Minireview

Reactive oxygen species and antioxidants in legume nodules

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Reactive oxygen species are a ubiquitous danger for aerobic organisms. This risk is especially elevated in legume root nodules due to the strongly reducing conditions, the high rates of respiration, the tendency of leghemoglobin to autoxidize, the abundance of nonprotein Fe, and the presence of several redox proteins that leak electrons to O_2. Consequently, nodules are particularly rich in both quantity and diversity of antioxidant defenses. These include enzymes such as superoxide dismutase (EC 1.15.1.1) and ascorbate peroxidase (EC 1.11.1.11) and metabolites such as ascorbate and thiol tripeptides. Nodule antioxidants have been the subject of intensive molecular, biochemical, and functional studies that are reviewed here. The emerging theme is that antioxidants are especially critical for the protection and optimal functioning of N_2 fixation. We hypothesize that this protection occurs at least at two levels: the O_2 diffusion barrier in the nodule parenchyma (inner cortex) and the infected cells in the central zone.

Abbreviations - APX, ascorbate peroxidase; ASC, ascorbate; GSH, glutathione; GSHS, glutathione synthetase; hGSH, homoglutathione; hGSHS, homoglutathione synthetase; Lb, leghemoglobin; ODB, oxygen diffusion barrier; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Aerobic metabolism is an inherently dangerous process for all organisms. The risks arise because, although O_2 itself is relatively nonreactive, it has the potential to be partially reduced to form reactive oxygen species (ROS), including the superoxide radical (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2). Metabolic processes involving electron transport, such as photosynthesis, respiration, and N_2 fixation, invariably generate ROS as by-products. For example, it is estimated that 10-20% of the electrons that flow through photosystem I reduces O_2 to H_2O_2 (Asada 1999), and that 2% of the O_2 consumed by mitochondria is used to generate H_2O_2 (Scandalios et al. 1997). Thus, plants and other aerobic organisms depend on a multifaceted antioxidant defense to avoid self destruction. In recent years there has been an upsurge of interest in ROS, antioxidants, and oxidative stress in plants, especially with regard to photosynthesis and plant-pathogen interactions. It is now clear that ROS are involved in the molecular damage observed in plants exposed to an impressive variety of adverse conditions including osmotic stress, strong light, UV light, low temperature, drought, air pollutants (especially ozone), herbicide treatment, and pathogen attack (Scandalios et al. 1997, Noctor and Foyer 1998).

Legumes and other N_2-fixing plants face oxidative risks beyond those associated with photosynthesis. As is the case with leaves, nodules are rich in strongly reducing compounds, polyunsaturated fatty acids, and O_2-labile proteins (most notably nitrogenase itself; *see "O_2-labile proteins"*), which can readily react with O_2 and generate ROS. Nodules have high rates of respiration due to the extensive energy demands of N_2 fixation, which results in a high flux of O_2 into the nodule and hence an elevated risk of ROS formation (Dalton 1995). This review will be restricted to the relevance of ROS and antioxidants in legume N_2 fixation. Although passing similarities will be made to broader aspects of ROS in plants, the reader is referred to other recent reviews for details (Bowler et al. 1994, Scandalios et al. 1997, Noctor and Foyer 1998, Asada 1999). It is also assumed that the reader is familiar with the essential features and terminology (Hirsch 1992) of the rhizobia-legume symbiosis. A basic distinction in two types of nodule anatomy and biochemistry, however, has to be advanced here to

facilitate comprehension to a wider readership. Nodules from legumes of tropical and subtropical origin (soybean, bean, cowpea) have a determinate growth pattern: they are usually spherical, do not have persistent meristems, and use ureides as the major export products of the fixed N_2 to the shoot. Nodules from legumes of temperate regions (pea, alfalfa, clover) have an indeterminate growth pattern: they are usually elongate and branched, have persistent meristems, and use amides as the main export products (Sprent 1980, Hirsch 1992).

Sources of ROS in nodules

The central (infected) zone of legume nodules is under rigorous physiological control with respect to O_2. Rhizobia, which are obligate aerobes, are able to derive energy from respiration while simultaneously operating the nitrogenase enzyme system which is extremely sensitive to O_2-induced denaturation. Nodules have a high capacity to produce ROS even though the concentration of free O_2 in the central zone is only 5 to 60 nM (Appleby 1984, Hunt and Layzell 1993). This high capacity for production of ROS arises from the elevated rates of respiration in bacteroids and mitochondria (not reviewed here), the abundance of leghemoglobin (Lb), O_2-labile proteins and nonprotein Fe, and the presence of enzymes forming O_2^- radicals and peroxides. ROS can also be formed by plant cells as part of a defense response against microbial infection, although a full hypersensitive response is not elicited during compatible reactions involving rhizobia.

Leghemoglobin

The main source of ROS in nodules is probably Lb (Fig. 1). This is a myoglobin-like hemoprotein of 16 kDa which can be found at concentrations of 1-5 mM in the cytosol of infected cells. It facilitates O_2 transport to the bacteroids at a low but constant flux, thus preventing O_2 inactivation of nitrogenase (Appleby 1984). Only the ferrous form of Lb (Lb^{2+}) is able to bind O_2, forming oxyleghemoglobin ($Lb^{2+}O_2$). Like other hemoglobins, $Lb^{2+}O_2$ spontaneously autoxidizes to form ferric Lb (Lb^{3+}) and O_2^-, especially at acidic pH.

The released O_2^- can subsequently oxidize other molecules of $Lb^{2+}O_2$ to Lb^{3+}, thus enhancing the overall rate of Lb^{2+} and $Lb^{2+}O_2$ inactivation (Puppo et al. 1982).

Both $Lb^{2+}O_2$ and Lb^{3+} can also be oxidized by H_2O_2 (Fig.1). At molar ratios of H_2O_2 to Lb^{3+} of 1:1 or 2:1, two Lb species are formed: ferryl Lb (Lb^{IV}), a relatively long-lived species, and at least one globin radical. The globin radical rapidly dissipates to more stable forms of Lb. These include a green compound, which is thought to arise by intramolecular heme-cross-linking, and a dimeric Lb, which is formed by intermolecular cross-linking (Moreau et al. 1995a). At molar ratios greater than 5:1, H_2O_2 causes breakdown of the heme group and release of free Fe (Puppo and Halliwell 1988), which can catalyze Fenton reactions *(see "Catalytic Fe").* These reactions are of special concern because they produce ·OH radicals which are the most destructive form of ROS (reviewed by Halliwell and Gutteridge 1990). Although ferryl Lb has not been detected in nodules, there is ample evidence that oxidation of Lb can be problematic in nodules. For instance, senescent nodules contain Lb^{3+} (Lee et al. 1995) and green pigments derived from the oxidative attack of Lb (Jun et al. 1994). These pigments probably represent initial degradation products of Lb since the heme (still containing the Fe) is partially oxidized but the apoprotein moiety remains intact (Jun et al. 1994). Young, healthy nodules contain ferric Lb reductase and flavins to keep Lb in the reduced, physiologically-active state (Becana and Klucas, 1990; Ji et al. 1991).

O_2-labile proteins

There are several important proteins in nodules that are capable of reacting with O_2 to generate ROS. The MoFe (dinitrogenase) protein, and especially the Fe (dinitrogenase reductase) protein and the FeMo cofactor of nitrogenase, are irreversibly damaged by O_2. This inactivation, which takes place within minutes, involves metal-S clusters and presumably the formation of ROS, since addition of superoxide dismutase (SOD) and catalase lessen the loss of activity (Robson and Postgate 1980). Ferredoxin, the proximal electron donor to nitrogenase, is also abundant in bacteroids. Based on the well-known ability of ferredoxins from bacteria and chloroplasts to reduce O_2 to O_2^- (Misra and Fridovich

1971), these powerful redox proteins (mid-point potential of around -430 mV) are another likely source of ROS in nodules. Hydrogenases are membrane-bound enzymes capable of partial or complete recycling of the H_2 gas produced by nitrogenase. The hydrogenase from soybean nodule bacteroids, like that from some other sources, is O_2-sensitive, with a half-life of 70 min when exposed to air (Arp and Burris 1979). Inactivation of hydrogenases by O_2 involves generation of O_2^- and probably other ROS, which may self-inactivate the protein, possibly through destruction of the Fe-S clusters (Schneider and Schlegel 1981).

Enzymatic formation of ROS

ROS can also be produced in nodules as natural products of enzyme activity. Xanthine oxidase (EC 1.1.3.22) and uricase (EC 1.7.3.3) are the first enzymes of the purine degradation pathway and produce O_2^- and H_2O_2, respectively. Both enzymes are localized in leaf peroxisomes (del Río et al. 1998) and uricase at least is also found in nodule peroxisomes. Immunolabeling indicates that uricase is confined to the large peroxisomes of uninfected cells in determinate nodules, where the enzyme catalyzes one of the last steps of ureide synthesis (VandenBosch and Newcomb 1986).

Lipoxygenase (EC 1.13.11.12) is another enzyme that generates ROS. This enzyme catalyzes the oxidation of polyunsaturated fatty acids by O_2 and produces hydroperoxides and O_2^- radicals (Lynch and Thompson 1984). At least five lipoxygenase genes are expressed in pea nodules, although none of them seems to be overexpressed in nodules relative to uninfected roots. All five genes are expressed at the nodule apex, which has led to the suggestion that lipoxygenase is involved in nodule development (Wisniewski et al. 1999). The numerous isozymes of SOD can produce H_2O_2 in different subcellular compartments. Co-localization of SODs and catalases or peroxidases to rapidly dispose of the resulting H_2O_2 is essential to avoid cell damage. For example, peroxisomes of nodules and leaves contain a MnSOD along with catalase in the matrix and ascorbate peroxidase (APX) in the membrane (Becana et al. 1989, Jiménez et al. 1997, del Río et al. 1998).

Catalytic Fe

Nodules are extremely rich in Fe as an essential constituent of nitrogenase, ferritin, and Lb (Ragland and Theil 1993). In considering the prooxidant properties of Fe, it is important to recognize that Fe bound up in proteins generally does not participate directly in the generation of ROS. On the other hand, free Fe and most chelated forms of Fe (including the Fe in heme) catalyze the decomposition of H_2O_2 to the ·OH radical and promote lipid peroxidation. For this reason, these forms of Fe are collectively referred to as "catalytic Fe". The ·OH radical can react virtually with all cellular components, including sugars, lipids, proteins, and DNA, in a site-specific manner (Halliwell and Gutteridge 1990). Fortunately, the concentration of catalytic Fe, especially free Fe and free heme, are kept very low in nodules (Becana and Klucas 1992), in part due to the presence of ferritin, a protein that stores Fe in an inactive form *(see "Ferritin").* In mitochondria of leaves and nodules, which need to import Fe for active heme synthesis, Fenton reactions may be avoided in part by maintaining very low levels of H_2O_2 due to the presence of specific APX isozymes *(see "ASC-GSH cycle").*

Antioxidants of nodules

Given the susceptibility of the process of N_2 fixation to oxidative damage, it is not surprising that legume nodules have evolved an elaborate and vast array of antioxidant defenses aimed to destroy ROS or prevent their formation. Antioxidant enzymes of nodules include SOD, catalase, and the components of the ascorbate-glutathione (ASC-GSH) cycle, and antioxidant metabolites include ASC, thiol tripeptides, and tocopherols.

Superoxide dismutases

The SOD family is composed of metalloproteins that catalyze the dismutation of O_2^- radicals to O_2 and H_2O_2. Three classes of SODs are known in plants depending on the active site metal cofactor (Mn, Fe, or Cu plus Zn). The MnSODs and FeSODs are structurally related, whereas CuZnSODs show no structural relationship to the others and are thought to have

evolved independently. The three enzymes exhibit distinct molecular properties, including differential sensitivity to inhibitors, and are located in different subcellular compartments. We will know review what is known on SODs from bacteroids and nodule host cells.

Free-living and symbiotic rhizobia may contain MnSOD and/or FeSOD isozymes. The bacteroids from bean nodules have a single homodimeric MnSOD of 41 kDa, which is expressed, in addition to an inducible FeSOD, in the corresponding free-living bacteria (Dimitrijevic et al. 1984). The bacteroids from *Sesbania rostrata* stem nodules contain both MnSOD and FeSOD (Puppo et al. 1986). An inducible FeSOD was the only isozyme detected in free-living *Bradyrhizobium japonicum* (Stowers and Elkan 1981), but in a subsequent survey of several free-living bacteria and bacteroids from agronomically relevant legumes, including soybean, only MnSOD was found (Becana and Salin 1989). A single SOD was detected in free-living *Sinorhizobium meliloti*. The complete sequence of the relevant gene *(sodA)* was recently obtained and found to encode an unusual (cambialistic) SOD that can use Fe or Mn as cofactor and that is resistant with either cofactor to H_2O_2 inactivation (Santos et al. 1999). Two isozymes were detected in the corresponding alfalfa nodule bacteroids, which, judging from their insensitivity to H_2O_2, were ascribed to the MnSOD type (Becana and Salin 1989).

All legume nodules contain CuZnSOD in the cytosol but additional isozymes of the CuZn form may occur in the plastids and other organelles. The enzyme from the soybean nodule cytosol is a homodimer of 32 kDa and, like other CuZnSODs, is inhibited by cyanide and diethyldithiocarbamate (Puppo et al. 1982). Cytosolic CuZnSOD activity is high in young nodules and may have an essential role in scavenging the O_2^- radical formed by the autoxidation of Lb (Fig. 1), thus protecting N_2 fixation (Puppo et al. 1982, Becana et al. 1989). An additional protection of N_2 fixation mediated by CuZnSODs occurs in *Sesbania* stem nodules. The abundant chloroplasts of the nodule cortex photosynthetically generate O_2 and O_2^-, which are toxic to nitrogenase. In this case, as occurs in leaves, the O_2^- radical is eliminated specifically by a chloroplastic CuZnSOD isozyme (Puppo et al. 1986). Sequences of genes encoding cytosolic *(sodCc)* or plastidic *(sodCp)* CuZnSODs of legume nodules have not been reported.

Nodule mitochondria contain MnSOD, on the basis of the resistance of the enzyme to inactivation by cyanide and H_2O_2. As with other higher plant MnSODs, the nodule enzymes are probably homotetrameric proteins in the range of 79 to 84 kDa (Puppo et al. 1987). The occurrence of FeSOD in nodule host cells, as in leaf tissue, has been more enigmatic. The FeSOD isozymes are resistant to cyanide but are inactivated by H_2O_2. Based on the use of these inhibitors on activity stained gels, FeSODs were initially found to be confined to a few families of vascular plants**.** However, the subsequent isolation of FeSOD-encoding genes *(sodB)* in plants of such diversity as *Nicotiana, Arabidopsis*, soybean, and rice suggested a widespread distribution of the enzyme in the plant kingdom (reviewed by Bowler et al. 1994). An FeSOD isozyme was also found in cowpea nodules (Becana et al. 1989) and the observation has now been extended to alfalfa, common bean, and mungbean nodules (M.C. Rubio and M. Becana, unpublished results). The enzyme could not be detected in lupine nodules whereas multiple FeSOD isozymes are expressed in soybean nodules.

Full-length cDNAs encoding FeSODs from cowpea and alfalfa nodules have been isolated in our laboratory (J.F. Moran, M.C. Rubio and M. Becana, unpublished results). The alfalfa enzyme is similar to those from photosynthetic tissues and is localized in the plastids. In leaves, FeSOD is induced in response to chloroplast-localized oxidative events (Tsang et al. 1991). The constitutive presence of FeSOD in healthy nodules suggests that this enzyme has a specific defensive role in nodule plastids. The importance of antioxidative processes in plastids is further underscored by the presence of ferritin and the first enzyme for biosynthesis of GSH (see below) in these organelles.

Catalases

Plant catalases (EC 1.11.1.6) are tetrameric hemoproteins that exist as multiple isozymes encoded by nuclear genes. They are located mostly in peroxisomes and glyoxysomes, although a specific isozyme, Cat3, is present in maize mitochondria (Scandalios et al. 1997). The catalase of soybean nodules is a typical homotetramer of 220 kDa (Puppo et al. 1982). This enzyme may be especially abundant in the peroxisomes of determinate nodules, where it

eliminates the H_2O_2 generated by uricase and possibly other oxidases (Kaneko and Newcomb 1987).

Bacteroids also contain high levels of catalase but no guaiacol peroxidases (Becana et al. 1989). Early studies (Francis and Alexander 1972) showed that effective white clover and soybean nodules have greater catalase activities than ineffective nodules. Bacteroids of *S. meliloti* apparently contain a single catalase isozyme, KatA, whereas two additional isozymes, KatB and KatC, are expressed in the free-living bacteria. Experiments with null KatA and double null KatAKatC mutant bacteria showed that KatA plays a minor protective role in N_2 fixation but that at least one of the two catalases is important for maintaining nodule function (Sigaud et al. 1999). The presence of catalases in the bacteroids seems paradoxical since these enzymes generate O_2, which is toxic to nitrogenase, and generally have very low affinity for H_2O_2. Peroxidases suffer from neither of these disadvantages so their absence from bacteroids is somewhat puzzling.

ASC-GSH cycle

The ASC-GSH cycle is one of the main antioxidant defenses in plants. This pathway has been reviewed extensively elsewhere (Dalton 1995, Noctor and Foyer 1998, Asada 1999) and is most widely recognized for its role in scavenging of H_2O_2 in chloroplasts. However, all the components of this pathway are also present in the mitochondria and peroxisomes of leaves (Jiménez et al. 1997), and in the cytosol of nodule cells (Dalton et al. 1986, Dalton 1995). The leading reaction of this pathway is catalyzed by APX and involves the reduction of H_2O_2 to water using the reducing power of ASC. The soybean nodule APX has recently been the subject of extensive biochemical studies (Jones et al. 1998). Subsequent reactions of the ASC-GSH pathway involve the regeneration of ASC by either NADH-dependent monodehydroascorbate reductase (EC 1.6.5.4) or coupled reactions that include dehydroascorbate reductase (EC 1.8.5.1) and a NADPH-dependent glutathione reductase (EC 1.6.4.2). At least some enzymes of the ASC-GSH cycle, including a membrane-bound APX,

are also present in mitochondria of soybean nodules (Dalton et al. 1993a) and pea leaves (Jiménez et al. 1997).

Several lines of evidence suggest that this pathway is important for maintaining N_2 fixation in nodules. Nodule effectiveness is highly correlated with the components of this pathway (Dalton et al. 1993b). APX is very abundant in nodules (1% of total soluble protein), especially in the cytosol of infected cells, which is consistent with its role in scavenging of H_2O_2 generated by Lb autoxidation (Dalton 1995). APX has been shown to enhance N_2 fixation up to 4.5-fold in an in vitro model system consisting of bacteria *(Azorhizobium)* or soybean nodule bacteroids; in this model system, Lb was also maintained in a more favorable oxygenation state (ie. a higher proportion of $Lb^{2+}O_2$) in the presence of ASC and APX (Ross and Dalton 1999).

In addition, APX transcript and protein levels are particularly abundant in the parenchyma (inner cortex) of nodules (Dalton et al. 1998). This same region also has elevated levels of respiratory activity (Dalton et al. 1998), phosphoenolpyruvate carboxylase (Robinson et al. 1996), and carbonic anhydrase (Coba de la Peña et al. 1997). It seems probable that the elevated levels of APX in the nodule parenchyma are a consequence of the increased need for antioxidant defenses due to respiratory H_2O_2 production. The aerobic respiration in this zone may function not only to fuel the high energy demands of N_2 fixation, but also to provide a type of respiratory protection in which the diffusion of O_2 into the nodule interior is limited. Furthermore, the increased CO_2 production in the parenchyma can be used to produce malate, following a series of reactions catalyzed by carbonic anhydrase, phosphoenolpyruvate carboxylase, and malate dehydrogenase (Fig. 2). Malic acid (coupled to K^+) has been suggested to play a role as osmoticum driving water into the intercellular spaces and thus regulating the extent of a variable O_2 diffusion barrier (ODB) localized in the nodule parenchyma (Minchin 1997). In addition, H_2O_2 can promote in vitro cross-linking of intercellular glycoproteins (N.J. Brewin, personal communication) and this material has been detected in the intercellular spaces of the nodule parenchyma. We believe that the ability of APX (coupled to respiratory activity) to fine tune the concentration of H_2O_2 in the parenchyma cell layers contributes to the operation of the ODB and can be considered as an

additional protective role of APX (beyond its role in infected cells) in the process of N_2 fixation.

Guaiacol and GSH peroxidases

Guaiacol peroxidases (EC 1.11.1.7) are glycosylated hemoproteins of approximately 50 kDa (horseradish peroxidase is a typical example) which are present as multiple isozymes in plant tissues and are involved in processes such as lignification and auxin metabolism. Several guaiacol peroxidases are present in nodules but their functions and subcellular localizations are unknown (Becana et al. 1989).

GSH peroxidases (EC 1.11.1.9) are commonly found in animal mitochondria and catalyze the GSH-dependent reduction of organic hydroperoxides (and H_2O_2) to the corresponding alcohols. Very recently, a cDNA has been isolated from pea leaf RNA that encodes a chloroplastic phospholipid hydroperoxide GSH peroxidase, and similar or additional isozymes may exist in other plants (Mullineaux et al. 1998). However, the physiological role of GSH peroxidase in plants has not been clearly defined. Because APX will not catalyze the reduction of organic peroxides, it would be important to know whether a GSH peroxidase similar to that detected in pea chloroplasts or another specific enzyme exists in the nodules to detoxify lipid and other organic hydroperoxides.

Ferritin

Ferritin is an antioxidant protein in its own right because of its ability to sequester and store up to 4,500 atoms of Fe per molecule in a catalytically-inactive form, thus preventing Fe-dependent oxidative damage. Nodules have a high demand for Fe due to the role of this element in many important enzymes involved with N_2 fixation (ferredoxin, nitrogenase, and Lb). In fact, ferritins are abundant early in nodulation and then decline concomitantly with the increases in nitrogenase and Lb, suggesting that ferritin provides Fe for the other Fe-proteins (Ragland and Theil 1993). The soybean nodule ferritin is a multimeric protein of

approximately 570 kDa and is positively correlated with nodule effectiveness (Ko et al. 1987). Ferritins are localized predominantly in the plastids and amyloplasts of uninfected cells (Lucas et al. 1998, Matamoros et al. 1999a).

Ascorbate

ASC (vitamin C) is a universal reductant and antioxidant of plants. It is found at concentrations of 1-2 mM in legume nodules (Dalton et al. 1986, Gogorcena et al. 1997, Matamoros et al. 1999a) and is positively correlated with nodule effectiveness (Dalton et al. 1993b). It is an essential metabolite for the operation of the ASC-GSH pathway but it also has beneficial effects that do not require the presence of APX. ASC can directly scavenge ROS and reduce ferric Lb and Lb^{IV} (Moreau et al. 1995b). It is also involved in the hydroxylation of proline, regulation of the cell cycle, and numerous fundamental processes of plant growth and development (Chinoy 1984, Noctor and Foyer 1998). The most definitive demonstration of the beneficial properties of ASC in nodules has come from studies in which exogenous ASC was supplied to soybean plants through direct infusion into stems. This caused a 4-fold increase in nitrogenase activity and a marked delay in nodule senescence (Bashor and Dalton 1999).

Thiols

The thiol tripeptide GSH (γGlu-Cys-Gly) is a versatile antioxidant that can directly scavenge ROS and participate in the ASC-GSH cycle for H_2O_2 removal in the chloroplasts and nodule cytosol (Dalton 1995). It is also involved in many other functions of plants, including the transport and storage of sulfur, stress tolerance, and the detoxification of heavy metals (Noctor and Foyer 1998). The pathway for GSH synthesis is shared by all organisms and involves two ATP-dependent steps catalyzed sequentially by γ-glutamylcysteine synthetase (γECS; EC 6.3.2.2) and glutathione synthetase (GSHS; EC 6.3.2.3). In plants, both enzymes

are localized to the chloroplasts and cytosol of leaves, and at least γECS is also localized to the proplastids of roots (Noctor and Foyer 1998).

The leaves, roots, and seeds of some legumes contain homoglutathione (hGSH; γGlu-Cys-βAla) instead of or in addition to GSH (Klapheck 1988). The synthesis of hGSH in the leaves proceeds through a specific homoglutathione synthetase (hGSHS) that is localized in both the chloroplasts (17%) and the extraplastidic (83%) compartment (Macnicol 1987, Klapheck et al. 1988). It is not known why hGSH is restricted to legumes and what specific role(s), if any, this thiol may play in nodule functioning. To address these questions, we conducted a preliminar survey of thiol composition in eight legumes of agronomic interest. This study showed that GSH is the major nonprotein thiol in indeterminate nodules, whereas hGSH is predominant in determinate nodules, except in those of cowpea, which only contain GSH (Matamoros et al. 1999b). The concentrations of GSH (0.4-1 mM) and hGSH (0.4-0.6 mM) in nodules are, on average, 2.2-fold and 6-fold greater, respectively, than in the leaves and roots of the same plants. The precursor thiols, cysteine (30-120 □M) and γ̃glutamylcysteine (7-50 □M), are also more abundant in nodules than in leaves and roots. The relative abundance of GSH and hGSH in different legume tissues and species is determined by the corresponding thiol tripeptide synthetases. Thiols and GSHS are especially abundant in the meristematic and infected zones of pea nodules. Thiols and γECS are also more abundant in the infected zone of bean nodules, but hGSHS is predominant in the cortex.These observations, along with the finding that the concentrations of thiol tripeptides in effective nodules are 3-4 fold higher than in ineffective nodules (Dalton et al. 1993b), strongly imply that GSH and hGSH play protective roles in N_2 fixation (Matamoros et al. 1999b).

The synthesis of GSH and hGSH in nodules was also examined at the molecular level. Sequence analyses of full-length cDNA clones encoding γECS proteins from legume nodules reveals that they are highly homologous to those from other higher plants. The N-terminal sequence contains a consensus motif that presumably targets the proteins to the plastids (Frendo et al. 1999, Matamoros et al. 1999b). Partial sequences encoding GSHS and hGSHS in *Medicago truncatula* have been isolated and the relative abundance of their transcripts in

different plant tissues is correlated with the concentrations of the corresponding tripeptides (Frendo et al. 1999). Furthermore, subcellular fractionation studies and sequence analyses of full-length cDNAs encoding γECS, GSHS and hGSHS from nodules of several legume species indicate that γECS is plastidic and hGSHS is cytosolic, and that there are GSHS isozymes in the cytosol and mitochondria (J.F. Moran, I. Iturbe-Ormaetxe, M.A. Matamoros, M.C. Rubio, M.R. Clemente, N.J. Brewin, and M. Becana, unpublished results).

Other antioxidant metabolites

Nodules contain other compounds with antioxidant properties. Soybean nodules contain 15 □g per g fresh weight of □tocopherol (Evans et al. 1999). This compound may serve to protect symbiosome membranes and other nodule membranes against lipid peroxidation. Polyamines are organic polycations involved in plant growth, development, and senescence. They are also potent ROS scavengers and inhibitors of lipid peroxidation (Drolet et al. 1986). This antioxidant activity is, however, more effective at concentrations greater than those (1-2 mM) presumably found in nodules (Fujihara et al. 1994). Uric acid, another potent antioxidant (Ames et al. 1981), is an intermediate in the pathways of purine degradation and ureide synthesis and is present in peroxisomes (Del Río et al. 1998). Flavonoids and other phenolics are abundant in nodules, where, despite their obvious role as signal molecules during nodule initiation, they can inhibit lipid peroxidation by intercepting the peroxyl radicals formed in nodule membranes (Moran et al. 1997).

Antioxidants and nodule formation

Plants respond to pathogens and elicitors with a rapid and transitory production of ROS referred to as the oxidative burst. Some signal molecules mediating this process may be salicylic acid, lipid peroxides, and some of their derivatives, but the mechanism remains to be clarified. Important similarities exist between nodulation and defense responses, including the up-regulation of enzymes of the phenylpropanoid pathway and the polymerization of hydroxyproline-rich glycoproteins (reviewed by Baron and Zambryski 1995). Martínez-

Abarca et al. (1998) demonstrated that legumes that have been inoculated with compatible strains of rhizobia do not accumulate salicylic acid in roots, whereas those inoculated with mutant strains unable to synthesize Nod factors do accumulate salicylic acid, thus indicating a defensive response. They proposed that compatible Nod factors suppress the defensive response of the legume, by an as yet unknown mechanism, thus avoiding rejection of rhizobia and allowing the infection process to proceed. Activities of several antioxidant enzymes (catalase, APX, SOD, and glutathione reductase) increase in the roots shortly after infection by compatible rhizobia (J. Olivares, personal communication). The induction of antioxidant enzymes may occur even earlier. Thus, a novel peroxidase gene is transiently expressed in alfalfa roots during the preinfection period and this induction can also be triggered by Nod factors (Cook et al. 1995). All these observations provide indirect evidence that ROS formation is involved in the initial stages of the rhizobial-plant interaction.

Antioxidants and nodule senescence

It is evident from what has been already reviewed that actively N_2-fixing nodules are particularly rich, both in content and diversity, in antioxidant defenses and that these are sufficient to cope with ROS toxicity. However, during the natural senescence (aging) of nodules, some antioxidants (GSH, hGSH, catalase), but not others (APX, ASC, $\tilde{\alpha}$tocopherol), significantly decline (Dalton et al. 1986, Swaraj et al. 1995, Evans et al. 1999, Matamoros et al. 1999b). Concomitantly, in aging nodules there is accumulation of catalytic Fe and oxidation of thiols, lipids, proteins, and DNA (Evans et al. 1999). Nevertheless, aging causes a 50% decrease of hGSH in soybean and bean nodules, and a 82% decrease of GSH in pea nodules, which strongly suggests that at least some antioxidant defenses are also compromised (Evans et al. 1999, Matamoros et al. 1999b). A similar decrease in thiol content is observed in mature pea nodules, which typically show an age gradient from the apex to the base (indeterminate growth pattern). The senescent zone have 49% less GSH than the meristematic and infected zones. The important decrease in thiol content with age may be

ascribed to both inhibition of thiol synthesis and augmented ROS-dependent thiol degradation (Matamoros et al. 1999b).

Nodule senescence can also be induced by exposure of plants to water or salt stress, excess nitrate, and prolonged darkness (Gogorcena et al. 1995, Escuredo et al. 1996, Comba et al. 1998, Swaraj et al. 1995 and references therein, Matamoros et al. 1999a). In most cases, along with the inhibition of nitrogenase activity and degradation of Lb, there is a marked decline in the major activities involved in H_2O_2 removal, APX and catalase, as well as in thiol and ASC content. The concentration of catalytic Fe was found to increase in water-stressed pea leaves and in stressed pea and bean nodules, leading to the hypothesis that catalytic Fe is involved in the oxidative damage of proteins, and probably other important biomolecules, in senescing plant tissues (reviewed by Becana et al. 1998).

The changes of some antioxidant defenses during nodule senescence, whether natural or induced, have been also examined using light and electron microscopy. Nitrate causes severe alterations in the ultrastructure of peroxisomes along with a marked decrease in the cytochemical staining of catalase in lupine nodules (Lorenzo et al. 1990). Nitrate and dark stress also lead to major decreases in the content of immunolabeled APX in the parenchyma and infected cells of bean and pea nodules (Matamoros et al. 1999a). Immunolocalization studies of ferritin in soybean and lupine nodules shows a decrease of the protein in infected cells but accumulation in the cortex (Lucas et al. 1998). Ferritin is hardly detectable in mature bean nodules but accumulates in the plastids and amyloplasts of the parenchyma and uninfected interstitial cells after treatment with nitrate (Matamoros et al. 1999a).

Conclusions and future directions

During the last few years, considerable effort has been devoted to identifying the main antioxidants of legume nodules and demonstrating that nodules experience oxidative stress during natural and induced senescence. Nodules are particularly rich in antioxidants, and these may play an essential protective role in N_2 fixation. Many enzyme antioxidants exist as multiple isoforms located in different nodule compartments, where they probably fulfill

specific defensive roles against ROS, which are formed by respiration and other enzymatic processes, by oxidation of Lb and O_2-labile proteins, and by Fe-catalyzed decomposition of peroxides.

The field is now ripe for the cellular and genetic dissection of the antioxidant pathways themselves and of the mechanisms by which these pathways are activated (or fail to be activated) in response to stress. The studies on the control of gene expression at the tissue, cell, and organelle levels will be greatly facilitated by the use of transgenic model and crop legumes. Likewise, production of rhizobial strains with improved antioxidant defenses may enhance symbiotic performance, particularly under nonoptimal conditions. Up and down modulation of important antioxidant genes of both symbiotic partners will therefore be of academic and agronomic interest.

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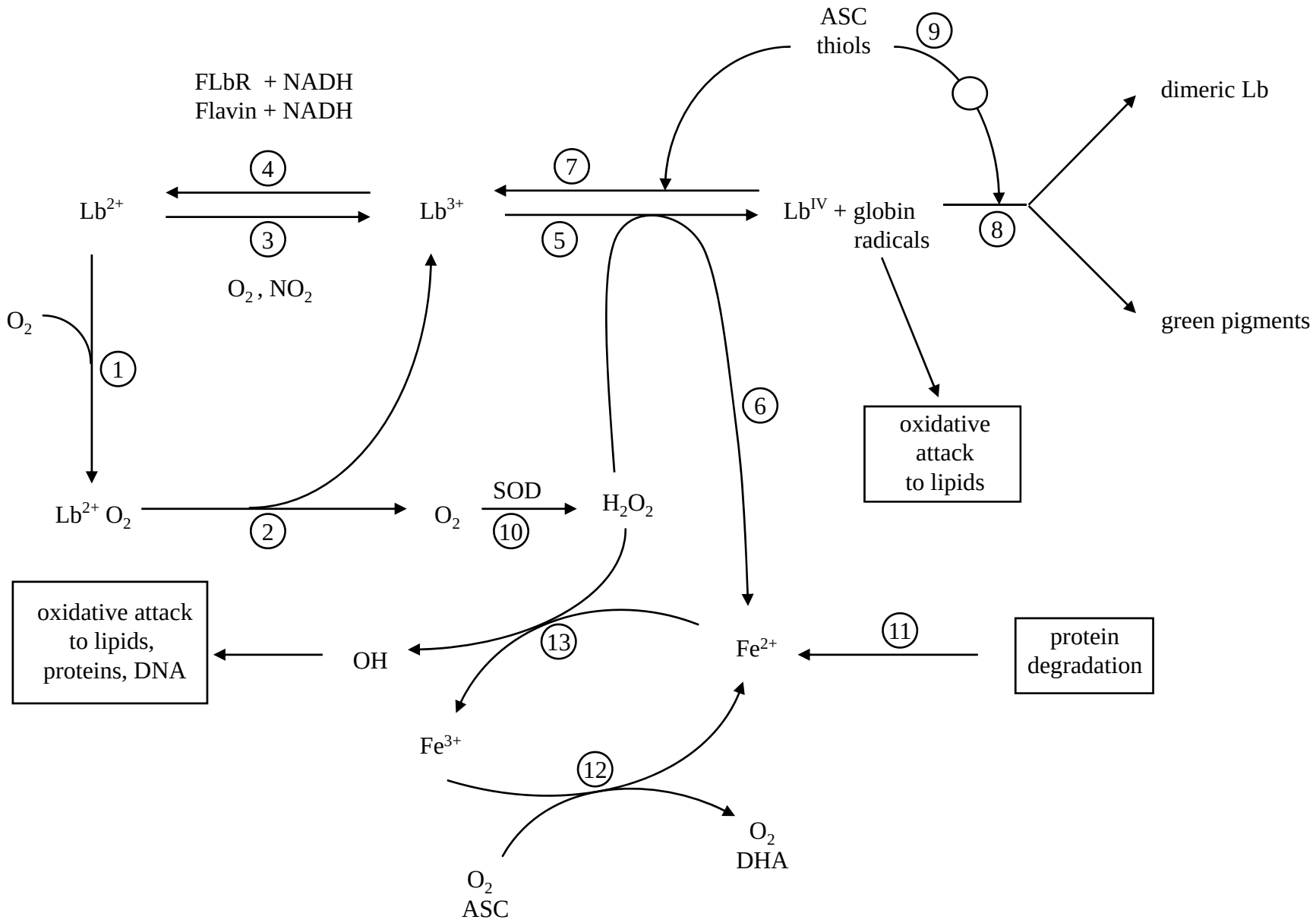
Fig. 1. Redox reactions involving Lb and formation of ROS. **(1)** Reversible oxygenation of Lb^{2+} to $Lb^{2+}O_2$. **(2)** Autoxidation of $Lb^{2+}O_2$ to Lb^{3+}, with formation of O_2^- radical. **(3)** Oxidation of Lb^{2+} to Lb^{3+} by the O_2^- radical or nitrite. **(4)** Reduction of Lb^{3+} to Lb^{2+} by ferric Lb reductase (FLbR) or flavins plus NADH. **(5)** Oxidation of Lb^{3+} to Lb^{IV} and globin radicals by moderate concentrations of H_2O_2. The resulting globin radicals may initiate lipid peroxidation. **(6)** Breakdown of the heme group of $Lb^{2+}O_2$ (not shown for clarity) and Lb^{3+} by high concentrations of H_2O_2, with release of Fe. **(7)** Reduction of Lb^{IV} by ASC and thiol compounds. **(8)** Quenching of globin radicals to dimeric Lb (protein-protein cross-linking) and green pigments (heme-protein cross-linking). **(9)** Inhibition of dimer and green pigment formation by ASC and thiols. **(10)** Dismutation of O_2^- radical to H_2O_2 catalyzed by SOD. **(11)** Release of Fe by degradation of Fe-proteins (or, for example, by ASC-dependent Fe mobilization of ferritin) during nodule senescence. **(12)** Recycling of Fe^{2+} from Fe^{3+} by the O_2^- radical (producing O_2) or by ASC (producing dehydroascorbate: DHA). **(13)** Reduction of H_2O_2 to ·OH radical catalyzed by Fe^{2+} (Fenton reaction). The resulting ·OH radical can oxidatively attack virtually all nodule cell components (lipids, proteins, DNA).

Fig. 2. Hypothesis linking ROS formation, carbon metabolism, and operation of the ODB in the nodule parenchyma. Based on determinations of O_2 concentration (Hunt and Layzell 1993, Minchin 1997) and localization of the following enzymes (activities, proteins, or transcripts): carbonic anhydrase (CA; Coba de la Peña et al. 1997), phosphoenolpyruvate carboxylase (PEPC; Pathirana et al. 1997), respiratory flavin dehydrogenases (Dalton et al. 1998), and APX (Dalton et al. 1998, Matamoros et al. 1999a). Malate formed in the nodule parenchyma would be used in connection with the ODB, but probably not as a respiratory substrate for bacteroids; for this purpose, malate is produced in the infected cells of the central zone. DHA, dehydroascorbate; MDH, malate dehydrogenase; OAA, oxaloacetate.

Fig. 1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FLbR + NADH
Flavin + NADH
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7
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Lb3+
LbIV + globin
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8
O2 , NO2
green pigments
O2
1
6
oxidative
attack
to lipids
Lb2+ O2
2
O2
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H2O2
oxidative attack
to lipids,
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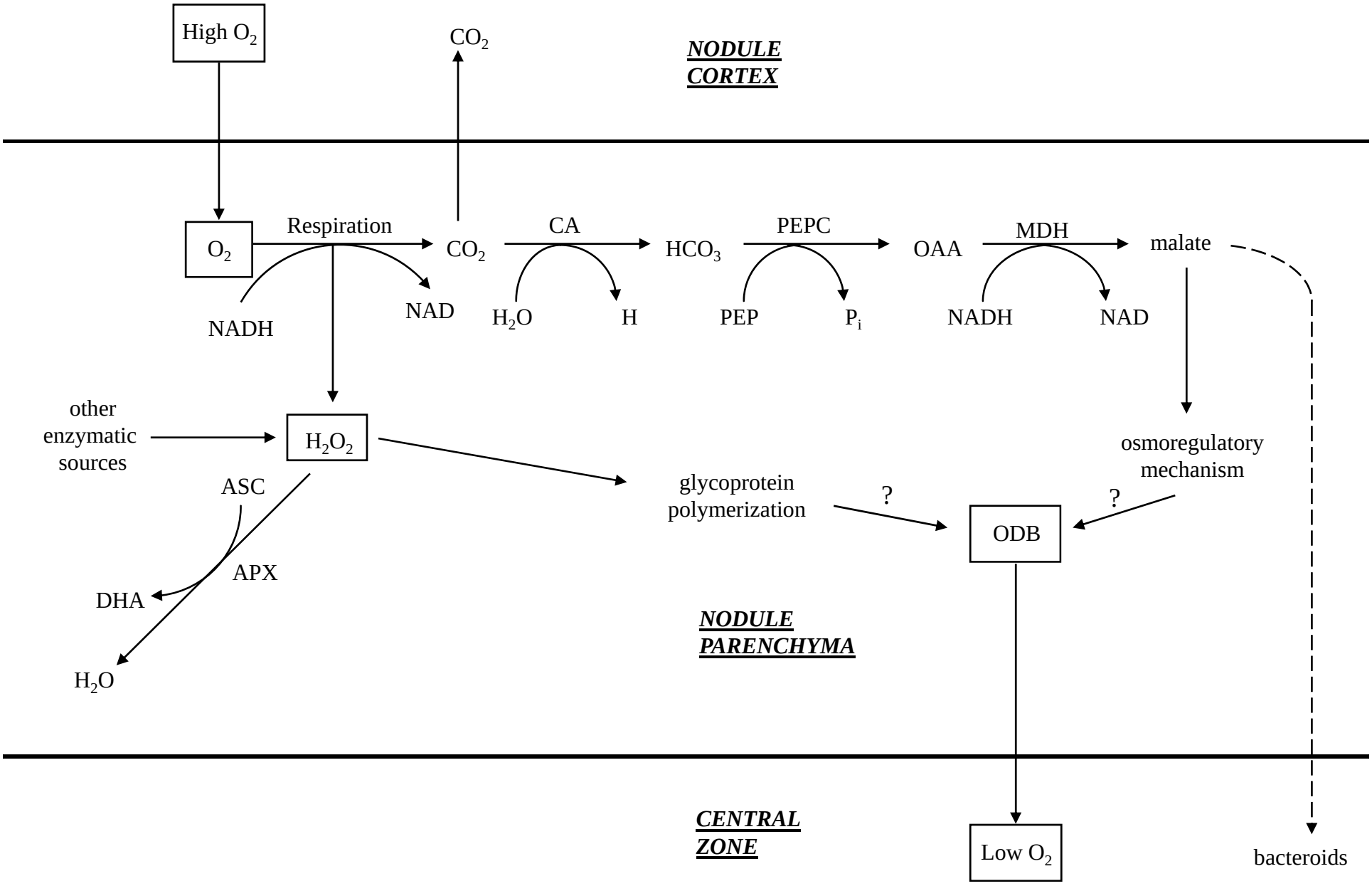
ASC

Fig. 2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malate
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NODULE PARENCHYMA
CENTRAL ZONE
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