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Ascorbate Peroxidase[1]

A Prominent Membrane Protein in Oilseed Glyoxysomes

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The glyoxysomes of growing oilseed seedlings produce H_2O_2, a reactive oxygen species, during the β-oxidation of lipids stored in the cotyledons. An expression library of dark-grown cotton (*Gossypium hirsutm* L.) cotyledons was screened with antibodies that recognized a 31-kD glyoxysomal membrane polypeptide. A full-length cDNA clone (1258 bp) was isolated that encodes a 32-kD subunit of ascorbate peroxidase (APX) with a single, putative membrane-spanning region near the C-terminal end of the polypeptide. Internal amino acid sequence analysis of the cotton 31-kD polypeptide verified that this clone encoded this protein. This enzyme, designated gmAPX, was immunocytochemically and enzymatically localized to the glyoxysomal membrane in cotton cotyledons. The activity of monodehydroascorbate reductase, a protein that reduces monodehydroascorbate to ascorbate with NADH, also was detected in these membranes. The co-localization of gmAPX and monodehydroascorbate reductase within the glyoxysomal membrane likely reflects an essential pathway for scavenging reactive oxygen species and also provides a mechanism to regenerate NAD^+ for the continued operation of the glyoxylate cycle and β-oxidation of fatty acids. Immunological cross-reactivity of 30- to 32-kD proteins in glyoxysomal membranes of cucumber, sunflower, castor bean, and cotton indicate that gmAPX is common among oilseed species.

Peroxisomes are single membrane-bound organelles found in almost all eukaryotic cells. They typically possess at least one H_2O_2-forming oxidase and catalase that degrades the peroxide (de Duve and Baudhuin, 1966). These organelles participate in a variety of tissue-specific metabolic pathways, including ether lipid biosynthesis, cholesterol and dolichol metabolism, β-oxidation of fatty acids, and the glyoxylate cycle (Van den Bosch et al., 1992). Peroxisomes involved in the latter two pathways are specifically called glyoxysomes (Breidenbach and Beevers, 1967). In humans, the critical metabolic role of peroxisomes is manifested in patients with Zellweger syndrome, a lethal disease characterized by defective peroxisome assembly that disrupts β-oxidation of fatty acids and plasmologen biosynthesis (Bioukar and Deschatrette, 1993).

Although many peroxisomal matrix enzymes are well characterized and their roles established, relatively few PMPs have been identified with assigned functions. For example, a 35-kD membrane protein (PAF-1) restored peroxisome assembly in peroxisome-deficient Chinese hamster ovary cells (Tsukamoto et al., 1991) and fibroblasts from a patient with Zellweger syndrome (Shimozawa et al., 1992). Proteins of approximately 70 kD serve as putative ATP-binding transport proteins (Kamijo et al., 1990, 1992) in the membranes of rat liver and human fibroblast peroxisomes. Channel or pore-forming properties were associated with the peroxisomal membranes of animals, yeasts, and plants (Van Veldhoven et al., 1987; Sulter et al., 1993b; Reumann et al., 1995); however, only one specific protein, a 31-kD protein purified from the yeast *Hansenula polymorpha*, was identified with those activities. The primary structure of PMP47 from *Candida boidinii* apparently has high sequence homology to a family of mitochondrial solute carrier proteins (Jank et al., 1993). Membrane-associated, protein-targeting receptors were reported in yeast peroxisomes (McCollum et al., 1993; Liu et al., 1995) and plant glyoxysomes (Wolins and Donaldson, 1994); small GTP-binding proteins were identified as rat liver PMPs (Verheyden et al., 1992). In plants, MDAR, a 32-kD protein referred to by the authors as ascorbate-free radical reductase, was enzymatically localized to the membrane of castor bean glyoxysomes (Bowditch and Donaldson, 1990). NADH-ferricyanide reductase activity was reported in the membranes of oilseed glyoxysomes and potato tuber peroxisomes (Luster et al., 1988; Struglics et al., 1993).

Sequence analyses of cDNAs encoding several of the animal and yeast proteins mentioned above (for reviews, see Causeret et al., 1993; Sulter et al., 1993a) played an important role in establishing functions for several of these proteins. Unfortunately, a cDNA sequence encoding a plant PMP has not been isolated. Only four plant polypep-

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Abbreviations: APX, ascorbate peroxidase; cAPX, cytosolic APX; CNBr, cyanogen bromide; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; gmAPX, glyoxysomal membrane-bound APX; GPX, guaiacol peroxidase; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; MDH, malate dehydrogenase; OAA, oxaloacetate; pCMB, *p*-chloromercuribenzoic acid; PMP, peroxisomal membrane protein; PVDF, polyvinylidene difluoride; sAPX, stromal APX; SOD, superoxide dismutase; tAPX, thylakoid APX.

tides, of 30 and 70 kD in cucumber (Corpas et al., 1994) and 28 and 31 kD in pumpkin (Yamaguchi et al., 1995b), were established as authentic PMPs via immunogold labeling of cotyledon glyoxysomes. Antibodies for the localization of the cucumber PMP70 and PMP30 were raised against two nondenatured PMP complexes of 290/270 and 67 kD, respectively. The 67-kD antiserum recognized similar molecular mass polypeptides (30–31 kD) in four oilseed species, including a 31-kD polypeptide in cotton (*Gossypium hirsutm* L.) seedlings. Yamaguchi et al. (1995a) proposed that the pumpkin 31-kD PMP was an APX based on amino acid sequence similarities between two fragments of this digested PMP and cAPX.

To further characterize and possibly elucidate the function of the 31-kD PMP in cotton, we screened a cotton cDNA library with the cucumber PMP67 antibodies, affinity purified to the 31-kD polypeptide. The deduced amino acid sequence of an isolated cDNA clone had high sequence similarity to cAPXs and possessed a unique, C-terminal membrane-spanning region. This APX would complement the peroxide-scavenging mechanism of matrix catalase and protect the membrane of this organelle from oxidative damage caused by this reactive oxygen species. These results were presented in a preliminary report (Bunkelmann and Trelease, 1995).

MATERIALS AND METHODS

Plant Material

Cotton (*Gossypium hirsutm* L. cv Coker 100A glandless [kindly supplied by Dr. Donald Hendrix, U.S. Department of Agriculture Western Cotton Research Laboratory, Phoenix, AZ]) seeds were germinated and grown in darkness for 48 h according to the procedure of Chapman and Trelease (1991).

Isolation of Organelles and Preparation of Membrane Proteins from Glyoxysome Fractions

Cotton glyoxysomes and membrane proteins were isolated from 48-h-old cotton cotyledons according to the method of Bunkelmann et al. (1995) with the following modifications. All homogenizing, Suc gradient, lysing, and washing buffers contained 4 mM ascorbate, and DTT was omitted from the homogenizing buffer. Glyoxysome-enriched fractions from Suc gradients were lysed with 100 mM potassium phosphate, 1 mM EDTA, 4 mM ascorbate, pH 7.1. After the samples were centrifuged at 100,000*g* (45 min), the supernatant (glyoxysomal matrix) was assayed for APX, MDAR, and catalase activities (see below). The pellet (glyoxysomal membrane fraction), with or without extraction in 100 mM Na_2CO_3 (pH 11.5), was resuspended in 100 mM potassium phosphate, 1 mM EDTA, 4 mM ascorbate, pH 7.1, and 1.5% (w/v) laurylmaltoside (dodecyl-β-D-maltoside, Calbiochem). After the sample was centrifuged (100,000*g* for 30 min), the supernatant possessed detergent-soluble membrane proteins. The insoluble pellet was resuspended in the same detergent solution described above.

Enzyme and Protein Assays

APX (EC 1.11.1.11) activity was assayed according to the method described by Amako et al. (1994). After H_2O_2 was added, the rate of ascorbate oxidation was estimated from the maximum linear decrease in A_{290} between 0 and 1.2 min at 20°C. Reaction mixtures were deoxygenated by bubbling with nitrogen gas prior to measuring APX activity. MDAR (EC 1.6.5.4) activity was assayed according to the method of Murthy and Zilinskas (1994) except that 0.5 unit of ascorbate oxidase (Sigma) was added in the reaction mixture and the change in A_{340} was recorded between 0 and 2 min (20°C). APX and MDAR activities were examined for inhibition by incubating the reaction mixtures with 50 μM pCMB (Sigma; 1 μL of 50 mM pCMB in DMSO) for 10 min prior to initiating the reaction with peroxide or ascorbate oxidase, respectively. Catalase assays were performed as described by Ni et al. (1990a). The Na_2CO_3 (pH 11.5) extract of the glyoxysomal membranes was neutralized with concentrated HCl prior to measuring enzyme activities. Protein concentrations in organelle or PMP fractions were determined with Bio-Rad (Coomassie method) or Pierce BCA protein assay reagents, respectively, according to the manufacturers' directions. Bovine plasma γ-globulin (Bio-Rad) was the standard.

Electrophoresis and Immunoblot Analysis of Proteins

Membrane proteins in the cotton glyoxysome fractions were separated by Gly SDS-PAGE (Laemmli, 1970) and electroblotted onto a PVDF membrane according to the method of Bunkelmann et al. (1995). The λ phage β-galactosidase-PMP31 fusion protein (described later) was expressed in *Escherichia coli* and isolated according to the method of Sambrook et al. (1989) with the following modification. After the *E. coli* lysate was diluted with 3 M NaCl, the sample was centrifuged at 12,000*g* and 4°C for 30 min. The supernatant containing the fusion protein was separated in a 10% T gel by Tricine SDS-PAGE as described by Schägger and Von Jagow (1987). The proteins were electroblotted onto a PVDF membrane using a semidry blotter (Bio-Rad Trans-Blot SD) according to the method of Schägger and Von Jagow (1991) except that the cathode buffer contained 0.1% (w/v) SDS. Protein blots were probed with antiserum or affinity-purified antibodies and visualized with Fast Red as described by Kunce and Trelease (1986) or as described in a modified protocol for enhanced chemiluminescence (Smith and Campbell, 1990).

With the latter protocol, the blots were immersed in blocking solution (20 mM Tris-HCl, pH 7.8, 180 mM NaCl, 3% nonfat dry milk) and probed with primary antibodies as described by Corpas et al. (1994). After thorough rinsing, the membranes were incubated in blocking solution containing goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma, affinity-isolated antigen-specific antibody) for 1 h at room temperature. Unbound secondary antibodies were removed by washing the membranes in blocking solution without milk. The blots were briefly rinsed in water prior to immersion in the chemiluminescence solution. This solution was prepared by adding 3.2 μL of 30%

H_2O_2 (immediately prior to use) to a mixture of 200 μL of 10 mg/mL 4-iodophenol (Aldrich) in DMSO (Sigma) and 8 mg of sodium luminol (Aldrich) dissolved in 20 mL of 150 mM NaCl, 50 mM Tris-HCl, pH 8.6 (Thorpe et al., 1985). Membranes were immersed in this solution for 1 min, drained, wrapped in plastic film, and exposed to Kodak X-Omat AR film for varying times, depending on the intensity of the luminescence.

Affinity Purification of Antibodies

Antiserum prepared against a cucumber PMP67 complex recognized prominent 30- and 31-kD membrane proteins in cucumber and cotton glyoxysome fractions, respectively. This antiserum was used to obtain affinity-purified antibodies to a 31-kD cotton PMP as described by Corpas et al. (1994). To affinity purify antibodies to the λ phage fusion protein, the Y1090r$^-$ strain of *E. coli* was infected with phage (Sambrook et al., 1989) containing the partial 31-kD cDNA (described later). Two 150-mm plates, each containing 10^5 plaque-forming units of the plaque-purified clone, were overlaid with nitrocellulose filters impregnated with 10 mM isopropylthio-β-D-galactosidase and incubated at 37°C for 14 h (an identical set of control plates contained nonspecific plaque-forming units from the original λgt11 library). The filters were thoroughly rinsed with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, blocked with 1% (w/v) nonfat milk in PBS (20 mM potassium phosphate, pH 7.5, 150 mM NaCl), and incubated for 3 h (room temperature) with the cucumber anti-PMP67 antiserum diluted 1:60 in PBS with 0.1% BSA (w/v, fraction V). The filters were washed extensively with PBS without milk and briefly rinsed in water. The antibodies were eluted from the membranes with 20 mL of 200 mM Gly-HCl, 1 mM EGTA, pH 2.7, and neutralized with an equal volume of 200 mM Tris-HCL, pH 8.0. To remove antibodies that have bound bacterial antigens, the neutralized antibody preparation was incubated with the control filters for 1 h and concentrated to 1 mL with a Centriprep 30 (Amicon). The antibodies were stored in 0.1% BSA, 0.02% sodium azide at 4°C.

EM and Immunocytochemistry

Cotton cotyledons (dark grown for 48 h) were sliced with a razor blade into 0.5-mm segments, immersed in 150 mM Suc as a cryoprotectant, and high-pressure frozen with an HPM 010 high-pressure freezing machine (Bal-Tec, Middlebury, CT). The tissue was freeze substituted in anhydrous acetone with a Bal-Tec FSU 010 unit for 76 h at −90°C, 6 h at −60°C, and 6 h at −30°C and warmed to room temperature. The acetone was substituted with 100% ethanol at 4°C (four times, 10 min each), infiltrated in LR White resin (EM Sciences, Fort Washington, PA), and cold-polymerized as previously described (Bunkelmann et al., 1995). Thin sections were probed according to the method of Bunkelmann et al. (1995) with antibodies (diluted 1:1) affinity purified to the β-galactosidase-PMP31 fusion protein or with cotton anti-catalase IgGs (diluted 1:100) purified on a protein A-Sepharose column (Kunce et al., 1988). After the primary antibodies were rinsed, the sections were probed with protein A-gold (15 nm, EY Laboratories, Inc., San Mateo, CA), poststained in 2% aqueous uranyl acetate (3 min), and examined at 80 kV with a Philips (Mahwah, NJ) EM 201 transmission electron microscope.

Immunoscreening of a λgt11 cDNA Library

A λgt11 expression library constructed from poly(A) RNA isolated from the cotyledons of 24-h dark-grown cotton seedlings (Ni et al., 1990b) was immunoscreened as described by Sambrook et al. (1989) with anti-cucumber PMP67 antibodies affinity purified to the cotton PMP31 using the following modifications. The nitrocellulose filters were immersed in a 3% (w/v) nonfat dry milk blocking buffer (20 mM Tris-HCl, pH 7.8, 180 mM NaCl), treated with primary and secondary antibodies, and visualized using enhanced chemiluminescence according to the protocol described for immunoblots. A positive plaque was purified through two additional rounds of screening. Both strands of a putative PMP31 cDNA (0.8 kb) were sequenced with the dideoxy chain termination method using Sequenase, version 2.0 (United States Biochemical). Only a partial clone was isolated from the library (the 5′ untranslated region was not present).

PCR Screening of the λgt11 Library via PCR

To obtain a full-length cDNA coding for the PMP31, the 5′ region of the PMP31 cDNA in the λgt11 library was amplified using PCR (Friedman et al., 1990). In the first round of PCR (94°C for 45 s, 55°C for 1 min, 72°C for 1.5 min for 38 cycles), the cDNA of interest from 3.5×10^6 plaque-forming units was amplified using a forward *lacZ* gene primer (5′-ACTTCCAGTTCAACATCAGCC-3′) and a reverse primer (5′-TTGGTCTAAGTTCTGCTA-3′) specific for the partial clone described above. The products of this reaction were diluted 1:100 with PCR buffer and reamplified in a second round of PCR (94°C for 15 s, 56°C for 25 s, and 72°C for 2 min for 30 cycles) using two nested primers, a *lacZ* gene oligonucleotide (5′-GGTGGCGACGACTCCTGGAGCCCG-3′) and a PMP31 cDNA oligonucleotide (5′-AGCGAGCTGAAGTGGGAG-3′). The products of this second reaction were electrophoresed in an agarose gel and the longest fragments were subcloned into the TA cloning vector (Invitrogen, San Diego, CA) according to the manufacturer's directions. Three of these clones were sequenced with an Applied Biosystems ABI Prism 377 DNA sequencer; each clone contained 5′ untranslated regions.

Sequence Analyses

Nucleotide and deduced amino acid sequence comparisons were done using BLAST (Altschul et al., 1990). Sequence alignment was done using GAP from the Genetics Computer Group Sequence Analysis Software Package (University of Wisconsin, Madison).

Protease Treatment of Intact Glyoxysomes

Intact glyoxysomes (200 μL) fractionated from a Suc gradient were incubated with 0, 25, 50, and 100 μg/mL

(w/v) trypsin (Worthington Biochemical, Freehold, NJ) at 4°C. In a control experiment, glyoxysomes were lysed with 0.1% (v/v) Triton X-100 (New England Nuclear) just prior to treatment with the protease. After 1 h, protease activity was inhibited by the addition of PMSF (Sigma) at a final concentration of 1 mM. The samples were separated by Gly SDS-PAGE and electroblotted, and the membrane was probed with antisera to the cucumber PMP67 complex and/or cotton catalase (both diluted 1:500).

CNBr Cleavage and Protein Sequencing

PMP31 was excised from 13 lanes (each containing 38 μg of cotton PMPs) of a Gly SDS-polyacrylamide gel stained (1 h) with 0.1% (w/v) Coomassie blue R, 10% methanol, 0.5% acetic acid (destained overnight in 10% methanol). The polypeptide was electroeluted (350 V, 4 h) with a laboratory-made apparatus into an Amicon Centricon 10 immersed in 2.5 mM Tris containing 19 mM Gly and 0.1% SDS (degassed). The electroeluted sample was concentrated by centrifugation (according to the manufacturer's instructions), precipitated with 90% acetone at −20°C (overnight), and centrifuged at 3000*g* (60 min, 4°C). The pellet was resuspended twice in cold 90% acetone, precipitated at −20°C for 30 min, and centrifuged as above for 45 min. The pellet was resuspended in cold 100% acetone, stored at −20°C for 30 min, and centrifuged for 20 min as above; this was repeated once. After the supernatant was discarded, the protein pellet was cleaved with CNBr according to the method of Matsudaira (1990), separated by 12% T Tricine SDS-PAGE, and electroblotted onto a PVDF membrane. The membrane was stained with Coomassie blue as described by Matsudaira (1990) and the N-terminal sequence of a polypeptide fragment was determined by automated Edman degradation as described by Chapman and Trelease (1992).

RESULTS

As illustrated by the silver-stained gel shown in Figure 1 (lane 1), several prominent polypeptides were present in the laurylmaltoside-soluble membrane fraction from isolated cottonseed glyoxysomes. The presence of these polypeptides in this fraction does not necessarily establish them as genuine PMPs. For example, we previously demonstrated with immunocytochemistry that the 26-kD polypeptide, the most prominent silver-stained band in lane 1 (Fig. 1), was a tonoplast membrane protein (Bunkelmann et al., 1995). The second most prominent polypeptide in this gel had a mass of 31 kD and was immunologically related to the cucumber PMP30 that was previously localized via immunogold EM to the membrane of cotyledon glyoxysomes (Corpas et al., 1994). The antiserum that recognized these two polypeptides (30 and 31 kD) was prepared against a nondenatured 67-kD cucumber PMP complex. This antiserum was used to affinity purify antibodies to the 31-kD polypeptide in cotton. The immunoblot in lane 2 (Fig. 1) shows that this polypeptide was specifically recognized by these antibodies. Lanes 3 and 4 of Figure 1 are referred to below.

A partial-length cDNA was isolated by immunoscreening a λgt11 cottonseed library with antibodies affinity purified to the cotton 31-kD protein. This cDNA lacked an untranslated 5′ end; however, a putative full-length cDNA (1258 bp) was obtained after screening the λgt11 library via PCR. This cDNA (GenBank accession No. U37060) possessed an open reading frame of 864 bp encoding a protein with a predicted molecular mass of 32 kD. Figure 2 shows the deduced amino acid sequence of this cDNA and its sequence comparison (74% similarity) with a tobacco cAPX. A putative membrane-spanning region (double underlined) was identified near the C terminus of this polypeptide. Evidence that this cDNA encoded the 31-kD polypeptide also is illustrated in Figure 2. The single underlined polypeptide sequence was identical with the sequence obtained from an N-terminal CNBr-cleaved fragment of the 31-kD polypeptide.

Additional evidence that the isolated cDNA encoded the 31-kD polypeptide was obtained with antibodies affinity purified to the β-galactosidase fusion protein that was expressed in *E. coli* infected with the λ phage containing the partial-length clone. Lanes 3 and 4 of Figure 1 show that these antibodies specifically recognized the 31-kD polypeptide and the fusion protein on immunoblots of cotton PMPs and lysates of *E. coli*, respectively. Thin sections of cotton cotyledons also were probed with these antibodies. As shown in the electron micrograph in Figure 3A, only the boundary membrane of the glyoxysome was labeled with gold particles. Gold particles were never observed on thylakoid membranes of plastids. Figure 3B is another representative but higher magnification micrograph of a thin section probed with the same fusion protein antibodies. The gold particles clearly were restricted to the glyoxysomal boundary membrane. Figure 3C shows thin sections probed with antibodies to catalase; immunogold labeling was distributed throughout the organelle matrix. Labeling

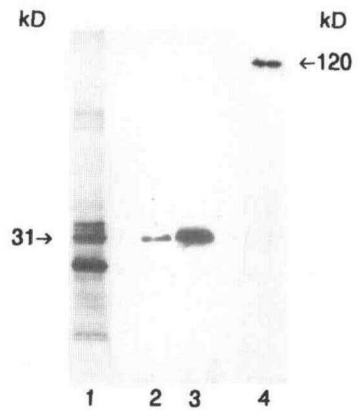

Figure 1. Silver-stained gel (lane 1) and immunoblots (lanes 2–4) of membrane proteins solubilized from isolated cotton glyoxysomes (lanes 1–3) and lysate from *E. coli* expressing β-galactosidase-PMP31 fusion protein (lane 4). Lane 1, Twenty micrograms of protein. Lane 2 (20 μg of protein), Membranes probed with cucumber antibodies affinity purified to the cotton 31-kD protein. Lanes 3 and 4 (20 and 28 μg of protein, respectively), Membranes probed with cucumber antibodies affinity purified to the β-galactosidase-PMP31 fusion protein.

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PMP31     1 ..MAFPVVDTEYLKEIDKARRDLRALIALKNCAPIMLRLAWHDAGTYDVS  48
tobac cAPX  1 MGKCYPTVSEEYLKAVDKCKRKLRGLIAEKNCAPLMLRLAWHSAGTYDVC  50
PMP31    49 TKTGGPNGSIRNEEEFTHGANSGLKIAIDFCEEVKAKHPKITYADLYQLA  98
tobac cAPX  51 SKTGGPFGTMRLKAEQGHGANNGIDIAIRLLEPIKEQFPILSYGDFYQLA  100
PMP31    99 GVVAVEVTGGPTIDFVPGRKDSNICPREGRLPDAKRGAPHLRDIFYR.MG  147
tobac cAPX 101 GVVAVEVTGGPDVPFHPGREDKTEPPVEGRLPDATKGSDHLRDVFVKQMG  150
PMP31   148 LSDKDIVALSGGHTLGRAHPERSGFDGPWTNEPLKFDNSYFLELLKGESE  197
tobac cAPX 151 LSDKDIVALSGGHTLGRCHKERSGFEGPWTTNPLIFDNSYFTELLSGEKE  200
PMP31   198 GLLKLPTDKALLDDPEFRKYVELYAKDEDAFFRDYAESHKKLSELGFTPT  247
tobac cAPX 201 GLLQLPSDKALLSDPAFRPLVEKYAADEDAFFADYAEAHLKLSELGFAEA* 250
PMP31   248 SARSKVMVKDSTVLAQGAVGVAVAAAVVILSYFYEVRKRMK*........  288
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Figure 2. Sequence homology between the deduced amino acid sequences of cDNAs encoding a putative 31-kD cotton PMP and tobacco (tobac) cAPX (accession No. U15933). A unique, putative membrane-spanning region in PMP31 is double underlined. The single underlined polypeptide sequence is identical with that obtained from a CNBr-cleaved fragment of the cotton 31-kD polypeptide. Single to double dots represent increasing similarity between amino acids; vertical lines indicate identical matches.

of glyoxysomes or any other organelle (or cytosol) was not observed when sections were probed with antibodies affinity purified to *E. coli* proteins infected with nonspecific phage from the cotton cDNA library (data not shown). These collective results show that the cDNA isolated from the cotton library encodes an authentic cotton glyoxysomal membrane protein (PMP31) that has substantial sequence homology to plant APXs.

The membrane topology of PMP31 was assessed by immunoblot comparisons of intact and permeabilized glyoxysomes treated with protease. Probing of blots with cucumber antiserum to the PMP67 complex revealed that an immunoreactive 28-kD fragment was detected only after permeabilized glyoxysomes were treated with 100 μg/mL trypsin (Fig. 4, compare lanes 4 and 8). Similar results were obtained with proteinase K (data not shown). Reprobing of the blot with catalase antiserum indicated an almost complete degradation of catalase (57 kD) at 100 μg/mL trypsin only in detergent-treated glyoxysomes (Fig. 4, compare lanes 4 and 8). Protease treatment of intact glyoxysomes did not generate a conspicuous 28-kD fragment or degrade catalase (Fig. 4, lanes 1–4).

As shown in Table I, APX activity was not measurable in the intact glyoxysomal matrix fractions because of interference that caused an increase in the A_{290}. The source of this interference was not determined. However, after the glyoxysomes were lysed and the membranes were pelleted, APX activity was reliably detected in the membrane pellet

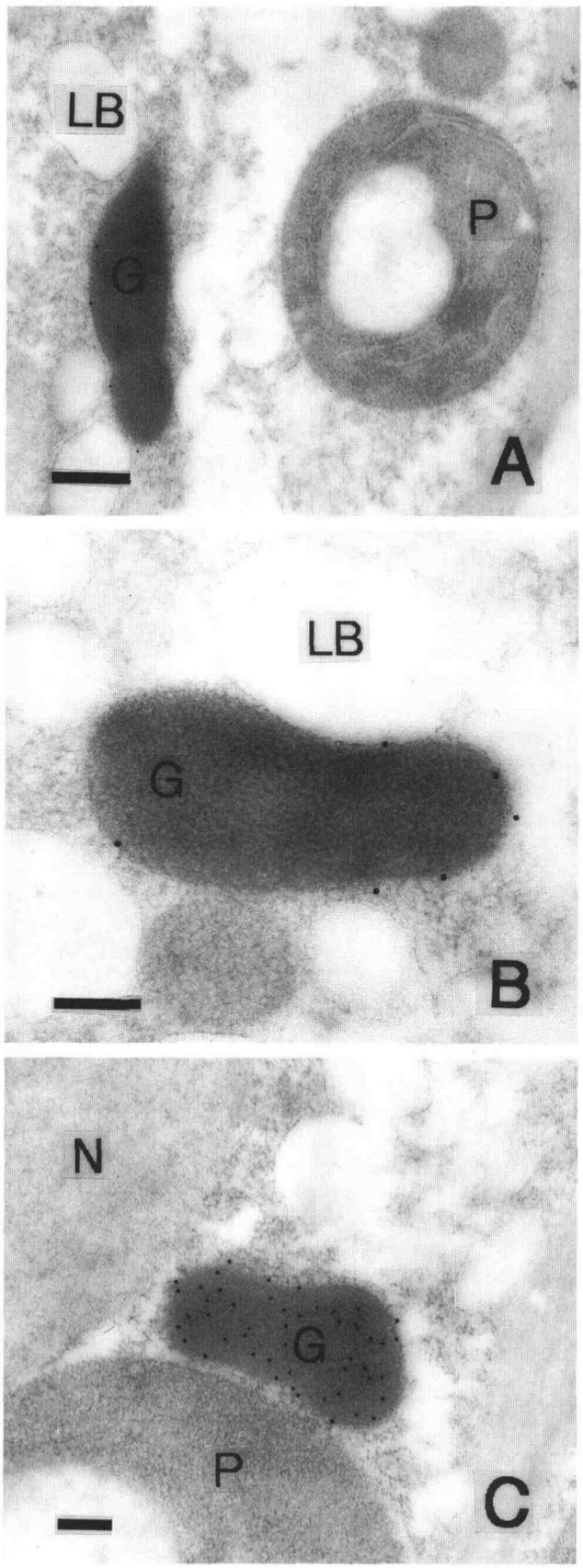

Figure 3. Immunogold labeling of glyoxysomal proteins in thin sections of high-pressure frozen, acetone freeze-substituted cotton cotyledons. A, Representative electron micrograph showing gold bound to the boundary membrane of the glyoxysome. B, Higher magnification micrograph of a thin section probed with the same antibodies as A. C, Thin section probed with catalase antibodies. A and B, Antibodies affinity purified to the β-galactosidase-PMP31-fusion protein. G, Glyoxysome; LB, lipid body; N, nucleus; P, plastid. A, Bar = 0.4 μm; B and C, bar = 0.2 μm.

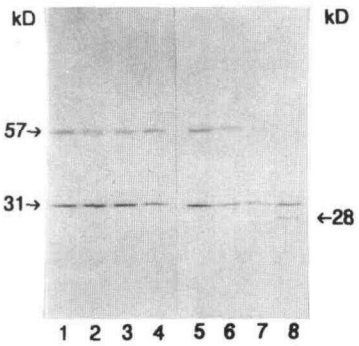

Figure 4. Immunoblots of nonpermeabilized (lanes 1–4) and detergent-permeabilized (lanes 5–8) glyoxysome fractions incubated with increasing concentrations of trypsin. Blots were first probed with cucumber antiserum to the PMP67 complex and then reprobed with cotton catalase antiserum (1:500 dilutions). Lanes 1 to 4, No detergent and 0, 25, 50, and 100 μg/mL (w/v) trypsin, respectively; lanes 5 to 8, 0.1% Triton X-100 and 0, 25, 50, and 100 μg/mL (w/v) trypsin. The SDS gel contained 31 μg of protein of the glyoxysome fraction per lane.

resuspended in detergent (glyoxysomal membranes). After centrifugation (100,000*g*) of the resuspended membranes, almost identical levels of activity were still measurable in the supernatant, the detergent-soluble membrane proteins. The solubilization of this enzyme in nonionic detergents (laurylmaltoside or octylglucoside) suggests that this APX is a membrane protein. More than 30% of this APX activity was retained with the membranes after Na_2CO_3 extraction to remove adherent matrix and peripheral membrane proteins. Although significant APX activity was lost, activity was not detected in the Na_2CO_3 extract.

Almost 75% of the MDAR activity measured in the glyoxysome fractions was localized to the detergent-soluble membranes (Table I). However, as observed for APX, Na_2CO_3 extraction of the membranes also decreased MDAR activity by approximately 70%. Likewise, MDAR activity was not detected in the Na_2CO_3 extract. Most of these decreases in APX and MDAR activities may have been caused by Na_2CO_3 inactivation rather than removal of these enzymes from the membranes. Both APX and MDAR activities were completely inhibited by preincubating the reaction mixtures with 50 μM pCMB, a thiol-modifying reagent (data not shown).

Catalase activity was predominantly localized to the matrix of glyoxysomes fractionated from the Suc gradients (Table I). Approximately 15 to 20% of the catalase activity measured in the glyoxysomes was present in the membrane fractions, and essentially all of this activity was removed from the membranes after extraction with Na_2CO_3 (trace activity was observed in the Na_2CO_3 extract).

DISCUSSION

PMP31 Is a gmAPX

Four types of APXs have been described in plants based on their subcellular location: cytosol, stroma, thylakoid membrane (cAPX, sAPX, and tAPX, respectively; for a review, see Asada, 1994), and the glyoxysome membrane (Bunkelmann and Trelease, 1995; Yamaguchi et al., 1995a). These enzymes also vary in their substrate specificity, pH optimum, lability in the absence of ascorbate, and molecular mass. PMP31 described in this paper is designated gmAPX based on its specific location in the glyoxysomal membrane of cotton cotyledons (Fig. 3).

The deduced amino acid sequence of gmAPX has a high degree of homology with several cAPXs including tobacco (Fig. 2), Arabidopsis (Kubo et al., 1992), pea (Mittler and Zilinskas, 1991), and spinach (Webb and Allen, 1995). Recently, a cDNA was isolated that encoded an isoenzyme (SAP1) of spinach APX and contained 60 amino acid residues beyond the typical C termini of cAPXs (Ishikawa et al., 1995). Although they reported that 40% of these residues were hydrophobic, little homology was observed between this region of SAP1 and the 41 amino acid residues at the C terminus of gmAPX. The authors did not determine or speculate concerning the subcellular location of SAP1. The N-terminal regions of spinach tAPX (Miyake et al., 1993) and tea sAPX (Chen et al., 1992) were sequenced by Edman degradation. We did not notice any significant sequence homology between the N termini of these chloroplastic APXs and the N terminus of gmAPX. None of the chloroplastic APXs have been cloned.

The peroxidase activity measured in the glyoxysomal membrane fraction (Table I) is not attributable to GPX, an enzyme that can utilize ascorbate as a substrate. GPX activity is not affected by the thiol-modifying agent pCMB (Amako et al., 1994), but preincubation of the detergent-solubilized PMPs with pCMB completely inhibited peroxidase activity with ascorbate. Likewise, several APXs, unlike GPX, are labile in ascorbate-free medium (Amako et al., 1994); gmAPX activity was measured only when ascorbate was included in all of the solutions used to isolate PMPs.

gmAPX likely protects the glyoxysomal membrane by scavenging H_2O_2 generated within the matrix of this organelle. During postgerminative growth, oilseeds convert stored lipids into carbohydrates via two glyoxysomal pathways, the β-oxidation of fatty acids and the glyoxylate cycle (Huang et al., 1983). H_2O_2 is generated by acyl-CoA oxidase and NADH by the multifunctional protein (Preisig-Müller et al., 1994) and MDH (Fang et al., 1987). Peroxide also is produced within glyoxysomes via the disproportionation of superoxide free radicals ($\cdot O_2^-$) by glyoxysomal SOD (Bueno et al., 1995). Superoxides are generated within the matrix of watermelon glyoxysomes (Sandalio et al., 1988) and apparently in the membranes of castor bean glyoxysomes (del Río and Donaldson, 1995). Although catalase in the matrix degrades most of the H_2O_2, its affinity for H_2O_2 is relatively low ($K_m = 0.047 \times 10^3$ to 1.1×10^3 mM; Halliwell, 1974). This would result in a lower concentration of H_2O_2 in the glyoxysome. At these lower levels, APX would scavenge H_2O_2 more efficiently than catalase because of its 5-fold lower K_m (3×10^{-2} mM for spinach sAPX; Nakano and Asada, 1987).

Based on results from the protease/detergent experiments (Fig. 4) and a putative membrane-spanning region

Table I. *Distribution of APX, MDAR, and catalase activities in fractions of isolated glyoxysomes*

Total enzyme activities were measured in eight glyoxysome-enriched fractions collected from four Suc gradients. The data presented are average values of three replicate assays. Similar patterns of data were observed in separate experiments, but statistical analyses are not included because of variations in absolute values between experiments. Activities were compared between duplicate fractions of glyoxysomal membranes that were untreated (−) or washed with Na_2CO_3 (+).

Fraction	APX		MDAR		Catalase	
	$-Na_2CO_3$	$+Na_2CO_3$	$-Na_2CO_3$	$+Na_2CO_3$	$-Na_2CO_3$	$+Na_2CO_3$
	$\mu mol\ min^{-1}$		$\mu mol\ min^{-1}$		$\mu kat\ min^{-1}$	
Glyoxysomes	nm[b]	nm	240	240	450	450
Glyoxysomal matrix	nm	nm	Trace	Trace	310	310
Na_2CO_3 extract	–[c]	nd[d]	–	nd	–	84
Glyoxysomal membranes	4500	1500	180	55	94	Trace
Detergent-soluble[a] membranes	4400	1400	170	50	74	Trace
Detergent-insoluble pellet	nd	nd	Trace	Trace	14	Trace

[a] Laurylmaltoside (1.5%). [b] nm, Not measurable. [c] –, Not measured. [d] nd, Not detected.

near the C-terminal end of the protein (Fig. 2), the majority of cotton gmAPX, including the active site, is predicted to be on the matrix side of the glyoxysome. In a model proposed by Yamaguchi et al. (1995a), however, the active site of the pumpkin gmAPX is exposed to the cytosolic side of the glyoxysome and scavenges H_2O_2 that diffuses out of this organelle. Their model is based on the latent activity of APX in isolated glyoxysomes and leaf-type peroxisomes.

Corpas et al. (1994) reported that cucumber PMP30 and cotton gmAPX (PMP31) were immunologically related and that the cucumber polypeptide had a native mass of 67 kD. These data suggest that gmAPX is a homodimer, a characteristic of cAPX (Patterson and Poulos, 1995).

Regeneration of Ascorbate

The scavenging of H_2O_2 by APX requires the continuous regeneration of ascorbate from MDA and/or DHA. In chloroplasts, PSI generates superoxide radicals that are disproportionated to O_2 and H_2O_2 by SOD. The H_2O_2 is scavenged by tAPX and sAPX, and ascorbate is regenerated from MDA directly by Fd or by NAD(P)H via stromal MDAR (Asada, 1994; Allen, 1995). If the MDA radical disproportionates to DHA, then stromal DHAR can catalyze the reduction of DHA using glutathione (Foyer and Halliwell, 1977; Nakano and Asada, 1981). The enzyme glutathione reductase would reduce the glutathione disulfide using NADPH. This latter pathway involving the regeneration of ascorbate from DHA also occurs in soybean root nodules (Dalton et al., 1986).

In castor bean glyoxysomes, a putative 32-kD membrane protein was shown to have MDAR activity with NADH (Bowditch and Donaldson, 1990). Within cotton glyoxysomes, the majority of MDAR activity also was measured in the detergent-soluble membrane fraction (Table I). The results from these two studies indicate that ascorbate is regenerated from MDA by a glyoxysomal membrane-bound MDAR. We did not investigate whether an ascorbate-glutathione pathway mentioned above for chloroplasts and root nodules also occurs in glyoxysomes, but Klapheck et al. (1990) reported that, at least in castor bean endosperm, APX, DHAR, MDAR, and glutathione reductase activities were mostly cytosolic. However, they also measured significant MDAR and glutathione reductase activities in organelle fractions of mitochondria and plastids, but not glyoxysomes.

Regeneration of Glyoxysomal NAD^+

Although NAD^+ is required in glyoxysomes for the continued β-oxidation of fatty acids and operation of the glyoxylate cycle, the mechanism of regenerating NAD^+ has not been resolved. Rat liver peroxisomes are apparently freely permeable to NADH/NAD^+ in vitro (Van Veldhoven et al., 1987), but this permeability does not seem to occur in yeast and plant peroxisomes. Van Roermund et al. (1995) concluded that *Saccharomyces cerevisiae* peroxisomes were impermeable to NADH in vivo and that MDH within these peroxisomes regenerated NAD^+ by reducing OAA to malate. They proposed that malate was shuttled to the cytosol and oxidized by cytosolic (or mitochondrial) MDH to OAA, and OAA was transported into the peroxisome.

Donaldson et al. (1981) reported that NADH was impermeable to the membranes of glyoxysomes isolated from castor bean endosperm. As discussed earlier, Bowditch and Donaldson (1990) proposed that a membrane-associated MDAR could oxidize NADH on the matrix side of the glyoxysomal membrane; however, they suggested that electrons were transferred to acceptors outside of the glyoxysomes. Because Klapheck et al. (1990) did not measure MDAR activity in castor bean glyoxysomes, they concluded that NADH must cross the glyoxysomal membrane for reoxidation. Mettler and Beevers (1980) proposed that a malate-aspartate shuttle between glyoxysomes and the mitochondria could account for the production of glyoxysomal NAD^+ without direct transport of NADH to mitochondria. In this pathway, glyoxysomal MDH would reduce OAA to malate and oxidize NADH to NAD^+.

We propose a pathway for the oxidation of NADH within the glyoxysome that does not involve transferring electrons across the glyoxysomal membrane. In the model illustrated in Figure 5, catalase and gmAPX scavenge H_2O_2 produced by SOD and the β-oxidation of fatty acids within the glyoxysome, whereas ascorbate is regenerated for gmAPX activity by the MDAR-catalyzed reduction of MDA using NADH. Although this model may not repre-

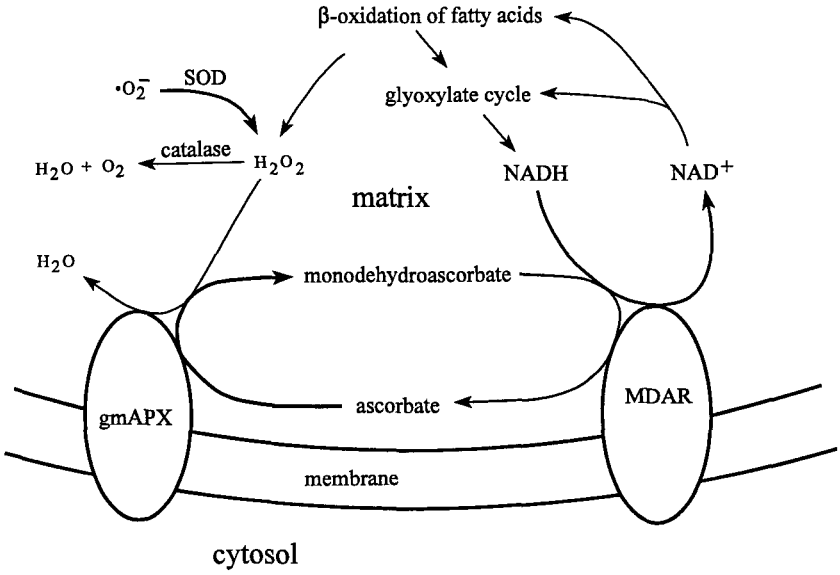

Figure 5. Proposed model illustrating H_2O_2 scavenging and reoxidation of NADH within oilseed glyoxysomes.

sent the primary mechanisms for peroxide scavenging and NAD^+ regeneration in glyoxysomes, it is likely an essential pathway for the protection of this membrane from reactive oxygen species and provides a coupled means for reducing H_2O_2 and regenerating NAD^+ necessary for sustaining seedling growth after germination.

gmAPX Is a Common Protein among Oilseed Species

In several species of oilseed seedlings, polypeptides of approximately 31 kD were identified as prominent glyoxysomal membrane proteins. A 31-kD PMP was reported for castor bean endosperm (Luster et al., 1988) and for sunflower (Jiang et al., 1994), cotton (Chapman and Trelease, 1992), and pumpkin cotyledons (Yamaguchi et al., 1995a, 1995b). A 30-/32-kD PMP was reported for cucumber cotyledons (Kruse and Kindl, 1982; Corpas et al., 1994). Corpas et al. (1994) immunocytochemically localized the cucumber PMP30 to the glyoxysomal membrane, and the same antiserum was used to localize the gmAPX to the cotton glyoxysomal membrane (Fig. 3). They also showed that the proteins listed above (except for pumpkin, which was not analyzed) were immunologically related on immunoblots. Therefore, we propose that all oilseed glyoxysomes possess a gmAPX that participates in the scavenging of H_2O_2, thereby protecting the membrane of this organelle during the mobilization of lipid reserves necessary for seedling growth and development.

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