



Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes

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Abstract

Peroxisomes are subcellular organelles with an essentially oxidative type of metabolism. Like chloroplasts and mitochondria, plant peroxisomes also produce superoxide radicals ($O_2^{\cdot-}$) and there are, at least, two sites of superoxide generation: one in the organelle matrix, the generating system being xanthine oxidase, and another site in the peroxisomal membranes dependent on NAD(P)H. In peroxisomal membranes, three integral polypeptides (PMPs) with molecular masses of 18, 29 and 32 kDa have been shown to generate $O_2^{\cdot-}$ radicals. Besides catalase, several antioxidative systems have been demonstrated in plant peroxisomes, including different superoxide dismutases, the ascorbate–glutathione cycle, and three NADP-dependent dehydrogenases. A CuZn-SOD and two Mn-SODs have been purified and characterized from different types of peroxisomes. The four enzymes of the ascorbate–glutathione cycle (ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase) as well as the antioxidants glutathione and ascorbate have been found in plant peroxisomes. The recycling of

NADPH from $NADP^+$ can be carried out in peroxisomes by three dehydrogenases: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and isocitrate dehydrogenase. In the last decade, different experimental evidence has suggested the existence of cellular functions for peroxisomes related to reactive oxygen species (ROS), but the recent demonstration of the presence of nitric oxide synthase (NOS) in plant peroxisomes implies that these organelles could also have a function in plant cells as a source of signal molecules like nitric oxide (NO^{\cdot}), superoxide radicals, hydrogen peroxide, and possibly S-nitrosoglutathione (GSNO).

Key words: Antioxidants, ascorbate–glutathione cycle, nitric oxide, peroxisomes, ROS.

Introduction

The first description of peroxisomes was carried out in 1954 in the course of electron microscopy studies in mouse kidney tubules (Rhodin, 1954) and these organelles were designated as 'microbodies', a morphological name not

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Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; ICDH, NADP-dependent isocitrate dehydrogenase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; perNOS, peroxisomal nitric oxide synthase; MDHAR, monodehydroascorbate reductase; PMPs, peroxisomal membrane polypeptides; CCRase, Cyt c reductase; EM, electron microscopy; NOS, nitric oxide synthase; EGTA, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; GSNO, S-nitrosoglutathione.

implying any biochemical function. But it was De Duve at the beginning of the 1960s who carried out the biochemical characterization of peroxisomes from mammalian tissues which led to their recognition as distinct cell organelles (De Duve *et al.*, 1960). These authors, by using differential and density-gradient centrifugation techniques, achieved the differentiation of the microbody fraction from lysosomes, microsomes, and mitochondria. By using combined biochemical and morphological methods the identity of microbodies and peroxisomes was unequivocally demonstrated. The presence of hydrogen peroxide-producing enzymes, like urate oxidase and D-amino acid oxidase, and the hydrogen peroxide-scavenging catalase in microbodies, led De Duve to propose the term 'peroxisomes' for these organelles (De Duve and Baudhuin, 1966). In the following years, these organelles were found to be present in almost all eukaryotic cells. Peroxisomes are organelles with diameters ranging from 0.1–1.7 μm , bounded by a single membrane, and contain a coarsely granular or fibrillar matrix that occasionally has amorphous or paracrystalline inclusions (Tolbert, 1981; Huang *et al.*, 1983). From a

physiological and biochemical viewpoint, peroxisomes can be broadly defined as ubiquitous subcellular organelles containing as basic enzymatic constituents catalase and H_2O_2 -producing flavin oxidases (De Duve and Baudhuin, 1966; Tolbert, 1981; Huang *et al.*, 1983). Although some authors postulated the presence of nucleic acids in peroxisomes (Gerhardt and Beevers, 1969; Ching, 1970; Osumi and Kazawa, 1978) today it is widely admitted that these organelles, unlike mitochondria and chloroplasts, do not have a genetic system and all their proteins are nuclear-encoded, synthesized on free polyribosomes and imported post-translationally into peroxisomes (Huang *et al.*, 1983; Lazarow and Fujiki, 1985; Reddy *et al.*, 1996; Kunau, 1998; Olsen, 1998; López-Huertas and Baker, 1999). Electron micrographs of plant peroxisomes from four different origins, including watermelon, pea, pepper, and olive, are presented in Fig. 1.

Initially, it was thought that the main function of peroxisomes was the removal by catalase of toxic hydrogen peroxide generated in the peroxisomal respiratory pathway by different oxidases. Peroxisomal oxidases

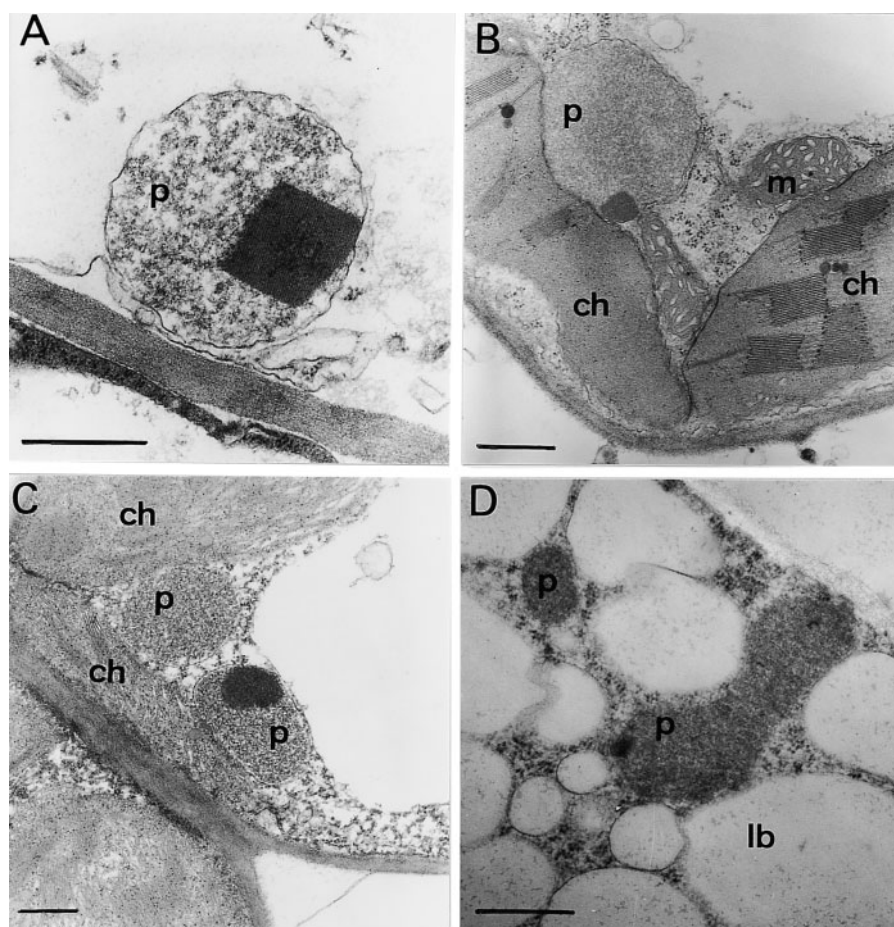


Fig. 1. Electron micrographs of peroxisomes from different plant tissues. (A) Pepper leaves (*Capsicum annuum* L.). (B) Pea leaves (*Pisum sativum* L.). (C) Olive tree leaves (*Olea europaea* L.). (D) Watermelon cotyledons (*Citrullus vulgaris* Schrad.). Sections of tissues were fixed, post-stained, and examined in an electron microscope: p, peroxisome; ch, chloroplast; m, mitochondrion; lb, lipid body. Bar = 0.5 μm .

are mainly flavoproteins, with the exception of urate oxidase, and include D-amino acid oxidase, acyl-CoA oxidase, glycolate oxidase, α -hydroxy acid oxidase, and glutaryl-CoA oxidase, among others, depending on the tissue origin (Huang *et al.*, 1983; Angermüller, 1989).

In recent years, it has been demonstrated that peroxisomes are involved in a range of important cellular functions in almost all eukaryotic cells (Fahimi and Sies, 1987; Van den Bosch *et al.*, 1992; Masters and Crane, 1995; Olsen, 1998; Reddy *et al.*, 1996; Subramani, 1998; Tabak *et al.*, 1999). These organelles have an essentially oxidative type of metabolism and can carry out different metabolic pathways depending on their source. Table 1 shows different functions that have been described so far for peroxisomes in plant and fungal cells. In plants, there are several types of peroxisomes which are specialized in certain metabolic functions. Glyoxysomes are specialized peroxisomes, occurring in the storage tissues of oilseeds, that contain the fatty acid β -oxidation and glyoxylate cycle enzymes to convert the seed reserve lipids into sugars which are used for germination and plant growth (Tolbert, 1981; Beevers, 1982; Huang *et al.*, 1983). Leaf peroxisomes are specialized peroxisomes present in photosynthetic tissues that carry out the major reactions of photorespiration (Tolbert, 1980; Huang *et al.*, 1983; Douce and Heldt, 2000). Another type of specialized peroxisomes are root-nodule peroxisomes from certain tropical legumes, in which the synthesis of allantoin, the major metabolite for nitrogen transport within these plants, is carried out (Schubert, 1986). The main metabolic processes responsible for the generation of H₂O₂ in different types of peroxisomes are the photorespiratory glycolate oxidase reaction, the β -oxidation of fatty acids, the enzymatic reaction of flavin oxidases, and the

disproportionation of superoxide radicals (Huang *et al.*, 1983; del Río *et al.*, 1992, 1996).

Studies on the compartmentation of the photorespiratory pathway in peroxisomes have led to the proposal that, in these organelles, unlike mitochondria and chloroplasts, the compartmentation of peroxisomal metabolism is, in major part, not caused by the boundary membrane but by the specific structure of the protein matrix. Apparently, the enzymes of the photorespiratory pathway are arranged in the peroxisomal matrix in the form of multienzyme complexes that allow efficient metabolite channelling (Douce and Heldt, 2000; Reumann, 2000), and transfer of metabolites proceeds across the peroxisomal membrane by porin-like channels (Reumann, 2000; Corpas *et al.*, 2000).

A characteristic property of peroxisomes is their metabolic plasticity since their enzymatic content can vary depending on the organism, cell/tissue-type and environmental conditions (Van den Bosch *et al.*, 1992; Mullen and Trelease, 1996). In animals, a variety of xenobiotics, mainly including hypolipidemic drugs, and certain herbicides and phthalate-ester plasticizers, induce in liver the proliferation of the peroxisomal population as well as the activity of the H₂O₂-producing acyl-CoA oxidase (Reddy *et al.*, 1987, 1996). In mussels, the proliferation of peroxisomes has been proposed as a specific biomarker of pollution by xenobiotics in marine and estuarine environments (Cancio and Cajaraville, 2000). In some yeast species, peroxisomes may be induced by growth on methanol, alkanes, fatty acids, D-alanine, and cadmium (Huang *et al.*, 1983; Chen *et al.*, 1995; Van der Klei and Veenhuis, 1997). In plants, the cellular proliferation of peroxisomes has also been reported both under natural and abiotic stress conditions. Table 2 illustrates the cases and the agents responsible for the induction of peroxisomes in different plant species. Very recently, the induction of peroxisome biogenesis genes (*PEX*) by H₂O₂ has been demonstrated in both plant and animal cells, indicating that the signal molecule H₂O₂ is responsible for the proliferation of peroxisomes (López-Huertas *et al.*, 2000). According to these authors, in plant wounding and pathogen attack a proliferation of peroxisomes takes place and, probably, this also happens in other stress situations that lead to an increased generation of H₂O₂ (López-Huertas *et al.*,

Table 1. Functions of peroxisomes in plant and fungus cells

Photorespiration
β -oxidation of fatty acids
Glyoxylate cycle
Metabolism of ureides
Metabolism of reactive oxygen species
Metabolism of methanol (fungi)
Oxalate synthesis (fungi)
Metabolism of amines (fungi)
Metabolism of alkanes (fungi)

Table 2. Peroxisome proliferation in plants

Plant tissue	Causative agent	Reference
Ryegrass leaves	Isoproturon (herbicide)	de Felipe <i>et al.</i> (1988)
Norway spruce needles	Ozone	Morré <i>et al.</i> (1990)
Pea leaves	Clofibrate (hypolipidemic drug)	Palma <i>et al.</i> (1991)
Carnation petals	Senescence	Droillard and Paulin (1990)
Pea leaves	Senescence	Pastori and del Río (1994)
Pea leaves	Cadmium	Romero-Puertas <i>et al.</i> (1999)
<i>Arabidopsis thaliana</i>	H ₂ O ₂	López-Huertas <i>et al.</i> (2000)

2000). However, it must be taken into account that quantitative evidence for the proliferation of the peroxisomal population, i.e. counting of the number of organelles per cell section of tissue, has only been reported in plants treated with isoproturon (de Felipe *et al.*, 1988), clofibrate (Palma *et al.*, 1991), cadmium (Romero-Puertas *et al.*, 1999) and during senescence of pea leaves (Pastori and del Río, 1994).

An example of the inducible nature of peroxisomal metabolism is the light-induced transition of glyoxysomes, the specialized peroxisomes of oilseeds, to leaf-type peroxisomes (Huang *et al.*, 1983; Masters and Crane, 1995; Mullen and Trelease, 1996). Likewise, during plant senescence and under the effect of abiotic stress by cadmium the reverse process is observed, that is the metabolic conversion of leaf peroxisomes into glyoxysomes (De Bellis *et al.*, 1990; Landolt and Matile, 1990; Nishimura *et al.*, 1993, 1996; Vicentini and Matile, 1993; Pastori and del Río, 1994; del Río *et al.*, 1998b, 2000). In these metabolic transitions of peroxisomes, endogenous proteases could be involved in the turnover of peroxisomal proteins. In plant peroxisomes, the presence of exo- and endo-proteolytic activity was reported for the first time in pea leaves, and an exo-peptidase was characterized as a leucine aminopeptidase, belonging to

Table 3. Proteases characterized in leaf peroxisomes from pea plants

Peroxisomes were purified from young (15-d-old) and senescent (50-d-old) leaves and proteases were characterized with different substrates and inhibitors (Corpas *et al.*, 1993a; Distefano *et al.*, 1997, 1999). +, present; -, absent.

Isoenzyme	Age of plant		Molecular mass (kDa)	Type of protease
	Young	Senescent		
Exo-peptidase	+	+	57	Leucine aminopeptidase
Endo-peptidases				
EP-1	-	+	220	Serine-proteinase
EP-2	+	+	88	Cysteine-proteinase
EP-3	-	+	76	Serine-proteinase
EP-4	+	+	64	Serine-proteinase
EP-5	+	+	50	?
EP-6	-	+	46	Cysteine-proteinase
EP-7	-	+	34	Metallo-proteinase

Table 4. Reactive oxygen-mediated metabolic functions of leaf peroxisomes

Function	Reference
Mechanism of toxicity of xenobiotics	Palma <i>et al.</i> (1991); del Río <i>et al.</i> (1992, 1996)
Senescence	del Río <i>et al.</i> (1998b); Jiménez <i>et al.</i> (1998b)
Mechanism of toxicity of heavy metals	del Río <i>et al.</i> (1996); Romero-Puertas <i>et al.</i> (1999)
Catabolism of purines	Corpas <i>et al.</i> (1993b)
Superoxide-generating PMPs	López-Huertas <i>et al.</i> (1997, 1999)
Ascorbate-glutathione cycle	Jiménez <i>et al.</i> (1997, 1998b)
Dehydrogenase-mediated NADPH recycling	Corpas <i>et al.</i> (1998b, 1999)
Source of ROS and NO [•] signal molecules	Barroso <i>et al.</i> (1999); Corpas <i>et al.</i> (2001)

the family of the serine-proteinases (Corpas *et al.*, 1993a). The different proteases characterized thus far in leaf peroxisomes are shown in Table 3. An increase in the total endo-proteolytic activity and in the number of endoprotease isoenzymes was found in peroxisomes purified from senescent pea leaves in comparison with peroxisomes from young leaves (Distefano *et al.*, 1997). In pea plants grown with cadmium an increase in the activity of four endoproteases of leaf peroxisomes was recently found (McCarthy *et al.*, 2001). In general, there are data that suggest the involvement of peroxisomal endoproteases in a regulated modification of proteins in this organelle (Distefano *et al.*, 1999).

In recent years, different experimental evidence has suggested the existence of cellular functions for leaf peroxisomes related to reactive oxygen species which are summarized in Table 4. These ROS-related roles add to the other well-established functions known for peroxisomes from plant cells. In this review, the production of reactive oxygen species (ROS), the different antioxidant systems and the generation of nitric oxide in peroxisomes will be analysed in the context of these new ROS-mediated functions of plant peroxisomes.

Production of oxygen radicals in peroxisomes

In plant cells, the production of ROS has been shown in chloroplasts, mitochondria, the plasma membrane and the apoplasmic space (Elstner, 1991; Asada, 1994; Bolwell, 1999). Peroxisomes also produce superoxide radicals (O₂^{•-}) as a consequence of their normal metabolism. In peroxisomes from pea leaves by biochemical and EPR methods the existence of, at least, two sites of O₂^{•-} generation was demonstrated: one in the organelle matrix, in which the generating system was identified as xanthine oxidase (XOD), and another site in the peroxisomal membranes dependent on NAD(P)H (Sandalio *et al.*, 1988; del Río *et al.*, 1989, 1992, 1998a). Xanthine oxidase catalyses the oxidation of xanthine and hypoxanthine to uric acid and is a well-known producer of superoxide radicals (Fridovich, 1986). The presence of xanthine and uric acid, substrate and product, respectively, of the xanthine oxidase reaction, as well as

allantoin, the product of the urate oxidase reaction, were detected in leaf peroxisomes by HPLC analysis (Corpas *et al.*, 1993b). The occurrence of xanthine, uric acid, and allantoin in leaf peroxisomes indicate a role for these organelles in the catabolism of xanthine produced as a result of the turnover of nucleotides, RNA and DNA in leaf cells (Corpas *et al.*, 1993b; del Río *et al.*, 1998a).

On the other hand, in the other peroxisomal site of O_2^- production, the peroxisomal membranes, a small electron transport chain similar to that reported in peroxisomal membranes from castor bean endosperm (Fang *et al.*, 1987) appears to be involved. This electron-transport chain is composed of a flavoprotein NADH:ferricyanide reductase of about 32 kDa and a Cyt *b* (measured as NADH:CCRase) (Fang *et al.*, 1987). The integral peroxisomal membrane polypeptides (PMPs) of pea leaf peroxisomes were identified by SDS-PAGE (López-Huertas *et al.*, 1995) and recently three of these membrane polypeptides, with molecular masses of 18, 29, and 32 kDa have been characterized and demonstrated to be responsible for O_2^- generation (López-Huertas *et al.*, 1997, 1999). The properties and possible identities of these polypeptides are shown in Table 5.

The main producer of superoxide radicals in the peroxisomal membranes was the 18 kDa PMP which was proposed to be a cytochrome, possibly belonging to the *b*-type group, and uses NADH as electron donor (López-Huertas *et al.*, 1997). The PMP32 is also NADH-dependent for superoxide production and, on the basis of its biochemical and immunochemical properties, very probably corresponds to the monodehydroascorbate reductase (MDHAR) (López-Huertas *et al.*, 1999) whose activity was previously detected in pea leaf peroxisomal membranes (Jiménez *et al.*, 1997). This indicates the participation of MDHAR in O_2^- production by peroxisomal membranes. Very recently, it has been reported that chloroplast MDHAR mediates the production of O_2^- in spinach thylakoid membranes (Miyake *et al.*, 1998). This shows that the chloroplastic and peroxisomal

membrane-bound MDHARs share the property of being O_2^- producers, although by different mechanisms. In chloroplast thylakoid membranes, O_2^- radicals are generated via O_2 reduction by MDHAR photoreduced at photosystem I (Miyake *et al.*, 1998), whereas in peroxisomal membranes O_2^- radicals are produced by MDHAR (PMP32) previously reduced by NADH (López-Huertas *et al.*, 1999). The third O_2^- -generating polypeptide, PMP29, is strictly dependent on NADPH as electron donor, is able to reduce cytochrome *c*, and could be related to the peroxisomal NADPH:cytochrome P-450 reductase.

Superoxide production by peroxisomal membranes may be an obligatory consequence of NADH re-oxidation by the peroxisomal electron-transport chain, in order to regenerate NAD^+ to be re-utilized in peroxisomal metabolic processes (del Río *et al.*, 1990, 1992; del Río and Donaldson, 1995). Under normal metabolic conditions, the O_2^- production by peroxisomal membranes is not dangerous to the cell, which is adequately protected against these radicals. However, under certain conditions of plant stress, the release of peroxisomal membrane-generated O_2^- radicals into the cytosol can be enhanced (del Río *et al.*, 1996), producing cellular oxidative stress situations mediated by reactive oxygen species. Since O_2^- radicals have a short half-life under physiological conditions and are rapidly converted into H_2O_2 and O_2 , the final result of these stress conditions will be an increase of H_2O_2 in the cell (del Río *et al.*, 1996). In recent years, H_2O_2 has been described as a diffusible transduction signal in different physiological processes and plant stress, leading to the induction of genes encoding different cellular protectants. Some functions that have been reported for H_2O_2 as a signal molecule are shown in Table 6.

In mammalian and yeast peroxisomes there is very little information on the production of oxygen radicals. In mouse peroxisomes, a 20 kDa membrane protein (Mpv17) has been reported to produce ROS. This peroxisomal membrane protein was identified by biochemical and genetic means, and is thought to be involved in kidney diseases like the glomerulosclerosis and nephrotic syndrome (Zwacka *et al.*, 1994). In *Arabidopsis thaliana*, a peroxisomal membrane protein (PMP22) was identified and characterized at the molecular level and had a 55% similarity with mammalian Mpv17 (Tugal *et al.*, 1999). However, the generation of ROS by this *Arabidopsis* PMP has not been studied.

Table 5. Peroxisomal membrane polypeptides involved in O_2^- generation in pea leaf peroxisomes (López-Huertas *et al.*, 1997, 1999)

Molecular mass (kDa)	Electron donor	Properties	Probable identity
32.0	NADH	Flavoprotein Haemoprotein Ferricyanide reductase activity	MDHAR
29.0	NADPH	Cyt <i>c</i> reductase activity	Related to Cyt P ₄₅₀ (?)
18.0	NADH	Cyt <i>c</i> reductase activity	<i>b</i> -type cytochrome

Antioxidant systems in peroxisomes

Superoxide dismutases

Superoxide dismutases (SODs; EC 1.15.1.1) are a family of metalloenzymes that catalyze the disproportionation

Table 6. Some functions reported for H₂O₂ in signal transduction pathways

Function	Organism	Reference
Activation of transcription factors (NF-κB, AP-1, etc.)	Mammalian cells	Schreck <i>et al.</i> (1991)
Gene regulation	Bacteria	Demple (1991)
Plant-pathogen interactions	<i>Arabidopsis</i> -fungi	Levine <i>et al.</i> (1994)
	<i>Arabidopsis</i> - <i>Ps. syringae</i>	Alvarez <i>et al.</i> (1998)
	Tobacco- <i>Ps. syringae</i>	Chamngopol <i>et al.</i> (1998)
Transcription induction of defence genes	Soybean cells	Levine <i>et al.</i> (1994)
Response to wounding	Chickpea	Rea <i>et al.</i> (1998)
ABA-mediated guard cell closure	<i>Arabidopsis</i>	Pei <i>et al.</i> (2000)
Osmotic stress	Maize	Guan <i>et al.</i> (2000)
Excess light stress	<i>Arabidopsis</i>	Karpinski <i>et al.</i> (1999)
		Willekens <i>et al.</i> (1997)
Induction of <i>PEX</i> genes in response to biotic stress	<i>Arabidopsis</i>	López-Huertas <i>et al.</i> (2000)

Table 7. Superoxide dismutases localized in peroxisomes

Source	SOD isozyme	Reference
Plants		
Pea	Mn-SOD	del Río <i>et al.</i> (1983)
		Sandalio <i>et al.</i> (1987)
Watermelon	CuZn-SOD, Mn-SOD	Sandalio and del Río (1988)
		Sandalio <i>et al.</i> (1997)
Carnation	Fe-SOD, Mn-SOD	Droillard and Paulin (1990)
Castor bean	Mn-SOD	del Río and Donaldson (1995)
Sunflower	CuZn-SODs	Corpas <i>et al.</i> (1998a)
Cucumber	CuZn-SOD, Mn-SOD	Corpas <i>et al.</i> (1998a)
Cotton	CuZn-SOD	Corpas <i>et al.</i> (1998a)
Tomato	SOD	Mittova <i>et al.</i> (2000)
Olive	CuZn-SOD	Valderrama <i>et al.</i> (unpublished results)
Humans		
Hepatoma cells	CuZn-SOD	Keller <i>et al.</i> (1991); Crapo <i>et al.</i> (1992)
Fibroblasts	CuZn-SOD	Keller <i>et al.</i> (1991)
Animals		
Rat liver	CuZn-SOD	Dhaunsi <i>et al.</i> (1992); Liou <i>et al.</i> (1993)
		Wanders and Denis (1992)
	Mn-SOD	Singh <i>et al.</i> (1999)
Fish liver	CuZn-SOD	Orbea <i>et al.</i> (2000)
Molluscs digestive gland	CuZn-SOD	Orbea <i>et al.</i> (2000)
Crustaceans digestive gland	CuZn-SOD	Orbea <i>et al.</i> (2000)
Rat brain	CuZn-SOD	Moreno <i>et al.</i> (1997)
Microorganisms		
<i>S. cerevisiae</i> (transformed cells)	CuZn-SOD	Keller <i>et al.</i> (1991)

of O₂⁻ radicals into H₂O₂ and O₂, and play an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments (Fridovich, 1986; Elstner, 1991; Halliwell and Gutteridge, 2000). SODs are distributed in different cell loci, mainly chloroplasts, cytosol, and mitochondria (Fridovich, 1986; del Río *et al.*, 1992; Bowler *et al.*, 1994; Halliwell and Gutteridge, 2000), but the presence of SOD in peroxisomes was demonstrated for the first time in plant tissues (del Río *et al.*, 1983; Sandalio *et al.*, 1987). Since then, the occurrence of SODs in isolated plant peroxisomes has been reported in at least nine different plant species (Table 7). And in five of these plants the presence of SOD in peroxisomes has been confirmed by immunogold electron microscopy using an antibody

prepared against peroxisomal CuZn-SOD from watermelon (Sandalio *et al.*, 1997; Corpas *et al.*, 1998a; Valderrama *et al.*, unpublished results).

Results obtained on the presence of SOD in plant peroxisomes were, years later, extended to human and animal cells, which were found to contain CuZn-SOD in peroxisomes and, more recently, Mn-SOD as well (Table 7). In mammals, a role for peroxisomes in oxidative stress-induced cellular pathophysiology has been proposed (Singh, 1996). In yeast cells, the gene of a human CuZn-SOD (*SOD1*) was expressed and immunocytoelectron microscopy of cells showed that human CuZn-SOD was accumulated in peroxisomes (Keller *et al.*, 1991). In an ectomycorrhizal fungus, the gene encoding a Mn-SOD was cloned, and the protein was found to

Table 8. Molecular properties of superoxide dismutases localized in plant peroxisomes

Source	SOD isozyme	M_r	Subunits (kDa)	pI	Intraperoxisomal locus	Reference
Pea leaves	Mn-SOD	92 000	4 × 27.0	5.5	Matrix	Palma <i>et al.</i> (1998)
Watermelon cotyledons	CuZn-SOD	33 000	2 × 16.5	4.0	Matrix	Bueno <i>et al.</i> (1995)
	Mn-SOD	108 000	4 × 27.0	5.7	Membrane	Sandalio and del Río (1988) Pastori <i>et al.</i> (1996)

contain a C-terminal peroxisomal localization peptide (PTS1) and lack an N-terminal mitochondrial transit peptide (Jacob *et al.*, 2001). This putative peroxisomal Mn-SOD appears to be involved in the cellular response of the fungus to cadmium stress (Jacob *et al.*, 2001).

Three SODs of peroxisomal origin have been purified and characterized, a CuZn-SOD and a Mn-containing SOD from watermelon cotyledons (Bueno *et al.*, 1995; Pastori *et al.*, 1996) and a Mn-SOD from pea leaves (Palma *et al.*, 1998). The molecular properties determined for these peroxisomal SODs and their intraorganellar localizations are summarized in Table 8. On the basis of determination of SOD latency in intact organelles and by solubilization assays with 0.2 M KCl and 0.1 M sodium carbonate, a Mn-SOD was found to be present in the external side of the membrane of peroxisomes from watermelon cotyledons (Sandalio and del Río, 1988; Sandalio *et al.*, 1997) (Table 8). A Mn-SOD was also localized in membranes of peroxisomes isolated from castor bean endosperm, and this isozyme was removed from the membranes by washing with 0.1 M sodium carbonate (del Río and Donaldson, 1995). Therefore, Mn-SOD appears to be a peripheral protein in those peroxisomal membranes, and this localization is pending to be ratified by immunoelectron microscopy using the specific antibody to Mn-SOD.

Ascorbate–glutathione cycle

The ascorbate–glutathione cycle, also called Foyer–Halliwell–Asada cycle, is an efficient way for plant cells to dispose of H_2O_2 in certain cellular compartments where this metabolite is produced and no catalase is present (Halliwell and Gutteridge, 2000). This cycle makes use of the non-enzymic antioxidants ascorbate and glutathione in a series of reactions catalysed by four antioxidative enzymes and has been demonstrated in chloroplasts, cytosol, and root nodule mitochondria (Foyer and Mullineaux, 1994). In peroxisomes purified from pea leaves, the presence of the four enzymes of the ascorbate–glutathione cycle, ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), and glutathione reductase (GR; EC 1.6.4.2) was demonstrated (Jiménez *et al.*, 1997). Likewise, in intact peroxisomes, the presence of

reduced ascorbate (ASC) and glutathione (GSH), and their oxidized forms, dehydroascorbate (DHA) and GSSG, respectively, was demonstrated by HPLC analysis (Jiménez *et al.*, 1997). The presence of the ascorbate–glutathione cycle enzymes has also been reported recently in peroxisomes of leaf and root cells of two species of tomato (Mittova *et al.*, 2000). The intraperoxisomal distribution of the four enzymes was studied by determining enzyme activity latency in intact organelles and by solubilization assays with 0.2 M KCl. On the basis of the results obtained, a model for the function of the ascorbate–glutathione cycle in leaf peroxisomes is shown in Fig. 2. DHAR and GR were found in the soluble fraction of peroxisomes, whereas APX activity was bound to the cytosolic side of the peroxisomal membrane. By Western blot analysis of peroxisomal membranes with a specific antibody against pumpkin APX, an integral membrane polypeptide of 35 kDa was identified as the APX of pea leaf peroxisomes (López-Huertas *et al.*, 1999). These results agree with recent findings of an APX isoenzyme in membranes of pumpkin and cotton peroxisomes (Yamaguchi *et al.*, 1995; Bunkelmann and Trelease, 1996). The substrate specificity of the membrane-bound peroxisomal APX from pea leaves was recently studied. The APX specific activity was much higher with pyrogallol than with ascorbate and the enzyme was more sensitive to incubation with Triton X-100 than the mitochondrial APX (Jiménez *et al.*, 1998a). By native-PAGE of peroxisomal membranes no APX activity could be detected probably due to enzyme inactivation. The low stability of this enzyme in the presence of Triton and after native-PAGE suggests that peroxisomal APX is more related to chloroplast APX than to mitochondrial and cytosolic APXs detected in pea leaves (Jiménez *et al.*, 1998a). cDNAs encoding peroxisomal APX have been isolated from cotton (Bunkelmann and Trelease, 1996), *Arabidopsis* (Zhang *et al.*, 1997; PM Mullineaux, personal communication), and spinach (Ishikawa *et al.*, 1998). The deduced amino acid sequence of peroxisomal APX has a high degree of identity with cytosolic APX, but it has a C-terminal amino acid extension containing a single, putative membrane-spanning region (Mullen *et al.*, 1999).

Two peroxisomal proteins from human and murine origin (HsPMP20 and MmPMP20) that exhibit antioxidant activity *in vitro* were characterized

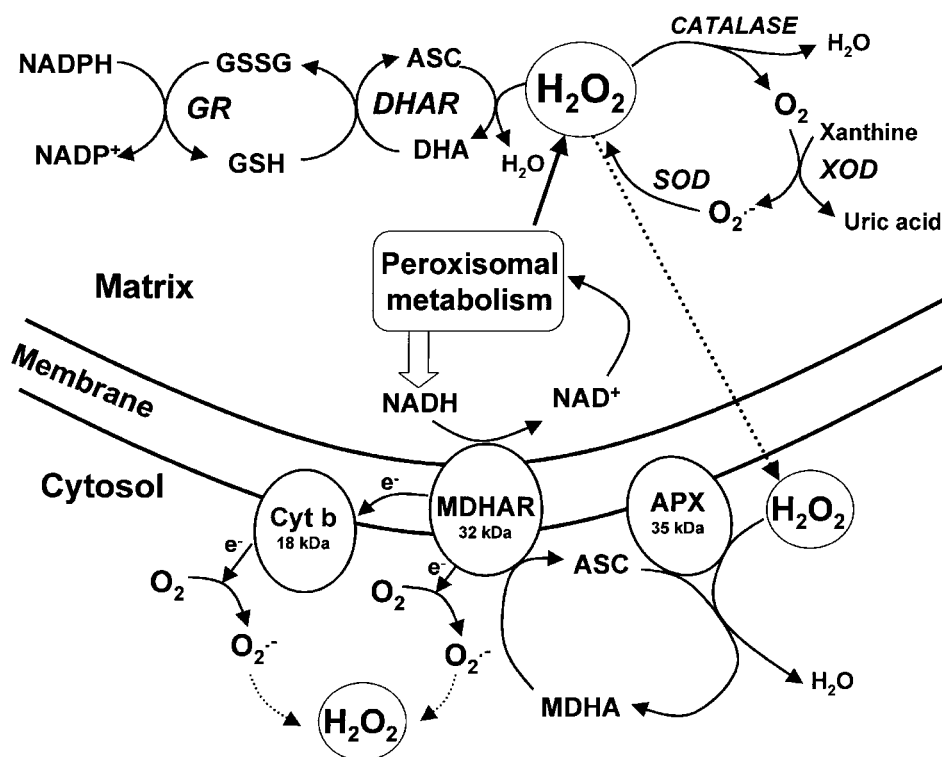


Fig. 2. Model proposed for the function of the ascorbate–glutathione cycle in leaf peroxisomes. The model is based on results recently described (Jiménez *et al.*, 1997) and those previously reported on the characterization of PMPs from pea leaves (López-Huertas *et al.*, 1997, 1999) and the NADH:MDHAR of glyoxysomal membranes from castor bean endosperm (Bowditch and Donaldson, 1990). ASC, ascorbate, reduced form; DHA, ascorbate, oxidized form (dehydroascorbate); MDHAR, monodehydroascorbate reductase; MDHA, monodehydroascorbate; GR, glutathione reductase; APX, ascorbate peroxidase; XOD, xanthine oxidase. (*Plant Physiology* **116**, 1198; 1998. Copyright 1998 American Society of Plant Physiologists, Rockville, MD.)

(Yamashita *et al.*, 1999). The PMP20 proteins were initially characterized in yeast and defined as membrane proteins (Garrard and Goodman, 1989). However, further research demonstrated that the PMP20 was released from the peroxisomal membrane, contained PTS1 sequences and was shown to be present in the peroxisomal matrix by immunocytochemistry (see references in Yamashita *et al.*, 1999). In spite of this evidence, these proteins are still termed peroxisomal membrane proteins (PMPs). The human HsPMP20 and murine MmPMP20 proteins can remove H_2O_2 by its thiol-peroxidase activity and have been proposed as novel members of the Ahp/thiol-specific antioxidant family, which might play a protecting role against oxidative stress in peroxisomes (Yamashita *et al.*, 1999).

Another peroxidase of putative peroxisomal localization could be thioredoxin-dependent peroxidase (peroxiredoxin). This enzyme has not been directly localized in peroxisomes, but an *E. coli* recombinant thioredoxin-dependent peroxidase has been recently characterized and was found to have a high homology with the C-terminal sequence of the peroxisomal protein PMP20 from *Candida boidini* (Verdoucq *et al.*, 1999). Additionally, in the human and rat protein AOEB166, a novel member of

the mammalian peroxiredoxin family, the amino acid analysis of their C-terminal domains revealed the existence of a peroxisomal targeting sequence (PTS1) (Knoops *et al.*, 1999). The presence of peroxiredoxins in peroxisomes would supply these organelles with another antioxidant enzyme system which would join catalase and the ascorbate–glutathione cycle in the control of the peroxisomal level of H_2O_2 .

MDHAR was also localized in the peroxisomal membranes and was highly latent in intact peroxisomes (Fig. 2). It has been proposed that the *trans*-membrane protein MDHAR can oxidize NADH on the matrix side of the peroxisomal membrane and transfer the reducing equivalents as electrons to the acceptor monodehydroascorbate on the cytosolic side of the membrane (Luster and Donaldson, 1987; Bowditch and Donaldson, 1990). In this process, molecular O_2 could also act as an electron acceptor, with the concomitant formation of $O_2^{\cdot-}$ (López-Huertas *et al.*, 1999).

The presence of APX and MDHAR in leaf peroxisomal membranes suggests a dual complementary function in peroxisomal metabolism of these membrane-bound antioxidant enzymes. The first role could be to reoxidize endogenous NADH to maintain a constant

supply of NAD⁺ for peroxisomal metabolism (Fig. 2), an idea that was originally proposed for the membrane-bound NADH dehydrogenase of glyoxysomes from castor bean endosperm (Fang *et al.*, 1987; Luster and Donaldson, 1987; Bowditch and Donaldson, 1990). A second function of the membrane antioxidative enzymes could be to protect against H₂O₂ leaking from peroxisomes, particularly when the catalase activity of peroxisomes is depressed and, as a result of it, the endogenous level of H₂O₂ is enhanced. Hydrogen peroxide can easily permeate the peroxisomal membrane, and an important advantage of the presence of APX in the membrane would be the degradation of leaking H₂O₂, as well as the H₂O₂ that is being continuously formed by dismutation of the O₂⁻ generated in the NADH-dependent electron transport system of the peroxisomal membrane (Fig. 2). It should be taken into account that although catalase in the peroxisomal matrix decomposes most of the H₂O₂ produced in these organelles, the catalase affinity for H₂O₂ is relatively low (Halliwell, 1974). However, at lower levels of H₂O₂ the ascorbate peroxidase activity of the ascorbate–glutathione cycle can scavenge H₂O₂ more efficiently than catalase owing to its much lower K_m (Nakano and Asada, 1981). This membrane scavenging of H₂O₂ could prevent an increase in the cytosolic H₂O₂ concentration during normal metabolism and under certain plant stress situations, when the level of H₂O₂ produced in peroxisomes can be substantially enhanced (del Río *et al.*, 1992, 1996).

NADP-dependent dehydrogenases

The reduced coenzyme NADPH is a basic electron donor in many biosynthetic and detoxification reactions of living cells. The main cellular sources of NADPH are the dehydrogenases of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), the malic enzyme, isocitrate dehydrogenase (ICDH), and chloroplastic ferredoxin-NADP⁺ reductase.

In recent years, different studies have shown that G6PDH has a relevant role in the mechanism of

protection against oxidative stress of bacteria, yeast and mammalian cells (for references see Corpas *et al.*, 1998b). In plant cells, NADPH has an important role in the protection systems against oxidative stress due to its involvement in the ascorbate–glutathione cycle of chloroplasts (Foyer and Halliwell, 1976; Asada, 1994). This evidence has supported the notion of G6PDH as an antioxidative enzyme which can be included in the group of catalase, SOD, APX and GR/peroxidase (Martini and Ursini, 1996). In higher plants, only two isoforms of G6PDH have been reported, which are localized in the cytosol and the plastidic stroma (Schnarrenberger *et al.*, 1995; Fickenscher and Scheibe, 1986).

In a recent work carried out in our laboratory, the presence of three NADP-dehydrogenases in leaf peroxisomes purified from pea plants was investigated. The dehydrogenases included: G6PDH, 6PGDH, and ICDH. The only NADP-dependent dehydrogenase that had been previously detected in peroxisomes was the ICDH present in trace amounts in spinach leaf peroxisomes (Tolbert, 1981) and in castor bean glyoxysomes (Donaldson, 1982). These three dehydrogenases were found to be present in the matrix of leaf peroxisomes and showed a typical Michaelis–Menten kinetic saturation curve (Table 9) (Corpas *et al.*, 1998b, 1999). The possible occurrence of the malic enzyme in pea leaf peroxisomes was investigated, but no traces of this NADP-dehydrogenase were found in peroxisomes (FJ Corpas *et al.*, unpublished results).

By isoelectric focusing four isoforms of ICDH, three isoforms of G6PDH, and one isoform of 6PGDH were detected in peroxisomal matrices (Corpas *et al.*, 1998b, 1999). The presence of G6PDH and ICDH in leaf peroxisomes was also checked by immunoblot analysis of peroxisomal matrices with antibodies against yeast G6PDH and pea leaf ICDH. Single cross-reactivity bands of 56 kDa and 46 kDa were detected for G6PDH and ICDH, respectively (Fig. 3). The localization of G6PDH and ICDH in pea leaf peroxisomes was also studied by immunogold electron microscopy using antibodies against yeast G6PDH and pea ICDH (Fig. 3). After embedding, electron microscopy immunogold labelling confirmed that G6PDH was localized in the peroxisomal

Table 9. Molecular and kinetic properties of glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and isocitrate dehydrogenase (ICDH) in peroxisomes purified from 50-d old pea leaves

Kinetic parameters were determined by using a non-linear-regression analysis program. *n*_H, Hill's coefficient (Corpas *et al.*, 1998b, 1999). n.d., not determined.

	G6PDH	6PGDH	ICDH
Molecular mass (kDa)	56.0	n.d.	46.0
Isoelectric point	5.5 5.3 4.8	5.1	6.0 5.6 5.4 5.2
Specific activity (m-units mg ⁻¹ of protein)	12.4	29.6	143
V _{max} (μ-units)	373.2	888.7	4771
K _m (μM)	107.3	10.2	19
Catalytic efficiency (V _{max} /K _m) (10 ⁶ μ-units M ⁻¹)	3.5	87.1	239
<i>n</i> _H	1.0	1.1	1.1

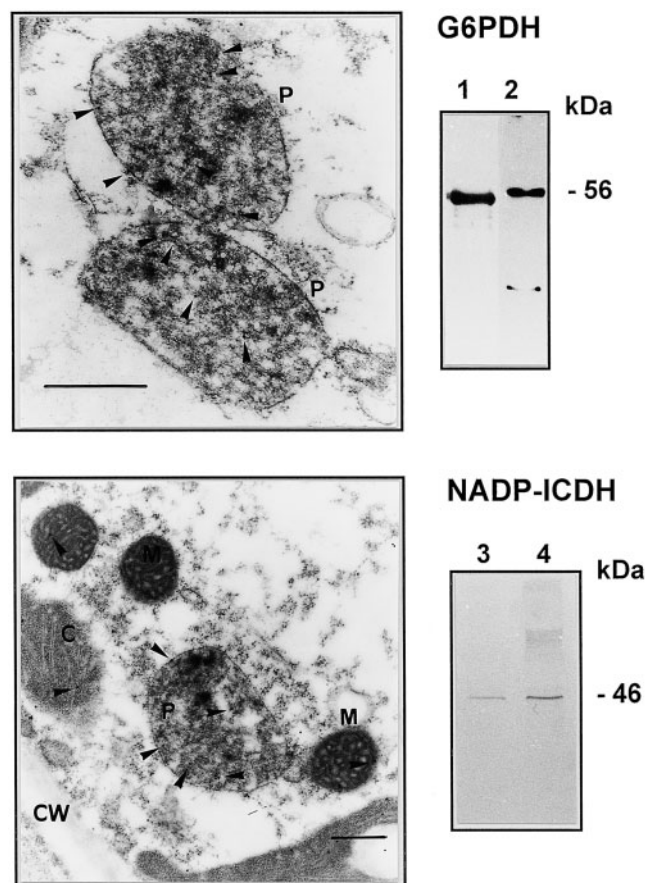


Fig. 3. Immunogold electron microscopy localization of G6PDH and ICDH in pea leaves (Corpas *et al.*, 1998b, 1999). The electron micrographs are representative of thin sections of leaves. Cell sections were probed separately with anti-yeast G6PDH (1:200 dilution) and anti-pea NADP-ICDH (dilution 1:500). Arrows indicate 15 nm gold particles; P, peroxisome; C, chloroplast; M, mitochondrion; CW, cell wall. Bars = 1.0 μ m. For Western blot analysis, peroxisomal matrices were subjected to SDS-PAGE and then transferred to PVDF membranes and incubated separately with polyclonal antibodies against yeast G6PDH (1:1000 dilution) and pea leaf ICDH (1:4000 dilution). 1, Yeast G6PDH (6.5 μ g protein); 2, peroxisomal matrices (45 μ g protein); 3, pig heart ICDH (5 μ g protein); 4, peroxisomal matrices (45 μ g protein).

matrices, with an average number of gold particles of 5.1, and also in chloroplasts (both thylakoid membranes and stroma) and cytosol (Corpas *et al.*, 1998b). The proportion of G6PDH in peroxisomes agrees with that reported for cellular fractions of mouse kidney, where 10% of the total G6PDH activity was also found in peroxisomes (Patel *et al.*, 1987). ICDH was also localized in the peroxisomal matrices, as well as in mitochondria, chloroplasts and cytosol, but the highest density of immunogold particles was found in peroxisomes and mitochondria (Corpas *et al.*, 1999) (Fig. 3).

The effect of plant senescence on the kinetic properties of the NADP-ICDH in pea leaf peroxisomes was analysed. No significant changes were observed in the specific activity or V_{\max} values of ICDH in the two

experimental situations. However, in peroxisomes from senescent leaves, the K_m of ICDH decreased almost 11-fold. This kinetic behaviour resulted in a catalytic efficiency approximately 12 times higher for peroxisomal ICDH from senescent leaves (Corpas *et al.*, 1999). However, the protein levels of ICDH in peroxisomes were not altered during senescence. The physiological significance of the change in the K_m of peroxisomal ICDH during senescence is probably double: first, to compete with isocitrate lyase, an enzyme of the glyoxylate cycle which is present in peroxisomes from senescent leaves, for the intracellular pool of isocitrate; and second, to provide a higher and constant supply of NADPH in order to eliminate, by the ascorbate-glutathione cycle, the excess of H_2O_2 produced during senescence when catalase activity decreases dramatically (Pastori and del Río, 1997).

The presence in peroxisomes of the two oxidative enzymes of the pentose phosphate pathway, G6PDH and 6PGDH, and ICDH implies that these organelles have the capacity to reduce $NADP^+$ to NADPH for its re-utilization in peroxisomal metabolism. However, the production of NADPH by peroxisomal ICDH is 13- and 5-fold higher than that due to peroxisomal G6PDH and 6PGDH, respectively (Corpas *et al.*, 1999). This fact, together with the observed fine control of ICDH during senescence, strongly indicates the importance of this enzyme in the enzymatic systems of NADPH recycling. The occurrence of NADP(H) in castor bean peroxisomes has been demonstrated (Donaldson, 1982). NADPH is necessary for the function of the NADPH:cytochrome *P*-450 reductases, whose presence has been detected in the membranes of castor bean peroxisomes (Alani *et al.*, 1990). On the other hand, one of the $O_2^{\cdot-}$ -generating polypeptides of peroxisomal membranes mentioned above, the PMP29, was clearly dependent on NADPH and was also able to reduce cytochrome *c* with NADPH as electron donor (López-Huertas *et al.*, 1999).

NADPH has been reported to protect catalase from oxidative damage (Kirkman *et al.*, 1999). This cofactor tightly binds to mammalian catalase and avoids the H_2O_2 -dependent conversion of the enzyme to an inactive state (compound II). But, additionally, NADPH may lead to the reduction of oxidizing states and internal groups of catalase other than the intermediate (Kirkman *et al.*, 1999). NADPH is also required for the maintenance of the ascorbate-glutathione cycle because it is necessary for the glutathione reductase activity which recycles reduced glutathione (GSH) from its oxidized form (GSSG) in order to be used for the scavenging of H_2O_2 in the cycle (Foyer and Halliwell, 1976; Halliwell and Gutteridge, 2000). Therefore, the three NADP-dependent dehydrogenases of peroxisomes (G6PDH, 6PGDH and ICDH) represent a very efficient system to recycle NADPH for its re-utilization in the peroxisomal metabolism, particularly

for the functioning of the ascorbate–glutathione cycle, which together with catalase represent an important antioxidant protection system against H_2O_2 generated in peroxisomes. An additional potential function for NADPH in peroxisomes could be related to the mechanism of protein import into these organelles. It was shown that the NADPH to $NADP^+$ ratio is important in peroxisomal protein import (Pool *et al.*, 1998).

Peroxisomes and nitric oxide

Nitric oxide (NO \cdot) is a widespread intracellular and intercellular messenger in vertebrates with a broad spectrum of regulatory functions in the central nervous, cardiovascular, and immune systems (Moncada *et al.*, 1991; Knowles and Moncada, 1994). As a consequence of the physiological importance of the free radical NO \cdot , numerous studies have been focused on the enzyme responsible for its endogenous production. Nitric oxide synthase (NOS; EC 1.14.13.39) catalyses the oxygen- and NADPH-dependent oxidation of L-arginine to NO \cdot and citrulline in a complex reaction requiring FAD, FMN, and tetrahydrobiopterin, and in some cases also calcium and calmodulin (Knowles and Moncada, 1994). NOSs have been characterized as functional homodimers with a subunit of ≥ 130 kDa that contain iron-protoporphyrin IX, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH $_4$) as prosthetic groups (Knowles and Moncada, 1994; McMillan *et al.*, 1996). Three NOS isoforms have been identified in mammalian tissues: neuronal NOS (nNOS or type I), inducible NOS (iNOS or type II), and endothelial NOS (eNOS or type III). While types I and II are present in the soluble fraction, type III has been shown to be membrane-associated and none of the isoforms has a strict tissue-specific expression pattern (Sessa *et al.*, 1992; McMillan *et al.*, 1996). In organelles from animal systems, as far as is known, the presence of NOS activity has only been detected so far in mitochondria of rat liver (Ghafourifar and Richter, 1997).

Research on NO \cdot in plants has gained considerable attention in recent years and there is increasing evidence of a role of this molecule in plant growth and development (Leshem *et al.*, 1998; Leshem and Haramaty, 1996; Ribeiro *et al.*, 1999). Ya'acov Leshem, the pioneer in the study of nitric oxide in plants, has proposed that NO \cdot should be classified as a phytohormone which may function as a gaseous endogenous plant growth regulator (Leshem, 2000). Moreover, different studies have suggested that, in plants, nitric oxide could also function as a signal molecule in the transduction pathway leading to the induction of defence responses against pathogens and in damage leading to cell death (Delledonne *et al.*, 1998;

Van Camp *et al.*, 1998; Bolwell, 1999; Durner and Klessig, 1999; McDowell and Dangel, 2000; Grant and Loake, 2000; Leshem, 2000; Pedroso *et al.*, 2000).

In plant extracts, NOS-like activity has been detected during the interaction of *Rhizobium*–legumes (Cueto *et al.*, 1996) and fungi–plants (Ninnemann and Maier, 1996), in soybean cell suspensions and *Arabidopsis* (Delledonne *et al.*, 1998), and in tobacco leaves (Durner *et al.*, 1998). The presence of NOS in the cytosol and nucleus of maize cells has also been reported (Ribeiro *et al.*, 1999).

Very recently, the presence of NOS in subcellular fractions of pea leaves was studied. Mitochondria and peroxisomes were purified from pea leaves by differential and sucrose density-gradient centrifugation. The assay of NOS activity throughout the gradient fractions by the citrulline assay showed an activity peak in the peroxisomal fractions of 170 pmol of L-[3H]citrulline $min^{-1} mg^{-1}$ protein coinciding with the maximum of catalase activity in the gradient (Barroso *et al.*, 1999). However, no NOS activity was detected in mitochondria. The specific activity of the peroxisomal NOS (perNOS) was reduced more than 70% in the absence of calcium and was strictly dependent on NADPH. The effect of seven archetype NOS inhibitors, including specific and unspecific inhibitors of different types of NOS isoforms was assayed. The results indicated a clear inhibition of the perNOS activity of 59–100%, L-aminoguanidine being the most effective inhibitor. The production of citrulline was specifically due to a NOS because a specific antibody against iNOS inhibited this reaction by more than 80% (Barroso *et al.*, 1999).

In the subcellular fractions of pea leaves the presence of NOS was also studied by Western blot analysis, and in intact leaves by electron microscopy immunogold-labelling, using an antibody against the peptide PT387 from the C terminus (Ac-Cys-residues 1131–1144) of the murine iNOS (Barroso *et al.*, 1999). The electron microscopy immunolocalization of NOS demonstrated the presence of the enzyme in the matrix of peroxisomes and also in chloroplasts (Fig. 4) (Barroso *et al.*, 1999). However, no immunogold labelling of NOS was detected in mitochondria. This contrasts with results obtained in mammalian tissues where NOS activity was found in mitochondria isolated from rat liver (Ghafourifar and Richter, 1997). The immunoblot analysis of the peroxisomal fractions revealed an immunoreactive band of about 130 kDa (Fig. 4), which is in the same molecular mass range described for most iNOS and eNOS (for references see Barroso *et al.*, 1999). Immunoreactive bands with similar mobility were obtained with the hepatic iNOS from lipopolysaccharide-induced rat and murine macrophage lysates used as positive controls (Fig. 4, lanes 2 and 3). A summary of the biochemical properties of NOS from pea leaf peroxisomes is presented in Table 10.

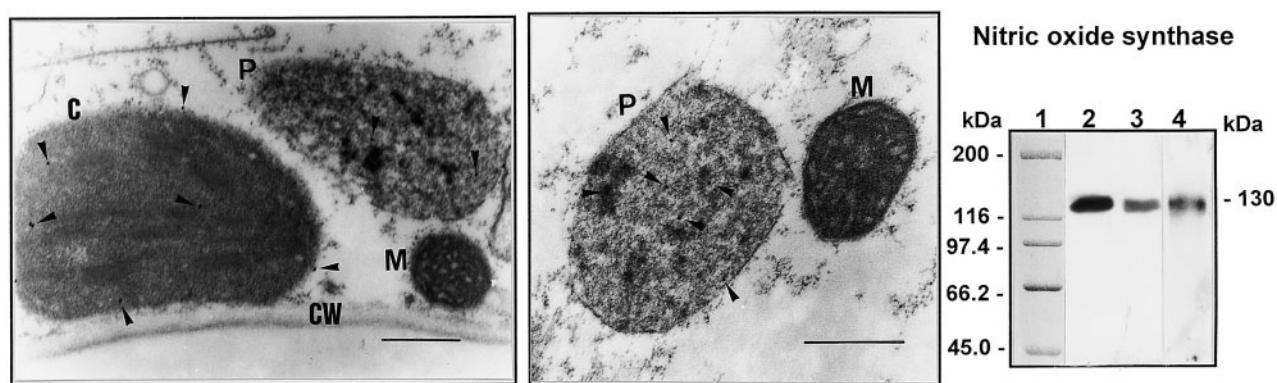


Fig. 4. Immunogold electron microscopy localization of NOS in pea leaves. The electron micrographs are representative of thin sections of pea leaves showing immunolocalization of NOS. Cell sections were probed with an antibody against iNOS (1/250 dilution) (Barroso *et al.*, 1999). Arrows indicate 15 nm gold particles. P, peroxisome; M, mitochondrion; C, chloroplast; CW, cell wall. Bars = 1.0 μ m. For immunoblot analysis, peroxisomal fractions were subjected to SDS-PAGE and then transferred to PVDF membranes and incubated with a polyclonal antibody against murine iNOS. 1, Molecular mass markers; 2, crude extracts of lipopolysaccharide-pretreated rat liver (30 μ g of protein); 3, murine macrophage control lysate (30 μ g of protein); 4, pea peroxisomal fraction (50 μ g of protein). A dilution of 1/2500 was used for lanes 2 and 3, and 1/500 for lane 4.

Table 10. Biochemical properties of the nitric oxide synthase (NOS) of pea leaf peroxisomes (Barroso *et al.*, 1999)

The different percentages of inhibition of the peroxisomal NOS activity produced by each inhibitor are indicated in brackets.

Subunit molecular mass	130 kDa
Cofactors	Ca ²⁺ NADPH (strict dependency)
Inhibitors	Aminoguanidine (100%) L-NAME (90%) L-NMMA (88%) Thiocitrulline (80%) Diphenyliodonium (60%) 7-nitroindazole (59%) L-N ⁵ -(1-Imenoethyl)-ornithine (59%)

Therefore, the biochemical, immunological, and immunocytochemical data provide evidence that plant peroxisomes contain nitric oxide synthase activity (perNOS) which is calcium-dependent, constitutively expressed, and immunorelated with the mammalian iNOS (Barroso *et al.*, 1999).

The presence of NOS in peroxisomes suggests that these organelles are a cellular source of NO[•] and also indicates possible interactions of NOS with other components of the metabolism of reactive oxygen species of leaf peroxisomes. A hypothetical model of this interaction is presented in Fig. 5. The NADP-dependent dehydrogenases found in the peroxisomal matrix (Corpas *et al.*, 1998b, 1999) could provide the necessary NADPH for the NOS reaction. But inside peroxisomes NO[•] could react with O₂⁻ radicals generated in the peroxisomal matrix by xanthine oxidase (Sandalio *et al.*, 1988; del Río *et al.*, 1992) to form the powerful oxidant peroxynitrite which (according to Sakuma *et al.*, 1997) could regulate the conversion of xanthine dehydrogenase into the superoxide-generating xanthine oxidase. Besides, the

information available on the proteolytic activity of peroxisomes from pea leaves suggests that peroxisomal endoproteases could, potentially, carry out the partial proteolysis which results in the irreversible conversion of xanthine dehydrogenase into xanthine oxidase (Distefano *et al.*, 1999). On the other hand, it is known that NO[•] in the presence of O₂ can react with reduced glutathione (GSH) to form S-nitrosoglutathione (GSNO), a reactive nitrogen oxide species (Wink *et al.*, 1996). As GSH occurs in leaf peroxisomes (Jiménez *et al.*, 1997) and NO[•] can be formed in these organelles (Barroso *et al.*, 1999), GSNO could be generated inside peroxisomes. In animal systems, GSNO has been reported to function as an intercellular and an intracellular NO[•] carrier, and in plants GSNO was found to be a powerful inducer of defence genes (Durner *et al.*, 1998). As hypothesized previously (Durner and Klessig, 1999), GSNO could function as a long-distance signal molecule, transporting glutathione-bound NO[•] throughout the plant. In this mechanism, leaf peroxisomes could participate through the endogenous production of GSNO which could diffuse to the cytosol.

NO[•] could also diffuse through the peroxisomal membrane, reacting with O₂⁻ produced in the cytosolic side of the membrane (del Río *et al.*, 1998a), thus generating the oxidant peroxynitrite in the cytosol. However, a modulation by NO[•] of the endogenous anti-oxidant enzymes of peroxisomes and the fatty acid β -oxidation enzymes cannot be ruled out. In animal systems, catalase and glutathione peroxidase activity are down-regulated by NO[•], whereas the activity of the peroxisomal β -oxidation is enhanced by NO[•]. This has been proposed as the mechanism responsible for the H₂O₂-associated cytotoxicity of NO[•] in peroxisomes, cytoplasm and mitochondria of animal cells (Dobashi *et al.*, 1997).

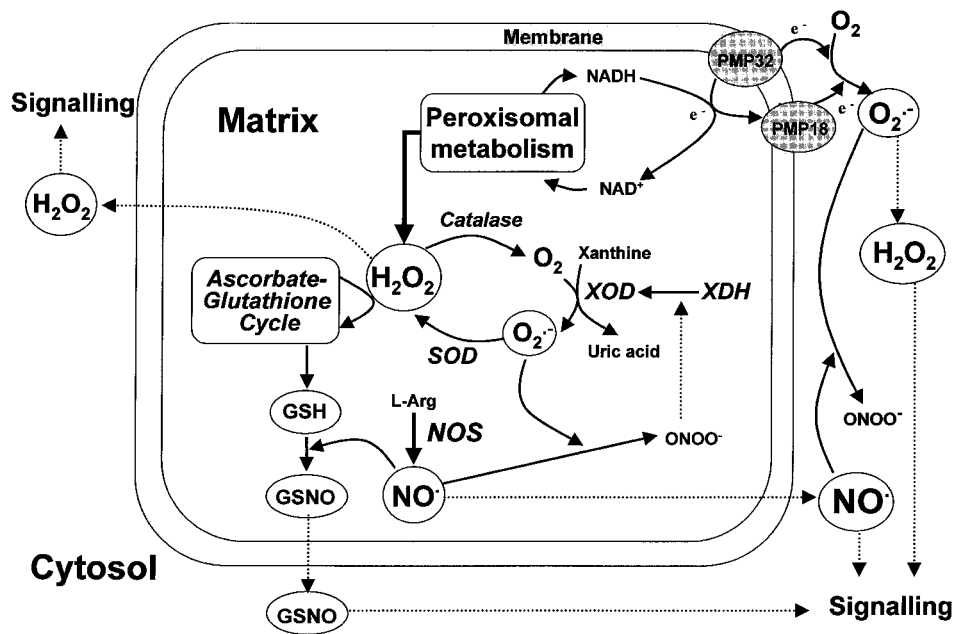


Fig. 5. Hypothetical model for the potential function of NOS in leaf peroxisomes and the role of these organelles in the generation of the signal molecules H_2O_2 , $O_2^{\cdot-}$, NO^{\cdot} , and GSNO (modified from Corpas *et al.*, 2001). PMP32, 32 kDa peroxisomal membrane polypeptide; PMP18, 18 kDa peroxisomal membrane polypeptide; XOD, xanthine oxidase; XDH, xanthine dehydrogenase; L-Arg, L-arginine; $ONOO^{\cdot-}$, peroxynitrite; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione.

Under normal physiological conditions, the production by peroxisomes of the ROS H_2O_2 and $O_2^{\cdot-}$ should be adequately controlled by catalase and ascorbate peroxidase, and SOD, respectively, which are present in peroxisomes. However, catalase is known to be inactivated by light and different stress conditions that suppress protein synthesis (Schäfer and Feierabend, 2000), and in these conditions an increase in the peroxisomal generation of H_2O_2 and $O_2^{\cdot-}$ can take place (del Río *et al.*, 1992, 1996). Superoxide radicals are known to inhibit catalase activity (Kono and Fridovich, 1982) and very recently it has been reported in tobacco plants that NO^{\cdot} and peroxynitrite inhibit catalase and APX activity, the two major H_2O_2 -scavenging enzymes of plant peroxisomes (Clark *et al.*, 2000). In addition, in animal cells an enhanced synthesis of nitric oxide was found to increase the peroxisomal H_2O_2 -producing β -oxidation. Taken together, these data mean that if, under any type of plant biotic and/or abiotic stress an induction of the peroxisomal production of $O_2^{\cdot-}$ and NO^{\cdot} radicals takes place, this can lead to the inhibition of catalase and APX activities and, possibly to an increase of the H_2O_2 level from the enhanced fatty acid β -oxidation. This breakdown of the peroxisomal antioxidant defences would originate an overproduction of H_2O_2 in peroxisomes and a toxic situation to the plant cell.

The rate of ROS and NO^{\cdot} generation in plant cells can have an ambivalent effect. A high cellular production of these active molecules can bring about extensive oxidative

damage (Halliwell and Gutteridge, 2000). On the contrary, numerous studies carried out in recent years have suggested that low levels of NO^{\cdot} and ROS, like H_2O_2 and $O_2^{\cdot-}$, are involved as signal molecules in many physiological processes, including the hypersensitive response to pathogens, growth and development (Bolwell, 1999; Van Camp *et al.*, 1998; Durner and Klessig, 1999). These messenger molecules are key mediators of pathogen-induced programmed cell death in plants and appear to function as part of a signal transduction pathway leading to the induction of defence responses against pathogens and cell death (Delledonne *et al.*, 1998; Van Camp *et al.*, 1998; Durner and Klessig, 1999; McDowell and Dangl, 2000; Grant and Loake, 2000).

The effects of nitric oxide on biological systems mentioned above are either regulatory or toxic (peroxynitrite formation), but a protective function for nitric oxide has also been proposed (Wink *et al.*, 1996). In animal systems, low concentrations of NO^{\cdot} have been reported to have protective antioxidant properties mainly by its effect diminishing metal-catalysed lipid peroxidation (Joshi *et al.*, 1999). Perhaps this is one of the mechanisms operating in plants, where nitric oxide is an endogenous maturation and senescence regulating factor (Leshem, 2000).

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 possibly GSNO, which can

contribute to achieve a more integrated communication among cell compartments both under normal physiological conditions and in response to biotic and abiotic stresses (Corpas *et al.*, 2001). This signal-producing function of plant peroxisomes is still more significant from a physiological viewpoint considering that the cellular population of these oxidative organelles can proliferate in plants during senescence and under different stress conditions, as indicated in Table 2. The study of the molecules that mediate the interorganellar communication within the cell is an important emerging area of plant organelle research, which can supply more information on how peroxisomal, mitochondrial, chloroplast and nuclear fractions are coupled throughout plant development and under different stress conditions by means of an elaborate signalling network (Mackenzie and McIntosh, 1999).

Concluding remarks

The existence of a reactive oxygen metabolism in plant peroxisomes and the presence in these organelles of a complex battery of antioxidative enzymes apart from catalase, such as SOD, the components of the ascorbate-glutathione cycle, the NADP-dehydrogenases, and the NO[•]-generating enzyme nitric oxide synthase, suggest novel roles for these organelles in cellular metabolism. Concomitantly, the important pool of new metabolites available in peroxisomes indicate that these organelles are involved in more metabolic pathways with other cell compartments, apart from the well-known photorespiratory role played with chloroplasts and mitochondria. This supports the idea postulated earlier (Tolbert *et al.*, 1987) on the effect of peroxisomal metabolism on metabolic pathways in other cell compartments. The flux of metabolites from and to peroxisomes are facilitated by pore-forming proteins (porins) in the peroxisomal membranes, whose existence has been demonstrated in peroxisomes in recent years (Reumann *et al.*, 1995; Reumann, 2000; Corpas *et al.*, 2000).

There are still many unanswered questions relating to the role of peroxisomes in the metabolism of plant cells. Further research on the cloning of genes for the biosynthetic enzymes of peroxisomal ROS metabolism and the characterization of mutants that are defective in stress responses, will throw more light on the physiological significance of peroxisomes in cellular metabolism and their function as a source of signalling molecules. This molecular information, apart from its importance from a basic research viewpoint, could be valuable to design molecular strategies directed to improve the tolerance of plants to different biotic and abiotic stresses. It seems reasonable to think that a ROS, NO[•] and GSNO signal molecule-producing function similar to that

postulated for plant peroxisomes could also be performed by human, animal and yeast peroxisomes.

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