Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species

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Abstract We examined the ability of plant nitrate reductase (NR) to produce nitric oxide (NO) using in vitro assays. Electrochemical and fluorometric measurements both showed that NO is produced by corn NR in the presence of nitrite and NADH at pH 7. The NO production was inhibited by sodium azide, a known inhibitor for NR. During the reaction, absorbance of 2′,7′-dichlorodihydrofluorescein increased markedly. This change was completely suppressed by sodium azide, glutathione or depletion of oxygen. We conclude that plant NR produces both NO and its toxic derivative, peroxynitrite, under aerobic conditions when nitrite is provided as the substrate for NR.

Key words: Nitrate reductase; Nitrogen assimilation; Nitrite; Nitric oxide; Peroxynitrite; Superoxide

1. Introduction

Nitrate assimilation is the major pathway for nitrogen supply in many plants and microorganisms. Because nitrate reduction is the rate-limiting step, nitrate reductase (NR) has been considered as the key enzyme for assimilatory nitrogen metabolism [1–3]. NR is known to be highly regulated by the complex transcriptional and post-translational mechanisms [1,2]. Degradation of NR is rapid and a half of the total enzyme disappears in 6 h of darkness [2,4]. It is not clear why plants possess such multiple systems to regulate NR [1,3].

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 [5]. Each redox center is associated with a functional domain of the enzyme that shows redox activity independent of the other domains. Dean and Harper found that the constitutive NR (cNR, EC 1.6.6.2) of soybean converts nitrite to nitric oxide (NO) probably at the Mo-pterin center [6]. Because the cNR is unique to *Leguminosae*, the NO production in plants was considered to be a phenomenon limited to this family [6]. Recently, Wildt et al. reported that plant species other than *Leguminosae*, including sunflower, sugar cane, corn, rape, spruce, spinach and tobacco, emit NO gas under certain conditions [7]. In a previous study, we showed that a corn NR produces NO via reduction of nitrite similar to that reported in the cNR [8]. These results suggest that inducible NR (iNR, EC 1.6.6.1), as well as the cNR, could have ability to produce NO.

In this study, the NR-dependent NO production was assayed in three ways: (1) measuring a liquid phase NO directly by an NO sensor; (2) monitoring the formation of green-fluorescent triazolofluorescein; (3) inhibition by sodium azide, an inhibitor of NR. Our results provide further evidence for the NO production catalyzed by iNR. We also demonstrate that the produced NO can be further converted to the extremely toxic compound peroxynitrite ($ONOO^-$) under aerobic conditions. Based on these results, a novel idea on the regulation of NR activity is proposed in association with the production of potentially toxic active nitrogen species.

2. Materials and methods

NO concentrations were measured by either an electrochemical or a fluorometric method. 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) [8]. The fluorometric detection of NO was carried out using the NO indicator, diaminofluorescein-2 (DAF-2) [9]. The fluorescence from DAF-2T, the reaction product of DAF-2 with NO, was measured with a Shimadzu fluorescence spectrophotometer (RF-5300PC, Shimadzu, Kyoto, Japan) at 25°C. The excitation and emission wavelengths for DAF-2T were 495 and 515 nm, respectively. Production of peroxynitrite was measured with a double beam spectrophotometer (UV-160A, Shimadzu, Japan) by monitoring the absorbance increase of 2′,7′-dichlorodihydrofluorescein (DCDHF) at 500 nm [10]. The reaction mixture (1 ml) for detecting NO and peroxynitrite contained 20 mM potassium phosphate (pH 7.0). Other experimental conditions are presented in the figure legends. NR purified from the corn seedlings was obtained from Molecular Biologische Technologie (Göttingen, Germany). The purity of NR was checked by an A_{280}/A_{413} ratio which showed less than 1.7. DAF-2 was purchased from Daiichi Pure Chemicals (Tokyo, Japan), DCDHF from Sigma-Aldrich (St. Louis, MO, USA) and all other reagents from Nacalai Tesque (Kyoto, Japan).

3. Results

Fig. 1A shows the time courses of NO production by NR using a Clark-type NO electrode. As we previously reported, NO was rapidly produced from nitrite (NO_2^-) and NADH by a corn NR [8]. This reaction was completely inhibited by sodium azide (NaN_3), a known NR inhibitor. The K_m value for nitrite estimated from NO production rate was 300 μM, which was 5-fold higher than that for nitrate (60 μM) determined from the NADH oxidation rate. NR-dependent NO production was further verified by another NO detecting system. Fig. 1B shows increases in fluorescence intensity of DAF-

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Abbreviations: DAF-2, diaminofluorescein-2; DCDHF, 2′,7′-dichlorodihydrofluorescein

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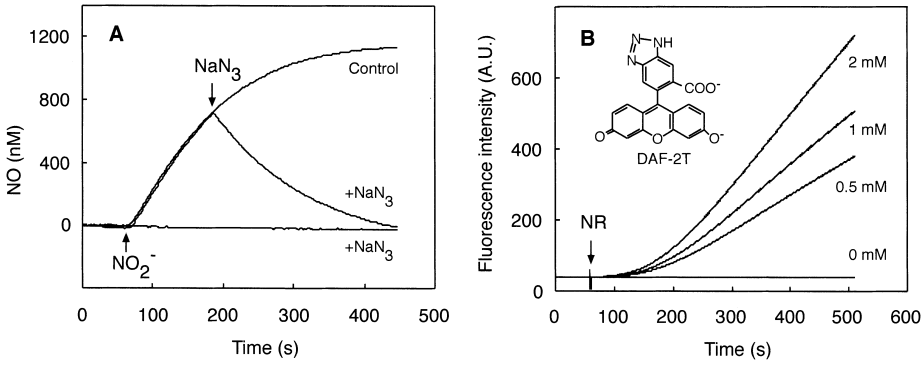

Fig. 1. The nitrite-dependent NO production by NR. A: Time courses for NO production measured with an NO electrode. The reaction mixture included 1 mM sodium nitrite, 100 μM NADH and 15 mU/ml NR. Sodium azide (1 mM) was added either before (lower trace) or during the reaction (middle trace). The reaction was initiated by adding sodium nitrite. B: Time courses for NO production measured by the fluorescence indicator DAF-2. The experimental conditions were similar to those in A except the presence of 10 μM DAF-2. Numbers beside each trace represent the concentration of added nitrite (mM).

2 which has been developed as a specific indicator for NO [9]. The increase in fluorescence intensity of DAF-2 corresponds to an NO-dependent conversion of non-fluorescent DAF-2 to green-fluorescent triazolofluorescein (DAF-2T), thereby indicating nitrite-dependent NO production by NR (Fig. 1B).

An excess of NADH enables NR to produce NO in conjunction with its normal substrate nitrate (NO_3^-) [8]. Unlike nitrite, the NO production observed with nitrate as the substrate showed a significant time-lag up to the onset of NO release (ex. 260 s at 100 μM NO_3^-). This time-lag, as well as the rate of NO production, depended on the concentration of supplemental NADH (Fig. 2), indicating that the reaction product nitrite is a direct substrate for the NO production. Detection of NO could be completely eliminated by the addition of hemoglobin, a strong quencher of NO. These results demonstrate that NR produces NO when nitrite is the substrate.

NO is recognized as both a signal molecule that regulates many enzyme activities [11–14] and as an agent of cytotoxicity [15]. The cytotoxic effects of NO can be largely ascribed to peroxynitrite that is produced by the diffusion-limited reaction of NO and superoxide (O_2^-) [16]. Barber and Kay reported that *Chlorella* NR produces O_2^- when NADH is provided under aerobic conditions [17]. They suggested that the Mo-pterin center of the enzyme is the site for the O_2^- production. Since corn NR also has the Mo-pterin center, a similar O_2^- production could occur and may produce peroxynitrite as a result. Fig. 3 shows absorbance changes of DCDHF, an indicator of peroxynitrite production [10]. When nitrite and NADH were added to a solution containing NR, a significant

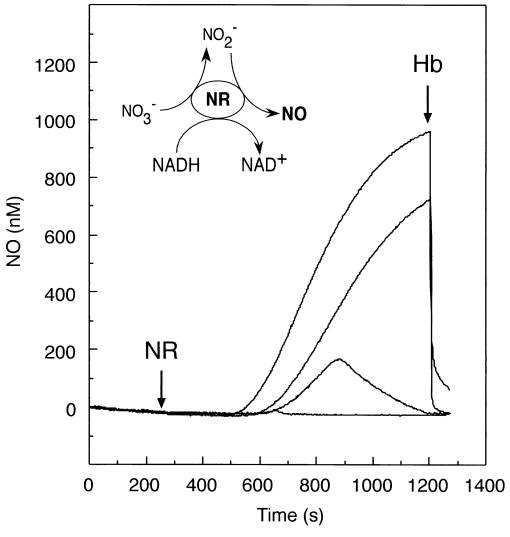

Fig. 2. The NO production induced by nitrate. The reaction mixture included 15 mU/ml corn NR and 50 μM sodium nitrate instead of nitrite. The NO production was measured with an NO electrode. Traces in order of decreasing signal correspond to time courses measured in the presence of 100, 50, 40 and 0 μM NADH, respectively. The reaction was initiated by adding NR at the arrow indicated. Hb, bovine hemoglobin (5 μM). Other conditions were similar to those in Fig. 1.

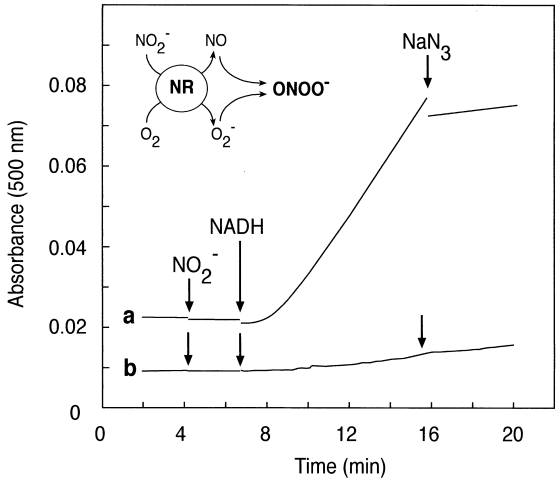

Fig. 3. Formation of peroxynitrite by NR. Production of peroxynitrite was monitored by the absorbance increase of DCDHF. The reaction medium included 100 μM DCDHF and 30 mU/ml corn NR. Nitrite (1 mM), NADH (1 mM) and NaN_3 (1 mM) were added at the arrows as indicated. a: Measured under ambient air; b: measured under a stream of nitrogen gas to remove oxygen. Other conditions were similar to those in Fig. 1.

increase in absorbance of DCDHF was detected (Fig. 3). This change was completely inhibited by NaN_3 (Fig. 3a). Glutathione, a scavenger for peroxynitrite, also inhibited the absorbance increase of DCDHF (data not shown). Because production of O_2^- requires molecular oxygen, a depletion of O_2 should eliminate the formation of peroxynitrite. Indeed, we failed to detect any absorbance changes under anaerobic conditions (Fig. 3b) although NO production was detectable (data not shown). These results strongly suggest that the NR produces in vitro peroxynitrite in addition to NO and O_2^-.

4. Discussion

4.1. Nitrite-dependent enzymatic pathway as a source of NO in plant cells

The present study has shown direct evidence for the NO production from nitrite which is catalyzed by the NR enzyme (Fig. 1). In soybean, the cNR deficient mutant was reported to loose its NOx ($NO+NO_2$) producing ability under ambient air but still it evolved small amounts of NOx under dark and anaerobic conditions [18]. Klepper pointed out that non-enzymatic reduction of nitrite can be responsible for NOx evolution in the mutant [18]. Because the NO production via chemical reduction of nitrite requires undissociated acid form of nitrite (HNO_2, $pK = 3.2$) [8,19], in vivo NO production via this pathway may be limited to special acidic compartments and tissues [19]. Thus, we consider that the NR-dependent enzymatic pathway would dominate the in vivo NO production observed under ambient air.

Normally, nitrite is efficiently converted to ammonia by the activity of nitrite reductase (NiR) in the chloroplasts [5], thereby being difficult to detect in healthy plant tissues in contrast with nitrate. Translocation of nitrite from cytosol into the chloroplasts requires ΔpH across the envelope [20] and the nitrite reduction by NiR needs the reduced ferredoxin [5], both of which can be established by the activity of photosynthetic electron transport on thylakoid membranes. Nitrite accumulation followed by NO production could be detected under conditions where these activities are absent or inhibited. In fact, high emissions of NO from plants have been observed in the dark [7,18,21] or in the presence of photosynthetic electron transport inhibitors [21]. Moreover, the NO emission from soybean leaves was reported to be suppressed by illumination [18,21]. In this context, photoinhibiting conditions, which can be obtained by an irradiation of excess light energy, may also induce NO production via the NR-dependent pathway.

4.2. Physiological implications of NR-dependent NO production in plants

It has long been known that nitrite is cytotoxic to plants, though the molecular mechanism is unknown. A complete inhibition of NiR activity is thought to be lethal for the plant cells probably due to an accumulation of nitrite [22]. A strong correlation between NO emission and nitrite content was reported in soybean leaf tissues [21]. An important finding of the present study is that NR further converts NO to peroxynitrite (Fig. 3). In animal cells, excessive production of peroxynitrite is known to cause oxidative damage leading to DNA damage, modification of enzymes and induction of lipid peroxidation [16,23]. Our results suggest that the nitrite cytotoxicity in plants is ascribable to the production of active nitrogen species including peroxynitrite through the NR-dependent pathway.

In mammalian inflammatory cells, peroxynitrite is produced by inducible NOS via NO production [23]. There has been accumulating evidence that NO is involved in plant signal transduction systems [12–14,24,25]. However, the source of NO is still controversial [26,27]. Although it is widely presumed that NO production is catalyzed by NOS, neither a gene nor a protein homologous to mammalian NOS has been isolated from plants to date [26,27]. The present study has clearly shown that NR possesses an NO producing ability, implicating a role for NR as a signal emitter in plant cells similar to that of mammalian NOS. Based on the apparent NO production rate, we estimated the K_m of NR for nitrite to be 5-fold higher than that for nitrate. Interestingly, the cNR enzyme of soybean was reported to have a lower K_m for nitrite than for nitrate [6], which raises a possibility that the enzyme can preferentially produce NO rather than the normal NR product. It is well appreciated that NR activity is highly regulated by transcriptional and post-translational mechanisms in response to many environmental conditions including nitrogen supply, light, pH, temperature, CO_2 and O_2 availability [1,2]. Although further physiological and biochemical evidence is required, we consider it plausible that such strict regulation of NR is beyond that needed for nitrogen assimilation but may be necessary for regulating production of active nitrogen species.

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