Mitochondrial and peroxisomal manganese superoxide dismutase: differential expression during leaf senescence

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Abstract

In pea (Pisum sativum L.) leaves, manganese superoxide dismutase (Mn-SOD) is mainly localized in mitochondria as well as in peroxisomes. In this work, the effect of leaf senescence on the peroxisomal and mitochondrial Mn-SOD was studied in detached leaves from pea plants which were incubated in the dark at 25 °C for 3–11 d. Northern blots hybridized with a cDNA encoding mitochondrial Mn-SOD from pea leaves and a mitochondrial Mn-SOD transit peptide-specific probe showed increased Mn-SOD transcript levels during leaf senescence, due in part to increased mitochondrial Mn-SOD mRNA. Recombinantly-expressed mitochondrial Mn-SOD was used to raise polyclonal antibodies which cross-reacted with Mn-SOD in peroxisomes purified from pea leaves. Western blot assays of crude extracts with the antibodies to pea mitochondrial Mn-SOD showed that the levels of total Mn-SOD protein gradually increased with leaf senescence. By native PAGE, the total Mn-SOD activity of pea leaves increased with senescence. EM immunocytochemistry was used to distinguish mitochondrial and peroxisomal Mn-SOD in senescent leaves. Increased Mn-SOD labelling in mitochondria was intensified, whereas the immunogold labelling of peroxisomes did not change with senescence. Overall, these results show that mitochondrial and peroxisomal Mn-SOD expression is regulated differently. The expression of mitochondrial Mn-SOD is induced during the senescence of pea leaves, whereas peroxisomal Mn-SOD could be post-translationally activated. Previously described results showing decreased mitochondrial Mn-SOD activity and increased peroxisomal Mn-SOD activity may be reflective of post-translational events regulating enzymatic activity during leaf senescence.

Key words: Differential expression, immunocytochemical localization, manganese superoxide dismutase, mitochondria, peroxisomes, Pisum sativum L., senescence, superoxide dismutase.

Introduction

Superoxide dismutases (SODs; EC 1.15.1.1) are a family of metalloenzymes that catalyse the disproportionation of superoxide (O₂⁻) radicals, and they play an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular loci (Fridovich, 1995; Halliwell and Gutteridge, 2000).

Manganese-containing superoxide dismutases have been characterized from a wide range of organisms including bacteria, algae, fungi, and animals (Bannister et al., 1987; Steinman, 1982), and also from several higher plants (Sevilla et al., 1980a, b, 1982; Fernández et al., 1982; Baum and Scandalios, 1981; Streller et al., 1994; Kröniger et al., 1995). In eukaryotic cells, Mn-SODs have been found to be localized mainly in mitochondria from different organisms (Fridovich, 1995; Halliwell and Gutteridge, 2000) with the exception of human and baboon liver where this enzyme is also present in the cytosol in considerable amounts (Steinman, 1982). In higher plants,
Mn-SODs are chiefly present in mitochondria (Steinman, 1982; Palma et al., 1986; del Río et al., 1992; Halliwell and Gutteridge, 2000), but also occur in different types of peroxisomes (del Río et al., 1992, 1998, 2002; Corpas et al., 1998). Using immunocytochemical and density-gradient centrifugation methods, a Mn-containing SOD was localized in peroxisomes from pea leaves (del Río et al., 1983; Sandalio et al., 1987).

Senescence of plant tissues is characterized by the loss of chlorophyll and protein, increased lipid peroxidation and membrane permeability, and enhanced metabolism of reactive oxygen that produces severe cellular damage (Kar and Feierabend, 1984; Thompson et al., 1987; Halliwell and Gutteridge, 2000). Experiments on detached pea (Pisum sativum L.) leaves induced to senesce in the dark, have shown that peroxisomes and mitochondria have a reactive oxygen-mediated function in the mechanism of leaf senescence (Pastori and del Río, 1994a, b, 1997; Jiménez et al., 1998). Ultrastructural studies of intact pea leaves showed that the population of peroxisomes and mitochondria increased significantly with senescence compared with young leaves (Pastori and del Río, 1994a). Peroxisomes isolated from dark-induced senescent pea leaves exhibited increased activity of O$_2^-$-producing xanthine oxidase, elevated H$_2$O$_2$ concentration and decreased catalase activity (Pastori and del Río, 1994a, b, 1997). The results obtained supported the idea that leaf senescence was associated with the reverse metabolic transition of leaf peroxisomes to glyoxysomes, with the channelling of acetyl-CoA through the glyoxylate cycle (del Río et al., 1998; McCarthy et al., 2001). Moreover, the specific activity of peroxisomal Mn-SOD was substantially increased by senescence (Pastori and del Río, 1994a, b, 1997). By contrast, mitochondria isolated from dark-induced senescent pea leaves also exhibited elevated H$_2$O$_2$ concentration, but significantly reduced Mn-SOD specific activity (Jiménez et al., 1998).

Mn-SODs efficiently remove superoxide radicals, but produce H$_2$O$_2$ as a by-product of their catalytic reaction (Fridovich, 1995). High concentrations of H$_2$O$_2$ are dangerous to the plant cell, but at low concentrations this metabolite also acts as a diffusible signalling molecule in signal transduction pathways that lead to the activation of gene expression (del Río et al., 1996, 2002; Bolwell, 1999; Van camp et al., 1998; Grant and Loake, 2000). Peroxisomal and mitochondrial Mn-SODs are responsible for a substantial part of the H$_2$O$_2$ produced in the respective cell organelles, and excess H$_2$O$_2$ may potentially diffuse into the cytosol. Although there is substantial information concerning the enzymatic activity of peroxisomal and mitochondrial Mn-SODs during leaf senescence, the molecular characterization is less defined for Mn-SODs of these cell organelles during the senescence process.

In this work, molecular techniques such as western and northern blot, and EM immunocytochemical analyses are employed to get deeper insights into the expression of peroxisomal and mitochondrial Mn-SOD during dark-induced senescence. Overall, these results suggest that mitochondrial and peroxisomal Mn-SOD expression is regulated differently.

**Materials and methods**

**Plant material**

Pea seedlings (Pisum sativum L., cv. Lincoln) were grown in vermiculite in a growth chamber under optimum conditions for 20 d, as previously described by del Río et al. (1985).

**Induction of senescence**

Senescence was induced by floating excised leaves from 20-d-old pea plants in ultrapure water in constant darkness at 25 °C for 0, 2, 5, 8, and 11 d (Pastori and del Río, 1994a). After the different incubation times, the leaves were washed with ultrapure water under dim light, weighed, frozen in liquid N$_2$, and stored at −70 °C.

**Generation of a mitochondrial Mn-SOD transit peptide-specific probe**

A mitochondrial Mn-SOD transit peptide-specific DNA probe of about 155 bp was generated by PCR (Friedman et al., 1990) using a λgt11 cDNA library constructed from mRNA from 10-d-old pea leaf tissue (Gantt and Key, 1986). Two oligonucleotides were synthesized corresponding to the amino-terminal 5'-GGGAATTCTAGAGTTTTTCAGAATCT-3' (primer A) - and carboxyl-terminal 5'-CCCTCTAGAACCGGGATTTGAAGTTGA-3' (primer B) - regions of the transit peptide of mitochondrial Mn-SOD of pea leaves (Wong-Vega et al., 1991). The PCR products were purified from an agarose gel using glassmilk (GeneClean, BIO 101, Inc.).

**Cloning of a pea mitochondrial Mn-SOD cDNA**

A cDNA encoding mitochondrial Mn-SOD of pea of 972 bp was generated by PCR (Friedman et al., 1990). Degenerate oligonucleotides were synthesized based on amino acid sequences of plant Mn-SODs and used as primers for PCR amplification. The template used was RNA from roots of 4-d-old pea plants (Altemare, 1992). A fragment of ~180 bp was amplified which had considerable identity with the amino acid sequences corresponding to maize and tobacco Mn-SODs (White and Scandalias, 1988; Bowler et al., 1992). The fragment was radiolabelled and used to screen a λgt11 cDNA library derived from mRNA from 10-d-old pea leaf tissue (Gantt and Key, 1986). A cDNA insert of 972 bp was purified and found to contain the entire coding sequence of the mature Mn-SOD protein, starting at nucleotide position 84 and ending at position 696 (Altemare, 1992). The amino acid sequence predicted from that of the cDNA encoding pea Mn-SOD was about 80% identical to the amino acid sequences of several plant Mn-SODs (Altemare, 1992). The nucleotide and deduced amino acid sequences of the 972 bp cDNA was nearly identical to another cDNA encoding mitochondrial Mn-SOD reported from pea leaf (Wong-Vega et al., 1991).

**Preparation of polyclonal antibodies to mitochondrial Mn-SOD**

The mitochondrial Mn-SOD cDNA was subcloned into the expression vector pMAL-c2 and used for the preparation of a maltose binding protein (MBP)-Mn-SOD fusion according to the protocol of New England Biolabs, Inc. (Altemare, 1992). The open reading frame encoding the pea mature mitochondrial Mn-SOD was fused to
the 3’ end of the E. coli MBP coding sequence to facilitate purification of pea Mn-SOD. The fusion protein was digested for 22 h at room temperature with a protease (factor Xa) that recognizes the amino acid sequence at the junction of the MBP-mitochondrial Mn-SOD fusion, releasing the mature Mn-SOD. The Mn-SOD was separated from the MBP by hydroxyapatite chromatography and affinity amylose chromatography, and finally the enzyme was concentrated by ultrafiltration on a PM-10 Amicon membrane. Polyclonal antibodies were raised in rabbits by four subcutaneous injections of 100 µg pure mitochondrial Mn-SOD/rabbit, at 14 d intervals. The first injection contained 100 µg of enzyme in PBS (phosphate buffered saline, 150 mM NaCl, 10 mM phosphate buffer, pH 7.4) and complete Freund’s adjuvant (1:1), and the other three booster injections contained 100 µg of enzyme in PBS and incomplete Freund’s adjuvant (1:1). The serum was obtained and polyclonal antibodies to mitochondrial Mn-SOD were affinity purified on nitrocellulose with bound mitochondrial Mn-SOD, and the specific antibody was eluted at pH 2.3. Protein concentration was estimated by the method of Bradford (Bradford, 1976) with BSA as standard.

Preparation of leaf extracts

All operations were carried out at 0–4 °C. Leaves were ground in ice-cold buffer (0.1 M sodium phosphate, pH 7.8, 0.1 mM EDTA) (1:5, w/v) with mortar and pestle. Homogenates were filtered through two layers of Miracloth (Calbiochem) and centrifuged at 30,000 g for 30 min. Aliquots of the supernatants were frozen in liquid N2 and stored at −70 °C.

Purification of peroxisomes

All operations were performed at 0–4 °C. Peroxisomes were purified from young and senescent pea leaves by differential and sucrose density-gradient centrifugation (35–60%, w/w), as described by López-Huertas et al. (1995). Peroxisomes purified by this method were essentially free of contamination by other cellular organelles (López-Huertas et al., 1995; Distefano et al., 1997). Peroxosomal soluble fractions were obtained by hypotonic shock and ultracentrifugation (López-Huertas et al., 1995).

Native PAGE, SDS-PAGE and immunoblot assays

SOD isoenzymes in pea leaf extracts were separated by PAGE in 15% polyacrylamide resolving gels, as described by Laemmli (1970). SOD activity bands were detected in gels by the photochemical NBT stain of Beauchamp and Fridovich (1971). The homogeneity of the affinity-purified mitochondrial Mn-SOD fusion protein was checked by SDS-PAGE on 12% and 15% polyacrylamide gels after heating the protein at 100 °C for 5 min in the presence of 2% (p/v) SDS and 5% 2-mercaptoethanol (v/v) (Laemmli, 1970). Gels were stained for proteins with Coomassie blue R-250 (Sigma). For immunoblotting, after SDS-PAGE on 15% polyacrylamide gels polypeptides were electroblotted to 0.1 µm nitrocellulose membranes from Schleicher & Schuell (Keene, NH) using a Trans-Blot cell from Bio-Rad. The blot was incubated overnight with affinity-purified antibodies to pea mitochondrial Mn-SOD, and then with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega), and was developed either with NBT and BCIP as substrates or by a chemiluminescence method (Bunkelmann and Trelease, 1996). The intensity of the immunoreactive bands in the NBT-BCIP reaction was calculated by scanning the nitrocellulose membranes at 560 nm on a Shimadzu CS-9000 densitometer.

Northern blot analysis

RNA was isolated from young and senescent leaves essentially as described by Chomczynski and Sacchi (1987) and quantitated spectrophotometrically. Twenty-five to 30 µg of total RNA was subjected to electrophoresis on a 1.3% agarose gel containing 2.2 M formaldehyde, transferred to Duralon-UV membranes (Stratagene) and hybridized with a 972 bp, nearly full-length 32P-labelled cDNA encoding mitochondrial Mn-SOD (Altomare, 1992) or the 155 bp PCR product which encodes the transit peptide of mitochondrial Mn-SOD. The filters were hybridized and washed at high stringency and then exposed to X-ray film. The same blot was subsequently hybridized to a pea 18S rRNA PCR-derived DNA probe (Mittler and Zilinskas, 1994) as an internal control to assess RNA loading. Hybridization signals were analysed using an Ultrascan XL laser densitometer (Pharmacia Biotech Inc.).

Electron microscopy and immunolabelling

Segments of young and senescent pea leaves (1 mm thick) were processed as described by Corpas et al. (1994). Pieces were fixed in 0.2% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 50 mM PIPES-KOH buffer (pH 7.4), for 1 h at 4 °C. The segments were dehydrated through a graded ethanol series (30–100%, v/v) and infiltrated in LR White resin. The embedded segments were polymerized at −20 °C. Sections were immunolabelled with affinity-purified anti-mitochondrial Mn-SOD and goat anti-rabbit IgG conjugated to 15 nm gold particles (Bio Cell) as the secondary antibody, according to Sandalio et al. (1997). Peroxisomes were also identified by double-labelling with antibodies against a peroxisomal marker enzyme, spinach glycolate oxidase (Nishimura et al., 1983) and, in this case, goat anti-rabbit IgG conjugated to 5 nm gold particles was used as secondary antibody. Preimmune serum was used as a control. Sections were poststained in 2% (w/v) aqueous uranyl acetate for 3 min and examined in a Zeiss EM 10C electron microscope. In mitochondria and peroxisomes from pea leaves incubated with anti-mitochondrial Mn-SOD the immunogold-labelled particles were counted. To obtain the mean number ± SEM of labelled organelles, 40 sectioned cells from each young and 8 d senescent leaves chosen at random were visualized. Differences from control values (young leaves) were significant at P <0.001.

Results

Pattern of SOD activity of pea leaves during senescence

Pea leaves mainly contain three electrophoretically distinct SODs, a Mn-containing SOD and two CuZn-containing SODs (I and II) (del Río et al., 1978). By subcellular distribution studies of SODs in pea leaves, the presence of Mn-SOD has been demonstrated in mitochondria and peroxisomes (del Río et al., 1983; Sandalio et al., 1987), whereas CuZn-SOD I is mainly localized in the cytosol (Duke and Salin, 1983), and CuZn-SOD II in the chloroplasts (Duke and Salin, 1983; Palma et al., 1986). The pattern of SOD isoenzymes of pea leaves during senescence is shown in Fig. 1 which is representative of the different native-PAGE runs carried out. In general, senescence produced an increase in the activity of all the isoenzymes. Densitometric analysis of different electrophoretic runs showed that the increase in activity after 11 d compared to the controls was maximum (77%) for the cytosolic CuZn-SOD I, followed by Mn-SOD (51%) and the chloroplastic CuZn-SOD II (20%).
Puriﬁcation of pea mitochondrial Mn-SOD by protein fusion

An SDS-polyacrylamide gel electrophoretogram of the puriﬁcation steps of the mitochondrial Mn-SOD from the MBP-Mn-SOD fusion protein is shown in Fig. 2. The puriﬁed mitochondrial Mn-SOD obtained had a subunit molecular mass of 27 kDa and was homogeneous by SDS-PAGE.

Western blot of total Mn-SOD of pea leaves during senescence

A polyclonal antibody was raised in rabbits against the puriﬁed pea mitochondrial Mn-SOD. By western blot assays the antibody was monospeciﬁc against the Mn-SOD of pea leaf extracts. This antibody was used to identify, by western blot, the Mn-SOD protein during dark-induced senescence of pea leaves (Fig. 3). This blot is representative of the different experiments carried out, and the results obtained showed that the levels of Mn-SOD protein gradually increased with the senescence of pea leaves, as checked by densitometric scanning of blots at the absorption wavelength of the NBT reduction product (560 nm).

Cross-reactivity of peroxisomes from pea leaves with anti-mitochondrial Mn-SOD

The cross-reactivity of the antibody raised against pea mitochondrial Mn-SOD with the peroxisomal Mn-SOD was studied in peroxisomes puriﬁed from young and senescent pea leaves by sucrose density-gradient centrifugation (Fig. 4). The antibody to pea mitochondrial Mn-SOD clearly recognized the Mn-SOD present in peroxisomes, and the intensity of the cross-reaction was similar in young and senescent leaves.

Analysis of pea leaf mitochondrial Mn-SOD transcript levels during leaf senescence

Mn-SOD expression was examined by Northern hybridization analysis using the 972 bp, near full-length cDNA
encoding mitochondrial Mn-SOD to probe RNA isolated from young and 3, 5, 8, and 11 d senescent pea leaves. After normalization of the northern blot data with respect to the 18S rRNA signal, it was found that the level of the pea Mn-SOD transcript increased with senescence and was maximum at 11 d (Fig. 5). In an attempt to distinguish transcripts that encode the mitochondrial Mn-SOD from those that encode the peroxisomal Mn-SOD, a PCR product corresponding to a sequence encoding only the mitochondrial Mn-SOD transit peptide was prepared. As the peroxisomal Mn-SOD lacks a mitochondrial targeting sequence, blots probed with the cDNA encoding the transit peptide were compared with those encoding the entire 972 bp cDNA to determine if any differences might be attributable to mitochondrial Mn-SOD transcript levels. The transcripts that hybridized under high stringency conditions with the PCR product corresponding to the transit peptide of pea mitochondrial Mn-SOD increased during the progression of senescence (Fig. 6) in much the same way as observed in RNA blots probed with the nearly full-length cDNA. This suggests that in pea leaves the mitochondrial Mn-SOD is induced in response to the senescence of the tissue.

**Immunocytochemical localization of Mn-SOD in young and senescent pea leaves**

To study the effect of leaf senescence on the Mn-SOD protein in mitochondria and peroxisomes, sections of young and senescent pea leaves were labelled with affinity-purified anti-mitochondrial Mn-SOD. On immunolabelled sections of young pea leaves, gold label was mainly localized within mitochondria, with 9.2±1.0 gold particles per organelle and, to a lesser extent, in peroxisomes where the mean labelling was 2.6±0.4 gold particles per organelle (Fig. 7). In senescent leaves, a significant increase (53%) of immunogold Mn-SOD labelling in mitochondria, compared to young leaves, was observed (14.1±1.5) whereas the labelling of peroxisomes did not change during senescence (2.3±0.2) (Fig. 7). In sections probed with preimmune serum no label was found.

**Discussion**

In this work, the effect of leaf senescence on peroxisomal and mitochondrial Mn-SOD was studied in excised leaves.
incubated in the dark, which is a good system for the rapid development of typical symptoms of senescence (Pastori and del Río, 1994a). Later works demonstrated that natural senescence causes the same changes in peroxisome-reactive oxygen metabolism as dark-induced senescence and, therefore, are characteristic of the physiological process of senescence (Pastori and del Río, 1997). Senescence brings about important alterations in the reactive oxygen metabolism of peroxisomes which are mainly characterized by the disappearance of catalase activity and an overproduction of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) (del Río et al., 1998). This accumulation of ROS can only be partly counteracted by the peroxisomal ascorbate–glutathione cycle, since this is also negatively affected by senescence. In mitochondria, senescence also produces a depression of the antioxidant defences, resulting in enhanced \( \text{H}_2\text{O}_2 \) production and membrane leakage (Jiménez et al., 1998).

The manganese superoxide dismutase from pea leaves was previously purified to homogeneity and characterized (Sevilla et al., 1980a, b, 1982; Fernández et al., 1982). In pea leaves, Mn-SOD is mainly localized in mitochondria and, to a lesser extent, in peroxisomes (del Río et al., 1983; Scandalios et al., 1987). It was thought that the mitochondrial and peroxisomal Mn-SODs must have extremely similar molecular properties, since, in their purification from pea leaves, they co-purified together and could not be differentiated either by native-PAGE or by SDS-PAGE (Sevilla et al., 1980a, b, 1982). More recently, peroxisomal Mn-SOD was purified to homogeneity from peroxisomes isolated from pea leaves. A 100% identity was found, over a 29 amino acid overlap, between the N terminus of the peroxisomal enzyme and the mitochondrial Mn-SOD, and high identity was exhibited with Mn-SODs from other plant species (Palma et al., 1998). Moreover, in this work it is shown that peroxisomal Mn-SOD is recognized by antibodies raised against mitochondrial Mn-SOD.

During leaf senescence a gradual increase in the activity of the three SODs of pea leaves was observed, although this increase was most significant in the case of cytosolic CuZn-SOD I (Fig. 1). In previous experiments conducted with isolated organelles from senescent pea leaves, a notable increase in the specific activity of peroxisomal Mn-SOD and a decay in the activity of mitochondrial Mn-SOD was determined (Pastori and del Río, 1994a, b, 1997; Jiménez et al., 1998). Based on these findings, it was hypothesized that the increase observed by native-PAGE in the activity of Mn-SOD from pea leaf extracts (Fig. 1) was probably attributed to the peroxisomal enzyme.

In an effort to distinguish molecularly between the mitochondrial and peroxisomal Mn-SODs, a cDNA corresponding to the mature form of mitochondrial Mn-SOD was used to examine the expression of the Mn-SODs using northern blot analysis of RNA from senescent pea leaves. It was expected that this cDNA would cross-react with transcripts corresponding to mitochondrial and peroxisomal Mn-SODs. However, these experiments showed a single transcript that increased during leaf senescence, and it was unclear if the cDNA probe was useful in differentiating between the expression of the mitochondrial Mn-SOD and peroxisomal Mn-SOD mRNAs due to similar mobility of the transcripts on agarose gel electrophoresis.

However, another cDNA corresponding to the transit peptide of pea leaf mitochondrial Mn-SOD also detected a transcript that increased during senescence. The specificity of this second cDNA probe indicates that the increased transcript levels were probably due to the induction of the mitochondrial Mn-SOD. The majority of the peroxisomal proteins, unlike mitochondrial proteins, do not have cleavable N-terminal presequences (transit peptides) (López-Huertas and Baker, 1999). The results with the transit peptide cDNA were similar to those obtained with the nearly full-length cDNA encoding the mitochondrial Mn-SOD and indicate that the mitochondrial Mn-SOD is induced during leaf senescence.

Increased mitochondrial Mn-SOD expression was also detected using EM immunocytochemical labelling of mitochondria with the antibody to mitochondrial Mn-SOD. These results agree with previous reports showing high levels of mitochondrial Mn-SOD mRNA in plant tissues with elevated respiratory activity (Zhu and Scandalios, 1993; Van Camp et al., 1996), which is one of the characteristic features of the dark-induced senescent pea leaves used in this work (Pastori and del Río, 1994a). This is in contrast to work conducted in mature-senescent...
detached barley leaves, where a decrease in the SOD transcript level and activity of the mitochondrial Mn-SOD was reported (Casano et al., 1994). This negative response could be due to the short dark-incubation times used by these authors (20 h) compared to those described in this work (up to 11 d).

In previous experiments carried out with mitochondria isolated from senescent pea leaves, subjected to the same experimental conditions described in this work, a large decrease of the mitochondrial Mn-SOD specific activity of about 5-fold, was determined (Jiménez et al., 1998). However, results found in this work show the existence of a negative correlation between the mitochondrial Mn-SOD transcript level and the Mn-SOD protein activity. This could be due to a senescence-induced inactivation of mitochondrial Mn-SOD, probably as a result of oxidative protein modification and/or proteolytic degradation, although a possible regulation of the mitochondrial Mn-SOD gene on transcriptional and translational levels cannot be discarded (Mittler and Zilinskas, 1994).

**Fig. 7.** Immunocytochemical localization of Mn-SOD in young and senescent pea leaves. Pea leaves were induced to senesce by dark-incubation for 8 d, and were labelled with affinity-purified anti-mitochondrial Mn-SOD from pea leaves. 15 nm gold particles represent the Mn-SOD labelling and 5 nm gold particles represent the glycolate oxidase labelling (peroxisomal marker). The immunogold labelling of Mn-SOD in peroxisomes is indicated by arrows. M, mitochondria; P, peroxisomes; C, chloroplasts; CW, cell wall. Bars: 0.5 μm: (A, B) young leaves, (C, D) senescent leaves.
During the purification of Mn-SOD from pea leaves the enzyme was found to be sensitive to oxidation (Sevilla et al., 1980a, 1982; Palma et al., 1998), and in pea plants under Cd-induced oxidative stress conditions the Mn-SOD protein was oxidatively modified and this was probably responsible for the reduction in its activity (Romero-Puertas et al., 2002). A similar non-correlated pattern has been reported in Scots pine needles for the chloroplastic and cytosolic CuZn-SOD transcript levels and activities in response to air pollutants (Karpinski et al., 1992).

It has been extensively reported in the literature that the response of SOD activity and other antioxidative enzymes to oxidative stress varies according to the environmental conditions, plant tissue, stage of development, etc (del Río et al., 1991; Bowler et al., 1992; Dat et al., 2000; Alscher et al., 2002). Recent work on Cd-induced oxidative stress has confirmed such a statement (Sandalio et al., 2001; McCarthy et al., 2001; Romero-Puertas et al., 2002; Schützendübel et al., 2002).

An intriguing question remains concerning the expression of peroxisomal Mn-SOD. While information is available on the expression of mitochondrial Mn-SOD in several plant species (Zhu and Scandalias, 1993; Van Camp et al., 1996), there are no reports on the expression of the peroxisomal enzyme in plants. Recently, the gene encoding Mn-SOD was cloned in a fungus and this protein could be a peroxisomal Mn-SOD since it was found to contain a C-terminal peroxisomal localization (PTS1) and lack an N-terminal mitochondrial transit peptide (Jacob et al., 2001). More information is available on another type of peroxisomal SOD, a CuZn-containing form, which is present in the matrix of oilseed peroxisomes (glyoxysomes) (del Río et al., 1992; Sandalio et al., 1997; Corpas et al., 1998). The peroxisomal CuZn-SOD from watermelon cotyledons has been characterized and its amino acid sequence determined by Edman degradation (Bueno et al., 1995). Comparison of its sequence with those reported for other plant SODs revealed homologies of about 90% with chloroplastic CuZn-SODs (Bueno et al., 1995). This suggested that chloroplast and peroxisomal CuZn-SODs could be encoded either by the same or closely related genes.

A similar situation could exist for mitochondrial and peroxisomal Mn-SODs, with both enzymes being encoded by the same gene. In the last decade, several proteins distributed in distinct cell loci have been reported to be produced from a single gene by alternative splicing (Harris et al., 1994; Srinivasan et al., 1994). In pumpkin, hydroxypropiuivurate reductase localizes to leaf peroxisomes and to the cytoplasm, and the two forms have been shown to be generated by light-controlled alternative splicing (Mano et al., 1999). The chloroplast stromal and thylakoid-bound ascorbate peroxidase of pumpkin has been shown to be produced by alternative splicing of the one gene (Mano et al., 1997). This has also been reported for the expression of the P gene of maize and some transcription units in plant DNA viruses (Edwards et al., 1994). In the case of the Mn-SOD from pea leaves, the differentially processed products of a unique gene could be targeted bidirectionally to mitochondria and peroxisomes. This could explain the extremely close molecular properties of the Mn-SODs from both oxidative organelles (Sevilla et al., 1980a, b.; Palma et al., 1998) and why the isolation of a cDNA from peroxisomal Mn-SOD has been so elusive thus far.

The immunolocalization of Mn-SOD in peroxisomes from pea leaves, described in this work, was previously reported in protoplasts isolated from pea leaves using an antibody against native Mn-SOD purified from pea leaves and the unlabelled antibody peroxidase-antiperoxidase (PAP) method (del Río et al., 1983). However, in this case IEM results did not detect any appreciable staining in mitochondria, the well-known cellular site for Mn-SOD in many eukaryotic organisms (Steinman, 1982; Palma et al., 1986; Fridovich, 1995; del Río et al., 1992; Halliwell and Gutteridge, 2000). This was probably due to a failure to permeabilize adequately the mitochondrial double-membrane by saponin in the IEM procedure used with pea leaf protoplasts (del Río et al., 1983), and this would have hindered the antibodies from getting into the mitochondria.

Our experiments showed that while the antibody to pea mitochondrial Mn-SOD clearly labelled mitochondria, the recognition of peroxisomal Mn-SOD was weaker. The low immunogold labelling detected in peroxisomes could be due to the lower concentration of Mn-SOD in peroxisomes compared to mitochondria (Sandalio et al., 1987), and the immunocytochemical reaction intensity could also be diminished by the low-avidity nature of the antibodies obtained by affinity chromatography (Larsson, 1988). Another possible explanation for the low immunogold labelling of peroxisomal Mn-SOD could be that the compartmentation of the enzyme within peroxisomes is such that the antisem-recognized epitopes were poorly accessible in EM sections. This could equally explain the low gold labelling also detected in mitochondria using this antibody. Given the inducible nature and remarkable metabolic plasticity of peroxisomes (Mullen and Trelease, 1996; del Río et al., 1992, 2002), a way to improve the sensitivity of the immunocytochemical assay could be to induce, by physiological means, the peroxisomal content of Mn-SOD.

In peroxisomes purified from senescent pea leaves, an increase in the specific activity of Mn-SOD of about 4-fold was demonstrated (Pastori and del Río, 1994a, b, 1997). The peroxisomal Mn-SOD was recognized by the antibody to mitochondrial Mn-SOD and the level of peroxisomal Mn-SOD protein was not affected by leaf senescence (Fig. 4). This latter result was also supported by the counting of immunogold particles in peroxisomes from senescent pea leaves. Therefore, the increase in the activity
of peroxisomal Mn-SOD during the senescence of pea leaves could be due to post-translational activation of the enzyme. In plant peroxisomes, the presence of exo- and endo-proteolytic activity was reported for the first time in pea leaves, and an exo-peptidase and different endo-peptidases have been characterized (Corpas et al., 1993; Distefano et al., 1997, 1999). There are data that suggest the involvement of peroxisomal endoproteases in a regulated modification of proteins in peroxisomes (Distefano et al., 1999) and these proteases perhaps could have a role in the post-translational activation of Mn-SOD.

In addition to H2O2, peroxisomes can also generate O2·− radicals and nitric oxide (del Río et al., 2002), and recently a role has been proposed for peroxisomes as a source of these messenger molecules which are involved in signal transduction pathways leading to specific gene expression (Corpas et al., 2001). In peroxisomes from senescent leaves there is a large increase of the H2O2 content and a severe depression of catalase activity (del Río et al., 1998). The senescence-induced H2O2 leaking from peroxisomes might act in the cytosol as a second messenger in signal transduction pathways. In this way, the senescence-induced post-translational activation of H2O2-producing Mn-SOD, could have a significant contribution in peroxisomes as an additional source of H2O2.

Results presented in this work illustrate the differential expression of mitochondrial and peroxisomal Mn-SOD during leaf senescence. Mitochondria, like peroxisomes, have also been proposed to play a role in the oxidative mechanism of senescence in pea leaves by favouring the leakage into the cytosol of O2·− and H2O2 (Jiménez et al., 1998). However, there are some differences between both organelles during senescence with respect to their antioxidant systems, particularly the ascorbate–glutathione cycle (Noctor and Foyer, 1998). In mitochondria, senescence produces a depression of this cycle, with the activities of DHAR, GR, APX, and MDHAR all being substantially decreased (Jiménez et al., 1998). The mitochondrial ascorbate and dehydroascorbate pools are reduced, whereas the oxidized glutathione levels are maintained. However, in peroxisomes from senescent leaves this negative effect is not so intense and only the APX and MDHAR activities are decreased, and the reduced and the oxidized glutathione pools are considerably enhanced (Jiménez et al., 1998). The differential response to senescence of the mitochondrial and peroxisomal ascorbate–glutathione cycle suggests that mitochondria could be affected by H2O2-derived oxidative damage earlier than peroxisomes. By contrast, these organelles are able to respond to ROS production with the increased synthesis of some antioxidant systems that could partly counteract the accumulation of ROS.

Further research is necessary to clone the cDNA encoding the peroxisomal Mn-SOD and to study the regulation of the enzyme’s expression during leaf senescence and under different plant stress conditions. Knowledge of the full amino acid sequence of peroxisomal Mn-SOD can supply very valuable information on the possible presence of topogenic signals (PTS) in this enzyme molecule responsible for its targeting to peroxisomes, as well as on the mechanism of import of Mn-SOD into these organelles.

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