Journal of
Experimental
Botany

Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*

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Abstract

NO (nitric oxide) production from sunflower plants (*Helianthus annuus* L.), detached spinach leaves (*Spinacia oleracea* L.), desalted spinach leaf extracts or commercial maize (*Zea mays* L.) leaf nitrate reductase (NR, EC 1.6.6.1) was continuously followed as NO emission into the gas phase by chemiluminescence detection, and its response to posttranslational NR modulation was examined *in vitro* and *in vivo*. NR (purified or in crude extracts) *in vitro* produced NO at saturating NADH and nitrite concentrations at about 1% of its nitrate reduction capacity. The K_m for nitrite was relatively high (100 μM) compared to nitrite concentrations in illuminated leaves (10 μM). NO production was competitively inhibited by physiological nitrate concentrations ($K_i = 50$ μM). Importantly, inactivation of NR in crude extracts by protein phosphorylation with MgATP in the presence of a protein phosphatase inhibitor also inhibited NO production. Nitrate-fertilized plants or leaves emitted NO into purified air. The NO emission was lower in the dark than in the light, but was generally only a small fraction of the total NR activity in the tissue (about 0.01–0.1%). In order to check for a modulation of NO production *in vivo*, NR was artificially activated by treatments such as anoxia, feeding uncouplers or AICAR (a cell permeant 5′-AMP analogue). Under all these conditions, leaves were accumulating nitrite to concentrations exceeding those in normal illuminated leaves up to 100-fold, and NO production was drastically increased especially in the dark. NO production by leaf extracts or intact leaves was unaffected by nitric oxide synthase inhibitors. It is concluded that in non-elicited leaves NO is produced in variable quantities by NR depending on the total NR activity, the NR activation state and the cytosolic nitrite and nitrate concentration.

Introduction

Nitric oxide (NO) is an inorganic free radical that acts as a signalling molecule with multiple biological functions in vertebrates. There it is produced mainly by nitric oxide synthase (NOS), which catalyses NO and L-citrulline formation from O_2 and L-arginine. The NOS activity was also detected in higher plant extracts (Cueto *et al*., 1996; Ninnemann and Maier, 1996; Ribeiro *et al*., 1999) and peroxisomes (Barroso *et al*., 1999). NO production by plant tissues was first observed by Klepper (Klepper, 1975*a*) with soybean plants treated with photosynthetic inhibitor herbicides (Klepper, 1978, 1979) or other chemicals (Klepper, 1990, 1991) as well as under dark anaerobic conditions (Klepper, 1987, 1990). It was suggested that this emission was due to chemical reactions of accumulated nitrite with plant metabolites such as salicylate derivatives or the chemical decomposition of HNO_2.

NO was also observed to be the predominant compound evolved during a purged *in vivo* assay with

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soybean nitrate reductase derived from accumulated nitrite (Harper, 1981). Results obtained with boiled leaflets (Harper, 1981) and the mutant soybean line nr_1 (Dean and Harper, 1986, 1988; Nelson *et al.*, 1983; Ryan *et al.*, 1983) further indicated that the enzymatic reaction of a constitutive NAD(P)H:nitrate reductase is responsible for the evolution of NO_x. Experiments with ^{15}N-labelled nitrate as the substrate for nitrate reduction showed that NO_x is produced from ^{15}N–NO_3^- (Dean and Harper, 1986). More recently, it was shown that NR from maize could also produce NO from nitrite plus NADH, and that this reaction could be prevented by azide (Yamasaki *et al.*, 1999; Yamasaki and Sakihama, 2000). These authors also found NO production with nitrate as the substrate, but in that case with a time-lag. They concluded that the actual substrate for NR-dependent NO production was nitrite, not nitrate.

It is known that different nitrate-nourished plant species have a compensation point for NO uptake from the atmosphere showing a net NO emission at low atmospheric NO concentrations (Rockel *et al.*, 1996; Wildt *et al.*, 1997).

Recent studies suggested that nitric oxide may play a role as a messenger in plant pathogen resistance (Pfeiffer *et al.*, 1994; Delledonne *et al.*, 1998; Durner and Klessig, 1999; Bolwell, 1999) by increasing cGMP and salicylic acid levels. It can further influence plant growth (Leshem and Haramaty, 1996; Beligni and Lamattina, 2000; Leshem, 2000), protect against cytotoxicity of reactive oxygen species (Beligni and Lamattina, 1999) and cause the accumulation of phytoalexin (Noritake *et al.*, 1996).

In spite of increasing evidence for important physiological roles of NO in plants, it is still unclear whether and under which conditions NO is produced by nitrate reductase (or by NOS), and how this is regulated. Here, evidence is provided that post-translational modulation of NR *in vitro* and *in vivo* also modulates the NO production rates. It is also shown (i) that NR-dependent NO production under some conditions may drastically exceed the published activities of NOS in leaves, (ii) that it has a relatively high K_m for nitrite of 100 μM, and (iii) that it is competitively inhibited by low nitrate concentrations ($K_i = 50$ μM) or by chlorate, both *in vitro* and *in vivo*.

Materials and methods

Plant material and growth

Sunflower (*Helianthus annuus* L. var. giganteus) and spinach (*Spinacia oleracea* L. var. Monnopa) were germinated in moist quartz sand at a 14 h photoperiod (280 μmol m^{-2} s^{-1}) with a temperature regime of 28/23 °C (day/night) and 90/80% relative humidity. After 1–2 weeks the plants were transferred to a hydroponic system (Rockel, 1997) and grown at 20 °C and 60% relative humidity during a 12 h photoperiod (350 μmol m^{-2} s^{-1}) and 17 °C and 80% relative humidity during darkness, respectively. The aerated nutrient solution was automatically adjusted

to pH 6.0 by the addition of 0.1 N NaOH (NH_4^+ nutrient solution) or 0.1 N H_2SO_4 (NO_3^- nutrient solution). Nutrient concentrations were kept constant ($\pm 5\%$) by the automated addition of a 10-fold stock solution (Rockel, 1997). The nutrient solution for nitrate-grown plants contained 0.2 mM KNO_3, 0.25 mM $Ca(NO_3)_2$, 0.05 mM $NaNO_3$, 0.05 mM KH_2PO_4, and 0.1 mM $MgSO_4$; the NH_4^+ nutrient solution contained 0.05 mM $(NH_4)_2HPO_4$, 0.35 mM $(NH_4)_2SO_4$, 0.125 mM K_2SO_4, 0.1 mM $MgSO_4$, and 0.25 mM $CaCl_2$; the N-free nutrient solution contained 0.05 mM KH_2PO_4, 0.125 mM K_2SO_4, 0.1 mM $MgSO_4$, and 0.25 mM $CaCl_2$. Micronutrient concentrations in all solutions were 10 μM KCl, 6.3 μM H_3BO_3, 4.0 μM FeH_2-EDTA, 0.4 μM $ZnSO_4.7H_2O$, 0.4 μM $MnSO_4.H_2O$, 0.1 μM $Na_2MoO_4.2H_2O$, and 0.1 μM $CuSO_4.5H_2O$.

Experimental set-up for gas phase NO measurements

Whole plants were transferred to the experimental set-up for NO measurements when they had reached a total leaf area of approximately 500–1000 cm^2.

The upper part of the plant was then placed into a continuously stirred tank reactor, a glass vessel of 164 l volume through which purified air was passed with a constant flow of 40 l min^{-1}. A teflon plate sealed the glass vessel from the environment and held the plant in position. Background concentrations of ozone, NO and NO_2 of the inflowing air were less than 100 ppt. Concentrations of NO and NO_2 in the air were measured by chemiluminescence detection (CLD AL ppt 770, Eco-physics, Dürnten, Switzerland, detection limit 20 ppt; 1 min time resolution). Water vapour and CO_2 were detected by an infrared analyser (Rosemount, Hanau, Germany, Binos 100). The ozone concentration was measured by UV absorption (Dasibi, Glendale, CA, USA, 1008 RS). All gas concentrations of the inflowing and outflowing air were measured alternately at 1 h intervals as described previously (Neubert *et al.*, 1993). NO emission fluxes were calculated from the concentration differences between the inlet and outlet of the chamber (Wildt *et al.*, 1997). All experiments were carried out with nitrate-grown plants unless otherwise stated.

Single leaf experiments

For single leaf experiments, the leaves were cut off from the plant and immediately placed in nutrient solution, where the petiole was cut off a second time below the solution surface. The leaves were then placed in a small glass chamber (vol. 5.0 l) with an airflow of 3–5 l min^{-1}. The CO_2 concentration and water vapour were continuously measured between the inlet and outlet. The NO concentration of the inflowing air was measured before and after each experiment, whereas the outflowing air was measured continuously during the experiments.

Preparation of leaf extracts

Leaves without midrib were weighed and immediately ground to a fine powder in liquid nitrogen. For desalted extracts, an extraction buffer containing 50 mM HEPES-KOH (pH 7.6), 1 mM DTT, 10 μM FAD, and 15 mM $MgCl_2$, was added to the frozen powder (2 ml g^{-1} FW). Where indicated in the legends, protein phosphatase inhibitors were added in order to maintain the actual state of NR phosphorylation. After centrifugation (16 000 *g*, 10 min, 4 °C), the supernatant was desalted on Sephadex G25 (Sigma, Steinheim, Germany) spin columns (9 ml gel volume, 3.5 ml extract, 4 °C) equilibrated with the extraction buffer. Two ml each of this extract were preincubated

at room temperature as described and used for the NO measurement.

NO measurement over solutions

For the measurement of NO production from crude extracts or purified NR, the solutions were placed in a Petri dish (diameter 5 cm) located in a sealed glass chamber (1.0 l volume). All experiments were performed in darkness at 25 °C under aerobic conditions. The sample was continuously stirred, and purified air was passed through the glass chamber with a constant flow of 3.0 l min^{-1}. NO was measured at the inlet and outlet of the chamber by chemiluminescence detection as described above. Effectors were added as indicated without disturbing the assay conditions. All compounds were also tested without NR or extract to exclude possible chemical NO formation.

Nitrate reductase activity and nitrite content

For separate determinations of NR activity, leaves without midrib were weighed and immediately ground in liquid nitrogen. Two ml of extraction buffer containing 50 mM HEPES-KOH (pH 7.6), 1 mM DTT, 10 μM FAD, and 15 mM MgCl$_2$, were added to 1 g of frozen powder. After centrifugation (16 000 *g*, 10 min, 4 °C), aliquots of the extract were directly used for the colorimetric determination of the original nitrite content of the leaves. The remaining supernatant was desalted on Sephadex G25 spin columns (1.5 ml gel volume, 0.650 ml extract, 4 °C) equilibrated with the extraction buffer. Aliquots (100 μl) were added to a reaction medium (900 μl, 50 mM HEPES-KOH (pH 7.6), 10 μM FAD, 1 mM DTT, 15 mM MgCl$_2$, 5 mM KNO$_3$, 0.2 mM NADH) and incubated for 3 min at 25 °C. After 3 min, the reaction was stopped by adding zinc acetate (125 μl, 0.5 M). Following centrifugation (16 000 *g*, 10 min), the supernatant was treated with 10 μM phenazine methosulphate (20 min, dark) to oxidize unreacted NADH, and used for colorimetric determination of nitrite formed as described earlier (Hageman and Reed, 1980).

Results

In vitro *NO production by NR can be measured as NO emission from solution into the gas phase*

In order to test whether the emission of NO into the gas phase from enzyme solutions could be used as a continuous measurement for the NO production, previous work carried out with a NO-analyser (Yamasaki and Sakihama 2000), using purified, commercially available maize leaf NAD(P)H-NR (Sigma, Deisenhofen, Germany. Lot Nr N 2397) was first repeated. The measurement of nitrite reductase activity in these preparations were negative, and addition of L-NNA to the assay indicated no contamination with NOS (data not shown). 10 mU maize leaf NR (according to the manufacturers specification) was added to 5 ml of a reaction medium containing nitrate (0.1 mM) plus NADH (0.1 mM), and the NO production was monitored continuously. Every 10 min, 100 μl aliquot was removed from the mixture for nitrite determination.

The results confirm recent data (Yamasaki and Sakihama, 2000) which were obtained by direct measurements of NO in solution (either with an NO analyser or by fluorescence indicators), and are only briefly summarized below. With nitrate as a substrate, NO emission began after a lag phase of 30 min when a nitrite concentration of between 50 and 60 μM had been reached. As already concluded (Yamasaki and Sakihama, 2000), NO was obviously not directly formed from nitrate, but from nitrite. In these experiments, where the maximum initial nitrite production rate (or NR activity) was 10 nmol min^{-1}, the maximum NO production rate (reached towards the end of the experiment) was only (0.09 nmol min^{-1}). Thus, the NO production by commercial purified maize NR was slightly less than 1% of the NR activity of the preparation. Similarly, the kinetics of NO emission were examined after the addition of nitrite, in the complete absence of nitrate (data not shown). The addition of 100 μM nitrite to 5 ml buffer solution containing NR and NADH as before resulted in an immediate onset of NO emission from the solution with similar rates as before, again confirming previous measurements (Yamasaki and Sakihama, 2000).

The substrate (nitrite) affinity of that commercial maize leaf NR for NO production was also examined (Fig. 1). The K_m for nitrite was approximately 100 μM. Consequently, it was found that NO production was competitively inhibited by low nitrate concentrations with a $K_i = 50$ μM. Chlorate as a well-known competitive (to nitrate) inhibitor of NR also caused a strong inhibition of NO production from nitrite (data not shown). In contrast, other anions, for example, 100 μM sulphate, had no effect on NO production.
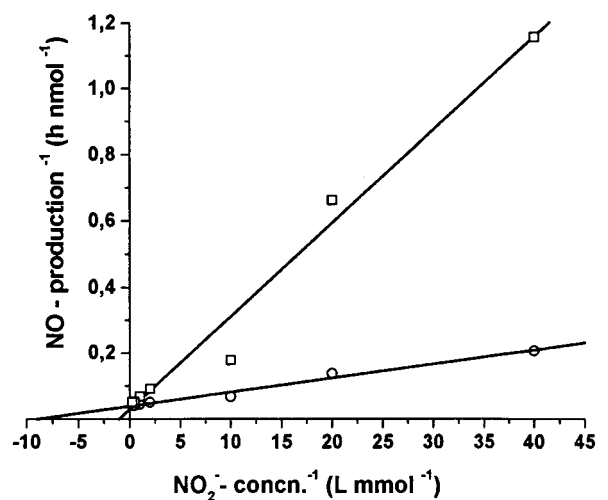

Fig. 1. Substrate (nitrite)-dependence of NO-production by purified maize NR, and its competitive inhibition by nitrate. 10 mU of NR were added to 2 ml reaction buffer (50 mM HEPES-KOH pH 7.6) containing 250 μM NADH and various nitrite concentrations in the absence (○) or presence of 50 μM nitrate (□).

Modulation of NO production by modulation of NR activity *in vitro*

As already mentioned, an aim of this study was to discover whether the well-known modulation of NR activity by reversible protein phosphorylation and 14-3-3-binding would also affect NO production with nitrite and NADH as substrates. It has previously been shown that the NR activity (measured in the presence of Mg^{2+}) can be rapidly modulated *in vitro* by preincubation of leaf extracts with MgATP or various effectors (for review see Kaiser *et al.*, 1999). In order to examine whether NO production would also be modulated by ATP preincubation, desalted leaf extracts were used instead of purified NR, as auxiliary enzymes (NR kinase, P-NR phosphatase and 14-3-3 proteins) are required for the modulation. For the experiment depicted in Fig. 2, a freshly prepared desalted extract from illuminated spinach leaves was preincubated with 5′-AMP plus EDTA, which causes the full activation of NR by dephosphorylation and release of 14-3-3-proteins (Kaiser *et al.*, 1999). Following the addition of NADH plus nitrite in 2 ml of a stirred reaction medium, NO was emitted into the gas phase above the solution.

Approximately 10 min after substrate addition, the NO emission decreased again. In contrast, preincubation of the extract with ATP, Mg^{2+} and protein phosphatase 2A inhibitor cantharidine, which inactivates NR by converting it into the catalytically inactive P-NR-14-3-3 complex (Kaiser *et al.*, 1999), caused an almost complete inhibition of nitrite-dependent NO production.

In the above example, the measured NRA in an aliquot of the extract was 10.3 μmol nitrite g^{-1} FW h^{-1} for the AMP pretreated samples and 1.4 μmol nitrite g^{-1}

FW h^{-1} after preincubation with ATP plus cantharidine. The rates of NO emission from nitrite of the fully activated enzyme on a fresh weight basis were 4.3 nmol NO g^{-1} FW h^{-1}, and thus again only a small fraction of NR activity in the crude extract, as observed with the commercial purified maize NR.

Regulation of NO emission *in vivo*

Sunflowers adapted to constant nitrate nutrition usually emitted no significant amounts of NO in the dark. In the light period up to 2 nmol NO g^{-1} FW h^{-1} were released. Figure 3 shows a typical dark–light cycle of intact nitrate-grown sunflower plants.

When detached sunflower leaves with the petioles in nitrate solution (10 mM) were illuminated in air, NO was emitted at a constantly low rate (Fig. 4). When the light was switched off, NO release transiently increased, but

Fig. 3. NO flux density (nmol NO g^{-1} FW h^{-1}) of sunflower plants in the exposure-chamber during a dark–light transient (six plants in the chamber, light intensity 450 μmol m^{-2} s^{-1}).
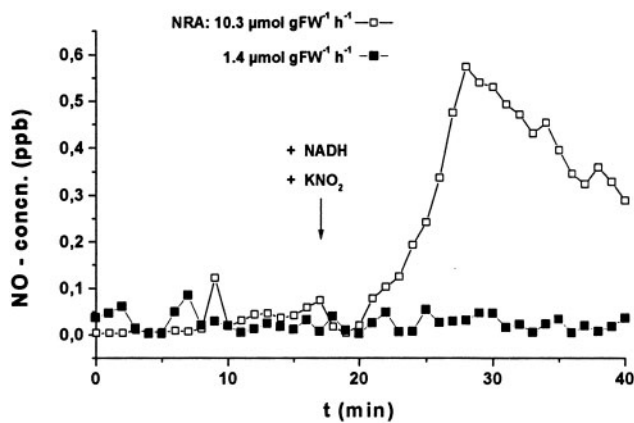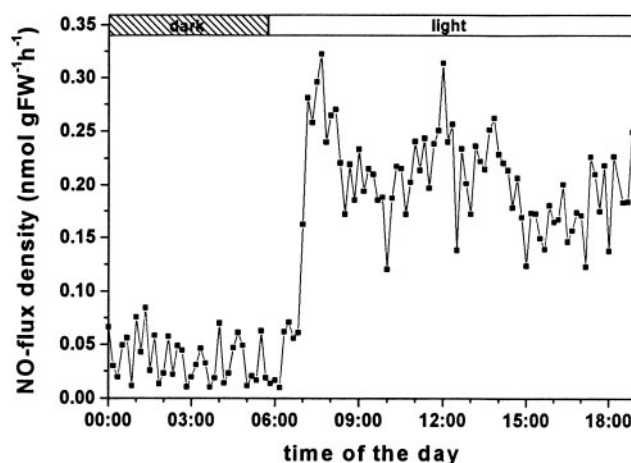

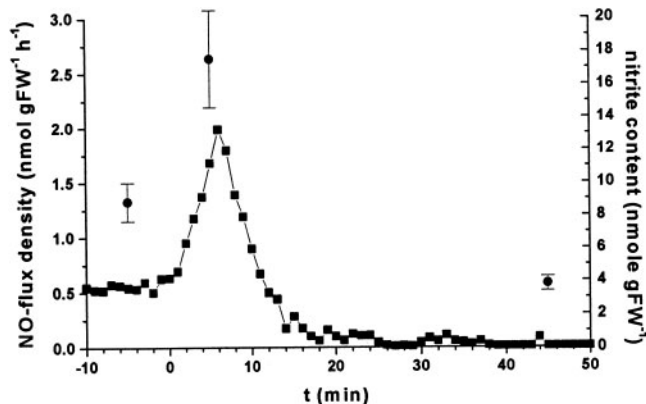**Fig. 2.** Modulation of NR *in vitro* also modulates NO production from nitrite plus NADH. Two ml of desalted spinach extract were pre-incubated for a total of 17 min with 5 mM AMP and 20 mM EDTA (open symbols) or with 5 mM ATP and 50 μM cantharidin (closed symbols) in a Petri dish placed into the reaction chamber (3.0 l air min^{-1}, 25 °C); NADH (250 μM) and KNO_2 (100 μM) were added as indicated.

Fig. 4. NO flux density (–■–) (nmol NO g^{-1} FW h^{-1}) and nitrite content (●) of a detached sunflower leaf in the exposure-chamber. At $t = 0$ light was switched off.

again settled at a very low steady-state value in the dark. This 'light-off burst' of NO emission was routinely observed not only with sunflower, but also with leaves or intact plants from other species like spinach or tobacco (data not shown). It was usually correlated with a transient nitrite accumulation (Fig. 4), (also compare Riens and Heldt, 1992).

The above *in vitro* experiments with desalted leaf extracts have shown that NO production by NR from nitrite follows the modulation of NR by protein phosphorylation/dephosphorylation. NR can also be modulated *in vivo*. The activity is usually high under good photosynthetic conditions and is decreased in the dark (Fig. 5). In the dark, NR can be activated to or above the light level by various treatments such as anoxia or by feeding uncouplers or cell permeable 5'-AMP analogues (Kaiser *et al.*, 1999). All these treatments also cause nitrite accumulation in darkened leaves or in roots (Kaiser *et al.*, 1993; Botrel and Kaiser 1997), and should therefore be expected to increase NO emission. This was indeed observed. One example is shown in Fig. 5, which gives NR activity $(+Mg^{2+})$, nitrite contents and NO emission by intact leaves.

As before, NRA in the dark was only about 50% of that in illuminated leaves, and the darkened leaves also contained 50% less nitrite than in the light. This was paralleled by NO emission. Under anoxia in the dark, NRA was strongly activated reaching approximately four times the activity of that in air. However, the leaf nitrite content increased more than 100-fold within 3 h, as did the NO emission (Fig. 5). As seen *in vitro*, even the maximum rate of NO release was only a small fraction $(\leq 1\%)$ of the NR activity in the extract, although nitrite concentrations in the tissue (up to 4.8 µmol g^{-1} FW, corresponding to a concentration in the water phase above 5 mM) were far above the K_m nitrite (100 µM).

Thus, NO release *in vivo* basically followed the pattern of NR activity, and was also related to the nitrite concentration in the tissue.

Table 1 summarizes the influence of various other methods of activating and inactivating NR in the dark. Dinitrophenol (DNP) is an uncoupler of oxidative phosphorylation and activates NR. Such activation of NR by uncouplers in the dark (air) has been shown to result in nitrite accumulation, similar to anoxia (Kaiser *et al.*, 1993). *In vitro*, inactive (phosphorylated) NR can be activated by 5'-AMP (Kaiser *et al.*, 1999). *In vivo*, this effect can be mimicked by feeding the cell permeable 5-aminoimidazole-4-carboxyamide 1-β-D-ribofuranoside (AICAR), which is converted inside the cell to the phosphorylated AMP-analogue ZMP (5-aminoimidazole-4-carboxyamide-1-β-D-ribofuranosyl 5'-monophosphate). Leaves fed with up to 20 mM AICAR in the dark show a very high activation state of NR (Huber and Kaiser, 1996). Further, the activation state of NR can be decreased by preventing dephosphorylation with okadaic acid, a specific inhibitor of PP2A phosphatases (Kaiser *et al.*, 1999). As expected, okadaic acid decreased the light-dependent NO emission.

The authors were also interested to examine whether nitric oxide synthase (NOS) would contribute to the NO emission from leaves. As the NOS reaction depends on the presence of oxygen, anoxia could not be used for inducing NO emission. Instead, the effect of the NOS inhibitor N-ω-nitro-L-arginine (L-NNA) was examined on the high NO emission in response to DNP. L-NNA had no inhibitory effect on the NO emission after DNP treatment.

In summary, all treatments which activated NR in the dark and also caused a nitrite accumulation to various degrees, led to increased NO emission, while okadaic acid completely prevented NO emission.

As nitrate was a very effective inhibitor of NO production *in vitro*, it was predicted that an increase in the cytosolic nitrate concentration would also decrease the rate of NO production in intact leaves. This was indeed confirmed, at least qualitatively. When a high rate of NO emission from intact leaves was initiated by anoxia in the
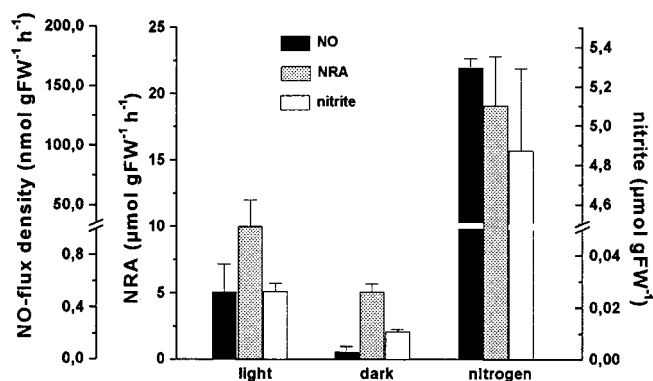

Fig. 5. NO flux density, actual nitrate reductase activity (NRA) and nitrite content of spinach leaves in light and dark (air) and dark (N$_2$). Samples for NR and nitrite determination were taken after 60 min in the light, after a further 30 min in the dark and after 180 min in dark+N$_2$ (mean of four samples for NRA and nitrite, for NO mean over 10 min around the moment of samples, error bars are standard deviation).

Table 1. *Response of steady-state NO emission from leaves to an activation or inhibition of NR*

Treatment	NO emission (nmol NO g^{-1} FW h^{-1}) ±SD (1σ)
Control, light	0.6±0.05
Control, dark	>0.2
+100 µM DNP, dark	7.9±0.13
+20 mM AICAR, dark	18.8±0.56
Okadaic acid, light	>0.2
+100 µM DNP +1 mM L-NNA dark	11.5±0.14

dark, feeding 50 mM KNO$_3$ through the leaf petiole caused an almost immediate decrease in NO emission (Fig. 6), which recovered slowly after a certain period of time. Here, cytosolic nitrate concentrations were unknown and therefore, the size and kinetics of the inhibition could not be predicted.

Discussion

These measurements confirm previous results that NO can be produced by NR from nitrite and NADH. To what extent different isoforms of NR are involved has not yet been examined, but in the leaves tested, NADPH-dependent activity was negligible (not shown).

NO may also be formed non-enzymatically. Mallick *et al.* assumed that NO formed from illuminated algae suspensions was at least partly due to a reaction of nitrite with reductants such as ascorbate or glutathione, as shown by NO production in pure nitrite solutions with these reductants (Mallick *et al.*, 2000). On the other hand, NO has been shown to react with glutathione to form *N*-nitrosoglutathione, which may even represent a storage form for NO (Singh *et al.*, 1999). Further, algal cells release nitrite to the medium, where it may be also photolysed to NO under high light intensities. Indeed, when leaves were fed with high nitrite concentrations in strong light, some NO emissions were found which did not respond to NR-inactivation (data not shown). However, in normal, nitrate-fed leaves NO production could be completely prevented by NR inactivation by feeding high nitrate or chlorate concentrations (see above). Thus, in the experiments depicted above, NO was exclusively formed by a reaction catalysed by NR.
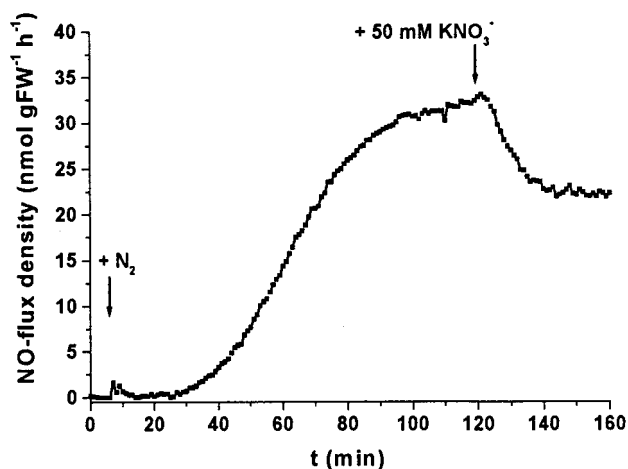

Fig. 6. NO flux density (nmol NO g^{-1} FW h^{-1}) of a detached sunflower leaf in the exposure-chamber in nitrate solution (750 μM). The chamber was flushed with nitrogen and KNO$_3$ (50 mM) was added to the solution as indicated.

Generally, even at saturating substrate concentrations, NO production appeared to be only a small fraction (1%) of the nitrate-reducing capacity of NR. Nitrate was not a direct substrate, but a highly efficient competitive inhibitor. The apparent K_m for nitrite (100 μM) was above the normal nitrite concentration found in illuminated leaves, which are approximately 10 nmol g^{-1} FW, but cytosolic nitrite concentrations are unknown. Nitrate concentrations in the cytosol of root cells are usually in the millimolar range (Miller and Smith, 1996), and similar concentrations have been found in leaf mesophyll cells (AJ Miller, personal communication). Since nitrite-dependent NO production is competitively inhibited by nitrate, NO emission rates from intact leaves must be very low, as observed (0.01–0.1% of the NR activity). In certain situations, however, for example, when NR was activated to such an extent that nitrite production exceeded the rate of nitrite reduction, the maximum NO emission rates from leaves came close to the maximum values (under substrate saturation) obtained with purified NR or leaf extracts *in vitro*.

NO$_2^-$ reduction to NO by NR is probably a one-electron-transfer reaction. So far, nothing is known about its molecular details. It is also unknown, why this reaction appears to be limited to such a low fraction of the nitrate reduction capacity of the enzyme. Due to that low capacity of NR for NO production it seems improbable that the reaction is suited to avoid excessive nitrite accumulation. Nevertheless, the system has an extremely broad response range, making it suitable, theoretically, for contributing to NO signalling. NO production by NOS, assayed with L-[^{3}H]-citrulline as substrate, was found to be 3 pmol mg^{-1} leaf protein min^{-1}, for example, in bacterial-infected tobacco leaves (Leshem, 2000). This is equivalent to about 1.8 nmol g^{-1} FW h^{-1} (a mean tobacco leaf protein concentration is 10–20 mg g^{-1} FW, not shown). In these experiments, NO production rates by NR *in vivo* were between 2–200 nmol g^{-1} FW h^{-1}. Thus, the capacity of the NR-system *in vivo* appears well in the range of NOS-activity, and may exceed it up to 100-fold under some conditions. Certainly participation of NR-derived NO in cellular signalling or interference with it would require strict regulation and sensitivity to effectors. It was therefore important to show here for the first time that the post-translational modulation of NR by serine phosphorylation and binding of a 14-3-3 dimer (for review see Kaiser and Huber, 1997) did indeed modulate NO production by NR, *in vivo* and *in vitro*. *In vivo*, the activation of NR would increase nitrite concentrations to the extent that nitrite reduction cannot cope, and this would increase NO production, because normal nitrite concentrations are far below the K_m. However, activation of NR also increased NO production from added (saturating) nitrite *in vitro*. Thus, the modulation of NR has a 2-fold effect on NO production: a

direct one by modulating the electron flow through the NR enzyme, and an indirect one by affecting the nitrite concentration. In addition, conditions changing the cytosolic nitrate concentration (which is a competitive inhibitor) or the rate of nitrite reduction must have drawbacks on the NR-dependent NO production. Thus, the system has multiple sites of interaction with photosynthesis and metabolism. Future work will focus on both the molecular mechanism and the regulation of NO production, on its physiological consequences. and its relation to the second NO-producing system, NOS.

Acknowledgement

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