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ORIGINAL ARTICLE

Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria

Maria Stoimenova · Abir U. Igamberdiev · Kapuganti Jagadis Gupta · Robert D. Hill

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Abstract Mitochondria isolated from the roots of barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.) seedlings were capable of oxidizing external NADH and NADPH anaerobically in the presence of nitrite. The reaction was linked to ATP synthesis and nitric oxide (NO) was a measurable product. The rates of NADH and NADPH oxidation were in the range of 12–16 nmol min^{-1} mg^{-1} protein for both species. The anaerobic ATP synthesis rate was 7–9 nmol min^{-1} mg^{-1} protein for barley and 15–17 nmol min^{-1} mg^{-1} protein for rice. The rates are of the same order of magnitude as glycolytic ATP production during anoxia and about 3–5% of the aerobic mitochondrial ATP synthesis rate. NADH/NADPH oxidation and ATP synthesis were sensitive to the mitochondrial inhibitors myxothiazol, oligomycin, diphenyleneiodonium and insensitive to rotenone and antimycin A. The uncoupler FCCP completely eliminated ATP production. Succinate was also capable of driving ATP synthesis. We conclude that plant mitochondria, under anaerobic conditions, have a capacity to use nitrite as an electron acceptor to oxidize cytosolic NADH/NADPH and generate ATP.

Keywords Anaerobiosis · Mitochondria · *Hordeum vulgare* · Nitric oxide · Nitrite · *Oryza sativa*

M. Stoimenova · A. U. Igamberdiev · R. D. Hill (✉)
Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, R3T 2N2 Canada
e-mail: rob_hill@umanitoba.ca

K. J. Gupta
Julius-von-Sachs-Institut fur Biowissenschaften, Lehrstuhl Botanik 1, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany

Abbreviations

AOX	Alternative oxidase
COX	Cytochrome *c* oxidase
DPI	Diphenyleneiodonium
ETC	Electron transport chain
FCCP	Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone
Hb	Hemoglobin

Introduction

Hypoxic stress has pronounced effects on mitochondrial function, both from the perspective of oxygen limitation and from increased production of compounds that compete at the oxygen-binding site, such as NO. Mitochondria cannot support oxygenic respiration below oxygen levels saturating terminal oxidases. The K_m for oxygen for the alternative oxidase (AOX) is in the millimolar range (Millar et al. 1994; Affourtit et al. 2001) limiting its function under low oxygen conditions. It can, however, play a role in nitric oxide tolerance since it is up-regulated by NO and under these conditions, prevents NO-induced cell death (Huang et al. 2002). The K_m value (140 nM) for cytochrome *c* oxidase (COX) would allow operation of some oxygenic respiration at low oxygen concentrations, but the saturating oxygen concentrations required may be higher than expected if NO is present due to the sensitivity of the oxidase to NO at both the heme and copper sites of the complex (Mason et al. 2006) occurring at nanomolar concentrations of NO (Brown and Cooper 1994; Cooper 2003). Since NO can accumulate under severe hypoxia (Dordas et al. 2003), COX may not be operative at submillimolar and even millimolar

oxygen concentrations. This may explain the observed decrease in metabolic activity in response to low oxygen (Geigenberger et al. 2000). The situation is further complicated as COX is capable of converting NO to nitrite (Cooper 2002; Pearce et al. 2002).

Cytochrome *c* oxidase can also catalyze nitrite reduction to NO (Walters and Taylor 1965; Brudvig et al. 1980; Paitian et al. 1985). This reaction was previously considered non-physiological but, at very low oxygen concentrations when NO conversion to nitrite is suppressed, animal mitochondria may convert up to 70% of supplied nitrite (Kozlov et al. 1999, 2005). This occurs via an arginine-independent pathway (Lasca et al. 2006) although the mitochondrial NO synthase, which is dependent on oxygen, has been identified in both animal and plant mitochondria (Guo and Crawford 2005). Either reduced ubiquinone at the level of complex III (Kozlov et al. 1999; Nohl et al. 2001) or COX (Paitian et al. 1985) may be involved in the nitrite: NO reductase activity of mitochondria. The production of nitric oxide from nitrite by COX is significant under hypoxic conditions, both in yeast and rat liver mitochondria (Castello et al. 2006). Mitochondrial biogenesis is stimulated by NO (Nisoli et al. 2004) suggesting possible involvement of these organelles in NO metabolism.

The similarity of major complexes of plant and animal mitochondrial electron transport chains (ETCs) (Siedow and Umbach 1995) suggests that the observations obtained with animal mitochondria-linked nitrite/NO metabolic reactions should also apply to plants. In fact, algal mitochondrial data (Tischner et al. 2004) and studies with higher plants (Planchet et al. 2005) confirm that plant mitochondria can readily convert nitrite to NO in anaerobic conditions. This conversion likely occurs in root mitochondria of higher plants but not in shoot mitochondria (Gupta et al. 2005).

There is abundant evidence that mitochondria can function under strict anoxic conditions (Kennedy et al. 1987, 1992; Fox and Kennedy 1991). Exposure to anoxia results in some changes in enzyme composition in mitochondria (Couee et al. 1992), but mitochondria preserve their ultrastructure and functionality, particularly when anaerobic plants are exposed to nitrate (Vartapetian and Polyakova 1999; Vartapetian et al. 2003). The latter observation was interpreted as an indication of the role of nitrate as a terminal electron acceptor under anoxia (Polyakova and Vartapetian 2003). Another interpretation is that nitrate is a part of a more extensive cycle where nitrite serves as an intermediate electron acceptor by supporting NADH oxidation (Igamberdiev and Hill 2004). An extension to this interpretation is the possibility that mitochondrial nitrite reduction to NO may be linked to ATP synthesis contributing to the functionality of these organelles in anoxic conditions (Igamberdiev et al. 2005).

In the present study, we explored the functionality of barley and rice mitochondria under argon/ nitrogen atmospheres. We demonstrate that mitochondria readily oxidize NADPH and NADH in anoxia via externally facing dehydrogenases, using nitrite as an electron acceptor and forming NO, resulting in the production of ATP.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L., cv Harrington) was germinated for 4–5 days and roots were used for the isolation of mitochondria. Rice (*Oryza sativa* L. ssp *japonica* cv Nihanmasari) was germinated by submerging in water for 2 weeks, at which point the roots were 4–6-cm long.

Isolation of mitochondria

The isolation procedure followed previously published methods (Nishimura et al. 1982; Vanlerberghe et al. 1995) with slight modifications. All steps were carried out at 4°C. Four day-old barley roots (40–50 g) were sliced and homogenized with 250 ml cold extraction buffer using a Polytron homogenizer (Brinkman Instruments, ON, Canada). Extraction buffer contained 0.4 M mannitol, 0.2% BSA (fatty acid free), 2 mM $MgCl_2$, 1 mM EDTA, 25 mM cysteine, 1% PVP, and 0.25 mM $CaCl_2$ in 0.1 M Hepes-KOH, pH 7.4. The bray was filtered through two layers of Miracloth (Calbiochem) and the extract was centrifuged twice at 1,000*g* for 10 min each time to remove the debris followed by centrifugation at 10,000*g* for 30 min. The resulting pellet was resuspended in 4 ml of the suspension buffer (0.1 M Hepes-KOH, pH 7.4 containing 0.3 M mannitol, 2 mM $MgCl_2$, 1 mM EDTA and 0.1 mM $CaCl_2$). The mitochondria were further purified on a discontinuous Percoll gradient composed of 6 ml of 60% (v/v), 10 ml of 45% (v/v), 10 ml of 28% (v/v) and 10 ml of 5% (v/v) Percoll, all layers containing 250 mM sucrose and 20 mM Hepes, pH 7.4. The mitochondria (2 ml) were layered on the top, and the gradient was centrifuged at 30,000*g* for 45 min. The mitochondrial fraction was collected from the interface between the 45 and 28% layers and washed three times in the suspension buffer to remove Percoll. To check mitochondrial functionality and respiratory control,

respiration was followed polarographically at 25°C in 2 ml suspension buffer containing 0.5 mM ADP and 0.5 mM KH_2PO_4 and mitochondrial protein (0.5 mg). In some experiments, in order to improve the stability of isolated mitochondria, the extraction and wash buffers contained superoxide dismutase and catalase (both 12,000 U l^{-1}) (Brewer et al. 2004). Oxygen uptake was initiated by addition of 0.5 mM NADH, NADPH or 10 mM succinate. Protein content of mitochondria was determined by the Bradford (1976) method.

Measurement of the mitochondrial functions in aerobic and anaerobic conditions

Aerobic measurements

Mitochondrial reactions were performed in suspension buffer containing 0.5 mM ADP, 0.15 mM NADH or NADPH or 10 mM succinate, 0.5 mM KH_2PO_4 and 50 μM P^1, P^5-di (adenosine 5′) pentaphosphate, to inhibit adenylate kinase. Reactions were initiated by the addition of 50 μl mitochondrial suspension (0.5–2 mg protein) to the suspension buffer to give a final volume of 2 ml. Nitrite (100 μM), uncouplers or ETC inhibitors were added as indicated in the tables and figures. The samples were mounted on a shaker running at 150 rpm to ensure optimal oxygen saturation. Reactions were terminated by adding an aliquot of the reaction mixture to 10% TCA, for ATP measurements, or 0.2 M KOH, for NADH/NADPH consumption measurements.

Anaerobic measurements

The reaction medium was identical to that for aerobic measurements, with the exception that 100 μM nitrite, uncouplers or ETC inhibitors were added as indicated in the text. Anaerobiosis was achieved prior to the addition of the mitochondrial suspension by degassing the medium for 15 min followed by equilibration with argon gas. This was accomplished by placing the samples in an air tight chamber mounted on a shaker running at 150 rpm. The chamber was connected to an argon gas tank via a humidifying system at a gas flow of approximately 1 l min^{-1}. The equilibration time was 30 min. After equilibration, 50 μl of argon pre-flushed mitochondrial suspension was injected into each sample. To estimate oxygen concentrations the absorbance at 580 nM was determined in reaction medium containing 50 μM sperm whale myoglobin (Sigma). Oxygen concentrations were estimated from the proportion of myoglobin present in the deoxy form (Nichols and Weber 1989). After argon flushing, 94 ± 3% ($n = 3$) of the myoglobin was in the deoxy form, indicating solution oxygen concentrations below 0.1 μM. The reaction was terminated by injecting an equal volume of 10% TCA (for ATP measurements) or 0.2 M KOH (for NADH or NADPH measurements) into the samples. The following inhibitors were used: rotenone (10 μM) to inhibit the mitochondrial complex I, oligomycin (1 μM) to inhibit the mitochondrial ATP synthase, antimycin A (25 μM) and myxothiazol (25 μM) to inhibit the complex III at different sites, KCN (2 mM) to inhibit cytochrome c oxidase, the uncoupler FCCP (10 μM), and diphenyleneiodonium (DPI) (10 μM) to inhibit non-proton pumping NAD(P)H dehydrogenases. In some experiments, 10 mM succinate was used as a substrate instead of NADH and NADPH.

NADH/NADPH measurements

The reaction of NADH or NADPH oxidation was allowed to proceed for 10–30 min after which the samples were fixed with 0.2 M KOH and compared to the samples incubated without nitrite. The NADH/NADPH content was measured spectrophotometrically at 340 nm after centrifugation of the samples at 15,000*g* for 10 min. The extinction coefficient of NADH and NADPH of 6.22 mM^{-1} cm^{-1} was used.

NO measurements

For the measurement of NO production from purified mitochondria, solutions were placed in small glass beakers of suitable size, located in a transparent lid chamber (1 l gas volume) mounted on a shaker. NO was measured by chemoluminescence detection as described (Planchet et al. 2005). In brief, a constant flow of measuring gas (purified air or nitrogen) of 1.5 l min^{-1} was pulled through the chamber and subsequently through the chemoluminescence detector (CLD 770 AL ppt; Eco-Physics, Dürnten, Switzerland; detection limit 20 ppt; 20 s time resolution) by a vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen (99%). The measuring gas (air or nitrogen) was made NO free by conducting it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm). Calibration was routinely carried out with NO free air (0 ppt NO) and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen; Messer Griesheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC−260; Tylan General, Eching, Germany) were used to adjust all gas flows.

The extent of NO production from mitochondrial contamination with nitrate reductase was determined by measuring nitrate reductase activity according to the method of Hageman and Reed (1980). Nitrite was assayed with Griess reagent after removing excess NADH with 10 μM phenazine methosulfate.

Nitrite determination

Nitrite was determined colorimetrically in mitochondrial suspensions subjected to boiling for 10 min. The vials containing approximately 4 ml of mitochondria (1.5 mg protein) were placed in a glass cuvette (1 l air volume) which was mounted on a rotary shaker. A constant flow of nitrogen 1.3 l min^{-1} was pulled through the cuvette. 0.5 mM nitrite and 1 mM NADH were added to the mitochondrial suspensions and at 0 and 30 min 1 ml of mitochondria were collected and reaction stopped by addition of 125 μl of 250 μM zinc acetate. The colorimetric determination of nitrite was carried out as described by Hageman and Reed (1980).

ATP assay

The 10% TCA-fixed mitochondrial suspensions were neutralized to pH 7.0, centrifuged at 10,000*g* for 5 min and the supernatant was diluted 1:10 with 100 mM Hepes-KOH (pH 7.4) for neutralization. An aliquot from each sample was placed in a luminometer tube containing 20 mM Hepes-KOH pH 7.4, 3 mM $MgCl_2$ and 10 μl luciferin–luciferase assay (Sigma, diluted according to the manufacturers instructions) to a total volume of 1 ml and measured using the Junior LB 9509 luminometer (Berthold Technologies, Germany) at a 10 s integration time.

Results

Aerobic properties of isolated mitochondria

The aerobic properties of mitochondria were tested polarographically for oxidation of NADH and NADPH. Mitochondria isolated from barley roots exhibited rates of NADH and NADPH dependent aerobic ATP synthesis of ~180 nmol min^{-1} mg^{-1} protein (Table 1). This rate was 70–80% inhibited by the ATP synthesis inhibitor, oligomycin, and reduced to very low values by addition of the uncoupler, FCCP. Respiratory control of Percoll-purified barley root mitochondria was 1.7–2.1, indicating that NADH and NADPH oxidation was relatively well coupled to ATP synthesis. Nitrite acted as a mild inhibitor of substrate-dependent ATP synthesis showing 15–20% inhibition (Fig. 1). In comparison to barley mitochondria, rice mitochondria exhibited much higher NADH and NADPH dependent aerobic ATP synthesis of ~600 nmol min^{-1} mg^{-1} protein (Table 1) with a similar respiratory control. Measurement of NADH oxidation spectrophotometrically and comparison of the rate of its oxidation with the rate of ATP synthesis resulted in the values of respiratory control of 1.7 ± 0.2 in barley and 1.8 ± 0.3 in rice ($n = 3$), comparable to the values obtained polarographically.

Table 1 Aerobic and anaerobic rates (nmol min^{-1} mg^{-1} protein) of ATP synthesis by barley and rice mitochondria in the presence of NADH, NADPH or succinate

Plant	Substrate	Aerobic	Anaerobic
Barley	NADH	183 ± 11	8.0 ± 1.7
	NADPH	165 ± 21	8.1 ± 0.3
	Succinate	105 ± 11	3.2 ± 1.8
Rice	NADH	674 ± 79	17.4 ± 1.7
	NADPH	605 ± 29	15.1 ± 2.2
	Succinate	433 ± 121	9.6 ± 2.4

Data are means ± SD of 3–4 independent measurements

Anaerobic NADH and NADPH oxidation

Anaerobic NADH and NADPH oxidation was dependent on nitrite. The rates of NADH and NADPH oxidation were similar for both plants, approaching 11–13 nmol min^{-1} mg^{-1} protein for barley and 12–16 nmol min^{-1} mg^{-1} protein for rice (Table 2). Rates of NADH and NADPH oxidation were highest in the presence of FCCP. There was no effect of rotenone on NADH oxidation, suggesting no involvement of complex I in the reaction (Table 2).

Nitrite disappearance and NO production

Nitrite disappearance in the presence of NADPH and NADH for barley and rice mitochondria under anaerobic conditions was 11 ± 4 nmol min^{-1} mg^{-1} protein ($n = 6$) for barley and 14 ± 5 nmol min^{-1} mg^{-1} protein ($n = 3$) for rice. Root mitochondria produced NO with NADH or NADPH and nitrite under anaerobic conditions. The results for rice are presented in Fig. 2, a similar graph for barley was presented earlier, see Gupta et al. (2005). The rates of NO production were two orders of magnitude lower than the rates of NAD(P)H oxidation, ATP synthesis or nitrite disappearance. Myxothiazol inhibited NO formation by 80% (Fig. 2a). KCN completely inhibited NO formation (Fig. 2b).

Nitrate reductase activity was not detectable in the mitochondrial preparations. Taking into account that the nitrite-dependent NO forming activity of nitrate

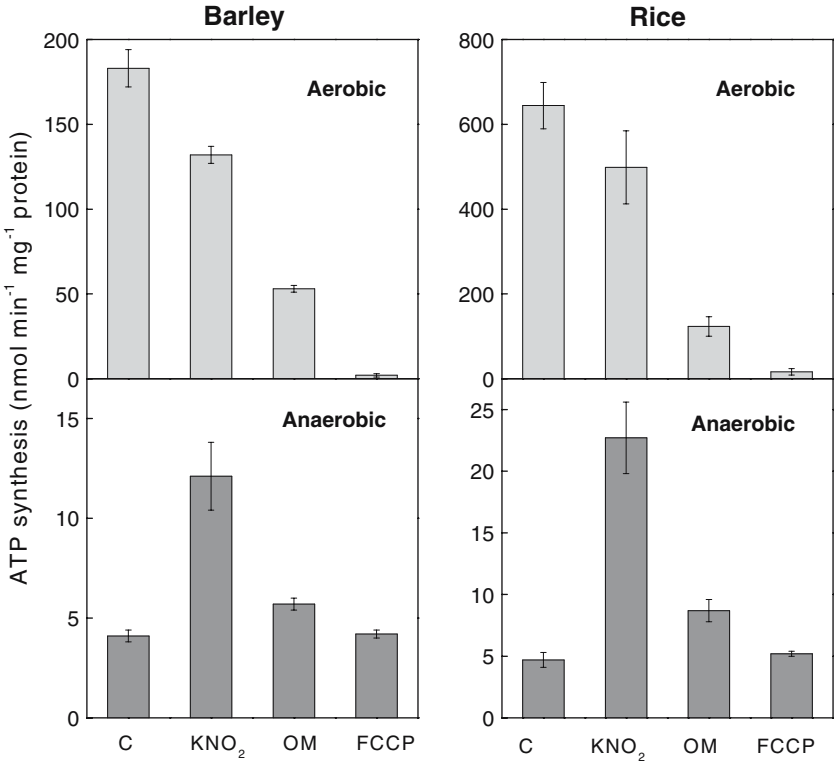

Fig. 1 Effects of 100 μM nitrite (KNO_2), 1 μM oligomycin (OM) and 10 μM FCCP on aerobic and anaerobic (argon) ATP synthesis by barley and rice mitochondria incubated with NADH

Table 2 Rates (nmol min^{-1} mg^{-1} protein) of NADH and NADPH oxidation by barley and rice mitochondria in anaerobic conditions using nitrite as electron acceptor

Plant	Substrate	No inhibitor	Rotenone	Oligomycin	FCCP
Barley	NADH	12.3 ± 1.4	11.5 ± 2.4	5.2 ± 0.5	21.0 ± 1.5
	NADPH	11.5 ± 0.8	9.2 ± 1.3	3.2 ± 0.9	12.9 ± 0.96
Rice	NADH	16.8 ± 2.8	16.1 ± 1.2	6.7 ± 1.9	22.1 ± 3.2
	NADPH	12.7 ± 2.7	11.8 ± 2.3	5.1 ± 2.1	14.9 ± 3.0

Data are means ± SD of 3–4 independent measurements

reductase is 1–2% of its activity with nitrate (Yamasaki and Sakihama 2000), it is highly unlikely that the mitochondrial NO production, obtained only under anoxia, results from nitrate reductase contamination of the mitochondria.

ATP synthesis

Barley and rice root mitochondria, incubated under argon, produced ATP during oxidation of NADPH and NADH. ATP synthesis was sustained by rice mitochondria for half an hour, while barley mitochondria ATP synthesis declined after 10–20 min (Fig. 3). There was no significant increase in ATP synthesis or mitochondrial stability when the mitochondria were isolated and incubated in the presence of superoxide dismutase and catalase. Whereas nitrite was required for anaerobic mitochondrial ATP synthesis, nitrite was partially inhibitory to aerobic mitochondrial ATP synthesis (Fig. 1). The basal rates of ATP production in the absence of nitrite were usually 3–5 nmol min^{-1} mg^{-1} protein. These rates were insensitive to the uncoupler FCCP (Fig. 1) and, therefore, not related to mitochondrial electron transport resulting from traces of oxygen but rather to incomplete inhibition of adenylate kinase by P^1, P^5-di (adenosine 5′) pentaphosphate or to other side reactions. In subsequent figures, basal ATP values have been subtracted. Nitrite-dependent net rates of ATP synthesis with either NADPH or NADH were 7–9 for barley mitochondria and 15–17 nmol min^{-1} mg^{-1} protein for rice mitochondria (Figs. 4, 5), resulting in approximately 0.7 ATP/ NAD(P)H for barley and 1.1 ATP/NAD(P)H for rice. Nitrite-dependent ATP synthesis was suppressed by the uncoupler, FCCP, or by the ATP synthase inhibitor, oligomycin. It was also inhibited by DPI, with the inhibition being stronger with NADPH, and myxothiazol. Rotenone had no effect. The anaerobic ATP synthesis of 2–4 nmol min^{-1} mg^{-1} protein observed in barley mitochondria with succinate as a substrate was low, but statistically significant (Table 1). In rice mitochondria it was in the range of 9–11 nmol min^{-1} mg^{-1} protein.

Discussion

Anaerobic use of nitrite as a terminal electron acceptor by mitochondria has been observed in some fungi, such

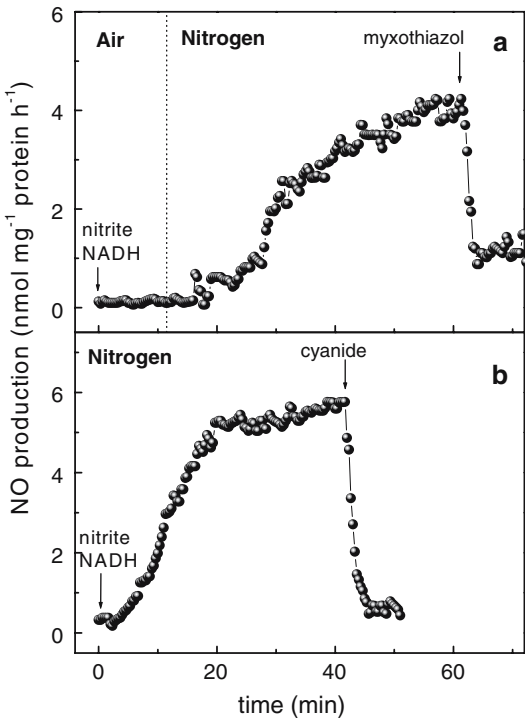

Fig. 2 Nitrite- (100 μM)- and NADH- (0.15 mM) dependent nitric oxide synthesis by rice mitochondria transferred to anoxic conditions. NO emission was measured in mitochondrial suspensions after addition of nitrite and NADH in air then switched to nitrogen (**a**) or NO emission was measured directly under nitrogen (**b**). Myxothiazol (25 μM) and cyanide (2 mM) were added as indicated

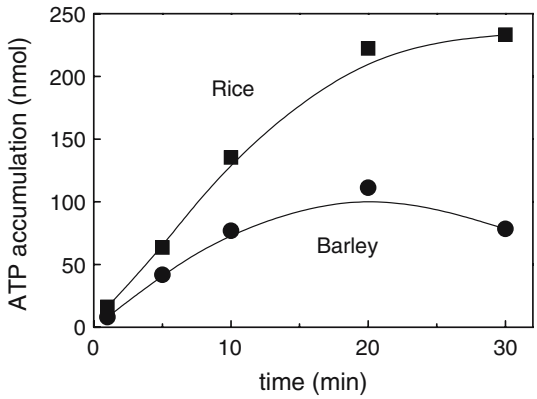

Fig. 3 Time course of nitrite-dependent anaerobic ATP accumulation by barley and rice mitochondria (1 mg mitochondrial protein)

as *Fusarium* (Kobayashi et al. 1996) and in ciliate protists (Finlay et al. 1983). These mitochondria use a nitrite reductase, which derives electrons from the cytochrome *c* pool (Tielens et al. 2002). In some species, the ubiquinone pool is the source of electrons for nitrate reductase (Zumft 1997). Nitrite reduction by the mammalian (Kozlov et al. 1999), algal (Tischner et al. 2004) and plant (Planchet et al. 2005) mitochondria

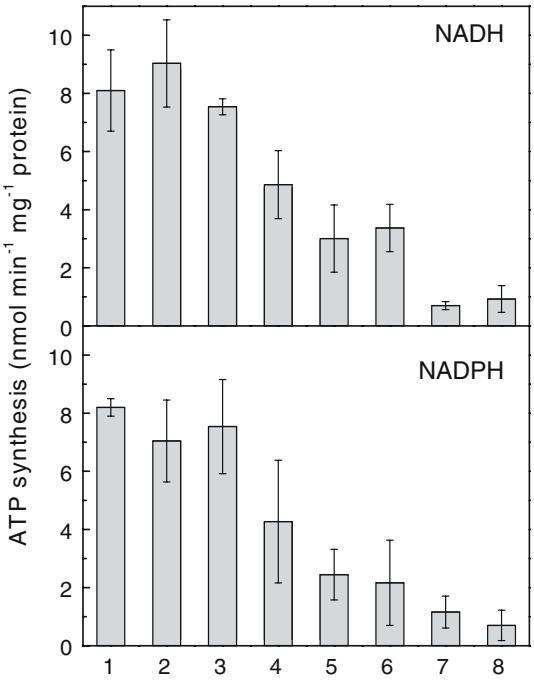

Fig. 4 Effect of inhibitors on nitrite-dependent ATP synthesis by barley mitochondria incubated under an argon atmosphere with NADH and NADPH as substrates. *1* none; *2* rotenone (10 μM); *3* antimycin A (25 μM); *4* myxothiazol (25 μM); *5* DPI (10 μM); *6* KCN (2 mM); *7* oligomycin (1 μM); *8* FCCP (10 μM). Values corrected for ATP synthesized in the absence of nitrite

is strongly anaerobic and likely does not involve additional enzymes associated with the mitochondrial ETC. For higher plant mitochondria, there were several indications of their functionality during anaerobiosis (reviewed in Kennedy et al. 1992) including anaerobic conditions of early germination (Logan et al. 2001). However no direct role of higher plant mitochondria in anaerobiosis has been suggested until it was demonstrated that root, but not shoot mitochondria, can produce NO using nitrite and NADH (Planchet et al. 2005; Gupta et al. 2005). This finding pointed to the mitochondria as a source of anaerobic NO and suggested that anoxic NADH and/or NADPH oxidation may also be carried out by mitochondria. The rates of nitrite disappearance are of the same order of magnitude as the rates of nitrite-dependent NADH and NADPH oxidation (Table 2) and ATP synthesis (Table 1; Figs. 4, 5). NO emission (Fig. 2) is affected by inhibitors in a similar fashion to ATP synthesis (Figs. 4, 5) and NAD(P)H oxidation (Table 2), indicating that NO is a product of the reaction.

The effect of inhibitors (Figs. 4, 5) shows that, similarly to anaerobic oxidation of substrates by animal mitochondria (Kozlov et al. 1999) and similarly to NO production by plant mitochondria (Planchet et al. 2005 and Fig. 2 of this paper), anaerobic nitrite-dependent

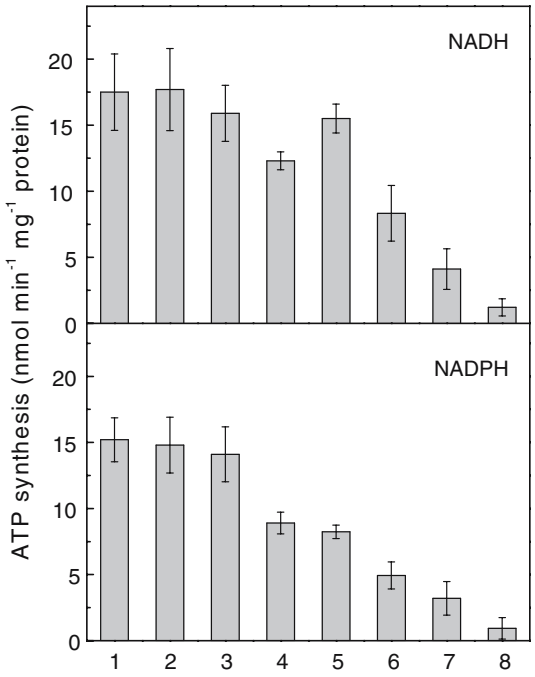

Fig. 5 Effect of inhibitors on nitrite-dependent ATP synthesis by rice mitochondria incubated under argon atmosphere with NADH and NADPH as substrates. Values corrected for ATP synthesized in the absence of nitrite. Abbreviations are the same as in Fig. 4

operation of plant mitochondria (NO production and ATP synthesis) is suppressed by myxothiazol and insensitive to antimycin A. The two inhibitors act at different sites of complex III. Myxothiazol binds to the Q_o site of the complex and prevents reduction of FeS protein from ubiquinol, while antimycin A inhibits electron transfer at the Q_i site, from heme b_H to oxidized Q in the Q cycle. In aerobic conditions, antimycin A bypass can result in superoxide formation (Muller et al. 2002) while under anoxia it can lead to nitrite reduction (Kozlov et al. 1999). The possibility of an antimycin A bypass in anaerobic conditions has been demonstrated (Muller et al. 2002). Nitrite reduction may also occur at cytochrome *c* oxidase in complex IV, since KCN inhibits ATP production and NO synthesis even more effectively (Figs. 2, 3, 4). The nitrite: NO reductase reaction of cytochrome *c* oxidase has been well established (Cooper 2002). The reaction becomes important during anaerobiosis when the protein is reduced (Castello et al. 2006). There are, thus, two plausible sites of nitrite reduction, one at complex III and the other at complex IV (Fig. 6). Both complexes are proton pumping. At the level of complex III, nitrite reduction may be accompanied by nitrite reaction with NO to give nitrous oxide, a reaction that has

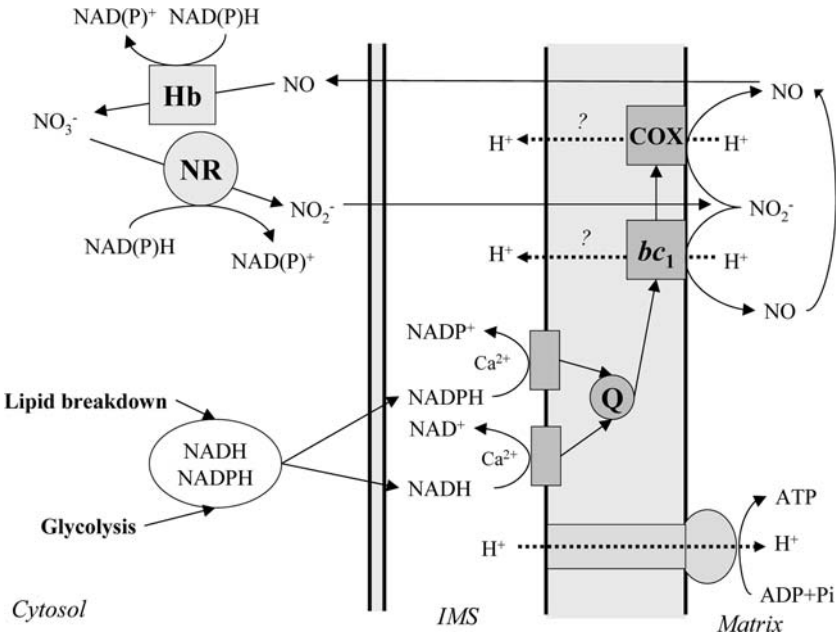

Fig. 6 Operation of plant mitochondria under hypoxic conditions. Glycolytic fermentation and lipid breakdown in hypoxia result in the increase of cytosolic NADH and NADPH. Externally facing Ca^{2+}-dependent mitochondrial dehydrogenases oxidize NADH and NADPH and transfer electrons to ubiquinone (*Q*). At levels of oxygen below saturation of cytochrome *c* oxidase (*COX*), nitrite can serve as an alternative electron acceptor at the sites of complex III and COX. Nitric oxide (*NO*) formed in this reaction is converted by hypoxically induced hemoglobin (*Hb*) to nitrate (NO_3^-). The latter is reduced to nitrite (NO_2^-) by hypoxically induced nitrate reductase (*NR*). ATP is synthesized due to proton pumping possibly at the sites of complex III (bc_1) and COX. *IMS* intermembrane space of mitochondria

been well characterized in bacterial systems (Stouthamer 1991). A reaction of this type could explain the observed lower yields of NO when compared to NAD(P)H oxidized and nitrite reduced.

There are two externally facing rotenone-insensitive and non-proton pumping dehydrogenases on the inner membrane of plant mitochondria, one specific to NADH and another to NADPH. They can be distinguished by sensitivity to DPI (Roberts et al. 1995) and nitrogen supply affects expression of both dehydrogenases (Escobar et al. 2006). Mitochondrial oxidation of cytosolic NADH and NADPH occurs via these dehydrogenases, excluding the possibility of a proton gradient forming at the site of electron transport from NAD(P)H to ubiquinone (Møller and Lin 1986; Møller 1997). We show that NAD(P)H oxidation and ATP synthesis is insensitive to rotenone, but sensitive to DPI, suggesting that complex I does not participate in anaerobic NAD(P)H oxidation. There is evidence to support the involvement of external NADPH and NADH dehydrogenases in mitochondrial electron transport (Roberts et al. 1995). Thus, a key feature of the operation of mitochondria under anoxia is their oxidation of extra-mitochondrial NAD(P)H. The high nucleotide K_m and Ca^{2+} dependence of externally-facing NADH and NADPH dehydrogenases (Møller 1997) suggests that they would have a major function when extramitochondrial NAD(P)H and Ca^{2+} concentrations are elevated. Such conditions are observed under hypoxia (Subbaiah et al. 1998). Furthermore, nitric oxide stimulates the release of Ca^{2+} from mitochondria (Richter 1997), while external NADH and NADPH oxidation by mitochondria plays an important role in seed germination under hypoxic conditions (Logan et al. 2001; Howell et al. 2006).

Although the oxidation of cytosolic NADPH and NADH is necessary to regulate the redox state of the anaerobic plant cell, the maintenance of ATP synthesis in anaerobic conditions is particularly important, since glycolytic ATP provides only a minor proportion of the ATP compared to that synthesized aerobically in the mitochondrial ETC. Oxidation of cytosolic NAD(P)H can generate sufficient membrane potential to allow ATP synthesis at rates that are nearly stoichiometrical to rates of NAD(P)H oxidation in rice, but with somewhat lower rates in barley mitochondria. This may be the result of the higher stability of rice mitochondria under anoxia leading to their more prolonged functionality and better coupling in anaerobic conditions.

The question arises as to whether ATP synthesis of ~8 nmol min^{-1} mg^{-1} mitochondrial protein for barley (Fig. 4) and ~17.5 nmol min^{-1} mg^{-1} for rice (with NADH) (Fig. 5) is significant, relative to the energetic demands of plant cells under hypoxic conditions. ATP turnover under anoxia has been estimated at 300–400 nmol h^{-1} mg^{-1} protein in rice coleoptiles and 260 nmol h^{-1} mg^{-1} protein in maize root tips (Greenway and Gibbs 2004). Let us assume an average rate of ATP turnover in germinating seedlings of 300 nmol h^{-1} mg^{-1} or 5 nmol min^{-1} mg^{-1} total protein. Mitochondrial protein represents 3.5% of the total protein in pea leaves (Neuburger et al. 1996) and 6–7% in heterotrophic plant cells (Douce 1985). The latter estimation is probably more realistic for root tissues since, in leaves, chloroplast protein accounts for ~65% of the total protein (Neuburger et al. 1996). Assuming a value of 7%, the hypoxic rates of ATP turnover will be ~70 nmol min^{-1} mg^{-1} mitochondrial protein. The rates of anaerobic, nitrite-driven mitochondrial ATP synthesis would, therefore, represent 11.5% of the ATP turnover rates in barley and 25% of those in rice during hypoxia. This contribution may even be higher since the mitochondrial volume in maize roots was determined to be 10% of the total cellular volume (Subbaiah et al. 1998). Taking into account the high protein content of mitochondria, mitochondrial protein could comprise as much as 10% of the total protein. This would increase the anaerobic mitochondrial contribution to ATP turnover to be 35% for rice.

The importance of nitrate reduction under hypoxic conditions has been questioned (Gibbs and Greenway 2003). The present results may help answer some of these questions. Anaerobic mitochondrial NAD(P)H oxidation and ATP synthesis as an alternative to glycolytic fermentation has some advantages, if it operates in conjunction with a means of removing potentially toxic products such as NO. The hemoglobin/NO cycle (Igamberdiev et al. 2004, 2006) could function in this regard. Anaerobic mitochondrial ATP synthesis, in conjunction with an operative hemoglobin/NO cycle, could contribute to the oxidation of glycolytic NADH, with the pyruvate being converted to alanine (Kennedy et al. 1992). However the citric acid cycle may be partially operative during anaerobiosis (Kennedy et al. 1987; Fox and Kennedy 1991), so if succinate and other citric acid cycle substrates can indeed be oxidized in anaerobic conditions in mitochondria, this may contribute to further oxidation of glycolytic substrates.

In conclusion, we have shown that plant mitochondria are capable of anaerobic ATP synthesis with NADH and NADPH as electron donors and nitrite as a terminal electron acceptor (Fig. 6). The anaerobic rate with the internal mitochondrial substrate (succinate) is detectable at least in rice mitochondria. The rates of anaerobic ATP production are only a few percent of the aerobic rate and the yield (in rice) is about 1

ATP per NAD(P)H oxidized while in barley it is lower (0.7), with ATP production linear for only 10 min. This suggests that mitochondrial operation under anaerobic conditions may be more sustainable in the hypoxia-resistant plant (rice) than in the hypoxia-sensitive plant (barley). This mitochondrial anaerobic process may contribute to the oxidation of reduced pyridine nucleotides formed in the cytosol during glycolysis and in lipid breakdown, but it may also contribute to the oxidation of the intramitochondrial substrates, particularly in rice. NO is, at least, one of the products of nitrite reduction.

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