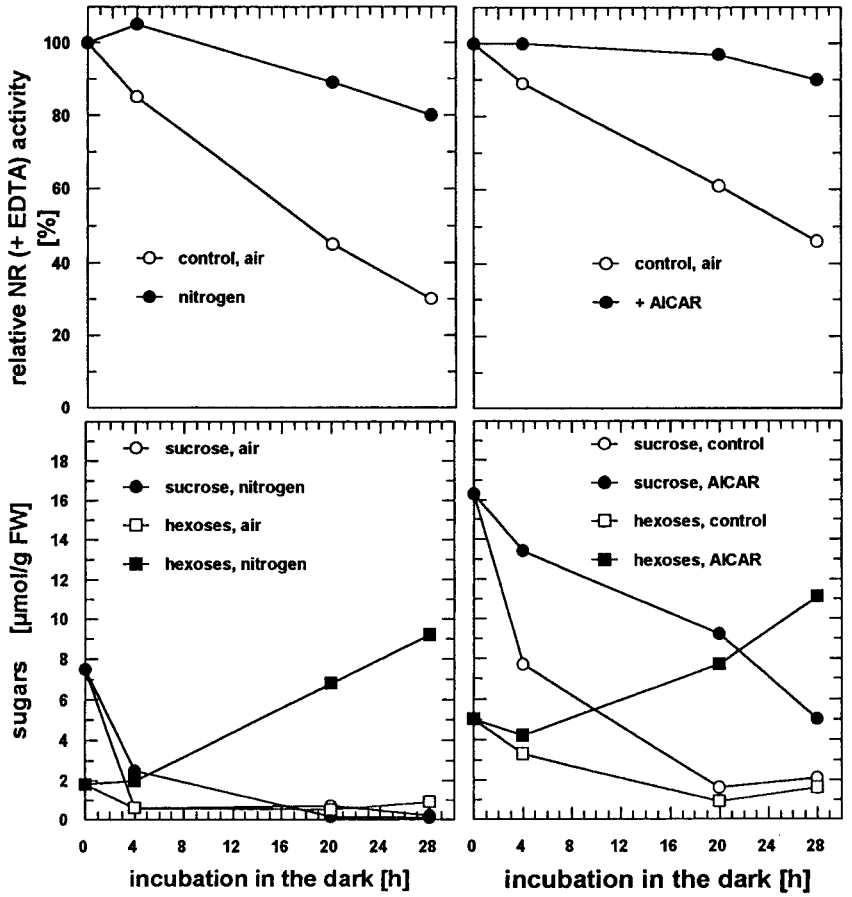

Fig. 2. Maximum NR activity (indicating the amount of functional NR protein) and sugar contents in detached spinach leaves (petiole in 10 mM nitrate-solution) in the dark. Sugars were determined in aqueous, boiled extracts by HPLC with pulsed amperometric detection. Under aerobic conditions (control, air), sucrose and hexose levels decrease strongly within 4 h, and NR activity disappears somewhat more slowly (50% in about 17 h), indicating NR protein degradation. Under anoxia, NR activity remains high, and while sucrose is still degraded, hexose levels increase. When leaves were fed in air with the 5′-AMP analog AICAR (5-aminoimidazole-4-carboxamide riboside, 20 mM) for 4 h, then transferred into fresh, AICAR-free solution), NR degradation was also prevented, and as before, sucrose was degraded whereas hexoses increased. It should be noted that the NR activation state was low in the dark in air, yet high with both anoxia or AICAR treatment (not shown). Further explanations in the text.

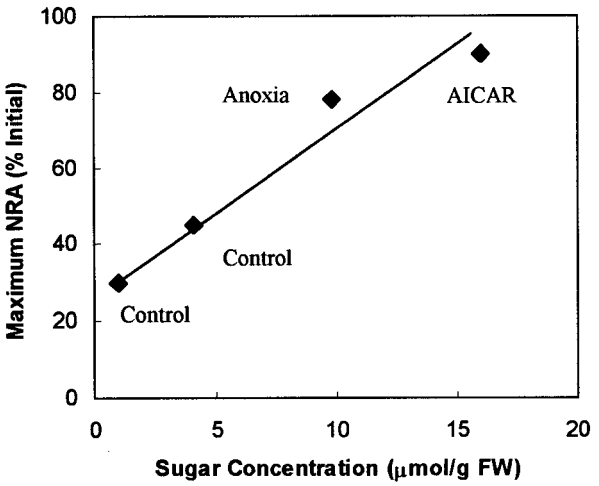

Fig. 3. Positive correlation between total sugar concentrations in leaves and the maximum NR activity. Data summarized from Fig. 2. Here, sugars are the sum of sucrose, glucose and fructose.

that sugar signalling may be involved in the control of NR proteolysis (Cotelle *et al.*, 2000). They do not resolve, however, the discrepancy about the role of 14-3-3s. A prediction from the overlay experiments of Cotelle *et al.* would be that in sugar-starved cells, NR should be more activated because 14-3-3 binding sites are lost, probably because of protein dephosphorylation (Cotelle *et al.*, 2000). However, NR activation was not observed (C MacKintosh, personal communication). It seems possible, however, that active NR (with 14-3-3s not bound) would be immediately degraded and would thus not show up as an increased activation state. Alternatively, one might speculate that different proteases exist which may degrade NR. A specific one would be active normally, for example, in the dark to degrade slowly inactive pNR bound to 14-3-3s. The other, less specific one might be induced or activated upon sugar starvation, using free NR as a substrate, not the pNR–14-3-3 complex. The multiple roles of sugars in the regulation

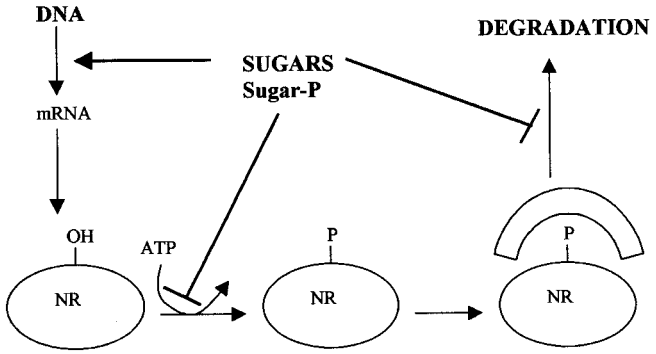

Fig. 4. Schematic diagram summarizing the multiple effects of sugars (and eventually sugar phosphates) on NR expression, NR activity and NR degradation. High sugar concentrations promote NR expression (together with nitrate), block (eventually as sugar phosphates) the protein kinase(s), thereby activating NR and prohibiting NR degradation. All three events lead to a synchronization of nitrate reduction with photosynthesis and carbohydrate availability, thereby avoiding a waste of energy and accumulation of toxic reduction products (nitrite and ammonium). One open and critical question is whether it is the 'form' of NR present that affects degradation, or whether the factors that affect the NR activation state also affect (independently) the induction or activation of a protease. Further explanations in the text.

of NR expression, NR modulation and NR degradation are summarized in Fig. 4.

Nitric oxide (NO) production by NR

It has long been recognized that NR can also catalyse the NAD(P)H-dependent reduction of nitrite to nitric oxide (NO) (Dean and Harper, 1988; Yamasaki *et al.*, 1999; Yamasaki and Sakihama, 2000). Facing the potential relevance of NO as a signalling molecule in plants, it is certainly important to know when and to what extent NR might contribute to NO production. NO production by purified NR has recently been investigated, and by detached leaves or whole plants (P Rockel, F Strube, A Rockel, J Wildt, WM Kaiser, unpublished results). Briefly, NO production by NR from NADH and nitrite comprised only a small percentage of the total nitrate reducing capacity (about 1%) of the enzyme. NO production followed the rapid modulation of NR described above, and was, in addition, strictly dependent on the nitrite concentration in the tissues (which, in turn, also depends on NR versus nitrite reductase (NiR) activity). NO production from nitrite (K_m 100 μM) was competitively inhibited by nitrate (K_i about 50 μM), and therefore, NO production by intact tissues was also dependent on the nitrate concentration. In addition to soluble NR, plasma membrane-bound enzymes may also contribute to NO production (Stöhr *et al.*, 2001). Nothing is known as yet about a possible regulation of NR-dependent NO production by environmental stress factors and, most importantly, by plant pathogens.

Is the above model for the regulation of NR valid for all higher plants?

Up to 15 higher plant species belonging to very different taxonomic groups have been examined and were found to follow the above-described general pattern of NR regulation. As far as is known, there is only one exception: NR in leaves from *Ricinus communis* L. differs from all others in several respects (Kandlbinder *et al.*, 2000). When the enzyme was essayed in crude extracts in the presence of 10 mM Mg^{2+}, it always appeared in an extremely low activation state, which was not much different in dark or light. In contrast, the maximum activity measured in the presence of EDTA was normal. Further, the enzyme from *Ricinus* could not be inactivated *in vitro* with MgATP. *Ricinus* NR ($+Mg^{2+}$) was highly active at pH 6.5, and almost completely inhibited at pH 8. At physiological pH (7.3), it was always very Mg^{2+}-sensitive, much more than other higher plant NRs. Meanwhile the enzyme has been cloned, and sequence analysis indicated that a serine-phosphorylation motive and 14-3-3 binding site were present as in many other deduced sequences for NR from terrestrial plants (CB Tsai, R Kaldenhoff, WM Kaiser, unpublished results). Thus, the molecular background and the physiological relevance of these deviating properties of *Ricinus* NR are still obscure. One possibility being considered is that the putative heavy-metal binding site on NR, responsible for inhibition of NR activity by metals such as Zn^{2+} (Campbell, 1999), is altered in sequence such that divalent cations such as Mg^{2+} can also bind and directly cause inhibition.

Open questions and perspectives

On the whole plant basis, it is still not understood why, at least in some situations, rates of nitrate reduction *in vivo* are different from NR activity as determined in leaf or root extracts. The present interpretation is that NADH may become a major rate-limiting factor. In leaves, this would couple nitrate reduction even more strictly to the export of reducing power from the chloroplast. Mutants or transformants disturbed in that transport may add to a better understanding of the substrate limitations of NR. It was expected that, at least under anoxia when plant tissues start alcoholic and lactic acid fermentation, NADH would not be limiting for nitrate reduction. However, this was not the case. Clearly, the physiological role of NR activation under anoxia is still obscure and requires more stringent investigations.

Also, it is not understood why, under conditions of extreme nitrate limitation, the residual NR appears no longer light/dark modulated. One speculative interpretation would be that the activity or expression of a NR protein kinase is controlled by nitrate availability.

It also remains to be determined to what extent NR contributes NO for pathogen/stress signalling under different conditions. It is thought that the inducible NO synthase (Foissner *et al*., 2000) is important for plant responses to pathogens, but NR could play a role as well. Comparisons of plants grown on nitrate (abundant NR) versus ammonium (no NR) in terms of pathogen responses may yield some insights as to the role that NR-catalysed NO production may play.

Another unresolved and critical question is whether it is the molecular form of NR present in leaves that affects its proteolytic degradation, or whether the factors that affect the NR activation state also independently affect the induction or activation of the protease(s) that degrade NR. Further, the 14-3-3s may either target proteins for degradation (Weiner and Kaiser, 1999) or may protect proteins from degradation (Cotelle *et al*., 2000) possibly by shielding critical residues. Further studies with NR may provide answers to these questions, which have direct implications for the regulation of other enzymes as well.

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