Characterization of the Heme Environment in *Arabidopsis thaliana* Fatty Acid α-Dioxygenase-1

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Abstract

Plant α-dioxygenases (PADOX) are hemoproteins in the myeloperoxidase family. We have used a variety of spectroscopic, mutagenic, and kinetic approaches to characterize the heme environment in *Arabidopsis thaliana* PADOX-1. Recombinant PADOX-1 purified to homogeneity contained 1 mol of heme bound tightly but noncovalently per protein monomer. Electronic absorbance, electron paramagnetic resonance, and magnetic circular dichroism spectra showed a high spin ferric heme that could be reduced to the ferrous state by dithionite. Cyanide bound relatively weakly in the ferric PADOX-1 heme vicinity (K_d ~10 mM) but did not shift the heme to the low spin state. Cyanide was a very strong inhibitor of the fatty acid oxygenase activity (K_i ~5 µM) and increased the K_m value for oxygen but not that for fatty acid. Spectroscopic analyses indicated that carbon monoxide, azide, imidazole, and a variety of substituted imidazoles did not bind appreciably in the ferric PADOX-1 heme vicinity. Substitution of His-163 and His-389 with cysteine, glutamine, tyrosine, or methionine resulted in variable degrees of perturbation of the heme absorbance spectrum and oxygenase activity, consistent with His-389 serving as the proximal heme ligand and indicating that the heme has a functional role in catalysis. Overall, *A. thaliana* PADOX-1 resembles a b-type cytochrome, although with much more restricted access to the distal face of the heme than seen in most other myeloperoxidase family members, explaining the previously puzzling lack of peroxidase activity in the plant protein. PADOX-1 is unusual in that it has a high affinity, inhibitory cyanide-binding site distinct from the distal heme face and the fatty acid site.

Fatty acid α-dioxygenases are hemoproteins that have been found in many plants, including tobacco, thale cress (*Arabidopsis thaliana*), rice, pea, and cucumber (1), and form one subfamily of the myeloperoxidase (MPO) family of proteins (2). The cyclooxygenases, also known as prostaglandin H synthases (PGHSs), and linoleate diol synthase represent two other MPO hemoprotein subfamilies known to have fatty acid oxygenase activity (2).
The plant dioxygenases convert a variety of fatty acids to the corresponding 2-hydroperoxides (Reaction 1).

\[
R-\text{CH}_2-\text{COOH}+\text{O}_2 \rightarrow R-(\text{OOH})-\text{COOH}
\]

**REACTION 1**

The 2-hydroperoxides undergo spontaneous decarboxylation to shorter aldehydes and fatty acids (3). The plant dioxygenase reaction differs from plant lipoxygenases and the cyclooxygenases in that the oxygen addition does not target an unsaturated region in the fatty acid (1). The initial fatty acid α-dioxygenase cloned from *A. thaliana*, termed PADOX-1 (for plant α dioxygenase-1), has about 20% amino acid identity with human PGHS-1 and −2 (4). A second isoform (PADOX-2) has been proposed recently (1), based on analysis of *A. thaliana* and other plant cDNA sequences. In the absence of crystallographic information for any PADOX, structural models for MPO itself (5) and the PGHS isoforms (6–8) provide a starting point for elucidating structural features of the PADOX proteins. Many residues crucial for activity in mammalian PGHS isoforms are conserved in the PADOX proteins, including the histidine residue serving as proximal heme ligand and that near the distal heme face (e.g. His-388 and His-207, respectively, in ovine PGHS-1, and His-389 and His-163 in *A. thaliana* PADOX-1) (4). However, substitution of glutamine for the putative proximal heme ligand in rice PADOX-1, His-383, did not abolish oxygenase activity (9), suggesting that this histidine might not actually be the proximal heme ligand. Many other residues in the heme vicinity in the mammalian PGHS isoforms are not conserved in the plant α-oxygenases (Fig. 1), suggesting that the heme environment in the plant enzymes differs from those in other MPO family members. Differences in the heme environment between other MPO family members and the PADOX enzymes are also indicated by the lack of detectable peroxidase activity in all but one PADOX examined so far (1) and by the markedly different heme spectroscopic properties reported for rice PADOX-1 (9) and mammalian PGHS (10–12), including a 12-fold weaker Soret band extinction coefficient and a very unusual ferrous heme spectrum for the rice enzyme. Given the importance of the heme in catalysis in other MPO subfamilies, especially in the fatty acid oxygenase activities of the PGH synthases and linoleate diol synthase (13, 14), it was of considerable interest to better characterize the heme environment in the plant α-oxygenases. We have expressed recombinant *A. thaliana* PADOX-1 in both prokaryotic and eukaryotic systems to characterize the heme prosthetic group, its interaction with various ligands, and its connection with oxygenase catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**

Ethyl hydrogen peroxide was from Polysciences (Warrington, PA). Fatty acids were from NuChek Preps (Elysian, MN). Isopropyl-β-D-thiogalactopyranoside was from Promega (Madison, WI). Immunoblotting reagents were from Bio-Rad. Reagents for DNA extraction and purification were from Qiagen (Valencia, CA). QuikChange site-directed mutagenesis kit and *Escherichia coli* strains DH5α, XL-10, and BL21(DE3)pLysS were from Stratagene (La Jolla, CA). Restriction enzymes and DNA-modifying enzymes were from New England Biolabs (Beverly, MA). Oligonucleotides were from Sigma-Genosys (Houston, TX) or Integrated DNA Technologies (Coralville, IA). pVL1392 plasmid and BaculoGold linearized baculovirus DNA were from Pharmingen (San Diego, CA). Sf9 insect cells, medium, and culture reagents were from Invitrogen. Goat anti-GST antibody and pGEX-4T-1 plasmid were from Amersham Biosciences. Thrombin, nuclease, and GSH-agarose were from Novagen (Madison, WI). The pCW vector was a kind gift from Dr. F. W. Dahlquist (University of
Oregon, Eugene, OR). Expressed sequence tag clone 218B16T7 was generously provided by Arabidopsis Biological Resource Center (Columbus, OH). Porphyrins and metalloporphyrins were from Porphyrin Products (Logan, UT). All other materials were from Sigma.

Recombinant DNA Techniques
Standard DNA manipulation procedures (15,16) were followed. Plasmid vectors pGEX-4T-1, pCW, and pVL1392 were used for DNA cloning, and E. coli DH5α cells were used for most in vivo DNA transformation experiments. Competent bacterial cells were prepared according to Inoue et al. (17). Restriction fragments were purified with the QIAquick gel extraction kit (Qiagen).

Constructs for Bacterial Expression
Construction of expression plasmid for GST-PADOX-1 fusion protein used PCR from a plasmid containing the A. thaliana PADOX-1 cDNA (218B16T7) and two primers: 5′-GATGAAAGTAATTACTTCCCTAAATCTC-3′ (sense) and 5′-CGCTCGAGTTAAGAGGGAATTCGGAGATAG-3′ (antisense). The antisense primer added an XhoI site (underlined) after the stop codon. The 1.9-kb product was digested with XhoI and inserted in pGEX-4T-1 to give the pGEX-aP10 vector, whose integrity was confirmed by sequencing. pGEX-aP10 was transformed into E. coli strain BL21(DE3)pLysS competent cells, and colonies were selected with ampicillin and chloramphenicol.

Construction of expression plasmid for full-length PADOX-1 protein used PCR from the 218B16T7 plasmid and two primers: 5′-GGTCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′ (sense) and 5′-GCTCTAGA-CAAATTCGAGACGCTGATTGGATTGATC-3′ (antisense). The sense primer introduced the initiation codon within an NdeI site (underlined). The antisense primer added an XbaI site (underlined) after the stop codon. The PCR product was digested with NdeI and XbaI and ligated into the pCW vector. The plasmid was sequenced and transformed into E. coli strain BL21(DE3)pLysS.

Site-directed Mutagenesis
Point mutations were introduced using the QuikChange kit (Stratagene) and the pGEX-aP10 expression plasmid with the following primer pairs (altered bases underlined): H163C sense, 5′-GGATTCAGTTCATGATC TGTGATTGGATTGATC-3′; H163C antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; H163M sense, 5′-GGATTCAGTTCATGATC ATGGATTGGATTGATCATC-3′; H163M antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; H163Q sense, 5′-GGATTCAGTTCATGATC CAGATCATGAACTGAATCC-3′; H163Q antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; H163Y sense, 5′-GGATTCAGTTCATGATC TACGATTGGATTGATCATC-3′; H163Y antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; Y386F sense, 5′-GGATTCAGTTCATGATC TGTGATTGGATTGATC-3′; Y386F antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; R387H sense, 5′-GGATTCAGTTCATGATC TGTGATTGGATTGATCATC-3′; R387H antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; H389C sense, 5′-GGATTCAGTTCATGATC TGTGATTGGATTGATCATC-3′; H389C antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; H389M sense, 5′-GGATTCAGTTCATGATC TGTGATTGGATTGATCATC-3′; H389M antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; H389Q sense, 5′-GGATTCAGTTCATGATC TGTGATTGGATTGATCATC-3′; H389Q antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′; H389Y sense, 5′-GGATTCAGTTCATGATC TGTGATTGGATTGATCATC-3′; H389Y antisense, 5′-GATCAATCCAATCTAGATCATGAACTGCATGATGAGACGCT-3′;
were transformed with the PCR product. Plasmid DNA was isolated from ampicillin-resistant colonies, screened by sequencing, and transformed into *E. coli* BL21(DE3)pLysS.

**Expression of Recombinant PADOX-1 Proteins in Bacteria**

Cells harboring the expression plasmid were grown at 37 °C in medium containing ampicillin and chloramphenicol to an *A*<sub>610</sub> of 0.7. After chilling to 23 °C, isopropyl-1-thio-β-D-galactopyranoside (1 mM) and δ-aminolevulinic acid (0.5 mM) were added, and culture was continued for 16 h at 23 °C with shaking at 200 rpm. Cells were harvested by centrifugation and stored at −70 °C.

**Expression of PADOX-1 Protein in Insect Cells**

A 1.9-kb fragment containing PADOX-1 cDNA was generated by PCR using the 218B16T7 plasmid and the following primer pair, sense 5′-GACTGCAGGCCAC-CATGAAAGTAAATTACTTCCCT-3′ and antisense 5′-GCTCTAGA-CAAATTAAGGGAATTCGGA-3′. These primers incorporated an upstream Kozak sequence (GCCACC) to optimize translation (18) and removed the 3′-untranslated region. The resulting 1.9-kb DNA was digested with PstI and XbaI, ligated into the pVL1392 vector, and used to transform *E. coli* DH5α cells. Transfer vector plasmid (PADOX/pVL1392) isolated from an ampicillin-resistant colony was verified by restriction digests and sequencing. For generation of recombinant baculovirus, adherent Sf9 cells were cotransfected with the PADOX/pVL1392 transfer vector and BaculoGold linearized baculovirus DNA, following the manufacturer’s instructions.

For production of recombinant PADOX-1 protein, Sf9 cells were grown in suspension at 27 °C to a density of 1.0–1.5 × 10<sup>6</sup> cells/ml. Recombinant baculovirus was added at a multiplicity of infection of 5–10, and culture was continued for 4 days. Cells were collected by centrifugation and washed once with phosphate-buffered saline. The cell pellets were resuspended in 0.1 M Tris, pH 7.8, 0.1% Tween 20 and disrupted by sonication. The sonicates were centrifuged at 1,000 × *g* for 10 min, and the sonication procedure was repeated with the pellets. The combined supernatants were centrifuged at 10,000 × *g* for 30 min to obtain a clarified supernatant with >95% of the total fatty acid oxygenase activity.

**Purification of Recombinant PADOX-1 and GST-PADOX-1 Proteins Expressed in Bacteria**

Purification was performed at 4 °C; the pH of each buffer was 7.8, and each buffer contained 0.05% Tween 20 unless otherwise specified. Cell pellets (20 g wet weight, from 5 liters of culture medium) were resuspended in 100 ml of 0.1 M Tris-HCl containing 0.2% Tween 20, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 µM bestatin, 1 µM pepstatin A, 20 µM leupeptin, and 20 µM chymostatin, frozen at −70 °C, thawed, and disrupted by sonication. Nuclease (0.5 units/ml) was added, and the material was centrifuged at 10,000 × *g* for 30 min. The supernatant was brought to 30% saturation with 5.2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 mM EDTA, and the mixture was centrifuged at 10,000 × *g* for 30 min. After discarding the pellet, the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> concentration of the supernatant was raised to 65% saturation before centrifuging again at 10,000 × *g* for 30 min. Precipitated proteins were solubilized by stirring with 50 ml of lysis buffer on ice for 1 h and then dialyzed against 0.1 M Tris-HCl, 2 mM EDTA. The dialyzed solution was centrifuged at 25,000 × *g* for 1 h, and the supernatant was concentrated by ultrafiltration. For purification of PADOX-1, the concentrated material was applied to a Sephacryl 300 HR column (2.5 × 90 cm) and eluted at 12 ml/h with 0.1 M Tris-HCl, 2 mM EDTA; active fractions were pooled and concentrated by ultrafiltration. For purification of the GST-PADOX-1, the concentrated material was applied to a GSH-agarose column (2.5 × 10 cm), washed with 5 volumes of 0.1 M Tris-HCl and 10 volumes of 0.1 M Tris-HCl, 0.5 M NaCl, and eluted with 0.1 M Tris-HCl, 15 mM GSH. Active, orange-colored fractions containing GST-PADOX-1 were pooled, concentrated, and then chromatographed on the Sephacryl 300 HR
column as described above for PADOX-1. Protein concentrations were determined with bovine serum albumin as the standard (19).

**Oxygenase Activity Assay**

Fatty acid oxygenase activity was determined at 30 °C using a YSI Inc. model 53 oxygen monitor (Yellow Springs, MD). Each reaction contained 3 ml of 0.1 M Tris-HCl, pH 7.8, with 100 µM fatty acid and was started by injection of enzyme. One unit of oxygenase activity gives a velocity of 1 nmol of O₂/min. Inhibition of oxygenase activity was measured by adding test compounds to the assay before the enzyme or by preincubating the enzyme with the test compound for 1 h at 4 °C prior to assay. Kₘ and Vₘₐₓ values were estimated by measuring oxygenase velocity with 5–300 µM fatty acid or 15–1100 µM oxygen and fitting the data to the Michaelis-Menten equation.

**Electrophoretic Analysis**

Proteins were analyzed by electrophoresis on 10% polyacrylamide gels under denaturing conditions (20). For immunoblotting, the proteins were transferred electrophoretically to nitrocellulose membranes and probed with antibody against GST (21).

**Heme Quantification**

Heme concentrations were determined from the reduced pyridine hemochrome, using an extinction coefficient difference (A₅₅₆–A₅₃₈) of 24.5 m⁻¹ cm⁻¹ (22).

**Heme Extraction**

This was carried out at 4 °C by a slight modification of Teale’s method (23). Purified PADOX-1 was dissolved in 50 mM Tris-HCl, pH 7.8, and diluted to ~25 µM (~2.5 mg/ml) with water. The pH was adjusted to 4.6 with 0.1 M HCl, and the solution was mixed with an equal volume of 2-butanol. Alternatively, the enzyme solution, pH 7.8, was directly mixed with an equal volume of butanol. In either case, the mixture was vortexed and then centrifuged to separate the orange colored organic phase containing the heme from the colorless lower aqueous phase containing apoprotein. The aqueous phase was dialyzed against 50 mM Tris-HCl, pH 7.8, to remove residual butanol and centrifuged to remove precipitate; heme was added before assay of oxygenase activity.

**UV-visible Absorbance Spectroscopy**

Spectra were recorded with a Shimadzu model 2101 PC dual beam spectrophotometer or an HP8453 diode array spectrophotometer. For examination of reduced enzyme, each sample was placed in a titrator cuvette and made anaerobic by five cycles of evacuation and argon replacement before addition of solid dithionite solution with a syringe (24). The reductant solution was prepared by dissolving solid dithionite powder in 0.1 M sodium pyrophosphate, pH 7.8; the sodium dithionite concentration was determined using lumiflavin-3-acetic acid (25). For titrations, spectra were recorded several minutes after each addition of ligand; equilibration was periodically confirmed by recording a second scan after an additional few minutes.

**Examination of Absorbance Changes during Reaction of GST-PADOX-1 with Fatty Acid**

The purified fusion protein (1 µM heme) in 0.1 M Tris, pH 7.8, with 0.025% Tween 20 at 22 °C was mixed with an ethanolic solution of 18:3n-3 (final concentrations: 20 µM 18:3n-3, 0.7% ethanol). Fatty acid consumption and absorbance changes were monitored in parallel reactions. For quantitation of fatty acid, the reaction was stopped by addition of 0.10 volumes of 1.2 M HCl, 0.016 volumes of 4.1 M 15:0 (internal standard) and 50 mg of NaCl. Lipids were
extracted with 3 volumes of toluene/ethyl acetate (1:1), transferred to screw cap vials, and the solvent evaporated under a stream of nitrogen gas. Fatty acids were derivatized with pyrenyl diazomethane (26). De-rivatized samples were analyzed by high pressure liquid chromatography on a Rainin Microsorb C18 column (150 × 4.6 mm) eluted at 1.5 ml/min with 95% methanol. The eluate was monitored by $A_{340}$ (the 15:0 and 18:3-derivatives eluted at 18.9 and 13.3 min, respectively). The 18:3n-3 concentration was calculated by multiplying the ratio of the high pressure liquid chromatography peak areas for the 18:3n-3 and 15:0 derivatives by the concentration of 15:0 added to the enzyme reaction. For quantitation of absorbance changes, enzyme and fatty acid were reacted in a quartz cuvette.

**EPR and MCD Spectroscopy**

EPR spectra were recorded on a Bruker EMX EPR spectrometer at liquid helium or liquid nitrogen temperature. The liquid helium system included an Oxford GFS 600 transfer line, an ITC 503 temperature controller, and an ESR 900 cryostat. The liquid nitrogen system included a BVT 3000 temperature controller. Quantitation was done by double integration with reference to a copper standard (22). MCD spectra were recorded at room temperature on a Jasco J-500C spectropolarimeter equipped with a 1.3-tesla electromagnet.

**RESULTS**

**Expression of Recombinant A. thaliana PADOX-1 in Bacteria and Insect Cells**

Recombinant PADOX-1 protein was expressed in active form in both prokaryotic (*E. coli*) and eukaryotic (Sf9 insect cells) systems. The yield of functional enzyme per liter of culture was about 10-fold higher for a GST-PADOX-1 fusion protein expressed in a bacterial system than for full-length PADOX expressed in either bacteria or insect cells. Thus, bacterial expression of the recombinant GST-PADOX-1 fusion protein was chosen as the primary method to prepare enzyme for characterization. A typical yield of the fusion protein (at the crude extract stage) was 34 mg/liter of bacterial culture.

Most of the fatty acid oxygenase activity of GST-PADOX-1 expressed in *E. coli* BL21(DE3) pLysS cells was recovered in the 10,000 × g supernatant after homogenization. The GST-PADOX-1 fusion protein tended to aggregate, and several detergents were tested for their ability to minimize aggregation without inhibiting the enzyme. Tween 20 (0.2%) was found to be effective and was routinely added during purification. After undergoing a combination of ammonium sulfate fractionation, dialysis, affinity chromatography, and gel filtration, the fusion protein preparation showed a single prominent SDS-PAGE band at 98 kDa (data not shown), very close to the predicted mass of 99 kDa (27 kDa for GST plus 72 kDa for PADOX-1). The fatty acid oxygenase specific activity of purified GST-PADOX-1 was typically around 11 µmol of O$_2$/min/mg of protein, with a pH optimum near 7.8. The enzyme utilized a variety of fatty acids of 18 carbons or less, with linolenate (18:3n-3), linoleate (18:2n-6), and oleate (18:1n-9) showing the highest activity and $K_m$ values of 15–20 µm.$^2$

Routine PADOX-1 assays used 18:2n-6 to maximize activity and minimize oxidative decomposition of the stock substrate.

Thrombin digestion of the GST-PADOX-1 fusion protein released full-length PADOX-1 that could be purified by gel filtration and that migrated as a 72-kDa species when analyzed by SDS-PAGE (data not shown). The UV-visible absorbance spectra of several such batches of PADOX-1 had Soret peaks at 413–415 nm, quite similar to the Soret peak position of 412–414 nm observed with the GST-PADOX-1 fusion protein, and the Soret peak position of 410–412 nm observed for enzyme was expressed as a full-length protein without the GST domain.

$^2$W. Liu and P. Fabian, unpublished results.
Further comparison of the GST-PADOX-1 fusion protein with PADOX-1 released by thrombin and with PADOX-1 expressed without the GST domain showed the three preparations had very similar values for the oxygenase $K_m$ for oleic acid, the oxygenase $K_i$ for cyanide, and the dissociation constant for cyanide calculated from spectrophotometric titrations, all described in more detail below (Table I). The close similarity of these several indices demonstrated that the presence of the GST fusion partner did not markedly affect the catalytic functioning or the heme environment of recombinant PADOX-1. However, PADOX-1 released by thrombin digestion lost activity more quickly during handling and storage than did the fusion protein, which retained almost 90% of its activity even after 6 months storage at 4 °C. This indicated that retaining the GST fusion partner considerably improved the stability of recombinant PADOX-1. Accordingly, the GST-PADOX-1 fusion protein was used for most of the detailed characterization (GST itself does not have fatty acid oxygenase activity or interfering chromophores).

**PADOX-1 Oxygenase Kinetics**

The oxygen consumption kinetic profiles were qualitatively similar for linolenate, palmitoleate, and myristate (Fig. 2). An initial lag, a classic hallmark of autoactivation in other fatty acid oxygenases (27,28), was followed by acceleration to a steady state and then an eventual decline as the concentration of oxygen consumed approached that of the fatty acid added. Cessation of activity was due to substrate depletion, as full oxygenase activity resumed if additional substrate was provided (data not shown). Oxygenase catalysis by PADOX-1 thus showed little indication of the self-inactivation characteristic of cyclooxygenase and lipoxygenase catalysis (29,30).

**PADOX-1 Peroxidase Kinetics**

Purified PADOX-1 was tested for peroxidase activity in steady state assays with 0.5 mM H$_2$O$_2$ and guaiacol (5 mM) or N,N,N',N'-tetramethyl-p-phenylene diamine (84 µM) as cosubstrate (31). No peroxidase activity was detected (data not shown), consistent with earlier reports for PADOX-1 from both *A. thaliana* and rice (3,4,9).

**Electronic Absorbance Spectra of Resting and Reduced PADOX-1**

The UV-visible absorbance spectrum of electrophoretically pure GST-PADOX-1 showed a Soret peak at 413 nm and a broad absorbance at 500–600 nm (Fig. 3). Addition of dithionite under anaerobic conditions resulted in a shift of the Soret peak to 426 nm and appearance of prominent bands at 530 and 560 nm. Subsequent introduction of air shifted the Soret peak toward that of the resting enzyme, although the original amplitude was not completely restored (Fig. 3). These results indicate that resting PADOX-1 has a ferric heme that can be reduced to the ferrous state, and that the ferrous state is readily reoxidized in air. There was a small batch-to-batch variation in the exact wavelength of the Soret peak (Table I). In addition, although most batches of full-length PADOX-1 and the GST-PADOX-1 fusion protein were completely reduced by reaction with dithionite within a few minutes, slower reaction with dithionite was observed on at least one occasion.

Pyridine hemochrome analysis of pure GST-PADOX-1 showed absorption maxima at 556 nm ($\alpha$) and 526 nm ($\beta$), identical with a Fe(III) protoporphyrin IX standard, establishing noncovalently bound heme as the prosthetic group. Quantitation of the heme content, together with protein assay and the Soret absorbance of the native, ferric enzyme, indicated a stoichiometry of 0.89 mol of heme/mol of PADOX-1 monomer and an extinction coefficient at 140 mM$^{-1}$ cm$^{-1}$. The extinction coefficient was used for subsequent determination of holoenzyme concentration. PADOX-1 thus has a single heme prosthetic group and appears to be a $b$-type cytochrome.
EPR Spectrum of GST-PADOX-1

The EPR spectrum of resting GST-PADOX-1 (Fig. 4A) showed that the ferric heme is mainly in the high spin state, with both axial ($g = 5.97$) and rhombic ($g = 6.20$ and $5.59$) components evident. There was also a small signal at $g = 4.26$, probably reflecting some rhombic ferric iron contamination of the sample, and a free radical signal at $g = 2.0$. The high field $g$ values, $g_{\text{min}}$, for both the axial and rhombic heme species are hard to define because of their small amplitude and potential overlap with background organic radical signals. The EPR spectrum did not change appreciably when severalfold excesses of ethyl hydroperoxide, 15-hydroperoxyeicosatetraenoic acid, or $80 \, \text{mM} \, \text{NaCN}$ were added (data not shown), indicating that the PADOX-1 heme did not react with either peroxide or with the potential axial ligand.

When the $g = 2$ region in the resting enzyme was examined further by EPR near liquid nitrogen temperature, the radical gave an isotropic signal with a 14-G overall line width centered at $g = 2.0037$; double integration indicated that the $g = 2$ signal was $<1\%$ of the PADOX-1 heme concentration (data not shown).

PADOX-1 treated with sodium nitrite in the presence of excess dithionite to generate NO in situ produced both 5-coordinate and 6-coordinate NO-ferrous heme EPR signals in place of the high spin heme EPR signals (Fig. 4B). The $g_z$ component of the 5-coordinate NO complex with 15–18-G hyperfine splitting caused by the nitrogen nucleus is centered at $g = 2.011$, whereas its $g_x$ and $g_y$ components are mixed with the $g_z$ and $g_y$ from the 6-coordinate NO complex. The key features are broad peaks located around $g = 2.08$ and 2.04 and a trough at 1.99. Thus, the PADOX-1 heme coordinates with NO (at least when in the ferrous state).

Electronic Absorption Spectrum Changes during PADOX-1 Oxygenase Catalysis

When GST-PADOX-1 was incubated with 20 eq of linolenate, the fatty acid was rapidly oxygenated, with half of the fatty acid consumed after about 5 s and no detectable substrate remaining at 50 s (Fig. 5A). The absorbance spectra showed only small changes, with the heme Soret peak at 413 nm losing about 2% of its initial intensity at the 4-s point, when oxygenase catalysis was underway (Fig. 5, A and B). The spectral changes were only partially reversed at the 39-s time point, when little substrate remained and oxygenase catalysis had almost ceased. A detailed examination of the kinetics of Soret spectral changes is shown in Fig. 5C. The small decrease in Soret absorbance occurred rapidly, during mixing, and persisted even after 25 s, when most oxygenase catalysis had ceased (Fig. 5A). In contrast, there were essentially no changes at a control wavelength (800 nm) at any time during reaction with the fatty acid (Fig. 5C). Similar results were obtained when linolenate was reacted with PADOX-1 expressed as a full-length protein without the GST fusion partner (data not shown). These results indicate that only a small fraction of the PADOX-1 heme accumulates in a chemically altered state during oxygenase catalysis and that much of this small change persists even after catalysis ceases.

Effects of Heme Supplementation on Oxygenase Activity

Fatty acid oxygenase activity was monitored with and without supplementation with heme or MnPPIX, with the metalloporphyrin present either during the preincubation or during the assay, for both PADOX-1 and the GST-PADOX-1 fusion protein (Table II). Preincubation of enzyme with heme or addition of MnPPIX to the assay mixture did not significantly affect the activity of either PADOX-1 or the fusion protein. Addition of heme to the assay did increase the oxygenase rate for both enzyme preparations, but the effect was quite modest (16–20%). No oxygen consumption occurred when fatty acid was incubated with heme or MnPPIX alone. The fact that preincubation with heme did not increase activity suggests that the increase seen with heme in the assay was not because of conversion of apoenzyme to holoenzyme but rather reflected some artifactual effect of heme bound nonspecifically to the protein.
Effects of Porphyrin or Metalloporphyrin Supplementation during GST-PADOX-1 Expression

In an effort to perturb oxygenase function, several attempts were made to replace the PADOX-1 heme with another metalloporphyrin by expressing recombinant GST-PADOX-1 in bacteria grown in medium supplemented with a porphyrin other than PPIX or a metalloporphyrin other than heme. The metalloporphyrins tested included Fe(III) mesoporphyrin, Fe(III) deuteroporphyrin, Mn(III)PPIX, and Zn(II) PPIX; the porphyrins tested included hematoporphyrin and deuteroporphyrin. In each case, the fusion protein was purified by affinity chromatography before characterization of oxygenase activity, Soret spectrum, and metalloporphyrin content (by pyridine hemochrome analysis). Each of the various supplementation conditions produced a level of recombinant oxygenase activity comparable with supplementation with heme. Some of the supplements resulted in purified GST-PADOX-1 protein with a slightly shifted Soret absorbance peak (the position ranged from 403 to 415 nm). However, the metalloporphyrin present in the fusion protein was found to be heme itself in every case. Thus, regardless of the porphyrin or metalloporphyrin added, the bacteria incorporated only heme into recombinant GST-PADOX-1.

Extraction of Heme from GST-PADOX-1

The heme was completely removed from the GST-PADOX-1 fusion protein by extraction with butanone under acidic conditions (pH 4.6) by using the procedure described under “Experimental Procedures.” However, no soluble apoprotein (examined by PAGE) or reconstitutable oxygenase activity (assayed after addition of fresh heme) remained after extraction, indicating that the apoprotein had become denatured and precipitated. At pH 7.8, the heme was also completely extracted from GST-PADOX-1 into the butanone phase, but the apoprotein remained in soluble form in the aqueous phase. Addition of fresh heme to the soluble apoprotein did not restore oxygenase activity, indicating that heme extraction from PADOX-1 was not reversible even at a pH mild enough to avoid complete denaturation.

Stability of Heme Environment to Denaturant

Titration of the fusion protein with guanidinium HCl led to progressive disruption of the heme environment, reflected in decreased intensity of the Soret absorbance peak at 412 nm, with half-maximal effect at about 1.7 M denaturant (Fig. 6). Half-maximal disruption of heme absorbance in myoglobin required a lower level of denaturant (Fig. 6), indicating that the heme environment was somewhat more stable in PADOX-1 than in myoglobin. The PADOX-1 oxygenase activity, assayed with or without heme, declined in parallel with the heme absorbance changes. This suggests that loss of oxygenase activity coincided with disruption of the heme environment, although it says nothing about a causative link between the two. Oxygenase activity was not recovered after disruption of the heme environment even after dilution of the denaturant into assay buffer containing fresh heme, indicating that denaturation with guanidinium HCl offers little promise as a strategy for prosthetic group exchange.

Effects of Mutation of Putative Heme Axial Ligands

Alignment of the PADOX-1 sequence with those of PGHS-1 and −2 identified His-389 as the likely proximal ligand for the heme and His-163 as the likely distal ligand (4). The effects of modifications of these residues on the heme environment and the catalytic activity were examined in purified recombinant GST-PADOX-1 fusion proteins (Table III). Substitution of the putative distal heme ligand, His-163, with either cysteine, glutamine, or tyrosine produced soluble recombinant proteins that showed little change in the position of the resting Soret peak. This indicated that these mutations had no gross effect on PADOX-1 folding or on the heme electronic structure. Substitution of His-163 with methionine, however, produced a decided red shift of the Soret peak to 420 nm. This suggests that the methionine at residue 163 binds as an axial heme ligand. Each of the His-163 substitutions decreased the oxygenase activity.
The H163Q substitution was the most disruptive, having less than 1% of wild type activity; the others showed significant oxygenase activity, with the H163M showing the most at 17% of the control. The Soret peak intensities of the mutants were roughly proportional to the amount of recombinant protein detected by immunoblot (data not shown), indicating that decreased activities were not because of decreased heme content. Clearly, modification of the structure of the putative distal heme ligand perturbed catalytic function and, in one case, perturbed the heme electronic structure as well.

Substitution of the putative proximal heme ligand His-389 with either cysteine, glutamine, methionine, or tyrosine had marked effects (Table III). The proteins with glutamine or tyrosine mutations were expressed in insoluble form, with no detectable activity. These mutations seem to disrupt proper folding of the protein, perhaps by interfering with heme binding to the apoprotein. The H389C and H389M mutants were expressed in soluble form, but their Soret peaks were blue-shifted to 396–400 nm, suggesting a 5-coordinate heme. Both of these mutants did retain some activity, about 8% of wild type. These mutations show that His-389 was not absolutely required for proper folding or catalytic function. Carbon monoxide did not shift the Soret peak of H389C even after reduction of the heme with dithionite (data not shown), indicating that if heme thiolate proximal ligation was present in this mutant, the distal pocket did not permit binding of CO to form the diagnostic P450-type spectrum.

Results from two control mutations near His-389 in the primary sequence are also included in Table III. The R387H mutation retained a normal heme spectrum and oxygenase activity; this negative result indicates that this segment of the PADOX-1 polypeptide is not inherently sensitive to perturbation by side chain substitutions. Mutation of Tyr-386 (corresponding to Tyr-385 in PGHS-1 and −2) to phenylalanine removed catalytic activity as expected but did not perturb the heme electronic spectrum. This result established that Tyr-386 is crucial for oxygenase activity, just as reported for the corresponding residue in rice PADOX-1 (9). The results with the His-163 and His-389 mutations provide strong evidence that His-389 is the proximal heme ligand in PADOX-1. They also suggest that His-163 is in the distal heme pocket and that the PADOX-1 heme has both structural and catalytic functions.

**Effect of Cyanide and Other Potential Ligands on GST-PADOX Electronic Absorbance Spectrum**

Titration of the fusion protein with cyanide produced a progressive shift in the Soret absorbance peak from 412 to 420 nm (Fig. 7). The maximal absorbance change was near 430 nm; plotting ΔA_{430} as a function of cyanide concentration revealed a saturable function with a K_d of 13 mM (Fig. 7, inset). As mentioned above, cyanide produced similar spectroscopic changes with PADOX-1 released from the fusion protein and with full-length PADOX-1 expressed using the pCW-based vector (Table I). These results demonstrate that cyanide binds relatively weakly in the heme vicinity in resting PADOX-1.

Addition of NaF at up to 65 mM produced no changes in the GST-PADOX-1 absorbance spectrum (data not shown), consistent with the presence of a high spin ferric heme. Titration of GST-PADOX-1 with up to 50 mM imidazole produced a uniform increase in absorbance across the spectrum with no shift of the Soret peak (data not shown), suggesting this compound did not coordinate with the heme iron but rather led to increased scattering, presumably due to protein aggregation. Another potential heme ligand, CO, did not shift the Soret peak of GST-PADOX-1 even after reduction of the heme with dithionite (data not shown), indicating that the distal face of the PADOX-1 heme was poorly accessible to CO.
Effects of Cyanide on MCD Spectrum of GST-PADOX-1

The MCD spectrum of resting GST-PADOX-1 showed a derivative feature with a zero crossing at 406 nm and a trough at 570 nm (Fig. 8). The position of the derivative feature is somewhat shifted from the Soret maximum of the ferric enzyme (Fig. 3), and the MCD intensity is consistent with a 6-coordinate high spin ferric heme (32). Addition of a low level of cyanide (20 µM) had no effect on the MCD spectrum of PADOX-1. Addition of a high level of cyanide (160 µM) shifted the zero crossing to 412 nm (Fig. 8), consistent with the red shift seen in the absorbance spectrum (Fig. 7). Cyanide binding is known to convert many ferric hemoproteins from high spin to low spin, accompanied by a very large increase in the intensity of the Soret MCD (33) and a shift in the MCD zero crossing to near the Soret maximum. For PADOX-1, however, even cyanide levels sufficient to shift the MCD zero crossing did not significantly increase the intensity of the MCD derivative feature (Fig. 8), indicating that binding of the ligand did not convert the heme to a low spin state. Thus, access to the distal face of the PADOX-1 heme appears to be restricted in such a way that cyanide does not bind as a conventional axial heme ligand.

Action of Cyanide on Fatty Acid Oxygenase Activity

Addition of cyanide to the assay inhibited the oxygenase activity of GST-PADOX-1, with essentially complete inhibition observed at 100 µM (Fig. 9). The concentration dependence was consistent with noncompetitive inhibition, with a $K_i$ value of about 5 µM. As mentioned earlier, the presence of GST in the fusion protein had no significant effect on inhibition of the oxygenase by cyanide (Table I). Thus, cyanide is a very potent inhibitor of PADOX-1 oxygenase catalysis even though the absorbance and MCD data show it has only weak binding to the PADOX-1 heme vicinity and does not coordinate to the heme iron (Figs. 7 and 8). A high affinity site for cyanide connected with the oxygenase inhibition can thus be clearly distinguished from the low affinity cyanide site connected to the heme spectral changes. Pre-incubating PADOX-1 with 25 µM cyanide at 4 °C for 1 h did not produce any inhibition when aliquots were subsequently diluted 500-fold for oxygenase activity assay, demonstrating that the inhibition by cyanide was readily reversible.

The possibility of interfering interactions of fatty acid and cyanide with PADOX-1 was examined by analyzing the dependence of oxygenase activity on the oleate level in the presence and absence of 7.5 µM cyanide (Fig. 10). This level of cyanide decreased the oxygenase $V_{max}$ value by about 60%, as expected from the results in Fig. 9, but did not produce a statistically significant change in the $K_m$ value for the fatty acid. Thus, cyanide binding at the high affinity site did not perturb the catalytic interaction with the fatty acid substrate.

The possibility of interfering PADOX-1 interactions with the second substrate, oxygen, and the cyanide inhibitor was also examined (Fig. 11). The $K_m$ value for oxygen was 109 µM in the control reaction. Addition of 7.5 µM cyanide increased the $K_m$ value by about 2-fold, to 236 µM oxygen, and decreased the $V_{max}$ value by over half (as expected from the results in Fig. 9). This fits the pattern for a mixed inhibitor and indicates that cyanide binding at the high affinity site interferes with interactions between the enzyme and oxygen.

Other potential heme ligands were tested for inhibitory actions on the PADOX-1 oxygenase. Imidazole inhibited the oxygenase activity of full-length PADOX-1 with a $K_i$ value of 121 µM. This is somewhat higher than the $K_i$ value of 46 µM reported for rice PADOX-1 (9). Several other imidazole analogs were tested for actions on the PADOX-1 oxygenase activity, including 4-phenylimidazole, 1-benzylimidazole, 2-methylimidazole, 2-aminoimidazole, 1-phenylimidazole, 1-(3-aminopropyl)-imidazole, 3,5-lutidine, and chlortrimazole. At a level of 1 mM, none of these imidazole analogs inhibited the oxygenase activity nor did they alter the heme Soret absorbance peak. Sodium azide did inhibit the oxygenase activity but only at a

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relatively high concentration \( (K_i = 0.73 \text{ mM}) \); however, even 34 mM azide had no effect on the Soret absorbance of the heme.

DISCUSSION

*A. thaliana* PADOX-1 belongs to subfamily 1 of the family of myeloperoxidase-related proteins (2). Sequence analysis indicates that almost all of the 16 subfamilies have a conserved heme-binding motif, and extensive characterization of bound heme has been done for some of the MPO subfamilies, including ovoperoxidase (subfamily 6), thyroid peroxidase (subfamily 11), myeloperoxidase and eosinophil peroxidase (subfamily 12), prostaglandin H synthase-1 and –2 (subfamily 15), and linoleate diol synthase (subfamily 16). The present results allow a more detailed comparison of the properties of the heme in PADOX-1 with those of the heme in other members of the MPO family.

Results from the pyridine hemochrome analysis described above show that PADOX-1 has one heme per monomer, as predicted from sequence analysis (4), and that the heme is dissociable; the latter aspect was confirmed by the butanone extraction studies. In contrast, the heme is known to be covalently attached to the protein in some MPO family members, including thyroid peroxidase, myeloperoxidase, lactoperoxidase, and eosinophil peroxidase (34–37). Although not covalently attached, the PADOX-1 heme is relatively tightly bound and did not dissociate during chromatography, much like the heme in linoleate diol synthase (38) and distinct from the heme in PGH synthase-1 and –2, which dissociates during purification (39,40). The PADOX-1 heme did dissociate during solvent extraction, although this led to irreversible loss of activity. Addition of heme, either before or during oxygenase assay, had little effect on PADOX-1 activity (Table II), indicating that any apoprotein in the preparation was unable to bind heme in a functional manner. This is very different from the behavior of PGHS-1, where the apoenzyme binds heme in a few seconds under assay conditions (41). The results from denaturation studies (Fig. 6) indicated that the PADOX-1 heme environment has a stability roughly comparable with that in myoglobin, with EC\textsubscript{50} values of 1.8 and 1.4 M Gdm, respectively.

The electronic and MCD absorbance spectra and the EPR characteristics show that the heme in resting *A. thaliana* PADOX-1 is in the 6-coordinate high spin ferric state (Figs. 3, 4, and 8). The Soret extinction coefficient for the ferric *A. thaliana* protein determined here (140 m\textsuperscript{M} cm\textsuperscript{−1}) falls in the range of 120–165 m\textsuperscript{M} cm\textsuperscript{−1}, typically seen for PGH synthase-1 (42,43), and is much more consistent with *b*-type cytochromes than the value of 11.6 m\textsuperscript{M} cm\textsuperscript{−1} reported for rice PADOX-1 (9). The EPR spectrum of resting PADOX-1 (Fig. 4) appears quite similar to those found for PGHS-1 and –2 (22,44), with major rhombic and axial high spin heme components evident. A significant amount of low spin heme has been observed near \( g = 3 \) in the EPR spectrum of concentrated PGHS-1, but such a signal was not seen in PADOX-1 at the enzyme concentration used (Fig. 4). The *A. thaliana* PADOX-1 ferric heme reacted with dithionite to produce the ferrous form, which readily re-oxidized in air to the ferric state (Fig. 3). This reversible reduction is much like that seen with PGH synthase-1 treated with stoichiometric amounts of dithionite (45). The electronic spectrum of ferric *A. thaliana* PADOX-1 (Fig. 3) is essentially the same as that reported for ferric PADOX-1 from rice (except for the 12-fold higher extinction coefficient for the *A. thaliana* protein). However the spectrum reported for the rice enzyme reacted with dithionite had unusual features (9), with the Soret peak being attenuated rather than red-shifted as commonly observed with hemoproteins undergoing a transition from the ferric to the ferrous state. The differences between the *A. thaliana* and rice PADOX-1 might be due to some structural divergence between the two proteins or to a difference in the rate of reaction with dithionite. In this connection, we did observe considerable variation in the rate of reduction by dithionite among batches of *A. thaliana* PADOX-1. Most batches converted to the ferrous state within a few minutes, but a
few reacted considerably more slowly; the reason for the difference is unclear. In any case, the 
present results make it clear that the unusual ferrous heme spectrum reported for rice PADOX-1
is not a characteristic of all plant PADOXs.

One aspect observed in this study of PADOX-1 that is atypical for myeloperoxidase family
members is the change in heme coordination that occurs upon reduction. Thus, the oxidized
enzyme contains the heme in a high spin state as judged by EPR and MCD criteria. However,
the optical spectrum of the dithionite-reduced protein exhibits the typical $\alpha, \beta$ optical transitions
at $\sim$560 and 530 nm, respectively; these transitions are diagnostic of low spin ferrous heme. It
would thus appear that conversion of the heme from the ferric to the ferrous state is
accompanied by the binding of a strong field ligand to the heme iron. This is most likely to be
His-163, the distal ligand that is not coordinated to the heme iron in the ferric enzyme. Reduced
PADOX is thus a bis-histidine heme compound, as are most $b$-cytochromes. However, whereas
bis-histidine cytochromes are unreactive with oxygen, reduced PADOX is atypical in that it is
readily reoxidized in air.

Another surprising observation with PADOX-1 is that only rather small changes occur in the
heme absorbance spectrum during turnover (Fig. 5). One potential concern is that these small
absorbance changes might reflect only a small fraction of active enzyme in the protein
preparation. Marked heme absorbance changes are observed in PGHS-1 and $-2$ during
cyclooxygenase and peroxidase turnover (12,46,47). The specific activity of recombinant
PGHS-2 preparations that produced prominent absorbance changes during turnover ranged
from 5 to 12 $\mu$mol of fatty acid/min/mg of protein or 400–900 $\mu$mol of fatty acid/min/mol of
enzyme (12,47), lower than the 11 $\mu$mol of fatty acid/min/mg of protein or 1100 $\mu$mol of fatty
acid/min/mol of enzyme for our purified GST-PADOX-1. The fact that heme spectral changes
are readily observed during catalysis for PGHS-2 but not for PADOX-1 of comparable specific
activity suggests that there are significant differences between the two MPO family members
in either rate constants (so that oxidized heme intermediates do not accumulate in PADOX-1
as they do in PGHS-2) or mechanism.

It has been reported previously that mutation of the predicted proximal or distal heme ligands
decreases the oxygenase activity of rice PADOX-1; surprisingly, considerable activity
remained when the putative proximal histidine was replaced with glutamine, a residue unlikely
to coordinate the heme iron (9). In the present study with the A. thaliana enzyme, substitution
of the putative proximal histidine, His-389, with either glutamine or tyrosine led to formation
of an insoluble, inactive form of the protein (Table III), consistent with a gross disruption of
folding as might accompany a failure to incorporate a structurally important heme. Substitution
of His-389 with cysteine or methionine, both plausible heme ligands, had considerably milder
effects, retaining about 8% of the oxygenase activity even though the heme spectral
characteristics were considerably altered (Table III). This behavior is also consistent with
His-389 being the proximal heme ligand, and further indicates that a proximal histidine is not
absolutely required for binding of heme in at least a partially functional fashion. The exact
nature of the heme proximal ligation in the H389C and H389M mutants is unclear. The H389C
mutant did not form a P450 type reduced CO compound, but authentic heme thiolate ligands
can be disrupted upon reduction (48). Substitutions of the putative distal heme ligand, His-163,
with cysteine, methionine, glutamine, or tyrosine resulted in soluble proteins, indicating that
proper folding of the protein was not particularly sensitive to changes at this residue (Table
III). Only methionine substitution at residue 163 significantly altered the heme absorbance
spectrum (Table III) and the MCD spectrum (data not shown). All His-163 substitutions did
affect the oxygenase activity, with less than 20% of wild type activity retained (Table III).
These results are entirely consistent with His-163 in PADOX-1 being located in the distal heme
pocket but not directly liganded to the ferric heme iron, just as seen for the corresponding
residue (His-207) in PGHS-1 and $-2$ (6–8).
Overall, the mutagenesis results strongly support the assignment of His-389 as the proximal heme ligand and His-163 as a distal heme pocket residue in A. thaliana PADOX-1, bearing out earlier predictions based on sequence analysis of this protein (4) and suggesting that the crystallographic structures available for other MPO family members (such as the PGH synthases and myeloperoxidase) are useful as rough guides to the general folding of the heme pocket in PADOX-1. The mutagenesis results also provide evidence that the PADOX-1 heme has a functional role in oxygenase catalysis, with several of the His-389 and His-163 mutants perturbing both the heme spectral characteristics and the oxygenase activity (Table III). This evidence is particularly important because many other experimental strategies, such as comparing the effects of compounds as heme ligands and as inhibitors, substitution of heme with other metalloporphyrins, and monitoring heme spectral changes during catalysis, were not useful. However, the case for heme functioning in oxygenase catalysis remains circumstantial until a direct, mechanistic role for the heme can be demonstrated.

One striking result from the current studies is the inability of a variety of small molecules to bind as distal axial ligands to the PADOX-1 heme, with the exception of NO. Cyanide did have a low affinity binding that shifted the heme electronic absorbance spectrum (Fig. 7), but even then cyanide did not convert the heme to a low spin state (Fig. 8) and thus did not coordinate the heme iron. There was no indication that imidazole or several substituted imidazole derivatives were able to even bind in the heme vicinity. The one exception was NO, which formed two different complexes with the ferrous PADOX-1 heme, a 5-coordinate complex with only NO bound and a 6-coordinate complex with both NO and histidine bound. The rather restricted access to the distal face of the PADOX-1 heme is in contrast with the situation for many other MPO family members, including PGH synthase, ovoperoxidase, MPO, and thyroid peroxidase, where small, charged molecules readily bind as distal heme ligands (49–52). These other MPO family members obviously have robust peroxidase activities, and the restricted access to the distal face of the heme observed in the present results provides a simple explanation for the previously puzzling lack of appreciable peroxidase catalysis by rice or A. thaliana PADOX-1 (4,9).

There are several possible explanations for the inability of small ligands to access the distal face of the PADOX-1 heme. The most straightforward explanation is steric inhibition. Sequence alignment indicates that PADOX-1 has a segment of some 40 residues just downstream of the “distal” histidine (His-163) that is not present in PGHS-1 and −2 or in linoleate diol synthase (2). This distal pocket “insert” might block access to the distal face of the heme in PADOX-1, although longer segments are present at the same position in other MPO family members with accessible distal heme pockets. The segment in question is poorly conserved between PADOX-1 and other MPO family members, and the distal pocket insert structure in PADOX-1 may well be distinct from those in other MPO family members. A second possible explanation for the limited access to the distal face of the PADOX-1 heme is the presence of a strong axial ligand. This is very unlikely given that the resting enzyme has a high spin heme and that most mutations of the candidate distal ligand, His-163, had little effect on the heme Soret peak (Table III). Local electrostatic repulsion might also impede ligand access to the distal heme face in PADOX-1. This is supported by the ability of NO to bind the heme but is not consistent with the inability of CO to bind. Understanding the restricted access to the heme in PADOX-1 will clearly require additional information.

The strong inhibitory effect of cyanide on PADOX-1 oxygenase activity revealed an unsuspected aspect of the structure of the protein. Cyanide binding to the distal heme face should inhibit any catalytic function that depended on the heme. Cyanide does bind very tightly to some MPO family members, including canine MPO ($K_d 0.5 \mu M$) (49). For PGHS-1, cyanide binds to the ferric heme ($K_d 0.1–0.2 \mu M$) (49,53) and inhibits the peroxidase at similar levels (53–55), although inhibition of the cyclooxygenase activity requires much higher cyanide.

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levels unless reductants are present (53,56). For linoleate diol synthase, the oxygenase activity was not inhibited by 1 mM cyanide (38). In the case of PADOX-1, the affinity for cyanide measured by heme spectral perturbation was low ($K_d \sim 13$ mM; Fig. 7), and so it was very surprising that the oxygenase activity was inhibited in a noncompetitive fashion by micromolar levels of the ligand ($K_i \sim 5$ µM; Fig. 9). Occupation of the high affinity inhibitory site by cyanide did not affect the kinetics of the enzyme with fatty acid but did alter the kinetics with oxygen. These results identify a novel, high affinity interaction of PADOX-1 with cyanide that is away from the heme iron and the parts of the protein responsible for binding fatty acid. There is precedent for anions that are classical heme ligands binding to nonmetal centers (57). Almost all substitutions of the putative proximal and distal heme ligands moderately decreased the inhibitory potency of cyanide (Table III), indicating that the high affinity cyanide site may be in the general vicinity of the heme pocket. The possible pathophysiological role of PADOX-1 inhibition by cyanide is an open question. It is interesting that cyanide is generated during biosynthesis of ethylene, a major signaling molecule in plants (58). Plants do have cyanide detoxification systems, and the exact intracellular cyanide levels remain controversial (59).

Summary

The present results reveal the basic similarities in the heme environment of *A. thaliana* PADOX-1 and other MPO family members, including the bis-histidine ligation scheme and the ferric resting state. PADOX-1 is clearly distinct from the other MPO family members studied in having quite restricted access to the distal face of the ferric heme, minimal heme redox changes during catalysis, and no detectable peroxidase activity. PADOX-1 also has a novel, high affinity inhibitory site for cyanide distinct from the heme. These findings establish important basic properties of the PADOX-1 heme environment and should serve as a useful starting point for elucidating the mechanistic role of the heme in oxygenase catalysis. Detailed comparison of the mechanistic strategies in the several MPO family fatty acid oxygenases will undoubtedly be useful in understanding the basis for the physiologically important cyclooxygenase activities in the PGHS isoforms.

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FIG. 1. Ovine PGHS-1 structure (Protein Data Bank code 1EQG (60)) in the heme vicinity showing residues conserved with *A. thaliana* PA-DOX-1 in yellow and unconserved residues in red.
FIG. 2. Oxygen consumption traces during reaction of PADOX-1 (full-length protein expressed with pCW vector; 63 nM heme) with 100 µM linolenate (solid line), palmitoleate (dashed line), or myristate (dotted line) in 0.1 M Tris, pH 7.8, containing 0.025% Tween 20 at 30 °C.
Purified GST-PADOX-1 (1.3 µM heme) in 0.1 M Tris, pH 7.8, containing 0.025% Tween 20 was equilibrated with argon before recording the resting spectrum. The enzyme was then reacted with excess dithionite to obtain the reduced spectrum. Finally, the cuvette was equilibrated with air to obtain the spectrum of the re-oxidized enzyme.

FIG. 3. Absorption spectra of native and dithionite-treated forms of GST-PADOX-1
FIG. 4. EPR spectra of resting GST-PADOX-1 and its ferrous NO complex

A, the spectrum of affinity-purified fusion protein (10 μM heme) in 0.1 m Tris, pH 7.8, containing 0.025% Tween 20 was recorded at 12 K, with a frequency of 9.609 GHz, a microwave power of 4 milliwatts, and a modulation amplitude of 10.9 G. B, PADOX (10 μM heme) was mixed with excess dithionite and sodium nitrite in an EPR tube at 24 °C until the purple pinkish color for ferrous NO heme complex persisted; the sample was then frozen in a dry ice/acetone bath. EPR conditions were essentially as above, except that the modulation amplitude was 2 G. The hyperfine splitting because of the NO nitrogen nuclei and the values of the major g components are indicated.
FIG. 5. Absorbance spectrum changes during reaction of GST-PADOX-1 (1 µM heme) with 18:3 n-3 (20 µM)
A, kinetics of fatty acid consumption. B, UV-visible difference spectra after 4 (thick line), 14 (thin line), and 39 s (dashed line) of reaction. C, kinetics of absorbance changes at 413 and 800 nm; initial downward deflections are mixing artifacts. Details are described under “Experimental Procedures.”
FIG. 6. Effects of denaturant on GST-PADOX-I heme spectrum and oxygenase activity and on myoglobin (Mb) heme spectrum
The enzyme (1.8 μM heme) was dissolved in 0.1 M Tris, pH 7.8, containing 0.025% Tween 20 and mixed with an equal volume of Gdm-HCl in the same buffer to give the indicated final Gdm-HCl concentrations. After 15 min of incubation on ice, the absorbance spectrum was recorded to determine the change in A_{412}, and the oxygenase activity was measured (150 μM oleate) with and without 1 μM heme in the assay buffer. A parallel experiment was run with horse heart myoglobin.
FIG. 7. Effect of cyanide on GST-PADOX-1 absorbance spectrum
The enzyme (1.5 µM) was dissolved in 0.1 M Tris-HCl, pH 7.8, containing 0.025% Tween 20 at 23 °C and titrated with 0–190 mm NaCN. Several minutes of equilibration was allowed after each addition of ligand before the spectrum was recorded. Arrows indicate the direction of absorbance change as the titration progressed. Inset, ΔA₄₃₀ increases are plotted as a function of the cyanide concentration; the line is a least squares fit of the data to a hyperbolic function.
FIG. 8. Effects of cyanide on the MCD spectrum of GST-PADOX-1
The enzyme (3.6 μM) was dissolved in 0.1 M Tris, pH 7.8, containing 0.025% Tween 20 at 23 °C. Spectra were recorded for the resting enzyme (Control) and after equilibration with 20 μM and 160 mM NaCN.
FIG. 9. Effect of cyanide on oxygenase activity of GST-PADOX-1

The assay mixtures contained enzyme (0.15 µM heme), 100 µM linolenate, and the indicated level of cyanide in 0.1 M Tris, pH 7.8, at 30 °C. Reaction was started by addition of enzyme. The line represents a nonlinear least squares fit to the data, assuming noncompetitive inhibition.
FIG. 10. Dependence of oxygenase activity on oleate concentration in the presence (triangles) and absence (circles) of 7.5 µM NaCN

Reaction mixtures contained PADOX-1 (0.12 µM heme) and the indicated levels of oleic acid in 0.1 M Tris, pH 7.8, at 30 °C. Reaction was started by addition of enzyme. The lines represent nonlinear least squares fits to the data.
FIG. 11. Dependence of PADOX-1 oxygenase activity on oxygen concentration in the presence (triangles) and absence (circles) of 7.5 μM NaCN.

Reaction mixtures contained enzyme (0.22 μM heme) and the indicated levels of dissolved oxygen in 0.1 M Tris, pH 7.8, at 23 °C. Reaction was started by addition of enzyme. The lines represent nonlinear regressions to the data.
| Recombinant protein | $K_m$ (18:1n-9) | $K_i$ (NaCN)$^a$ | $K_d$ (NaCN)$^b$ | Soret peak$^c$ |
|---------------------|-----------------|-----------------|-----------------|-----------------|
| GST-PADOX-1$^d$     | 19 ± 4          | 4.9 ± 0.6       | 12.9 ± 2.1      | 412–414         |
| PADOX-1$^e$         | 14 ± 4          | 4.6 ± 0.9       | 8.2 ± 1.6       | 413–415         |
| PADOX-1$^f$         | 24 ± 5          | 3.2 ± 0.4       | 14.3 ± 2.3      | 410–412         |

$^a$Determined with 100 µM 18:3n-3 as substrate.

$^b$Determined from shifts in the heme Soret absorbance peak in titrations with NaCN.

$^c$Ranges indicate the variation observed in at least four measurements for each recombinant protein.

$^d$Expressed in *E. coli*.

$^e$Cleaved from the GST-PADOX-1 fusion protein by thrombin and purified by gel filtration.

$^f$Expressed in *E. coli* without a GST domain, using the pCW vector.
Table II
Effect of metalloporphyrin supplementation on oxygenase activity measured in 0.1 M Tris, pH 7.8, with 0.025% Tween 20 and 150 μM 18:1n-9 at 30 °C

| Metalloporphyrin added to preincubation<sup>a</sup> | Metalloporphyrin added to assay<sup>b</sup> | PADOX-1 relative oxygenase rate<sup>c</sup> | GST-PADOX-1 relative oxygenase rate<sup>d</sup> |
|---------------------------------------------------|--------------------------------------|---------------------------------|---------------------------------|
| None                                              | None                                 | 1.00               | 1.00               |
| Heme (5.0 μM)                                     | None                                 | 1.02 ± 0.04         | 1.03 ± 0.01         |
| None                                              | Heme (1 μM)                          | 1.20 ± 0.09         | 1.16 ± 0.04         |
| None                                              | MnPPIX (1 μM)                        | 1.01 ± 0.05         | 1.00 ± 0.02         |

<sup>a</sup>The concentration of holoenzyme added to the preincubation (calculated from A<sub>410</sub> using an extinction coefficient of 140 mM<sup>-1</sup> cm<sup>-1</sup>) was 3.5 μM for PADOX-1 and 1.8 μM for GST-PADOX-1.

<sup>b</sup>Any metalloporphyrin in the preincubation was diluted 600-fold in the assay.

<sup>c</sup>Control activity was 4.1 ± 0.1 nmol of O<sub>2</sub>/min.

<sup>d</sup>Control activity was 9.6 ± 0.2 nmol of O<sub>2</sub>/min.
Table III
Heme characteristics and oxygenase activities of PADOX-1 with mutations in putative axial heme ligands

Soluble recombinant GST-PADOX-1s with mutations in either the putative proximal heme ligand (His-389) or the putative distal heme ligand (His-163) were purified by affinity chromatography before their absorbance spectra were recorded, and their oxygenase activity was measured with linoleate. Activities were normalized to the amount of recombinant protein using immunoblot densities. $K_d$ values for cyanide binding were calculated from absorbance increases near 430 nm as a function of the cyanide concentration, as done for the experiment in Fig. 7. $K_i$ values were calculated from the decreases in activity as a function of cyanide concentration, as for the experiment in Fig. 9.

| Construct | Resting Soret peak position | Oxygenase specific activity | $K_d$ for CN$^-$ | $K_i$ for CN$^-$ |
|-----------|----------------------------|----------------------------|-----------------|-----------------|
|           | nm | % wild type | $\mu$ | $\mu$ |
| Wild type | 413 | 100 | 13 ± 2 | 5 ± 1 |
| H163C     | 414 | 6 | 1.6 ± 0.3 | 40 ± 5 |
| H163M     | 420 | 17 | >280a | 127 ± 11 |
| H163Q     | 414 | <1 | >240a | NDb |
| H163Y     | 414 | 12 | 66 ± 12a | 18 ± 2 |
| Y386F     | 413 | <1 | 11 ± 1 | NDb |
| R387H     | 414 | 99 | 15 ± 2 | 31 ± 6 |
| H389C     | 400 | 8 | 75 ± 10d | 32 ± 4 |
| H389M     | 396 | 8 | 60 ± 6d | 30 ± 4 |
| H389Q     | Nonec | Nonec | Nonec |
| H389Y     | Nonec | Nonec | Nonec |

a No isosbestic point observed (not a simple $E + CNS^- \rightleftharpoons E - CNS^-$ transition).
b Not determined.
c Recombinant protein expressed in insoluble form.