S21-003

Nitrate reductase as a producer of nitric oxide in plants: temperature-dependence of the enzymatic active nitrogen formation

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Keywords: C/N balance, nitrate reductase, nitric oxide, oxidative stress, temperature stress

Introduction

Nitric oxide (NO) has long been recognized as a harmful air pollutant that can be produced through industrial activities. After the discovery of NO synthase (NOS, EC 1.14.13.39) that produces NO during the conversion of L-arginine to L-citrulline, our view of NO has been drastically changed from a harmful pollutant to an important signal messenger in animal cells (Yamasaki, 2000). In contrast to the large body of knowledge on functions of NO in animal systems, however, little is known in plant systems. Recent studies have suggested that NO is involved in a broad spectrum of physiological responses, including pathogen response, programmed cell death, germination, phytoalexin production and ethylene emission (Bolwell, 1999; Wendehenne, 2001). Although NOS inhibitor experiments using *N*-nitro-L-arginine (L-NNA), N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arigine-methyl ester (L-NAME) have suggested the existence of a mammalian-type NOS in plants, no plant NOS gene has been conclusively identified to date. Furthermore, no homologue of mammalian-type NOS has been found in the genome of *Arabidopsis thaliana*. Thus, the presence of mammalian-type NOS in plants remains a subject to be clarified and the mechanism for NO production in plant cells has not yet been confirmed.

It has been sometimes reported that several plant and algal species emit NO when nitrate or nitrite is supplied in darkness. In the legume plant *Glycine max*, the constitutive nitrate reductase (cNR) was identified to produce NO (Dean and Harper, 1986). Harper and coworkers have suggested that NO would be produced from nitrite by the activity of cNR (Dean and Harper, 1988). Normally, NR is the rate-limiting step enzyme of nitrate assimilation in plants and algae, catalyzing the reduction of nitrate (NO_3^-) to nitrite (NO_2^-). The NO producing activity had been considered a unique characteristic of cNR that is only distributed in the Phaseolus tribe of the family leguminosae (Dean and Harper, 1988).

We have recently shown in vitro evidence that maize inducible NR (iNR) is also capable of producing NO through one electron reduction of nitrite (Yamasaki et al., 1999). A similar nitrite-dependent NO production catalyzed by NR has been reported in bacteria and fungi (Yamasaki, 2000). These results suggest that NO producing activity of NR is a more general feature than was previously thought (Yamasaki, 2000). Here we demonstrate that production of active nitrogen species (NO and $ONOO^-$) by NR is strongly temperature-dependent.

Materials and methods

Nitric oxide concentrations were measured by an electrochemical method (Yamasaki and Sakihama 2000). The electrochemical detection of NO was carried out with a Clark-type NO electrode (ISO-NOP, WPI, Sarasota, USA) in conjunction with an ISO-NO Mark II and Duo.18 data acquisition system (WPI, USA). Production of $ONOO^-$ was measured with a double beam spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan) by monitoring the absorbance increase of 2',7'-dichlorodihydrofluorescein (DCDHF) at 500 nm (Yamasaki and Sakihama 2000). The reaction mixture (1 ml) for detecting NO and $ONOO^-$ contained 20 mM potassium phosphate (pH 7.0). Nitrate reductase purified from the corn seedlings was obtained from Molecular Biologische Technologie (Göttingen, Germany). The purity of NR was checked by an A_{218}/A_{413} ratio which showed less than 1.7. DCDHF was purchased from Sigma-Aldrich (St. Louis, USA) and all other reagents from Nacalai Tesque (Kyoto, Japan).

Results

Figure 1A shows the time courses of NO production by NR using a Clark-type NO electrode. NO was rapidly produced from nitrite (NO_2^-) and NADH by a maize NR. The addition of sodium azide (NaN_3), a known NR inhibitor, completely eliminated NO production (Yamasaki and Sakihama 2000). KCN was also effective to abolish NO production (not shown). The apparent NO production was also completely suppressed by the addition of hemoglobin (Hb), a strong quencher of NO. These results clearly demonstrate that NR produces NO when nitrite is the substrate.

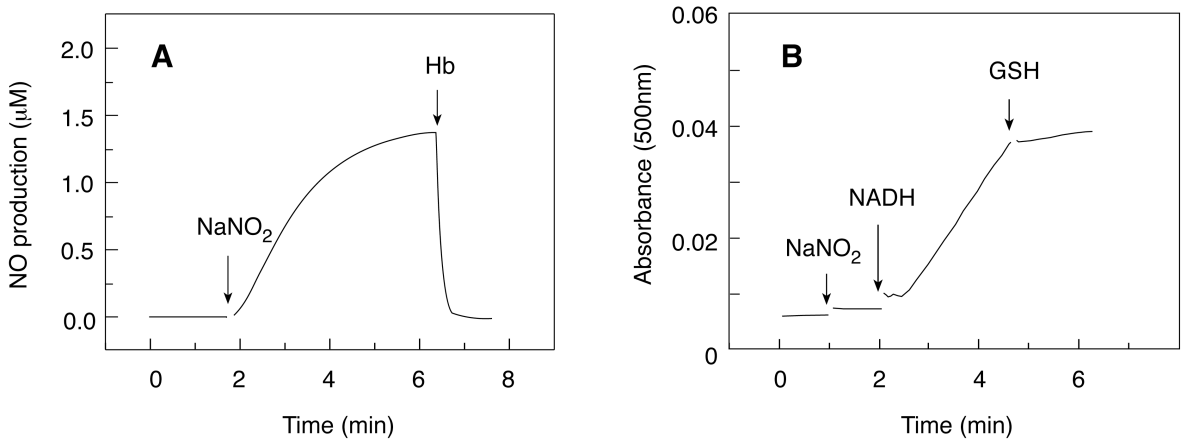

Figure 1. Active nitrogen production by a maize NR. A, Nitrite-induced NO production by NR measured with an NO electrode. B, Nitrite-induced $ONOO^-$ production by NR monitored with DCDHF. The reaction was initiated by 1 mM nitrite. The reaction mixtures for NO detection contained NR (15 mU/ml), NADH (0.1 mM) and nitrite (0.1 mM) and that for $ONOO^-$ measurement included NR (30 mU/ml), NADH (1 mM), nitrite (1 mM). Hb, hemoglobin (10 μM); GSH, reduced form of gluthathione (5 mM).

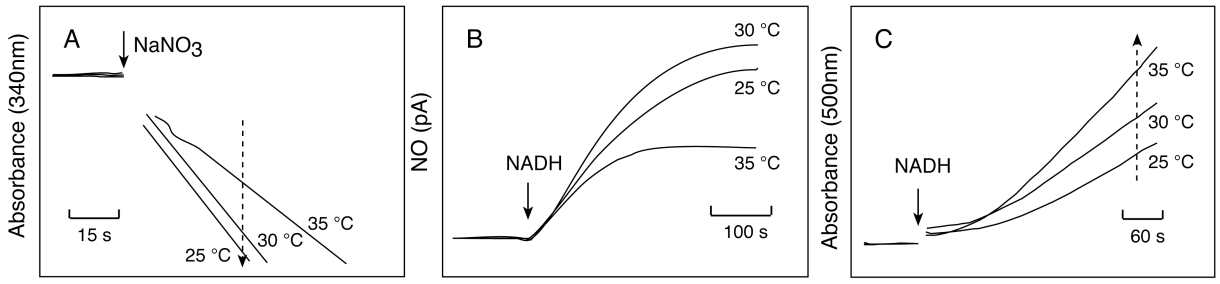

Figure 2. The activities of NR measured at different temperatures. A, Nitrate-induced oxidation of NADH. B, Nitrite-induced NO production. C, Nitrite-induced $ONOO^-$ production. The reaction mixtures in (A) contained NR (15 mU/ml), NADH (0.1 mM) and nitrate (0.1 mM). Others were similar to those in Fig. 1.

NO is recognized both as a signaling molecule that regulates many enzyme activities and as an agent of cytotoxicity. The cytotoxic effects of NO can be largely ascribed to peroxynitrite ($ONOO^-$) that is produced by the diffusion-limited reaction of NO and superoxide (O_2^-):

$$NO + O_2^- \rightarrow ONOO^-. \qquad (1)$$

Figure 1B shows absorbance changes of DCDHF, an indicator of $ONOO^-$ production (Yamasaki and Sakihama 2000). When nitrite and NADH were added into a solution containing NR, a significant increase in absorbance of DCDHF was detected. This change was completely inhibited by NaN_3 (Yamasaki and Sakihama 2000). Glutathione (GSH), a scavenger for $ONOO^-$, also inhibited the absorbance increase of DCDHF (Fig. 1B). Depletion of oxygen from the reaction mixture resulted in the suppression of absorbance decrease of DCDHF because the production of O_2^- requires molecular oxygen. These results strongly suggest that NR produces in vitro $ONOO^-$ in addition to NO and O_2^- (Yamasaki and Sakihama 2000).

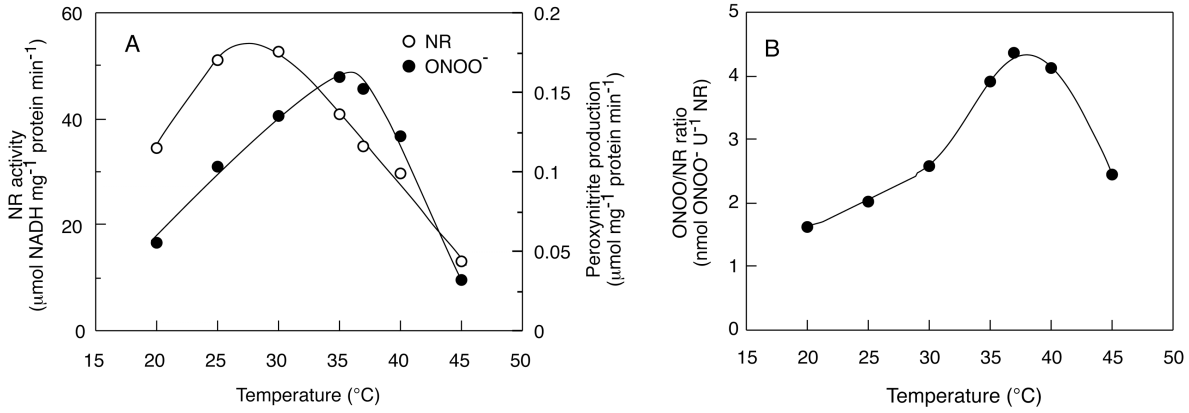

Figure 3. Dependence on temperature of nitrate-dependent NADH oxidizing activity and nitrite-dependent $ONOO^-$ producing activity.

NR transfers electrons from NAD(P)H to nitrate via three redox centers including two prosthetic groups (FAD and heme) and a Mo-pterin center. Each redox center is associated with a functional domain of the enzyme that shows redox activity independent of the other domains. NO could be produced by one electron reduction of nitrite through electron leakage from a redox center of NR. It has long been known that NR activity is extremely sensitive to high temperature. Interestingly, the temperature sensitivity of each redox activity has been reported to be different. It is plausible that NO production may not require whole electron transport process, thereby showing different temperature dependency from that of nitrate reducing activity. In fact, we observed that the temperature dependencies were different (Figs. 2 and 3). Figure 2A shows NR activity represented by the oxidation rate of NADH. With increase in temperature (25, 30 and 35 °C), the rate of NADH oxidation decreased. In contrast, the rate of $ONOO^-$ production increased in response to temperature increase (Fig. 2C). NO production showed the optimum temperature at 30 °C (Fig. 2B).

Figure 2 has shown that temperature response of the active nitrogen producing activity is different from normal NR activity. Figure 3 shows temperature dependency of NR activity and $ONOO^-$ producing activity. As previously reported NR activity showed the maximum value around 30 °C and decreased sharply at temperatures above the optimum (Fig. 3A). In contrast, the apparent optimum temperature for $ONOO^-$ production was 35 °C (Fig. 3A). Figure 3B summarizes temperature dependence of efficiency of $ONOO^-$ production by NR. On the basis of the unit activity of NR, $ONOO^-$ was produced the most efficiently around 37 °C.

Discussion

Although the capability of NO production itself was found in plants earlier than the discovery of that in animals, until recently only the legume plants were demonstrated to emit NO and NO_2 (Klepper, 1979). New methodologies for the detection of NO enable us to measure small NO emissions from living organisms. Until now, NO producing activities in vivo have been reported in a various species of plants and algae: soybean, pea, sunflower, spruce, rape, maize, sugarcane, spinach, tobacco, *Arabidopsis thaliana*, *Taxus brevifolia*, *Kalanchoë daigremontiana*, *Lupinus albus, Mucuna hassjoo*, *Anabaena doliolum*, *Scenedesmus obliquus*. These studies have established that plants and algae possess the capability to produce NO under certain conditions. However, the molecular mechanism for NO production in plants remains to the subject of controversy—whether NOS, NR or nonenzymatic reaction?

Together with the previous pioneering works by Harper and colleagues, our in vitro studies have confirmed that NR is a plant NO producing enzyme. Figure 4 illustrates our present working hypothesis for the nitrite-dependent NR-catalyzed NO production in plants and algae (Yamasaki, 2000). When photosynthetic electron transport systems supply enough of the reduced form of ferredoxin (Fd_R) for the NiR reaction, nitrite is efficiently assimilated into glutamine via the GS/GOGAT pathway. Thus, no accumulation of nitrite and no NO production occurs in the cells. However, a decline of reduced ferredoxin causes a decrease in the conversion rate of nitrite, resulting an accumulation of nitrite in the cytosol. Under this condition, NR would convert nitrite to NO. Because NO is membrane permeable, any NO produced in the cytosol could easily diffuse into the stroma of chloroplasts as well as mitochondria. If the photosynthetic electron transport system produces O_2^- at PS I under illumination (Asada 1999), $ONOO^-$ may be produced in the reaction between NO and O_2^- (reaction 1). Recently, we have found that NR-less mutant of the green alga *Chlamydomonas reinhardtii* does not produce NO in response to the addition of nitrite

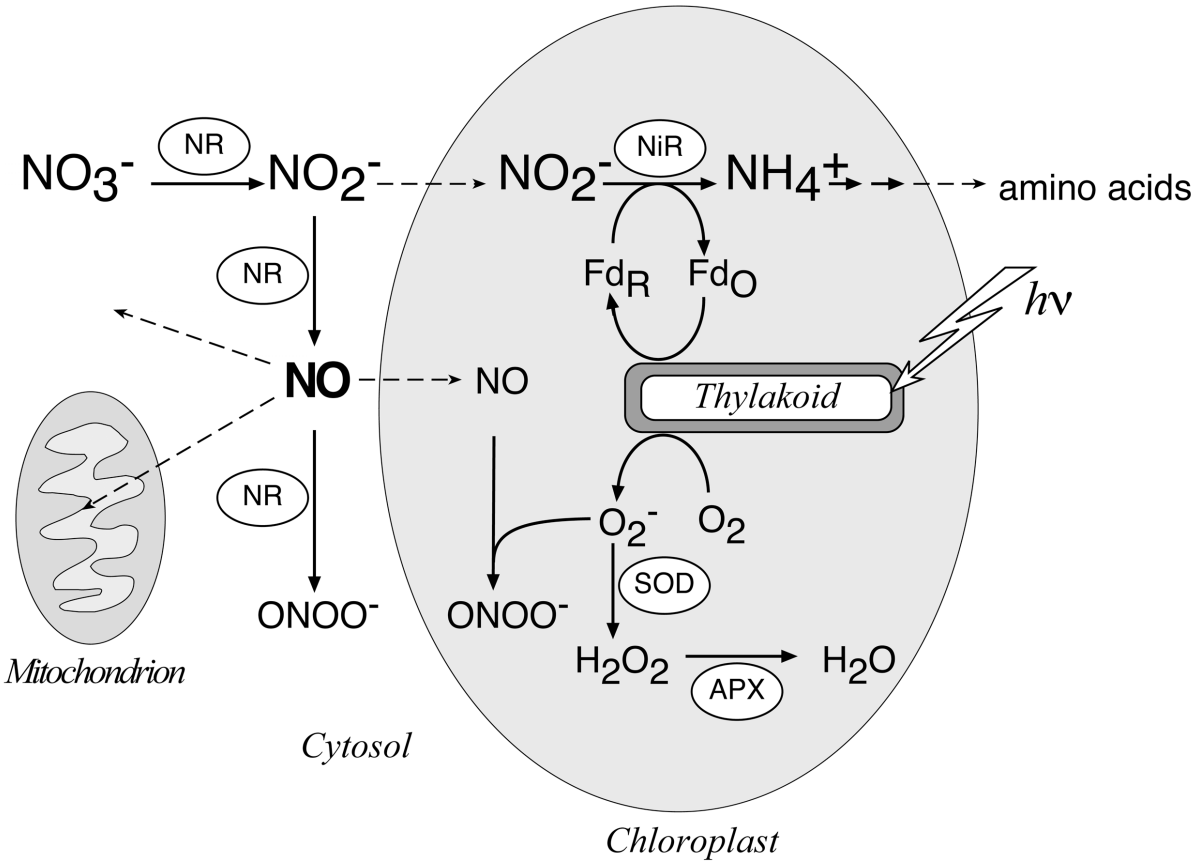

Figure 4. Hypothetical scheme representing the nitrite-dependent NO production pathway.

(Sakihama et. al 2001), direct evidence supporting the presence of NR-dependent NO production pathway.

In *C. reinhardtii*, the gene encoding the alternative oxidase (AOX) of mitochondria has been found in the gene cluster of nitrogen assimilation including NR, NiR, nitrate and nitrite transporters (Quesada et al., 2000). AOX is a termial oxidase for the cyanide-insensitive respiration pathway and its function is not yet clear. We have shown substantial evidence that AOX is also resistant to NO produced by NR and can prevent formation of active oxygen (Yamasaki et al., 2001). The requirement for harmonic expression of NR and AOX supports the hypothesis that AOX can play an important role in avoiding oxidative damage induced by NO (Millar and Day, 1996).

It seems that nitrite-dependent NR-mediated NO production pathway has been postulated. Yet, physiological significance of the NO production pathway is largely unknown. We have suggested that overproduction of NO by NR may be a cause of oxidative damage of plants, in particular under abiotic and biotic stress (Yamasaki et al. 1999; Yamasaki 2000). When O_2^- is produced in plant cells under stress conditions, $ONOO^-$ is potentially formed as the result of reaction (1). $ONOO^-$ and hydroxyl radical (•OH) are the most cytotoxic radical species, which can oxidize lipid, DNA and proteins. In the presence of CO_2, $ONOO^-$ could convert tyrosine residue of enzymes to nitrosyl-tyrosine (Yamasaki 2000). Moreover, singlet oxygen ($^1O_2$) may be produced by a reaction between $ONOO^-$ and H_2O_2. These reactions of $ONOO^-$ may lead dysfunction of cellular metabolism. The results presented in Figs. 2 and 3 imply that those

unfavorable reactions mediated by NR would be pronounced at high temperatures.

Photosynthesis is the most heat-sensitive metabolism in plants. In many temperate plant species, temperatures ranging from 35 ℃ to 45 ℃ inhibit photosynthesis. Although rubisco activase, a carbon metabolism enzyme, has been proposed as a heat-sensitive enzyme, the primary temperature-sensitive site or enzyme of plant cells is controversial (Sharkey 2000). The in vitro data presented here (e.g. Fig. 3B) would give us a unique aspect to consider another view of thermal stress on plants, i.e. a possible involvement of active nitrogen production by NR in the mechanism for heat-induced dysfunction of cellular metabolism.

Acknowledgement

We are grateful to Dr. M. F. Cohen for critical reading of the manuscript. This work was supported by Grant-in-Aid for Scientific Research (B) and (C) from Japan Society for the Promotion of Science.

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