The cyclooxygenase (COX) activity of prostaglandin endoperoxide H synthases (PGHSs) catalyzes the committed step in prostaglandin (PG) formation (1–3).

PGHSs are the primary source of free radicals in mammalian cells (1–3). These peroxidases (POX) catalyze the heterolytic cleavage of primary and secondary hydroperoxides with much more readiness than H2O2, but the basis for this specificity has been unresolved. Several large amino acids form a hydrophobic “dome” over part of the heme, but when these residues were mutated to alanines there was little effect on Compound I formation from H2O2 or 15-hydroperoxyeicosatetraenoic acid, a surrogate substrate for PGHS. Ab initio calculations of heterolytic bond dissociation energies of the peroxyl groups of small peroxides indicated that they are almost the same. Molecular Dynamics simulations suggest that PGG2 binds the POX site through a peroxyl-iron bond, a hydrogen bond with His-207 and van der Waals interactions between the peroxyl group and the protoporphyrin IX. We speculate that these latter interactions, which are not possible with H2O2, are major contributors to PGHS POX specificity. The distal Glu-203 four residues removed from His-207 have been thought to be essential for Compound I formation. However, Q203V mutants catalyzed heterolytic cleavage of peroxides and exhibited native COX activity. PGHSs are homodimers with each monomer having a POX site and COX site. Cross-talk occurs between the COX sites of adjoining monomers. However, no cross-talk between the POX sites of monomers was detected in a PGHS-2 heterodimer comprised of a Q203R monomer having an inactive POX site and a G533A monomer with an inactive COX site.

Prostaglandin endoperoxide H synthases (PGHSs) catalyze the committed step in prostaglandin (PG) formation (1–3).

There are two PGHS isoforms, PGHS-1 and PGHS-2, which are also known as cyclooxygenase-1 and -2 (COX-1 and -2). PGHSs catalyze two reactions including a COX reaction in which arachidonic acid is converted to prostaglandin G2 (PGG2) and a peroxidase (POX) reaction in which PGG2 is reduced to PGH2 (1–3). Both PGHS-1 and PGHS-2 are located on the luminal side of the endoplasmic reticulum and nuclear envelope (4–7). In general, PGHS-1 is constitutively expressed, whereas PGHS-2 is inducibly expressed in many cell types (8, 9).

The COX and POX reactions occur at structurally distinct but functionally interconnected sites of PGHSs (1–3). In the POX reaction the Fe3+-protoporphyrin IX (PPIX) is first oxidized to an oxoferryl heme radical \( \pi \)-cation, referred to as Compound I, which is similar to Compound I of horseradish peroxidase (10–13). Compound I can either be reduced by exogenous reductants to an oxoferryl heme form (Compound II), or it can undergo intramolecular reduction, involving transfer of an electron from Tyr-385, forming a Compound II-like spectral intermediate and a tyrosyl radical (1–3, 14, 15). This latter complex is known as Intermediate II and is analogous to the intermediate ES of cytochrome \( c \) peroxidase (16–19). Intermediate II is the COX active form of the enzyme. When arachidonic acid is present in the COX site, the Tyr-385 radical reduces a hydrogen atom from C-13 of arachidonate, triggering the COX reaction (20). Intermediate II is regenerated within the COX site when PGG2 is produced. PGHS reconstituted with Mn3+-PPIX proceeds through a COX catalytic cycle analogous to that of Fe3+-PPIX PGHS, but formation of the manganese-Compound I-like species is much slower (21–23).

Activation of the COX activity of PGHS-2 is triggered at a hydroperoxide concentration that is about 10 times lower than that required for PGHS-1 (24). This may be important in cells co-expressing PGHS-1 and PGHS-2, where PGHS-2 COX activity can function when PGHS-1 COX activity is latent (25, 26).

PGG2 is considered to be a natural substrate for PGHS POX activity, but other peroxides are also substrates. The identity of the peroxide that initiates COX activity in vivo is not known. PGHS-1 is reported to prefer primary and secondary alkyl hydroperoxides to \( \mathrm{H}_2\mathrm{O}_2 \) or bulky peroxides like \( \mathrm{rBuOOH} \). The molecular basis for this sub-
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FIGURE 1. POX site of ovPGHS-1 as observed in the crystal structure (27). His-388 is the proximal ligand group. His-207 is the distal histidine. Glu-203 is the distal glutamine. Val-291, Leu-294, Leu-295, Leu-408, and Phe-409 are the residues that compose the hydrophobic dome. In this surface model of the POX site, carbon is shown in gray, nitrogen in blue, and oxygen in red. In the ball and stick model, carbon is yellow, nitrogen is blue, and oxygen is red.

strate preference is not known. Examination of the crystal structures of the POX active sites in ovine (ov) PGHS-1 (Fig. 1) and murine PGHS-2 indicate that the distal surface of the heme group is open to the solvent and that the site is sufficiently spacious to accommodate large and linear hydroperoxides coming directly from the aqueous environment (27, 28). A dome comprised of mostly hydrophobic amino acids lies above the distal surface of the heme, and Molecular Dynamics modeling has suggested that these residues can interact with alkyl chains of alkyl hydroperoxides related to PGG2 (29, 30). Harrison has suggested that these residues can interact with alkyl the distal surface of the heme, and Molecular Dynamics dome comprised of mostly hydrophobic amino acids lies above coming directly from the aqueous environment (27, 28). A spacious to accommodate large and linear hydroperoxides and murine PGHS-2 indicate that the distal surface of the heme structures of the POX active sites in ovine (ov) PGHS-1 (Fig. 1)strate preference is not known. Examination of the crystal buffer (20 mM Tris-HCl, pH 8.0, 5% glycerol, 150 NaCl and 

EXPERIMENTAL PROCEDURES

Materials—15-Hydroperoxyeicosatetraenoic acid (15-HPETE) was synthesized as described previously (33) or purchased from Cayman Chemical Company. Ethyl hydroperoxide (EtOOH) was from Polyscience. t-BuOOH, H2O2, guaiacoil, phenol, and NaCN were from Sigma. Polyoxyethylene-6-decyl ether (C10E6) was from Anatrace. Fe3+-protoporphyrin 1X (PPIX) and Mn3+-PPIX were from Frontier Scientific. Arachidonic acid, 15-hydroxyeicosatetraenoic acid (15-HETE), 15- ketoehicosatetraenoic acid (15-KETE), and 5-phenyl-4-pentenyl alcohol (PPA) were from Cayman Chemical Co.. Hexanes, isopropanol, and acetic acid were HPLC grade from Fisher. Other chemicals were analytical grade from Sigma.

Mutagenesis, Protein Expression, and Purification—A cDNA for ovPGHS-1 containing a hexahistidine (His6) tag at the N terminus (34, 35) was subcloned into pFastBac plasmid (Invitrogen). The QuikChange site-directed mutagenesis protocol (Stratagene) was used to construct the mutants. pFastBac plasmids were used for transposition of DH10Bac Escherichia coli cells following Bac-to-Bac expression system protocols (Invitrogen). Mutants were identified by antibiotic resistance and blue/white screening. DNA was isolated and used to transfect Spodoptera frugiperda (SF-21) insect cells (Invitrogen) as a Cellfectin (Invitrogen) lipid-bacmid DNA complex. Baculovirus was precipitated from media with polyethylene glycol (Mr, 3350), and the DNA fragments containing mutant PGHSs were amplified by PCR for further sequence verification. Media containing baculovirus with correctly mutated sites were harvested and used for cell infection.

SF-21 cells were infected with a multiplicity of infection of 0.01, and cell pellets were harvested 4 days later when cell viability had dropped below 85%. Cell pellets were resuspended in 20 mM Tris-HCl, pH 8.0, and broken by sonication. Cell lysates were solubilized for 1 h with 0.8% C10E6. The supernatant after ultracentrifugation at 158,000 × g x 2 h was incubated with 4 ml of Ni-NTA fast flow-agarose (Qiagen) per liter of cell culture in the presence of 5% glycerol, 500 mM NaCl, and 5 mM imidazole. The slurry was poured into a column and washed with washing buffer (20 mM Tris-HCl, pH 8.0, 5% glycerol, 500 mM NaCl, 20 mM imidazole, and 0.1% C10E6). Bound PGHS was eluted with three column volumes of 250 mM imidazole. These latter eluates were pooled, concentrated, and desalted using a Sephadex G-25 (Sigma) spin column, which was pre-swelled in desalting buffer (20 mM Tris-HCl, pH 8.0, 5% glycerol, 150 NaCl and 0.02% C10E6).

A G533A/Q203R-huPGHS-2 heterodimer was constructed and expressed using procedures described previously (32). Mutated FLAG-tagged G533A-huPGHS-2 cDNA was cleaved from the pFastBac vector with Stul and Kpnl and inserted downstream of Promoter p10 in pFastBac Dual vector that had been treated with Smal and Kpnl. Mutated His6-tagged Q203R-huPGHS-2 was cloned into Promoter PH of pFastBac Dual using the same strategy except that the EcoRI and HindIII were used to digest the plasmid. The correct orientation and positions of the inserts were confirmed by sequencing and restriction digestion. The heterodimer was expressed in the baculovirus system as described above and purified by a combination of
Ni-NTA and anti-FLAG-agarose chromatography (32). The purity was determined by SDS-PAGE and by Western blot analysis using anti-His<sub>6</sub> and anti-FLAG antibodies (Sigma).

**POX Activity Assays**—POX reactions were conducted in 100 µl of filtered and degassed buffer, 100 mM Tris-HCl, pH 8.0, and 100 mM NaCl. Heme-reconstituted PGHS (76 nm) containing 9.0 mm guaiacol was mixed with an equal volume of a peroxide substrate solution using a stopped-flow apparatus (SX-60 HiTech Instruments). Formation of the guaiacol oxidation product 3,3'-dimethoxydiphen-4,4'-quinone was monitored at 436 nm ($\epsilon_{436} = 6,390 \text{ M}^{-1} \text{ cm}^{-1}$ (36)). The initial velocity $v$ was determined when guaiacol oxidation was linear with time. Plots of the initial velocities as a function of hydroperoxide substrate concentrations were used in the Michaelis-Menten equation $v = V_{\text{max}}[S]/(K_m + [S])$ to determine $V_{\text{max}}$ and $K_m$ values; $k_{\text{cat}}$ is the activity/mmol of enzyme.

**COX Activity Assays**—COX reaction mixtures contained 3 ml of 0.1 M Tris-HCl, pH 8.0, 100 µm arachidonic acid, 1 mM phenol, and 5 µm hematin equilibrated in a glass chamber at 37 °C. Reactions were initiated by adding enzyme to the assay chamber. A Yellow Springs Instruments Model 53 oxygen monitor was used to monitor O<sub>2</sub> consumption by native or mutant PGHSs with kinetic traces recorded using DasyLab (DasyTec) software. The rates reported are maximal rates occurring after a lag phase. One unit of COX activity is defined as 1 µmol of O<sub>2</sub> consumed/min/mg of enzyme at 37 °C in the assay mixture. The lag time is defined as the time required for the COX activity to reach a maximum after initiating the reaction.

**Identification of POX Spectral Intermediates and Kinetic Analysis**—Presteady state analysis of the POX reactions was performed with a rapid mixing and scanning technique using a stopped-flow apparatus equipped with double grating monochromators (DX-60 HiTech Instruments). ApoPGHS was reconstituted with Fe<sup>3+</sup>-PPIX or Mn<sup>3+</sup>-PPIX at a stoichiometry of 0.8 per enzyme monomer. The final heme concentrations were usually 1–2 µm, and substrate concentrations were 2–4 µm 15-HPETE or 50–100 µm H<sub>2</sub>O<sub>2</sub>. In single wavelength experiments at least 10-fold higher substrate concentrations were used to ensure steady state kinetics. Both the enzyme and substrate solutions were prepared in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.02% C<sub>10</sub>E<sub>6</sub>. The enzyme and substrate solutions in individual syringes were mixed rapidly by triggering them into a mixing chamber driven through an optical cell and stopped with a third syringe. Different oxidation states of Fe-PPIX or Mn-PPIX were monitored spectroscopically. In the case of Fe<sup>3+</sup>-PPIX, the signal decay at 411 nm is due to the consumption of resting enzyme and the formation of Compound I. A peak shift to 420 nm represents the formation of Compound II and Intermediate II; these two species have the same UV-visible spectral features. There are two isobestic points in the spectral scans; 427 nm between resting enzyme and Compound I and 410 nm between Compound I and Compound II-Intermediate II. The kinetic traces were extracted from spectra at isobestic points and fitted to exponential equations to obtain pseudo-first-order rate constants ($k_{\text{obs}}$). For the second-order reaction of Compound I formation, $k_1$ was the slope from the linear part of a plot of pseudo-first-order rate constants $k_{1(\text{obs})}$ versus hydroperoxide substrate concentrations. For Intermediate II formation, $k_2$ was numerically equal to the maximum $k_{2(\text{obs})}$ at saturating substrate concentrations for the intramolecular two-species reaction system.

With ovPGHS-1 reconstituted with Mn<sup>3+</sup>-PPIX, the resting enzyme (3 µm) when mixed with 50 µm 15-HPETE showed decreases in absorbance at 372, 472, and 561 nm and a new peak at 417 nm. A consecutive three-species model (SpecFit, BioLogic Science Instruments) based on a singular value decomposition algorithm was exploited to resolve intermediates derived from Mn<sup>3+</sup>-PPIX ovPGHS-1. Kinetic traces were collected at 417 nm and fitted to two exponential equations. A three-exponential equation was applied for higher substrate concentrations when side reactions (e.g., reduction of oxidized heme intermediates or suicide inactivation) may be involved.

**Cyanide Binding**—Purified ovPGHS-1 proteins (1–3 µm) were reconstituted with 0.8 mol of Fe<sup>3+</sup>-PPIX/protein monomer and incubated at room temperature for 30–60 min. The protein solution was centrifuged at 10,000 × g for 10 min, and 1 ml of the supernatant was transferred to a quartz cuvette. Aliquots of a concentrated NaCN stock solution were added to the protein solution to yield final cyanide concentrations of 0, 0.08, 0.16, 0.24, 0.32, 0.40, 0.65, 1.15, 1.65, 2.90, 4.15, 6.65, 11.65, 16.65, and 26.65 mM. The protein-ligand mixture was incubated for 5 min for each concentration of cyanide. The absorbance changes measured at 430 nm where the maximum change occurs were fitted to ΔAU = ΔAU<sub>max</sub> [CN<sup>-</sup>]/(K<sub>d</sub> + [CN<sup>-</sup>]) to calculate a dissociation constant (K<sub>d</sub>).

**Analysis of 15-HPETE Reaction Products**—POX reactions were conducted in a 500-µl reaction mixture containing 50 nM native ovPGHS-1 or Q203V-ovPGHS-1 dimer, 50 nM hematin, and 4.5 mM phenol in 100 mM Tris-HCl, pH 8.0, at room temperature. The reactions were initiated by adding 15-HPETE (final concentration of 5 µm) and terminated at 3 min by adding 1.5 ml of a pre-cooled (4°C) mixture of diethyl ether, methanol, 0.2 M citric acid (30:4:1). PPA (5 µm) was added as an internal control. The organic phase was shaken with 380 µl of a saturated NaCl solution at 4 °C to remove H<sub>2</sub>O. The upper organic layer was transferred to a clean tube and evaporated under N<sub>2</sub>. The products were dissolved in 100 µl of hexane:isopropanol: acetic acid (987:12:1; running solution) and resolved by HPLC on a Nucleosil Silica column (5 µm, 250 × 4.6 mm, PJ Cobert Associates) mounted on a Shimadzu HPLC system equipped with diode array detector. The bound products were eluted with running solution at a flow rate of 1 ml/min. 15-HPETE and 15-HETE were monitored at 235 nm, and 15-KETE was at 279 nm. PPA was eluted after the other standards; it has a peak absorbance at 249 nm but was monitored at 235 nm. HPLC tracings were converted to bitmap format, and peak areas were quantified using WinDIG 2.5 digitizing freeware.

**MP2-level Calculations of the Heterolytic Bond Dissociation of an O–O Bond**—The quantum chemical calculations were carried out by using the second-order Møller-Plesset perturbation method (MP2) as implemented in the Gaussian98 software package (37). All the structures were geometry-optimized and their vibrational frequencies calculated using the 6-311+ + G** basis set in which polarization and diffuse functions are included.

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Hydroperoxides and Cyclooxygenase

Molecular Dynamics (MD) Simulation of PGG2, Binding to the POX Site—The protocol for creating a model of PGG2, inserting it in ovPGHS-1, and running molecular dynamics on the complex is described in detail in our previous work on a similar substrate (29). A brief summary is as follows. A molecular structure for PGG2 was generated using the MOE software package (38), and the resulting atom coordinates were optimized with use of Gaussian98 (37). Atom-centered charges for the electrostatic part of the force field for MD were then generated with the Merz-Kollman method (37). The coordinates of PGHS were obtained from the crystal structure (Protein Data Bank code 1CQE) of ovPGHS-1. The PGG2 structure was docked to PGHS by using a modified simulated annealing protocol, which found space for PGG2 by a fragment-based search procedure. The coordinates of the best-docked PGG2-PGHS complex, based on an energy criterion, were used to initiate a 1-ns MD simulation. The MD was carried out with the SANDER module of AMBER7 (39) using explicit solvent (~5000 waters) in a 80 × 80 × 80-Å3 box, with the ionization states of all residues set appropriate to pH 7.

RESULTS

Hydroperoxide Substrate Specificity of the POX Activity of ovPGHS-1—Previous studies have established that ovPGHS-1 catalyzes guaiacol oxidation efficiently with several primary and secondary alkyl hydroperoxide substrates (i.e. PGG2, 5-phenyl-4-pentenyl-1-hydroperoxide, and 15-HPETE (2, 3, 24, 40). However, because the POX activity undergoes rapid suicide inactivation (35, 41, 42), it has been not possible to obtain accurate rate constants using conventional steady state spectroscopic assays. We developed and exploited a rapid mixing stopped-flow assay to determine ovPGHS-1 POX activity more precisely. Oxidation of the reducing substrate guaiacol was monitored at 436 nm immediately after the reaction components were mixed. This method permits steady-state product formation to be quantified before enzyme suicide inactivation affects the rates significantly. The initial rates of guaiacol oxidation as a function of substrate concentrations are shown for representative hydroperoxides in Fig. 2. The \( k_{cat}/K_m \) values for guaiacol-dependent hydroperoxide reduction by ovPGHS-1 were 2.7, 0.23, 0.01, and 0.0023 \( \mu M^{-1} s^{-1} \) for 15-HPETE, EtOOH, \( H_2O_2 \), and \( t-BuOOH \), respectively. Thus, as observed previously (1–3, 43), primary and secondary lipophosphores (i.e. 15-HPETE and EtOOH) are much better substrates than \( H_2O_2 \) or \( t-BuOOH \). Notably, there was no detectable inhibition of ovPGHS-1 POX activity with a variety of alkyl alcohols including 5-HETE and 15-HETE even at alcohol to hydroperoxide ratios of more than 1000.

Rate Constants for Compound I Formation with Different Hydroperoxides—Native ovPGHS-1 reconstituted with Fe\(^{3+} \)-PPIX has a major peak at 411 nm. It forms a Compound I spectral intermediate when incubated with 15-HPETE or EtOOH also at 411 nm as illustrated in Fig. 3A for 15-HPETE. This is followed by an accumulation of a Compound II-Intermediate II spectral intermediate with a peak at 420 nm, which is resolved using singular value decomposition analysis (Fig. 3A, center panel). There are two other minor bands at about 530 and 560 nm in the visible region. Compound I was formed from 15-HPETE or EtOOH with ovPGHS-1 with second-order rate constants in the range of \( 10^{-6}–10^{-7} M^{-1} s^{-1} \) (Table 1). Native ovPGHS-1 showed spectral changes only with high concentrations of \( H_2O_2 \). After mixing 50 \( \mu M \) \( H_2O_2 \) and 1 \( \mu M \) ovPGHS-1, the Soret peak at 411 nm slowly decreased (Fig. 3B). There was a slight red shift (~4 nm) after a short time that occurred concomitant with the appearance of Compound II-Intermediate II-like spectral features at longer wavelengths (Fig. 3B). However, the heme appeared to be decomposing (bleaching) as evidenced by decreased absorbance at later time points. Similar phenomena have been reported previously (15, 44). Bleaching is commonly seen with heme proteins in their reactions with peroxides and compromise the resolution of active intermediates (45, 46). Because of this we were unable to identify an appropriate model to resolve pure species of Compound I with \( H_2O_2 \). Listed in Table 1 is a second order rate constant for Compound I formation from ovPGHS-1 with \( H_2O_2 \) reported by others where the value was determined by fitting the early phases of the decay of resting enzyme to a two-exponential equation, assuming a consecutive two-step reaction mechanism for \( H_2O_2 \) reduction by ovPGHS-1 (21). Table 1 shows that the efficiency of hydroperoxides as heme oxidants follows the same trend as their steady state activities. \( H_2O_2 \) and \( t-BuOOH \) had \( k_1 \) values 100–1000 times lower than those of 15-HPETE and EtOOH.
TABLE 1  
Presteady state rate constants for Compound I formation by Fe$^{3+}$-PPIX ovPGHS-1 with different hydroperoxides

| Hydroperoxides | $k_1$ | References |
|----------------|-------|------------|
| 15-HPETE       | $5.3 \times 10^7$ | Present study |
| EtOOH          | $7.1 \times 10^7$ | 24, 43 |
| HOOH           | $2.5 \times 10^6$ | Present study |
| t-BuOOH        | $9.9 \times 10^5$ | 21, 24 |

COX Activation by Different Hydroperoxides—$O_2$ consumption during the COX reaction with arachidonic acid is sigmoidal (47); plots of activity versus time show a lag before reaching maximal velocity. In a typical COX assay, the COX activity of PGHS is activated by trace amounts of hydroperoxide contaminants commercial fatty acid substrate preparations (48). The first few COX reaction turnovers then generate sufficient PGG$_2$ to support a burst of oxygen consumption. NaCN binds to the heme group of ovPGHS-1 and interferes with the reduction of 15-HPETE by guaiacol with a $K_i$ of 0.11 mM (data not shown). The presence of 10 mM cyanide in the COX assays by slowing peroxide reduction and comonitant heme oxidation leads to longer lag times in the COX reaction (49). In our hands, lipid hydroperoxides (i.e., 15-HPETE and EtOOH) shortened the lag times that were prolonged by cyanide, but H$_2$O$_2$ and t-BuOOH had little or no effects at the concentrations tested (Table 2).

**Mutations of Hydrophobic Residues in the POX Dome**—Data on the steady state catalytic efficiencies of PGHS POX activity and the rate constants for Compound I formation with different hydroperoxides along with the abilities of various hydroperoxides to activate PGHS COX functioning are consistent with ovPGHS-1 POX preferring lipid hydroperoxides to hydrophilic or bulky substrates. The molecular basis for this specificity has not been determined. Forming a hydrophobic dome over the distal site of the heme of PGHS are side chains of leucine, valine, and phenylalanine residues (Fig. 1). Molecular dynamic simulations suggest that these side chains interact with alkyl side chains of peroxides (29, 30). Alanine substitutions at some of the sites caused some alterations of POX activity toward 15-HPETE (Table 3). However, there was no obvious pattern to the changes in rates. About half of the mutations caused qualitatively similar changes with 15-HPETE versus H$_2$O$_2$, and there were no changes in $k_{cat}$ values that were more than 10-fold. A second group of mutations involved replacements of one of the residues in the dome, Leu-294 with alanine (i.e., L294D and L294E) showed much reduced turnover numbers and catalytic efficiencies with 15-HPETE. The L294E mutant had a $k_{cat}$ value that was less than 2% that of native ovPGHS-1; however, the UV-visible spectra of native and L294E-ovPGHS-1 reconstituted with heme were indistinguishable, and as described below, native and L294E-ovPGHS-1 bound heme equally well.

Rate Constants for Compound I Formation by Fe$^{3+}$-PPIX ovPGHS-1 Variants with Different Hydroperoxides—Several alanine-substituted mutant enzymes were selected to examine rates of formation of heme spectral intermediates. Table 4 compares the rate constants for Compound I and Compound II-Intermediate II formation by native, L294A, and V291A/L294A ovPGHS-1 with 15-HPETE or H$_2$O$_2$. These enzymes formed...

### TABLE 2  
Oxygen consumption and lag times in COX activation

| Components       | Lag time | Maximum rate |
|------------------|----------|--------------|
| NaCN, 10 mM      | 10       | 48 ± 1.8     |
| NaCN, 100 mM     | 72       | 4.9 ± 2.3    |
| NaCN, 200 mM     | 24       | 12 ± 0.36    |
| NaCN, 500 mM     | 26       | 9.9 ± 0.54   |
| NaCN, 1 mM       | 66       | 6.1 ± 0.07   |
| NaCN, 10 mM      | 78       | 15 ± 0.07    |

**FIGURE 3.** UV/visible spectra of Compound I and Compound II-Intermediate II complex formation. A, 1 μM Fe$^{3+}$-PPIX ovPGHS-1 with 2 μM 15-HPETE. B, 2 μM Fe$^{3+}$-PPIX ovPGHS-1 with 50 μM H$_2$O$_2$. Experiments were performed as described under "Experimental Procedures." Vertical arrows indicate the directions of peak changes after ovPGHS-1 was mixed with hydroperoxide. Formation of Intermediate II-Compound II as judged by the formation of a peak at 420 nm was resolved using singular value decomposition analysis as shown in the center panel in A.
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Compound I from 15-HPETE at about the same rate as native ovPGHS-1 when reconstituted with Fe\(^{3+}\)-PPIX. These results indicate that substituting residues in the dome with a smaller amino acid does not affect Compound I formation; however, as judged by \(k_{cat}\) values for POX catalysis (Table 3), the expanded POX active site may in some cases affect electron transfer to the heme group is structurally the same as heme \(b\) except for the metal ion, and it is a reasonable analog with which to study heme behaviors when the heme is labile (21–23). Mn\(^{3+}\)-PPIX ovPGHS-1 exhibits strong Soret absorption, and its oxidized variants can be easily detected spectroscopically; moreover, the slower POX reaction rates facilitate observation of oxidized intermediates.

Native ovPGHS-1 reconstituted with Mn\(^{3+}\)-PPIX showed three major peaks at 372, 472, and 561 nm (data not shown). After mixing with 15-HPETE, absorption at these wavelengths decayed, and a new peak formed at 417 nm as described by others (21–23). Kinetic traces were collected at 417 nm where maximum absorbance changes occurred and fit to two exponential equations to calculate rate constants for formation of the intermediates. Similar to the pattern of \(k_i\) values for Fe\(^{3+}\)-PPIX ovPGHS-1 forms, the rate constants for Compound I formation from 15-HPETE by Mn\(^{3+}\)-PPIX ovPGHSs showed no significant changes with any of the mutant enzymes except for L294E-ovPGHS-1. The \(k_i\) value for this latter mutant was 2 orders of magnitude lower than that of native enzyme. Thus, introduction of a large side chain having a negative charge reduces the rate of Compound I formation significantly.

### Table 3

| ovPGHS-1 | 15-HPETE | \(K_m\) | \(k_{cat}/K_m\) |
| --- | --- | --- | --- |
| Native | \(270 \pm 2.7\) | \(14 \pm 0.5\) | \(19\) |
| V291A | \(300 \pm 18\) | \(31 \pm 5\) | \(9.8\) |
| L294A | \(730 \pm 28\) | \(22 \pm 2\) | \(33\) |
| L295A | \(230 \pm 68\) | \(460 \pm 170\) | \(0.5\) |
| L408A | \(35 \pm 0.6\) | \(20 \pm 1.4\) | \(1.7\) |
| F409A | \(670 \pm 32\) | \(98 \pm 8.4\) | \(6.8\) |
| V291A/L294A | \(300 \pm 18\) | \(28 \pm 5\) | \(11\) |
| L294A/L295A | \(580 \pm 39\) | \(70 \pm 15\) | \(5.5\) |
| L294A/F409A | \(480 \pm 26\) | \(66 \pm 7.5\) | \(7.3\) |
| L295A/L408A | \(72 \pm 7.8\) | \(33 \pm 8.0\) | \(2.2\) |
| V291A/L294A/L295A | \(290 \pm 7\) | \(120 \pm 5\) | \(2.5\) |
| L294G | \(2000 \pm 68\) | \(140 \pm 10\) | \(10\) |
| L294S | \(2100 \pm 170\) | \(210 \pm 25\) | \(40\) |
| L294D | \(39 \pm 5.6\) | \(110 \pm 34\) | \(0.4\) |
| L294E | \(5.2 \pm 0.15\) | \(17 \pm 1.6\) | \(0.3\) |
| Q203V | \(135 \pm 2.0\) | \(42 \pm 2.2\) | \(3.2\) |
| Q203N | \(51 \pm 2.4\) | \(80 \pm 14\) | \(0.7\) |
| Q203R | \(0 \pm N/A\) | \(0\) | N/A |

### Table 4

| ovPGHS-1 | 15-HPETE | \(K_m\) | \(k_{cat}/K_m\) |
| --- | --- | --- | --- |
| Native | \(5.3 \pm 0.2\) | \(460 \pm 50\) | \(0.9\) |
| V291A | \(6.2 \pm 1\) | \(460 \pm 60\) | N/A |
| L294A | \(8.5 \pm 0.3\) | \(200 \pm 40\) | \(7.0 \pm 0.2\) |
| L294G | \(7.6 \pm 0.9\) | \(140 \pm 6\) | \(3.9 \pm 0.6\) |

### Table 5

| ovPGHS-1 | 15-HPETE | \(K_m\) | \(k_{cat}/K_m\) |
| --- | --- | --- | --- |
| Native | \(9.3 \pm 1.1\) | \(5.3 \pm 0.0049\) | \(510 \pm 59\) |
| L294A | \(8.4 \pm 0.7\) | N/A | \(270 \pm 13\) |
| V291A | \(6.6 \pm 1.4\) | \(14 \pm 1.0\) | \(24 \pm 1.3\) |
| V291A/L294A | \(6.0 \pm 0.47\) | \(3.3 \pm 7.8\) | \(79 \pm 3.7\) |
| L294G | \(13 \pm 2.7\) | \(6.7 \pm 0.0\) | \(140 \pm 16\) |
| L294E | \(0.997 \pm 0.16\) | \(0.21 \pm 0.050\) | \(89 \pm 4.4\) |
| L294D | \(3.1 \pm 0.26\) | N/A | \(65 \pm 11\) |
| Q203V | \(3.5 \pm 0.85\) | \(8.1 \pm 0.53\) | N/D |

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\(a\) Estimated value (81).
TABLE 6
Effect of Val-291, Leu-294, and Gln-203 on CN− binding to the Fe3+-PPIX ovPGHS-1 group of ovPGHS-1

| ovPGHS-1   | K_dCN− | μM   |
|------------|---------|------|
| Native     | 0.41 ± 0.04 |      |
| V291A      | 0.06 ± 0.004 |      |
| L294A      | 0.33 ± 0.002 |      |
| V291L294A  | 0.14 ± 0.03 |      |
| L294E      | 0.59 ± 0.05 |      |
| Q203V      | 1.01 ± 0.05 |      |
| Q203R      | 0.83 ± 0.08 |      |

Based on the information in Tables 4 and 5, we conclude that hydrophobic residues of the distal heme active site are not particularly important in Compound I formation from 15-HPETE. As shown in Table 6, the dissociation constants (K_d) for the binding of CN− to the heme group of native and several mutant PGHS were similar with the possible exception of V291A ovPGHS-1. This is consistent with the idea that hydrophobic residues of the dome have no major effects on the heme group.

In contrast to what was observed with 15-HPETE, Compound I formation from H2O2 by Mn3+-PPIX ovPGHS-1 was affected by the V291A mutation by a factor of about 20 (Table 5). This raises the possibility that although Val-291 may not affect the binding and reduction of alkyl hydroperoxides, it may actually facilitate the use of H2O2 as a substrate. It is unclear if this is related to the effect of Val-291 on the heme group as identified by the effect on CN− binding.

**COX Activation of ovPGHS-1 Mutants by Hydroperoxides**

At low concentrations of hydroperoxides, the lag time in COX catalysis is determined by the efficiency of Compound I formation and its conversion to Intermediate II. For native ovPGHS-1, a typical lag time in our assays was 10 s at a protein concentration of 23 nm. Substitutions of hydrophobic residues in the ovPGHS-1 POX dome with glycine, alanine, or aspartate had no influence on the lag times for O2 consumption by ovPGHS mutants (Table 7). L294E-ovPGHS-1 had a lag time three times longer than for native ovPGHS-1, consistent with L294E-ovPGHS-1 having a much lower rate of Compound I formation.

**Mutations of Gln-203**—The role of Gln-203, which is located four amino acids away from the distal histidine and is conserved as either a glutamine or a basic residue in all peroxidasases, has been reported to be essential for Compound I formation (50). Gln-203 is thought to stabilize a developing charge on the distal oxygen atom of hydroperoxide, thereby facilitating heterolytic cleavage of the O–O bond. Indeed, eliminating the amide group of the Gln-203 side chain has been reported to eliminate the POX activity of muPGHS-2 (50). Thus, we were surprised to find that Q203V ovPGHS-1 had functional POX (Table 3) and COX (Table 7) activities. Although the COX activity is essentially the same for native and Q203V-ovPGHS-1, the k_cat/K_m for the POX activity of Q203V-ovPGHS-1 is only 17% that of the native enzyme. The results of our COX assays are not inconsistent with what has been observed with other PGHS mutants that have low POX activity but retain near maximal COX activity (e.g. Ref. 50). Q203V-ovPGHS-1 had the same lag time and specific activity as native enzyme, indicating that Gln-203 is not needed to activate the COX activity of ovPGHS-1.

Consistent with previous studies with murine PGHS-2 (50), a conservative substitution of Gln-203 to asparagine yielded a Q203N-ovPGHS-1 having 20 and 7% of native POX activity with 15-HPETE and H2O2, respectively, whereas a Q203R-ovPGHS-1 mutant was inactive. Because of the apparent discrepancy between our data with Q203V-ovPGHS-1 and previous data on murine PGHS-2 (50), we expressed, isolated, and tested Q203V mutants of ovPGHS-1, human PGHS-2, and murine PGHS-2 prepared from the same starting insect cells; the COX-specific activities of the three proteins were 31, 31, and 39 units/mg, respectively.

The UV-visible spectrum of Fe3+-PPIX Q203V-ovPGHS-1 was indistinguishable from that of native Fe3+-PPIX ovPGHS-1, and native and Q203V-ovPGHS-1 bound CN− equally well (Table 6). However, no Compound I formation could be detected in stopped-flow assays when Fe3+-PPIX Q203V-ovPGHS-1 was incubated with either 15-HPETE or H2O2 (data not shown). Moreover, all of the Mn3+-PPIX ovPGHS-1 variants tested, Mn3+-PPIX Q203V-ovPGHS-1 produced the smallest change in absorbance at 417 nm when incubated with 15-HPETE and no change in absorbance with H2O2 (Table 5). In short, the Mn3+-PPIX version of Q203V-ovPGHS-1 showed a trace of a Compound I-like spectral species, but the Fe3+-PPIX form did not.

To determine whether Q203V-ovPGHS-1 catalyzed a one- or two-electron reduction of 15-HPETE, we analyzed the products formed from 15-HPETE. Heterolytic cleavage of 15-HPETE occurring through a 2-electron reduction yields the alcohol (15-HETE), whereas homolytic cleavage leads to a number of products including the ketone (15-KETE) (50–53). As shown in Fig. 4, normal phase HPLC chromatograms of products generated by native and Q203V-ovPGHS-1 with 15-HPETE and phenol were virtually identical. When normalized to PPA, the peak areas for 15-HPETE as well as to that of 15-HPETE in the sample with 15-HPETE alone. The proteins were reconstituted with 1 mol of heme/mol of dimer, so little or no free heme should be present; however, it should be noted that when 15-HPETE alone was incubated with 50 nm heme, 75–80% of the 15-HPETE was lost. A peak eluting at 6.5 min was seen in all samples that contained heme plus 15-HPETE.
whether or not enzyme was present; the identity of the material eluting at this position, which has prominent peaks at 211, 229, and 268 nm was not determined. There was no detectable 15-KETE based on the absorbance at 279 nm with either native or Q203V-ovPGHS-1 (data not shown); in the case of Q203V-ovPGHS-1, there was a small doublet peak at 279 nm with a retention time of 11.6 min. We did not pursue the identification of these latter products because all of the 15-HPETE product could be accounted for as 15-HETE. Overall, our results showing equal amounts of 15-HETE formation by native and Q203V-ovPGHS-1 led us to conclude that Gln-203 is not required for heterolytic cleavage of hydroperoxide substrates by PGHSs.

Energetic Properties of Hydroperoxide Heterocleavage—Our experimental work suggested that the hydroperoxide substrate specificity of PGHS POX might simply be determined by the intrinsic reactivity of hydroperoxides instead of, as predicted, by key residues in the hydrophobic heme dome. A potential difference could arise from the bond dissociation energies (BDE) for the hydroperoxyl groups of different hydroperoxides. The calculated gas phase BDEs were 374, 374, 370, and 364 kcal/mol for H$_2$O$_2$, methyl hydroperoxide, ethyl hydroperoxide, and 2-hydroperoxyl-3-trans-pentene (a surrogate for 15-HPETE), respectively, for heterolytic cleavage of the O–O bond. The calculated gas phase BDEs for homolytic cleavage were 46, 43, and 45 kcal/mol for H$_2$O$_2$, methyl hydroperoxide, and ethyl hydroperoxide, respectively.

These values that, where available, are in agreement with literature values (54, 55) show that heterolytic O–O bond breakage requires significantly higher activation energy than homolysis (e.g. ~360 versus ~40 kcal/mol). For small hydroperoxides the calculations can be carried out with a large basis set using the MP2 method. The calculations indicate that no significant differences exist between the BDEs of the small hydroperoxides, although what we refer to as Model HPETE, with the indicated conjugation, does show some evidence of a lowered heterolytic BDE. Of course, to obtain heterolytic BDEs of a realistic size, electron donation from the porphyrin ring and the iron and protonation by His-207 aided by the proximal histidine is required, as suggested in the classic push-pull mechanism (56, 57). Thus, we can only conclude that it is not the intrinsic O–O bond strength that is responsible for differences in substrate specificity between HPETE and H$_2$O$_2$. A more likely explanation resides in the physical aspects of docking substrates to the enzyme.

**MD Simulation of PGG$_2$ Binding to the POX Site**—We previously reported on the interaction between a PGG$_2$ analog (pseudo-PGG$_2$) that does not include the endoperoxide group and ovPGHS-1 that was carried out by first docking pseudo-

![FIGURE 4](image-url)

**FIGURE 4.** Native and Q203V-ovPGHS-1 catalyze heterolytic cleavage of 15-HPETE. POX assays were performed at room temperature for 2 min, and the products were extracted and separated by normal phase HPLC as detailed under “Experimental Procedures.” Unless otherwise indicated, reaction mixtures contained 50 nM hematin and 4.5 mM phenol. Tracings shown in panels B–H are for 235 nM. Quantitation of peak areas was performed using WinDIG freeware and normalized to the internal standard PPA. A, composite curves showing the location of standards. 1, 15-KETE (279 nm); 2, 15-HETE (235 nm); 3, 15-HPETE (235 nm); 4, PPA (235 nm). B, native ovPGHS-1 (50 nM) plus 15-HPETE (5 μM). C, native ovPGHS-1 (50 nM). D, 15-HPETE (5 μM). E, Q203V-ovPGHS-1 (50 nM) plus 15-HPETE (5 μM). F, Q203V-ovPGHS-1 (50 nM). G, extract of sham reaction mixture. H, extract of reaction mixture containing 15-HPETE (5 μM) and no hematin.
PGHS-2, we constructed, expressed, and purified a FLAG-

FIGURE 5. Contacts of PGG2 with key residues of ovPGHS-1. The dome residues on the distal side of the heme plane are displayed in space-filling representation. PGG2 is in stick representation with the endoperoxide group, the five-membered ring is in yellow, and the Fe3⁺-protoporphyrin IX (heme) is in ball and stick representation. The α oxygen of the endoperoxide is ligated to the iron as indicated by the solid line. Two hydroperoxide hydrogen bonds, to His-207 and Gln-203, are indicated with dashed lines.

PGG2 to ovPGHS-1 followed by MD on the complex (29). The same procedure was performed with PGG2. The interactions between PGG2 and ovPGHS-1 are displayed in Fig. 5. The hydroperoxide is ligated to the iron (α Oxygen-iron distance ~2.1 Å). The hydroperoxide hydrogen is hydrogen-bonded to His-207 and its β-terminal oxygen is hydrogen-bonded to Gln-203. The ω-carbon chain containing carbons C13-C20 interacts with the dome residues Lys-211, Gln-289, and Val-291. Its carboxylate chain (containing carbons C1-C7) interacts with residues, and the (ionized) carboxylate group interacts with solvent. The endoperoxide ring is sequestered by the hydrophobic residues Val-291, Leu-294, Leu-295, Leu-408, and Phe-409. Thus, this and our pseudo-PGG2 simulation (29) along with another recent PGG2 simulation (30) all show that the alkyl chains of PGG2 can make van der Waals contacts with residues in the hydrophobic dome. Importantly, there are contacts between the first 2–3 carbons on either side of the carbon bearing the hydroperoxyl group and the protoporphyrin IX group. All the mutations listed in Table 3 are of residues that interact with PGG2 (Fig. 5). Although the MD suggests that hydrogen bonding occurs between PGG2 and Gln-203, this interaction is clearly not essential for POX catalysis.

Cross-talk between Monomers of huPGHS-2 Heterodimers—The COX activity of PGHSs exhibits half of the site activity with only one site able to catalyze an oxygenation reaction at any given time (32). Related to this, it has been proposed that PGHS-1/PGHS-2 heterodimers can form in vivo and in vitro and that the POX activity of the PGHS-2 subunit can activate the COX activity of the partner PGHS-1 subunit (58). To test for cross-talk between the POX and COX active sites of PGHS-2, we constructed, expressed, and purified a FLAG-tagged G533A-/His6-tagged Q203R-huPGHS-2 heterodimer; the purification involved using sequential Ni-NTA and anti-FLAG affinity chromatography as described previously for COX active site mutants (32). As positive controls, we prepared and analyzed His6-tagged native huPGHS-2, His6-tagged G533A huPGHS-2 and His6-tagged Q203R-huPGHS-2 homodimers. All of the recombinant proteins were more than 95% pure as determined by SDS-PAGE (data not shown). As expected, all of the proteins were recognized by the antibodies against the His6 tag and PGHS-2, whereas only the heterodimer was reactive with the anti-FLAG antibody (32) (Fig. 6).

As reported previously, the POX activity of the G533A huPGHS-2 homodimer was about the same as that of native PGHS-2 (Fig. 5; Ref. 32). Moreover, as observed with Q203R-ovPGHS-1 (Table 3), the Q203R-huPGHS-2 homodimer lacked POX activity (Fig. 5). The G533A/Q203R-huPGHS-2 heterodimer, lacking POX activity in one monomer, had 20–30% of the POX activity of the native huPGHS-2 homodimer, which is less than the predicted 50%. Although, there are other explanations, we suspect that the mutant monomers do not interact as well in the heterodimer as identical monomers of a homodimer. Nonetheless, despite having significant POX activity, the G533A/Q203R-huPGHS-2 heterodimer exhibited no COX activity; moreover, the addition of exogenous 15-HPETE did not cause activation of the COX activity of the heterodimer. The results indicate that the POX site of the G533A monomer is unable to activate the COX site of the Q203R monomer. From this, we conclude that oxidation of the heme group in the POX site of one monomer does not lead to oxidation of the Tyr-385 group in the COX site of the partner monomer comprising a PGHS homodimer.

DISCUSSION

It is well established that peroxides are required for the COX activity of PGHSs (1–3, 49, 59, 60). Peroxides function by oxidizing the heme group at the POX site of PGHSs, which in turn leads to oxidation of Tyr-385 in the COX site, and the Tyr-385 radical abstracts a hydrogen from arachidonic acid in the rate-determining step in COX catalysis (1–3). There is a subtle difference in the peroxide requirements of PGHS-1 and PGHS-2, the biological importance of which remains to be resolved. PGHS-2 is activated at about a 10-fold lower lipid hydroperoxide concentration than PGHS-1 (61–63). In principle, this would permit PGHS-2 to operate under conditions where PGHS-1 is not activated (64). It is known that PGHS-2 can function at low arachidonic acid concentrations in NIH 3T3 cells when PGHS-1 is latent (25), that this could be due to the negative allosterism behavior of PGHS-1 (26, 65), and that at least in vitro the negative allosterism can be overcome by the addition of peroxides (63).

The physiological hydroperoxide initiator of the COX reaction is not known, although both peroxynitrite and organic peroxides are the most likely candidates depending on the cell type (66). Lipid peroxides can be formed both nonenzymatically from reactive oxygen species and polyunsaturated fatty acids in membrane lipids (67) and enzymatically through the actions of 5-, 12-, and 15-lipoxygenases, which typically oxygenate free polyunsaturated fatty acids.
Hydroperoxides and Cyclooxygenase

A

Coomassie-stained

Anti-PGHS-2

Anti-HIS

Anti-FLAG

B

FIGURE 6. POX and COX activities of PGHS-2 heterodimers. Hexahistidine-tagged (His6) native and G533A- and Q203R-huPGHS-2 homodimers and FLAG-tagged G533A/His6-Q203R-huPGHS-2 heterodimer were expressed and purified as described under “Experimental Procedures.” A, the purity was confirmed by Coomassie-stained SDS-PAGE and Western blotting using antibodies to PGHS-2, hexahistidine or FLAG. B, normalized POX and COX activities of native and G533A and Q203R homodimers and G533A/Q203R-huPGHS-2 heterodimer. PGHS-2 POX reactions were performed at 4 °C in 0.1 ml of 100 mM Tris-HCl, pH 8.0, containing 70 pM of PGHS monomers, 1 mM phenol, and 100 µM 15-HPETE at different concentrations (4, 8, 16, 32, 64, and 128 µM). The initial rates of guaiacol oxidation were determined as described in the legend to Fig. 2. Rate-concentration simulations using the Michaelis-Menten equation yielded a kcat of 253 s−1 and a Km of 88 µM for native huPGHS-2. C, specific COX activities were determined in 3 ml of 100 mM Tris-HCl, pH 8.0, containing 70 pm of PGHS monomers, 1 mM phenol, and 100 µM arachidonic acid. O2 consumption was monitored using an O2 electrode probe, and initial rates were calculated using DasyLab software.

H2O2 also has the potential to activate the POX activity of PGHSs, but the concentration of H2O2 is expected to be relatively low in the lumen of the endoplasmic reticulum where PGHSs reside (68–70).

POX Peroxide Substrate Specificity—Previous studies by other laboratories have indicated that primary and secondary alkyl hydroperoxides are the best POX substrates (1–3,43), and the results of our POX assays have confirmed and extended this by providing more accurate rate measurements.

The basis for the peroxide substrate specificity has been an enigma because the POX site of PGHSs is relatively open to solvent (28, 31, 71, 72). Modeling studies, including our own, have suggested that the alkyl groups of secondary hydroperoxides (i.e. PGG2 derivatives) can interact with residues in the hydrophobic dome that overlies a portion of the distal surface of the heme group at the PGHS POX site (29, 30). However, we find that modifications of these residues have relatively small effects on POX catalytic efficiency or on the rate constants for of Compound I formation from 15-HPETE. The relatively small effects seen with some mutants having substitutions of dome residues can be attributed to differences in the rates of reduction of oxidized heme intermediates by guaiacol. Accordingly, we conclude that the hydrophobic dome does not greatly influence lipid hydroperoxide binding or heterolysis. We considered the possibility that the hydroperoxide substrate specificity resides in the different reactivities of the O–O bonds of different hydroperoxides toward heterolytic cleavage. However, calculation of gas phase BDE failed to support this concept.

Although our MD studies indicate that there are interactions between portions of PGG2 and various dome residues, changes of up to three of the hydrophobic residues in the dome (e.g. V291A/L294A/L295A (Table 3) and V291A/L294A (Table 4)) do not appear to have a major effect on PGHS POX catalysis. Moreover, POX catalysis appears to be reasonably independent of interactions involving the endoperoxide group and 5-membered ring of PGG2, that are predicted by MD because the ki values for Compound I formation from 15-HPETE and PGG2 are quite similar (24, 73).

The ki value for Compound I formation from EtOOH is a tenth of that for 15-HPETE, and the ki for H2O2 is less than a hundredth of that for 15-HPETE (74, 75). This suggests that it is the nature of the first few atoms neighboring the hydroperoxide group that is the most important determinant of the rate of Compound I formation. Having 2–3 carbons in an alkyl chain neighboring a primary hydroperoxyl group would not be expected to have strong interactions with residues in the dome. However, there could well be interactions with the protoporphyrin IX group that would not be present with H2O2.

Gln-203 in POX Catalysis—One of the most surprising findings of this study was that Gln-203 was not essential for POX catalysis as had been indicated in earlier studies (50). Analysis of the products of 15-HPETE reduction by native ovPGHS-1 and Q203V-ovPGHS-1 indicated that this substrate underwent a two-electron reduction in both cases. However, with Q203V-ovPGHS-1, we were able to detect traces of a Compound I-like spectral intermediate only with the Mn3+-PPIX form of this mutant enzyme. We speculate that in the case of Fe3+-PPIX Q203V-ovPGHS-1 plus 15-HPETE, Compound I is short-lived because in the stopped-flow experiments the oxidized heme intermediates are rapidly reduced by 15-HPETE itself. 15-HPETE is a reasonably efficient reductant of oxidized heme species with native ovPGHS-1 (76) but perhaps more efficient with Q203V-ovPGHS-1.
Cross-talk between POX and COX Active Sites—Radical migration has been of interest in the context of the PGHS reaction mechanism. Radicals may migrate within or between proteins including PGHS-1 (77), DNAs, or lipids (78, 79). For example, electrons can be transferred from a human myoglobin molecule to a tyrosyl radical in another nearby molecule (80). Funk and coworkers (58) have suggested that a PGHS-2 lacking molecule to a tyrosyl radical in another nearby molecule (80).

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