Characterization of membrane polypeptides from pea leaf peroxisomes involved in superoxide radical generation

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The production of superoxide radicals ($O_2^{\cdot-}$) and the activities of ferricyanide reductase and cytochrome *c* reductase were investigated in peroxisomal membranes from pea (*Pisum sativum* L.) leaves using NADH and NADPH as electron donors. The generation of $O_2^{\cdot-}$ by peroxisomal membranes was also assayed in native polyacrylamide gels using an *in situ* staining method with NitroBlue Tetrazolium (NBT). When peroxisomal membranes were assayed under native conditions using NADH or NADPH as inducer, two different $O_2^{\cdot-}$ -dependent Formazan Blue bands were detected. Analysis by SDS/PAGE of these bands demonstrated that the NADH-induced NBT reduction band contained several polypeptides (PMP32, PMP61, PMP56 and PMP18, where PMP is peroxisomal membrane polypeptide and the number indicates molecular mass in kDa), while the NADPH-induced band was due exclusively to PMP29. PMP32 and PMP29 were purified by preparative SDS/PAGE and

electroelution. Reconstituted PMP29 showed cytochrome *c* reductase activity and $O_2^{\cdot-}$ production, and used NADPH specifically as electron donor. PMP32, however, had ferricyanide reductase and cytochrome *c* reductase activities, and was also able to generate $O_2^{\cdot-}$ with NADH as electron donor, whereas NADPH was not effective as an inducer. The reductase activities of, and $O_2^{\cdot-}$ production by, PMP32 were inhibited by quinacline. Polyclonal antibodies against cucumber monodehydroascorbate reductase (MDHAR) recognized PMP32, and this polypeptide is likely to correspond to the MDHAR reported previously in pea leaf peroxisomal membranes.

Key words: cytochrome *b₅* reductase, cytochrome *c* reductase, ferricyanide reductase, monodehydroascorbate reductase, peroxisomal membrane protein.

INTRODUCTION

Peroxisomes are subcellular respiratory organelles that contain catalase and H_2O_2 -producing flavin oxidases as their basic enzymic constituents [1]. These organelles have different metabolic pathways depending upon their origin, but they share an essentially oxidative type of metabolism [2,3]. The superoxide dismutases (SODs; EC 1.15.1.1) are a family of metalloenzymes that catalyse the disproportionation of superoxide radicals ($O_2^{\cdot-}$), and play an important role in protecting cells against the toxic effects produced by these oxygen radicals [4,5]. In the last few years, the presence of SOD has been demonstrated in different types of plant peroxisomes [6,7], as well as in peroxisomes of human and animal origin [8–11].

$O_2^{\cdot-}$ radicals are produced in aerobic organisms as a consequence of oxygen metabolism [4]. In cellular and subcellular systems, the generation of $O_2^{\cdot-}$ has been reported in neutrophils, monocytes and macrophages [12], mitochondria [13,14], chloroplasts [15], microsomes [16,17], nuclei [18] and plasma membranes [19]. The NADH-induced production of $O_2^{\cdot-}$ in peroxisomal membranes has been demonstrated in watermelon cotyledons [20], castor bean endosperm [21] and pea leaves [22]. This $O_2^{\cdot-}$ production could be due to a short peroxisomal electron transport chain that has been described by several authors in the peroxisomal membranes of castor bean endosperm [23–25]. This electron transport chain consists of two proteins: a flavoprotein and cytochrome *b₅* (Cyt *b₅*). The flavoprotein is an NADH:Cyt *b₅* reductase, and transfers the electrons from the peroxisomal

matrix NADH to Cyt *b₅* on the cytosolic side of the peroxisomal membrane. Finally, Cyt *b₅* transfers the electrons to physiological acceptors or to cytochrome *c* [23–25]. The flavoprotein can also reduce ferricyanide *in vitro* [NADH:ferricyanide reductase (FCRase) activity] [24]. In previous work, we have proposed that dissolved molecular oxygen in the vicinity of the cytosolic side of the peroxisomal membrane could compete with other physiological acceptors for the electrons donated by Cyt. *b₅*, with the production of $O_2^{\cdot-}$, to an extent depending on the concentrations of the competing acceptors [6].

Recently, the integral peroxisomal membrane polypeptides (PMPs) of pea leaf peroxisomes have been characterized by SDS/PAGE analysis of carbonate-washed peroxisomal membranes [26]. An 18 kDa PMP (PMP18) was purified from pea leaf peroxisomal membranes and evidence was obtained showing that this PMP is involved in $O_2^{\cdot-}$ generation by these membranes [27]. However, at present, except for PMP18, no direct information is available on the $O_2^{\cdot-}$ -producing system of peroxisomal membranes. This information is important in order to advance our knowledge of the role of oxygen free radicals in peroxisomal and cellular metabolism, and also to obtain deeper insights into the function of peroxisomes in situations of oxidative stress.

In the present work, PMPs of 29 and 32 kDa are shown to generate $O_2^{\cdot-}$. These polypeptides were purified from pea leaf peroxisomal membranes by preparative SDS/PAGE and characterized by using specific inhibitors and antibodies.

Abbreviations used: CCRase, cytochrome *c* reductase; Cyt. *b₅*, cytochrome *b₅*; FCRase, ferricyanide reductase; MDHAR, monodehydroascorbate reductase; NBT, NitroBlue Tetrazolium; $O_2^{\cdot-}$, superoxide radical; PHMB, *p*-hydroxymercuribenzoate; PMP29 (etc.), peroxisomal membrane polypeptide of 29 kDa (etc.); SOD, superoxide dismutase.

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MATERIALS AND METHODS

Plant material

Pea (*Pisum sativum* L., cv. Lincoln) seeds, 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 allowed to germinate in vermiculite. Seedlings were grown in a growth chamber (Convion PGW-36) [26] in aerated optimum nutrient solutions [28] for 30 days.

Preparation of organelles and membrane fractions

All operations were performed at 0–4 °C. Peroxisomes were purified from pea (*Pisum sativum* L.) leaves by differential and sucrose-density-gradient (35–60%, w/w) centrifugation, as described by López-Huertas et al. [26]. Purified peroxisomes were practically free of chloroplasts and mitochondria, as confirmed by measuring the activities of appropriate marker enzymes [26]. Peroxisomes were recovered from the gradient tubes by puncture with a syringe, and peroxisomal membranes were obtained by osmotic shock of the organelles and centrifugation at 120 000 *g* for 30 min in a Beckman 60 Ti rotor. The resulting pellet was washed twice with 0.1 M Na₂CO₃ (pH 11.5) and, after centrifugation at 120 000 *g* for 30 min, the peroxisomal membranes were resuspended in 50 mM potassium phosphate buffer (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, 1 mM leupeptin and 1 mM PMSF, and were stored at –20 °C. Alternatively, membrane pellets were resuspended in the sample buffer used for SDS/PAGE, which consisted of 47 mM Tris/HCl (pH 7.8), 1.5% (w/v) SDS, 7.5% (v/v) glycerol, 3.75% (v/v) β -mercaptoethanol and 0.002% (w/v) Bromophenol Blue.

Electrophoresis and Western blotting

Carbonate-washed peroxisomal membranes were subjected to SDS/PAGE to separate the integral membrane polypeptides using a Bio-Rad Mini-Protein II or Protean II (20 cm plates) slab cell (Bio-Rad, Richmond, CA, U.S.A.), with the discontinuous buffer system of Laemmli [29] in 15% (w/v) polyacrylamide separating gels and 4% (w/v) stacking gels. Samples were heated at 95 °C for 5 min in the sample buffer mentioned above and spun at 15 000 *g* for 2.5 min, and supernatants were loaded in the gels. Mini-gels were run for 45 min at constant voltage (200 V) and were silver stained (Sigma Silver Staining Kit). Alternatively, peroxisomal membrane proteins were separated by native PAGE in 2.5–6.0% (w/v) polyacrylamide gradient gels with 0.05% Triton X-100 or in 7.5% (w/v) polyacrylamide gels. Electrophoretic transfer of proteins from SDS/PAGE or native PAGE on to PVDF membranes (Immobilon P transfer membranes; Waters) was carried out using the procedure of Corpas and Trelease [30]. After transfer, membranes were used for cross-reactivity assays with polyclonal antibodies against ascorbate peroxidase from pumpkin cotyledons [31], monodehydroascorbate reductase (MDHAR) from cucumber fruits [32] and soybean root nodules [33], and Cyt. *b₅* reductase from human erythrocytes [34]. For immunodetection, an enhanced chemiluminescence method using luminol [7] was carried out using affinity-purified goat anti-(rabbit IgG)–horseradish peroxidase conjugate (Bio-Rad).

Electroelution and reconstitution

PMPs from carbonate-washed peroxisomal membranes (50 μ g) were separated by SDS/PAGE in 15% (w/v) polyacrylamide preparative gels. After electrophoresis, the positions of PMP32

and PMP29 were determined by silver staining of a 2 cm-wide strip of gel containing standards. Then the horizontal strips containing PMP32 and PMP29 were cut out and placed into the glass tubes of a Bio-Rad 422 Electro-Eluter Module. Elution was carried out at 10 mA/tube, at constant current for 5 h in 25 mM Tris/192 mM glycine buffer (pH 8.3) containing 0.1% (w/v) SDS, with membrane caps with a cut-off of 3500 Da. After elution, the solution was replaced by 25 mM Tris/192 mM glycine buffer (pH 8.3) containing 0.05% (v/v) Triton X-100, and electrophoretic dialysis was carried out at 10 mA/tube for 24 h with five volume changes. Finally, the electroeluted proteins were concentrated 8-fold.

O₂^{•-} determination

For the determination of O₂^{•-}, the SOD-inhibitable reduction of ferricytochrome *c* was monitored [20,21]. The effect of 60 μ M *p*-hydroxymercuribenzoate (PHMB) on O₂^{•-} production by carbonate-washed peroxisomal membranes was evaluated.

The effect of quinacrine on O₂^{•-} production by the electroeluted PMP32 was assayed. The reconstituted protein was incubated with 0.5 mM quinacrine for 10 min at room temperature, and the NADH-dependent generation of O₂^{•-} was determined as described above.

The O₂^{•-}-producing capability of peroxisomal membranes was also assayed on polyacrylamide gels by a NitroBlue Tetrazolium (NBT) reduction method (L. M. Sandalio, E. López-Huertas and L. A. del Río, unpublished work). Proteins were separated by native PAGE in either 7.5% (w/v) polyacrylamide gels or 2.5–6.0% (w/v) polyacrylamide gradient gels with 0.05% (v/v) Triton X-100. Gels were incubated in the dark with 2 mM NBT for 20 min and then with either 1 mM NADH or 1 mM NADPH until the appearance of Blue Formazan bands was observed. The reaction was stopped by immersion of the gels in distilled water. In parallel, control membranes were incubated with NBT in spectrophotometer cuvettes and, after addition of NADH or NADPH, NBT reduction was determined at 560 nm in the presence and absence of 12 μ M CuZn-SOD (Sigma Chemical Co.). The bands of NBT reduction activity were cut out and equilibrated for 10 min at 60 °C with sample buffer, and were then subjected to SDS/PAGE to separate the proteins involved in NBT reduction.

Enzymic assays

NADH:FCRase and NAD(P)H:cytochrome *c* reductase (CCRase) activities were measured according to Luster et al. [23]. The effects of 60 μ M PHMB on both activities in peroxisomal membranes were assayed. The effects of 0.5 mM quinacrine on NADH:FCRase and NAD(P)H:CCRase activities were studied in reconstituted PMP32, under the same conditions as described for measurement of O₂^{•-} production. In native polyacrylamide gels, NADH:FCRase was assayed as described by Kuwahara et al. [35], and flavoproteins were detected as described by Segura-Aguilar et al. [36].

Other assays

Proteins were measured by the method of Bradford [37], using BSA as standard.

RESULTS

On the basis of previous work on the generation of O₂^{•-} in membranes from plant peroxisomes [20–22], NADH and NADPH were tested as inducers of O₂^{•-} production in mem-

Table 1 O₂^{•-} production, NADH:FCRase activity and NADH:CCRase activity of carbonate-washed peroxisomal membranes

Carbonate-washed membranes were incubated for 10 min at room temperature with 60 µM PHMB before the addition of NADH and cytochrome *c*. O₂^{•-} production is expressed as nmol of O₂^{•-}·min⁻¹·mg of protein⁻¹, FCRase activity as nmol of ferricyanide·min⁻¹·mg of protein⁻¹, and CCRase activity as nmol of cytochrome *c*·min⁻¹·mg of protein⁻¹.

	O ₂ ^{•-} production (nmol·min ⁻¹ ·mg ⁻¹)	NADH:FCRase (nmol·min ⁻¹ ·mg ⁻¹)	NADH:CCRase (nmol·min ⁻¹ ·mg ⁻¹)
Membranes	50	5900	310
Membranes + PHMB	0	1770	0

Figure 1 Identification in peroxisomal membranes of PMPs involved in NAD(P)H-dependent O₂^{•-} generation

Proteins were resolved by native PAGE in 2.5–6.0% (w/v) polyacrylamide gradient gels, and their ability to carry out O₂^{•-}-dependent NBT reduction was assayed by using 1 mM NADPH (lane 1) or 1 mM NADH (lane 2) as inducers; lanes 3 and 4 were stained for flavoproteins and FCRase activity respectively. The Blue Formazan bands obtained were cut out and subjected to a second electrophoretic dimension (SDS/15% PAGE), and proteins were stained with silver nitrate. Lane 5, carbonate-washed peroxisomal membranes; lane 6, NADH-dependent NBT reduction band; lane 7, NADPH-dependent NBT reduction band.

Table 2 O₂^{•-} production and CCRase activity of PMP29 electroeluted from carbonate-washed peroxisomal membranes

PMP29 was isolated from carbonate-washed peroxisomal membranes by preparative SDS/PAGE and electroelution. For both determinations, 100 mM NADH or NADPH was used as electron donor. O₂^{•-} production is expressed as nmol of O₂^{•-}·min⁻¹·mg of protein⁻¹, and CCRase activity as nmol of cytochrome *c*·min⁻¹·mg of protein⁻¹.

Electron donor	O ₂ ^{•-} production (nmol·min ⁻¹ ·mg ⁻¹)	CCRase activity (nmol·min ⁻¹ ·mg ⁻¹)
NADPH	95.3	460
NADH	0	0

branes from pea leaf peroxisomes, and also as electron donors of oxidoreductases purified from peroxisomal membranes.

When carbonate-washed peroxisomal membranes were assayed for O₂^{•-} production with NADH as inducer, the maximum rate obtained was 50 nmol of O₂^{•-}·min⁻¹·mg of protein⁻¹; production was completely suppressed upon incubation of membranes with the -SH inhibitor PHMB (Table 1). NADH:FCRase and NADH:CCRase reductase activities were detected in peroxisomal membranes, and these were inhibited by PHMB by 70% and 100% respectively. This suggests that thiol groups are

Table 3 O₂^{•-} production, NADH:FCRase activity and NADH:CCRase activity of PMP32 electroeluted from carbonate-washed peroxisomal membranes

PMP32 was isolated from carbonate-washed peroxisomal membranes by preparative SDS/PAGE and electroelution. Samples were incubated for 10 min at room temperature with 0.5 mM quinacrine before the addition of NADH and cytochrome *c*. O₂^{•-} production is expressed as nmol of O₂^{•-}·min⁻¹·mg of protein⁻¹, FCRase activity as nmol of ferricyanide·min⁻¹·mg of protein⁻¹ and CCRase activity as nmol of cytochrome *c*·min⁻¹·mg of protein⁻¹.

	O ₂ ^{•-} production (nmol·min ⁻¹ ·mg ⁻¹)	NADH:FCRase (nmol·min ⁻¹ ·mg ⁻¹)	NADH:CCRase (nmol·min ⁻¹ ·mg ⁻¹)
PMP32	81.5	2870	64.4
PMP32 + quinacrine	0	0	0

involved in O₂^{•-} production by, and in the FCRase and CCRase activities of, peroxisomal membranes (Table 1).

The generation of O₂^{•-} by peroxisomal membranes was also assayed in native polyacrylamide gels using an *in situ* staining method with NBT. This method allowed detection of the production *in situ* of O₂^{•-} by different inducers. Controls were carried out by measuring NADH-induced NBT reduction by peroxisomal membranes in the presence and the absence of SOD. The reduction of NBT was 80% inhibited by CuZn-SOD, indicating that the Blue Formazan bands detected in the gels were due to NBT reduction by O₂^{•-} radicals. When carbonate-washed peroxisomal membranes were assayed under native conditions using NADPH or NADH as inducer, two different O₂^{•-}-dependent bands were detected (Figure 1, lanes 1 and 2). The polypeptide composition of these formazan bands was analysed by SDS/PAGE. The NADH-induced formazan band yielded silver-staining bands mainly corresponding to PMP18, PMP32, PMP56 and PMP61 (Figure 1, lane 6). The identification of different PMPs in SDS/polyacrylamide gels as components of peroxisomal membrane proteins that have been resolved by native PAGE is well documented [23,38–40]. The NADPH-induced formazan band yielded one silver-staining band of 29 kDa (Figure 1, lane 7). Flavoproteins have been reported to generate O₂^{•-} in different cell compartments [13–19], and a flavoprotein of approx. 32 kDa has been described to be responsible for the NADH:FCRase activity of peroxisomal membranes from castor bean endosperm [23]. To characterize further the formazan bands obtained with the NBT staining method, carbonate-washed peroxisomal membrane proteins were subjected to native PAGE in polyacrylamide gradient gels and stained specifically for flavoproteins and NADH:FCRase activity. Two bands of flavoproteins (Figure 1, lane 3) and one band of FCRase activity (Figure 1, lane 4) were detected. One of the flavoprotein bands and the FCRase activity migrated identically in the native gels, and both coincided with the NADH-dependent formazan band.

To obtain direct evidence for the participation of PMP32 and PMP29 in O₂^{•-} generation by peroxisomal membranes, the purification of both PMPs by preparative SDS/PAGE and electroelution was carried out. In reconstituted PMP29, the rate of O₂^{•-} production was 95.3 nmol of O₂^{•-}·min⁻¹·mg of protein⁻¹ when NADPH was used as inducer, whereas no O₂^{•-} production was detected with NADH (Table 2). PMP29 also exhibited CCRase activity which was specifically dependent on NADPH as electron donor. The isolated and reconstituted PMP32 exhibited NADH:FCRase activity and some NADH:CCRase activity, and was able to generate O₂^{•-} with NADH as electron donor (Table 3). The reductase activities and O₂^{•-} production were inhibited by quinacrine (Table 3). This compound is a well

Figure 2 Western blot analysis of membrane polypeptides from pea leaf peroxisomes

PMPs were separated by SDS/PAGE (15% polyacrylamide gels) of peroxisomal membranes (20 µg of protein per lane), and were transferred on to a PVDF membrane and probed with polyclonal antibodies. Lanes: 1, Coomassie Blue-stained molecular mass markers (kDa); 2, Coomassie Blue staining; 3, anti-MDHAR antibodies (1:250 dilution); 4, anti-(Cyt *b*₅ reductase) antibodies (1:1000 dilution); 5, anti-(ascorbate peroxidase) antibodies (1:500 dilution).

Figure 3 Identification of the protein involved in NADH-dependent O₂⁻ generation in peroxisomal membranes

Proteins were resolved by native PAGE in 7.5% (w/v) polyacrylamide gels, and their ability to carry out O₂⁻-dependent NBT reduction was assayed by using 1 mM NADH as inducer (lane 1). Proteins were then transferred on to a PVDF membrane and probed with anti-MDHAR antibodies (1:500 dilution) (lane 2).

known inhibitor of the flavoprotein complex of the neutrophil NADPH oxidase system [41]. The effect of quinacrine on PMP32 suggests that this polypeptide is a flavoprotein, which is in accordance with previous reports on PMP32 from castor bean endosperm [23,25,42]. All these lines of evidence suggest that PMP32 is responsible for the formation of the O₂⁻-dependent formazan band obtained in the native gels (Figure 1, lane 2).

To obtain a deeper insight into the nature of PMP32, Western blot assays were carried out in peroxisomal membranes, using antibodies against ascorbate peroxidase from pumpkin cotyledons, MDHAR from cucumber and soybean root nodules, and Cyt. *b*₅ reductase from human erythrocytes (Figure 2). PMP32 cross-reacted with antibodies against Cyt. *b*₅ reductase and MDHAR from cucumber (Figure 2, lanes 3 and 4), whereas the antibody against MDHAR from soybean nodules did not recognize PMP32 (results not shown). The antibody against ascorbate peroxidase, however, recognized a polypeptide with a molecular mass of 35 kDa (Figure 2, lane 5).

To investigate the possible involvement of MDHAR in O₂⁻-dependent NBT reduction, peroxisomal membrane proteins were separated by native PAGE (7.5% polyacrylamide gels). After

NBT staining with NADH as inducer (Figure 3, lane 1), proteins were transferred on to PVDF membranes and assayed for cross-reactivity with the antibody against cucumber MDHAR. As shown in Figure 3 (lane 2), a cross-reactivity band overlapped the NBT reduction band obtained by native PAGE.

DISCUSSION

In previous work, the integral polypeptides present in membranes from pea leaf peroxisomes were characterized [26]. At least twelve PMPs were identified by SDS/PAGE analysis of carbonate-washed peroxisomal membranes. The most prominent polypeptides had molecular masses of 61, 56, 32 and 18 kDa, although slight discrepancies could be observed in the relative proportions of each polypeptide using the Coomassie Blue or silver nitrate staining methods. This can be explained by the fact that silver ions bind to ionic side-chains of amino acids, and the colour intensity obtained depends on the proportions of these particular amino acids in the proteins rather than on the protein concentration itself [43]. In contrast, Coomassie Blue binding to many proteins is a more concentration-dependent function, although this dye is less sensitive than silver nitrate for protein staining [43].

The presence of NADH:FCRase and NADH:CCRase activities was detected in peroxisomal membranes from pea leaves, as was found previously in membranes of castor bean [42] and potato tuber peroxisomes [44]. The NADH:FCRase activity of pea leaf PMP32 was more than twice that reported for castor bean peroxisomes [42], and 33 times that reported for potato tuber peroxisomes [44]. However, the NADH:CCRase activity of pea leaf PMP32 was of the same order as that found in castor bean peroxisomal membranes [42].

The ability of peroxisomal membranes to carry out NBT reduction, mediated by O₂⁻, was assayed by an *in situ* method in gels under non-denaturing conditions. When NADH was used as inducer, only one Blue Formazan band was detected as a result of NBT reduction by O₂⁻. Analysis of this band by SDS/PAGE showed the presence of PMP32, together with other PMPs (mainly the 18, 56 and 61 kDa polypeptides) (Figure 1, lane 6). The co-purification of several polypeptides in native polyacrylamide gels occurs commonly with membrane proteins [23,38–40]. In castor bean endosperm, the co-purification of PMP57 and PMP66 with PMP32 has been described, and it was proposed that these polypeptides may be structurally or functionally related membrane components [23].

The replacement of NADH by NADPH gave a different Formazan Blue band on native PAGE, consisting of a single PMP of 29 kDa (Figure 1, lane 7). This NBT method in native gels permitted the identification, in a simple way, of two different O₂⁻-generating systems in peroxisomal membranes that use specifically either NADH or NADPH as an inducer of O₂⁻ production.

To obtain more information about these peroxisomal membrane proteins, PMP32 and PMP29 were separated by SDS/PAGE, electroeluted and reconstituted by dialysis. Due to the instability of the NADH:FCRase and NADH:CCRase activities, the peroxisomal proteins were electroeluted immediately after the purification of pea leaf peroxisomes. Under these conditions, the specific activities of NADH:FCRase and NADH:CCRase, and the O₂⁻ production capacity of the isolated PMPs, were considerably enhanced in comparison with carbonate-washed peroxisomal membranes.

The reconstituted PMP29 used cytochrome *c* and O₂ as electron acceptors and was specific for NADPH as electron donor. This polypeptide may be related to the NADPH:cytochrome *P*-450

reductase described in castor bean glyoxysomes [45], which apparently is involved in processes of xenobiotic detoxification [46]. This would explain the observed increase in the content of PMP29 in pea leaves after treatment of plants with 1 mM clofibrate [26]. Recently, the capacity of pea leaf peroxisomes to reduce NADP⁺ by endogenous glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase for the re-utilization of NADPH in peroxisomal metabolism has been demonstrated [47]. NADPH is particularly required for the ascorbate/glutathione cycle, which has been found to be present in plant peroxisomes [48]. PMP35, as judged by its immunoreactivity with the antibody to ascorbate peroxidase, appears to be the membrane-bound ascorbate peroxidase that participates in the ascorbate/glutathione cycle and whose activity was detected previously in pea leaf peroxisomal membranes [48].

PMP32 showed specificity for NADH as electron donor and can use different electron acceptors, such as O₂, cytochrome *c* and ferricyanide. The addition of quinacrine abolished O₂^{-•} production and cytochrome *c* reduction by PMP32. Quinacrine is a well known inhibitor of the NADPH oxidase of plasma membranes through its effect on the flavin moiety of the oxidase complex [41].

The presence of membrane-associated MDHAR activity has been demonstrated in peroxisomal membranes from castor bean endosperm and pea leaves [48,49]. The MDHAR characterized from pea leaves has a peroxisomal targeting sequence in its C-terminus, i.e. Ser-Lys-Ile [50]. In castor bean endosperm, MDHAR activity is due to PMP32, which was responsible for the FCRase activity characterized previously in these membranes [23]. This suggests that PMP32 of pea leaves, reported in the present work, is the MDHAR activity detected previously in pea leaf peroxisomal membranes [48]. This is further supported by the fact that PMP32 is clearly recognized by an antibody to cucumber MDHAR (Figure 2, lane 3) and also shares properties with MDHAR, including sensitivity to SH-group inhibitors, and specificity for electron acceptors and for NADH as electron donor [32,50]. The participation of MDHAR in O₂^{-•} production by peroxisomal membranes was also supported by the recognition of the NBT reduction band in native PAGE by the antibody against cucumber MDHAR (Figure 3). Very recently, Miyake et al. [51] have reported that chloroplast MDHAR mediates the production of O₂^{-•} in spinach thylakoid membranes. This indicates that the chloroplastic and peroxisomal membrane-bound MDHARs share the property of being O₂^{-•} producers, although by different mechanisms. In chloroplast thylakoid membranes, O₂^{-•} radicals are generated via O₂ reduction by MDHAR photoreduced at photosystem I [51], whereas in peroxisomal membranes O₂^{-•} radicals are produced by MDHAR (PMP32) previously reduced by NADH.

In conclusion, in peroxisomal membranes from pea leaves there are at least three O₂^{-•}-generating membrane polypeptides, with molecular masses of 18, 29 and 32 kDa. The main producer of O₂^{-•} in the peroxisomal membranes is PMP18, which is probably Cyt. *b₅* [27]. The O₂^{-•}-producing PMP32 is also NADH-dependent, and probably corresponds to the MDHAR reported previously in peroxisomal membranes. In contrast, the third O₂^{-•}-generating polypeptide, PMP29, is strictly dependent on NADPH as electron donor, and could be related to the peroxisomal NADPH:cytochrome *P-450* reductase.

O₂^{-•} production by peroxisomal membranes may be an obligatory consequence of NADH re-oxidation by the peroxisomal electron-transport chain, in order to regenerate NAD⁺ to be re-utilized in peroxisomal metabolic processes [6,21,48]. Under normal metabolic conditions, O₂^{-•} production by peroxisomal membranes is not dangerous to the cell, which is adequately

protected against these radicals. However, under certain conditions of plant stress, the release of peroxisomal membrane-generated O₂^{-•} radicals into the cytosol can be enhanced [52], producing cellular oxidative stress situations mediated by activated oxygen species. In mouse peroxisomes, a 20 kDa membrane protein has been reported to produce reactive oxygen species, and this protein is thought to be involved in the glomerulosclerosis and nephrotic syndrome [53]. Since O₂^{-•} free radicals have a short half-life under physiological conditions and are rapidly converted into H₂O₂ and O₃, the final result of these stress conditions will be an increase of H₂O₂ in the cell [52]. In recent years, H₂O₂ has been described as a diffusible transduction signal for the induction of genes encoding different cellular protectants [54]. The cytosolic production of O₂^{-•} by the peroxisomal membrane proteins, reported in the present work, also suggests an important role for peroxisomes as a source of activated oxygen transduction signals, such as O₂^{-•} and H₂O₂, that can lead to specific gene expression.

This work was supported by the DGES (grant PB95-0004-01) and the Junta de Andalucía (research group CVI 0192), Spain, and by grant CHR-CT94-0605 from the European Union. We acknowledge Dr. M. Nishimura (National Institute for Basic Biology, Ozaki, Japan), Dr. T. Yubisui (Kochi University, Japan), Dr. K. Asada (Fukuyama University, Japan) and Dr. D. A. Dalton (Reed College, Portland, OR, U.S.A.) for their donation of antibodies against pumpkin ascorbate peroxidase, Cyt. *b₅* reductase, MDHAR from cucumber and MDHAR from soybean root nodules respectively. E.L.-H. acknowledges a Ph.D. fellowship (PFPI) from the Ministry of Education and Culture, Spain.

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Received 2 June 1998/19 October 1998; accepted 12 November 1998