

Reactive Oxygen Species and Reactive Nitrogen Species in Peroxisomes. Production, Scavenging, and Role in Cell Signaling¹

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Peroxisomes can be broadly defined as subcellular organelles bounded by a single membrane that contain as basic enzymatic constituents catalase and hydrogen peroxide (H₂O₂)-producing flavin oxidases and occur in almost all eukaryotic cells (Baker and Graham, 2002). In recent years, it has become increasingly clear that peroxisomes carry out essential functions in eukaryotic cells (Baker and Graham, 2002; del Río et al., 2002; Igamberdiev and Lea, 2002; Titorenko and Rachubinski, 2004). The peroxisome of plant cells is a highly dynamic compartment that is dependent upon the actin cytoskeleton, not microtubules, for its subcellular distribution and movements (Minorsky, 2002). These organelles have an essentially oxidative type of metabolism, and a characteristic property of peroxisomes is their metabolic plasticity, because their enzymatic content can vary depending on the organism, cell/tissue-type, and environmental conditions (Baker and Graham, 2002).

Plant peroxisomes also play a significant role in photomorphogenesis (Hu et al., 2002), degradation of branched amino acids, biosynthesis of the plant hormones jasmonic acid and auxin, and the production of the compatible osmolyte Glycyl betaine (Minorsky, 2002; Reumann et al., 2004). Moreover, evidence for the existence of regulatory proteins in peroxisomes, like heat shock proteins, kinases, and phosphatases, is just emerging (Hayashi and Nishimura, 2003; Reumann et al., 2004). In plants, the cellular population of peroxisomes can proliferate during senescence and under different stress conditions produced by xenobiotics,

ozone, cadmium, and H₂O₂ (del Río et al., 1998, 2002; Romero-Puertas et al., 1999; Nila et al., 2006). Peroxisome proliferator-activated receptor, the transcription factor involved in peroxisomal proliferation and induction of peroxisomal fatty acid β -oxidation in animal tissues, recently was demonstrated to be functional in transgenic tobacco (*Nicotiana tabacum*) plants (Nila et al., 2006).

A total of 286 peroxisomal genes coding for putative peroxisomal proteins have been identified in the Arabidopsis (*Arabidopsis thaliana*) genome (Hayashi and Nishimura, 2003). About 220 and 60 proteins were identified that carry a putative peroxisome targeting signal PTS1 or PTS2, respectively (Reumann et al., 2004). The information on subcellular targeting prediction, homology, and in silico expression analysis for these Arabidopsis proteins was compiled in the public database AraPeroX (<http://www.araperox.uni-goettingen.de>; Reumann et al., 2004).

In recent years, different experimental evidence has indicated the existence of cellular functions for peroxisomes related to reactive oxygen and nitrogen species (ROS and RNS).

PRODUCTION OF ROS IN PEROXISOMES

In plant cells, as in most eukaryotic organisms, peroxisomes are probably the major sites of intracellular H₂O₂ production. In more recent years, it has been demonstrated that superoxide (O₂^{•-}) and nitric oxide (NO[•]) radicals are also produced in peroxisomes. The main metabolic processes responsible for the generation of H₂O₂ in different types of peroxisomes are the photorespiratory glycolate oxidase reaction, the fatty acid β -oxidation, the enzymatic reaction of flavin oxidases, and the disproportionation of O₂^{•-} radicals (Baker and Graham, 2002; del Río et al., 2002; Foyer and Noctor, 2003).

Peroxisomes, like mitochondria and chloroplasts, produce O₂^{•-} radicals as a consequence of their normal metabolism. In peroxisomes from pea (*Pisum sativum*) leaves and watermelon (*Citrullus vulgaris*) cotyledons, there are at least two sites of O₂^{•-} generation: one in the organelle matrix, in which the generating system was

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identified as xanthine oxidase, and another site in the peroxisomal membranes dependent on NAD(P)H (del Río et al., 2002; Fig. 1). Xanthine oxidase catalyzes the oxidation of xanthine and hypoxanthine to uric acid and is a well-known producer of $O_2^{\cdot-}$ radicals (Halliwell and Gutteridge, 2000).

The other site of $O_2^{\cdot-}$ production is the peroxisomal membrane, where a small electron transport chain appears to be involved (Fig. 1). This is composed of a flavoprotein NADH:ferricyanide reductase of about 32 kD and a cytochrome *b* (del Río et al., 2002). Three integral peroxisomal membrane polypeptides (PMPs) of pea leaf peroxisomes, with molecular masses of 18, 29, and 32 kD, have been characterized and demonstrated to be responsible for $O_2^{\cdot-}$ generation (López-Huertas et al., 1999). The main producer of $O_2^{\cdot-}$ radicals in the peroxisomal membrane was the 18-kD PMP, which was proposed to be a cytochrome *b* (López-Huertas et al., 1999). While the 18- and 32-kD PMPs use NADH as electron donor for $O_2^{\cdot-}$ production, the 29-kD PMP was dependent on NADPH, and was able to reduce cytochrome *c* with NADPH as electron donor (López-Huertas et al., 1999; del Río et al., 2002). The PMP32 very probably corresponds to the monodehydroascorbate reductase (MDAR), and the third $O_2^{\cdot-}$ -generating polypeptide, PMP29, could be related to the peroxisomal NADPH:cytochrome P450 reductase (López-Huertas et al., 1999).

PRODUCTION OF RNS IN PEROXISOMES

In plants, there is increasing evidence of a role of NO^{\cdot} as an endogenous plant growth regulator as well as a signal molecule in the transduction pathways leading to

the induction of defense responses against pathogens and in damage initiating cell death (Delledonne et al., 1998, 2001; Durner et al., 1998; Klessig et al., 2000). The enzyme NO^{\cdot} synthase (NOS) catalyzes the oxygen- and NADPH-dependent oxidation of L-Arg to NO^{\cdot} and citrulline in a complex reaction requiring different co-factors (Alderton et al., 2001). The occurrence of this NO^{\cdot} -producing enzyme in isolated peroxisomes was first demonstrated in plant tissues, in leaves of pea plants (Barroso et al., 1999). This Arg-dependent enzyme required NADPH, BH_4 , calmodulin, and calcium, was sensitive to archetypical inhibitors of mammalian NOSs, and its NO^{\cdot} production was inhibited by an antibody against mouse iNOS (Barroso et al., 1999; Corpas et al., 2004a). In pea leaves, olive (*Olea europaea*) leaves, and sunflower (*Helianthus annuus*) cotyledons, the presence of the enzyme in the matrix of peroxisomes was demonstrated by immunocytochemistry (Barroso et al., 1999; Corpas et al., 2004b). The specific activity of the peroxisomal Arg-dependent NOS was very similar to that reported for the NOS characterized in Arabidopsis (AtNOS1 protein), which has been localized in mitochondria of this plant species (Guo and Crawford, 2005). The presence of NOS in plant peroxisomes was extended years later to animal peroxisomes (Stolz et al., 2002).

The production of NO^{\cdot} in peroxisomes purified from pea leaves was demonstrated by fluorometric analysis and electron paramagnetic resonance spectroscopy with the spin trap $Fe(MGD)_2$ (Corpas et al., 2004a). S-nitrosoglutathione (GSNO) is another RNS that can be formed in peroxisomes by the reaction of reduced glutathione with NO^{\cdot} , which has been recently immunolocalized in pea leaf peroxisomes using an antibody to GSNO (L.M. Sandalio, unpublished data).

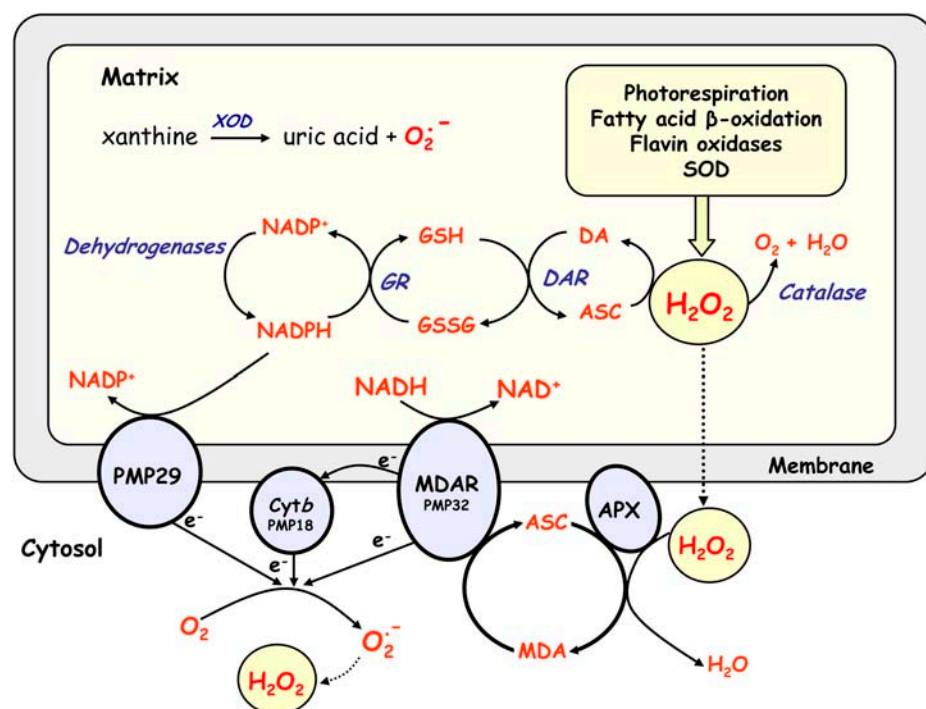


Figure 1. Model proposed for the production of $O_2^{\cdot-}$ radicals and function of antioxidative enzymes of the ascorbate-glutathione cycle in leaf peroxisomes. The model is based on results described by different authors (del Río et al., 1998, 2002; López-Huertas et al., 1999). ASC, Ascorbate, reduced form; DA, dehydroascorbate, ascorbate oxidized form; DAR, dehydroascorbate reductase; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; MDA, monodehydroascorbate.

ANTIOXIDANT SYSTEMS IN PEROXISOMES

The occurrence of $O_2^{\cdot-}$ dismutases (SODs) in isolated plant peroxisomes has been reported in at least nine different plant species (del Río et al., 2002). Results obtained concerning the presence of SOD in plant peroxisomes were extended years later to human and animal peroxisomes (del Río et al., 2002). Three SODs of peroxisomal origin have been purified and characterized (del Río et al., 2002).

The ascorbate-glutathione cycle that occurs in chloroplasts, cytoplasm, and mitochondria (Noctor and Foyer, 1998) has also been demonstrated in peroxisomes. The four enzymes of the cycle, ascorbate peroxidase (APX), MDAR, dehydroascorbate reductase, and glutathione reductase (GR) are present in peroxisomes purified from pea leaves and tomato (*Lycopersicon esculentum*) leaves and roots (del Río et al., 1998; Mittova et al., 2004; Kuźniak and Skłodowska, 2005). The intraperoxisomal distribution of the ascorbate-glutathione cycle was studied in pea leaves, and a model for the function of the ascorbate-glutathione cycle is shown in Figure 1. The peroxisomal GR of pea leaves has been recently purified and characterized (Romero-Puertas et al., 2006). MDAR was also localized in the matrix of peroxisomes (Leterrier et al., 2005; Lisenbee et al., 2005) and the genomic clone of this antioxidative enzyme has been recently characterized (Leterrier et al., 2005). The occurrence of another peroxidase activity, glutathione peroxidase, has been reported in leaf peroxisomes of tomato plants (Kuźniak and Skłodowska, 2005). The presence of APX and MDAR in leaf peroxisomal membranes could protect against H_2O_2 leaking from peroxisomes, as well as the H_2O_2 that is being continuously formed by dismutation of the $O_2^{\cdot-}$ generated in the NAD(P)H-dependent electron transport system of the peroxisomal membrane (López-Huertas et al., 1999; del Río et al., 2002).

In isolated plant peroxisomes, the presence of three NADP-dehydrogenases was demonstrated, including Glc-6-P dehydrogenase, 6-phosphogluconate dehydrogenase, and isocitrate dehydrogenase (del Río et al., 2002). The presence in peroxisomes of these dehydrogenases implies that these organelles have the capacity to reduce $NADP^+$ to NADPH for its reutilization in their metabolism. NADPH is necessary for the function of GR activity, the NADPH:cytochrome P450 reductase (Baker and Graham, 2002) and the $O_2^{\cdot-}$ -generating polypeptide of peroxisomal membranes, PMP29 (López-Huertas et al., 1999), as well as for the reduction of double bonds of unsaturated fatty acids by 2,4-dienoyl-CoA reductase (Reumann et al., 2004). The peroxisomal NO-producing enzyme, NOS, also requires NADPH for its activity.

Peroxioredoxins (Prxs) are a family of thioredoxin-dependent peroxidases (Horling et al., 2002). A putative Prx with a molecular mass of 60 kD was localized in the matrix of pea leaf peroxisomes (Corpas et al., 2003), and in mammalian cells a Prx (Prx V) was also localized in these organelles (Seo et al., 2000). In

Arabidopsis, it has been suggested that two Prxs (Prx II B and Prx II C) could have a cytosolic or peroxisomal distribution (Horling et al., 2002). The localization of Prxs in peroxisomes would supply these organelles with another antioxidant enzyme system that would join catalase and the ascorbate-glutathione cycle in the control of the peroxisomal level of H_2O_2 .

FUNCTION OF PEROXISOMES IN OXIDATIVE STRESS

In most biotic and abiotic stress conditions, an overproduction of ROS has been demonstrated and these species are thought to be responsible for the oxidative damage associated with plant stress (Dat et al., 2000; Mittler, 2002). Under normal physiological conditions, the production by peroxisomes of ROS should be adequately controlled by the antioxidative enzymes present in peroxisomes. However, the risk of serious cellular damage can arise when, under stress conditions, the peroxisomal generation of ROS is enhanced and the protective antioxidative systems of the organelle are depressed.

Peroxisomes appear to have a ROS-mediated role in the oxidative reactions characteristic of senescence. The senescence-induced changes in the reactive oxygen metabolism of peroxisomes are mainly characterized by the disappearance of catalase activity and an overproduction of $O_2^{\cdot-}$ and H_2O_2 and a strong decrease of APX and MDAR activities (del Río et al., 1998). On the other hand, in peroxisomes from senescent pea leaves, the enzymatic production of NO[•] from L-Arg (NOS activity) was down-regulated by 72%, and this led to the proposal that peroxisomal NO[•] could be involved in the process of senescence of pea leaves (Corpas et al., 2004a). Since $O_2^{\cdot-}$ radicals under physiological conditions quickly dismutate into H_2O_2 and O_2 , the final result of senescence is a buildup in leaf peroxisomes of the more stable metabolite H_2O_2 , which can diffuse into the cytosol. This represents a serious situation for peroxisomes and other cell organelles such as mitochondria, nuclei, and chloroplasts, due to the possible formation of the strongly oxidizing $\cdot OH$ radicals by the metal-catalyzed reaction of H_2O_2 with $O_2^{\cdot-}$ (Halliwell and Gutteridge, 2000).

In leaf peroxisomes from plants subjected to stress conditions by xenobiotics, like clofibrate (ethyl- α -*p*-chlorophenoxyisobutyrate) and the herbicide 2,4-dichlorophenoxyacetic acid, an oxidative stress mechanism mediated by ROS, was demonstrated to be involved (Baker and Graham, 2002; del Río et al., 2002). In peroxisomal membranes, treatment of pea plants with the hypolipidemic drug clofibrate induced the 29-kD polypeptide (PMP29) and depressed the content of PMP32 (Baker and Graham, 2002) and also induced a proliferation of the peroxisomal population of pea and tobacco leaves (del Río et al., 2002; Nila et al., 2006), a similar effect to that previously described in rodents by Reddy et al. (1982).

Peroxisomal MDAR1 transcripts were induced in pea leaves sprayed with the herbicide 2,4-dichlorophenoxyacetic acid (Leterrier et al., 2005).

Leaf peroxisomes are also involved in heavy metal toxicity. In leaf peroxisomes from plants treated with cadmium, an enhancement of the H_2O_2 concentration as well as the oxidative modification of some endogenous proteins was found (Romero-Puertas et al., 1999, 2002). A slight increase of the peroxisomal population of pea leaves by cadmium was also observed (Romero-Puertas et al., 1999). Cadmium induces senescence symptoms in peroxisomes and, probably, a metabolic transition of leaf peroxisomes into glyoxysomes, with a participation of the peroxisomal proteases in all these metabolic changes (McCarthy et al., 2001; Palma et al., 2002). Peroxisomes responded to cadmium toxicity by increasing the activity of antioxidative enzymes involved in the ascorbate-glutathione cycle and the NADP-dehydrogenases located in these organelles.

In peroxisomes of leaves and roots from salt-tolerant tomato plants, there was an up-regulation of the antioxidative systems in response to salt-induced oxidative stress (Mittova et al., 2004). In Arabidopsis plants, salt stress induced the expression of three peroxisome-associated genes, including thiolase (*PEP1*), *PEX10*, and *PEX1*, and required components of the ethylene, jasmonate, and abscisic acid signaling pathways (Charlton et al., 2005).

Stress by H_2O_2 in tobacco plants with 10% of wild-type catalase activity showed that catalase was crucial for maintaining the redox balance during oxidative stress (Willekens et al., 1997). In transformed Arabidopsis plants, a model was proposed whereby diverse stresses that generate H_2O_2 as a signaling molecule result in peroxisome proliferation via the up-regulation

of peroxisome biogenesis genes (*PEX*). According to this model, the peroxisome proliferation by H_2O_2 might be a common mechanism of protection against oxidative stress, by making use of the antioxidants of peroxisomes (López-Huertas et al., 2000).

A ROS-dependent involvement of plant peroxisomes in fungal infection has been proposed in tomato plants (Kuzniak and Skłodowska, 2005). In addition, in the response of Arabidopsis plants to compatible fungal infections, epidermal peroxisomes appear to have a role in degrading ROS generated at penetrating sites (Koh et al., 2005).

ROLE OF PEROXISOMES AS A SOURCE OF ROS AND RNS SIGNAL MOLECULES

Considering the presence of NOS in peroxisomes and the ROS generating systems and diverse antioxidants of these organelles, a model for the function of peroxisomes as a source of the signal molecules H_2O_2 , $O_2^{\cdot-}$, NO^{\cdot} , and GSNO is shown in Figure 2. The RNS GSNO is a powerful inducer of defense genes (Durner et al., 1998), and GSNO could function as a long-distance signal molecule, transporting glutathione-bound NO^{\cdot} throughout the plant (Klessig et al., 2000). The presence and expression of the enzyme GSNO reductase in pea leaves has been recently reported (Barroso et al., 2006). Should GSNO reductase be present in peroxisomes, this would imply that these organelles could modulate the amount of GSNO exported to the cytosol to participate in diverse signaling pathways.

NO^{\cdot} can diffuse through the peroxisomal membrane to the cytosol, but a modulation by NO^{\cdot} of the endogenous enzymes catalase and glutathione peroxidase and the H_2O_2 -producing β -oxidation cannot be ruled

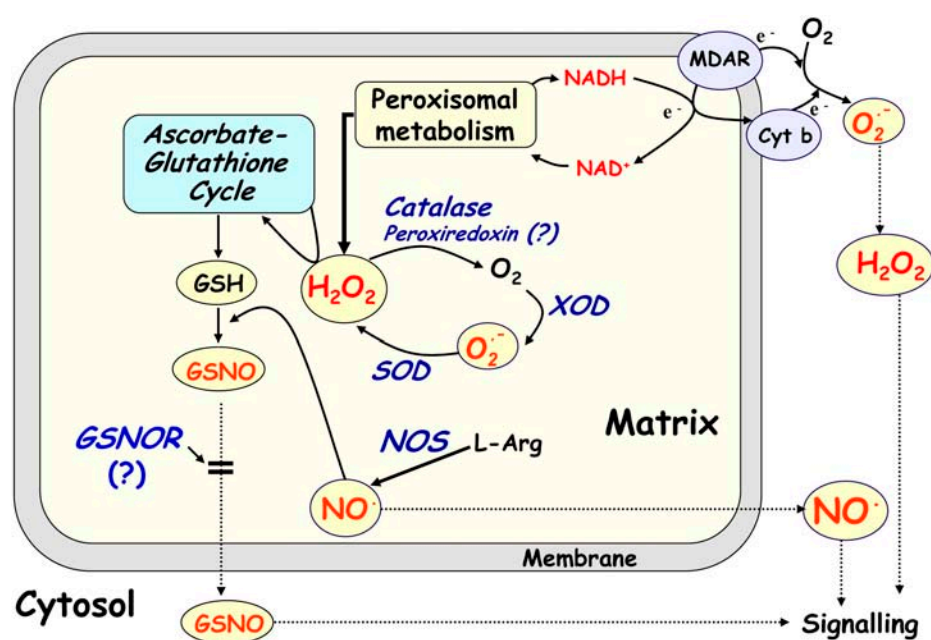


Figure 2. Hypothetical model of the role of peroxisomes in the generation of the signal molecules H_2O_2 , $O_2^{\cdot-}$, NO^{\cdot} , and GSNO. Cytochrome *b*, a *b*-type cytochrome (PMP18); GSNO reductase, GSNO reductase; XOD, xanthine oxidase. Broken arrows indicate signaling.

out (del Río et al., 2002). Catalase activity is known to be inactivated by $O_2^{\cdot-}$ radicals (Halliwell and Gutteridge, 2000) and NO^{\cdot} and peroxynitrite can inhibit catalase and APX activity (Klessig et al., 2000). Accordingly, if under any type of plant stress an induction of the peroxisomal generation of $O_2^{\cdot-}$ and NO^{\cdot} takes place, this can lead to the inhibition of catalase and APX activities. This breakdown of the peroxisomal antioxidant defenses would eventually originate an overproduction of H_2O_2 in peroxisomes, leading to cellular oxidative damage and possibly cell death.

Nevertheless, the rate of ROS and RNS generation in plant cells has opposing effects. A high cellular production of these active molecules can bring about extensive oxidative damage, but low levels of RNS and ROS are involved as signal molecules in the transduction pathways leading to the induction of defense responses against pathogens and cell death (Klessig et al., 2000; Delledonne et al., 2001).

Accordingly, peroxisomes should be considered as cellular compartments with the capacity to generate and release into the cytosol important signal molecules such as $O_2^{\cdot-}$, H_2O_2 , NO^{\cdot} , and GSNO, which can contribute to a more integrated communication among cell compartments and tissues (Corpas et al., 2001). This signal-producing function of plant peroxisomes is still more significant considering that the population of these oxidative organelles can proliferate in plants during senescence and under different stress conditions (del Río et al., 2002; Nila et al., 2006).

CONCLUSION

The existence of a reactive oxygen and nitrogen metabolism in plant peroxisomes and the presence in these organelles of a complex battery of antioxidative enzymes, emphasizes the importance of these organelles in cellular oxidative metabolism.

Plant peroxisomes have a ROS- and RNS-mediated metabolic function in leaf senescence and certain types of abiotic stress. Until recent years, mitochondria and chloroplasts were considered to be almost exclusively responsible for the intracellular oxidative damage induced by different stresses. However, peroxisomes can have two antagonistic roles in cells, as oxidative stress generators and as a source of ROS and RNS signal molecules. These organelles could act as subcellular indicators or sensors of plant stress and senescence by releasing signaling molecules to the cytosol and triggering specific changes in defense gene expression. A ROS and RNS signal molecule-producing function similar to that postulated for plant peroxisomes perhaps could also be performed by animal and fungal peroxisomes.

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