Minireview

Light regulation of nitrate reductase in green leaves of higher plants

Cathrine Lillo

Light stimulates de novo synthesis, as well as activation of higher plant nitrate reductase at the protein level. In green leaves, the light effects are mediated through photosynthetically active light by products of the Calvin cycle. A daily, light-induced increase in nitrate reductase (NR) mRNA, protein, and activity is observed in various higher plants. Glucose or sucrose can replace light in eliciting the increase in nitrate reductase mRNA accumulation, and some metabolite from nitrate assimilation exerts a negative feedback on transcription of the nitrate reductase genes. The positive feed-forward induced by sugars, and the negative feedback from nitrogen compound(s) apparently result in the circadian rhythms of NR mRNA observed in various plants. Nitrate reductase shows hysteretic behaviour, converting between a low and a high activity form in response to NAD(P)H. The low activity form is inhibited by calcium/magnesium, as opposed to the high activity form that is not inhibited. Examination of nitrate reductase activity in crude extracts made from plants before and after a light-dark transition suggests the existence of two different forms of nitrate reductase; a 'light form' with a pH optimum of 7.8 in potassium phosphate buffer that is not inhibited by calcium or magnesium, and a 'dark form' with a pH optimum of 7.5 that is strongly inhibited by calcium or magnesium. It is postulated that a transient lack of NADH as a result of decreased illumination can convert nitrate reductase into the less active form in situ.

Key words – Calcium, hysteresis, light, magnesium, nitrate reductase.

C. Lillo, Rogaland Univ. Center, Box 2557 Ullandhaug, N-4004 Stavanger, Norway.

Introduction

Nitrate reductase catalyzes the reduction of nitrate to nitrite using NAD(P)H as an electron donor. The enzyme has FAD, cytochrome b_{557} and a molybdenum cofactor as prosthetic groups (Warner and Kleinhofs 1992). Both inducible and constitutive isoforms have been found in higher plants. Inducible NR is regulated by several environmental factors, availability of nitrate and light being the most important (Solomonsen and Barber 1990, Redinbaugh and Campbell 1991). Higher plant NR is located in the cytosol, but has several regulatory characteristics in common with plastid proteins (Mohr et al. 1992, Lillo 1993b). This review is concerned with the inducible higher plant NR, and focuses on light regulation of synthesis as well as activation/deactivation of the enzyme.

Abbreviation – NR, nitrate reductase.

Signal perception

In etiolated leaves light perceived by the phytochrome system induces an increase in NR activity in a wide range of higher plants, and this increase in NR activity depends on transcription of NR mRNA and synthesis of NR protein (Melzer et al. 1989, Mohr et al. 1992). However, light absorbance by phytochrome was not related to the diurnal variation of NR activity observed in green leaves of wheat and barley, because in contrast to etiolated leaves, pulses of red light did not lead to any increase in NR activity (Lillo and Henriksen 1984). Also, at the nucleic acid level, no light response mediated through the phytochrome system could be found in green leaves, but in etiolated leaves a positive response was clearly shown for NR mRNA (Melzer et al. 1989). These results illustrate a more general feature, namely as stated by

Light
↓
Chlorophyll
↓
Photosynthesis
Calvin Cycle
↓
Glucose
↓
⊕↓
NR genes → NR mRNA → NR enzyme ⇄ NR enzyme
⊖↑   inactive ←   active
↑
Glutamine

Light
↓
Chlorophyll
↓
Photosynthesis
Calvin Cycle
↓
C_3/C_4 shuttle
↓
NADH
↓⊕
Hysteretic
behaviour ↑⊖
Transient lack of NAD(P)H
and presence of Mg^{2+}/Ca^{2+}
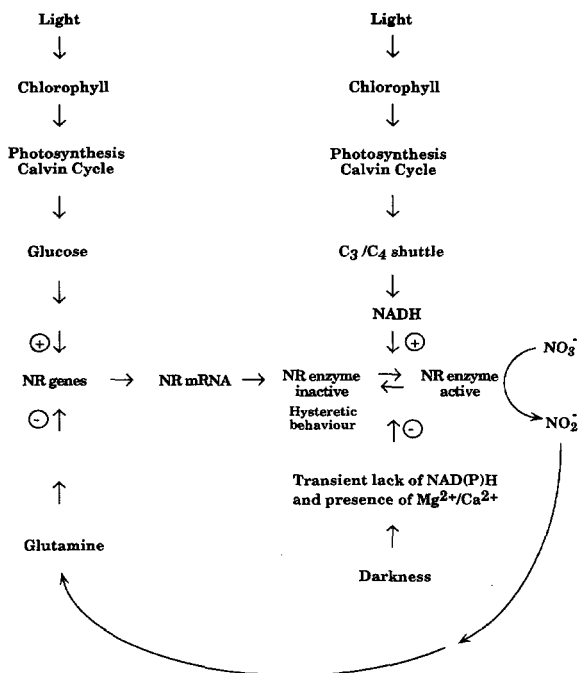↑
Darkness

NO_3^-
NO_2^-

Fig. 1. Scheme for light effects on NR in green leaves. Light absorbed by chlorophyll increases transcription of the NR genes, the effect being mediated by products of the Calvin cycle, such as glucose. Some product(s) of nitrate metabolism, probably glutamine, exerts a negative feed-back on transcription. The NR enzyme is interconverted between a less active and more active form in response to NAD(P)H provided from the chloroplasts by the shuttle mechanism. In the presence of magnesium a presumably transient decrease in NAD(P)H in response to darkness can rapidly convert NR into the less active form.

Thompson and White (1991), that phytochrome apparently plays a minor role in light responses of green tissue.

Importance of blue light receptors such as flavins for regulation of NR activity has been suggested (de la Rosa et al. 1989). In vitro, blue light reactivates NR inactivated by, for example, cyanide. To date, an inactive NR-cyanide complex has not been isolated from a higher-plant source. However, since the physiological system is very complex, and hydroxylamine, superoxide or possibly other metabolites could also play an important role in formation of an reversible inactive form of NR in vivo, regulation by blue light cannot be ruled out (Solomonsen and Barber 1990).

In Perilla leaves, Kannangara and Woolhouse (1967) showed that induction of NR was achieved only in the presence of CO_2. Photosynthetic fixation of CO_2 is often necessary for maximal NR activity (Klepper et al. 1971, Kaiser and Brendel-Behnisch 1991). Therefore, in green leaves the crucial light effect on NR is caused by photosynthetically active light absorbed by chlorophyll. Both slow (hours) and rapid (minutes) changes in NR activity are mediated by photosynthetic light, and the light effects are observed at two different levels (Lillo 1991): (1)

mRNA-level/de novo synthesis of the enzyme, and (2) protein-level/activity modulation of the enzyme.

Light regulation of the NR mRNA-level/de novo synthesis of the enzyme

Generally, in various higher plants NR activity, protein, and mRNA levels are high in the early part of the photoperiod and then decrease during the afternoon even when the plants are still exposed to high light intensities (Lillo 1984b, Galangau et al. 1988, Deng et al. 1989, Lillo and Ruoff 1989). In some experiments, an increase in NR mRNA or activity is also observed during the night (Lillo 1984a, Galangau et al. 1988, Deng et al. 1989). Starch degradation and mobilization from the chloroplasts starting 6–8 h into the dark period (Gordon et al. 1980) could cause such increases in NR activity and mRNA level. However, the influence of temperature shifts or uncontrolled greenhouse conditions sometimes complicate the interpretation of light effects. In experiments with corn plants subjected to a constant day/night temperature, samples were harvested every hour or every half-hour, and allowed observation of a sudden light-dependent increase in NR mRNA. The NR mRNA level reached a maximum approximately 1 h after the light was turned on but then fell off, although the plants were still kept in the light (Lillo 1991). Nuclear run-off experiments indicated that the mRNA increase was caused by increased transcriptional activity (Lillo 1991). The light induction of NR mRNA is apparently mediated through products of CO_2-fixation (Fig. 1). Evidence for this is from experiments showing that sucrose or glucose could replace light in the induction of NR mRNA in Arabidopsis leaves. The sucrose/glucose response was confined to a 2.7-kilobase region of the 5' flanking sequence of the NR gene as demonstrated by a reporter gene (Cheng et al. 1992).

NR mRNA level, NR protein and activity show light-induced circadian rhythms (Lillo 1984b, Lillo and Ruoff 1989). Light up-regulates the NR mRNA level; however, there is also a negative feedback that down-regulates the NR mRNA level. This suggests that when some metabolite(s) of nitrate assimilation has reached a certain concentration in the cell, further synthesis of NR mRNA is slowed down. This assumption is supported by experiments with mutants of Nicotiana plumbaginifolia that were defective in molybdenum cofactor and therefore had no NR activity (Pouteau et al. 1989). Nitrate assimilation was not possible in these mutants and, indeed, the oscillations in NR mRNA were abolished. Evidence for a negative feedback by some metabolites of nitrate has been obtained from experiments with tobacco leaves fed with tungsten, thereby giving a non-functional NR enzyme. In these leaves, NR mRNA was continuously expressed, i.e. the decrease in NR mRNA otherwise observed did not occur (Deng et al. 1989). Glutamine has been suggested as a compound that may exert such a negative control on NR expression (Callaci and Smarrelli 1991, Deng et al. 1991, Shiraishi et al. 1992). The light-dependent positive

feedback on NR mRNA and the negative feedback caused by glutamine assure that the NR genes are not overexpressed, and fulfil the necessary requirements of a self-sustained rhythm.

Light modulation of NR activity at the protein level

pH

It has long been assumed that the light-dependent pH increase in the chloroplast's stroma can contribute to increased activity of several enzymes (Buchanan 1980). It is also likely that pH in the cytosol increases by 0.2 to 0.5 units in response to a dark-light transition (Kurkdjian and Guern 1989). Although pH was considered to be without physiological effect on NR activity in some investigations (e.g. Kaiser and Brendle-Behnisch 1991), recent studies have shown that the pH optimum for NR is higher (7.8) in potassium phosphate buffer than in HEPES buffer (7.5) for both squash and barley (Lillo 1994). Since free and bound phosphate is abundant in the cell, the potassium phosphate buffer may give results that reflect the physiological pH-optimum better than the HEPES buffer. An increase in pH of for example 0.5 units will result in increased NR activity by approximately 100% (Lillo 1994).

Influence of adenine and pyridine nucleotides

NADH usually has been considered as an inhibitor of NR activity. However, Aryan and Wallace (1985) showed that the inhibition of NR activity by NADH was mediated by evolution of superoxide radicals by certain enzymes in the extracts. Inhibition by NADH was otherwise found in combination with cyanide or hydroxylamine, which blocked the Mo-containing subunit (Solomonsen and Barber 1990). Recently, NAD(P)H has been shown to activate NR, and interconversion between a low activity form and a form with about twice the activity depended on the presence of NAD(P)H (Lillo and Ruoff 1992).

The adenine nucleotides influence NR activity in several, sometimes very complex ways. ADP is an inhibitor of NR, and is usually found to inhibit NR activity by about 50% (Nelson and Ilan 1969, Eaglesham and Hewitt 1975, Sanchez and Heldt 1990). ATP inactivates NR in crude extracts of spinach (Kaiser and Spill 1991, Huber et al. 1992a), squash, barley and corn (C. Lillo, unpublished results) as well as partially purified preparations from spinach (MacKintosh 1992). The inactivation of NR by ATP is suggested to be due to phosphorylation of the enzyme, and is assumed to occur in vivo in response to darkness. The inactivation of the enzyme was only recognizable in the presence of Mg^{2+} or Ca^{2+}. Huber et al. (1992a) obtained evidence for in vivo phosphorylation of NR in spinach leaves, but the physiological significance was ambiguous since total phosphorylation of NR was about the same in light and darkness. However, when examining specific peptides, two sites were heavier phosphorylated in the dark than in the light (Huber et al. 1992b).

Calcium and magnesium inhibition

NR activity in crude extracts of spinach or squash leaves made from plants after transfer to darkness for some hours, or even for only a few minutes, is inhibited by 5 mM Mg^{2+} or Ca^{2+}. This is opposed to extracts made a couple of hours after start of the daily photoperiod which showed no, or only slight inhibition of NR activity by calcium or magnesium (Huber et al. 1992b, Kaiser et al. 1992, Riens and Heldt 1992, Lillo 1994). NR in extracts from leaves harvested during the photoperiod had a pH optimum of 7.8, and NR in extracts from plants transferred to darkness had a pH optimum of 7.6 (Lillo 1994). Such results suggest the existence of two different forms of NR in light and darkness. Calcium and magnesium also inhibited purified NR, but only the form that showed delayed product formation (Lillo 1993b). Comparison of the characteristics of purified NR and NR in extracts from dark or light exposed plants, showed that the dark form corresponds to the form of NR showing delayed product formation (hysteretic behaviour), and the light form corresponds to the form of NR showing linear product formation (Lillo 1994).

Calcium has long been recognized as an important second messenger in animal cells, and more recently in plants (Roberts and Harmon 1992). However, NR is not as sensitive to transient changes in calcium as calmodulin or other Ca-regulated proteins possessing a special domain (Roberts and Hammond 1992). NR responds to Ca^{2+} in the 100 μM range (Lillo 1993b). Furthermore calcium can be replaced by magnesium, and is therefore in this respect also different from calmodulin. Since Mg^{2+} is present in the cytoplasm of plant cells in a much higher concentration than Ca^{2+}, Mg^{2+} is probably the more important inhibitor.

Model for light/dark influence on NR activity

After reduction of nitrate to nitrite in the cytosol, nitrite is converted to ammonium by nitrite reductase in the chloroplasts. Reduction of nitrite is light-dependent, since ferredoxin reduced by photosynthetic electron transport is the electron donor. Unless NR activity is rapidly inactivated upon a drop in irradiation, toxic NO_2^- would build-up in the cell. However, a build-up of NO_2^- is not observed and it was shown that NR activity decreased rapidly to about 15% of the control with a half-life of only 2 min after darkening of spinach leaves (Sanchez and Heldt 1992). Several different factors, such as decreased pH, increased ADP and phosphate concentrations (Sanchez and Heldt 1990) and possibly cyanide or superoxide (de la Rosa et al. 1989, Solomonsen and Barber 1990) may contribute to reduced NR activity in the dark. It has been argued that such factors cannot provide sufficient inactivation of NR (Sanchez and Heldt 1990).

When NR is depleted of NAD(P)H in vitro, it is converted to a less active form showing hysteretic behaviour during approximately 30 min (Lillo and Ruoff 1992). This would be too slow to explain the half-life of 2 min reported for NR deactivation. However, the enzyme is

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immediately strongly inhibited by calcium or magnesium under conditions when the enzyme *starts* converting into the form showing hysteretic behaviour. Transiently (minutes), a decrease in ATP and an increase in ADP are observed in *Avena* protoplasts when light is turned off (Hamp et al. 1982, Raymond et al. 1987). Possibly, an analogous decrease in NADH may occur when light is turned off since the supply of ATP and NADH depends on the same shuttle mechanism bringing Calvin cycle intermediates such as triose phosphates from the chloroplast to the cytosol. A transient lack of NADH would apparently rapidly inactivate NR in the presence of physiological concentrations of magnesium. NR activity would then be reactivated when another NADH-supply is established (Fig. 1). The hysteretic behaviour of the enzyme would cause a delay in the reactivation and assure that nitrite does not build up. This hysteretic delay in reactivation would probably allow sufficient time for degradation of NR to establish a stable decreased level of NR activity adjusted to the reduced nitrite reduction in the dark.

Outlook

For a more complete understanding of regulation of de novo synthesis of NR, the promotor-regulating proteins need to be identified. The signal transduction pathways to the transcription level from the sugar(s) that up-regulates, and from the nitrate metabolite(s) that downregulates NR mRNA must be elucidated. The physiological significance of phosphorylation/dephosphorylation for adjustment of NR activity should be further investigated. The enzymes responsible for phosphorylation/dephosporylation, and the mechanism of signal perception by the phosphorylation/dephosphorylation system need to be characterized. Further information on the concentration of different metabolites, like NADH, in response to light-dark transitions is necessary for evaluation of the physiological significance of calcium/magnesium inhibition related to the hysteretic properties of nitrate reductase.

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Edited by P. Böger and J. J. S. van Rensen