Peroxisomal Copper, Zinc Superoxide Dismutase

Characterization of the Isoenzyme from Watermelon Cotyledons

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The biochemical and immunochemical characterization of a superoxide dismutase (SOD, EC 1.15.1.1) from peroxisomal origin has been carried out. The enzyme is a Cu,Zn-containing SOD (CuZn-SOD) located in the matrix of peroxisomes from watermelon (Citrullus vulgaris Schrad.) cotyledons (L.M. Sandalio and L.A. del Río [1988] Plant Physiol 88: 1215–1218). The amino acid composition of the enzyme was determined. Analysis by reversed-phase high-performance liquid chromatography of the peroxisomal CuZn-SOD incubated with 6 M guanidine-HCl indicated that this enzyme contained a noncovalently bound chromophore group that was responsible for the absorbance peak of the native enzyme at 260 nm. The amino acid sequence of the peroxisomal CuZn-SOD was determined by Edman degradation. Comparison of its sequence with those reported for other plant SODs revealed homologies of 70% with cytosolic CuZn-SODs and of 90% with chloroplastic CuZn-SODs. The peroxisomal SOD has a high thermal stability and resistance to inactivation by hydrogen peroxide. A polyclonal antibody was raised against peroxisomal CuZn-SOD, and by western blotting the antibody cross-reacted with plant CuZn-SODs but did not recognize either plant Mn-SOD or bacterial Fe-SOD. The anti-SOD-immunoglobulin showed a weak cross-reaction with bovine erythrocytes and liver CuZn-SODs, and also with cell-free extracts from trout liver. The possible function of this CuZn-SOD in the oxidative metabolism of peroxisomes is discussed.

SODs (EC 1.15.1.1) are a family of metalloenzymes that catalyze the disproportionation of superoxide (O$_2^-$) radicals, and they play an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular loci (Fridovich, 1986; Halliwell and Gutteridge, 1989). In recent years the presence of different types of SOD has been demonstrated in peroxisomes from several plant species. Peroxisomes are subcellular respiratory organelles that contain catalase and hydrogen peroxide-producing flavin oxidases as basic enzymatic constituents (Tolbert, 1981; Huang et al., 1983). These organelles have an essentially oxidative type of metabolism, and in recent years it has become increasingly clear that peroxisomes carry out essential functions in almost all eukaryotic cells (Fahimi and Sies, 1987; Van den Bosch et al., 1992; Manns and Van Veldhoven, 1993).

By using immunocytochemical and density-gradient centrifugation methods, a Mn-containing SOD was localized for the first time in peroxisomes from pea leaves (del Río et al., 1983; Sandalio et al., 1987). The presence of SOD was also demonstrated in peroxisomes from watermelon (Citrullus vulgaris Schrad.) cotyledons, but in this case two isozymes were detected, a Cu,Zn- and a Mn-containing SOD (Sandalio and del Río, 1987). The peroxisomal CuZn-SOD represented about 18% of the total SOD activity of the plant tissue and was the major SOD isozyme in these organelles (Sandalio and del Río, 1987). The study of the intraorganellar distribution of SOD in plant peroxisomes showed that the CuZn-SOD was present in the matrix of these organelles, whereas the Mn-SOD isozyme was apparently bound to the external side of the peroxisomal membrane (Sandalio and del Río, 1988). The occurrence of SOD isoforms in peroxisomes from cucumber, cotton, and sunflower cotyledons also has recently been reported (Corpas et al., 1994). In recent years, the presence of SOD in plant peroxisomes has been extended to human and animal cells. Human fibroblasts and hepatoma cells were found to contain CuZn-SOD activity (Keller et al., 1991; Crapo et al., 1992), and the occurrence of CuZn-SOD was also demonstrated in rat liver peroxisomes (Dhaunsi et al., 1992; Wandsers and Denis, 1992). The generation of O$_2^-$ radicals, the substrate of SOD, in matrices and membranes of plant peroxisomes has been reported and the generating systems of superoxide have been partially characterized (Sandalio et al., 1988; del Río et al., 1989; López-Huertas et al., 1994). These findings together with other experimental evidence led to the proposal of the existence of new functions for peroxisomes related to activated oxygen species that could

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Abbreviations: CuZn-SOD, copper, zinc-containing superoxide dismutase; SOD, superoxide dismutase.
have important effects on cellular metabolism (del Río et al., 1990, 1992).

Recently, the peroxisomal CuZn-SOD from watermelon cotyledons was purified to homogeneity (Bueno and del Río, 1992). Although there is abundant information on SODs from cytosolic, mitochondrial, and chloroplastic origin (Steinman, 1982; Parker et al., 1984; Bannister et al., 1987, 1991; Halliwell and Gutteridge, 1989; Asada, 1992), until now no SOD has been characterized from any type of peroxisome of a eukaryotic organism. The characterization of the peroxisomal CuZn-SOD is important to study its function in these oxidative cell organelles and also to establish comparative biochemical and evolutionary studies with SODs present in other cellular compartments from different organisms, such as mitochondria, cytosol, chloroplasts, and lysosomes.

To our knowledge, ours is the first report of the biochemical and immunochemical characterization of a SOD from peroxisomal origin, and the molecular properties of this enzyme are compared with those of other SODs distributed in different cellular compartments.

MATERIALS AND METHODS

Plant Material

Seeds of watermelon (Citrullus vulgaris Schrad. cv Sugar Baby) were obtained from Fitó (Barcelona, Spain). Seeds were germinated at 30°C under dark conditions, as described (Bueno and del Río, 1992). From the seedlings obtained, the cotyledons (first leaves) were used for enzyme purification.

Purification of Peroxisomal CuZn-SOD

The enzyme was purified from 10-d-old watermelon cotyledons by the procedure previously described (Bueno and del Río, 1992). A yield of 8 μg enzyme per g cotyledon was obtained, and the peroxisomal CuZn-SOD was homogeneous by native PAGE and SDS-PAGE (Bueno and del Río, 1992).

Amino Acid Composition

The amino acid composition was determined by reversed-phase HPLC in a Waters apparatus (Millipore) using the Pico-Tag method (Cohen et al., 1989). Phenylthio-carbamyl-derivatized amino acids were detected at 254 nm and quantitated using an amino acid standard solution from Pierce.

Denaturation and Reversed-Phase HPLC

The enzyme (21–230 μg) was incubated with 6 M guanidine-HCl for 5 min and loaded onto a Vydac (Separations Group, Hesperia, CA) C-4 reversed-phase column equilibrated with 0.1% TFA, and eluted with a 0 to 100% linear gradient of acetonitrile:H₂O (2:1) in 0.1% TFA over 30 min. Pure CuZn-SODs from bovine liver and erythrocytes and from horseradish (Sigma) were also subjected to the same treatment.

Amino Acid Sequence

The internal sequence was determined by proteolytic digestion of intact protein with Staphylococcus aureus V-8 protease and pancreatic trypsin (Boehinger-Mannheim), as described (Gimenez-Gallego et al., 1985, 1986). Peptides were separated by HPLC on a Vydac C-18 reversed-phase column and were subjected to sequence analysis by automated Edman degradation in an Applied Biosystems pulse-liquid sequenator (model 477A). Phenylthiohydantoin-derivatized amino acids were detected using an online phenylthiohydantoin analyzer from Applied Biosystems (model 120A).

Inhibition by Hydrogen Peroxide and Cyanide

Potassium cyanide and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Potassium cyanide was prepared in 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, and was added to the SOD assay mixture. Hydrogen peroxide was preincubated at 25°C with the protein for 10 to 60 min, and aliquots were taken for assay. SOD activity was determined by its ability to inhibit the superoxide-mediated reduction of ferricytochrome c by the superoxide radicals produced by xanthine/xanthine oxidase (McCord and Fridovich, 1969). SOD control reactions were carried out in the presence of each concentration of inhibitor, and reactions were initiated by adding an appropriate dilution of xanthine oxidase so that the generation of O₂⁻ radicals was constant.

Thermal Stability

The stability of peroxisomal CuZn-SOD was studied at 25, 50, 70, 80, and 100°C. Enzyme solutions of 30 μg mL⁻¹ in 50 mM potassium phosphate buffer (pH 7.8) were incubated at those temperatures, and 10-μL aliquots were withdrawn at different times for the SOD activity determination. The ranges of incubation times were 1 h to 60 min at 25°C, 10 to 120 min at 50°C, 5 to 60 min at 80°C, and 15 min at 100°C.

Preparation of a Polyclonal Antibody against Peroxisomal CuZn-SOD

Two New Zealand rabbits were immunized by four subcutaneous injections of 100 μg pure CuZn-SOD/rabbit, at 14-d intervals. The first injection contained 100 μg of enzyme in PBS 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 rabbits were bled by cardiac puncture 1 week after the last injection. Blood was allowed to clot and then was centrifuged at 750g for 20 min at 4°C. A total volume of serum of 42 mL was obtained. Serum immunoglobulins were isolated by ammonium sulfate precipitation (33% saturation) at pH 7.8, three times, and were dissolved in 19 mL of borate-buffered saline and dialyzed overnight at 4°C against 0.0175 M sodium phosphate buffer (pH 6.3) (buffer A). The antisera solution was purified by anion-exchange chromatography in a Mono Q column HR 10/10 equilibrated with buffer A, using a fast protein liquid chromatography sys-
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Table 1. Amino acid composition of peroxisomal CuZn-SOD

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues/mol of Enzyme</th>
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<tr>
<td>Lys</td>
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<td>His</td>
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<td>Leu</td>
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<td>Tyr</td>
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<td>Phe</td>
<td>10</td>
</tr>
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<td>Trp</td>
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* a Based on a molecular mass of 33,000 D.
* b Determined as cysteic acid after performic acid oxidation.
* c Determined fluorimetrically.

SDS-PAGE and Immunoblotting

For SDS-PAGE, proteins were heated at 100°C for 5 min in the presence of 2% SDS and 5% mercaptoethanol. Electrophoresis was carried out on 12% acrylamide-SDS gels, as described by Laemmli (1970), using a Bio-Rad Mini-Protein II slab cell. For immunoblotting, the proteins from the SDS-PAGE were electrophoretically blotted onto Immobilon nitrocellulose sheets from Millipore using a Bio-Rad Transblot apparatus, and were detected with anti-peroxisomal CuZn-SOD as the primary antibody (1/1000 dilution), goat anti-rabbit IgG with horseradish peroxidase (Bio-Rad) as the secondary antibody, and 4-chloro-1-naphthol as the stain (Bio-Rad Immunoblot instruction manual).

Preparation of Cell-Free Extracts

For antigenic cross-reactivity assays of peroxisomal CuZn-SOD antibody, cell-free extracts from watermelon (C. vulgaris Schrad.) cotyledons, pea (Pisum sativum L.) leaves, spinach (Spinacia oleracea L.) leaves, sorghum (Sorghum vulgare L.) leaves, clover (Trifolium repens L.) leaves, lettuce (Lactuca sativa L.) leaves, erythrina (Erythrina poep-}

pigiana) leaves, and broad bean (Vicia faba L.) leaves, and soluble fraction from soybean (Glycine max L.) nodules and trout (Salmo trutta L.) liver were used. Except for the soluble fraction of soybean nodules, which was prepared as described (Becana et al., 1989), all the other cell-free extracts were prepared by homogenization in 50 mM potassium phosphate buffer (pH 7.8), containing 1 mM DTT, 8% (w/v) polyvinylpolypyrrolidone, 1 mM PMSF, and 0.1% (v/v) Triton X-100 (31, w/v). Homogenates were filtered through six layers of nylon cloth and centrifuged at 34,000g for 30 min. The supernatants obtained were used for the assays.

Other Assays

Protein content was determined either by the method of Lowry et al. (1951) or Murphy and Kies (1960), and BSA (fraction V) was used as a standard.

RESULTS

The amino acid composition of peroxisomal CuZn-SOD purified from watermelon cotyledons is shown in Table I. The most abundant amino acids were Gly, Ala, Glu, Thr, Val, and Asp, and there were low contents of His, Cys, and Met. Only one residue of Tyr and Trp was detected in the enzyme. The UV absorption spectrum of purified peroxisomal CuZn-SOD is characterized by a weak absorption maximum at 260 nm and a low absorption at 280 nm (Fig. 1). In preliminary experiments for the determination of the amino acid sequence, the peroxisomal CuZn-SOD was incubated with 6 M guanidine-HCl and then analyzed by reversed-phase HPLC. Under these conditions, a polypeptide with the molecular mass and amino acid composition of peroxisomal CuZn-SOD and a nonproteinaceous chromophore were separated (Fig. 2A). The chromophore ac-
counted for most of the absorbance peak at 260 nm, previously detected in the native enzyme, whereas the polypeptide did not present any significant absorption at this wavelength (Fig. 2B). In the light of these results, the CuZn-SODs from bovine erythrocytes and liver and from horseradish were also subjected to denaturation with 6 M guanidine-HCl followed by reversed-phase HPLC. Results showed only the appearance of a polypeptide band, and no chromophore group absorbing at 260 nm was detected (results not shown). Attempts were made to identify the nature of the noncovalently associated chromophore group detected in the peroxisomal CuZn-SOD from watermelon cotyledons, and using an HPLC method specific for nucleotides, nucleosides, and major purine bases (Wynants and Van Belle, 1985), it was found that the chromophore group apparently was not a nucleotide (results not shown).

The sequence of peroxisomal CuZn-SOD was determined by N-terminal Edman degradation of the whole enzyme, and a set of peptides was generated by digestion with several peptide hydrolases. Overlapping of the sequence of the N terminus of the protein and several of the peptides provided an uninterrupted stretch of sequence of 40 amino acids (Fig. 3). Another set of peptides that overlapped produced an additional uninterrupted stretch of 50 amino acids. Comparison of these two fragments and of peptides V-5, T-3, and V-6 with other SODs (Fig. 4) led to the establishment of the overall sequence for peroxisomal CuZn-SOD shown in Figure 4. Comparison of the partial amino acid sequence of peroxisomal CuZn-SOD (118 residues) with the sequences of different cytosolic and chloroplastic CuZn-SOD, showed a maximum homology of about 70% with the cytosolic enzymes from pine, maize, and tomato, and of about 90% with chloroplastic CuZn-SODs from spinach, petunia, tomato, and pea. The highest homology (93%) was that found with the chloroplast CuZn-SOD from spinach.

Cyanide at a 1-mM concentration completely inhibited the CuZn-SOD activity, as expected for a SOD belonging to this metalloenzyme family (Fridovich, 1986). Hydrogen peroxide inhibited peroxisomal CuZn-SOD activity at 25°C...
Figure 4. Alignment of amino acid residues for peroxisomal CuZn-SOD, chloroplastic CuZn-SODs from spinach (Kitagawa et al., 1986), petunia (Tepperman et al., 1988), tomato (Perl-Treves et al., 1988), pea (Scioli and Zilinskas, 1988), and pine (Karpinski et al., 1992), and cytosolic CuZn-SODs from spinach (Kanematsu and Asada, 1990), cabbage (Steffens et al., 1986), tomato (Perl-Treves et al., 1988), maize (Cannon et al., 1987), and pine (Karpinski et al., 1992). Proteins were visually aligned. Since homologies were very high, no statistical methods for alignment and assessment of significance were used. Alignments were made to maximize homology. Residues that are identical to peroxisomal CuZn-SOD are boxed, and X indicates an unidentified residue. Residues coordinating Cu and Zn are indicated with asterisks.
in a concentration- and time-dependent manner (Fig. 5). At a 0.5-mM hydrogen peroxide concentration the enzyme retained about 45% of its initial activity after 1 h of incubation. A similar remaining activity was observed after incubation with 1 mM hydrogen peroxide for about 20 min, and after 30 min at this concentration the CuZn-SOD activity was completely inhibited. Hydrogen peroxide concentrations of 2.5 and 5 mM produced the total loss of activity after 10 min.

The thermal stability of peroxisomal CuZn-SOD was studied at 25, 50, 70, 80, and 100°C. After 6 d at 25°C the CuZn-SOD retained 65% of its initial activity, and after 4 h of incubation at 50°C the enzyme still kept more than 60% of its activity (results not shown). The minimum time at which the CuZn-SOD activity was reduced to 50% after heating at 70 and 100°C was about 15 and 3 min, respectively (Fig. 6).

A polyclonal antibody was raised in rabbits against peroxisomal CuZn-SOD. The titration of the antibody in the immune serum by the ELISA method indicated an antibody titer of about 1/2,000,000. The specificity of purified IgG to peroxisomal CuZn-SOD was assayed by western blotting against watermelon cotyledon crude extracts and homogenous peroxisomal CuZn-SOD from watermelon cotyledons. The incubation of the nitrocellulose sheets with 1/1,000 dilution of anti-peroxisomal CuZn-SOD revealed a single 16.5-kD band that corresponded to the peroxisomal CuZn-SOD subunit (Bueno and del Río, 1992) (Fig. 7). In crude extracts when the incubation times and antibody dilutions in the western blot assays were forced, occasionally a weak band of high M_r was detected. This band, however, was also produced under the same conditions by the preimmune serum (results not shown). The cross-reactivity of anti-peroxisomal CuZn-SOD against pure SODs from different origins was studied (Fig. 8). The antibody did not recognize either the Mn-SOD from pea leaves nor the Escherichia coli Fe-SOD, but showed a strong cross-reactivity against the 15-kD subunit of Sigma horseradish CuZn-SOD. In this case, different bands also appeared in the high M_r region of nitrocellulose sheets, probably due to the presence of abundant cross-reactive impurities in the commercial enzyme, which was overloaded (10 µg) in the SDS-PAGE gels. The incubation of the anti-peroxisomal CuZn-SOD with animal CuZn-SODs showed a weak cross-reaction with the 16-kD polypeptide band of this CuZn-SOD subunit (Steinman, 1982), apart from other impurity bands. Different cell-free extracts from plant and animal

Figure 5. Effect of hydrogen peroxide on the activity of peroxisomal CuZn-SOD. The enzyme (30 µg mL\(^{-1}\)) was preincubated at 25°C in 50 mM potassium phosphate buffer, pH 7.8, containing different hydrogen peroxide concentrations (0.5, 1.0, 2.5, and 5.0 mM). At different incubation times, aliquots were withdrawn and their SOD activity was determined.

Figure 6. Temperature stability of peroxisomal CuZn-SOD. The purified enzyme (30 µg mL\(^{-1}\)) in 50 mM potassium phosphate buffer, pH 7.8, was incubated at the temperatures indicated. Aliquots were withdrawn at time intervals and assayed for residual SOD activity.

Figure 7. Western blot of watermelon peroxisomal CuZn-SOD and crude extracts from watermelon cotyledons. After SDS-PAGE on 12% gels and transfer to nitrocellulose sheets, the peroxisomal CuZn-SOD subunit was detected by incubation with a 1/1,000 dilution of anti-peroxisomal CuZn-SOD, followed by the second antibody and staining with 4-chloro-1-naphthol. A, Watermelon cotyledons extract. B, Homogenous peroxisomal CuZn-SOD from watermelon cotyledons (Bueno and del Río, 1992). C, Molecular mass standards.
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CuZn-SODs from bovine erythrocytes and liver and from 260 responsible for the A peak of the native enzyme. Pure contains a noncovalently bound chromophore group that is CuZn-SOD incubated with 6 M guanidine-HCl indicated g-atom Cu and 2 g-atom Zn/mol (Puget and Michelson, which has a molecular mass of 33,100 D and contains 1 SODs from animal and plant origin contain 2 g-atom Cu and 2 g-atom Zn/mol dimer (Bueno and del Rio, 1992). Most CuZn-SODs, contains only 1 g-atom Cu and 1 g-atom Zn/mol dimer (Steinman, 1982; Fridovich, 1986; Bannister et al., 1987, 1991). The comparison with peroxisomal CuZn-SOD showed the existence in the P. leiognathi enzyme of extended regions of nonhomologous amino acid sequence and also an insert of 12 amino acids, and provided evidence for the absence of evolutionary relationships between peroxisomal CuZn-SOD and the P. leiognathi CuZn-SOD. However, comparison of the partial amino acid sequence determined for peroxisomal CuZn-SOD with those reported for chloroplastic and cytosolic CuZn-SODs showed high similarities with respect to length and alignment of the homologous regions, and evidenced a high homology (about 90%) with chloroplastic CuZn-SODs. However, there are several amino acid replacements that differentiate peroxisomal CuZn-SOD from most plant SODs. Pro (P) at position 40 is conserved in all cytosolic and chloroplastic CuZn-SODs (Fig. 4), but in the peroxisomal enzyme it is substituted for by Glu (E), as in the bovine, human, sheep, pig, rat, and mouse CuZn-SOD (Bannister et al., 1991). Other amino acids that are nearly specific for origin were examined by western blotting for reactivity to purified IgG to peroxisomal CuZn-SOD. Results showed varying degrees of cross-reactivity (Fig. 9). The intensity of the CuZn-SOD band in the immunoblot was greatest for pea leaves, followed by clover leaves, broad bean leaves, and erythrina leaves, whereas cell-free extracts from soybean nodules and spinach and lettuce leaves did not show any reaction (Fig. 9). Cell-free extracts of trout liver were recognized by the peroxisomal CuZn-SOD antibody, with a weak band of about 16 kD being detected.

DISCUSSION

As we have previously reported (Bueno and del Rio, 1992), peroxisomal CuZn-SOD from watermelon cotyledons has a molecular mass of 33,000 D and is composed of two equal subunits of 16.5 kD. The peroxisomal enzyme has a pI of 4.0 (Sandalio and del Rio, 1987) and, unlike other CuZn-SODs, contains only 1 g-atom Cu and 1 g-atom Zn/mol dimer (Bueno and del Rio, 1992). Most CuZn-SODs from animal and plant origin contain 2 g-atom Cu and 2 g-atom Zn/mol dimer (Steinman, 1982; Fridovich, 1986; Bannister et al., 1987, 1991), and apparently the only exception is the CuZn-SOD from Photobacterium leiognathi, which has a molecular mass of 33,100 D and contains 1 g-atom Cu and 2 g-atom Zn/mol (Puget and Michelson, 1974).

Analysis by reversed-phase HPLC of the peroxisomal CuZn-SOD incubated with 6 M guanidine-HCl indicated that this peroxisomal enzyme, unlike other CuZn-SODs, contains a noncovalently bound chromophore group that is responsible for the A_260 peak of the native enzyme. Pure CuZn-SODs from bovine erythrocytes and liver and from horseradish also show a UV band around 260 nm, but treatment of these SODs with 6 M guanidine-HCl followed by reversed-phase HPLC showed that the chromophore responsible for the absorption at 260 nm was covalently bound to the protein. The only data known thus far on the peroxisomal CuZn-SOD chromophore is that it has an absorption maximum at 260 nm, it has a low molecular mass, probably lower than 1000 D, and that it is not a nucleotide. The full characterization of the peroxisomal CuZn-SOD chromophore group using MS and ^1H-NMR techniques is now under way in our laboratory.

Attempts were made to compare the amino acid sequence of peroxisomal CuZn-SOD with that reported for CuZn-SOD from P. leiognathi, which has molecular mass and copper content similar to those of peroxisomal CuZn-SOD (Puget and Michelson, 1974). It is well established that the CuZn-SOD from P. leiognathi has a low degree of amino acid sequence homology with most of the CuZn-SODs known (Steffens et al., 1983; Steinman, 1987; Bannister et al., 1991). The comparison with peroxisomal CuZn-SOD showed the existence in the P. leiognathi enzyme of extended regions of nonhomologous amino acid sequence and also an insert of 12 amino acids, and provided evidence for the absence of evolutionary relationships between peroxisomal CuZn-SOD and the P. leiognathi CuZn-SOD. However, comparison of the partial amino acid sequence determined for peroxisomal CuZn-SOD with those reported for chloroplastic and cytosolic CuZn-SODs showed high similarities with respect to length and alignment of the homologous regions, and evidenced a high homology (about 90%) with chloroplastic CuZn-SODs. However, there are several amino acid replacements that differentiate peroxisomal CuZn-SOD from most plant SODs. Pro (P) at position 40 is conserved in all cytosolic and chloroplastic CuZn-SODs (Fig. 4), but in the peroxisomal enzyme it is substituted for by Glu (E), as in the bovine, human, sheep, pig, rat, and mouse CuZn-SOD (Bannister et al., 1991). Other amino acids that are nearly specific for
peroxisomal CuZn-SOD are Ile's (I) at positions 58 and 78; Ser (S) at position 102 is present only in cytosolic CuZn-SODs and not in the chloroplastic enzymes. Some of the ligands coordinating the Cu and Zn that were sequenced in the peroxisomal enzyme (His71, His90, Asp85, and His120) appear to be conserved, as they are among all known CuZn-SODs. It is known that the chloroplastic CuZn-SOD is much more conserved than the cytosolic isozyme (Kwiatkowski and Kaniuga, 1986; Perly-Treves et al., 1988; Sciolli and Zilinskas, 1988; Kanematsu and Asada, 1990; Karpiniski et al., 1992), and this indicates that the rate of mutation of the cytosolic CuZn-SOD is higher than that of the chloroplastic CuZn-SOD (Kwiatkowski and Kaniuga, 1986; Kanematsu and Asada, 1990). It has been suggested that the high rate of production of superoxide radicals in chloroplasts would have a lethal effect if mutations in CuZn-SOD bring about a decrease or loss of their activity to scavenge superoxide radicals (Kanematsu and Asada, 1990). From a functional viewpoint, plant peroxisomes are closely related to chloroplasts, and both organelles participate in conjunction in the oxidative carbon cycle of photorespiration (Huang et al., 1983). Moreover, in peroxisomes as in chloroplasts, the production of superoxide radicals takes place as a result of their oxidative metabolism (del Río et al., 1992). The high homology (90%) of the peroxisomal CuZn-SOD amino acid sequence with the chloroplastic CuZn-SOD could be explained by the close functional affinity between these two organelles, and strongly suggests that chloroplast and peroxisomal CuZn-SODs could be encoded by the same or closely related genes.

Other remarkable features of peroxisomal CuZn-SOD are its thermal stability and resistance to hydrogen peroxide inactivation. The peroxisomal enzyme has half-life values at 50 and 70°C of more than 4 h and 15 min, respectively, which are higher than those reported for most CuZn-SODs (Steinman, 1982; Kwiatkowski and Kaniuga, 1986). Peroxisomal CuZn-SOD was inhibited by hydrogen peroxide, as expected for a CuZn-SOD, but in the presence of 0.5 mM hydrogen peroxide the enzyme had a half-life of nearly 60 min, a value that is higher than that shown by many CuZn-SODs (Kwiatkowski and Kaniuga, 1986; Kanematsu and Asada, 1990). The higher resistance of the peroxisomal enzyme to hydrogen peroxide could be due to its lower metal content, since hydrogen peroxide apparently inactivates CuZn-SODs by destroying the His ligands of Cu and Zn in the protein (Kwiatkowski and Kaniuga, 1986). Considering that peroxisomes are the main cellular site of hydrogen peroxide production (Huang et al., 1983; Fahimi and Sies, 1987; Van den Bosch et al., 1992), the increased resistance to hydrogen peroxide inactivation of peroxisomal CuZn-SOD could represent a protective adaptation mechanism of the enzyme to the peroxisomal environment rich in hydrogen peroxide.

An interesting property of the purified antibody to plant peroxisomal CuZn-SOD is its cross-reactivity with the phylogenetically distant animal CuZn-SODs. The antibody to spinach CuZn-SOD does not cross-react with bovine erythrocyte CuZn-SOD, and the antibodies to the cytosolic CuZn-SODs do not recognize the chloroplastic enzymes and vice versa (Asada et al., 1977; Kanematsu and Asada, 1990). Human and animal CuZn-SODs were traditionally considered as exclusive cytosolic enzymes, but recent reports have demonstrated the presence of CuZn-SOD in peroxisomes from human and animal cells (Keller et al., 1991; Crapo et al., 1992; Dhaunsi et al., 1992; Wanders and Denis, 1992). The weak cross-reactivity observed between the antibody to plant peroxisomal CuZn-SOD and animal CuZn-SODs could be due to the antibody recognition of the animal CuZn-SOD isozyme localized in peroxisomes.

Research carried out on plant peroxisomes in our laboratory in recent years has allowed us to postulate that oxyradical metabolism may be a common metabolic property of peroxisomes of eukaryotes (del Río et al., 1990, 1992). The main function of CuZn-SOD in peroxisomes could be the protection of these cell organelles against toxic effects derived from superoxide radicals generated therein. An example could be the protection of catalase from its inhibition by superoxide radicals (Kono and Fridovich, 1982). Under certain conditions of depressed catalase activity induced by different plant stress situations (del Río et al., 1992), $\text{O}_2^{-}\text{-}$ radicals produced in peroxisomes could react with hydrogen peroxide and give rise to the vastly more reactive hydroxyl radicals (OH) (Halliwell and Gutteridge, 1989; Elstner, 1990). This strong oxidizing species damages biological membranes and reacts with most of the compounds present in biological systems, including DNA (Halliwell and Gutteridge, 1989; Elstner, 1990; Packer and Glazer, 1990).

The availability of the antibody against peroxisomal CuZn-SOD will allow us to carry out immunolocalization studies of this enzyme in plant peroxisomes. Additionally, molecular biology studies will be conducted in our laboratory to isolate a cDNA clone for this SOD and to study the mechanism of import of the enzyme into peroxisomes.

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