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Superoxide Radical Generation in Peroxisomal Membranes: Evidence for the Participation of the 18-kDa Integral Membrane Polypeptide

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Peroxisomes were isolated from pea (*Pisum sativum* L.) leaves and the peroxisomal membranes were purified by treatment with Na_2CO_3. The production of superoxide radicals ($O_2^{\cdot-}$) induced by NADH was investigated in peroxisomal membranes from intact organelles incubated with proteases (pronase E and proteinase K). Under isoosmotic conditions, in the presence of pronase E, the production of $O_2^{\cdot-}$ radicals was inhibited by 80%. SDS-PAGE of peroxisomal membranes after protease treatment demonstrated a decrease in the 18-kDa PMP. This suggests that this polypeptide has a small fragment exposed to the cytosolic side of the peroxisomal membrane which is essential for $O_2^{\cdot-}$ production. The 18-kDa PMP was purified by preparative SDS-PAGE and in the reconstituted protein the NADH-driven production of $O_2^{\cdot-}$ radicals was investigated. The isolated polypeptide showed a high generation rate of superoxide (about 300 nmol $O_2^{\cdot-} \times mg^{-1}$ protein $\times$ min^{-1}) which was completely inhibited by 50 mM pyridine. The 18-kDa PMP was recognized by a polyclonal antibody against Cyt b_5 from human erythrocytes. The presence of *b*-type cytochrome in peroxisomal membranes was demonstrated by difference spectroscopy. Results obtained show that in the NADH-dependent $O_2^{\cdot-}$ radical generating system of peroxisomal membranes, the 18-kDa integral membrane polypeptide, which appears to be Cyt b_5, is clearly involved in superoxide radical production.

Keywords: Activated oxygen, cytochrome b_5, pea, peroxisome, peroxisomal membrane polypeptide (PMP), superoxide

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; DETAPAC, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; PMP, peroxisomal membrane polypeptide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; SOD, superoxide dismutase.

INTRODUCTION

Peroxisomes are subcellular respiratory organelles which contain catalase and H_2O_2-producing flavin oxidases as basic enzymatic constituents.[1] These organelles have an essentially oxidative type of metabolism and have different metabolic pathways depending upon their origin.[2,3] Superoxide dismutases (EC 1.15.1.1) are a family of metalloenzymes that catalyze the disproportionation of superoxide radicals ($O_2^{\cdot-}$), and play an important role in protecting cells against the toxic effects pro-

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duced by these activated oxygen species.[4,5] In recent years, the presence of SOD has been demonstrated in different types of plant peroxisomes including those from pea leaves,[6,7] watermelon cotyledons,[8,9] carnation petals,[10] castor bean endosperm[11] and cotton, cucumber and sunflower cotyledons.[12]

Superoxide radicals ($O_2^{\cdot-}$) are produced in aerobic organisms as a consequence of oxygen metabolism.[5] In cellular and subcellular systems, the generation of $O_2^{\cdot-}$ radicals has been reported in neutrophils, monocytes and macrophages,[13] mitochondria,[14,15] chloroplasts,[16] microsomes,[17–19] nuclei,[20] and plasma membranes.[21] Recently, the generation of $O_2^{\cdot-}$ radicals in peroxisomes from watermelon cotyledons, castor bean endosperm and pea leaves has been demonstrated.[11,22,23] In the peroxisomal matrix, the production of superoxide radicals is induced by purines and the responsible system is xanthine oxidase (EC 1.1.3.22).

In peroxisomal membranes, NADH induces the generation of superoxide radicals and the electron transport chain occurring in the membranes of certain types of peroxisomes could be responsible.[24] In glyoxysomes, a type of specialized peroxisomes of oilseeds, this electron transport chain consists of a flavoprotein NADH:ferricyanide reductase and Cyt b_5 (measured as NADH:cytochrome *c* reductase activity).[25–27]

In phagocytic cells, mitochondria, microsomes, and chloroplasts, the generation of superoxide radicals is very well-established and the mechanisms involved in this radical production have been characterized.[5,13,14,16,19] However, in peroxisomes no direct information is available on the $O_2^{\cdot-}$-producing system of peroxisomal membranes and there is no conclusive evidence on the identity and molecular properties of the redox protein(s) involved in this superoxide radical generation. This information is important to advance our knowledge of the role of oxygen free radicals in the peroxisomal and cellular metabolism, and also to get deeper insights into the function of peroxisomes in oxidative stress situations.

In this work, the production of superoxide radicals in membranes from pea leaf peroxisomes and its latency after protease treatment of intact organelles, was studied. The presence of *b*-type cytochromes was investigated by differential spectroscopy and using an antibody against Cyt b_5, and the generation of $O_2^{\cdot-}$ radicals by the 18-kDa PMP purified by electroelution was demonstrated.

MATERIALS AND METHODS

Chemicals

Acrylamide, Bis, SDS, Tris, glycine, and molecular mass markers were from Bio-Rad Laboratories. Potassium phosphate, potassium chloride, EDTA, sodium carbonate, methanol, acetic acid and sucrose were from Merck. BSA, DTT and Triton X-100 were from Boehringer-Mannheim. Cytochrome *c* type III, leupeptin, PMSF, and pyridine were from Sigma Chemical Company.

Plant Material

Pea seeds (*Pisum sativum* L., cv. Lincoln), obtained from Royal Sluis (Enkhuizen, Holland), were surface-sterilized with 3% (v/v) commercial bleaching solution for 3 min and then were washed with distilled water and germinated in vermiculite. Seedings were grown in a growth chamber (Conviron PGW-36) in aerated optimum nutrient solutions[28] for 30 days.

Preparation of Organelles and Membrane Fractions

Peroxisomes were purified from pea leaves (*Pisum sativum* L.) by differential and sucrose density-gradient centrifugation (35–60%; w/w), as described by López-Huertas *et al.*[29] Purified peroxisomes were practically free of chloroplasts, mitochondria, and ER, as checked by using appropriate marker enzymes.[29] Peroxisomes were recovered from the gradient tubes by punc-

tion with a syringe, and peroxisomal membranes were obtained by osmotic shock and washed two times with 0.1 M Na_2CO_3 (pH 11.5), as described by López-Huertas *et al.*[29] After centrifugation at 120,000 g for 30 min, the resulting pellet was resuspended in 50 mM K-phosphate buffer (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, 1 mM leupeptin, and 1 mM PMSF, and was stored at –20°C.

Protease Treatment

Purified intact peroxisomes were incubated under isoosmotic conditions with pronase E (3.7 µg/mg protein) for 10 min at 30°C, and the reaction was stopped by 3-fold dilution with 100 mM K-phosphate (pH 7.5), followed by addition of 0.5 mM PMSF. Peroxisomes were also incubated with proteinase K (0.1 mg/mg protein) for 45 min at 0°C, and the reaction was stopped by addition of 1 mM PMSF. In both cases, peroxisomes were then broken by osmotic shock and the peroxisomal membranes were washed with Na_2CO_3, as described above, and PMPs were differentiated by SDS-PAGE and silver staining.

SDS-PAGE

Carbonate-washed peroxisomal membranes were subjected to SDS-PAGE to separate the integral membrane polypeptides using a Bio-Rad Mini-Protean II slab cell (Bio-Rad, Richmond, CA), with the discontinuous buffer system of Laemmli[30] in 15% separating gels, with 4% stacking gels. Samples were heated at 100°C for 5 min in sample buffer consisting of 47 mM Tris-HCl (pH 7.8), 1.5% (w/v) SDS, 7.5% (v/v) glycerol, 3.75% (v/v) 2-β-mercaptoethanol, and 0.002% (w/v) bromophenol blue. Gels were run for 45 min at constant voltage (200 V) and were silver stained (Sigma Silver Staining Kit).

Electrophoretic Transfer of Proteins

Electrophoretic transfer of proteins onto nitrocellulose membranes was carried out according to the procedure of Small *et al.*[31] After transfer, nitrocellulose sheets were used for cross-reactivity assays with a polyclonal antibody against Cyt b_5 from human erythrocytes, generously donated by Dr. T. Yubisui,[32] Kochi University, Japan. Goat anti-rabbit lgG with horseradish peroxidase (Bio-Rad, Richmond, CA) was used as the second antibody, and 4-chloro-1-naphthol as the stain (Bio-Rad Immunoblot Instruction Manual).

Superoxide Determination

For the determination of $O_2^{\cdot-}$ radicals, the superoxide dismutase-inhibitable reduction of ferricytochrome *c* was followed.[11,22] The assays were carried out at 25°C in a Beckman DU-7 spectrophotometer, and the reaction mixture (1.1 ml) contained: air-saturated 50 mM K-phosphate buffer (pH 7.8); 0.1 mM DETAPAC; 23 µM ferricytochrome *c*; 0.1 mM KCN; and carbonate-washed peroxisomal membranes (5–15 µg protein). The reaction was started by adding 100 µM NADH (final concentration) and was followed at 550 nm for 1–2 min. At this time, bovine erythrocyte CuZn-SOD (Sigma) was quickly added to the reaction mixture (1 µM final concentration) and the slope change, due to the $O_2^{\cdot-}$-independent reduction of Cyt *c* was recorded. Controls with identical composition but without sample or NADH were used to compensate for possible nonenzymatic Cyt *c* reduction. The amount of $O_2^{\cdot-}$ radicals produced was calculated as described by Asada,[16] using a $\Delta\varepsilon_{550}$ of 19×10^3 M^{-1} cm^{-1} for Cyt *c*.[33] The effect of pyridine on the $O_2^{\cdot-}$ production by the electroeluted 18-kDa PMP was assayed. Membranes were incubated with 50 mM pyridine for 10 min, at room temperature, and the NADH-dependent generation of $O_2^{\cdot-}$ radicals was determined as mentioned above.

Cytochrome Spectroscopy

Carbonate-washed peroxisomal membranes were resuspended in 1.4 ml of 0.05 M Tricine (pH 7.5), 50% (v/v) glycerol, and were analyzed for

cytochrome content by difference spectroscopy using a Perkin-Elmer Lambda 5 double-beam spectrophotometer. Sodium dithionite (2 µl of a saturated solution) was added to the sample cuvette and the absorption spectrum from 380 to 600 nm was scanned versus a reference cuvette containing the same sample without reductant.[34]

Electroelution

The PMPs of carbonate-washed peroxisomal membranes (50 µg) were separated by SDS-PAGE in a 15% preparative gel. After electrophoresis, the position of the 18-kDa PMP was determined by silver staining of a 2 cm wide strip of gel with the standards. Then, the horizontal strip containing the polypeptide was cut out and placed into glass tubes of a Bio-Rad 422 Electro-Eluter Module, following the indications of the Bio-Rad Instruction Manual. The elution was carried out at 10 mA/glass tube, at constant current for 5 h in 25 mM Tris and 192 mM glycine buffer (pH 8.3) containing 0.1% (w/v) SDS, with membrane caps with a cut off of 3,500 daltons. After elution, the solution was replaced by 25 mM Tris and 192 mM glycine buffer (pH 8.3), containing 0.05% (v/v) Triton X-100, and electrophoretic dialysis was carried out at 10 mA/glass tube for 24-h with 5 volume changes. The electroeluted protein (400 µl) was 8-fold concentrated and $O_2^{\cdot-}$ production was measured as indicated above.

Other Assays

Proteins were measured according to Bradford[35] using BSA as standard. The integrity of peroxisomes was estimated by latency assays of hydroxypyruvate reductase activity (EC 1.1.1.29), as described by Sandalio *et al.*[7] The hydroxypyruvate reductase activity was determined in a reaction medium containing 0.45 M sucrose (isotonic medium) and in the same medium without sucrose and containing 0.025% (v/v) Triton X-100 (hypotonic medium).[7]

RESULTS

On the basis of previous results obtained in our laboratory on the generation of $O_2^{\cdot-}$ radicals in peroxisomal membranes from pea leaves and watermelon cotyledons,[22,23] NADH was tested as inducer of $O_2^{\cdot-}$ production in sodium carbonate-washed peroxisomal membranes from pea leaves. As shown in Table I the superoxide radical generation obtained for leaf peroxisomal membranes was of 20.5 nmol × mg^{-1} protein × min^{-1}. To obtain information on the orientation of the proteins involved in the $O_2^{\cdot-}$ generation system of the peroxisomal membrane, limited proteolysis assays of intact peroxisomes were carried out. Under isoosmotic conditions and in the presence of pronase E, a strong inhibition (80%) of the NADH-induced generation of $O_2^{\cdot-}$ was found (Table I). The integrity of peroxisomes after protease treatment was tested by the latency of the hydroxypyruvate reductase activity, which is a well-known soluble enzyme located in the matrix of peroxisomes.[2] The latency of hydroxypyruvate reductase activity was almost the same in both untreated and pronase E-treated peroxisomes (Table I). This indicated that the protease did not penetrate into the peroxisomal membrane and proteolysis occurred only on the cytoplasmic side of the membrane. SDS-PAGE of the peroxisomal membranes after treatment with pronase E demonstrated a decrease in the 18-kDa PMP and the appearance of a new 12-kDa PMP, while the rest of PMPs remained unchanged (Fig. 1). Cleavage of peroxisomal membranes by proteinase K produced similar results and the $O_2^{\cdot-}$ radical production was also strongly inhibited (results not shown).

To get more information on the identity of the 18-kDa PMP, the cross-reactivity of peroxisomal membranes with the antibody against Cyt b_5 from human erythrocytes was assayed. As shown in Figure 2, the antiserum recognized the 18-kDa PMP which indicates that this polypeptide could very likely be Cyt b_5. The other band of 61-kDa that cross-reacted with the antibody against cytochrome b_5 was also recognized by other anti-

TABLE I Intactness of purified leaf peroxisomes incubated with and without pronase E, and superoxide radical production by carbonate-washed peroxisomal membranes from intact and protease-treated peroxisomes.

	Isotonic medium	Hypotonic medium + 0.025% Triton X-100	% Intactness	$O_2^{\bullet-}$ production
	μmol NADH $\times$ min^{-1} $\times$ ml^{-1}			nmol $O_2^{\bullet-}$ $\times$ mg^{-1} protein $\times$ min^{-1}
Control peroxisomes	51.2	325.4	84.3	20.5
Peroxisomes treated with pronase E	40.2	322.0	87.5	4.1

Peroxisomes were incubated in isotonic medium with or without pronase E (3.7 μg/mg protein) for 10 min at 30°C. The integrity of peroxisomes was determined from latency assays of hydroxypyruvate reductase activity (μmol NADH $\times$ min^{-1} $\times$ ml^{-1}), as described in Materials and Methods. The peroxisomal membranes were isolated and treated with sodium carbonate, and the production of $O_2^{\bullet-}$ radicals induced by NADH was determined. Values are means of two experiments using different batches of peroxisomal membranes.

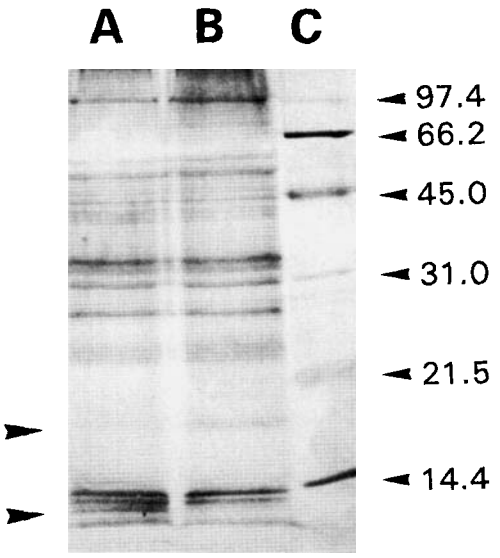

FIGURE 1 SDS-PAGE of carbonate-washed membranes from pea leaf peroxisomes incubated with pronase E. Intact peroxisomes were purified by sucrose density-gradient centrifugation and were incubated in isotonic medium with pronase E at 30°C for 10 min. Then, the peroxisomal membranes were isolated and treated with sodium carbonate, and the PMPs were separated by SDS-PAGE (15% acrylamide gels) and localized by silver staining. A, peroxisomes incubated with pronase E (5 μg proteins). B, control peroxisomes (5 μg proteins). C, molecular mass markers in kDa.

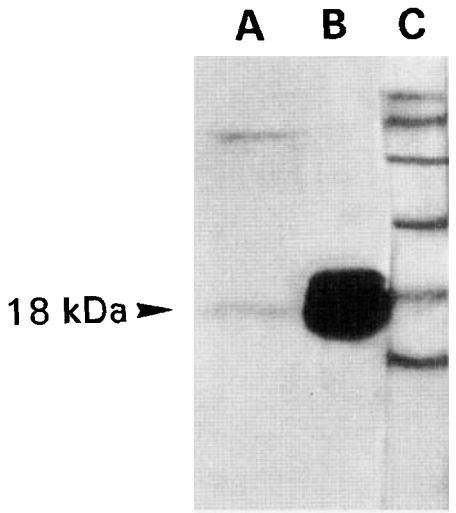

FIGURE 2 Western blot analysis of PMPs of pea leaf peroxisomes with an antibody against Cyt b_5. Proteins were separated by SDS-PAGE (15% acrylamide gels), transferred onto a nitrocellulose sheet and probed with a polyclonal antibody against Cyt b_5 from human erythrocytes.[32] A, PMPs from pea leaf peroxisomes (5 μg proteins). B, purified Cyt b_5 from human erythrocytes. C, molecular mass markers.

bodies against different peroxisomal membrane proteins from animal origins (results not shown). Therefore, the 61-kDa band appears to be a cross-reactive protein of leaf peroxisomal membranes which is nonspecifically recognized by the cytochrome b_5 antibody. The presence of *b*-type cytochromes in carbonate-washed membranes was studied by difference spectroscopy using sodium dithionite as reductant. In the presence of dithionite, peroxisomal membranes had an absorption maximum at 556 nm in comparison with oxidized membranes (Fig. 3). This absorption spectrum indicates the presence of a *b*-type cytochrome in the peroxisomal membranes.[36] However, we could not identify the type of Cyt *b* present, mainly due to the high amount of membrane proteins necessary to carry out these differential spectroscopy experiments and the extremely low yields obtained in the isolation of carbonate-washed peroxisomal membranes from pea leaves. To obtain the difference spectrum shown in Figure 3, 500 μg of peroxisomal membrane proteins had to be used which were prepared from 2.5 kg of pea leaves through the use of 62 different sucrose density-gradients.

In order to have direct evidence of the participation of the 18-kDa PMP in the generation of superoxide radicals by peroxisomal membranes, the purification of this polypeptide by preparative SDS-PAGE and electroelution was carried out. In the reconstituted protein, the rate of $O_2^{\cdot-}$ radical production was 308 nmol $O_2^{\cdot-} \times mg^{-1}$ protein $\times$ min^{-1} (Table II), which is 15-times the rate of $O_2^{\cdot-}$ radical generation determined in carbonate-washed membranes from control leaf peroxisomes. This superoxide generation was completely suppressed by pyridine, a well known inhibitor of the cytochrome *b* component of the neutrophil NADPH oxidase system.[37]

DISCUSSION

The study of membrane proteins from leaf peroxisomes has a great limitation derived from the

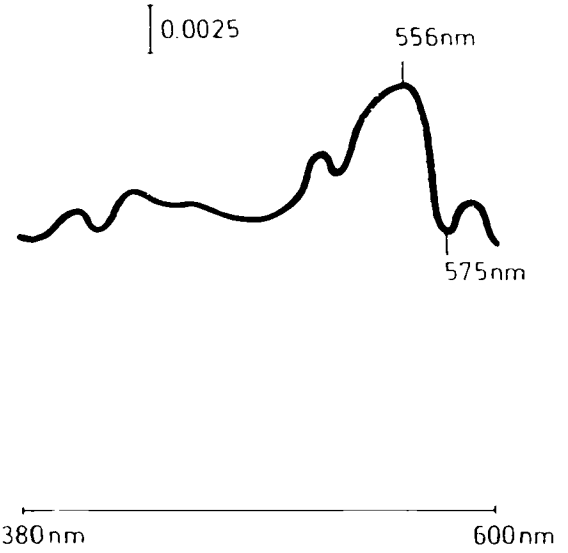

FIGURE 3 Difference spectrum of carbonate-washed peroxisomal membranes from pea leaves. Peroxisomes were purified by sucrose density-gradient centrifugation. The peroxisomal membranes were isolated and treated with sodium carbonate. Samples (0.25 mg protein per cuvette) were analyzed for cytochrome content by reduced *versus* oxidized difference spectroscopy at 25°C. Sodium dithionite was added to the sample cuvette and the absorption spectrum from 380 to 600 nm was scanned versus a reference cuvette with the sample without reductant.

methodological difficulties involved in the isolation of intact leaf peroxisomes and their considerable lability.[1,2] Moreover, the final purification of peroxisomal membranes by sodium carbonate treatment is a very convenient method to remove all peripheral proteins keeping the integral components of the peroxisomal membranes[38] but it supplies very low final yields. Under optimum conditions, the yield of PMPs obtained from 1 kg of pea leaves (about 20 density-gradient preparative tubes) was only 0.2 mg of membrane proteins.

In previous works conducted in our laboratory we demonstrated the NADH-induced $O_2^{\cdot-}$ radical production in peroxisomal membranes,[22,23] and the involvement of an electron transport chain similar to that described in glyoxysomal membranes by Fang *et al.*[27] was hypothesized.[24] This chain consists on a flavoprotein NADH:reductase and Cyt b_5 which would be responsible for the NADH:Cyt *c* reductase activity detected in these organelles.[27] In our hypothetical model, Cyt b_5 might transfer electrons to O_2 with the production of $O_2^{\cdot-}$. To test the validity of this model, we first carried out the characterization by SDS-PAGE of the PMPs of peroxisomal membranes washed with sodium-carbonate.[29]

Results reported in this work show that the rate of $O_2^{\cdot-}$ production in pea leaf peroxisomal membranes was of the same order to that described for glyoxysomal membranes from castor bean endosperm[11] and in both cases was considerably higher than those rates found in peroxisomal membranes untreated with sodium-carbonate.[22,23] This could be due to the complete removal by sodium-carbonate of traces of peroxisomal soluble Mn-SOD and/or other $O_2^{\cdot-}$-reacting proteins what would avoid their catalytic scavenging of $O_2^{\cdot-}$ radicals locally produced.[9,23] In any case, these results also evidence the stability of the $O_2^{\cdot-}$-generating system in leaf peroxisomal mem-

TABLE II The NADH-dependent superoxide radical production by the 18-kDa peroxisomal membrane polypeptide.

	nmol $O_2^{\cdot-} \times mg^{-1}$ protein $\times min^{-1}$
18-kDa PMP	308
18-kDa PMP + 50 mM pyridine	0

The 18-kDa PMP was isolated from carbonate-washed peroxisomal membranes (50 µg proteins) by preparative SDS-PAGE and electroelution. The production of $O_2^{\cdot-}$ radicals induced by NADH was determined. Samples were incubated for 10 min at room temperature with pyridine before the addition of NADH and Cyt *c*. Values are means of two experiments using different batches of peroxisomal membranes.

branes during the exposure to the alkaline pH of 0.1 M sodium carbonate used for the membrane purification (pH 11.5).

To judge by limited proteolysis assays, the production of oxygen free radicals takes place outside of the membrane, facing to the cytosol, and the 18-kDa PMP is involved, particularly a small fragment of this PMP which is oriented to the cytosol. There are several lines of evidence suggesting that the 18-kDa PMP is the Cyt b_5. Its molecular mass is close to that described for Cyt b_5 from different origins, which is in the range 15–20 kDa,[39–42] and the 18-kDa PMP also cross-reacted with an antibody against animal Cyt b_5. This is not surprising if it is considered that Cyt b_5 from different origins share similarities in molecular mass, optical spectrum, isoelectric point, and amino acid sequence.[39,41] All these evidences suggest an evolutionary significance for this electron transport protein and also the conservation of surface recognition epitopes. Recently, we have demonstrated the cross-reactivity of some peroxisomal PMPs from pea leaves with antibodies against PMPs from rat liver peroxisomal membranes.[29] An additional evidence in favour of the identity of the 18-kDa PMP as Cyt b_5 is the fact that pyridine completely abolishes the NADH-driven production of $O_2^{\cdot-}$ by the 18-kDa PMP. Pyridine is a demonstrated inhibitor of the superoxide production by the NADPH-oxidase from neutrophils[37] and plasma membranes[43] at the level of a *b*-type cytochrome.

The results of protease treatment, in terms of partial degradation of the 18-kDa PMP, as well as the strong inhibition of superoxide radical production are consistent with other results described in the literature. In glyoxysomes from castor bean endosperm, Luster and Donaldson[26] described the abolition of the NADH:Cyt *c* reductase activity after treating the glyoxysomal membranes with trypsin, and they suggested the degradation of Cyt b_5 as responsible of this inhibition. In cauliflower floret microsomes, Cyt b_5 was also cleaved by trypsin with production of two polypeptides with molecular masses of 13- and 2-kDa.[42] The Cyt b_5 from animal ER is a protein of about 16-kDa with a small fragment of about 3-kDa which is faced to the cytosol and is susceptible to cleavage by trypsin.[44] This hydrophilic fragment is the amino-terminal heme-containing catalytic region which participates in electron-transfer functions.[44] In view of these facts, it seems reasonable to think that the protease degradation of the 18-kDa PMP brings about the loss of the $O_2^{\cdot-}$ production capacity of pea leaf peroxisomal membranes due to the removal of the protein fragment responsible for the univalent reduction of O_2.

Cytochromes *b* and their reductases are components of membranes from different cellular compartments both in animal and plant cells, where they participate in electron transfer pathways. They have been especially associated with ER, mitochondria, and plasma membranes.[45,46] Other *b*-type cytochrome, the Cyt b_{558}, is also an important component of the NADPH oxidase of neutrophils which is responsible for the respiratory burst associated to the phagocitosis process.[5,13,47] The pea leaf peroxisomal membranes used in our work were practically free of contamination by mitochondria, chloroplasts, and ER, as verified by using appropriate marker enzymes.[29] Likewise, possible contamination by plasma membranes can be discarded since these membranes do not sediment at the differential centrifugation speed of 12,000 g used for the isolation of peroxisomes.[29,48] Moreover, plasma membranes have a density on sucrose gradients much lower[49] than that of leaf peroxisomes[2,29] what makes it that they separate very well from these organelles by density-gradient centrifugation.

The presence of a *b*-type cytochrome in peroxisomal membranes from pea leaves described in this work is in agreement with data recently reported by other authors. In castor bean endosperm, Fang *et al.*[27] described the participation of Cyt b_5 in the electron transport chain present in glyoxysomal membranes, where it is involved in the NADH:Cyt *c* reductase activity detected in these membranes. More recently, Struglics et al[50] have also reported a short redox chain in mem-

branes from potato tuber peroxisomes, consisting of a Cyt *b* and a Cyt *c* reductase.

The participation of a *b*-type cytochrome in the NADH-dependent $O_2^{\cdot-}$ generation by peroxisomal membranes is consistent with the hypothetical model previously proposed for peroxisomal membranes,[24] and strongly suggests the involvement of superoxide radicals in the peroxisomal metabolism. However, more work is still necessary to fully characterize the *b*-type cytochrome of leaf peroxisomal membranes and to identify the electron donor(s) of Cyt *b* in these membranes. This study is now under way in our laboratory.

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