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Nitric Oxide as an Alternative Electron Carrier During Oxygen Deprivation

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Abstract Plant cells exposed to anaerobic stress generate copious amounts of the gaseous free radical nitric oxide (NO). At this time, the concomitant expression of the ubiquitous class 1 plant hemoglobins establishes one component of a soluble terminal NO dioxygenase system, which yields nitrate ions via reaction of oxyhemoglobin with NO. Class 1 hemoglobin expression also enhances the cellular energy status, redox status, and NO metabolism of plant cells exposed to hypoxic stress. The ability of class 1 hemoglobins to ligate oxygen at concentrations two orders of magnitude lower than cytochrome *c* oxidase suggests that hemoglobin and NO may serve as components of an alternative type of respiration that is operative during conditions that impair the operation of mitochondrial terminal oxidases. We suggest that, under hypoxic conditions, NO can be formed by anaerobic reduction of nitrite by a portion of the mitochondrial electron transport chain. NADH and NADPH, accumulated due to glycolytic fermentation and lipid breakdown, contribute electrons to the process, generating a chemiosmotic potential capable of generating ATP. The overall anaerobic reaction sequence is referred to as the Hb/NO cycle.

1 Introduction

There is a wealth of evidence to support the role of nitric oxide (NO) as a signaling molecule in biological systems. There is also an equal abundance of literature dealing with the production of NO and other nitrogen oxides by soil microbes existing under, or exposed to, oxygen deprivation. Nitrogen oxide emission is frequently associated with soil waterlogging where nitrogenous compounds, existing in higher oxidation states, act as alternative electron acceptors to oxygen, producing gaseous nitrogen oxides that are mainly released into the atmosphere (McKenney and Drury 1997).

In this chapter, we review the recent progress demonstrating that NO is formed during hypoxia in plants and that NO production assists in maintaining the redox and energy status of the hypoxic plant cell. In addition, we present a schematic model of the components constituting the hemoglobin-assisted NO turnover (defined as Hb/NO cycle). We also discuss the role and physiological relevance of individual components of the Hb/NO cycle with respect to cellular NO levels and plant energy and/or redox status.

2
Nitric Oxide Formation During Hypoxia

Nitric oxide is produced in significant quantities in plants exposed to oxygen deprivation (Rockel et al. 2002; Dordas et al. 2003a, 2004). Rates of NO formation under hypoxia are in the order of 10–50 nmol g^{-1} FW h^{-1} and are approximately 50-fold higher than the levels generated under aerobic conditions. A significant amount of NO produced is immediately scavenged, so real rates of NO production under hypoxia may potentially differ by at least one order of magnitude (Vanin et al. 2004). The various pathways by which NO may be formed in plants have been discussed in other portions of the monograph. In this chapter, our discussion will be limited to those pathways by which the compound is likely to be produced under hypoxic conditions.

As nitrate has been a known alleviator of plant flooding (hypoxic) stress for many years (Arnon 1937), and nitrate metabolism is linked to both aerobic and anaerobic plant NO generation, it is worthwhile summarizing the beneficial aspects of nitrate to hypoxic plant metabolism as a prelude to NO generation in hypoxic plants. From existing studies, the beneficial effects of nitrate, thus far, have been linked to pH regulation (Fan et al. 1997), carbohydrate utilization (Reggiani et al. 1986; Saglio et al. 1988; Fan et al. 1997), and NAD^+ regeneration (Reggiani et al. 1985). Ultrastructural studies also suggest that the integrity of hypoxically stressed mitochondria are extended in the presence of exogenous nitrate. More recent studies utilizing transgenic plants deficient in nitrate reductase (Stoimenova et al. 2003a,b) implicate products of nitrate metabolism to positively contribute towards flooding tolerance, energy metabolism, and pH regulation of plant during hypoxia.

Cytosolic nitrate reductase (cNR) serves as an attractive enzymatic catalyst of plant NO generation during hypoxic stress. In phosphorylated form, cNR exhibits low activity due to interactions with 14 – 3 – 3 proteins (Huber et al. 2002). However, the dephosphorylation of cNR via cNR phosphatases has been shown to correlate with the activation and stability of cNR and bears particular relevance for hypoxic NO generation (Huber et al. 2002). Degradation and half-life of cNR is also affected by cNR phosphorylation and 14-3-3 binding, with cNR activation being positively correlated with protein stability (Kaiser and Huber 2001). The drop in cytosolic pH under anoxic conditions triggers cNR dephosphorylation leading to a 2.5-fold activation of the enzyme (Botrel and Kaiser 1997). Moreover, when cNR is activated, a shift away from lactate and ethanol formation probably occurs, which is supported by the observation that nitrate reductase-lacking tobacco plants produce substantially more ethanol and lactate during anaerobiosis (Stoimenova et al. 2003b). In Arabidopsis root cultures, two nitrate reductase genes are induced under low-oxygen (5%) pressure. One (NR1) shows moderate induction after 0.5–4 h of hypoxia and strong induction after 20 h, while the other (NR2) is strongly activated in 2–4 h and even more after 20 h (Klok et al. 2002). The

potential maximum activity of activated nitrate reductase, although lower than alcohol dehydrogenase, exceeds the rate of hypoxic ethanol formation by more than threefold (Botrel and Kaiser 1997).

Although nitrate reductase is activated under hypoxia, nitrite reduction is limited at the nitrite reductase step (Botrel et al. 1996). This limitation is explained both by a suppressive effect of cellular acidification and by increased flux through nitrate reductase (Botrel and Kaiser 1997; Botrel et al. 1996). When nitrite accumulates, it can also be used by cNR as a substrate to produce NO. The cNR reaction rate with nitrite to produce NO is probably only 1–2% of the maximal cNR reaction in leaves (Yamasaki et al. 2001; Rockel et al. 2002; Sakihama et al. 2002), so its physiological importance is still questionable.

In addition to cNR, the plasma membrane-bound nitrate reductase (PM-NR) and nitrite-NO reductase (Ni-NOR), have also been implicated in hypoxic NO generation (Ward et al. 1988, 1989; Meyerhoff et al. 1994). In the case of the latter, Ni-NOR, it appears as though atmospheric oxygen reversibly inhibits enzyme activity and NO production (Stöhr and Stremlau 2006). Such observations implicate Ni-NOR in hypoxic NO generation. However, it must be noted that the pH optimum, electron donor preference, and response to nitrate are markedly different for Ni-NOR when compared to that of cNR (Stöhr and Stremlau 2006, see also the contribution by Stöhr in this volume).

NO synthase (AtNOS1) from Arabidopsis is the only arginine-dependent nitric oxide synthase identified thus far in plants, and appears to generate the NO required for pathogen response, hormonal signaling, flowering, and regulation of reactive oxygen species (Guo et al. 2003). It is an unlikely candidate for plant NO synthesis under hypoxia for two reasons: first, NO synthase consumes oxygen and thus competes with other processes requiring oxygen in the hypoxic cell, and second, the nitrogen of NO is provided by nitrate during hypoxic NO production in maize cells (Dordas et al. 2004).

In addition to enzymatic sources of NO generation in plants, there is also evidence implying that non-enzymatic sources contribute to NO generation. For instance, in acidic and reducing environments, non-enzymatic reduction of nitrite produces nitrous acid, which can then react with ascorbate to produce dehydroascorbate and NO (Weitzberg and Lundberg 1998). This reaction has been demonstrated in cereal aleurone layers (Bethke et al. 2004).

While all of the above reactions for NO formation can contribute to the hypoxic NO pool, recent studies indicate that NO can be formed in a mitochondrial nitrite:NO reductase reaction associated with the electron transport chain (Tischner et al. 2004; Gupta et al. 2005; Planchet et al. 2005), a reaction possible in anaerobic plant mitochondria (Igamberdiev et al. 2005, for details see the chapter by Kaiser in this volume). Such a reaction may be conserved among species as both fungal (Kobayashi et al. 1996) and protist mitochondria (Finlay et al. 1983) were capable of operating during anaerobiosis in concert with associated nitrite reductase(s). The reaction can involve

electrons from either the cytochrome *c* (Tielens et al. 2002) or the ubiquinone pool (Zumft 1997). In higher plants, the capacity of nitrite reduction to NO is associated with root mitochondria, while in leaf mitochondria it is likely absent (Gupta et al. 2005).

Although nitrite reductase is generally not associated with either plant or animal mitochondria, considerable amounts of nitrite are reduced to NO by hypoxic animal or plant mitochondria (Kozlov et al. 1999, 2005). This reaction can be catalyzed by anaerobic cytochrome *c* oxidase in its reduced state at the heme iron site (Brudvig et al. 1980; Paitian 1985; Mason et al. 2006). Six additional plant-specific subunits of cytochrome *c* oxidase (Millar et al. 2004) have also been implicated in this reaction. There is also the possibility of a transfer of electrons to nitrite from ubiquinol at the level of complex III (Kozlov et al. 1999; Nohl et al. 2001; Lacsa et al. 2006). In animal tissues, the major source of hypoxic NO production is arginine-independent and linked to the mitochondrial electron transport chain (Lacsa et al. 2006). Recent studies of plant and algal mitochondria suggest that they can readily convert nitrite to NO under anaerobic conditions at a higher rate than other previously mentioned reactions (Tischner et al. 2004; Planchet et al. 2005).

3
Hypoxia-Induced Hemoglobin Synthesis in Plants

The expression of a hemoglobin gene accompanying hypoxia was first demonstrated in barley (Taylor et al. 1994), following work on the existence of hypoxia-induced hemoglobin in non-nodulating plant species (Bogusz et al. 1988). The properties of the Hb protein (Duff et al. 1997) indicated that it probably did not function as a carrier, store or sensor of O_2 (Hill 1998). Barley class 1 hemoglobin is a homodimer with a monomeric molecular weight of 18 kDa (Duff et al. 1997). Its O_2 dissociation constant (2–3 nM) indicates that it remains oxygenated at extremely low O_2 tensions. The K_m for oxygen of cytochrome *c* oxidase is 140 nM. At concentrations where oxyhemoglobin dissociates, therefore, cytochrome *c* oxidase is effectively non-functional in utilizing oxygen. Other class 1 hemoglobins possess similar properties (Arredondo-Peter et al. 1997; Hargrove et al. 1997; Kundu et al. 2003). The unique features of class 1 hemoglobins result from the hexacoordination of the heme moiety during oxygen ligation, in comparison to the pentacoordination occurring in leghemoglobins, erythrocyte, and muscle hemoglobins. This hexacoordination of the heme pocket results in tighter oxygen binding, which is a basis for a conserved high fidelity NO dioxygenation mechanism (Gardner et al. 2006).

Class 1 hemoglobin is expressed in roots and other tissues within 2 h of exposure to hypoxia (Taylor et al. 1994). Strong hypoxic induction of the Hb gene, comparable to the induction of alcohol dehydrogenase, occurs in Arabidopsis root cultures in concert with the induction of enzymes of nitrogen

metabolism, including nitrate reductases (Klok et al. 2002). Hb is also expressed in germinating seeds, which can become highly hypoxic at certain stages. In barley grain, Hb mRNA is detectable within 2 h of imbibition, and its expression continues to increase up to the point of radicle elongation, i.e., the completion of germination (Guy et al. 2002). Limiting oxygen availability increases the expression of the Hb gene in the embryos (Guy et al. 2002).

Oxygen deficiency by itself does not trigger Hb gene expression. A possible link between decreasing ATP levels and Hb synthesis has been observed (Nie and Hill 1997). Further investigation showed that both protein dephosphorylation and anaerobic elevation in cytosolic Ca^{2+} are factors in Hb gene expression (Nie et al. 2006). Hb induction is also observed in response to nitrate (Nie and Hill 1997), nitrite, and NO treatment (Ohwaki et al. 2005), implicating Hb expression with these nitrogenous compounds.

4
Nitric Oxide Turnover by Class 1 Hemoglobin

Class 1 hemoglobins can effectively convert NO to nitrate at nanomolar concentrations of O_2. In the course of this reaction, the ferrous form of hemoglobin is oxidized to the ferric form. Reduction back to the ferrous form to sustain the catalytic cycle can be achieved by a reductase using the appropriate reducing agent. In microorganisms, NO is scavenged by NO dioxygenase (NOD), which is a flavohemoglobin possessing two domains, one of which is hemoglobin and the other is an NAD(P)H-dependent reductase (Gardner et al. 1998). The NOD reaction is described by the equation

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

When the reductase domain is lacking, the rate of reaction is exceedingly slow (Frey et al. 2002). Even leghemoglobin in its ferric form can be reduced non-enzymatically by NADH, reduced glutathione, or ascorbate, but with a much lower rate than with the help of methemoglobin reductase (Becana and Klucas 1990). Our studies with a mutant, class 1 barley Hb demonstrate that Hb alone is incapable of sustaining physiologically significant NAD(P)H-dependent NO-degrading activity (Igamberdiev et al. 2006a). We have purified and identified a cytosolic monodehydroascorbate reductase that acts in conjunction with the class 1 Hb to sustain turnover of NO (Igamberdiev et al. 2006a). Its proposed role is the removal of monodehydroascorbate formed in the reduction of methemoglobin by ascorbate. Correlations between ascorbate levels and hemoglobin expression indicate that the suggested ascorbate-dependent mechanism of methemoglobin reduction may be operative in vivo (Igamberdiev et al. 2006b).

The expression of a class 1 hemoglobin has a direct effect on the level of NO found under hypoxic conditions (Dordas et al. 2003a, 2004). Cytoplasmic ex-

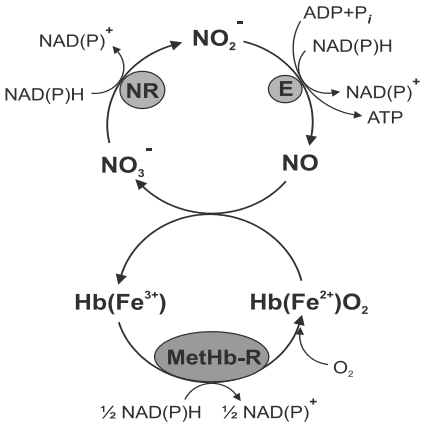

Fig. 1 Suggested scheme of hypoxic respiratory pathway involving Hb and NO (Hb/NO cycle) resulting in regeneration of NADH and anaerobic ATP synthesis. *NR* nitrate reductase. *E* nitrite:NO reductase activity of mitochondria. NO is oxidized to nitrate by oxyhemoglobin [$Hb(Fe^{2+})O_2$], which is converted to metHb [$Hb(Fe^{3+})$]. MetHb is reduced by a MetHb reductase (*MetHb-R*), such as the ascorbate/monodehydroascorbate reductase system. The high affinity of $Hb(Fe^{2+})$ for O_2 results in its immediate oxygenation even at very low (nanomolar) O_2 concentration. Nitrite reduction to NO in mitochondria at the sites of complex III and IV is coupled to NAD(P)H oxidation by external NADH and NADPH dehydrogenases, producing a chemiosmotic potential resulting in ATP synthesis. Adapted from Igamberdiev et al. (2005)

tracts of alfalfa root cultures have NO dioxygenase activity that is dependent on hemoglobin and NADH or NADPH. The activity is flavin- and sulfhydryl-dependent, exhibiting a broad pH optimum and a strong affinity to NADH and NADPH. The K_m is 3 μM for both nucleotides, while the maximum rate with NADH is 2.5 times higher than with NADPH (Igamberdiev et al. 2004). These properties are consistent with the reported properties of a monodehydroascorbate reductase (Hossain and Asada 1985). The sequence of reactions involving nitrate reductase-catalyzed nitrate conversion to nitrite, NO production from nitrite, and Hb-dependent NO conversion to nitrate in conjunction with the ferric Hb reduction is defined as the Hb/NO cycle (Dordas et al. 2003b; Igamberdiev and Hill 2004; Igamberdiev et al. 2005) (Fig. 1).

5
Maintenance of Redox and Energy Status by Hemoglobin During Hypoxia

The hypoxic or anoxic cell must deal with two critical metabolic conditions: a highly reductive environment due to accumulation of NAD(P)H, and a reduced efficiency of ATP synthesis due to the cessation of oxidative phosphorylation. It is generally accepted that glycolysis and fermentation are the

major routes by which the cell adapts to these conditions. We suggest that the Hb/NO cycle is another route by which cells react to the situation.

Hb overexpression in anoxic maize cell cultures results in the maintenance of cell energy status, as evidenced by higher ATP levels and decreased utilization of fermentative pathways (Sowa et al. 1998). The Hb/NO cycle (Fig. 1) would predict greater turnover of NO in a reducing environment, diverting metabolism away from fermentative pathways involving alcohol dehydrogenase. NADH/NAD and NADPH/NADP ratios in plants overexpressing hemoglobin (Igamberdiev et al. 2004) are not significantly affected by hypoxia, while in plant lines downregulating Hb the ratios increase significantly under low oxygen tensions. The expression of hemoglobin in hypoxic cells, therefore, in addition to maintaining energy status, helps to maintain the redox status of the cell.

Mitochondrial localization of yeast flavohemoglobin has recently been reported (Cassanova et al. 2005). However, the nucleotide sequence of the barley class 1 Hb gene suggests that it is not imported into cell organelles (Taylor et al. 1994) and is probably localized mainly in the cytosol (Igamberdiev et al. 2004). A lack of localization within mitochondria should not be a significant limitation as rapid diffusion of NO from mitochondria to the cytosol would permit NO scavenging in the cytosolic compartment. Figure 2 shows how the expression of barley hemoglobin in alfalfa roots correlates with NO levels, ATP/ADP, and NADH/NAD ratios. These results are based on previously published data (Dordas et al. 2003a; Igamberdiev et al. 2004). The most pronounced effects of Hb expression are visible under hypoxic conditions. Hb expression strongly decreases NO levels, NADH/NAD ratios, and increases the ATP/ADP ratio, indicating an important metabolic role for Hb in the process of NO removal linked to NADH oxidation and ATP production. NAD(P)H oxidation in the Hb/NO cycle occurs at three sites in the Hb/NO cycle (Fig. 1), accounting for the observed changes in the NADH/NAD ratio. The possible implications of ATP production linked to the Hb/NO cycle are described in the next section.

6
Anoxic Mitochondrial ATP Synthesis Driven by Nitrite and NAD(P)H

The data presented in Fig. 2, showing hemoglobin participation in the maintenance of redox and energy state of hypoxic plant cells, indicate that its involvement in NO removal is linked to NADH oxidation and ATP synthesis. NO removal by itself can make mitochondria more functional via relief from the inhibitory effects of NO. Even under highly anoxic conditions, however, the energy state of cells improves (Sowa et al. 1998). There is sufficient evidence to conclude that mitochondria are capable of operating under highly anoxic conditions. Exposure to anoxia results in some changes in enzyme

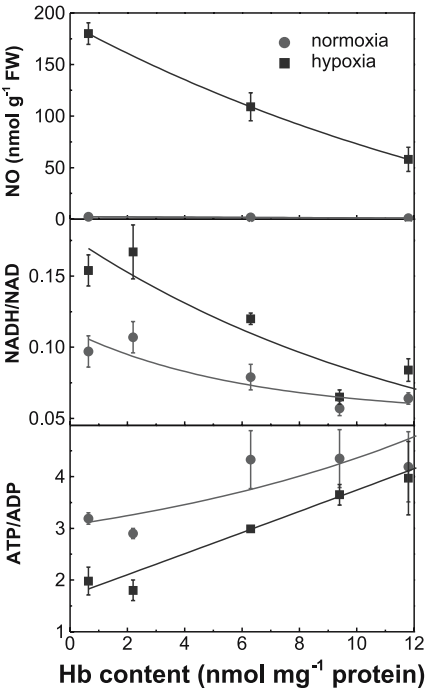

Fig. 2 Effects of differential expression of barley hemoglobin in alfalfa roots on NO levels, ATP/ADP and NADH/NAD ratios under normoxic (40 kPa O_2) and hypoxic (3 kPa O_2) conditions. Two underexpressing Hb, two overexpressing Hb, and a control line were used. Based on the data of Dordas et al. (2003a) and Igamberdiev et al. (2004)

composition in mitochondria (Couee et al. 1992; Kennedy et al. 1987), but they remain functional and preserve their ultrastructure for a prolonged time, if nitrate is present (Vartapetian and Polyakova 1999; Vartapetian et al. 2003). This evidence led to the suggestion that nitrate might serve as a terminal electron acceptor under anoxia (Polyakova and Vartapetian 2003). No evidence has been presented for nitrate reductase activity associated with plant mitochondria.

A key feature of mitochondrial operation under anoxia is the oxidation of extra-mitochondrial NAD(P)H. The high K_m, low pH optimum, and Ca^{2+} dependence of externally facing NADH and NADPH dehydrogenases (Edman et al. 1985; Møller 1997) suggests that they operate when the cytosolic NAD(P)H and Ca^{2+} concentrations are elevated and the cytosolic pH is decreased, as observed under hypoxia (Roberts et al. 1992; Subbaiah et al. 1998).

We have shown that anaerobic plant mitochondria can synthesize ATP using nitrite and NAD(P)H, forming NO and $NAD(P)^+$ (Stoimenova et al. manuscript in preparation). The reaction was insensitive to rotenone and antimycin A, partially sensitive to diphenyleneiodonium and myxothiazol,

and strongly sensitive to oligomycin and uncouplers. Oxidation of cytosolic NADH and NADPH via externally facing dehydrogenases precludes the formation of a proton gradient at the site of electron transport from NAD(P)H to ubiquinone. Inhibition of ATP synthesis and NO production by myxothiazol and the absence of inhibition by antimycin A is similar to the inhibition pattern of nitrite reduction observed in mammalian mitochondria (Kozlov et al. 1999). The effect is analogous to a single electron leak to dioxygen, indicating a one-electron mechanism of nitrite reduction due to an oxidized state of ubiquinol at the cytochrome bc_1 complex. We cannot, however, exclude the possibility that nitrite reduction may also occur at a later step and involve cytochrome *c* oxidase, since the reactions of NO production and ATP synthesis are sensitive to cyanide (Fig. 3).

Anaerobic mitochondrial production of ATP is approximately 5% of the aerobic rate, yielding at a minimum 0.4–0.6 ATP per NAD(P)H oxidized, indicating that membrane potential generation is limited (Stoimenova et al. manuscript in preparation). The reaction involves the release of two electrons by one NADH and the reduction of a single nitrite by one electron. While the observed rate of ATP synthesis may be low relative to aerobic respiration, it is important to note that the rate is comparable to ATP generation by glycolytic substrate phosphorylation.

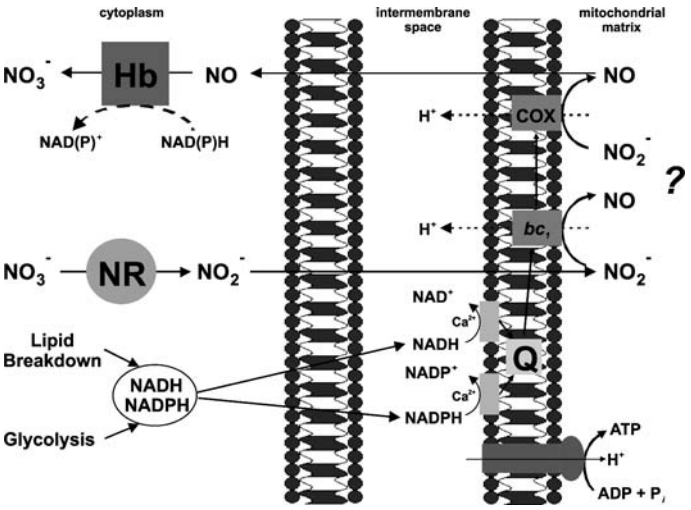

Fig. 3 Operation of plant mitochondria under oxygen deprivation. Glycolytic fermentation and lipid breakdown in hypoxia result in the increase of cytosolic NADH and NADPH. Externally facing mitochondrial dehydrogenases oxidize NADH and NADPH. At levels of oxygen well below saturation of cytochrome *c* oxidase, nitrite serves as an alternative electron acceptor at the sites of complex III and/or complex IV. Nitric oxide formed in this reaction is converted by hypoxically induced hemoglobin (*Hb*) to nitrate. The latter is reduced to nitrite by hypoxically induced nitrate reductase (*NR*)

7
Possible Link of Hemoglobin-Dependent NO Turnover to Nitrite-Dependent ATP Synthesis by Mitochondria

Theoretically, NO formed by mitochondria can be recycled back to nitrite via a corresponding side reaction with cytochrome *c* oxidase (Cooper 2002). The cytochrome *c* oxidase under low oxygen concentrations will be present in a reduced form, however, which makes it very unlikely that this reaction occurs at the copper site of the protein (Cooper 2002). When the cytochrome *c* oxidase is reduced, nitrite binds at the heme iron center of the protein and a reverse reaction of NO formation from nitrite is more likely (Cooper 2002). A plausible alternative is the operation of a hemoglobin/NO cycle (Igamberdiev and Hill 2004) converting NO to nitrate, needing only nanomolar traces of oxygen present, even in highly anaerobic conditions, to recycle NO formed by mitochondria back to nitrate (Fig. 1), while the operation of mitochondria with nitrite as a terminal acceptor does not need oxygen at all (Fig. 3).

8
Conclusions

We present a case for a pathway operating during oxygen deprivation, in which ATP is synthesized in mitochondria, linked to NAD(P)H-driven NO formation from nitrite. Both mitochondrial complex III and cytochrome *c* oxidase could serve as putative sites of nitrite reduction to NO. Accumulation of NO under hypoxic conditions results in its further metabolism in which hypoxically induced class 1 hemoglobin converts NO to nitrate. Nitrate is reduced to nitrite by nitrate reductase, an enzyme also induced under hypoxia. NO, nitrate, and nitrite serve as electron carriers in this cyclic process with oxygen being the terminal electron acceptor. The oxygen is made available through a class 1 hemoglobin, which exists in the oxygenated form at very low oxygen tensions. This hemoglobin- and nitric oxide-related respiration is considered as an alternative to classic fermentation pathways and serves to maintain the redox and energy levels of hypoxic cells.

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