Plant seed peroxygenase is an original heme-oxygenase with an EF-hand calcium binding motif*

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A growing body of evidence indicates that phytoxylipins play important roles in plant defense responses. However, many enzymes involved in the biosynthesis of these metabolites are still elusive. We have purified one of these enzymes, the peroxygenase (PXG), from oat microsomes and lipid droplets. It is an integral membrane protein requiring detergent for its solubilization. Proteinase K digestion showed that PXG is probably deeply buried in lipid droplets or microsomes with only about 2 kDa at the C-terminal region accessible to proteolytic digestion. Sequencing of the N terminus of the purified protein showed that PXG had no sequence similarity with either a peroxidase or a cytochrome P450 but, rather, with caleosins, i.e. calcium-binding proteins. In agreement with this finding, we demonstrated that recombinant thale cress and rice caleosins, expressed in yeast, catalyze hydroperoxide-dependent mono-oxygenation reactions that are characteristic of PXG. Calcium was also found to be crucial for peroxygenase activity, whereas phosphorylation of the protein had no impact on catalysis. Site-directed mutagenesis studies revealed that PXG catalytic activity is dependent on two highly conserved histidines, the 9 GHz EPR spectrum being consistent with a high spin pentacoordinated ferric heme.

Plant peroxygenase (PXG) is a versatile oxygenase that is strictly hydroperoxide-dependent for its activity. First discovered as an hydroxylase (1), it has been subsequently identified as a sulfoxidase (2) and an epoxidase (3, 4). But detailed studies have revealed that only a single mechanism accounted for these different activities. In brief, peroxygenase catalyzes the direct transfer of one oxygen atom from a hydroperoxide, which is reduced into its corresponding alcohol to a substrate which will be oxidized (5). Accordingly, peroxygenase catalyzes hydroxylation reactions of aromatics, sulfoxidations of xenobiotics, or epoxidations of unsaturated fatty acids (Fig. 1). In mammals, most of these reactions are performed by cytochrome P450 monoxygenases. But it was shown that in contrast to such enzymes, PXG activity does not require any cofactor such as NAD(P)H and does not use molecular oxygen, excluding that PXG is encoded by a classical P450 monoxygenase (1, 3). Presently, the function of PXG is not fully established. Associated with epoxide hydrolase, it composes “the peroxygenase pathway,” which is involved in the oleic acid cascade leading to the synthesis of the C18 cutin monomers (6, 7). However, PXG was also found to be very active in microsomes of plants such as soybean that possess cuticles poor in these C18 cutin precursors, therefore suggesting additional physiological role(s) for this enzyme (7). Accordingly, it has been suggested that the peroxygenase pathway also leads to the formation of epoxy- and trihydroxy derivatives of linoleic acid (8, 9), which are involved in defense responses against fungal infection in rice or tomato (10–12). The peroxygenase pathway also constitutes one branch of the so-called “lipoxygenase pathway,” where, as the first step, lipoxygenase catalyzes oxygenation of unsaturated fatty acids (C18:2, C18:3, or C16:3), yielding the corresponding trihydroxy derivatives of linoleic acid (8, 9), which result in the production of jasmonates and volatile aldehydes, respectively. In contrast, little is known at the molecular level on the enzymes involved in the other branches of the lipoxygenase pathway. Although the molecular mechanism of the peroxygenase is now better defined, the gene encoding this protein and the nature of the gene product are still unknown. Thus, one primary goal of the present investigation was to identify the PXG gene and to compare the encoded protein with other oxygenases such as cytochromes P450 and peroxidases.

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§ The abbreviations used are: PXG, peroxygenase; LD, lipid droplet; MOPS, 4-morpholinepropanesulfonic acid; HPLC, high performance liquid chromatography; CHAPS, [3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ER, endoplasmic reticulum; 13-HPOD, 13-hydroperoxyoctadec-9,11-dienoic acid; AIF-1, allograft inflammatory factor-1.
Plant Peroxygenase as a Calcium-dependent Enzyme

Materials—Commonly used chemicals and reagents were of the highest purity available. Purified oligonucleotides were provided either by Invitrogen, Eurogentec, or by Sigma Genosys. [1-14C]Oleic acid (52 Ci/mol) and [1-14C]Linoleic acid (55 Ci/mol) were purchased from PerkinElmer Life Sciences. Rice seeds (Oryza sativa, var. indica, cv. IR64) soaked for 2 h in water were germinated on two layers of cloth saturated with water under a photoperiod of 16 h. Plants were harvested 7 days later, frozen in liquid nitrogen, and kept at −80 °C until use.

Preparation of Oat Subcellular Fractions—Isolation of microsomal fraction from oat seeds soaked overnight in water was performed essentially as described previously for soybean seedlings (3). During this procedure (after the second centrifugation step at 100,000 × g) a floating layer consisting of lipid droplets was collected from the top of the tubes with a pipette. The crude lipid droplet fraction was carefully washed with 100 mM potassium pyrophosphate buffer that contained 0.1 M sucrose (pH 7.4). After centrifugation at 100,000 × g for 45 min, the lipid droplet fraction was then resuspended in 10 mM potassium phosphate buffer containing 0.1 M sucrose (pH 7.4) and centrifuged at 100,000 × g for 45 min. LDs were finally resuspended in 10 mM Tris-HCl buffer containing 1% glycerol (pH 8).

Purification of Oat Peroxygenase—All the subsequent steps were performed at 4 °C. Washed microsomes or LDs (30 mg protein) resuspended in 5 ml of a 10 mM Tris-HCl buffer (pH 8) containing 10% glycerol (buffer A) were treated with emulphogene (polyoxyethylene 10 tridecyl ether from Sigma, final concentration 0.2% v/v) for 45 min. The mixture was then centrifuged at 100,000 × g for 45 min. The supernatant was applied to a 1 × 2-cm column of DEAE-Trisacryl M (BioSepra) equilibrated with buffer B (buffer A containing 0.2% emulphogene v/v)). After the column was washed with buffer B to eliminate the first protein peak, peroxygenase activity was eluted with a linear NaCl gradient (0 → 1 M) in buffer B (2 × 30 ml). The flow rate was 0.5 ml/min, and 5 ml fractions were collected. The fractions containing the peak of peroxygenase were pooled and dialyzed overnight against 2 × 2 liters of 10 mM sodium acetate (pH 5.5) containing 10% glycerol and 0.2% (v/v) emulphogene buffer C. The dialyzed fraction was applied on a 1 × 10-cm column of CM-Sepharose CL-6B (GE Healthcare) equilibrated with buffer C. After the column was washed with buffer C, peroxygenase activity was eluted with a linear NaCl gradient (0 → 1 M) in buffer C (2 × 30 ml). The flow rate was 0.5 ml/min, and 5 ml fractions were collected. This purification protocol was repeated three times to isolate sufficient amounts of protein. The fractions containing the peak of peroxygenase activity were pooled and dialyzed overnight against buffer C and applied on a column of CM-Sepharose CL-6B. Peroxygenase activity was eluted as described above. The total amount of proteins was measured after each purification step by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

Subcloning of AtPXG1, AtPXG2, and OsPXG—Full-length AtPXG1 (At4g26740) and AtPXG2 (At5g55240) were amplified from an Arabidopsis cDNA library by PCR using primers AtPXG1F and AtPXG1R and primers AtPXG2F and AtPXG2R.
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Table 1

| Name       | Sequence (5’ to 3’)                      |
|------------|------------------------------------------|
| OsPXGFR    | GCTCTAGATTATTTATCATCATCATTTATAA          |
| OsPXGFR    | GCTCTAGATTAGTGGTGGTGGTGGTGGTGGTAG        |
| H52V       | GCCAACACCAGAGAACAGAAGAGTTTTCTAACAGAAGGCC |
| H59V       | CGCACTTGCAGCTGTTTCTCTGTTCTCTTTCAATGATC |
| H70V       | CTTAGGTTCCTACATCTGTGTTGTTCTCCTCTTCTCATGTC |
| T116VF     | CCTGACCCTTGCATTGGGTTGTTGGTCCTAGGCTGACC |
| H313V      | CCTTGTCTCTTCTTTATCTCTTTACACAACAGATCAAAGC |
| H134V      | CCCCATTATATATACACAACAATATTTTTAAA         |
| H138V      | CAAGATGATGATGATGATGATGATGATGATGATGATGAT |
| K196VF     | GGAATGATGATGATGATGATGATGATGATGATGATGAT |
| C221G      | GCTCTAGATTAGTGGTGGTGGTGGTGGTGGTAG        |
| C230G      | GCTCTAGATTATTTATCATCATCATTTATAA          |

respectively (Table 1). To facilitate cloning, BamHI or XbaI restriction sites were added to the primers. For the further purification of AtPXG1, PCR was performed using primers AtPXG1NH and AtPXG1CH to add a His tag at either the N- or C-terminal ends of the PXG gene. We used also PCR for purification of AtPXG1, PCR was performed using primers (modified codons are underlined, and the nucleotide changes are indicated in bold in Table 1).

Enzymatic Activities—Peroxygenase activity was routinely measured (e.g. during the purification procedure) with aniline as substrate (2). Sulfoxidase activity was assayed by using either methyl p-tolyl sulfoxide or thiobenzamide as substrates, as previously described (19, 20). Epoxidation of [1-14C]oleic and linoleic acids was performed according to Bléo and Schuber (3). The metabolism of 13-hydroperoxy-[1-14C]octadecadienoic acid was studied as described before (5).

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit of Stratagene with the sense mutations primers described in the PCR primers sections (modified codons are underlined, and the nucleotide changes are indicated in bold in Table 1).

Heme Content Determination—The heme staining procedure was carried according to Noordermeer et al. (21). Hemin (from Sigma) was used as standard for the quantification of heme at 370 nm.

Oat Antibodies and Western Blot Analysis—The production of rabbit polyclonal antibodies was performed using standard immunization protocols. Proteins were fractionated by 15% SDS-PAGE and electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a mini-transblot transfer cell apparatus (Bio-Rad). High precision Protein™ Standards (Bio-Rad) were used as molecular weight markers. For detection of AtPXG:His, a mouse monoclonal anti-His antibody and an anti-mouse antibody conjugated to peroxidase were used at 1:500 and 1:5000 dilutions, respectively. Blots were developed using the ECL kit from Pierce.

N-terminal Sequencing of PXGs—After separation by SDS-PAGE using a 12.5% polyacrylamide gel, the proteins were transferred to a polyvinylidene difluoride membrane. Edman degradation was performed with an automated sequenator (Applied Biosystems 492 Procise).

Proteolysis—Microsomes were washed in 50 mM Tris/HCl buffer (pH 8) containing 10 mM CaCl₂ and resuspended in an equal volume of this buffer. Aliquots of microsomes (100 μl containing 1.5 mg proteins) were incubated with increased quantities of protease K (from 15 to 750 μg) in a total volume of 175 μl at 37 °C overnight. Aliquots of 50 μl were removed for immediate measure of PXG residual activity. The proteinase was then inhibited by the addition of 1 mM phenylmethylsulfo-
nyl fluoride. A similar protocol was used for proteinase K treatment of LDs.

Phosphorylation Experiments—Proteinase-treated microsomes were incubated in the presence of 0.1 unit of casein kinase II and 4 µCi of [γ-35S]ATP in 100 mM KH2PO4 buffer (pH 7.5) containing 8 mM MgCl2 for 4 h at 27 °C. Proteins and peptides separated by SDS/PAGE were transferred to a polyvinylidene difluoride membrane for immuno- and radio-detections. Similar protocol was used for the phosphorylation of purified AtPXG1 except that this fraction was incubated for 1 h at 4 °C to preserve enzymatic activity.

Synthesis of Radiolabeled Substrates—Racemic 9,10-[1-14C]epoxystearic acid (1.9 GBq/mmol) and 13(S)-[1-14C]-hydroperoxyoctadeca-9(Z),11(E)-dienoic acid were synthesized as previously described (3).

Electron Paramagnetic Resonance (EPR) Spectroscopy—Conventional 9-GHz EPR measurements were performed using a Bruker ER 300 spectrometer with a standard TE102 cavity equipped with a liquid helium cryostat (Oxford Instrument) and a microwave frequency counter (Hewlett Packard 5350B).

RESULTS

Purification of PXG from Oat Seed Microsomes—Peroxygenase activity was purified from oat seeds and was found to be localized to microsomal fractions. The membrane-bound peroxygenase could not be released by treatment with 3 M KCl, confirming that the enzyme is an integral membrane protein (19). We, therefore, tested various detergents (CHAPS, BIG-CHAP, octylglucoside, Triton X-100, emulphogene) for their ability to solubilize the enzyme. Emulphogene (0.2%) was among the most effective. The solubilized membrane extract was purified according to the protocol described under “Experimental Procedures.” The instability of the peroxygenase caused severe problems especially during dialysis and concentration of the pooled fractions, but the addition of glycerol (10%) contributed to some extent to stabilize PXG (Table 2).

Further purification using strong cationic exchanger (Mono S) or hydrophobic column (alkyl-Superose) resulted in a loss of enzyme activity. It is unclear whether this loss of activity is the result of inactivation or irreversible binding of the enzyme to the columns. Analysis on SDS-PAGE of the final fractions containing PXG activity showed a bulk of proteins of low molecular weight near the migration front but also a band around 27 kDa, whose intensity was correlated with peroxygenase activity (Fig. 2). The N terminus sequence of this protein (AVVVSDAMSS) belongs to a small family called caleosin (24) because they contain a calcium binding domain and seem to possess similar structural features with oleosins, which are found in lipid droplets. This apparent localization of caleosin (24) because they contain a calcium binding domain and seem to possess similar structural features with oleosins, which are found in lipid droplets. This apparent localization of caleosin prompted us to examine if lipid droplets display PXG activity.

Purification of PXG from Oat Seed Lipid Droplets—Purified lipid droplets isolated from oat seeds were able to perform co-oxidation reactions known to be catalyzed by peroxygenases. For example, they actively oxidized thiobenzamide to its sulfone (65.8 nmol/min/mg of protein) and oleic acid into 9,10-epoxy-stearate (51.4 nmol/min/mg of protein). They should be noted that these activities were about four times higher than those determined in microsomal fractions.

Consequently, we performed the purification of PXG from lipid droplets following the same protocol used for the microsomal fraction. Detergent was required to solubilize the peroxygenase activity from lipid droplets, suggesting that the protein was buried into the phospholipid monolayer or lipid core of
these organelles. Silver nitrate staining of the purified enzyme fraction separated by SDS-PAGE showed two major bands at about 40 and 27 kDa. But only the intensity of the latter was correlated with the activity of the peroxygenase. The N terminus sequence of this 27-kDa protein (AVVVSDAMSSVAKGAPVTAQRPVXXD) was identical to that of the protein isolated from oat seeds microsomes (but extended the sequence by some amino acids) and, thus, also showed homologies with caleosins (48 and 43% with ATS1 and EF27, respectively). Considering that most of caleosins identified so far have a molecular mass around 25–29 kDa, we hypothesized that the purified 27-kDa protein might be a caleosin supporting peroxygenase activity.

Expression of Caleosins in Yeast and Identification as Plant Peroxygenases—To validate that PXG was indeed a caleosin, we have expressed in yeast the first caleosin identified in Arabidopsis (At4g26740, also named ATS1 or AtClo1 (22, 25)). Crude extracts of yeast expressing the recombinant protein catalyzed co-oxidation reactions typical of peroxygenase such as sulfoxidation of thiobenzamide (5.8 nmol/min/mg of protein), hydroxylation of aniline (4.5 nmol/min/mg of protein), or epoxidation of oleic acid (1.5 nmol/min/mg of protein). Importantly, all these activities were strictly hydroperoxide-dependent. Yeast crude extracts were then subfractionated by differential centrifugations into 100,000 g supernatant, microsomes, and lipid droplets. Whereas microsomes and lipid droplets actively catalyzed co-oxidation reactions (for example, 62 and 124 nmol/min/mg of protein of thiobenzamide sulfoxide formed, respectively), the soluble fraction was found inactive. Neither extract from wild type WA6 nor yeast transformed with an empty vector showed any catalytic activity. Based on these experiments we annotate At4g26740 encoding peroxygenase as AtPXG1.
sponding genes (At5g55240 and X89891, respectively) were expressed in yeast. Fig. 3 shows that both microsomal and oleosomal fractions prepared from the transformed yeasts catalyzed the oxidation of thiobenzamide, aniline, and oleic acid with similar efficiency as AtPXG1. Taken together, these results show that plant peroxygenases are caleosins.

Characterization of Purified AtPXG1—To further characterize AtPXG1 in regard to PXG activity, a His$_6$-tagged version of AtPXG1 was expressed in yeast and was used for purification by affinity chromatography on a Ni$^{2+}$ column. SDS-PAGE of the purified enzyme showed one single band at 32 ± 4 kDa as revealed by silver nitrate staining and Western blot analysis using an anti-His-tag antibody. The purity of AtPXG1 was 98% as assessed by scanning densitometry. The recombinant enzyme is somewhat larger than predicted, suggesting that it might be covalently modified, as was previously reported for this protein but also for an oleosin (22, 27). To tentatively identify this modification, we used site-directed mutagenesis. First we replaced threonine residues at positions 15 and 116 and the asparagine at position 109 by a valine. These positions were predicted to be possible sites of $O$- and $N$-glycosylation by the programs NetOgly 1.0, YinOyan, and Net-N-Glyc, respectively. These mutations did not affect the molecular weight of the recombinant proteins.

Another posttranslational modification is SUMOylation of proteins, resulting in particular in an enhancement of their stability (28). Analysis of AtPXG1 sequence revealed that it possessed a high sumoylation potentiality at the position Lys-196 (SUMOplot™ Prediction). However, replacement of Lys-196 with a valine resulted in a protein with the same mass as the wild type enzyme. Together, it seems that glycosylation or sumoylation processes were not responsible of the modification of the mass of the recombinant protein. To ensure that we have purified a peroxygenase rather than another oxidase, we used the purified AtPXG1 to provide additional proof of the identity of the 32-kDa recombinant protein as a peroxygenase.

AtPXG1 Is a Hemoprotein—We had previously demonstrated that the soybean peroxygenase contains an iron protoporphyrin IX (heme) that supports catalytic activity (3). Thus, we looked for the presence of a heme in AtPXG1, although there is no report on the presence of a prosthetic group in caleosins.

First, a peak at 407 nm, representative of the Soret band of the hemoproteins, could be detected in the light absorbance spectra of the purified fraction of AtPXG1. Second, the addition of cumene hydroperoxide (Fig. 4, inset) resulted in a gradual decrease of this Soret band. As found for the soybean peroxygenase (3), such an effect was correlated with a decline of hydroperoxide supported oxidations by AtPXG1. Fig. 4 shows the clear correlation between these two phenomena. Both the decrease in the absorbance at 407 nm and the enzyme inactivation follow pseudo-first order kinetics with similar half-lives (about 3.5 min). Together, these results confirmed that AtPXG1 contains a heme responsible of its enzymatic activity.

AtPXG1 Catalyzed Intramolecular Oxygen Transfer—We next verified that purified AtPXG1 was capable of catalyzing the intramolecular transfer of oxygen in fatty acid hydroperoxide in the absence of other oxidizable compound as we found previously for the partially purified soybean peroxygenase (5). For this purpose, purified AtPXG1 was incubated in the sole presence of 13-[$^{14}$C]HPOD, and the products of the reaction were analyzed by radio-HPLC (Fig. 5). After 1 h of incubation at 26 °C, about 80% of the fatty acid hydroperoxide was transformed into a polar compound (peak 1, elution time 3.3 min). The mass spectra of its methyl ester-trimethyl silyl ether derivative was identical to those of Me$_3$Si derivative of methyl 9,10,11-trihydroxyoctadecenoate (29). This compound likely derived from the spontaneous chemical hydrolysis of 9,10-epoxy-11-octadecenoic acid, which was found unstable at acidic pH. Peak 2 (elution time, 31.3 min) was 13-hydroxy-octadec-9,11-dienoic acid, the methyl ester-trimethylsilyl ether derivative of this compound co-chromatographed with authentic standard, and the mass spectra of the two compounds are identical. Peak 3 represented the residual substrate. Thus, AtPXG1, like the soybean peroxygenase, was capable of catalyzing the reduction of fatty acid hydroperoxide with the concomitant formation of an epoxyalcohol.

AtPXG1 Catalyzed Stereospecific Oxidation Reactions—Another characteristic of the soybean peroxygenase is the high
stereospecificity of the catalyzed alkylaryl sulfide oxidation and unsaturated fatty acid epoxidation. We found that when AtPXG1 was incubated in the presence of methyl p-tolyl sulfide and cumene hydroperoxide as an oxygen donor, the (S)-sulfoxide was formed with ~60% enantiomeric excess. If oleic acid was used as substrate instead of sulfide, AtPXG1 produced cis-9,10-epoxyoctadecenoic acid, which consisted largely in the 9(R),10(S) enantiomer (70%). Thus, AtPXG1 catalyzed asymmetric co-oxidation reactions.

Inhibition of AtPXG1—Characterization of the soybean peroxynase has revealed that it is sensitive to some inhibitors. Of these, we have tested β-mercaptoethanol and the organophosphorus terbufos. The sulfoxidation of thiobenzamide, catalyzed by AtPXG1, was completely abolished in the presence of 1 mM β-mercaptoethanol, which probably acts as a competitive inhibitor. In contrast, terbufos is a suicide substrate for plant peroxynases, and at a concentration of 3 mM, it effectively inactivated the activity of AtPXG1. Together, these results conclusively demonstrate that AtPXG1 is a peroxynase.

Characterization of the Heme Microenvironment Using EPR Spectroscopy and Site-directed Mutagenesis—The way heme proteins interact with their prosthetic group largely determines their catalytic activity and biological functions. In cytochrome P450, a cysteine thiolate was identified to be the heme axial ligand, whereas a histidine is coordinated to the heme in horseradish peroxidase. The 9 GHz EPR spectrum of peroxidases reflects structural features of the heme active site and correlates to the oxidation and spin states of the heme iron as well as the coordination number of the iron (for review, see Ref. 31). The EPR signal with two main resonances at $g_{\text{eff}}^x \approx 6$ and $g_{\text{eff}}^z \approx 2$ are characteristic of ferric heme iron ($S = 5/2$) in the high spin state. In particular, horseradish peroxidase shows a rhombically distorted EPR signal with $g_x = 6.44$, $g_y = 5.05$, and $g_{Bz} = 1.96$ (Fig. 6). In agreement, the crystal structure of this enzyme showed that the heme iron is pentacoordinated, with His-170 being the axial ligand, and His-42 being the catalytically relevant residue in the distal heme side. We have used low temperature EPR spectroscopy to characterize the heme site in the oat peroxynase. The spectrum (Fig. 6) agrees well with those reported for pentacoordinated high spin heme enzymes, with resonances at $g_x = 6.22$, $g_y = 5.53$, and $g_{Bz} = 1.98$. The small but significant difference in rhombicity of the EPR signal of peroxynase as compared with horseradish peroxidase is not surprising since the distal side residues (and/or their relative position to the heme iron) are not necessarily the same in these enzymes. It was shown that the ferric 9 GHz EPR spectrum is very sensitive to changes in the distal heme side in mono- and bifunctional peroxidases. Moreover, even among peroxidases, such differences in the rhombicity of the signal was observed. For example, one of the isoforms of turnip peroxidases showed an EPR signal (see Fig. 1 in Ref. 33) very similar to the one observed in AtPXG1.

4 E. Bleé, unpublished results.

FIGURE 5. Reverse phase-HPLC analysis of the products formed after incubation of 13-HPOD in presence of purified AtPXG1. 13-HPOD (60 μM, 50 × 10⁶ cpm) was incubated in presence of purified AtPXG1 (100 μg of protein) in 300 μl of acetate buffer (0.1 M (pH 5.5)) for 1 h. Products of the reaction were detected by radioactivity (a and b) and at 234 nm (c). Peak 1, 9,10,13-trihydroxy-11-octadecenoic acid; peak 2, 13-hydroxy-octadeca-9,11-dienoic acid; peak 3, 13-HPOD.
His-138 resulted in a complete loss of detectable peroxygenase activity. Mutations of the highly conserved His-70 and the resulting proteins after their purification by affinity chromatography. Only two of these mutations affected the hydroxylase activity of the resulting proteins, indicating strongly that these residues were not involved in the coordination of the distal histidine as the sixth ligand to the heme iron as in the case of cytochromes (see Ref. 35 and references therein).

To assess the identity of the axial ligand in plant peroxygenases, we have used site-directed mutagenesis on AtPXG1. First we have ruled out any implication of cysteine in heme coordination by mutating Cys-221 and Cys-230, the only two Cys residues present in the primary sequence of AtPXG1. Their point mutation to glycine did not modify the enzymatic activities of the mutated proteins, indicating strongly that these residues were not involved in the coordination of the heme in AtPXG1. Sequence alignment of plant caleosins shows that a certain number of histidines are conserved, including those corresponding to His-52, His-59, His-70, His-131, His-134, and His-138 of AtPXG1. To investigate the role of these histidines, we have constructed mutants in which each of these residues was replaced by valine. In addition, to avoid any interference, a FLAG peptide (DYKDDDDK) was inserted for purification of the mutant H70V, devoid of its prosthetic group and probably misfolded, was more sensitive to destruction by the yeast proteasome. Obviously, the exact role(s) of His-70 and His-138 need further clarification.

**FIGURE 6.** The 9-GHz EPR spectra of oat peroxygenase (top) and horseradish peroxidase (HRP) (bottom) in the resting (ferric) state. Experimental conditions: temperature, 4.2 K; microwave frequency, 9.42 GHz; modulation amplitude, 4 G; modulation frequency, 100 kHz; microwave power, 2 milliwatts.

![Diagram](image)

**FIGURE 7.** Requirement of calcium for PXG activity. A, conservation of the EF-hand loop in AtPXG1, AtPXG2, EFA 27, tescalin, and allograft inflammatory factor-1. The consensus EF-hand domain consists of a long helix-loop-helix array. Only the EF-hand loop was aligned for AtPXG1 (Arabidopsis thaliana, At4g26740), AtPXG2 (A. thaliana, At5g55240), EFA 27 (O. sativa, X 89891), Tsc (Mus musculus, AAH19492), and AIF-1 (Homo sapiens U49392). The most common amino acids that bind Ca²⁺ at the X, Y, and Z positions include aspartate (D) and asparagine (N). The residue at position Y donates a carbonyl oxygen, whereas a glutamate (E) or an aspartate (D) is present at position Z. The glycine at position G permits a bend in the EF-hand loop, and the residue at position I is hydrophobic (isoleucine, valine) (59). The arrow indicates the aspartate at position X of AtPXG1 that was mutated to valine to obtain a putative Ca²⁺ binding-deficient mutant (D75V). B, Ca²⁺ dependence of AtPXG1 activity. Oxidations activities: thiobenzamide sulfoxidation (black dots) aniline hydroxylation (black stripes), and oleic acid epoxidation (gray) were measured for AtPXG1 (1), for AtPXG1 after extensive dialysis of the fraction containing the purified enzyme (2), for AtPXG1 after dialysis followed by the addition of 1 mM CaCl₂ in the medium (3), and for the mutant D75V (4).

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**Plant Peroxygenase as a Calcium-dependent Enzyme**

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**AtPXG1**

D I D D D N G I I Y P W E T

**AtPXG2**

D I D D D N G I I Y P W E T

**EFA 27**

D I D D D N G I I Y P W E T

**Tsc**

Y D S D S D G R I T L E E Y

**AIF-1**

D I D D D N G I I Y P W E T
domains within AtPXG1 that may coordinate \( \text{Ca}^{2+} \) ions (Fig. 7A). Accordingly, the caleosin EFA27 was found to bind calcium in rice (23). To define a possible role of calcium in the structure and/or activity of AtPXG1, we have modified the EF-hand domain of AtPGX1 by site-directed mutagenesis. It was shown that mutation of the first amino acid of the EF-hand loop, an Asp coordinating \( \text{Ca}^{2+} \), abrogates calcium binding by AIF-1 and Tsc (41, 42). On this basis, we replaced the Asp at position X of the EF-hand with Val as shown by the arrow in Fig. 7A. This point mutation did not alter the peroxygenase activity (Fig. 7B), possibly suggesting that calcium had only an insignificant role in the structure/activity of plant peroxygenases. To substantiate this point, we have extensively dialyzed AtPXG1 against chelating agent (EDTA) to remove any trace of metal in the protein. This treatment completely abolished co-oxidation properties of AtPXG1, which could be restored (up to 70%) by adding 1 mM \( \text{CaCl}_2 \) to the medium (Fig. 7B). From these experiments, it appears that \( \text{Ca}^{2+} \) ions are present in AtPXG1 and are required for its structural integrity, but that mutation of the first aspartate in the EF-hand motive is not sufficient to abolish function of AtPXG1.

**Phosphorylation of AtPXG1 Does Not Modify Its Catalytic Activity**—Although AtPXG1 has several putative phosphorylation sites, it is unclear whether it binds phosphate and, if so, how it affects the enzyme activity. We verified the phosphate binding potential of AtPXG1 by incubating the protein in the presence of casein kinase and \(^{35}\text{S}\)ATP. A similar reaction without casein kinase served as a negative control. Fig. 8 shows that AtPXG1 does bind phosphate and that casein phosphorylation sites are responsible for this binding. However, phosphorylation did not result in any change in the sulfoxidation of thiobenzamide catalyzed by AtPXG1. This indicated that phosphorylation is probably not a required modification for catalytic activity.

**Association of PGX with Membranes**—PXG can only be extracted from oat membranes (lipid droplets or microsomes) with detergents, raising the question of its mode of association with lipid mono- or bilayers. It has been speculated that caleosins might adopt different tertiary structures depending whether they bind to the ER or LDs (43). Caleosins binding to ER were suggested to adopt a type I orientation, i.e. with the N-terminal domain on the lumen side and the C terminus facing the cytosol. According to this hypothesis, the active site of PXG would face the ER lumen, whereas the phosphorylation sites would be in contact with the cytosol. When bound to lipid droplets, it was suggested for thermodynamic reasons that caleosins expose both their polar N- and C-terminal domains to the cytosol (43, 44). To experimentally test these hypotheses, we used digestions with a protease to identify the fragments of PXG that are proteolytically protected and, hence, presumably buried within the ER lumen or in the core of the LDs. We have first used a proteinase K versus microsomes (or lipid drop-
lets) ratio of 0.04 because such conditions were previously shown to completely degrade caleosins incorporated in artificial lipid droplet fractions, suggesting that only a small fragment of about 2 kDa was accessible to proteolytic action. This fragment is presumably located at the C terminus of PXG for the following reasons: (i) the 25-kDa truncated PXG can no more be phosphorylated, (ii) a putative phosphorylation site for casein kinase II (SLFE), which is strongly conserved among caleosins, is located at position 225–230 in AtPXG1, and (iii) the molecular mass of the peptide corresponding to the Ser-225–C terminus sequence in AtPXG1 is about 2.4 kDa (Fig. 9, a and b). The fact that the truncated 25-kDa protein cannot be further degraded even in the presence of high amounts of proteinase K (Fig. 9, a and b) indicates that most of PXG is inaccessible because it is presumably deeply buried within membranes, in the ER lumen, or in the core of lipid droplets. A protein band with a molecular mass of 54 kDa was also revealed by Western blot both in microsomes and lipid droplets (Fig. 9a). This value corresponds closely to that expected for a dimeric form of PXG. Intriguingly, the disappearance of this band upon proteolytic treatment was correlated with the loss of PXG activity. These results may, thus, indicate that PXG is catalytically active as a dimer only.

DISCUSSION

PXG Is a Unique Oxygenase—Our data identify a plant peroxygenase as a caleosin and consequently ascribe an enzymatic function to this class of proteins. This study revealed also that caleosins are hemoproteins. Such results were unforeseen because caleosins were mostly typified by the presence of a single EF-hand calcium binding domain that is particularly conserved in all plant and fungal species described to date (25). No function has been attributed to these proteins, although it has been speculated that they might play a role in membrane fusion and lipid trafficking through Ca2+–mediated processes (46). We have demonstrated here that calcium was indispensable for the enzymatic activity of PXG. Because this activity was not modulated in vitro by addition of calcium (not shown), our results are instead consistent with a role for this ion in preserving a catalytically active conformation of the protein. Such a function in...
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maintaining the structure in the heme environment has already been shown for one of the archetypal heme-containing peroxidases; that is, the horseradish peroxidase, where only one Ca\(^{2+}\) is essential for the protein structure (47, 48). PXG does not share any significant sequence similarity with peroxidases nor with any member of the large cytochrome-P450 family. However, these three classes of hemoproteins share similarities in their catalytic mechanisms. For example, all three are thought to use an oxo-ferryl intermediate in their reactions that might be held by the pressure of a peptide on one pyrrole ring as a result of conformational change during freezing, for example. The fact that PXG has a histidine as the axial ligand but is a "pure" peroxygenase raises some intriguing questions concerning its mechanism. The distal machinery of both peroxidases and cytochrome P450s is thought to be critical in determining whether these enzymes act as oxygenases or peroxidases (53–56). Therefore, the further identification of structural elements and crucial residues of the active site of PXG should help in better understanding the mechanistic features of this atypical hemoprotein.

Structural Considerations—The name caleosin originates from the putative oleosin-like association of these proteins with lipid droplets (24, 25). From a structural point of view, oleosins are characterized by a long hydrophobic central domain, which adopts a hairpin conformation and which, after crossing the outer phospholipid monolayer, is embedded into the neutral lipid core of the lipid droplet. In contrast, the amphipathic N- and C-terminal domains of the oleosins are located at the surface of the LDs where they interact with polar head groups of the phospholipids (57). Caleosins also possess these three domains, but two of them are characterized by additional key features; the N-terminal region contains a calcium binding EF-hand motif, and the C-terminal region has putative protein kinase phosphorylation sites (24, 43). Accordingly, we could localize the active site of the Ca\(^{2+}\)-dependent PXG within the N terminus and also establish that the enzyme could be phosphorylated by casein kinase II. Concerning the hydrophobic domain, in silico analysis of the hydropathy profile of 20 caleosins revealed that they can be divided into three classes depending on the location of this domain in the primary structure of these proteins (Fig. 10b). Although located centrally in class I, the hydrophobic region is shifted to the N terminus in class II and appears randomly located in fungal caleosins (Fig. 10b). Strikingly, these three subfamilies perfectly match the three classes determined by constructing a phylogenetic tree with the full-length sequences of the caleosins (Fig. 10a). Class I groups most of plant caleosins exhibiting the key structural domains of oleosins. These caleosins are present in seeds but also in vegetative tissues, stems, or siliques (14, 23, 39). Some of them are inducible by abiotic stress or abscisic acid (23, 39). On the other hand, very little is known on plant caleosins from Class II. Their hydrophobic domain is closer to the N-terminal region, but this does not necessarily imply that these proteins are not membrane-bound since AtPXG5 was recently found in the pollen coat (58). The gene encoding this latter caleosin was placed in tandem with At1g23250 on chromosome 1, probably resulting from a fairly recent duplication. A similar duplication might also have occurred to give rise to AtPXG4 and At1g70680 on the same chromosome 1. Finally, the last class comprises yet uncharacterized caleosins from fungi. Although the significance of such a classification remains to be elucidated, it raises intriguing possibilities related to PXG specificities and functions. All classes of caleosins share two histidine residues and the calcium binding site required for PXG activity, except At1g 23250 from Class II. We have demonstrated that three members of Class I act as peroxygenases. Catalytic activity as PXG of fungal caleosins and members of Class II remains to be confirmed. The possibility that fungal caleosin could catalyze the formation of anti-fungal epoxy and hydroxy fatty acid derivatives raises the question of the physiological relevance of such compounds in fungi and of the regulation of their biosynthesis during plant-pathogen interaction. The present identification of the gene encoding plant peroxygenase should give a firm basis for forthcoming studies on the physiological importance of this enzyme.

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REFERENCES

1. Ishimaru, A., and Yamazaki, I. (1977) J. Biol. Chem. 252, 6118–6124
2. Bélè, E., and Durst, F. (1987) Arch. Biochem. Biophys. 254, 43–52
3. Bélè, E., and Schuber, F. (1990) J. Biol. Chem. 265, 12887–12894
4. Hamberg, M., and Hamberg, G. (1990) Arch. Biochem. Biophys. 283, 409–416
5. Bélè, E., Wilcox, A. L., Marnett, L. J., and Schuber, F. (1993) J. Biol. Chem. 268, 1708–1715
6. Bélè, E., and Schuber, F. (1993) Plant J. 4, 113–123
7. Lequeu, J., Fauchon, M.-L., Chambon, A., Bronner, R., and Bélè, E. (2003) Plant J. 36, 155–164
8. Hamberg, M., and Hamberg, G. (1996) Plant Physiol. 110, 807–815
9. Bélè, E. (1996) in Lipoxgenase and Lipoxgenase Pathway Enzymes (Piazza, G. J., ed) pp. 138–161, AOCS Press, Champaign, IL.
10. Ohta, H., Shida, K., Peng, Y.-L., Furuwasa, I., Shishiyama, J., Aibara, S., and
