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Evidence for the Presence of the Ascorbate-Glutathione Cycle in Mitochondria and Peroxisomes of Pea Leaves

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The presence of the enzymes of the ascorbate-glutathione cycle was investigated in mitochondria and peroxisomes purified from pea (*Pisum sativum* L.) leaves. All four enzymes, ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (EC 1.6.5.4), dehydroascorbate reductase (EC 1.8.5.1), and glutathione reductase (EC 1.6.4.2), were present in mitochondria and peroxisomes, as well as in the antioxidants ascorbate and glutathione. The activity of the ascorbate-glutathione cycle enzymes was higher in mitochondria than in peroxisomes, except for APX, which was more active in peroxisomes than in mitochondria. Intact mitochondria and peroxisomes had no latent APX activity, and this remained in the membrane fraction after solubilization assays with 0.2 M KCl. Monodehydroascorbate reductase was highly latent in intact mitochondria and peroxisomes and was membrane-bound, suggesting that the electron acceptor and donor sites of this redox protein are not on the external side of the mitochondrial and peroxisomal membranes. Dehydroascorbate reductase was found mainly in the soluble peroxisomal and mitochondrial fractions. Glutathione reductase had a high latency in mitochondria and peroxisomes and was present in the soluble fractions of both organelles. In intact peroxisomes and mitochondria, the presence of reduced ascorbate and glutathione and the oxidized forms of ascorbate and glutathione were demonstrated by high-performance liquid chromatography analysis. The ascorbate-glutathione cycle of mitochondria and peroxisomes could represent an important antioxidant protection system against H_2O_2 generated in both plant organelles.

Activated O_2 species, such as superoxide radicals ($O_2^{\cdot -}$), H_2O_2, and singlet oxygen ($^1O_2$), are produced in reactions that take place in different compartments of plant cells. The electron transport chains of chloroplasts and mitochondria are two important sources of $O_2^{\cdot -}$ radicals and H_2O_2 (Boveris, 1984; Elstner, 1991). Recently, the generation of $O_2^{\cdot -}$ in peroxisomes has been demonstrated to be caused by xanthine oxidase in the peroxisomal matrix and to an NADH-dependent electron transport chain in the peroxisomal membranes (del Río et al., 1992, 1996; López-Huertas et al., 1996b).

Superoxide and H_2O_2 can react by a metal-catalyzed Haber-Weiss reaction that generates hydroxyl radicals ($^{\cdot}OH$), which can rapidly attack all types of biomolecules, leading to irreparable metabolic dysfunction and cell death (Halliwell and Gutteridge, 1989). To keep these harmful reactions to a minimum, plants possess enzymatic and nonenzymatic antioxidative defense systems. SODs catalyze the dismutation of $O_2^{\cdot -}$ radicals to molecular O_2 and H_2O_2, thus playing a key role in this defense (Fridovich, 1986), and CAT and various peroxidases remove H_2O_2 (Asada, 1994). An increased production of O_2-free radicals has been involved in plant damage by biotic or abiotic stresses, as well as in the metabolic events associated with leaf and petal senescence and aging and fruit ripening (Thompson et al., 1987; Bowler et al., 1992; del Río et al., 1992; Morán et al., 1994; Pastori and del Río, 1994, 1997).

Scavenging of H_2O_2 by APX is the first step of the ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Asada, 1994). DHAR, MDHAR, and GR also participate in this cycle, which has been clearly demonstrated in the chloroplasts of pea (*Pisum sativum*) and spinach (*Spinacea oleracea*) (Nakano and Asada, 1981; Asada, 1994; Foyer et al., 1994). The ascorbate-glutathione cycle has also been suggested for H_2O_2 removal in *Euglena gracilis*, root nodules, and endosperm of germinating seeds (Shigeoka et al., 1980; Klapheck et al., 1990; Dalton et al., 1993).

The protection against O_2 radicals by mitochondrial Mn-SOD is well documented (Droillard and Paulin, 1990; del Río et al., 1991; Bowler et al., 1992; Hernández et al., 1993; Streller et al., 1994), and a protective role of Mn-SOD in copper-induced oxidative stress in pea leaf peroxisomes has been proposed (Palma et al., 1987). However, there is scarce information concerning the mitochondrial H_2O_2 scavenging systems. The localization of CAT, GPX, and Cyt peroxidase activities in mitochondria from maize (*Zea mays* L.) seedlings has been reported (Scandalios et al., 1980; Prasad et al., 1995). MDHAR and GR have been found in

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Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate, reduced form; CAT, catalase; CCO, Cyt *c* oxidase; DHA, ascorbate, oxidized form (dehydroascorbate); DHAR, dehydroascorbate reductase; G6PDH, Glc-6-P dehydrogenase; GPX, guaiacol peroxidase; GR, glutathione reductase; HPR, hydroxypyruvate reductase; MDHAR, monodehydroascorbate reductase; Mn-SOD, manganese-containing superoxide dismutase; pCMS, *p*-chloromercurisulfonic acid; SOD, superoxide dismutase.

potato (*Solanum tuberosum*) and pea mitochondria, respectively (Arrigoni et al., 1981; Edwards et al., 1990). However, the occurrence of APX and DHAR activities and the identification of ascorbate in leaf mitochondria have not been reported.

In peroxisomes H_2O_2 is produced mainly in the photorespiration glycolate pathway, in fatty acid β-oxidation, in the enzymatic reaction of flavin oxidases, and in the disproportionation of $O_2^{\cdot -}$ radicals (Huang et al., 1983; Elstner, 1987; del Río et al., 1992, 1996). The scavenging of H_2O_2 was thought to be carried out exclusively by CAT, which is quite abundant in peroxisomes, and, therefore, the ascorbate-glutathione cycle was not expected to be localized in peroxisomes. However, an APX isoenzyme has been recently identified in membranes of pumpkin (*Cucurbita pepo*) glyoxysomes and peroxisomes (Yamaguchi et al., 1995) and cotton (*Gossypium hirsutum*) glyoxysomes (Bunkelmann and Trelease, 1996), and the presence of MDHAR activity was also detected in membranes of oilseed glyoxysomes (Bowditch and Donaldson, 1990; Bunkelmann and Trelease, 1996). Nevertheless, neither the occurrence of ascorbate and glutathione nor a mechanism for the production or regeneration of ascorbate (ascorbate-glutathione cycle) in leaf peroxisomes has been reported.

In recent work carried out in our laboratory, total glutathione and total ascorbate were detected in mitochondria and peroxisomes from pea leaves (Jiménez et al., 1996). We carried out the present study to characterize the H_2O_2 scavenging systems of both cell organelles. As a result, evidence was obtained suggesting that the ascorbate-glutathione cycle could be operative in both mitochondria and peroxisomes from pea leaves.

MATERIALS AND METHODS

Pea (*Pisum sativum* L. cv Lincoln) seeds were obtained from Royal Sluis (Enkhuizen, The Netherlands). Pea plants were cultured in a growth chamber in aerated nutrient solutions under optimum conditions for 20 d, as described by Hernández et al. (1995).

Purification of Cell Organelles

Mitochondria and peroxisomes were isolated from pea leaves by differential and density-gradient centrifugation, as described by Struglics et al. (1993), but with some minor modifications. Leaves (40 g) were chopped with a polytron (Kinematica Krienz, Luzern, Switzerland) into a medium containing 30 mM Mops (pH 7.3), 3 mM EDTA, 25 mM Cys, 0.3 M mannitol, and 0.3% (w/v) BSA, using 4 volumes of medium g^{-1} fresh weight, and the homogenates were filtered through two layers of Miracloth (Calbiochem). The chloroplasts were sedimented by centrifugation at 1,600*g* for 5 min in a centrifuge using a JA-14 rotor (Beckman). The supernatant was centrifuged at 10,000*g* for 10 min, and the supernatant obtained was considered the cytosolic fraction. The pellet was resuspended in a wash medium (10 mM Mops, pH 7.2, 1 mM EDTA, 0.3 M mannitol, and 0.1% [w/v] BSA) and centrifuged at 1,050*g* for 5 min. The mitochondria, peroxisomes, and contaminating chloroplasts were finally pelleted at 12,000*g* for 15 min. The total enzyme activity in the organelle fraction was determined in the pellet that had been resuspended in a small volume of the wash medium.

Separation of cell organelles was achieved by a self-generated Percoll gradient. For this purpose, 2.5 mL of a suspension of the 12,000*g* pellet in the wash medium was layered on a gradient composed at the bottom of 13 mL of 28% (v/v) Percoll in 0.3 M Suc, 10 mM Mops, pH 7.2, 0.1% (w/v) BSA. On top of that 18.5 mL of the same solution was layered but contained 0.3 M mannitol instead of 0.3 M Suc. The gradients were centrifuged at 41,400*g* for 35 min in an ultracentrifuge, using a 50.2 Ti rotor (Beckman) at 4°C. Fractions of 1.5 mL were collected dropwise by puncturing the bottom of the tube using a fractionator (model 185, Isco, Lincoln, NE), which was equipped with an absorbance detector and a fraction collector. Enzyme activity was measured in the different fractions collected. The density of the fractions was determined at room temperature with a refractometer (Atago, Tokyo, Japan) . The integrity of the outer mitochondrial membrane was estimated from the succinate:Cyt *c* oxidoreductase activity, as described by Hernández et al. (1993).

The mitochondrial and peroxisomal fractions were pooled, diluted 10 times with the wash medium without BSA, and spun down at 17,000*g* three times for 10 min each. Finally, the pellets were carefully resuspended in a small volume of the same medium. The yield of mitochondria and peroxisomes from 40 g of pea leaves was approximately 1.0 and 0.4 mg of protein of each organelle, respectively.

For studies of APX activity, an independent organelle isolation procedure was used; 20 mM sodium ascorbate was added to the extraction medium and all of the other solutions contained 1 mM ascorbate to prevent possible inactivation of APX.

Enzyme Assays

Unless otherwise indicated, the activities of all enzymes were assayed in organelle samples diluted 2- to 5-fold with 50 mM potassium phosphate buffer, pH 7.8, containing 0.1% (v/v) Triton X-100. APX (EC 1.11.1.11) was determined at 25°C according to the method of Hossain and Asada (1984) from the decrease in A_{290} due to the ASC oxidation by H_2O_2 (ϵ_{290} = 2.8 mM^{-1} cm^{-1}). The oxidation rate of ASC was estimated between 1.0 and 60 s after starting the reaction with the addition of H_2O_2. The reaction mixture (1 mL) contained 50 mM Hepes-NaOH (pH 7.6), 0.22 mM ascorbate, 0.3 mM H_2O_2, and an enzyme sample. Correction was made for the low, nonenzymatic oxidation of ascorbate by H_2O_2. Pyrogallol specificity of the peroxidase was tested using 0.1 mM H_2O_2 and 20.0 mM pyrogallol. The H_2O_2-dependent oxidation of the donor was followed at 430 nm (due to purpurogallin, ϵ_{430} = 2.47 mM^{-1} cm^{-1}) (Klapheck et al., 1990). Mitochondrial and peroxisomal samples were disrupted by osmotic shock with 50 mM potassium phosphate buffer, pH 7.0, and 0.1% (v/v) Triton X-100, and were incubated separately for 5 to 10 min, with 5 mM aminotriazole, an inhibitor of CAT activity, and with 0.5 mM pCMS, a specific inhibitor of APX

activity (Mittler and Zilinskas, 1993). After the incubation with pCMS, samples were assayed for residual APX and GPX activity using ascorbate and pyrogallol as electron donors, and the recommended procedure for separate assays of APX and GPX was followed (Amako et al., 1994).

MDHAR (EC 1.6.5.4) was assayed by the decrease in A_{340} due to the NADH oxidation (ϵ_{340} = 6.22 mM^{-1} cm^{-1}) (Arrigoni et al., 1981). Monodehydroascorbate was generated by the ascorbate/ascorbate oxidase system (Arrigoni et al., 1981). To determine the MDHAR activity, the rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the initial monodehydroascorbate-dependent NADH oxidation rate (with ascorbate and ascorbate oxidase).

DHAR (EC 1.8.5.1) was determined according to the method of Dalton et al. (1993) by following the increase in A_{265} due to ascorbate formation (ϵ_{265} = 14 mM^{-1} cm^{-1}) and using N_2-bubbled buffer. The reaction rate was corrected for the nonenzymatic reduction of DHA by GSH. A factor of 0.98, to account for the small contribution to the absorbance by GSSH, was also considered.

GR (EC 1.6.4.2) was assayed by the decrease in A_{340} due to the NADPH oxidation (ϵ_{340} = 2.8 mM^{-1} cm^{-1}), as described by Edwards et al. (1990). The reaction rate was corrected for the small, nonenzymatic oxidation of NADPH by GSSH.

SOD (EC 1.15.1.1) was assayed by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich, 1969). SOD isozymes (Cu,Zn-SODs and Mn-SOD) were separated by PAGE on 10% gels, as described by Hernández et al. (1994).

CAT (EC 1.11.1.6) and HPR (EC 1.1.1.29) were used as markers of peroxisomes, and CCO (EC 1.9.3.1) and fumarase (EC 4.2.1.2) were used as markers of mitochondria. Marker enzymes were assayed as previously described (Hernández et al., 1993; Pastori and del Río, 1994). G6PDH (EC 1.11.1.49) was assayed according to the method of Doehlert et al. (1988).

Determinations of Enzyme Latency

In purified, intact preparations of mitochondria and peroxisomes the latency of enzyme activity was determined by carrying out the activity assays in a medium containing 0.3 M mannitol, in the absence and in the presence of 0.01% (v/v) Triton X-100. Total organelle activity was measured after lysis of the organelle by the addition of 0.01% (v/v) Triton X-100 (final concentration). The percentage of latency of activity was calculated using the formula of Burgess et al. (1985).

Solubilization of APX, MDHAR, DHAR, and GR

The method of Sandalio and del Río (1988) with some modifications was followed. Suspensions of purified mitochondria and peroxisomes were separated into two identical fractions, each of which were pelleted by centrifugation at 12,000*g* for 10 min and suspended in 200 μL of 10 mM Hepes-KOH buffer (pH 7.2), 1 mM EDTA, 10% (v/v) glycerol, with and without (control) 0.2 M KCl, and maintained on ice for 30 min, with shaking every 10 min in a vortex mixer. The suspensions were centrifuged at 62,000*g* for 30 min, and the pellets were resuspended in 200 μL of the same buffer with and without (control) 0.2 M KCl. For solubilization studies of APX activity, 1 mM ascorbate was included in the suspension buffers. The supernatants and resuspended pellets were assayed for APX, MDHAR, DHAR, and GR activities and proteins.

Determination of Total Glutathione and Total Ascorbate

Both antioxidants were extracted at 0°C from leaf tissue, and from mitochondria and peroxisomes, which were previously isolated using media without ascorbate and Cys. For each antioxidant, mitochondria and peroxisomes obtained from two density gradients were used. GSH and GSSH were extracted by mixing 260 μL of the sample with an equal volume of 12% perchloric acid containing 2 mM bathophenanthroline disulfonic acid. The resulting acid extract was frozen, thawed, and centrifuged at 12,000*g* for 5 to 10 min. Derivation of the supernatant was carried out with 2,4-dinitrofluorobenzene (Farris and Reed, 1987) using γ-Glu-Glu as an internal standard. Samples were lyophilized and resuspended in 50 μL of 70% methanol/H_2O. Less than 0.7% of the GSH content was converted to GSSH using these assay conditions, as determined by HPLC analysis. However, to prevent this oxidative artifact, an aliquot of the sample (250 μL) was routinely treated with the acid solution containing 40 mM *N*-ethylmaleimide before its derivation. HPLC profiles of these samples were compared with the profiles of samples not treated with *N*-ethylmaleimide (Asensi et al., 1994).

Ascorbate was extracted from 200 μL of the sample (leaf crude extracts, mitochondria, and peroxisomes) by mixing with an equal volume of 10% *m*-phosphoric acid and incubation for 30 min. The mixture was diluted with distilled H_2O to give a final concentration of 2% *m*-phosphoric acid and was centrifuged at 12,000*g* for 10 min. ASC in the supernatant was determined immediately by HPLC, as described by Castillo and Greppin (1988). DHA was separated from ASC by incubating the samples for 24 h at room temperature with 1 mM DTT (final concentration). The DHA concentration was measured as ASC following rechromatography. The system was calibrated with different concentrations of ascorbic acid as a standard curve.

Other Analytical Methods

Chlorophyll and proteins were quantified as described by Hernández et al. (1995).

RESULTS

When pea leaves were subjected to cell fractionation by differential centrifugation, the activity of the marker enzymes indicated a relatively clean separation of organelle fractions (chloroplasts, mitochondria, and peroxisomes) from the cytosolic fraction. The activities of the enzymes of the ascorbate-glutathione cycle were substantially higher in crude chloroplasts (2,200*g* pellet) and cytosol (12,000*g*

supernatant) than in the crude mitochondrial and peroxisomal fractions (12,000*g* pellet; Table I). Most of the chloroplasts were recovered in the 2,200*g* pellet, which contained approximately 40% of the total APX activity and 13% of the activity of the marker enzyme G6PDH. Three to 4% of the APX activity was located in the 12,000*g* pellet, which had about 67% of the mitochondrial marker CCO and also contained the peroxisomal markers HPR (15%) and CAT (13%) and 14% of the chlorophyll. The remainder of the APX activity (42%) was in the 12,000*g* supernatant, which contained most of the G6PDH activity (78%; Table I). The distribution of the other enzymes of the ascorbate-glutathione cycle (GR, MDHAR, and DHAR) was similar to that of APX activity, with about 3% of the total activity being recovered in the 12,000*g* mitochondrial and peroxisomal fractions.

To examine further the activity of the ascorbate-glutathione cycle enzymes present in the 12,000*g* pellet, mitochondria and peroxisomes were purified by centrifugation on Percoll density gradients (Fig. 1). Peroxisomes were identified in the gradient by the main peaks of CAT and HPR and were in the fractions 4 to 8, with a maximum equilibrium density of 1.07 g cm^{-3}. These organelles were well separated from intact mitochondria, which as shown by the CCO activity, banded in fractions 10 to 13. The majority of the chloroplasts were removed in the first differential centrifugation step of the method used, and the broken chloroplasts, mitochondria, and peroxisomes remained near the top of the gradient.

The purity of isolated peroxisomes and mitochondria is illustrated in Table II. In mitochondria, on the basis of the specific activity of HPR, there was contamination by peroxisomes of about 13%. Similarly, the mitochondrial contamination of peroxisomes was lower than 10%. No G6PDH activity was detected in either of the two purified organelles. The intactness of the outer mitochondrial membrane of purified mitochondria was in the range of 70 to 90% and the intactness of the purified peroxisomes was about 65%. All four enzymes of the ascorbate-glutathione cycle (APX, MDHAR, DHAR, and GR) were present in the mitochondria and peroxisomes, although the activity levels were, in general, higher in the mitochondria than in the peroxisomes (Table III). In the density gradients, the APX activity was positively correlated with both the mitochondrial and the peroxisomal marker enzymes (Fig. 1).

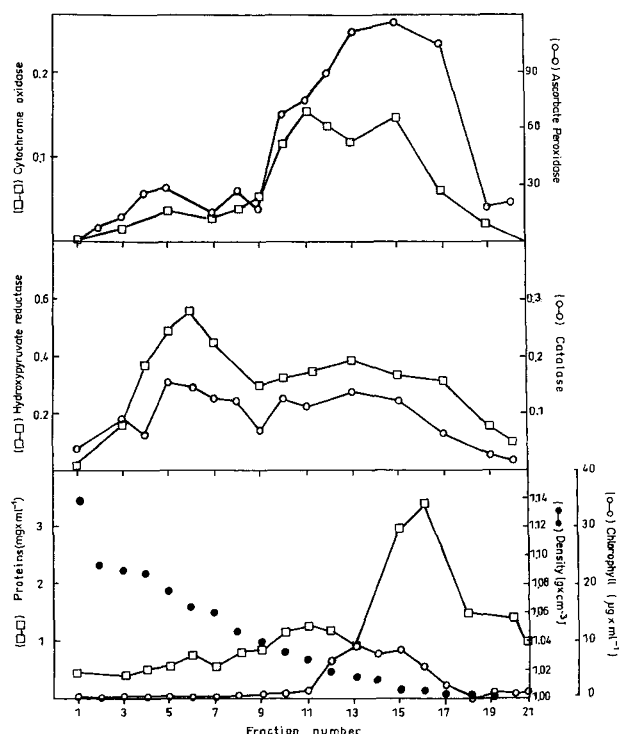

Figure 1. APX in organelles isolated from pea leaves by Percoll density-gradient centrifugation. CAT and HPR were used as marker enzymes for peroxisomes, and CCO was used for mitochondria. Chlorophyll was used as the chloroplast marker. Protein content is expressed as mg mL^{-1}. APX activity is expressed as nmol min^{-1} mL^{-1} and all other enzyme activities are expressed as μmol min^{-1} mL^{-1}.

When the APX and GPX activities of mitochondria were assayed using ascorbate or pyrogallol as the substrates, respectively, results showed that the thiol reagent pCMS inhibited the oxidation of ascorbate by 76%, and under the same conditions, the rate of pyrogallol oxidation was 45% inhibited (Table IV, reactions B and C, respectively). The pCMS-insensitive oxidation of pyrogallol was due to the GPX activity (reaction C).

The thiol reagent pCMS produced a similar inhibition effect in the peroxisomal oxidation of ascorbate as that of mitochondria (about 72%), whereas the pyrogallol oxida-

Table I. *Distribution of marker enzymes and of enzymes of the ascorbate-glutathione cycle in different subcellular fractions from pea leaves*

Total enzyme activities in the crude extract as μmol min^{-1} were: G6PDH, 6.35 ± 0.3; CCO, 8.84 ± 0.2; CAT, 25.2 ± 2.3; HPR, 86 ± 9; APX, 247 ± 14; GR, 6.6 ± 0.5; DHAR, 2.7 ± 0.3; and MDAR, 2.5 ± 0.3.

Fraction[a]	Distribution of Total Enzyme Activity							
	G6PDH	CCO	CAT	HPR	APX	GR	MDHAR	DHAR
	%							
Crude extract	100	100	100	100	100	100	100	100
2,200*g* Pellet (crude chloroplasts)	13	21	17	15	43	68	51	76
12,000*g* Supernatant (cytosolic fraction)	78	12	70	60	42	20	48	22
12,000*g* Pellet (crude mitochondrial and peroxisomal fraction)	1.5	66.5	13	15	3.5	3.5	2.5	3.5

[a] The different cellular fractions were obtained by centrifugation of crude homogenates as described in "Materials and Methods." Values are the means of at least four independent experiments.

Table II. *Specific activity of marker enzymes in mitochondria and peroxisomes from pea leaves*

Specific activity is expressed as μmol min^{-1} mg^{-1} protein. The percentage activity was calculated by dividing the specific activity in each organelle by the sum of activities in the mitochondria and peroxisomes. Values are the means of three independent experiments.

Organelle	CCO		HPR	
	Specific activity	Percentage activity	Specific activity	Percentage activity
Mitochondria	0.18	90.5	0.12	13
Peroxisomes	0.019	9.5	0.81	87

tion was inhibited by 73% (Table V). Incubation of the purified peroxisomes with aminotriazol (5 mM) inhibited the ascorbate oxidation rate by about 29% (Table V).

The suborganellar localization of APX in mitochondria and peroxisomes showed that most of the APX activity in mitochondria and peroxisomes was in the KCl-insoluble fraction (Table VI). However, treatment with 0.01% (v/v) Triton X-100 effectively solubilized the APX activity in both organelles (data not shown). Latency values of APX in intact mitochondria and peroxisomes (Table VIII) were not significant (6–7%) and were much lower than the intactness values of both organelles (95% for mitochondria and 65% for peroxisomes).

As shown in Table VIII, with Hepes buffer GR and DHAR activities were recovered mainly in the soluble mitochondrial fraction. However, the KCl treatment of mitochondria seemed to produce a partial sedimentation of the DHAR activity present in the supernatant, since about 50% of the DHAR activity was detected in the mitochondrial membranes after osmotic lysis with KCl (Table VIII). In contrast to GR and DHAR activities, almost 70% of the mitochondrial MDHAR was present in the membrane fraction, and this activity was retained in the membranes after KCl treatment. The latency values of GR and MDHAR in intact mitochondria were very high, 97 and 89%, respectively (Table VIII). In peroxisomes almost all of the GR activity and about 70% of the DHAR activity were released by KCl treatment (Table IX). However, as in the case of mitochondria, the amount of DHAR activity attached to the peroxisomal membrane fractions was apparently increased after the KCl treatment. On the other hand, the MDHAR activity was found predominantly in the peroxisomal membrane fractions. The latency values of GR and MDHAR were close to 100% and were very similar to the latency values found for these enzymes in mitochondria, whereas latency of DHAR activity was close to 50%.

In crude extracts and intact mitochondria and peroxisomes, the content of ASC and GSH and their oxidized forms DHA and GSSH was analyzed (Table X). In mitochondria, as in crude extracts, the ascorbate pool was kept mainly in the reduced form, whereas in peroxisomes there was only 40% ASC. About 97% of the total glutathione in mitochondria and peroxisomes was in the reduced form, and the ratio of GSH/GSSH was close to 30. This pattern also occurred in crude extracts, although the ratio GSH/GSSH was higher than it was in mitochondria and peroxisomes.

DISCUSSION

Three major types of APX have been described based on their subcellular location (thylakoid-bound, stromal chloroplastidic, and cytosolic) and molecular properties (Chen and Asada, 1989; Mittler and Zilinskas, 1991; Asada, 1994). Recently, a novel isoenzyme of APX was localized in membranes from oilseed glyoxysomes (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996) and leaf peroxisomes (Yamaguchi et al., 1995). In the latter study the presence of APX in mitochondria and peroxisomes from pea leaves was demonstrated, as well as the occurrence of all of the other enzymatic activities responsible for the ascorbate regeneration (the ascorbate-glutathione cycle). Likewise, the occurrence in mitochondria and peroxisomes of ASC and GSH, and the oxidized forms DHA and GSSH, was shown.

In matrix and membrane fractions of mitochondria and peroxisomes, lysed by hypotonic shock in the presence and in the absence of 0.2 M KCl, about 90 and 95% of the APX activity was associated with the membrane fractions of mitochondria and peroxisomes, respectively. This solubilization pattern suggests that APX is membrane-bound in mitochondria and peroxisomes, since the enzyme was not removed from the membrane by the KCl treatment, which is a method commonly used to distinguish between peripheral and integral membrane proteins (Thomas and Mcnamee, 1990). This situation is similar to that described for membrane-bound APXs in spinach thylakoids and pumpkin glyoxysomes and peroxisomes (Miyake and Asada, 1992; Yamaguchi et al., 1995), which were not solubilized from the membranes with 0.2 M KCl.

The evidence for the presence of APX activity in peroxisomal membranes has been supported by recent results obtained in our laboratory. Western analyses of sodium carbonate-washed peroxisomal membranes from pea leaves with the antibody against APX of pumpkin peroxisomal membranes (Yamaguchi et al., 1995) revealed a strong cross-

Table III. *Specific activity of Mn-SOD and the ascorbate-glutathione cycle enzymes in mitochondria and peroxisomes purified from pea leaves*

Data are the means of at least four different experiments.

Fraction	Mn-SOD	APX	GR	MDHAR	DHAR
	units mg^{-1} protein		*nmol min^{-1} mg^{-1} protein*		
Crude extract	7.9 ± 0.8	188 ± 9	4.2 ± 0.9	48.6 ± 7.2	11.3 ± 2.4
Mitochondria	189 ± 12	161 ± 11	22.3 ± 3.2	98.6 ± 10.5	210 ± 40
Peroxisomes	28.5 ± 2.1	281 ± 43	12.7 ± 2.3	32.8 ± 3.6	25.0 ± 3.5

Table IV. *Effect of inhibitors on ascorbate and/or pyrogallol oxidation by mitochondrial APX*

Purified mitochondria, previously disrupted by osmotic shock, were used to assay the oxidation rate of ASC under the standard conditions for APX (reaction A). The oxidation rates of ASC (reaction B) and pyrogallol (reaction C) were determined after the incubation of lysed mitochondria at 25°C with 0.5 mM pCMS during 10 min. The pCMS-sensitive oxidation rate of ASC (A and B) give the activity of APX in terms of ascorbate oxidation. The pCMPS-insensitive oxidation rate of pyrogallol (C) represents the activity of GPX in terms of the pyrogallol oxidation, as indicated in "Materials and Methods." Incubations with 5 mM aminotriazol were carried out at 25°C for 10 min. Data are the means of three different experiments.

Substrate	Specific Activity	Inhibition	Reaction Type
	nmol ASC min^{-1} mg^{-1} protein	%	
Ascorbate	151		A
+0.5 mM pCMS	36	76	B
+5 mM Aminotriazol	89	41	
Pyrogallol	271		
+0.5 mM pCMS	149	45	C

reactive band of about 35 kD (López-Huertas et al., 1996a). This indicates that the 35-kD peroxisomal membrane polypeptide of pea leaf peroxisomes could be the membrane-bound APX.

Intact mitochondria and peroxisomes showed a low latency of APX activity, and this indicates that APX is bound to the outer side of the external mitochondrial membrane and to the cytosolic side of the peroxisomal membrane. However, the presence of ascorbate/DHA transporters has been demonstrated in chloroplasts (Beck et al., 1983) and in protoplasts and vacuole membranes (Rautenkranz et al., 1994). In this case, if in the latency assays ascorbate is transported rapidly across the membrane, the APX would have access to its substrate regardless of its location. Should ascorbate/DHA transporters also occur on the bounding membranes of mitochondria and peroxisomes, then the presence of APX on either the inner face of the outer membrane or the outer face of the inner mitochondrial membrane and on the matrix side of the peroxisomal membrane could not be ruled out.

Table V. *Effect of inhibitors on ascorbate and/or pyrogallol oxidation by peroxisomal APX*

Purified peroxisomes, previously disrupted by osmotic shock, were used to assay the oxidation rate of ASC under the standard conditions for APX (reaction A). The incubation conditions were as described in Table IV. Data are the means of two different experiments.

Substrate	Specific Activity	Inhibition	Reaction Type
	nmol ASC min^{-1} mg^{-1} protein	%	
Ascorbate	709		A
+0.5 mM pCMS	202	72.0	B
+5 mM Aminotriazol	506	29.0	
Pyrogallol	2980		
+0.5 mM pCMS	804	73.0	C

Table VI. *Intraorganellar localization of APX in mitochondria and peroxisomes purified from P. sativum leaves*

Experiments were conducted in 10 mM Hepes-KOH buffer (pH 7.2) containing 10% (v/v) glycerol, 1 mM EDTA, and 1 mM sodium ascorbate in the absence (control) and in the presence of 0.2 M KCl. The purified mitochondrial and peroxisomal preparations used had APX activities of 165 and 281 nmol ASC min^{-1} mg^{-1} protein, respectively, and 132 μg of mitochondrial protein and 122 μg of peroxisomal protein were processed as described in "Materials and Methods." The percentage of solubilization was calculated by dividing the specific activity in the supernatant by the sum of the specific activities in the supernatant and in the pellet. Data are the means of two different experiments.

Organelle	Hepes Buffer	Hepes Buffer plus 0.2 M KCl
	% solubilization	
Mitochondria	13.6	12.8
Peroxisomes	nd[a]	8.9

[a] nd, Not detectable.

With regard to peroxisomes, the results reported in this work agree with those recently described by Yamaguchi et al. (1995), who did not find any latent APX activity in leaf peroxisomes and glyoxysomes from pumpkin, and concluded that the active site of APX was exposed to the cytosol. On the contrary, in glyoxysomes from cotton cotyledons on the basis of protease/detergent assays and the deduced amino acid sequence, a matrix side location for the active site of APX has been proposed (Bunkelmann and Trelease, 1996).

MDHAR activity was also tightly associated with the membranes of mitochondria and peroxisomes from pea leaves after solubilization with 0.2 M KCl. In glyoxysomal membranes, MDHAR appears to have a *trans*-membrane orientation similar to that found on the outer mitochondria membranes in chromaffin vesicles (Bowditch and Donaldson, 1990; Njus and Kelley, 1993). Latency experiments of intact pea mitochondria and peroxisomes showed a high latency of MDHAR activity (about 90%) in both organelles, suggesting that the electron acceptor and donor sites of MDHAR activity are not in a *cis* orientation in the external side of the outer mitochondrial membrane and the cytosolic side of the peroxisomal membrane.

The latency of GR activity in intact peroxisomes and mitochondria was very high (97 and 100%, respectively), and most was released in the soluble fraction when both organelles were lysed by hypotonic shock. All of these data indicate that GR is a matrix-associated enzyme in mitochondria and peroxisomes from pea leaves. These results agree with those previously described by Edwards et al. (1990), who found high latent GR activity in pea mitochondria. A similar suborganellar localization was found for DHAR activity, which was predominantly recovered in the soluble fractions from lysed mitochondria and peroxisomes. The degree of association of DHAR activity to the membranes of both organelles after KCl treatment was similar to that shown by the mitochondrial matrix-associated and peroxisomal matrix-associated fumarase and CAT activities, respectively. Latency values of DHAR

Table VII. *Latency of APX activity in isolated mitochondrias and peroxisomes from pea leaves*

Enzyme activities are expressed as μmol min^{-1} mg^{-1} protein. The latency was determined by performing the activity assays in an isotonic medium with 0.3 M manitol in the absence and in the presence of 0.01% (v/v) Triton X-100. The percentage of latency was calculated as described in "Materials and Methods." CCO and HPR were used as marker enzymes of mitochondria and peroxisomes, respectively. Data are the means of three different experiments.

Organelle	APX Activity	Percentage of APX Latency	CCO Activity	Percentage of CCO Latency	HPR Activity	Percentage of HPR Latency
Mitochondria	0.16	6	0.01	95	–[a]	–
+Triton X-100	0.17		0.22		–	
Peroxisomes	0.26	7	–	–	0.26	65
+Triton X-100	0.28		–		0.75	

[a] –, No activity detected.

activity in intact mitochondria and peroxisomes suggest the presence of a fraction of this activity on the external side of the outer mitochondrial membrane and on the cytosolic face of the peroxisomal membrane. However, the latency values of DHAR could also be due to a preferential transport of the DHA across the membranes of mitochondria and peroxisomes, as has been suggested for potato tuber mitochondria and other cell membranes (Washko et al., 1993; de Leonardis et al., 1995). Therefore, the question of whether a fraction of DHAR activity in mitochondria and peroxisomes is an integral membrane protein or the result of nonspecific adsorption of cytosolic DHAR to the membranes remains to be seen.

In pea leaf mitochondria, it was found that APX had a slightly lower specific activity with ascorbate than with pyrogallol by using pCMS as a specific inhibitor to discriminate between APX and GPX (Mittler and Zilinskas, 1993; Amako et al., 1994). In pea peroxisomes APX showed much higher specific activity with pyrogallol than with ascorbate. This agrees with what has been reported for other APX isozymes (Chen and Asada, 1989; Mittler and Zilinskas, 1991). In its substrate specificity, the mitochondrial and the peroxisomal APX seem to be different from the chloroplastic APX isozyme from pea leaves, which is highly specific for ascorbate as an electron donor (Mittler and Zilinskas, 1993), but they are more similar to the pea cytosolic APX (Mittler and Zilinskas, 1991) and to the APX I from tea leaves (Chen and Asada, 1989) and root nodules (Dalton et al., 1987). All of these APX isozymes have a broader spectrum of donor specificity than the chloroplastic APX. The inhibition of mitochondrial and peroxisomal APX by aminotriazole has also been observed in other APX isozymes (Foyer et al., 1994).

In glyoxysomal membranes from castor bean endosperm, Bowditch and Donaldson (1990) demonstrated the presence of membrane-associated MDHAR activity and found that this activity was due to the 32-kD glyoxysomal membrane protein responsible for the ferricyanide reductase activity previously characterized in those membranes (Luster et al., 1988). Recently, the integral membrane polypeptides of pea leaf peroxisomes were characterized (López-Huertas et al., 1995), and the 32-kD membrane polypeptide was identified as a flavoprotein with NADH: ferricyanide reductase activity, which is also able to generate NADH-dependent $O_2^{\cdot -}$ radicals (López-Huertas et al., 1996b). This suggests that the MDHAR activity detected in this work in the matrix side of pea leaf peroxisomal membranes is the 32-kD membrane polypeptide previously characterized in the same membranes.

Table VIII. *Latency and intraorganellar localization of MDHAR, DHAR, and GR in mitochondria purified from P. sativum leaves*

Experiments were conducted in 10 mM Hepes-KOH buffer (pH 7.2) containing 10% (v/v) glycerol, 1 mM EDTA, and 1 mM sodium ascorbate in the absence (control) and in the presence of 0.2 M KCl as described in Table V. Soluble fractions and membranes from mitochondria were isolated as described in "Materials and Methods." The percentage of solubilization was calculated by dividing the total activity in the supernatant by the sum of the activities in the supernatant and in the pellet. CCO and fumarase were used as marker enzymes of mitochondria.

Enzymes	Enzyme Latency	Hepes Buffer	Hepes Buffer plus 0.2 M KCl
		%	
MDHAR[a]	89	36	30
DHAR	56	78	48
GR	97	98	98
Fumarase	98	68	67
CCO	91	59	42

[a] Assay conditions were as in "Materials and Methods," except that 1 mM KCN was included to inhibit mitochondrial NADH oxidation.

Table IX. *Latency and intraorganellar localization of MDHAR, DHAR, and GR in peroxisomes purified from P. sativum leaves*

Experiments were conducted in 10 mM Hepes-KOH buffer (pH 7.2) containing 10% (v/v) glycerol, 1 mM EDTA, and 1 mM sodium ascorbate in the absence (control) and in the presence of 0.2 M KCl. Soluble fractions and membranes from peroxisomes were isolated as described in "Materials and Methods." The percentage of solubilization was calculated by dividing the total activity in the supernatant by the sum of the activities in the supernatant and in the pellet. Catalase was used as a marker enzyme of peroxisomes.

Enzymes	Enzyme Latency	Hepes Buffer	Hepes Buffer plus 0.2 M KCl
		%	
MDHAR	96	11	22
DHAR	46	66	40
GR	100	100	40
Catalase	63	72	53

Table X. *Ascorbate and glutathione contents in crude extracts and intact mitochondria and peroxisomes purified from pea leaves*

The antioxidants were extracted and determined as described in "Materials and Methods." For each antioxidant, mitochondria and peroxisomes obtained from two density gradients were combined and processed. Values are the means of at least four different experiments.

Fraction	Total Protein	ASC	DHA	GSH	GSSH
	mg	*μg mg⁻¹ protein*		*nmol mg⁻¹ protein*	
Crude extracts	1627	7.10	1.70	9.70	0.02
Mitochondria	1.03	3.81	0.41	5.92	0.21
Peroxisomes	0.15	0.65	0.94	4.15	0.15

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 be an electron acceptor, with the concomitant formation of $O_2^{\cdot -}$ radicals (López-Huertas et al., 1996b). The evidence reported in this work of 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 function could be to reoxidize endogenous NADH to maintain a constant supply of NAD^+ for peroxisomal metabolism, an idea that was originally postulated for the membrane-bound NADH dehydrogenase of glyoxysomes from castor bean (*Ricinus communis*) endosperm (Fang et al., 1987; Luster and Donaldson, 1987; Bowditch and Donaldson, 1990). A second function of the membrane antioxidant enzymes could be protection against H_2O_2 leaking out of peroxisomes. H_2O_2 can easily permeate the peroxisomal membrane (Boveris et al., 1972), and an important advantage of the presence of APX in the membrane would be the degradation of H_2O_2 from that source, as well as the H_2O_2 that is being continuously formed by disproportionation of the $O_2^{\cdot -}$ radicals generated in the NADH-dependent electron transport system of the peroxisomal membrane (del Río et al., 1992, 1996; López-Huertas et al., 1996b). This membrane scavenging of H_2O_2 could avoid an increase of the cytosolic H_2O_2 concentration during normal metabolism and under plant-stress situations, when the level of H_2O_2 produced in peroxisomes can be substantially enhanced (del Río et al., 1996).

In leaf mitochondria the MDHAR activity could also have a *trans*-membrane orientation, and the two membrane-bound antioxidant enzymes APX and MDHAR, such as in peroxisomes, could couple the oxidation of internal mitochondrial NADH to the scavenging of H_2O_2 outside of the mitochondrial membrane, since H_2O_2 can easily permeate it (Boveris, 1984). Likewise, the mitochondrial metabolism through the MDHAR activity could also be coupled to other cytosolic metabolic reactions in which ascorbate regeneration is required, such as in the case of the secretory vesicles (Njus and Kelley, 1993).

The data reported in this work show that mitochondria and peroxisomes from pea leaves contain a complete complement of the ascorbate-glutathione cycle enzymes, as well as glutathione and ascorbate, and therefore this cycle could be functional in these two organelles for the scavenging of H_2O_2. The presence of the antioxidants ascorbate and glutathione in the soluble fractions of peroxisomes and mitochondria imply their participation as endogenous defenses against H_2O_2 and other oxidants, including superoxide radicals, overproduced inside these organelles. Because DHAR and GR are soluble enzymes, they could participate in the regeneration of ASC from DHA. This way, ASC and GSH could collaborate in the control of peroxisomal and mitochondrial H_2O_2 concentration and avoid its reaching toxic levels.

However, H_2O_2 may also function as a specific chemical messenger in cellular signal transduction pathways (Baeuerle et al., 1996; del Río et al., 1996). The ascorbate-glutathione cycle demonstrated in leaf peroxisomes and mitochondria from pea plants perhaps could have a role in those signal transduction processes that lead to specific gene expression by regulating the cytosolic concentration of the diffusible signaling molecule, H_2O_2.

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LITERATURE CITED

Amako K, Chen GX, Asada K (1994) Separate assays for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol **35:** 497–504

Arrigoni O, Dipierro S, Borraccino G (1981) Ascorbate free radical reductase: a key enzyme of the ascorbic acid system. FEBS Lett **125:** 242–244

Asada K (1994) Production and action of active oxygen species in photosynthetic tissues. *In* CH Foyer, PM Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense System in Plants. CRC Press, Boca Raton, FL, pp 77–103

Asensi M, Sastre J, Pallardó FV, Estrela JM, Viña J (1994) Determination of oxidized glutathione in blood: high-performance liquid chromatography. Methods Enzymol **234:** 367–371

Baeuerle PA, Rupec RA, Pahe HL (1996) Reactive oxygen intermediates as second messengers of a general pathogen response. Pathol Biol **44:** 29–35

Beck E, Burkert A, Hofmann M (1983) Uptake of L-ascorbate by intact spinach chloroplasts. Plant Physiol **73:** 41–45

Boveris A (1984) Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. Methods Enzymol **105:** 429–435

Boveris A, Oshino N, Chance B (1972) Cellular production of hydrogen peroxide. Biochem J **128:** 617–630

Bowditch MY, Donaldson RP (1990) Ascorbate free-radical reduction by glyoxysomal membranes. Plant Physiol **94:** 531–537

Bowler C, Van Montagu M, Inzé D (1992) Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol Plant Mol Biol **43:** 83–116

Bunkelmann JR, Trelease RN (1996) Ascorbate peroxidase. A prominent membrane protein in oilseed glyoxysomes. Plant Physiol **110:** 589–598

Burgess N, Beakes GW, Thomas DR (1985) Separation of mitochondria from microbodies of *Pisum sativum* L. cv. Alaska cotyledons. Planta **166:** 151–155

Castillo FJ, Greppin H (1988) Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure. Environ Exp Bot **28:** 231–238

Chen G-X, Asada K (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic properties. Plant Cell Physiol **30:** 987–998

Dalton DA, Baird LM, Langeberg L, Taugher CY, Anyan WR, Vance CV, Sarath G (1993) Subcellular localization of oxygen defense enzymes in soybean (*Glycine max* L. Merr.) root nodules. Plant Physiol **102:** 481–489

Dalton DA, Hanus FJ, Russell SA, Evans HJ (1987) Purification, properties, and distribution of ascorbate peroxidase in legume root nodules. Plant Physiol **83:** 789–794

de Leonardis S, de Lorenzo G, Borraccino G, Dipierro S (1995) A specific ascorbate free radical reductase isozyme participates in the regeneration of ascorbate for scavenging toxic oxygen species in potato tuber mitochondria. Plant Physiol **109:** 847–851

del Río LA, Palma JM, Sandalio LM, Corpas FJ, Pastori GM, Bueno P, López-Huertas E (1996) Peroxisomes as a source of superoxide and hydrogen peroxide in stressed plants. Biochem Soc Trans **24:** 434–438

del Río LA, Sandalio LM, Palma JM, Bueno P, Corpas FJ (1992) Metabolism of oxygen radicals in peroxisomes and cellular implications. Free Radical Biol Med **13:** 557–580

del Río LA, Sevilla F, Sandalio LM, Palma JM (1991) Nutritional effect and expression of superoxide dismutases: induction and gene expression, diagnostics, prospective protection against oxygen toxicity. Free Radical Res Commun **12:** 819–828

Doehlert DC, Kuo TM, Felker FC (1988) Enzymes of sucrose and hexose metabolism in developing kernels of two inbreds of maize. Plant Physiol **86:** 1013–1019

Droillard MJ, Paulin A (1990) Isozymes of superoxide dismutase in mitochondria and peroxisomes isolated from petals of carnation (*Dianthus caryophyllus*) during senescence. Plant Physiol **94:** 1187–1192

Edwards EA, Rawsthorne S, Mullineux PM (1990) Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum* L.). Planta **180:** 278–284

Elstner EF (1987) Metabolism of activated oxygen species. *In* DD Davies, ed, The Biochemistry of Plants, Vol 11: Biochemistry of Metabolism. Academic Press, San Diego, CA, pp 253–315

Elstner EF (1991) Mechanisms of oxygen activation in different compartments of plant cells. *In* EJ Pell, KL Steffen, eds, Active Oxygen Species, Oxidative Stress, and Plant Metabolism. The American Society of Plant Physiologists, Rockville, MD, pp 13–25

Fang TK, Donaldson RP, Vigil EL (1987) Electron transport in purified glyoxysomal membranes from castor bean endosperm. Planta **172:** 1–13

Farris MW, Reed DJ (1987) High-performance liquid chromatography of thiols and disulfides: dinitrophenol derivates. Methods Enzymol **143:** 101–109

Foyer CH, Halliwell B (1976) Presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta **133:** 21–25

Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. Physiol Plant **92:** 696–717

Fridovich I (1986) Superoxide dismutases. Adv Enzymol Relat Areas Mol Biol **58:** 61–97

Halliwell B, Gutteridge JMC (1989) Free Radicals in Biology and Medicine, Ed 2. Oxford University Press, Oxford, UK

Hernández JA, Corpas FJ, Gómez M, del Río LA, Sevilla F (1993) Salt-induced oxidative stress mediated by activated oxygen species in pea leaf mitochondria. Physiol Plant **89:** 103–110

Hernández JA, del Río LA, Sevilla F (1994) Salt stress-induced changes in superoxide dismutase isozymes in leaves and mesophyll protoplasts from *Vigna unguiculata* (L.) Walp. New Phytol **126:** 37–44

Hernández JA, Olmos E, Corpas FJ, Sevilla F, del Río LA (1995) Salt-induced oxidative stress in chloroplasts of pea plants. Plant Sci **105:** 151–167

Hossain MA, Asada A (1984) Inactivation of ascorbate peroxidase in spinach chloroplasts on dark addition of hydrogen peroxide: its protection by ascorbate. Plant Cell Physiol **25:** 1285–1295

Huang AHC, Trelease RN, Moore TS (1983) Plant Peroxisomes. Academic Press, New York

Jiménez A, Hernández GM, Pastori GH, del Río LA, Sevilla F (1996) Active oxygen metabolism in the senescence of pea leaves: ascorbate and glutathione content in different cell compartments. Biochem Soc Trans **24:** 198S

Klapheck S, Zimmer I, Cosse H (1990) Scavenging of hydrogen peroxide in the endosperm of *Ricinus communis* by ascorbate peroxidase. Plant Cell Physiol **31:** 1005–1013

López-Huertas E, Corpas FJ, Sandalio LM, del Río LA (1996a) Isolation and characterization of peroxisomal membrane polypeptides involved in superoxide radical production (abstract 234–1). Presented at the VIIIth Biennial Meeting of the International Society for Free Radical Research, Barcelona, Spain

López-Huertas E, Sandalio LM, del Río LA (1995) Integral membrane polypeptides of pea leaf peroxisomes: characterization and response to plant stress. Plant Physiol Biochem **33:** 295–302

López-Huertas E, Sandalio LM, del Río LA (1996b) Superoxide radical generation in plant peroxisomal membranes: characterization of redox proteins involved. Biochem Soc Trans **24:** 195S

Luster DG, Bowditch MI, Eldridge KM, Donaldson RP (1988) Characterization of membrane-bound electron transport enzymes from castor bean glyoxysomes and endoplasmic reticulum. Arch Biochem Biophys **265:** 50–61

Luster DG, Donaldson RP (1987) Orientation of electron transport activities in the membrane of intact glyoxysomes isolated from castor bean endosperm. Plant Physiol **85:** 796–800

McCord JM, Fridovich I (1969) Superoxide dismutase: an enzymic function for erythrocuprein. J Inorg Biochem **244:** 6049–6055

Mittler R, Zilinskas BA (1991) Purification and characterization of pea cytosolic ascorbate peroxidase. Plant Physiol **97:** 962–968

Mittler R, Zilinskas BA (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate dependent reduction of nitroblue tetrazolium. Anal Biochem **212:** 540–546

Miyake C, Asada K (1992) Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. Plant Cell Physiol **33:** 541–553

Morán JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV, Aparicio-Tejo P (1994) Drought induces oxidative stress in pea plants. Planta **194:** 346–352

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol **22:** 867–880

Njus D, Kelley M (1993) The secretory-vesicle ascorbate-regenerating system: a chain of concerted H^+/e^--transfer reactions. Biochim Biophys Acta **1144:** 235–248

Palma JM, Gómez M, Yáñez J, del Río LA (1987) Increased levels of peroxisomal active oxygen-related enzymes in copper-tolerant pea plants. Plant Physiol **85:** 570–574

Pastori GM, del Río LA (1994) An activated-oxygen-mediated role for peroxisomes in the mechanism of senescence of *Pisum sativum* L. leaves. Planta **193:** 385–391

Pastori GM, del Río LA (1997) Natural senescence of pea leaves: an activated oxygen-mediated function for peroxisomes. Plant Physiol **113:** 411–418

Prasad TK, Anderson MD, Steward CR (1995) Localization and characterization of peroxidases in the mitochondria of chilling-acclimated maize seedlings. Plant Physiol **108:** 1597–1605

Rautenkranz AAF, Li L, Mächler F, Märtinoina E, Oertli JJ (1994) Transport of ascorbic and dehydroascorbic acids across protoplasts and vacuole membranes isolated from barley (*Hordeun vulgare* L. cv Gerbel) leaves. Plant Physiol **106:** 187–193

Sandalio LM, del Río LA (1988) Intraorganellar distribution of superoxide dismutase in plant peroxisomes (glyoxysomes and leaf peroxisomes). Plant Physiol **88:** 1215–1218

Scandalios JG, Tong W-F, Roupakias DG (1980) Cat 3, a third gene locus coding for a tissue-specific catalase in maize: genetics, intracellular location and some biochemical properties. Mol Gen Genet **179:** 33–41

Shigeoka S, Nakano Y, Kitaoka S (1980) Metabolism of hydrogenperoxide in *Euglena gracilis* by L-ascorbic acid peroxidase. Biochem J **186:** 377–380

Streller S, Kromer S, Winsgle G (1994) Isolation and purification of mitochondrial Mn-superoxide dismutase from the gymnosperm *Pinus sylvestris* L. Plant Cell Physiol **35:** 859–867

Struglics A, Fredlund KM, Rasmusson AG, Moller IM (1993) The presence of a short redox chain in the membrane of intact potato tuber peroxisomes and the association of malate dehydrogenase with the peroxisomal membrane. Physiol Plant **88:** 19–28

Thomas TC, Mcnamee MG (1990) Purification of membrane proteins. Methods Enzymol **182:** 499–520

Thompson JE, Ledge RL, Barber RF (1987) The role of free radicals in senescence and wounding. New Phytol **105:** 317–344

Washko PW, Wang Y, Levine M (1993) Ascorbic acid recycling in human neutrophils. J Biol Chem **268:** 15531–15535

Yamaguchi K, Mori H, Nishimura M (1995) A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. Plant Cell Physiol **36:** 1157–1162