A dehydrogenase-mediated recycling system of NADPH in plant peroxisomes

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The presence of the two NADP-dependent dehydrogenases of the pentose phosphate pathway has been investigated in plant peroxisomes from pea (*Pisum sativum* L.) leaves. Both enzymes, glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), were present in the matrix of leaf peroxisomes, and their kinetic properties were studied. G6PDH and 6PGDH showed a typical Michaelis–Menten kinetic saturation curve, and had specific activities of 12.4 and 29.6 mU/mg protein, respectively. The K_m values of G6PDH and 6PGDH for glucose 6-phosphate and for 6-phosphogluconate were 107.3 and 10.2 μM, respectively. Dithiothreitol did not inhibit G6PDH activity. By isoelectric focusing of peroxisomal matrices, the G6PDH activity was resolved into three isoforms with isoelectric points of 5.55, 5.30 and 4.85. The isoelectric point of peroxisomal 6PGDH was 5.10. Immunoblot analyses of peroxisomal matrix with an antibody against yeast G6PDH revealed a single cross-reactive band of 56 kDa. Post-embedment, EM immunogold labelling of G6PDH confirmed that this enzyme was localized in the peroxisomal matrices, the thylakoid membrane and matrix of chloroplasts, and the cytosol. The presence of the two oxidative enzymes of the pentose phosphate pathway in plant peroxisomes implies that these organelles have the capacity to reduce $NADP^+$ to NADPH for its re-utilization in the peroxisomal metabolism. NADPH is particularly required for the ascorbate–glutathione cycle, which has been recently demonstrated in plant peroxisomes [Jiménez, Hernández, del Río and Sevilla (1997) Plant Physiol. **114**, 275–284] and represents an important antioxidant protection system against H_2O_2 generated in peroxisomes.

INTRODUCTION

The reduced coenzyme NADPH is a basic electron donor in numerous biosynthetic and detoxification reactions of living cells. The main cellular sources of NADPH are the dehydrogenases of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), the malic enzyme, and the isocitrate dehydrogenase. Plant cells have an additional source of NADPH in the ferredoxin-$NADP^+$ reductase of chloroplastic photosystem I [1].

In recent years, different studies have shown that G6PDH has a relevant role in the mechanism of protection against oxidative stress of bacteria, yeast and mammalian cells [2–6]. In plants cells, NADPH has an important role in the protection system against oxidative stress due to its involvement in the ascorbate–glutathione cycle of chloroplasts [7,8]. These evidences have supported the notion of G6PDH as an antioxidative enzyme which can be included in the group of catalase, superoxide dismutase, ascorbate peroxidase and glutathione reductase/peroxidase [9]. In higher plants, only two isoforms of G6PDH have been reported, which are localized in the cytosol and the plastidic stroma [10–12], and both forms have been purified and characterized from potato tuber [13,14].

Peroxisomes are subcellular organelles that have an essentially oxidative type of metabolism, and have catalase and H_2O_2-producing flavin oxidases as basic enzymic constituents [15,16]. Leaf peroxisomes are present in photosynthetic tissues and carry out the major reactions of the oxidative cycle of photorespiration and also contain the glycolate-pathway enzymes [15]. The presence of superoxide dismutases and the production of superoxide radicals ($O_2^{\cdot -}$) in peroxisomes was first demonstrated in plant tissues [17–21]. Different evidences found in recent years have shown that leaf peroxisomes can be responsible for a variety of induced oxidative stress situations [18,22], and also have a role in the oxidative mechanism of leaf senescence [23]. Very recently, the occurrence in pea leaf peroxisomes of the enzymes of the ascorbate–glutathione cycle (ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) has been reported [24].

In this work, using peroxisomes purified from pea leaves, the presence of the oxidative enzymes of the pentose phosphate pathway, G6PDH and 6PGDH, was demonstrated and their kinetic properties were studied. This finding implies that plant peroxisomes have the capacity to reduce $NADP^+$ to NADPH for its re-utilization in the peroxisomal metabolism, particularly in the antioxidative ascorbate–glutathione cycle of these organelles.

EXPERIMENTAL

Plant material and growth conditions

Pea (*Pisum sativum* L., cv. Lincoln) seeds, obtained from Fitó (Barcelona, Spain), were surface-sterilized with 3% (v/v) commercial bleaching solution for 3 min, and then were washed with distilled water, and germinated in vermiculite for 15 days. Healthy and vigorous seedlings were selected and grown in the greenhouse in nutrient solutions under optimum conditions [25] for 50 days.

Purification of peroxisomes

All operations were performed at 0 to 4 °C. Peroxisomes were purified from pea leaves by differential and sucrose density-

Abbreviations used: DTT, dithiothreitol; ER, endoplasmic reticulum; G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; IEF, isoelectric focusing; 6PG, 6-phosphogluconate; 6PGDH, 6-phosphogluconate dehydrogenase; PMS, phenazine methosulphate; TBST, 10 mM Tris/HCl, pH 7.6, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20, and 0.02% (w/v) NaN_3.

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gradient centrifugation (35–60 %, w/w) by the method described by López-Huertas and co-workers [26]. The 12000 ***g*** pellet, obtained in the differential centrifugation, was loaded on top of a sucrose density-gradient with the following composition: 3 ml of 60 %, 6 ml of 57 %, 9 ml of 51 %, 9 ml of 47 %, 6 ml of 42 %, and 3 ml of 35 % sucrose (w/w). Gradients were centrifuged at 83000 ***g*** for 80 min in a Beckman ultracentrifuge, using a VTi50 vertical rotor. Peroxisomes were detected in the gradients by measuring catalase activity as marker enzyme, and had intactness percentages of almost 90 % [26]. To assess possible contamination by other organelles, fumarase, acid phosphatase, cytochrome *c* reductase and fructose-1,6-bisphosphatase were used as markers for mitochondria, vacuoles, endoplasmic reticulum (ER), and chloroplasts, respectively. Peroxisomes were recovered from the gradient tubes by puncture with a syringe, and were diluted 5-fold with 100 mM potassium phosphate, pH 7.5, containing 1 mM EDTA, and were incubated on ice for 60 min with gentle magnetic stirring. The suspensions were centrifuged at 120000 ***g*** for 30 min in a Beckman 60Ti rotor, and supernatants were recovered and concentrated by ultrafiltration using a PM-10 membrane (Amicon). Under these conditions, a concentration of the peroxisomal soluble fractions of about 1.8–4.5 μg of protein/μl was obtained.

Enzyme assays and kinetic analysis

Catalase activity was determined according to Aebi [27], cytochrome *c* reductase was assayed as described by Lord [28], fumarase activity was measured by the method of Walk and Hock [29], acid phosphatase activity was determined according to Wagner [30], and fructose-1,6-bisphosphatase activity was assayed according to Lázaro and co-workers [31]. The G6PDH activity was determined spectrophotometrically by recording the sreduction of $NADP^+$ at 340 nm [32]. Assays were performed at 25 °C in a reaction medium (1 ml) containing 50 mM Hepes, pH 7.6, 2 mM $MgCl_2$ and 0.8 mM $NADP^+$, and the reaction was initiated by the addition of 5 mM glucose 6-phosphate (G6P). One milliunit of activity was defined as that amount of enzyme required to reduce 1 nmol of $NADP^+$/min at 25 °C. This activity was corrected for 6PGDH activity [32]. For the determination of 6PGDH, the reaction mixture was similar to that described for G6PDH but the substrate was 5 mM 6-phosphogluconate (6PG) [32]. For kinetic studies of both G6PDH and 6PGDH the range of concentration of each substrate was from 0.001 to 10 mM.

The kinetic data of G6PDH and 6PGDH activities were analysed by a non-linear regression method based on the rectangular hyperbola described by the Michaelis–Menten equation [33]. This non-linear plot was constructed with the aid of a computer program (Enzfiter, Elsevier Biosoft). For illustrative and comparative analyses, data were also presented as linear double-reciprocal plots. The activity ratio was defined as the relationship between enzyme activity at a substrate-subsaturating concentration and maximum velocity. The catalytic efficiency, defined as the ratio between the enzyme activity and its K_m for each substrate, was determined at saturating substrate concentrations. This parameter is an indication of the relationship between the total enzyme activity and the degree of interaction between the enzyme and its substrate.

Electrophoretic methods

Non-denaturing PAGE was performed on 5 % acrylamide gels according to Davis [34]. Samples for electrophoresis were prepared in 20 % (v/v) glycerol containing 8 mM $NADP^+$ and gels were run at a constant current of 1.5 mA/gel. Isoelectric focusing (IEF) was carried out in a Mini-Protean II slab cell (Bio-Rad) using a pH gradient of 3.5 to 7.0, as described by Palma and co-workers [35]. Samples were prepared in a solution containing 15 % (w/v) sucrose, 2.3 % (w/v) ampholytes, and 8 mM $NADP^+$. The isoforms of G6PDH and 6PGDH were visualized by incubating the gels in a solution consisting of 50 mM Tris/HCl, pH 7.6, 0.8 mM $NADP^+$, 5 mM EDTA, 2 mM $MgCl_2$, 0.24 mM Nitro Blue Tetrazolium and 65 μM PMS, which contained 10 mM G6P for G6PDH and 10 mM 6PG for 6PGDH. When blue formazan bands appeared over a colourless background (approx. 15 min) the reaction was stopped by immersing the gels in 7 % (v/v) acetic acid. The isoenzymic activity in the gels was recorded by measuring the relative absorbance of bands at 560 nm in a densitometer (model CS9000, Shimadzu, Columbia, MD, U.S.A.).

For immunoblot analyses, SDS/PAGE was carried out in 10 % acrylamide slab gels. Samples were heated at 95 °C for 5 min in 62.5 mM Tris/HCl, pH 6.8, containing 2 % (w/v) SDS, 10 % (v/v) glycerol and 10 mM dithiothreitol (DTT). Polypeptides were separated by SDS/PAGE using a Bio-Rad Mini-Protean II slab cell, and were transferred on to PVDF membranes (Immobilon P, Millipore Corp., Bedford, MA) using a semidry transfer apparatus (Novablot electrophoretic transfer unit, LKB) with 10 mM 3-(cyclohexylamino)-1-propanesulphonic (CAPS) buffer, 10 % methanol, pH 11.0, at 1.5 mA/cm² for 2 h. The same transfer procedure was used for Western blot assays of native gels. For immunodetection of G6PDH a polyclonal antibody against *Saccharomyces cerevisiae* G6PDH from Sigma was used. This antibody was affinity-purified with yeast G6PDH (Sigma), as described by Corpas et al. [36]. Goat anti-rabbit horseradish peroxidase was used as the second antibody and blots were developed by an enhanced chemiluminescence method using luminol [37]. Polyclonal antibodies against spinach glycolate oxidase [38] and pumpkin catalase [39] were used.

Electron microscopy and immunocytochemistry

Pea leaf segments of approximately 1 mm² were fixed, dehydrated and embedded in LR White resin according to Corpas and co-workers [36]. Immunolabelling was performed as indicated by Sandalio and co-workers [40]. Ultrathin sections were incubated for 2 h with either affinity-purified IgG to yeast G6PDH or IgG against glycolate oxidase and catalase, all diluted in TBST [10 mM Tris/HCl (ph 7.6)/0.9 % (w/v) NaCl/0.05 % (v/v) Tween 20/0.02 % (w/v) NaN_3] buffer. The sections were then incubated for 45 min with goat anti-rabbit IgG conjugated to 15 nm gold particles (Bio Cell) diluted 1:40 in TBST plus 2 % (w/v) BSA. Double-labelling experiments were done using both faces of the ultrathin sections. Sections were poststained in 2 % (v/v) uranyl acetate for 3 min and examined in a Zeiss EM 10C transmission electron microscope.

Other assays

Proteins were determined according to Bradford [41] using BSA as standard, and the density of the gradient fractions was calculated from the refractive index of the fractions, which was measured at room temperature using an Atago refractometer.

RESULTS

Purification of pea leaf peroxisomes

The purification of peroxisomes from pea leaves by sucrose density-gradient centrifugation is shown in Figure 1. Peroxisomes (fractions 18–22) were identified by the peak of catalase activity, the peroxisomal marker enzyme used, and were well separated

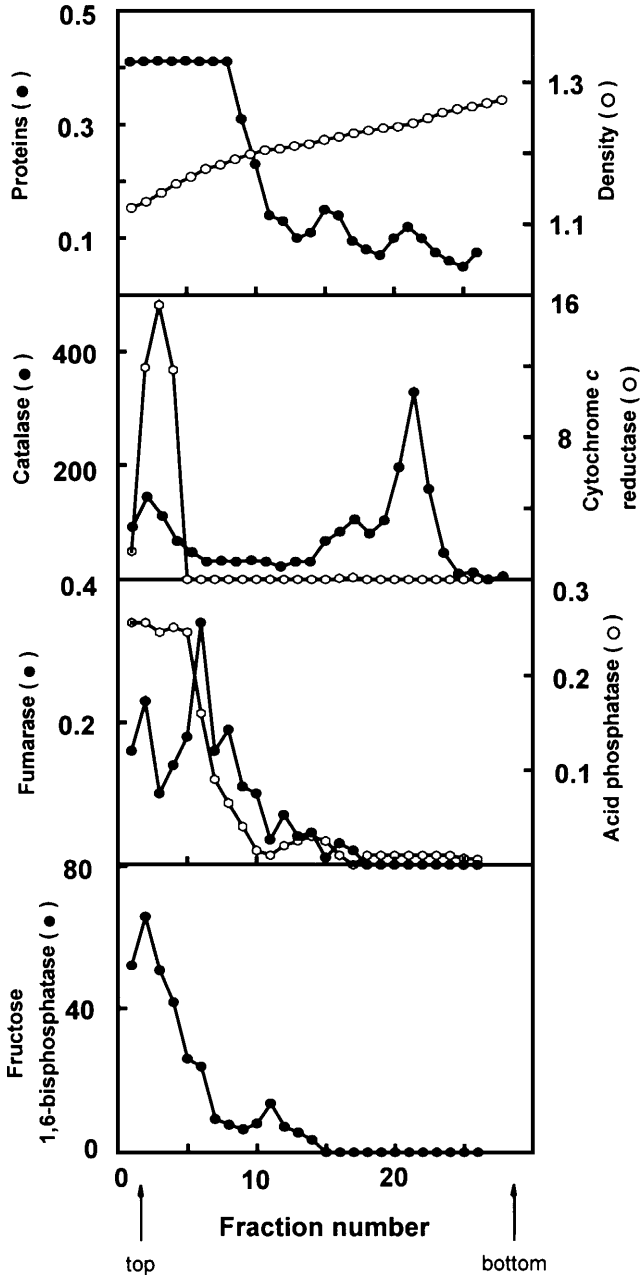

Figure 1 Purification of peroxisomes from pea leaves

Cell organelles were purified from 50-day-old pea leaves by differential and sucrose density-gradient centrifugations, as described by López-Huertas and co-workers [26]. Gradient fractions of 1.5 ml were eluted with a gradient fractionator and assayed for specific marker enzymes to localize cell organelles in the gradient: fumarase for mitochondria, cytochrome *c* reductase for ER, acid phosphatase for vacuoles, and fructose-1,6-bisphosphatase for chloroplasts. Acid phosphatase, cytochrome *c* reductase and fructose-1,6-bisphosphatase activities are expressed in nmol · min^{-1} · ml^{-1}, and catalase and fumarase in μmol · min^{-1} · ml^{-1}. Proteins were expressed as mg/ml and density as g/cm^3.

from other organelles and protein peaks. The peroxisomal fraction banded at an average equilibrium density of 1.24 g/cm^3, characteristic for these intact organelles in sucrose solutions [16,26]. The purity of peroxisomal fractions was evaluated by measuring the activity of marker enzymes of mitochondria (fumarase), vacuoles (acid phosphatase), ER (cytochrome *c* reductase) and chloroplasts (fructose-1,6-bisphosphatase). Re-

Table 1 Percentage of recoveries of each of the marker enzymes in the organelle fractions isolated by sucrose density-gradient centrifugation

Pea leaves were processed as indicated in the Experimental section, and the 12000 ***g*** particulate pellet was centrifuged in a sucrose gradient as described in Figure 1. The soluble fraction mainly contains broken chloroplasts, mitochondria, vacuoles, and ER. The percentage recovery in the subcellular fractions was calculated from the total activity of each marker enzyme in the 12000 ***g*** pellet.

		Distribution of total enzyme activity (%)			
	Catalase	Fumarase	Cytochrome *c* reductase	Acid phosphatase	Fructose-1,6-bisphosphatase
Peroxisomes (fractions 18–22)	49	0	0		0
Mitochondria (fractions 6–10)	8	80	0	16	12
Soluble fraction (fractions 1–5)	29	23	80	52	90

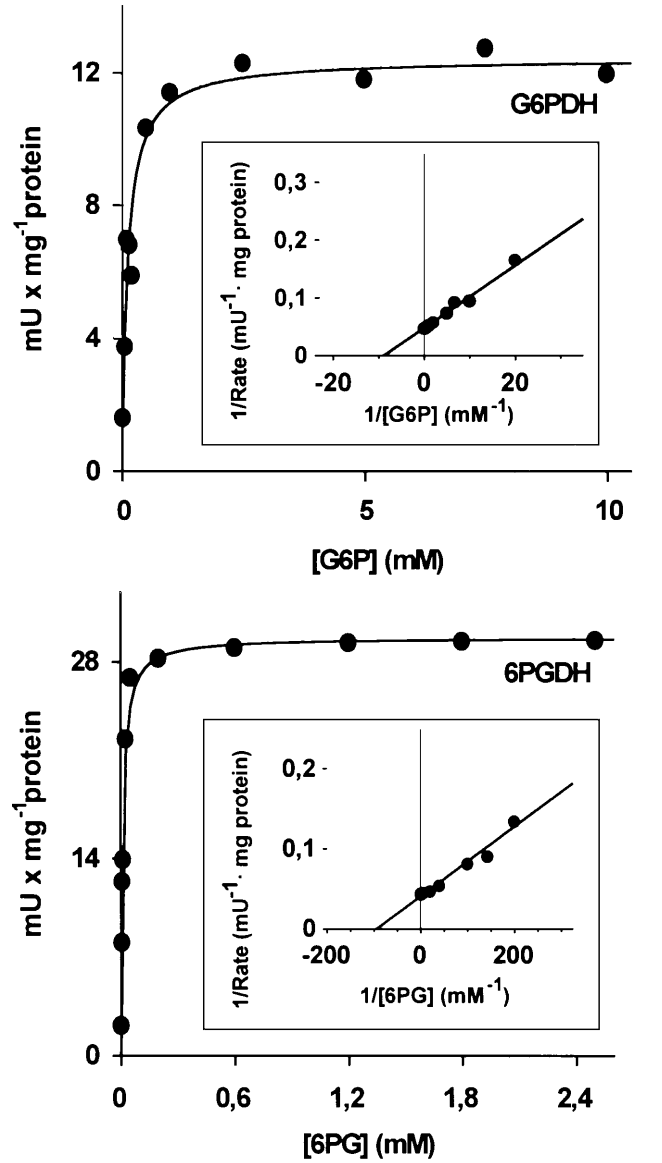

Figure 2 Effect of G6P and 6PG concentrations on the G6PDH and 6PGDH activities of peroxisomes from pea leaves

Insert graphs show the Lineweaver–Burk plot of the kinetic data. Key: ',' = '.'

Table 2 Kinetic parameters of G6PDH and 6PGDH dehydrogenase in peroxisomes purified from pea leaves

Kinetic parameters were determined by using a non-linear-regression analysis program. The quotients $V_{0.05}/V_{max}$ and $V_{0.005}/V_{max}$ were used for the activity ratio of G6PDH and 6PGDH, respectively.

Kinetic parameters	G6PDH	6PGDH
Specific activity (m-units/mg of protein)	12.4	29.6
V_{max} (μ-units)	373.2	888.7
K_m (μM)	107.3	10.2
Activity ratio	0.3	0.3
Catalytic efficiency (10^6 μ-units/M)	3.5	87.1
h	1.0	1.1

sults showed that purified peroxisomes were essentially free of contamination by mitochondria, vacuoles, ER or chloroplasts (Table 1).

Kinetic behaviour of G6PDH and 6PGDH

In peroxisomal-soluble fractions (matrices) the presence and the kinetic behaviour of the enzymes G6PDH and 6PGDH were studied by measuring the formation of NADPH, and their saturation curves and double-reciprocal plots are depicted in Figure 2. The initial rates of NADPH formation were measured as a function of G6P (from 0.01 mM to 10 mM) and 6PG (from 0.001 to 10 mM) with excess of the substrate $NADP^+$ (0.8 mM).

Both G6PDH and 6PGDH showed typical Michaelis–Menten saturation curves and the double-reciprocal plot of the data yielded a single straight line. This seems to indicate the existence in both enzymes of one active site of high affinity for its substrate [42]. Furthermore, these linear plots do not show a sigmoid shape, indicating no cooperative effect of the substrate, thus suggesting that it is unlikely that any random-order mechanism operates under steady-state conditions [43].

Since double-reciprocal plots tend to emphasize the values obtained at low substrate concentration where the degree of error is likely to be greatest [44], the data from these experiments were analysed by a simple least-squares fitting of the untransformed data to a rectangular hyperbola [33]. The kinetic parameters determined are summarized in Table 2.

There were significant differences in the specific activity, V_{max} and K_m values of both hexose monophosphate dehydrogenases. The specific activity and V_{max} of G6PDH were 2.4 times lower than those of 6PGDH, whereas the K_m value for G6PDH was about 10 times higher than that of 6PGDH. The kinetic behaviour of both enzymes shows that the catalytic efficiency of 6PGDH was about 25 times higher than that of G6PDH. Hill coefficient (h) values of G6PDH and 6PGDH were about 1, thus indicating the absence of cooperativity in the kinetics of both enzymes. The most important consequence of this kinetic behaviour is that G6PDH is a rate-limiting factor and, therefore, has a higher control in the flux of NADPH production.

After 2 h of incubation with 1 mM DTT, the peroxisomal G6PDH suffered a loss of activity of about 20% in the first 15 min and then the enzyme activity remained steady throughout the incubation time (results not shown).

Electrophoretic and immunoblot analyses of G6PDH

The G6PDH activity of yeast G6PDH, used as control, and purified peroxisomes from pea leaves was analysed by native PAGE (Figure 3a). In both cases an unique band of G6PDH activity was detected (lanes 1, and 2). The cross-reactivity by western blot of peroxisomes, after native and denaturing PAGE, with the antibody against yeast G6PDH is shown in Figures 3(b) and 3(c). The proteins recognized by the antibody in the native-PAGE blot had the same mobility as the activity bands detected in the native-PAGE gel (Figures 3a and 3b; lanes 1 and 2). In the SDS/PAGE blot of peroxisomal matrices, the antibody cross-reacted with a polypeptide of 56 kDa (Figure 3c, lane 2), and with a polypeptide of 54 kDa in the case of yeast G6PDH which was used as a control (Figure 3c, lane 1).

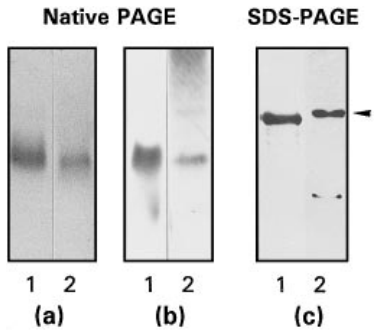

Figure 3 Isoenzyme activity of G6PDH and Western blot analysis of peroxisomes from pea leaves

Gels were stained for G6PDH activity as described in the Experimental section. For Western blot assays, peroxisomal matrices were subjected to native-PAGE and SDS-PAGE and then were transferred to PVDF membranes and incubated with a polyclonal antibody against yeast G6PDH (1/1 000 dilution). Panel **a**, native gels stained for G6PDH activity. Panels **b** and **c**, native-PAGE and SDS-PAGE blots, respectively, incubated with anti-yeast G6PDH. Lane 1, G6PDH from yeast (Sigma) (6.5 μg protein); lane 2, peroxisomal matrices (45 μg protein).

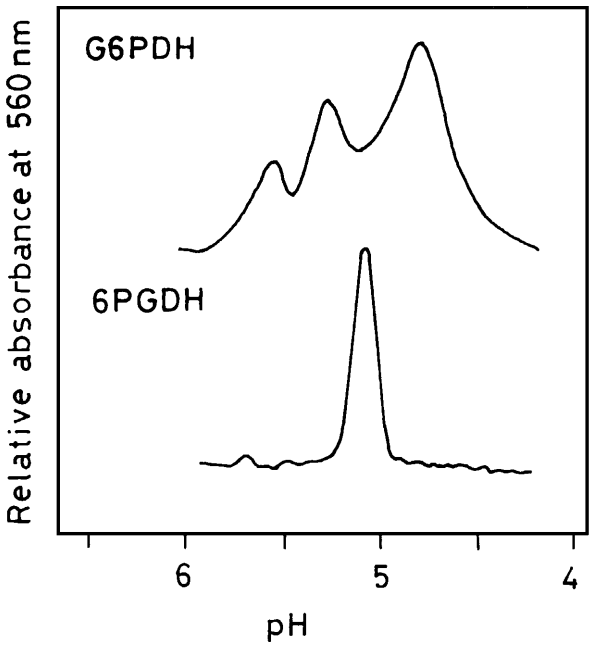

Figure 4 Densitograms of G6PDH and 6PGDH isoforms in pea leaf peroxisomes

Samples of peroxisomal matrices (180 μg protein) were subjected to IEF in a pH gradient of 3.5–7.0. G6PDH and 6PGDH isoforms were identified by activity staining, and gels were scanned at 560 nm.

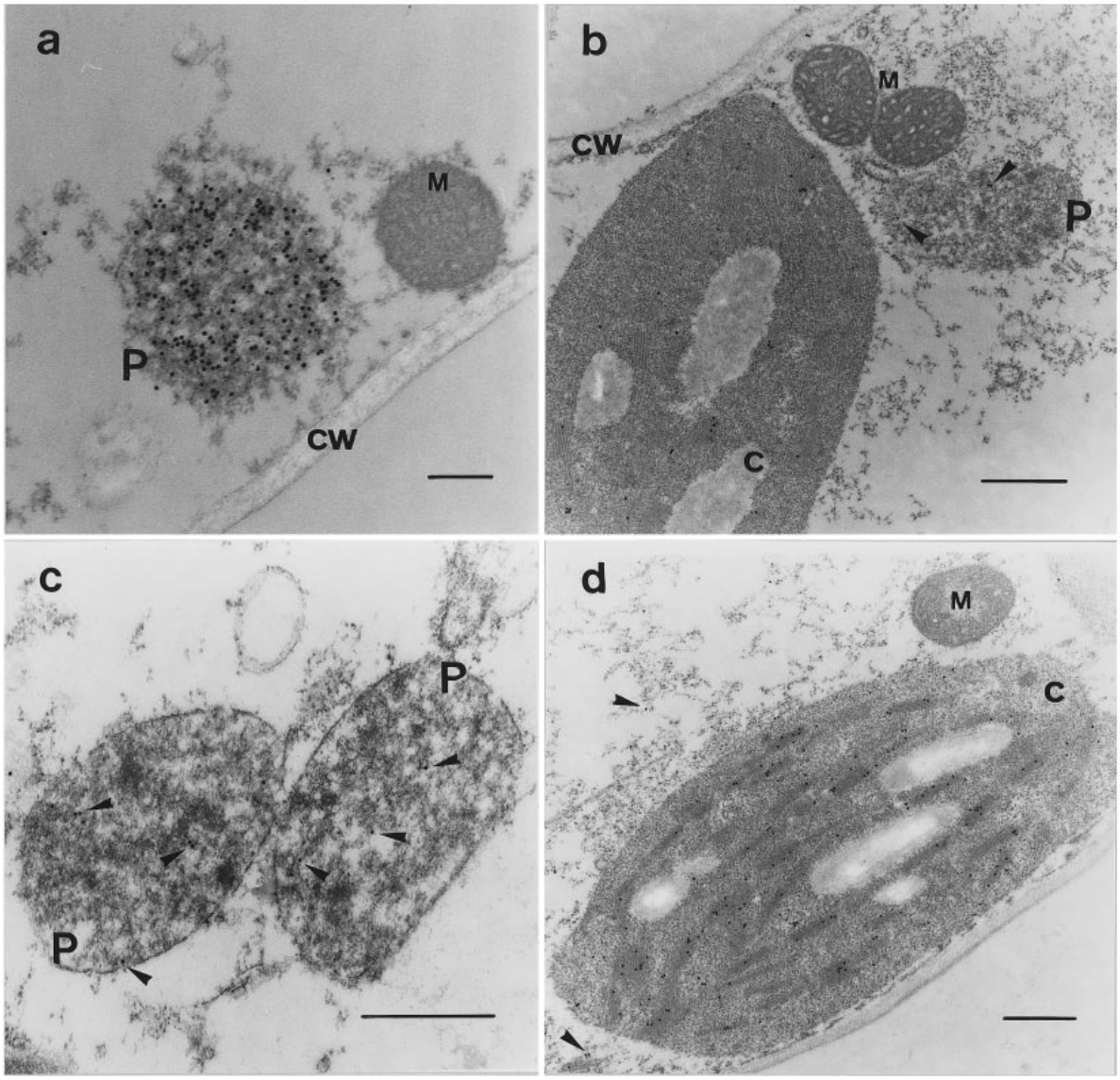

Figure 5 EM immunocytochemical localization of G6PDH in pea leaves

The electron micrographs are representative of thin sections of pea leaves showing immunolocalization of glycolate oxidase, catalase, and G6PDH. (**a**) Identification of peroxisomes by double immunogold labelling with anti-pumpkin glycolate oxidase (1/300 dilution) and anti-pumpkin catalase (1/100 dilution) with 15 nm and 5 nm gold particles, respectively. (**b**) Cell section probed with affinity-purified IgGs to yeast G6PDH (1:100 dilution). (**c**) and (**d**) Cell sections probed with anti-yeast G6PDH (1:200 dilution). Arrows indicate 15 nm gold particles. C, chloroplast; CW, cell wall; M, mitochondrion; P, peroxisome. Bars = 1.0 μm.

IEF analysis of G6PDH and 6PGDH was carried out in peroxisomal matrices. The densitometric scan of G6PDH and 6PGDH activity-stained IEF gels is presented in Figure 4. In the range of pH used (pH 3.5–7.0), three G6PDH isoforms were detected in peroxisomal matrices with pIs of 5.55, 5.30 and 4.85. However, under the same conditions only one isoform of 6PGDH was found in peroxisomes, with a pI of 5.10.

Immunocytochemical localization of G6PDH

The localization of G6PDH in pea leaf peroxisomes was also studied by EM immunocytochemistry. Representative electron micrographs of thin sections of pea leaves are shown in Figure 5. Peroxisomes have a single membrane and a granular matrix and were clearly identified by double labelling with antibodies against the peroxisomal marker enzymes catalase and glycolate oxidase (immunogold particles of 5 and 15 nm, respectively) (Figure 5a). A wide view of cellular sections labelled with affinity purified IgG to yeast G6PDH is shown in Figure 5(b). Immunogold particles clearly appeared in several cellular compartments, including peroxisomes, chloroplasts and cytoplasm (Figures 5b and 5d). A high magnification of two peroxisomes is shown in Figure 5(c). The average number of gold particles per unit area (μm^2) in each cellular compartment of pea leaf cells is shown in Table 3. Peroxisomes and chloroplasts had an area of 1.7 and 12.6 μm^2, respectively. Considering, the results of Table 3, the number of gold particles detected in the matrix of peroxisomes was on average 5.1. However, chloroplasts had an average of 127.3 immunogold particles and about 75% of them were found in the thylakoid membranes, the remaining gold particles being located

Table 3 Immunocytochemical labelling intensity of G6PDH in pea leaf cells

G6PDH-labelling density is given as the number of gold particles per μm^2. Two separately embedded blocks were used to cut sections, each of which were photographed from random fields. An average number of 15 photographs were used for quantitative analysis, and results are given as the means $\pm$ S.E.M.

Cellular compartment	No. gold particles/μm^2
Chloroplast	10.1 ± 0.8
Peroxisome	3.0 ± 0.4
Cytoplasm	0.6 ± 0.1

in the chloroplast stroma (Figure 5d). The number of gold particles found in sections treated with preimmune serum was less than 5 per cell, and were mainly located in cell walls and chloroplasts.

DISCUSSION

The results reported in this paper show for the first time the presence in purified plant peroxisomes of the oxidative enzymes of the pentose phosphate pathway, G6PDH and 6PGDH. The isolation of intact leaf peroxisomes with good yields has methodological difficulties owing to the normally small population of peroxisomes in leaf cells and their considerable lability [16,26]. The sucrose density-gradient centrifugation method used supplied adequate yields of highly purified intact peroxisomes for all the enzymic, kinetic and immunological assays of G6PDH and 6PGDH. For this study, about 4 mg of peroxisomal-soluble proteins (peroxisomal matrices) had to be used, which were prepared from 1.6 kg of pea leaves through the use of 40 different sucrose density-gradients.

The analysis of the kinetic properties of the hexose-monophosphate dehydrogenases in soluble fractions from pea leaf peroxisomes showed the presence of G6PDH and 6PGDH, and suggested that these enzymes might play a relevant role in the regulation of the NADPH production flux in the plant peroxisome. The control of the pentose phosphate shunt and other metabolic pathways in which G6PDH participates has been intensively investigated in many organisms and there is general agreement that, under most physiological conditions, G6PDH catalyses a rate-limiting reaction in the pentose phosphate pathway [45,46].

The kinetic properties of G6PDH and 6PGDH have been studied in organisms from different origins [13,32,46–52]. However, the kinetic parameters obtained for the dehydrogenases of plant peroxisomes differ from those previously reported for other organisms, and this could be a reflection of the specific roles that these enzymes can play in each cellular compartment. Important differences between the chloroplastic and peroxisomal G6PDH were observed when the activity was measured in the presence of the reducing agent DTT. It has been reported that the chloroplastic G6PDH isoform from different sources is nearly completely inhibited after 10 min incubation with DTT concentrations lower that 1 mM [48,53]. In contrast, in this work we found that 80% of initial activity of the peroxisomal G6PDH remained after 2 h incubation with 1 mM DTT. In its stability in the presence of DTT, the peroxisomal enzyme behaves in a similar way to the cytosolic form of G6PDH [13].

The analysis of the peroxisomal G6PDH by IEF showed the presence of three isoforms. In plants, the occurrence of multiple enzymic isoforms in the same cellular compartment is not uncommon. In pea leaves, glutathione reductases purified from chloroplasts and mitochondria have been resolved in five and three isoforms, respectively [54]. Another example is the cytosolic copper, zinc superoxide dismutase purified from water-melon cotyledons and separated by IEF into two isoforms [35]. In rat liver and rabbit erythroid cells three forms of G6PDH with different electrophoretic mobilities were distinguished [55–57]. Grigor [55] proposed that the different G6PDHs corresponded to reduced, oxidized and intermediate forms of the protein. At a subcellular level, the degree of oxidation of G6PDH could reflect its level of proteolytic susceptibility and might be related to the turnover of this enzyme [58]. In higher plants, the cytosolic and plastidic G6PDHs from potato have a molecular mass of 55 kDa [13] and 56 kDa [14], respectively.The molecular mass determined for the pea peroxisomal G6PDH subunit was 56 kDa, which is in the range reported for other G6PDHs from animals, microorganisms and plants [59]. The antibody to yeast G6PDH cross-reacted with peroxisomal matrices and also recognized a lower-molecular-mass band (Figure 3b, lane 2) which could be a proteolytic product of the native G6PDH. This is a reasonable possibility considering that exo- and endo-proteases are present in pea leaf peroxisomes [60,61].

The immunogold labelling techniques for electron microscopy are one of the main tools to the accurate localization of macromolecules in cell compartments [62]. The EM immunocytochemical results reported in this paper confirmed the localization of G6PDH in pea leaf peroxisomes. In animals, the peroxisomal localization of G6PDH in mouse kidney [63] and in rat liver [47] has been reported. However, to our knowledge, the EM immunocytochemical localization of G6PDH has not been carried out thus far for any organism. In higher plants, by cell fractionation methods G6PDH has been shown to be present in the cytoplasm and stroma of chloroplasts [10,11]. Results obtained in this work showed that the proportion of each G6PDH and 6PGDH activities in peroxisomes, cytosol and chloroplasts of pea leaves was about 10, 10 and 80%, respectively (results not shown). The proportion of G6PDH in peroxisomes agrees with that reported for cellular fractions of mouse kidney, where 10% of the total G6PDH activity was also found in peroxisomes [63].

In chloroplasts, G6PDH has a double localization, the thylakoidal membrane and stroma (Figure 5d). The occurrence of G6PDH in chloroplast thylakoid membranes, reported for the first time in this work, represents a new enzymic source of NADPH for these chloroplast membranes where most of the NADPH is generated by reduction from the photosystem I electron acceptor [8]. In thylakoid membranes, G6PDH could be forming complexes such as has been recently reported for several enzymes of the reductive pentose phosphate pathway [64,65]. Apparently, there are metabolic advantages to be gained from the association of the reductive pathway enzymes with the thylakoidal membrane of chloroplasts [64]. The fact that chloroplast G6PDH is inactivated by light and only active in the dark [48] means that the chloroplast thylakoid can have the advantage of a G6PDH-mediated source of NADPH for immediate metabolic requirements. The presence of G6PDH in the thylakoidal membrane and matrix of chloroplasts could be another example of the multiple compartmentation of an enzyme into chloroplasts as has been shown for copper,zinc superoxide dismutase [66] and ascorbate peroxidase [8].

The presence of the two oxidative enzymes of the pentose phosphate pathway, G6PDH and 6PGDH, in plant peroxisomes implies that these organelles have the capacity to reduce $NADP^+$ to NADPH for its re-utilization in the peroxisomal metabolism. The occurrence of NADP(H) in castor bean peroxisomes has

been demonstrated by Donaldson [67]. NADPH is necessary for the function of the NADPH:cytochrome *P*-450 reductases, whose presence has been detected in the membranes of castor bean peroxisomes [68]. Recently, in peroxisomal membranes from pea leaves three polypeptides (PMPs) with molecular masses of 18, 29 and 32 kDa have been shown to generate superoxide radicals ($O_2^{\bullet -}$) [19,21]. While the 18 and 32 kDa PMPs use NADH as electron donor for $O_2^{\bullet -}$ production, the 29 kDa polypeptide was clearly dependent on NADPH and was also able to reduce cytochrome *c* with NADPH as electron donor [19,20].

In the ascorbate–glutathione cycle, NADPH is required for the glutathione reductase activity of the cycle [7]. Up until now, the ascorbate–glutathione cycle has only been found in plants, mainly located in chloroplasts and cytosol, and is an efficient system of plant cells for the scavenging of H_2O_2 which is thus prevented from reaching toxic concentration levels [7,8,69]. Catalase in the peroxisomal matrix decomposes most of the H_2O_2 produced in these organelles as a result of their oxidative metabolism, but the catalase affinity for H_2O_2 is relatively low [70]. However, at lower levels of H_2O_2 the ascorbate peroxidase activity of the ascorbate–glutathione cycle can scavenge H_2O_2 more efficiently owing to its much lower K_m [71]. The antioxidative ascorbate–glutathione cycle has been very recently demonstrated to be present in plant peroxisomes where two of the cycle enzymes (ascorbate peroxidase and monodehydroascorbate reductase) are membrane bound [24]. A function proposed for these membrane antioxidant enzymes is the protection against H_2O_2 that might diffuse out of peroxisomes, and also of the H_2O_2 that is being continuously formed by disproportionation of the $O_2^{\bullet -}$ radicals generated in the NADH-dependent electron transport system of the peroxisomal membranes [18,19,22,24]. However, for the operativity of the ascorbate–glutathione cycle, $NADP^+$ has to be reduced to NADPH, and this recycling function could be carried out by the NADP-dependent G6PDH and 6PGDH. These two pentose phosphate pathway enzymes could be essential for the maintenance of the ascorbate–glutathione cycle operativity, which together with catalase represent an important antioxidant protection system against H_2O_2 generated in peroxisomes. This could avoid increases in the cytosolic H_2O_2 concentration during normal metabolism and under plant-stress conditions when the level of H_2O_2 generated in peroxisomes can be notably enhanced [22].

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