REVIEW ARTICLE

Nitrate, NO and hemoglobin in plant adaptation to hypoxia: An alternative to classic fermentation pathways

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Abstract

The role of nitrate reduction to produce nitric oxide (NO) and its subsequent oxidation by oxyhemoglobin as a mechanism to maintain plant cell energetics during hypoxia is examined. Nitrate reduction in hypoxic conditions can be considered as an alternative respiratory pathway with nitrate as an intermediate electron acceptor, contributing to oxidation of NADH. NO, produced in the reaction, does not accumulate due to the induction of hypoxia-induced (class 1) hemoglobins. These hemoglobins remain in the oxyhemoglobin form, even at oxygen tensions two orders of magnitude lower than necessary to saturate cytochrome *c* oxidase. They act, likely in conjunction with a flavoprotein, as NO dioxygenases converting NO back to nitrate, consuming NAD(P)H in the process. The overall system oxidizes 2½ moles of NADH per one mole of nitrate recycled during the reaction, leading to the maintenance of redox and energy status during hypoxia and resulting in reduced production of ethanol and lactic acid.

Abbreviations: cNR: cytosolic nitrate reductase; Hb: hemoglobin; Ni-NOR: nitrite: NO reductase; NO: nitric oxide; PM-NR: plasma membrane-bound nitrate reductase

Key words: glycolysis; hemoglobin; hypoxia; nitrate reductase; nitrite reductase; nitric oxide

Introduction

ATP synthesis, required to maintain plant growth and viability in an aerobic environment, is achieved by oxidation of carbon sources, using oxygen as an electron acceptor. Under conditions that limit oxygen availability, complete substrate oxidation is restricted by the lack of an electron acceptor. Production of ethanol and lactic acid through well-known fermentation pathways is one mechanism organisms use to provide glycolytic substrate oxidation and ATP synthesis, maintaining short term cell viability under hypoxic conditions. There is evidence now of a second process operating that may be a critical factor for plant survival in hypoxic environments (Dordas *et al.* 2003a,b, 2004; Igamberdiev *et al.,* 2004b). The process (Figure 1) involves a stress-induced (class 1) hemoglobin, with a high affinity for oxygen, and nitric oxide, produced via nitrite reduction. NADH oxidation is achieved in the reactions forming NO and its subsequent oxidation back to nitrate. The high affinity of the hemoglobin for oxygen facilitates the cyclic process, even at extremely low oxygen tensions. In this review, we summarize the experimental evidence that lends support to this hypothesis.

Nitrate uptake and anaerobiosis

The switch that occurs from the aerobic tricarboxylic acid cycle to fermentation pathways, forming lactate and ethanol, under hypoxic or anoxic conditions is accompanied by a decline in pH due to ATP hydrolysis and lactate accumulation (the latter may be out of step with cytoplasmic

acidification and even not occur in some hypoxic tissues) (Saint-Ges *et al.,* 1991; Kennedy *et al.,* 1992; Ratcliffe, 1995; Gout *et al.,* 2001). The decline in pH slows the rate of lactate formation (Hanson and Jacobsen, 1984) and activates pyruvate decarboxylase, diverting glycolytic carbon flow to ethanol formation (Kennedy *et al.,* 1992). Alanine, formed via amination of pyruvate, is second only to ethanol as a product of glycolytic flux during hypoxia (Gibbs and Greenway, 2003). Unlike ethanol formation, formation of alanine does not consume NADH, potentially leading to a decline in glycolytic flux due to the unavailability of an electron acceptor.

A portion of the requirement for an electron acceptor may be supplied via nitrate reduction, providing the NH_4^+ required in the transamination reaction forming alanine. Evidence that ^{15}N, supplied as exogenous $^{15}NO_3^-$, appears in amino acids (Reggiani *et al.,* 1993; Fan *et al.,* 1997), the *de novo* synthesis of enzymes of the NO_3^- reduction pathway (Mattana *et al.,* 1994) and the activation of nitrate reductase during hypoxia as a result of a decline in pH (Botrel and Kaiser, 1997) suggest that nitrate reduction plays an important role during hypoxia (Botrel *et al.,* 1996). Alkalization of the medium observed during nitrate uptake (Steffen *et al.,* 2001) may be linked to prevention of acidification of the cytosol in hypoxic conditions.

Estimates of the end products of nitrate reduction suggest that only 1.3-4% of NADH recycled during hypoxia is connected with the reduction of nitrate to NH_4^+ (Gibbs and Greenway, 2003). The same authors argue that products, such as alanine, have not been detected in sufficient amounts to consider them as alternatives to the classic fermentation products in accounting for glycolytic flux. There are, however, experimental estimates of

30% NAD recycling, possibly as a result of nitrate reduction (calculated from the observed decrease in ethanol production in nitrate-fed cells) (Fan *et al.,* 1997). In the very anoxia-tolerant *Echinochloa phyllopogon*, ethanol and lipid synthesis account for only 34% oxidation of NAD(P)H, while the rest can be linked to nitrate reduction (Fox *et al.,* 1994). A lower rate of ethanol production in rice coleoptiles supplied with NO_3^- compared to those supplied with NH_4^+ has been shown (Fan *et al.,* 1997). Considering NO as a possible product of nitrite reduction from nitrate reductase (Figure 1), it is likely that only a minor part of the nitrite formed during hypoxia will undergo reduction to ammonia (Figures 1 and 2) since nitrite reductase is inhibited under these conditions (Botrel *et al.,* 1996).

Electron microscopic studies of rice mitochondria during anaerobiosis clearly show a protective role of nitrate in maintaining membrane ultrastructure (Vartapetian and Polyakova, 1999; Vartapetian *et al.,* 2003). These ultrastructural observations show the positive effect of nitrate reduction on the functionality of the anaerobic plant cell. It has been noted that ATP is maintained in potato cells at a higher level under hypoxia in nitrate as opposed to ammonium ion medium (Oberson *et al.,* 1999; Rawyler *et al.,* 2002). These findings suggest that during inhibition of the mitochondrial electron transport chain by lack of oxygen, the presence of nitrate may have an influence, however indirect, on the functional state of mitochondria.

Alanine formation is strongly induced during hypoxia. There is no consumption of NADH in the process, while the 70% excess NADH due to alanine synthesis might have been recycled via nitrate reduction to ammonium (Gibbs and Greenway, 2003, assessed from Reggiani *et al.,*

1995). Ammonium ion, from nitrate reduction, can be incorporated into glutamate either via the GS-GOGAT system consuming NADPH and ATP or via reverse glutamate dehydrogenase consuming only NAD(P)H, a more favourable reaction in hypoxic conditions (Gibbs and Greenway, 2003). A portion of the glutamate can be converted to γ-aminobutyric acid, suggested to be an important component for pH regulation (Crawford *et al.,* 1994). The concentration of 2-oxoglutarate is maintained via a partial TCA cycle operating at elevated reduction levels in mitochondria (Igamberdiev and Gardeström, 2003) that are characteristic under hypoxic conditions. The decrease of 2-oxoglutarate dehydrogenase activity in anaerobically grown rice and *Echinochloa* while other TCA cycle enzymes were present at high levels (Fox and Kennedy, 1991) is in favor of 2-oxoglutarate accumulation. Figure 2 summarizes the metabolic pathways linked to nitrite reduction and the formation of ammonium ion. It should be noted that alanine appears as a product of nitrite reduction to ammonium ion and its formation strongly depends on the operation of nitrite reductase which is inhibited under hypoxia (Botrel *et al.,* 1996).

Regulation of nitrate reductases and NO formation

In roots, two distinct types of nitrate reductase are present, one located in the cytosol (cNR) and the other attached to the plasma membrane and facing the apoplast (PM-NR) (Stöhr and Ullrich, 1997; Stöhr and Mäck 2001). Estimates show that in general only one-third of root nitrate reduction can be attributed to cNR, while two-thirds may be due to PM-NR (Gojon *et al.,* 1986).

There is a 2.5 fold activation of cNR during exposure of plant roots to hypoxia (Botrel and Kaiser, 1997), with nitrite reduction being suppressed at the nitrite reductase step (Botrel *et al.,* 1996; Ferrari and Varner, 1971). The limitation of nitrite reduction is connected both with cellular acidification and with increased flux through nitrate reductase (Botrel and Kaiser, 1997; Botrel *et al.,* 1996). The potential maximum activity of activated nitrate reductase, although lower than alcohol dehydrogenase, exceeds the rate of hypoxic ethanol formation by more than three fold (Botrel and Kaiser, 1997). In Arabidopsis root cultures, two nitrate reductase genes were induced under low-oxygen (5%) pressure. The NR1 gene showed moderate induction after 0.5-4 h of hypoxia and strong induction after 20 h. The NR2 gene was strongly activated in 2-4 h and even more after 20 h (Klok *et al.,* 2002).

Nitrate reductase is inactivated by interaction of the phosphorylated form of the enzyme with 14-3-3 proteins (Huber *et al.,* 2002), while the enzyme is activated by dephosphorylation, catalyzed by cNR phosphatase. Changes in cNR activity measured *in vitro* are not always associated with changes in nitrate reduction rates *in vivo*, suggesting that the cNR can be under strong substrate and/or cofactor limitation. The degradation and half-life of the cNR protein also appears to be affected by cNR phosphorylation and 14-3-3 binding, as cNR activation always correlates positively with its stability (Kaiser and Huber, 2001).

In aerated roots, cNR is highly phosphorylated and largely inactive. It is partly dephosphorylated (activated) by anoxia or by cellular acidification (pH 4.8 plus propionic acid) (Botrel and Kaiser, 1997). Anaerobic activation of NR is about 2.5 fold greater at acidic external pH than at slightly alkaline pH,

although ATP levels decrease and AMP levels increase at pH 5 and at pH 8 to the same extent (Kaiser *et al.,* 1999). Thus, rapid changes in the cNR-phosphorylation state in response to anaerobiosis are not directly triggered by the adenylate pool, but rather by cytosolic pH. Although some authors state that an increase in cNR activity does not prevent ethanol formation (Botrel and Kaiser, 1997), the nitrate reductase-lacking plants of tobacco produced substantially more ethanol and lactate during anaerobiosis (Stoimenova *et al.* 2003) indicating that cNR may have the effect of directing hypoxic metabolism away from lactate and ethanol formation.

Plasma membrane nitrate reductase (PM-NR) activity was initially demonstrated by Huffaker's group (Ward *et al.,* 1988; Ward *et al.,* 1989; Meyerhoff *et al.,* 1994). It is present only in root tissue where it exceeds the activity of cNR, particularly during the night (Stöhr and Mäck, 2001). It can use both succinate and NADH, but succinate is the preferred electron donor. Taking into account succinate accumulation during hypoxia (Fan *et al.,* 2003) and the possibility of fumarate reduction back to succinate by succinate dehydrogenase in cooperation with complex I under accumulation of reduced ubiquinone (Cecchini, 2003), there is the possibility that the plasma membrane may have an important role in nitrate reduction during hypoxic conditions.

Plasma-membrane bound nitrite-NO reductase (Ni-NOR) is the likely enzyme that converts nitrite to NO rather than PM-NR. NiNOR faces the apoplast and has an activity sufficient to convert all of the nitrite formed by PM-NR to NO (Stöhr *et al.,* 2001). Ni-NOR uses reduced cytochrome *c* for nitrite conversion to NO (Stöhr *et al.,* 2001). Since

participation of cytochrome *c* at the plasma membrane is unlikely, it is possible that the physiological electron donor for this reaction could be either another cytochrome or Hb, induced under hypoxic condition (Figure 1). A hemeprotein oxidized during this reaction can be reduced by a protein possessing cytochrome reductase activity. The pH optimum of Ni-NOR is favourable for hypoxic conditions (6.1), and it can utilize even low amounts of nitrite (V_{max} is reached at a nitrite concentration of 100 μM) (Stöhr *et al.,* 2001).

There is a possibility that plant Ni-NOR may perform a similar function to the respiratory nitrite reduction of microorganisms (Stöhr and Ullrich, 2002) and mammalian mitochondria (Kozlov *et al.,* 1999). It is known that NO is a potent inhibitor of the mitochondrial cytochrome *c* oxidase (complex IV) (Zottini *et al.,* 2002). The alternative cyanide-resistant oxidase is NO-resistant, however its participation in hypoxic operation of mitochondria is unlikely because of its low affinity to oxygen (Huang *et al.,* 2002).

Modulation of the redox state of the plasma membrane in hypoxia is indicated by the formation of hydrogen peroxide, detectable by electron microscopy, indicating the possible operation of an NADPH oxidase (Blokhina *et al.,* 2001). Nitrate uptake can be regulated by plasmalemma redox activity (Steffen *et al.,* 2001). The H^+-ATPase of the plasma membrane and cytosolic nitrate reductase are both regulated by 14-3-3 proteins (Finnie *et al.,* 1999). Although it is not clear how nitrate uptake, NO formation and scavenging at the site of the plasma membrane can be related to the maintenance of ATP and NADH levels during hypoxia, there may be similar systems operating in plants to those that have been suggested in microorganisms (Jormakka *et al.,*

2003). Protonmotive force generation via a redox loop mechanism on the plasma membrane may include NAD(P)H oxidases linked putatively to PM-NR and/or Ni-NOR via cytochrome b_5 and phylloquinone found in plasmalemma preparations (Bridge *et al.,* 2000). Phylloquinone function may be similar to the function of mitochondrial ubiquinone (Lochner *et al.,* 2003). The presence of multiple redox proteins in plasma membrane vesicles (Bérczi and Møller, 2000) is in favour of such a hypothesis. Apart from the consideration of the development of a protonmotive force, there is the problem of utilizing that protonmotive force to generate of ATP. No ATP synthase has been identified in eukaryote plasma membranes. Furthermore, the ATPase of plasma membranes is a P-type ATPase, an unlikely candidate for ATP synthesis.

When nitrite accumulates, it can also be used by cNR as a substrate to produce NO. The K_m of cNR for nitrite is about 100 µM, which is higher than that of Ni-NOR, and the reaction is competitively inhibited by nitrate (K_i 50 µM) (Rockel *et al.,* 2002). There are controversial statements about the cNR reaction rate with nitrite to produce NO. The rate in legume nodules may even exceed the rate of nitrate reduction to NH_4^+ (Dean and Harper, 1988). Others show that the rate of NO formation is only 1-2% of the maximal cNR reaction in leaves (Yamasaki *et al.,* 2001; Rockel *et al.,* 2002; Sakihama *et al.,* 2002). When Ser^{521} is replaced by Asp, cNR is not phosphorylated and is permanently active. The rates of NO emission from tobacco plants expressing this mutated protein are tens times higher (in darkness and under hypoxia) than those in control plants (Lea *et al.,* 2004) suggesting that the reaction of the native protein is tightly regulated.

NO is an important metabolite, acting as a signal molecule in most biological systems. In particular, it is produced in animals (Bredt and Snyder, 1994) and plants (Dordas *et al.,* 2003b, 2004) during hypoxic stress. NO was shown to be formed in fairly large quantities in both alfalfa root cultures and maize cell cultures under hypoxic stress. In *Chlamydomonas reinhardtii* cells, addition of nitrite causes formation of NO with an NR reaction rate of 60 nmol mg^{-1}Chl min^{-1} (Sakihama *et al.,* 2002).

There are other possible, but less significant, sources of nitrate/nitrite reduction and NO formation during hypoxia. In acidic and reducing environments, NO can be formed by non-enzymatic reduction of nitrite to nitrous acid. The latter reacts with ascorbate producing dehydroascorbate and NO (Weitzberg and Lundberg, 1998). It was shown that in aleurone layers the conditions are favorable for non-enzymatic formation of NO (Bethke *et al.,* 2004). Xanthine oxidoreductase located in peroxisomes (Corpas *et al.,* 2001) is also an enzyme producing NO (Harrison, 2002). NO-generating activity of xanthine oxidoreductase with nitrite is increased at low oxygen tensions (Millar *et al.,* 1997; Godber *et al.,* 2000). It is likely that this enzyme produces superoxide at high oxygen concentrations, and NO at low oxygen concentrations.

In animals and microorganisms, the main source of NO is the enzyme NO synthase (Bredt and Snyder, 1994). The formation of NO during hypoxia via the NO synthase reaction is unlikely, since this enzyme consumes O_2. Evidence of the presence of NO synthase in plants has been controversial until recently (Butt *et al.,* 2003), when it was shown that a modified version of the P-protein of the glycine decarboxylase complex exhibits this activity

(Chandok *et al.,* 2003). The activity, however, is low and may be present only in tissues such as green leaves where glycine decarboxylase is abundant. Its role, therefore, likely involves signalling during pathogen attack. Another NO synthase involved in hormonal signalling has been identified, with no sequence similarity to any mammalian isoform (Guo *et al.,* 2003).

The rate of NO formation is reported to be in the range of 10-50 nmol g^{-1} FW h^{-1} under hypoxic conditions and 0.2-0.5 nmol g^{-1} FW h^{-1} under dark, aerobic conditions (Rockel *et al.,* 2002; Dordas *et al.,* 2003b). Ni-NOR rates in roots are above 0.5 (or even 5-10) µmol g^{-1} FW h^{-1} (estimated from Stöhr *et al.,* 2001), well above the range necessary to explain the observed NO rates of formation. The ability of plant cells to scavenge NO can be as high as 4-5 µmol NO g^{-1} FW min^{-1} measured in root extracts at 1 µM NO concentration (Igamberdiev *et al.,* 2004b), suggesting that measurements of NO formation *in situ* are likely to be underestimated. It also suggests that there is an effective system to break down NO in plant tissue, which has been proposed to involve hemoglobin (Dordas *et al.,* 2003a). It is also possible that under anaerobiosis, the mitochondrial cytochrome *c* oxidase can use NO as an electron acceptor. It has been suggested that the enzyme evolved from the anaerobic NO reductase family (De Vries and Schröder, 2002) and in higher organisms, including mammals, it can exhibit NO oxidase and peroxynitrite reductase activities (Borutaite and Brown, 1996; Pearce *et al.,* 2002). The importance of these activities in plant systems for maintaining anaerobic ATP synthesis has not been investigated. In anoxia-tolerant *Echinochloa*, there are indications of the possibility of nitrate reduction by mitochondria, similar to that in bacteria using cytochrome *d* that transfers electrons to nitrate (Fox *et al.,* 1994). A

corresponding absorbance band for such a cytochrome was identified, but these investigations have not been continued.

Role of hemoglobin

The expression of a hemoglobin gene accompanying hypoxia was first demonstrated in barley (Taylor *et al.,* 1994), in conjunction with the initial work on the existence of this type of hemoglobin in monocots. The properties of the Hb protein (Duff *et al.,* 1997) indicated that it did not likely function as a carrier, store or sensor of O_2 (Hill, 1998). While the main physiological function of the gene probably relates to conditions of oxygen starvation, Hb gene induction is more directly related to cell ATP status than to oxygen levels (Nie and Hill, 1997). Expression of the gene during hypoxia likely affects cell survival as overexpression of Hb in hypoxic maize cell cultures resulted in the maintenance of cell energy status (Sowa *et al.,* 1998). Barley class 1 hemoglobin is a homodimer with a monomeric molecular weight of 18 kDa (Duff *et al.,* 1997). Its affinity for O_2 is two orders of magnitude higher (2-3 nM) than that of cytochrome *c* oxidase (140 nM), evidence that it remains oxygenated at extremely low O_2 tensions. Other class 1 hemoglobins possess similar properties (Arredondo-Peter *et al.,* 1997; Hargrove *et al.,* 1997; Kundu *et al.,* 2003a). The unique features of class 1 hemoglobins result from the hexacoordination of the heme moiety in comparison to the pentacoordination which exists in hemoglobins such as erythrocyte, muscle hemoglobins and leghemoglobins. Hexacoordinate hemoglobins are capable of reversible

intramolecular coordination of the ligand binding site by way of an amino acid side chain from within the heme pocket (Trent *et al.,* 2001).

In addition to maintenance of cell energy status during hypoxia, class 1 hemoglobin expression leads to lower NAD(P)H/NAD(P)) ratios within the hypoxic cell (Igamberdiev *et al.,* 2004b). An involvement of hemoglobin in oxidation of NAD(P)H is very plausible. There are flavohemoglobins that catalyze the oxygenation of NO such as in *E. coli* (Poole and Hughes, 2000) or yeast (Zhu and Riggs, 1992), with the flavin domain providing the catalytic site for oxidation of reduced flavin and, indirectly, reduced pyridine nucleotide. These proteins, with pentacoordinated heme, have a relatively high oxygen affinity, in comparison to plant class 1 hemoglobins, and form nitrate only under aerobic conditions. There is no evidence of a flavin-binding domain in plant hemoglobins. Any analogous system in plants would, therefore, require hemoglobin to react in concert with another protein, either individually or as part of a dioxygenase complex. Oxyhemoglobin would donate negatively charged dioxygen to NO, forming nitrate and methemoglobin, a known reaction of oxyhemoglobin (Di Iorio, 1981). The reduction of methemoglobin to hemoglobin can occur in a number of ways. A methemoglobin reductase has been demonstrated in nodules of leguminous plants (Topunov *et al.,* 1980). A number of diaphorase-type enzymes, such as cytochrome b_5 reductase of the endoplasmic reticulum (Hagler *et al.,* 1979) or dihydrolipoamide dehydrogenase (Moran *et al.*, 2002; Igamberdiev *et al.,* 2004a), have methemoglobin reductase activity. Another possibility is the presence of this reaction in the hemoglobin molecule itself.

No example has yet been observed of an organism that has genes or expresses a flavohemoglobin with hexacoordinate heme properties (Kundu *et al.,* 2003b). Cyanobacteria*, Chlamydomonas,* all plants and most animal species contain hexacoordinate hemoglobins and no flavohemoglobins. This has led to the suggestion that hexacoordinate hemoglobins in these species may serve a similar role as that of flavohemoglobins in bacteria and yeast (Kundu *et al.,* 2003b). Participation of a truncated Hb which is hexacoordinated at alkaline pH is likely in NO scavenging in *Chlamydomonas* (Couture *et al.,* 1999).

In microorganisms, NO is scavenged by NO dioxygenase (NOD), which is a flavohemoglobin possessing NAD(P)H-dependent enzymatic activity (Gardner *et al.,* 1998; Gardner *et al.,* 2000). The NOD reaction is described by the equation

$$2NO + 2O_2 + NAD(P)H \rightarrow 2NO_3^- + NAD(P)^+ + H^+$$

In animal tissues, dioxygen-dependent metabolism of NO is likely connected with heme- and flavoprotein (Gardner *et al.,* 2001), however, the identity of a particular hemoglobin and flavin remains undetermined.

Anoxic maize cells overexpressing class 1 hemoglobin have lower ADH activity compared to wild type and to lines down regulating hemoglobin (Sowa *et al.,* 1998). Higher hemoglobin levels would result in a greater turnover of NO in the Hb/NO cycle, which oxidizes NADH (Figure 1), replacing to some extent the requirement for ADH activity. Lower NADH/NAD and NADPH/NADP ratios in plants overexpressing hemoglobin have been

observed (Igamberdiev *et al.,* 2004b). These ratios are not affected significantly by hypoxia in Hb overexpressing lines, while in the lines downregulating Hb the ratios increase drastically under low oxygen tensions. It is evident that the expression of hemoglobin in hypoxic cells, in addition to maintaining energy status, also assists in maintaining the redox status of the cell. Whether it does so solely through fermentation pathways and the operation of an Hb/NO cycle or through a membrane-associated electron transport is an unresolved question.

Strong hypoxic induction of the Hb gene, comparable to the induction of alcohol dehydrogenase, occurs in Arabidopsis root cultures in concert with induction of enzymes of nitrogen metabolism, including nitrate reductases (especially nitrate reductase-2 which may be PM-NR) (Klok *et al.,* 2002). Several protein kinases have a similar profile of induction (Klok *et al.,* 2002) indicating a possible link between decreasing ATP levels and Hb synthesis (Nie and Hill, 1997). Hb induction is also observed in response to nitrate (Nie and Hill, 1997), nitrite and NO treatment (Ohwaki *et al.,* 2003).

Ascaris hexacoordinated hemoglobin reacts with NO to produce nitrate, with the oxidation of NADPH, presumably without participation of any additional protein (Minning *et al.,* 1999). Some NADH-dependent NO-degrading activity has also been demonstrated for bacterial hemoglobins lacking a flavoprotein subunit or corresponding domain (Frey *et al.,* 2002). This activity is increased 4-5 fold by insertion of the reductase domain. Even leghemoglobin can be reduced non-enzymatically by NADH, reduced glutathione or ascorbate, but with a lower rate than with the help of methemoglobin reductase (Becana and Klucas, 1990). Since the above

studies use relatively large concentrations of hemoglobin, from an enzyme standpoint, to demonstrate that the hemoglobin has NO dioxygenase activity, the results must be treated with caution. A small impurity in the hemoglobin preparation that possesses reductase activity could account for all of the observed activity. Our own unpublished studies with a mutant barley Hb, in which the only cysteine in the monomer has been modified to a serine (Cys^{79} to Ser), have demonstrated that the NO-dioxygenase activity associated with the mutant hemoglobin involves the participation of a component that is sensitive to sulfhydryl reagents, indicating that Hb alone is incapable of sustaining physiologically significant NAD(P)H-dependent NO-degrading activity.

The expression of a class 1 hemoglobin has a direct effect on the level of NO found under hypoxic conditions. Transformed plant tissues (alfalfa roots, maize cells) overexpressing hemoglobin have approximately half the NO levels of control plants under hypoxic conditions, whereas those underexpressing the protein have about twice the NO levels of control plants (Dordas *et al.,* 2003b, 2004; Igamberdiev *et al.,* 2004b). Cytoplasmic extracts of alfalfa root cultures have NO dioxygenase activity that is dependent on hemoglobin and NAD(P)H, thus, supporting the mechanism proposed in Figure 1. The activity exhibited a broad pH optimum and a strong affinity to NADH and NADPH, with a K_m = 3 μM for both nucleotides (Igamberdiev *et al.,* 2004b).

The evidence we have presented indicates that class 1 hemoglobins modulate NO levels within the plant cell. From the mounting data of the involvement of NO in many signal transduction pathways (Wendehenne *et al.,*

2001; Desikan *et al.,* 2002; Hoeberichts and Woltering, 2003; Neill *et al.,* 2003), it is apparent that the presence of this molecule would severely interfere with signal transduction. This, therefore, provides a reasonable argument as to why this gene is only expressed under specific conditions in which cell energy levels are depressed.

The Hb/NO cycle and carbon metabolism

A major unanswered question is how Hb induction during hypoxia maintains plant energy status. An obvious route is via increased substrate level phosphorylation through increased glycolytic flux (Dordas et al, 2003). Figure 3 summarizes the sequence of events resulting in the induction of Hb during plant adaptation to hypoxia. Oxygen deficiency causes a decrease of mitochondrial respiration, which is partly compensated by increased glycolytic flux. As a result, NADH levels increase and ATP levels decrease. ATP decline causes nitrate reductase activation (Stöhr and Mäck, 2001) and Hb gene induction (Nie and Hill, 1997). Upon accumulation of nitrite, NO production increases, and Hb participates in NO oxidation to nitrate. Associated with the oxidation of NADH and the Hb/NO cycle during hypoxia/anoxia is the maintenance of the redox and energy status of the cell. In their study of the products of anaerobic nitrate and ammonium ion metabolism in rice coleoptiles, Fan *et al.* (1997) found an approximately 55% higher production of glycolytic products in the presence of ammonium ion in comparison to nitrate under anaerobic conditions. This would favour an argument that the observed effects on NO, ATP and NADH levels during hypoxia in plants

overexpressing hemoglobin are not the result of increases in glycolytic flux. This type of study requires revisiting under conditions where the expression of Hb is controlled to determine what part glycolytic flux plays in the overall process.

Evidence has been accumulating of a putative nitrate respiratory pathway, capable of generating a proton motive force, in bacteria (Jormakka *et al.,* 2003). The presence of such a pathway in plants, potentially generating ATP more efficiently, might explain why Fan *et al.* (1997) observe less carbon flow under nitrate than under ammonium under anoxia. This, however, is remote since the known ATPases present in plasma membrane fractions are not of a type that are capable of utilizing chemiosmotic gradients.

There is clearly a need for further study to unravel the mechanism by which hemoglobin overexpression results in enhanced energy status in anoxic plants.

Conclusion

We have presented a case for the role of nitrate, NO and hemoglobin in maintaining plant cell viability under anoxic stress. Nitrate can be viewed as an intermediate electron acceptor under conditions of oxygen deficiency. NO is formed from nitrite by nitrate reductase. NO is oxygenated by a hypoxically -induced class 1 hemoglobin with an extremely high oxygen avidity. The turnover of this reaction is maintained by linkage to a methemoglobin reductase likely inherent to a separate flavoprotein. This cyclic reaction helps to maintain the redox status of the cell at very low oxygen tensions and may

reduce the need for fermentative pathways to generate glycolytic energy (Figures 1 and 3).

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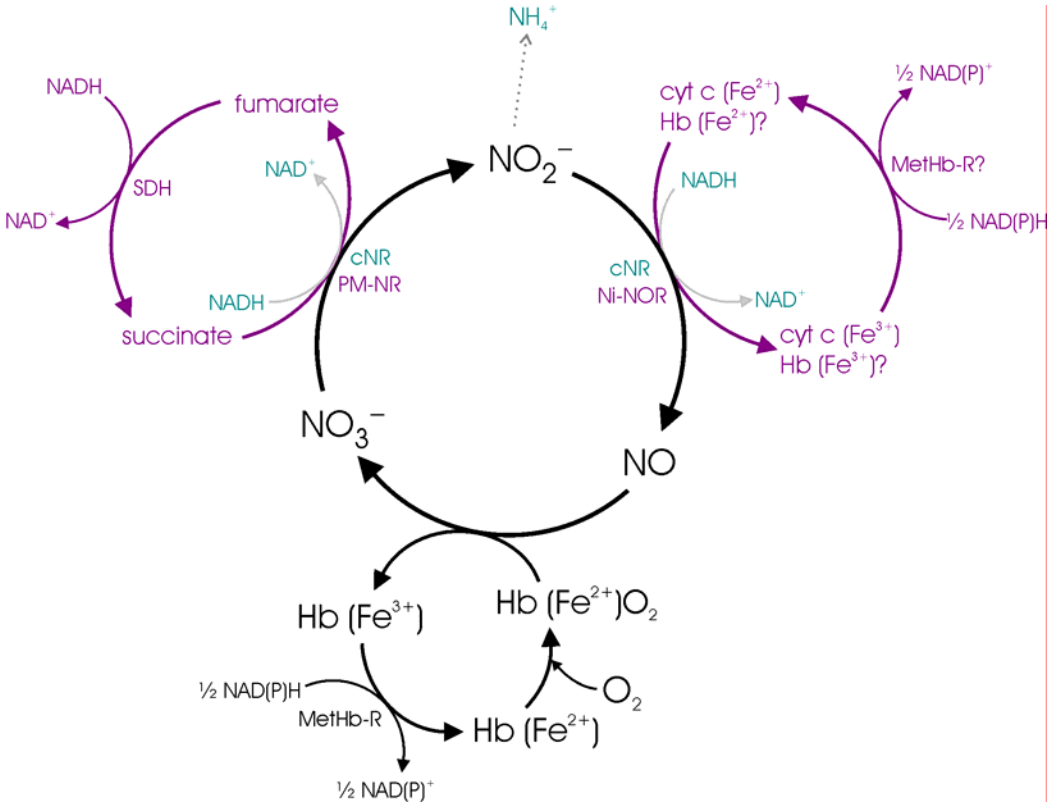
References

Legends to the figures

Fig. 1. Hb/NO cycle participation in NAD(P)H oxidation during hypoxia. Nitrate can be reduced to nitrite either by the cytosolic nitrate reductase (cNR), or by the plasma membrane nitrate reductase (PM-NR). Only a part of nitrite formed can be reduced to ammonia (green line, see Fig. 2 for details). A side reaction of cNR or the plasma membrane nitrite-NO reductase (Ni-NOR) oxidize nitrite to NO. NO is reduced to nitrate by oxyhemoglobin [$Hb(Fe^{2+})O_2$], which turns to metHb [$Hb(Fe^{3+})$]. High affinity of $Hb(Fe^{2+})$ to O_2 results in its immediate oxygenation even at very low (nanomolar) O_2 concentration. Reactions linked to cNR are shown in blue, those linked to PM-NR and Ni-NOR are shown in red. Abbreviations: SDH: succinate dehydrogenase; MetHb-R: methemoglobin reductase.

Fig. 2. Scheme illustrating the connection of nitrite reduction to fermentation pathways operating under hypoxia. The connection of ethanolic fermentation and NiR catalysed formation of ammonia occurs mainly via alanine production. Other pathways include lactate formation, link from pyruvate to 2-oxoglutarate (OG) via partial TCA cycle and formation of γ-aminobutyric acid (GABA). Glutamate formation takes place either in GS-GOGAT system (linked to ATP breakdown) or in the reverse glutamate dehydrogenase reaction.

Fig. 3. Role of Hb induction in NO scavenging, NADH recycling and ATP formation during hypoxia.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PM-NR
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NAD+
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Hb (Fe3+)?
½ NAD(P)+
MetHb-R?
½ NAD(P)H
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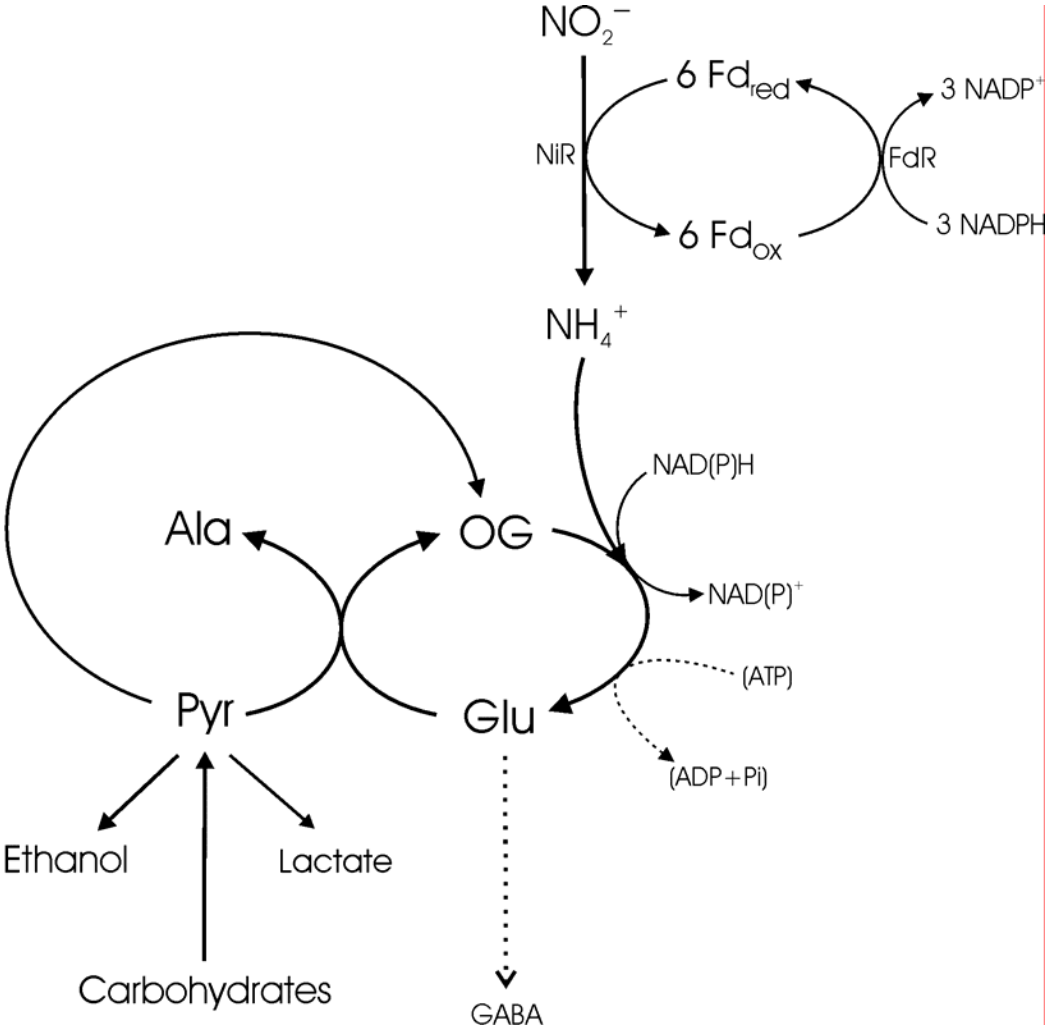
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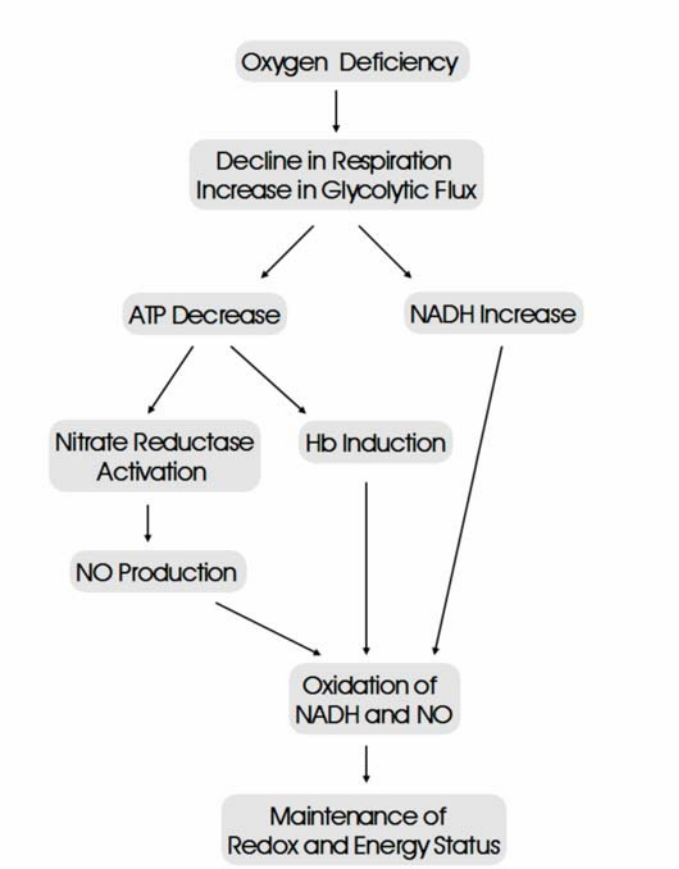
Oxygen Deficiency
Decline in Respiration
Increase in Glycolytic Flux
ATP Decrease
NADH Increase
Nitrate Reductase Activation
Hb Induction
NO Production
Oxidation of NADH and NO
Maintenance of Redox and Energy Status