Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria

A. Harvey Millar, David A. Day*

Division of Biochemistry and Molecular Biology, and The Cooperative Research Centre for Plant Science, Australian National University, Canberra 0200, Australia

Received 15 October 1996

Abstract **Oxygen consumption via the cytochrome pathway in isolated soybean (*Glycine max* [L.] Merr.) cotyledon mitochondria was inhibited by nitric oxide (NO) while respiration via the cyanide-insensitive alternative oxidase was not significantly affected. Inhibition of cytochrome pathway activity was rapidly reversible upon depletion of the added NO. NO production was also detected in solutions of $NaNO_2$ plus ascorbate and the extent of cytochrome pathway inhibition was dependent on the NO_2^- concentration. Little inhibition of alternative pathway respiration was observed under similar conditions. The alternative oxidase may play a role in nitric oxide tolerance in higher plants and in organisms such as trypanosomes which contain a plant-like alternative oxidase.**

Key words: Plant respiration; Respiratory inhibitor; *Glycine max*

1. Introduction

Nitric oxide (NO) is an important physiological messenger in animals but at high concentrations it becomes a potent toxin inhibiting respiration and other cellular functions [1,2]. Nitric oxide affects respiration by acting as a competitive inhibitor of cytochrome *c* oxidase [3–5], and it has been suggested that NO functions as an endogenous regulator of mitochondrial electron transport and oxidative phosphorylation in mammalian cells [6]. The effect of NO on the respiration of plant mitochondria has not been addressed to date.

In addition to cytochrome oxidase, plant mitochondria possess a second terminal oxidase, termed the alternative oxidase (AOX). Unlike the cytochrome pathway, which is coupled to oxidative phosphorylation via proton translocation, electron transport from ubiquinol to AOX is non-phosphorylating and releases energy as heat [7]. These two terminal oxidases compete for electrons in plant mitochondria, with inhibition of one pathway redirecting flux to the other, when AOX is activated by pyruvate [8,9]. The two pathways can be differentiated by inhibitors such as cyanide, antimycin A, myxothiazol or carbon monoxide (acting on the cytochrome pathway) and *n*-propyl gallate (nPG) or SHAM (acting on AOX).

Measurements of gaseous emissions from soybean and pea leaves have demonstrated that NO can be synthesised in plant cells [10–13]. NO formation has also been detected in soybean root nodules through the formation of NO-leghaemoglobins [14]. NO is produced enzymatically from NO_2^- in leaves of soybean and other leguminous species by the constitutive NAD(P)H nitrate reductase (NR) enzyme [12]. Soybean mutants and plant species (e.g. wheat, barley, pea) that lack this form of constitutive NR do not appear capable of enzymatic conversion of NO_2^- to NO, but do produce lower levels of NO either via a non-enzymatic route [11] or via a putative mammalian-like L-arginine NO synthase [13,15]. NO is, therefore, a potential effector of respiration in plants as in mammals.

In this paper, we investigate the effect of NO on the regulation of plant mitochondrial electron transport in mitochondria isolated from soybean cotyledons. NO was found to inhibit reversibly the cytochrome pathway but had little effect on AOX activity.

2. Materials and methods

Soybean seedlings (*Glycine max* [L.] Merr. cv. Stevens) were grown in a controlled environment cabinet (28°C/20°C 14 h/10 h day/night) in trays of vermiculite. Mitochondria were isolated from cotyledons after 7 days as described by Day et al. [16]. Oxygen uptake in liquid phase was measured using a Rank Brothers O_2 electrode in 2 ml of standard reaction medium (containing 0.3 M sucrose, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% (w/v) bovine serum albumin (BSA), 10 mM TES pH 7.2) at 25°C. Maximum rates of AOX activity were ensured by including pyruvate (1 mM) in the reaction medium, 0.25–0.5 mg mitochondrial protein was used in each assay. Stable NO solutions were prepared by bubbling the gas evolved from Ar-purged 0.1 M H_2SO_4+0.1 M KI solutions through Ar-purged distilled water in a rubber-sealed scintillation vial. NO concentration in solutions was 0.2–0.5 mM depending on the preparation and was stable for over 24 h. Sodium nitroprusside stock solutions (50 mM) were made immediately before experiments and kept in the dark. Small aliquots of NO or nitroprusside were added by syringe injection to the reaction chamber. Nitric oxide concentrations were measured with an ISO-NO 2 mm electrochemical sensor (World Precise Instruments, Sarasota, USA) inserted into the reaction chamber. The ISO-NO sensor was calibrated by the conversion of known aliquots of $NaNO_2$ to NO in a solution of 0.1 M KI and 0.1 M H_2SO_4 according to the manufacturer's instructions. The protein content of mitochondrial samples was estimated according to the method of Lowry et al. [17]. Percoll was purchased from Pharmacia Biochemicals Inc (Uppsala, Sweden). All other reagents were purchased from Sigma (Sydney, Australia).

3. Results

In soybean cotyledon mitochondria oxidising NADH, O_2 uptake was partially inhibited by an added pulse of NO but recovered upon NO depletion (Fig. 1A). Oxygen consumption via the cytochrome pathway (respiration in the presence of nPG) was inhibited almost completely during this pulse of NO (Fig. 1B). On the other hand, when electron flow occurred via AOX (respiration in the presence of myxothiazol), only slight inhibition of O_2 consumption was observed (Fig. 1C). Oxygen consumption during NO inhibition in Fig. 1A was

*Corresponding author. Fax: (61) (6) 249 0313.
E-mail: David.Day@anu.edu.au

Abbreviations: nPG, *n*-propyl gallate; AOX, alternative oxidase

PII S0014-5793(96)01230-6

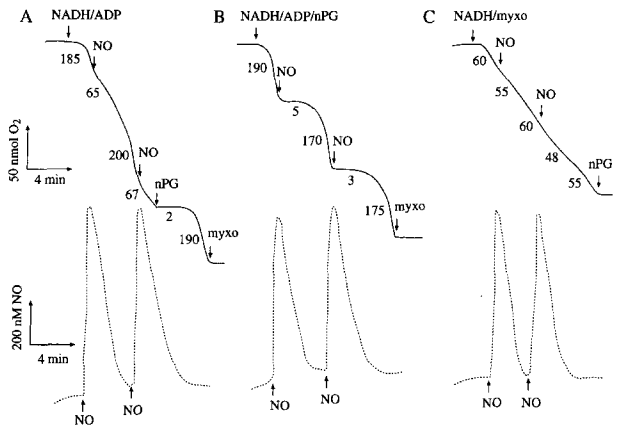

Fig. 1. Reversible effect of NO on cytochrome and alternative pathway activity in soybean cotyledon mitochondria. Oxygen consumption and NO production measured as described in Section 2. (A) Uninhibited respiration (+ADP); (B) cytochrome pathway respiration in the presence of 50 μM nPG (+ADP); (C) alternative pathway respiration in the presence of 1 μM myxothiazol (myxo) and 1 mM pyruvate. NADH was added at 1 mM; rates on traces are in nmol O_2 min^{-1} mg^{-1} protein. Typical traces of one of three experiments are shown.

fully inhibited by added nPG, showing that the flux of electrons in the presence of NO was directed through AOX. Similar results were obtained when comparing the NO sensitivity of cytochrome and alternative pathway respiration in soybean root mitochondria and sweet potato mitochondria, and with other respiratory substrates (data not shown). Similar results were also obtained using sodium nitroprusside (0.3 mM) that decays forming NO in the presence of light (data not shown).

Respiration via the cytochrome pathway in the presence or absence of ADP was substantially inhibited by NO in the nM range (Fig. 2). Half inhibition of respiration in the presence of ADP was apparent at approx. 50 nM NO and in the absence of ADP at approx. 150 nM NO. In contrast, respiration via the alternative oxidase was much less inhibited, with 80% of the control rate observed at 900 nM NO (Fig. 2). All inhibitions were fully reversed after the complete decay of NO in the chamber. All measurements were taken at 100–150 μM dissolved O_2 as the extent of NO inhibition is dependent on the O_2 concentration (data not shown).

Nitric oxide is produced in plant cells when nitrite accumulates [10–12]. Addition of $NaNO_2$ (5 mM) to a suspension of soybean mitochondria had little effect on O_2 consumption rate via the cytochrome pathway or NO concentration in the reaction chamber (Fig. 3A). Subsequent addition of the common cellular reductant, ascorbate (10 mM), caused an increase in NO concentration in the reaction chamber to 60 nM and a gradual decrease in O_2 consumption. A further addition of $NaNO_2$ (15 mM) lowered O_2 consumption to 12% of the initial rate as the NO concentration rose to 230 nM. In similar assays, oxygen consumption rate via both the cytochrome and the alternative pathway was measured at a standard O_2 concentration (100–150 μM) before and 5 min after the addition of ascorbate (10 mM) in the presence of varying $NaNO_2$ (0–20 mM) (Fig. 3B). A small (17%) decrease in cytochrome pathway respiration was observed over the $NaNO_2$ concentration range in the absence of ascorbate, while AOX activity was unaffected. In the presence of ascorbate, 20 mM $NaNO_2$ inhibited O_2 consumption via the cytochrome pathway by 85% but had only a slight effect on AOX activity.

4. Discussion

Cytochrome pathway activity in plant mitochondria was found to be inhibited to a much greater extent than AOX activity by NO (Figs. 1–3). The degree of inhibition of cytochrome oxidase depended on dissolved oxygen concentration as observed in mammalian mitochondria [3]. The full reversal of this inhibition upon decay of NO has also been observed previously in animal mitochondria. Irreversible effects of NO on mitochondrial dehydrogenases were not observed here and have recently been shown to be due to peroxynitrite formation in vivo rather than NO per se [5]. The specificity of NO is analogous to the well-known effect of carbon monoxide (CO) as a competitive inhibitor of the cytochrome oxidase but not the AOX in plant mitochondria [18]. The greater inhibition of cytochrome pathway activity in the presence than in the absence of ADP at low NO concentrations (Fig. 3) is probably due to a greater control coefficient for cytochrome *c* oxidase during oxidative phosphorylation than when proton leak rate becomes a controlling factor in the absence of ADP. Similar conclusions have been drawn from observations of NO inhibition in rat heart mitochondria [19].

Inhibition of the cytochrome pathway in plant mitochondria is known to re-direct electron flow to the alternative

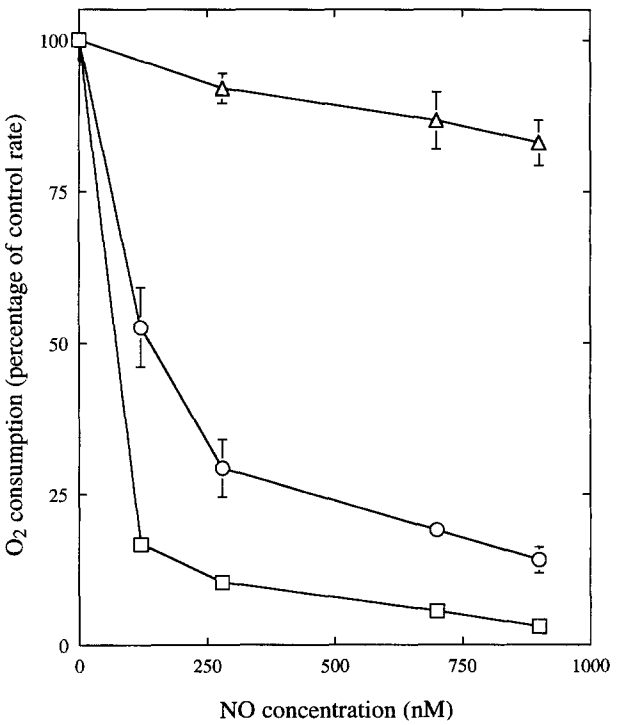

Fig. 2. Effect of varying NO concentrations on cytochrome and alternative pathway activity in soybean cotyledon mitochondria. Oxygen consumption and NO production measured as described in Section 2. (□) Cytochrome pathway respiration in the presence of 50 μM nPG (+ADP); (○) cytochrome pathway respiration in the presence of 50 μM nPG (−ADP); (△) alternative pathway respiration in the presence of 5 μM myxothiazol and 1 mM pyruvate. Control rates (nmol O_2 min^{-1} mg^{-1} protein) were as shown in Fig. 1. NADH was added at 1 mM. Data points are mean values ± S.D. ($n = 3$); S.D. is smaller than point symbol where error bars are not visible.

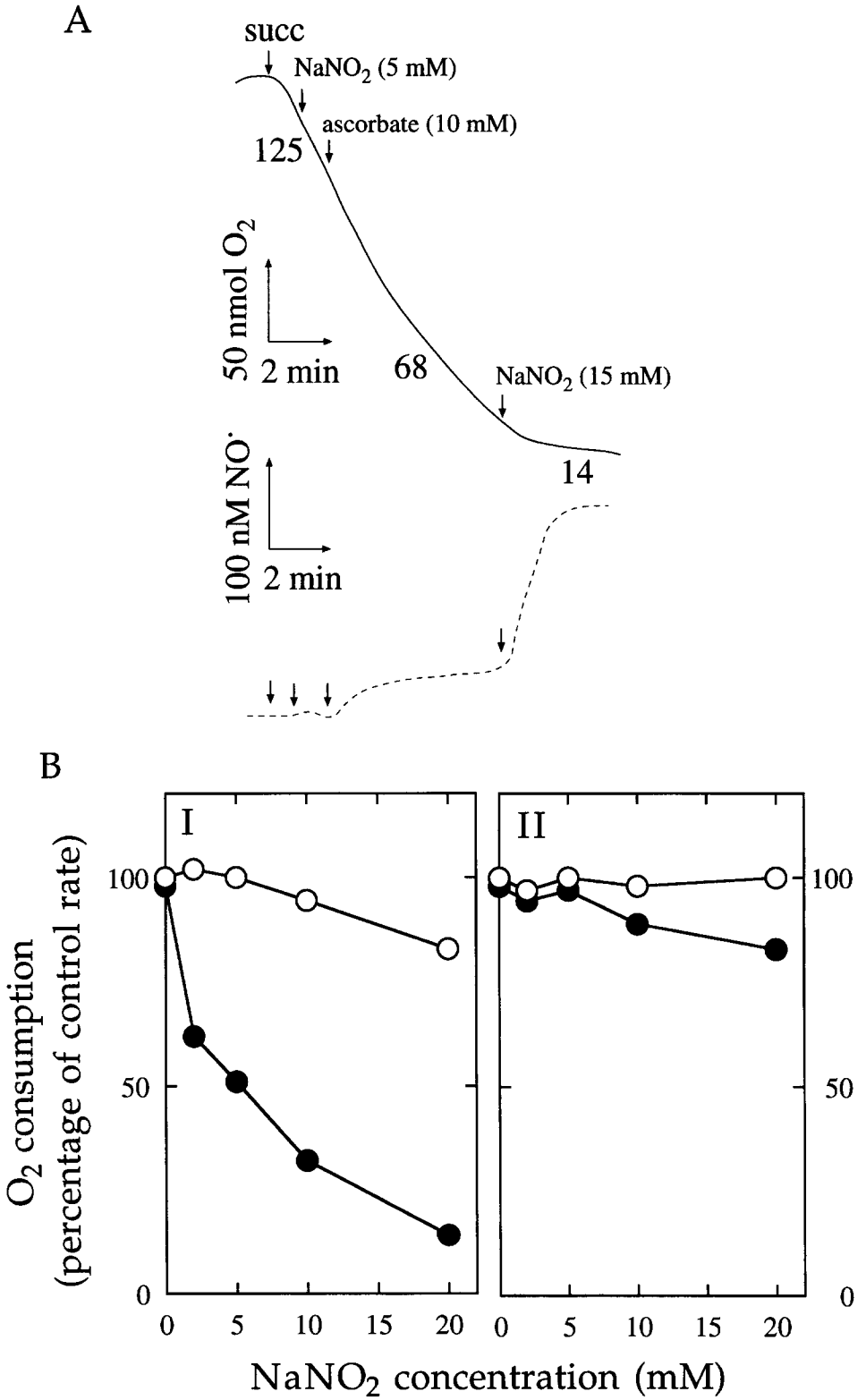

Fig. 3. Effect of NO_2^- and ascorbate on NO production and respiration via the cytochrome and alternative respiratory pathways in soybean cotyledon mitochondria. Oxygen consumption and NO production measured as described in Section 2. (A) Trace of typical experiment on the cytochrome pathway; numbers are nmol O_2 min^{-1} mg^{-1} protein. (B) Inhibition of cytochrome (I) and alternative (II) pathway O_2 consumption as a function of $NaNO_2$ concentration (0–20 mM) in the presence (●) and absence (○) of ascorbate (10 mM). Control rates were 125 nmol O_2 min^{-1} mg^{-1} protein in (I) and 55 nmol O_2 min^{-1} mg^{-1} protein in (II). All assays included succinate (10 mM) and ATP (0.1 mM) and either nPG (50 μM) (cytochrome pathway assays) or myxothiazol (1 μM) and pyruvate (1 mM) (alternative pathway assays).

pathway by an increased reduction of the ubiquinone pool [20]. In Fig. 1A, oxygen consumption in the presence of NO occurred clearly via AOX, as shown by its sensitivity to added nPG. The production of NO in plants could shift partitioning

of respiratory electron transport towards AOX and thereby alter the efficiency of ATP synthesis. Partial inhibition of cytochrome pathway in vivo could also induce de novo synthesis of AOX, since treatments which inhibit cytochrome pathway activity, such as antimycin A, cold and inhibitors of mitochondrial protein synthesis, are known to induce synthesis of AOX in plants [21].

Our understanding of NO production by plants is very limited. In most cases NO production has been linked to the accumulation of nitrite in plant tissues [10–12]. NO can be formed non-enzymatically from nitrite by the action of common cellular reductants such as NADH and ascorbate [22]. The latter is found in millimolar concentrations in the chloroplast as well as the cytosol of plant cells [23,24]. In vitro, NO formation from millimolar nitrite concentrations in the presence of ascorbate is sufficient to inhibit cytochrome oxidase in isolated plant mitochondria (Fig. 3). Oxidised nitrogen gases (NO_x) such as NO_2 and NO are also found in many environments as atmospheric pollutants derived from industrial and natural sources [25,26]. These atmospheric $NO(_x)$ have largely negative effects on plant growth [26], and inhibition of respiration and oxidative phosphorylation may be one mechanism of this growth retardation. Future study of NO production by plants under physiological stress conditions and/or the impact of exogenous pollutant NO is needed to determine the significance of NO as a novel effector of plant growth and development.

The results observed here with plant mitochondria may have wider implications for organisms possessing homologous alternative oxidases. For example, parasitic trypanosomes have a KCN-insensitive alternative oxidase (TAO) which has a high degree of homology to the plant AOX sequences [27]. In procyclic trypanosomes, a single cytochrome oxidase acts as the terminal electron acceptor. Upon infection, trypanosomes differentiate into a specialised bloodstream-form, in which the classical cytochrome pathway is lost and replaced with TAO [28]. During infection, NOS activity is induced in host macrophages and NO levels increase rapidly [29]. Thus, TAO may be induced as an NO-insensitive oxidase during trypanosomiasis, and may be important for the ability of the parasite to avoid the host's immune response.

Acknowledgements: We would like to thank Dr. Ian Clark (ANU, Canberra, Australia) for the generous loan of the ISO-NO electrochemical sensor for the duration of this research. Dr. O.K. Atkin, Dr F.J. Bergersen and Professor J.T. Wiskich are thanked for helpful discussions. This research was supported by grants from the Australian Research Council to D.A.D., as well an Australian Postgraduate Award and a Ph.D. Assistantship from the Cooperative Research Centre for Plant Science held by A.H.M.

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