Mutational Analysis of the Role of the Distal Histidine and Glutamine Residues of Prostaglandin-Endoperoxide Synthase-2 in Peroxidase Catalysis, Hydroperoxide Reduction, and Cyclooxygenase Activation*

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Site-directed mutants of prostaglandin-endoperoxide synthase-2 (PGHS-2) with changes in the peroxidase active site were prepared by mutagenesis, expressed in Sf-9 cells, and purified to homogeneity. The distal histidine, His193, was mutated to alanine and the distal glutamine, Glu198, was changed to asparagine, valine, and arginine. The guaiacol peroxidase activities of H193A, Q189V, and Q189R were drastically reduced to levels observed in the absence of protein; only Q189N retained wild-type PGHS-2 (wtPGHS-2) activity. The mechanism of hydroperoxide reduction by the PGHS-2 mutants was investigated using 15-hydroperoxyeicosatetraenoic acid (15-HPETE), a diagnostic probe of hydroperoxide reduction pathways. The hydroperoxide reduction activity of Q189V and Q189R was reduced to that of free Fe(III) protoporphyrin IX levels, whereas Q189N catalyzed more reduction events than wtPGHS-2. The percentage of two-electron reduction events was identical for wtPGHS-2 and Q189N. The number of hydroperoxide reductions catalyzed by H193A was reduced to ~60% of wtPGHS-2 activity, but the majority of products were the one-electron reduction products, 15-KETE and epoxyalcohols. Thus, mutation of the distal histidine to alanine leads to a change in the mechanism of hydroperoxide reduction. Reaction of wtPGHS-2, Q189N, and H193A with varying concentrations of 15-HPETE revealed a change in product profile that suggests that 15-HPETE can compete with the reducing substrate for oxidation by the peroxidase higher oxidation state, compound I. The ability of the PGHS-2 proteins to catalyze two-electron hydroperoxide reduction correlated with the activation of cyclooxygenase activity. The reduced ability of H193A to catalyze two-electron hydroperoxide reduction resulted in a substantial lag phase in the cyclooxygenase assay. The addition of 2-methylimidazole chemically reconstituted the two-electron hydroperoxide reduction activity of H193A and abolished the cyclooxygenase lag phase. These observations are consistent with the involvement of the two-electron oxidized peroxidase intermediate, compound I, as the mediator of the activation of the cyclooxygenase of PGHS.

Peroxidases are enzymes found in plants, fungi, bacteria, and animals that catalyze the reduction of hydroperoxides (1, 2) (Reaction 1). They serve not only to reduce hydroperoxides but to generate oxidized intermediates with numerous cellular functions. For example, the higher oxidation state of myeloperoxidase oxidizes chloride ion to the bacteriostatic hypochlorous acid (3), whereas the higher oxidation state of soybean peroxidase epoxidizes unsaturated fatty acids to plant defense molecules (4).

ROOH + XH2 → ROH + XHX2O

REACTION 1

Heme-containing peroxidases from evolutionally diverse sources retain a characteristic folding pattern and contain structurally equivalent helices surrounding the heme prosthetic group (2, 5). The crystal structures of seven peroxidases have been solved: yeast cytochrome c peroxidase (6), fungal lignin peroxidase (7, 8), Arthromyces ramosus peroxidase (9), peanut peroxidase (10), fungal chloroperoxidase (11), canine and human myeloperoxidase (12, 13), and ovine, murine, and human prostaglandin-endoperoxide synthases (PGHSs) (5). Six of these peroxidases contain a histidine residue as the fifth ligand to the heme iron and a histidine on the distal side of the heme 4–5 Å above the iron.2 An additional residue is present on the distal side of the heme that is believed to participate with the distal histidine in two-electron reduction of the hydroperoxide substrate (14). This residue is arginine for the plant, fungal, and yeast peroxidases and glutamine for the mammalian peroxidases (Fig. 1).

Peroxidases reduce hydroperoxides by two electrons to generate the corresponding alcohol and a spectroscopically detectable higher oxidation state called compound I (Reaction 2) (15–18). The spectral properties of compound I of mammalian peroxidases are consistent with the Fe(IV)=O porphyrin cation radical (19) (1, 18). One-electron reduction of compound I by an

1 The abbreviations used are: PGHS, prostaglandin-endoperoxide synthase (EC 1.14.99.1); wtPGHS, wild-type PGHS; Fe-PPIX, Fe(III) protoporphyrin IX; 15-HPETE, 15-hydroperoxyeicos-5,8,11,13-tetraenoic acid; 10-OH-18:3, 10-hydroperoxyoctadec-9:11,13-trienoic acid; PPHP, 5-phe

2 Chloroperoxidase contains a cysteine thiolate as the fifth ligand to the heme iron.
distal histidine, H193 to alanine or glutamine abolishes peroxidase and cyclooxygenase activity (30). Extensive investigation of the role of active site residues in PGHS catalysis has been hindered by the absence of a high level expression system. For example, expression of PGHS-1 in SF-9 cells produces mainly inactive protein (31). Recently, several groups reported successful expression of milligram quantities of active PGHS-2 in SF-9 cells using baculovirus vectors (32–34). Therefore, we constructed site-directed mutants of PGHS-2, designed to assess the role of the distal peroxidase residues in catalysis, and the mutated proteins were expressed in SF-9 cells. In this manner, sufficient amounts of the purified mutants were available for characterization of their biochemical properties. The distal glutamine of PGHS-2, Glu189, was changed to asparagine, valine, and arginine. In addition, the distal histidine, His193 was mutated to alanine. The effects of these mutations on peroxidase activity, the mechanism of hydroperoxide reduction, and cyclooxygenase activity were assessed and are described here.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled arachidonic acid and linolenic acid were from Nu-Chek Prep, Inc. (Elysian, MN). [1-14C]Arachidonic acid (57 mCi/mmol) and [1-13C]linolenic acid (53 mCi/mmol) were from NET Inc. Life Science Products. Soybean lipoxigenase (type I-B), hematin (Fe-PPIX), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), epinephrine, butylated hydroxyanisole, and diethyldithiocarbamate were from Sigma. Guaiacol and 2-methylimidazole (2-MeIm) were from Aldrich. 5-phenyl-4-pentenyl-1-hydroperoxide (PPHP) was synthesized as described (35). 10-Hydroxyprostaglandin dehydrogenase (10-OH-PGDH) was prepared as described (36, 37).

*Human PGHS-2 Mutagenesis and Expression*—The coding region of human PGHS-2 was subcloned into the plasmid pALTER-1, and in vitro mutagenesis was performed using an Altered Sites® II kit according to the manufacturer's instructions (Promega, Madison, WI). The final mutagenesis reaction was transformed into competent BMHI 71–18 mutS cells (Clonetech, Palo Alto, CA), and the cells were grown overnight in LB broth without ampicillin. Plasmid DNA was isolated and used to transform competent DH10B cells (Life Technologies, Inc.). Plasmid DNA from ampicillin-resistant colonies was screened for the desired mutation by sequencing. Human PGHS-2 inserts containing the desired mutation with no secondary misincorporations were isolated with Qiaex resin (Qiagen, Chatsworth, CA) and subcloned into the baculovirus transfer vector pVL 1393 (Pharmingen, San Diego, CA) for expression in insect (SF-9) cells.

SF-9 cells (from Spodoptera frugiperda) were grown in spinner flasks at 27 °C in a humidified incubator with 1% trypsin phosphate broth containing 10% fetal bovine serum. Recombinant baculoviruses were isolated by transfecting 5 μg of plasmid DNA with 400 ng of linearized baculovirus DNA (Pharmingen, San Diego, CA) using the calcium phosphate method (38). Using the linearized DNA, the transfection mixture yields >99% recombinants. A stock of the recombinant baculoviruses was made from the transfection supernatant. SF-9 cells were grown to 0.5 × 10^6 cells/ml (1-liter spinner flask) in serum-containing medium at 27 °C. The cells were infected with PGHS recombinant baculovirus inoculum at a multiplicity of infection of 0.1 and harvested after 4 days.

*Human PGHS-2 Purification from Infected SF-9 Cells*—wtPGHS-2 and the PGHS-2 mutants were purified as described previously by Gierse et al. (32). Further purification of the mutant proteins was achieved by anion exchange chromatography on a Mono Q column (Pharmacia). PGHS-2 mutants were eluted from the Mono Q with a linear gradient from 0 to 500 mM NaCl in 25 mM Tris-HCl, 0.1% CHAPS, pH 8.0. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis. The PGHS-2 mutants were stored at −80 °C in 25 mM Tris-HCl, 150 mM NaCl, 0.1% CHAPS, pH 8.0. The specific activity of wtPGHS-2 was 10–12 μmol of arachidonic acid min⁻¹ mg⁻¹.

**Cyclooxygenase Activity**—Cyclooxygenase activity was determined by measuring oxygen consumption at 37 °C with a Gilson model 568

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**Fig. 1. The peroxidase active site of PGHS-1**.

Electron donor leads to formation of compound II, a Fe(IV)=O species with the porphyrin fully covalent (Reaction 3). One-electron reduction of compound II regenerates the resting Fe(III) state of the enzyme (Reaction 4).

**REACTIONS 2–4**

Fe IV-PPIX + ROOH → O=Fe IV-PPIX + ROH

O=Fe IV-PPIX + e⁻ → O=Fe IV-PPIX

O=Fe IV-PPIX + e⁻ → Fe III-PPIX + H₂O

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**Expression of site-directed mutants of PGHS-1 in COS cells** has shown that mutation of the proximal histidine His388 or the distal histidine, H207 to alanine or glutamine abolishes peroxidase and cyclooxygenase activity (30). Extensive investigation of the role of active site residues in PGHS catalysis has been hindered by the absence of a high level expression system. For example, expression of PGHS-1 in SF-9 cells produces mainly inactive protein (31). Recently, several groups reported successful expression of milligram quantities of active PGHS-2 in SF-9 cells using baculovirus vectors (32–34). Therefore, we constructed site-directed mutants of PGHS-2, designed to assess the role of the distal peroxidase residues in catalysis, and the mutated proteins were expressed in SF-9 cells. In this manner, sufficient amounts of the purified mutants were available for characterization of their biochemical properties. The distal glutamine of PGHS-2, Glu189, was changed to asparagine, valine, and arginine. In addition, the distal histidine, His193 was mutated to alanine. The effects of these mutations on peroxidase activity, the mechanism of hydroperoxide reduction, and cyclooxygenase activity were assessed and are described here.

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**Cyclooxygenase Activity**—Cyclooxygenase activity was determined by measuring oxygen consumption at 37 °C with a Gilson model 568
oxygraph equipped with a Clark electrode and a thermostatted cuvette (1.3 ml) (Gilson Medical Electronics, Inc., Middleton, WI). Enzyme aliquots (150–200 nL) were added to 0.1 M Tris-HCl, pH 8.0, containing 500 μM phenol. Oxygen uptake was initiated by the addition of 100 μM arachidonic acid, and the initial reaction velocity was determined from the linear portion of the oxygen uptake curve.

The cyclooxygenase activity of wtPGHS-2 and the PGHS-2 mutants was also assayed by thin layer chromatography. Standard reaction mixtures (200 μL) contained 22 mM PGHS-2, 500 μM epinephrine, 100 μM diethyldithiocarbamate, and 12 μM [1-14C]arachidonic acid in 0.1 M Tris-HCl, pH 8.0. Prior to the addition of [1-14C]arachidonic acid, PGHS-2 was reconstituted with 2 eq of Fe-PPIX for 2 min. Reactions were terminated after 10 min by solvent extraction with 200 μL of ethyl ether, methanol, 1 M citric acid, pH 4.0 (30:4:1), containing 10 μg of butylated hydroxyanisole and 10 μg of unlabeled arachidonic acid. The organic layers were spotted onto silica gel plates, and the plates were developed with ethyl acetate/methylene chloride/glacial acetic acid (75:25:1) at 4°C. Radiolabeled products were quantitated with a radioactivity flow detector (IN/US Systems, Products were eluted isocratically in hexane/2-propanol/glacial acetic acid (20:80:0.005). The level of purity of the PGHS-2 mutants was assessed by comparative HPLC on a Zorbax Rx-Sil column (5-μm, 25 cm × 4.6 mm). The PGHS-1 proteins were detergent-extracted from the membrane fraction of insect cell lysates and partially purified by preparative HPLC on a Zorbax Rx-Sil column (5-μm silica, 4.6 cm × 25 cm). wtPGHS-1 and apo-PGHS-1 were reconstituted with 0.5 eq of Fe-PPIX for 2 min in 0.5 mM Tris-HCl, pH 8.0, containing 500 μM guaiacol. The peroxidase reaction was initiated by the addition of radiolabeled hydroperoxide in methanol. Following incubation at room temperature for varying periods of time, the reaction was quenched with 1 M HCl, and the lipid products were extracted with 2 × 2 vol of ether. When low concentrations of [1-14C]15-HPETE were used, 14 nM of unlabeled 15-HPETE was added to the quenched reactions to reduce degradation during the extraction steps. The ether layers were dried with anhydrous MgSO4, and the solvent was evaporated under nitrogen. The dried lipid products were resuspended in hexane and subjected to HPLC analysis using a Zorbax Rx-Sil analytical column (5-μm silica, 4.6 cm × 250 mm). Products were eluted isocratically in hexane/2-propanol/glacial acetic acid (98:2:1) at a flow rate of 2.0 mL/min. The eluent was monitored at 235 nm, and the 15-HPETE was collected manually. The solvent was evaporated, and the 15-HPETE was stored at −20°C in methanol. [1-14C]13-OOH-18:3 was prepared enzymatically from [1-14C]linolenic acid using soybean lipoxygenase (0.6 mg). [1-14C]15-HPETE was purified by preparative HPLC as described for 15-HPETE (41). The concentrations of [1-14C]15-HPETE and [1-14C]13-OOH-18:3 were determined from the UV absorption at 235 nm using a molar absorptivity of 23,000 M−1 cm−1 (42).

Hydroperoxide Reduction Assays—The PGHS-2 mutants, wtPGHS-2, and apo-PGHS-1 were reconstituted with 0.5 eq of Fe-PPIX for 2 min in 50 mM of 0.1 M Tris-HCl, pH 8.0, containing 500 μM guaiacol. The peroxidase reaction was initiated by the addition of radiolabeled hydroperoxide in methanol. Following incubation at room temperature for varying periods of time, the reaction was quenched with 1 M HCl, and the lipid products were extracted with 2 × 2 vol of ether. When low concentrations of [1-14C]15-HPETE were used, 14 nM of unlabeled 15-HPETE was added to the quenched reactions to reduce degradation during the extraction steps. The ether layers were dried with anhydrous MgSO4, and the solvent was evaporated under nitrogen. The dried lipid products were resuspended in hexane and subjected to HPLC analysis using a Zorbax Rx-Sil analytical column (5-μm silica, 4.6 cm × 250 mm). Products were eluted isocratically in hexane/2-propanol/glacial acetic acid (98:2:1) at a flow rate of 1 mL/min. The radiolabeled products were quantitated using a radioactivity flow detector (IN/US Systems, Inc., Tampa, FL).

Guaiacol Peroxidase Assay—Peroxidase assays were performed using a Shimadzu UV1601 spectrophotometer. PGHS protein (100 nM or 200 nM) was reconstituted with 1 eq of Fe-PPIX in 100 mM Tris-HCl, pH 8.0, containing 500 μM guaiacol. The reaction was initiated with the addition of 100 μM PPHP or 100 μM 15-HPETE in methanol. The rate of guaiacol oxidation was monitored at 436 nm (ε436 = 6390 M−1 cm−1) (43). To determine Km and Vmax values for wtPGHS-2 and Q189N, ABTS was used as the reducing substrate. Peroxidase assays contained 100 nM PGHS protein, 100 nM Fe-PPIX, 500 μM ABTS, and varying concentrations of 15-HPETE in 0.1 M Tris, pH 8.0. The molar absorptivity used to calculate mol ABTS oxidized was 34,700 M−1 cm−1 at 420 nm (44).

RESULTS

PGHS-2 mutants with changes in the peroxidase active site were prepared by site-directed mutagenesis and expression in SF9 cells. The PGHS-2 protein was detergent-extracted from the membrane fraction of insect cell lysates and partially purified by anion exchange chromatography. Several of the mutants, H193A, Q189V, and Q189R, were rechromatographed on a Mono Q column in the presence of 0.1% CHAPS. The PGHS-2 mutants eluted as a broad band between 200 and 250 mM NaCl. The level of purity of the PGHS-2 mutants was assessed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Purification to homogeneity was achieved for wtPGHS-2, Q189N, and H193A, whereas Q189R and Q189V were purified to ~60% homogeneity.

Peroxidase Activity—The effect of amino acid substitution on the guaiacol peroxidase activity of PGHS-2 was measured with PPHP or 15-HPETE as the hydroperoxide substrate. Anion exchange chromatography removes any protein-bound Fe-PPIX; therefore, the proteins were reconstituted with 1 eq of Fe-PPIX. Fe-PPIX in Me2SO was added to PGHS protein in Tris buffer containing guaiacol and allowed to reconstitute for 2 min prior to addition of the hydroperoxide substrate. Control assays were performed in the absence of PGHS protein to correct for the low peroxidase activity of free Fe-PPIX. The rates of guaiacol oxidation for wtPGHS-2 and the PGHS-2 mutants are reported in Table I. The guaiacol peroxidase activity with both hydroperoxide substrates of H193A was reduced drastically to approximately the level of activity observed in the absence of protein. Likewise, Q189V and Q189R had no guaiacol peroxidase activity above Fe-PPIX alone with either hydroperoxide substrate. Only Q189N displayed guaiacol peroxidase activity with both hydroperoxide substrates equivalent to that of wtPGHS-2.

Peroxidase assays were also performed using ABTS, rather than guaiacol, as the reducing substrate. The ABTS cation radical absorbs strongly at 420 nm (ε420 = 34,700 M−1 cm−1), which provides for greater sensitivity. PGHS undergoes rapid self-inactivation; therefore, the maximal number of ABTS oxidation events (mol of ABTS oxidized/mol of PGHS), rather than the rate of ABTS oxidation, was used to reflect total enzyme activity. In the ABTS peroxidase assay, H193A had detectable peroxidase activity above Fe-PPIX levels. H193A (100 nM protein reconstituted with 50 nM Fe-PPIX) catalyzed 78 mol of ABTS oxidized/mol of Fe-PPIX versus 49 mol of ABTS oxidized/mol of Fe-PPIX for Fe-PPIX alone at 50 μM 15-HPETE.

Q189N displayed guaiacol peroxidase activity equivalent to wtPGHS-2 when assayed with 100 μM PPHP or 150 μM 15-HPETE (Table I). To further probe the peroxidase activity of Q189N and wtPGHS-2, these proteins were assayed over a range of 15-HPETE concentrations with 500 μM ABTS as reducing substrate. Fig. 3 plots mol of ABTS oxidized/mol of PPHP versus 49 mol of ABTS oxidized/mol of Fe-PPIX for Fe-PPIX alone at 50 μM 15-HPETE.
observed when guaiacol was used as the reducing substrate. From these data, $K_m$ and $V_{\text{max}}$ values were calculated. $V_{\text{max}}$ represents the maximal number of turnovers catalyzed by the peroxidase at saturating concentrations of peroxide. Previous studies have shown this to be an accurate reflection of $V_{\text{max}}$ (45). The $K_m$ and $V_{\text{max}}$ values were 4.9 $\mu$M and 132 mol of ABTS oxidized/mol of PGHS for wtPGHS-2 and 10.9 $\mu$M and 215 mol of ABTS oxidized/mol of PGHS for Q189N.

Hydroperoxide Reduction Activity—The above data reflect the effect of mutation on the entire peroxidase catalytic cycle i.e. reduction of the hydroperoxide and reduction of the porphyrin higher oxidation states by guaiacol. To further dissect the peroxidase catalytic cycle, the initial hydroperoxide reduction step was characterized. The protein component of peroxidases facilitates formation of the peroxidase higher oxidation states and defines the mechanism of hydroperoxide reduction by the heme prosthetic group. Whereas peroxidases catalyze exclusively two-electron reduction of a hydroperoxide, free Fe-PPIX catalyzes predominantly one-electron-electron hydroperoxide reduction. Thus, mutations in the peroxidase active site could affect the mechanism of hydroperoxide reduction by PGHS.

Our laboratory has developed several diagnostic molecules to probe the mechanism of hydroperoxide reduction by metal complexes. Following reaction of a diagnostic hydroperoxide with a metal complex or peroxidase, the products are separated by HPLC, characterized, and quantitated to determine the mechanism of hydroperoxide reduction. The mechanism of hydroperoxide reduction by the PGHS-2 mutants was investigated using [1-14C]15-HPETE. 15-HPETE is an analog of the PGHS substrate, PGG$_2$, and can be readily synthesized from arachidonic acid using soybean lipoxygenase. The sole product of two-electron reduction of 15-HPETE is the corresponding alcohol, 15-HETE. Following one-electron reduction of 15-HPETE, the resulting alkoxyl radical can be further oxidized to 15-KETE, or it can cyclize to form an epoxyallylic radical that eventually forms epoxyhydroperoxides and epoxyalcohols (Scheme 1).

To determine the mechanism of hydroperoxide reduction, wtPGHS-1, wtPGHS-2, and the PGHS-2 mutants were incubated with [1-14C]15-HPETE and guaiacol or ABTS. Several measures were taken to normalize reaction conditions. The purified apoproteins were reconstituted with 0.5 eq of Fe-PPIX to minimize the presence of unbound Fe-PPIX in the assay mixtures. Free Fe-PPIX will react with hydroperoxides; therefore, control reactions with 50 nM Fe-PPIX and 50 $\mu$M [1-14C]15-HPETE were performed. Because the presence of CHAPS in the protein preparations can enhance free Fe-PPIX reactivity with hydroperoxides, the final concentration of CHAPS in all incubations was normalized to 0.02%.

The radioactivity product profiles obtained for the reaction of equimolar wtPGHS-1, wtPGHS-2, and H193A with 50 $\mu$M [1-14C]15-HPETE are shown in Fig. 4. The reaction of wtPGHS-1 with [1-14C]15-HPETE consistently generated the two-electron reduction product, 15-HETE (95–98%), and a minor product, 15-KETE (2–5%). The reaction of [1-14C]15-HPETE with wtPGHS-2 generated 15-HETE (60–65%) and additional products, 15-KETE and epoxyalcohols, potentially derived from one-electron reduction of 15-HPETE to an intermediate alkoxyl radical (Scheme 1). This suggests that wtPGHS-2 catalyzes both one- and two-electron hydroperoxide reduction. The reaction of free Fe-PPIX with [1-14C]15-HPETE generated products with the same retention times as 15-KETE and the epoxyalcohols under identical chromatographic conditions. H193A catalyzed fewer turnovers of [1-14C]15-HPETE than wtPGHS-2, but, notably, the majority of products were the one-electron reduction products, 15-KETE and the epoxyalcohols. Thus, replacement of the distal histidine with an alanine leads to a substantial change in the mechanism of hydroperoxide reduction.

Table II summarizes the number of hydroperoxide molecules reduced and the number of two-electron reduction events catalyzed by the PGHS proteins at 50 $\mu$M [1-14C]15-HPETE. The maximal number of turnovers is used to reflect total enzyme activity. Comparison of the number of enzyme turnovers and the number of two-electron reduction events for the wild-type PGHS enzymes shows that wtPGHS-1 catalyzed predominantly two-electron reduction (95%) as previously reported, whereas wtPGHS-2 catalyzed 60% two-electron reduction at 50 $\mu$M 15-HPETE (however, see below). In addition, ovine PGHS-1 catalyzed 3-fold more turnovers than recombinant wtPGHS-2. The difference in hydroperoxide reduction activity between wtPGHS-1 and wtPGHS-2 may reflect actual differences in catalytic efficiencies between the two enzymes. However, they also may be due to differences in glycosylation between the native ovine PGHS-1 and the recombinant human PGHS-2 or...
to different sensitivities to hydroperoxide-dependent inactivation (see below).

The hydroperoxide reduction activity of H193A was ~60% of wtPGHS-2 activity; however, this mutant catalyzed only 40% two-electron reduction. Therefore, the ability of the H193A enzyme to catalyze two-electron reduction of 15-HPETE was 60% less than wtPGHS-2 at 50 μM 15-HPETE. The ability of H193A to reduce 15-HPETE was approximately twice that of Fe-PPIX alone. Q189N catalyzed more hydroperoxide reduction than wtPGHS-2, but the percentage of two-electron reduction was comparable. The number of enzyme turnovers and two-electron reduction events catalyzed by Q189R and Q189V was nearly the same as the free Fe-PPIX control. Higher concentrations of Q189R and Q189V (up to 1 mM) were incubated with [1-14C]15-HPETE, but no significant conversion of 15-HPETE to products above that observed with Fe-PPIX alone was detected.

To further examine the mechanism of hydroperoxide reduction, wtPGHS-2 was incubated with varying concentrations of [1-14C]15-HPETE. Representative chromatograms obtained for the reaction of wtPGHS-2 with 2.5 μM and 50 μM 15-HPETE are shown in Fig. 5. At 2.5 μM 15-HPETE, wtPGHS-2 consistently generated >97% 15-HETE, the two-electron reduction product, and 15-KETE as the only additional product. The product profile for wtPGHS-2 at 2.5 μM 15-HPETE is identical to the profile obtained for the reaction of wtPGHS-1 with 50 μM 15-HPETE shown in Fig. 3. However, reaction of wtPGHS-2 with 50 μM 15-HPETE yielded 15-HETE, 15-KETE, a rearranged hydroperoxide and epoxyalcohols. The two-electron reduction product, 15-HETE, comprised only 50% of the total products. As the concentration of 15-HPETE increased, the percentage of one-electron products increased from 3% at 2.5 μM to 63% at 100 μM. The maximal number of hydroperoxide reduction events catalyzed by wtPGHS-2 also increased as the concentration of 15-HPETE increased.

Q189N also was assayed over a range of 15-HPETE concentrations. As the concentration of 15-HPETE increased, the percentage of one-electron reductions and the maximal number of hydroperoxide reduction events catalyzed also increased as observed for wtPGHS-2. The product profile data, coupled with the ABTS peroxidase activity data for wtPGHS-2 and Q189N shown in Fig. 3, suggest that 15-HPETE can compete with the reducing substrate, ABTS or guaiacol, for reaction with the peroxidase higher oxidation state, compound I. Compound I can oxidize 15-HPETE to a peroxyl radical intermediate, and following combination of two peroxyl radicals and loss of molecular oxygen, alkoxyl radicals derived from 15-HPETE would be formed. As the concentration of 15-HPETE increases, this pathway appears to become more prevalent, and products derived from the alkoxyl radical, 15-KETE, and epoxyalcohols increase.

Table III summarizes the number of hydroperoxide molecules reduced and the number of two-electron reduction events catalyzed by the PGHS-2 proteins following reaction at 2.5 μM 15-HPETE, a concentration where the products appear to accurately mirror the initial heme-hydroperoxide interaction. Q189N consistently catalyzed nearly 2-fold greater turnovers than wtPGHS-2, and both proteins catalyzed 97% two-electron reduction of 15-HPETE. H193A catalyzed substantially fewer turnovers than wtPGHS-2, and at 2.5 μM 15-HPETE, the percentage of two-electron reduction events was 61%. In contrast, H193A catalyzed only 40% two-electron reduction at 50 μM.
Table II
Hydroperoxide reduction by FGHS enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Maximal turnover</th>
<th>Two-electron reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol product/mol enzyme</td>
<td>mol ROH/mol enzyme</td>
</tr>
<tr>
<td>wtPGHS-1</td>
<td>1518 ± 10</td>
<td>1437 (95%)</td>
</tr>
<tr>
<td>wtPGHS-2</td>
<td>500 ± 40</td>
<td>302 (60%)</td>
</tr>
<tr>
<td>H193A</td>
<td>314 ± 30</td>
<td>127 (40%)</td>
</tr>
<tr>
<td>Q189N</td>
<td>772 ± 62</td>
<td>503 (65%)</td>
</tr>
<tr>
<td>Q189R</td>
<td>148 ± 40</td>
<td>50 (34%)</td>
</tr>
<tr>
<td>Q189V</td>
<td>129 ± 8</td>
<td>47 (36%)</td>
</tr>
<tr>
<td>Fe-PPIX</td>
<td>164 ± 32</td>
<td>61 (37%)</td>
</tr>
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</table>

15-HPETE reduction by wtPGHS-2. wtPGHS-2 was reacted with [1-14C]15-HPETE in 0.1 M Tris-HCl, pH 8.0, containing 500 μM ABTS (1 ml reaction volume). A, 20 nM wtPGHS-2 reconstituted with 10 nM Fe-PPIX incubated with 2.5 μM [1-14C]15-HPETE. B, 100 nM wtPGHS-2 reconstituted with 50 μM Fe-PPIX incubated with 50 μM [1-14C]15-HPETE. The reactions were quenched after 3 min with 30 μl of 1 N HCl and the products were extracted with ether. Elution was performed with hexane/2-propanol/acetic acid (987:12:1) at a flow rate of 1 ml/min using a Zorbax Rx-Sil column (5 μm silica, 4.6 x 250 mm), and the products were quantitated using a radioactivity flow detector.

15-HPETE (Table II). Therefore, the ability of the H193A enzyme to catalyze two-electron reduction of 15-HPETE was 72% less than wtPGHS-2. The only other product detected following reaction of H193A with 2.5 μM 15-HPETE was 15-KETE (39%). In this case, 15-KETE arises by direct one-electron reduction of 15-HPETE to form the alkoxyl radical, followed by reoxidation to 15-KETE. Thus, mutation of the distal histidine to alanine affects both the rate and mechanism of hydroperoxide reduction.

Newmeyer and Ortiz de Montellano recently reported a distal histidine to alanine mutant of horseradish peroxidase (H42A) with drastically reduced peroxidase activity (46). The peroxidase activity of H42A could be rescued by incubating the peroxidase with 2-substituted imidazoles. Therefore, the hydroperoxide reduction activity of H193A was measured in the presence of 5 mM 2-MeIm. The maximal number of turnovers catalyzed by H193A was stimulated nearly 2-fold by 2-MeIm (Table III). In addition, the number of two-electron reduction events increased 2-fold. The addition of 5 mM 2-MeIm to wt-PGHS-2 did not stimulate hydroperoxide reduction but instead inhibited slightly (5–10%).

H193A was incubated with two additional fatty acid hydroperoxides, 10-OH-18:2 and 13-OH-18:3, to confirm that H193A catalyzed substantial one-electron hydroperoxide reduction. These hydroperoxides have been employed in our laboratory to characterize one-electron and two-electron reduction pathways (36, 41, 47). Alkoxyl radicals derived from one-electron reduction of 10-OH-18:2 undergo β-scission to 10-oxodec-8-enoic acid. Alkoxyl radicals derived from 13-OH-18:3 undergo β-scission to 13-octoxide-9,11-dienoic acid or cyclization to an epoxyallylic radical. The total number of hydroperoxide molecules reduced (mol product/mol H193A) and the number of two-electron reduction events (mol of ROH/mol of H193A) were determined for each of the three hydroperoxides (Table IV). The number of moles of H193A used to catalyze maximal turnovers was determined from the moles Fe-PPIX used to reconstitute the protein. The incubations with 13-OH-18:3 and 10-OH-18:2 were performed at high hydroperoxide concentrations (50 or 100 μM); therefore, some of the products derived from an alkoxyl radical may have been obscured.
Fig. 6. Oxygen uptake curves for wtPGHS-2 and H193A. PGHS-2 protein (150 nM reconstituted with 5 eq of Fe-PPIX) was added to 0.1 M Tris, pH 8.0, containing 500 μM phenol in a 37 °C-thermostatted oxygraph cell (1.3 ml) equipped with an oxygen electrode. Oxygen uptake was initiated by the addition of 100 μM arachidonic acid.

![Graph showing oxygen uptake curves for wtPGHS-2 and H193A.](image_url)

Table V

<table>
<thead>
<tr>
<th>Protein</th>
<th>Maximal rate&lt;sup&gt;a&lt;/sup&gt; (μmol 20:4 mg min/μmol enzyme)</th>
<th>Total O&lt;sub&gt;2&lt;/sub&gt; uptake</th>
<th>[1-14C]O&lt;sub&gt;2&lt;/sub&gt; conversion&lt;sup&gt;b&lt;/sup&gt;</th>
<th>mol PGE&lt;sub&gt;2&lt;/sub&gt; + PGD&lt;sub&gt;2&lt;/sub&gt;/ mol enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtPGHS-2</td>
<td>8.8 (5 s)</td>
<td>59</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>H193A</td>
<td>2.7 (15 s)</td>
<td>60</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Q189N</td>
<td>4.8/5 (s)</td>
<td>58</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>Q189R</td>
<td>0.3/75 (s)</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Q189V</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> PGHS protein (200 nM protein reconstituted with 5 eq of Fe-PPIX) was added to 0.1 M Tris-HCl, pH 8.0, containing 500 μM phenol in a 37 °C-thermostatted oxygraph cell (1.3 ml) equipped with an oxygen electrode. Oxygen uptake was initiated by the addition of 100 μM arachidonic acid (20:4), and the maximal rates of oxygen uptake were determined from the linear portion of the uptake curve. The lag phase (in seconds) before maximal uptake is presented in parentheses.

<sup>b</sup> [1-14C]arachidonic acid conversion assays (200 μM) contained 22 nM PGHS protein reconstituted with 2 eq of Fe-PPIX, 500 μM epinephrine, 100 μM diethyldithiocarbamate, and 12 μM [1-14C]arachidonic acid. Higher concentrations of Q189R and Q189V (100 nM) reconstituted with 2 eq of Fe-PPIX) were required to detect conversion to products. Reactions were terminated after 10 min at room temperature with 200 μl of ethyl ether, methanol, 1 μl citric acid, pH 4.0 (30:4:1), containing 10 μg of butylated hydroxyanisole and 10 μg of unlabeled arachidonic acid. The radiolabeled products were separated by TLC and quantitated with a radioactivity scanner. Authentic standards of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) were applied to the TLC plate to confirm the identity of the prostaglandin products.

<sup>c</sup> ND, not determined.

Formed by the reaction of hydroperoxide with compound I, H193A catalyzed predominantly one-electron reduction of the hydroperoxides, with the percentage of two-electron events ranging from only 16% for 13-OOH-18:3 to 40% for 15-HPETE and 10-OOH-18:2. The maximal number of turnovers was highest for 15-HPETE.

Cyclooxygenase Activity—The peroxidase activity of PGHS is required to activate the protein’s cyclooxygenase activity (48, 49). Therefore, we probed for the effect of the mutations on cyclooxygenase catalysis. The cyclooxygenase activity of the PGHS-2 mutants was assessed by monitoring the maximal rates of oxygen uptake and the total extent of oxygen uptake (μmol O<sub>2</sub>) with an oxygen electrode. In addition, the lag phase, defined as the time between the addition of arachidonic acid and the development of maximal oxygen uptake, was determined for each protein. PGHS-2 proteins were reconstituted with 5 eq of Fe-PPIX in Tris buffer containing phenol as the reducing substrate. Free Fe-PPIX cannot react with arachidonic acid to catalyze oxygen uptake or prostaglandin formation, so the excess was not a problem.

The time courses for O<sub>2</sub> uptake for equimolar amounts of H193A and wtPGHS-2 are shown in Fig. 6. Whereas H193A exhibited no detectable guaiacol peroxidase activity (Table I), the extent of oxygen uptake for H193A was identical to wt-PGHS-2. However, H193A exhibited a substantial lag phase prior to the onset of maximal oxygen uptake. The observed lag phase for H193A was consistently 15–20 s, and the maximal rate of oxygen uptake was one-third the rate of wt-PGHS-2. Oxygen uptake data for H193A and the other PGHS-2 proteins is summarized in Table V. The maximal rate of oxygen uptake and the total oxygen uptake for Q189N were 55–60% of wt-PGHS-2 levels. Oxygen uptake by Q189R and Q189V were very low or undetectable.

To confirm the results of the oxygen uptake experiments and to determine the pattern of products generated, the PGHS-2 mutants were incubated with [1-14C]arachidonic acid and epinephrine in Tris buffer, and the conversion of arachidonic acid to prostaglandin products, prostaglandin E<sub>2</sub> and prostaglandin D<sub>2</sub>, was quantitated (Table IV). In this assay, H193A catalyzed 80% of wt-PGHS-2 conversion to prostaglandins, and Q189N catalyzed identical amounts of conversion as wt-PGHS-2. When Q189R and Q189V were assayed under identical conditions (22 nM protein), no conversion was observed; therefore, the concentration of Q189R and Q189V was increased 5-fold to 110 nM. Q189R catalyzed low levels of conversion to prostaglandins, but no prostaglandins were detected for Q189V. Whereas Q189N had wt-PGHS-2 activity in the [1-14C]arachidonic acid conversion assay, only 55–60% of wt-PGHS-2 activity was observed in the oxygen uptake assay. The two assays utilize different reducing substrates; therefore, a conversion assay was performed using phenol rather than epinephrine. Q189N activity with phenol in the conversion assay was reduced to 60% of wt-PGHS-2 activity, consistent with the oxygen uptake results.

The lag phase for H193A observed in Fig. 6 is attributed to decreased peroxidase activity. Cyclooxygenase activation of PGHS proteins is thought to occur by reaction of trace fatty acid hydroperoxides (in the arachidonic acid) with the resting enzyme. Under conditions of low hydroperoxide substrate (2.5 μM 15-HPETE), 2-MeIm stimulated the hydroperoxide reduction activity of H193A 2-fold and increased the percentage of two-electron reduction from 61 to 70%. Therefore, it seemed plausible that 2-MeIm may overcome the lag phase of H193A.
and H193A in the presence of 5 mM 2-MeIm. The addition of 2-MeIm greatly stimulated product formation at 10 and 30 s but did not affect the extent of product formation. No lag phase was observed in the time course for O₂ uptake by H193A in the presence of 5 mM 2-MeIm. The addition of 5 mM 2-MeIm to wtPGHS-2 did not affect product formation.

**DISCUSSION**

We have used site-directed mutagenesis to explore the role of active site residues on the distal side of the heme in the reaction of PGHS with fatty acid hydroperoxides and the activation of the enzyme’s cyclooxygenase activity. The amino acid substitutions made were based on the proposed mechanism of peroxidase catalysis. Dramatic losses in peroxidase activity were observed for the histidine to alanine (H193A), glutamine to arginine (Q189R), and glutamine to valine (Q189V) mutants (Table I). Only the glutamine to asparagine mutant (Q189N) appeared to retain wild-type activity, although there were some kinetic differences between the wild-type and mutant enzymes (Fig. 3). Overall, the changes in enzyme activity are consistent with the proposed role of the histidine and glutamine residues in peroxidase catalysis (Scheme 2). For example, the distal histidine is proposed to facilitate heterolytic hydroperoxide reduction by removing a proton from the hydroperoxide as it ligates the iron atom then transferring the proton to the incipient alkoxide ion generated by hydroperoxide reduction (14). Mutation of this residue to an alanine drastically reduced peroxidase activity. Similar losses in activity have been reported as a result of mutation of the distal histidine of several heme peroxidases including horseradish peroxidase and cytochrome c peroxidase (28, 46, 50, 51).

The distal glutamine residue may stabilize the developing negative charge on the alkoxide through a hydrogen-bonding interaction. The properties of the Gln189 mutants are consistent with an important role for an amide side chain at this position (Gln or Asn), but they do not enable us to define what this role is. Mutation of glutamine 189 to valine, a residue unable to participate in hydrogen bonding, abolished peroxidase activity. However, mutation of the glutamine to arginine, the residue found in plant, yeast and fungal peroxidases, also abolished peroxidase activity. This was somewhat surprising, because arginine would be expected to stabilize a negative charge more effectively. Although no detectable hydroperoxide reduction was observed for Q189R or Q189V, low levels of cyclooxygenase activity were detected for Q189R (Table V). Based on the current model for peroxidase-dependent activation of cyclooxygenase activity, Q189R must have some capacity to catalyze two-electron reduction of a hydroperoxide to form compound I (see below). However, the magnitude of this activity appears to be no greater than that of Fe-PPIX alone, which makes it undetectable in our peroxidase assay.

Concomitant with a decrease in peroxidase activity, we noted a decrease in the ability of the mutant enzymes to catalyze heterolytic scission of the hydroperoxide bond (Tables II and III). If a distal histidine is not present to serve as a general acid-base catalyst, then the mechanism of oxygen-oxygen bond cleavage changes to homolytic. Homolytic scission, which is equivalent to one-electron hydroperoxide reduction, generates a one-electron oxidized heme equivalent to compound II. The addition of high concentrations of 2-MeIm chemically reconstituted the ability of the H193A mutant to effect heterolytic hydroperoxide cleavage as evidenced by increases in the maximal turnovers and the percentage of two-electron reduction of 15-HPETE (Table III). This is consistent with the exogenous 2-MeIm providing the general acid-base catalyst that is missing in the H193A mutant.

In the course of our studies with diagnostic hydroperoxides, we discovered what appears to be induced decomposition of the fatty acid hydroperoxides triggered by the higher oxidation states of the PGHS peroxidase. The induced decomposition was manifest as a change in product profile with increasing hydroperoxide concentration. At low hydroperoxide concentration, the two-electron reduction product was virtually the exclusive product, whereas at higher hydroperoxide concentrations, alkoxyl radical-derived products were detected in increasing amounts. Scheme 3 depicts a reaction sequence to explain the concentration-dependent formation of 15-KETE and epoxyalcohols from 15-HPETE. The key event is oxidation of 15-HPETE by compound I to produce a peroxy radical that
goes on to form an alkoxyl radical. The latter is converted to 15-KETE and epoxyalcohols. The oxidation of hydroperoxides by heme-oxo complexes is well preceded from the work of Traylor and co-workers (52–54) with simple iron porphyrin complexes. Dunford (55) recently reported spectroscopic evidence suggesting that compound I of PGHS could react with hydroperoxides to form compound II and a peroxyl radical. This mechanistic scheme also provides an explanation for the decrease in ABTS peroxidase activity of Q189N above 25 m

The ability of 15-HPETE to effectively compete with guaiacol and ABTS for reduction of compound I or II may seem surprising but is actually a reflection of the high affinity of PGHS for fatty acid hydroperoxide substrate. The $K_m$ values for the reaction of PGHS with fatty acid hydroperoxides is in the range of 5–20 \( \mu \text{M} \), whereas the $K_m$ of this enzyme for $\text{H}_2\text{O}_2$ is approximately 300 \( \mu \text{M} \) (56–58). Obviously, the organic functionality of the natural substrates contributes significantly to their binding to PGHS. These same interactions may contribute to the binding of fatty acid hydroperoxide to the higher oxidation states, so that when the heme oxidation state is accessible, as it appears to be in PGHS-2, the hydroperoxide can be oxidized. The $K_m$ values for many of the peroxidase-reducing substrates are in the range of 150–2000 \( \mu \text{M} \), so the fatty acid hydroperoxides compete effectively with them for reduction of the higher oxidation states (59).

The results of our experiments with the distal histidine mutant are strongly supportive of a role for the peroxidase activity in activating the PGHS cyclooxygenase activity. The lag phase that precedes maximal cyclooxygenase activity for H193A reflects the diminished ability of this mutant to reduce hydroperoxide impurities present in arachidonic acid. The $K_m$ for activation of wild-type PGHS by hydroperoxides is approximately 10 \( \mu \text{M} \), so trace amounts of arachidonate hydroperoxides are sufficient to react with the peroxidase to activate the cyclooxygenase (58). Since the reaction of the H193A mutant with hydroperoxides is much slower, cyclooxygenase activation by contaminating hydroperoxides is much less efficient. Indeed, the addition of 50 \( \mu \text{M} \) 15-HPETE to the H193A mutant abolishes the lag phase. The addition of 2-MeIm also abolishes the lag phase by increasing the rate of two-electron reduction by this mutant. Since 2-MeIm appears to selectively increase two-electron hydroperoxide reduction, its ability to eliminate the lag phase further implicates the two-electron oxidized enzyme, compound I, as the intermediate required for cyclooxygenase activation.

It has been previously reported that the PGHS-1 mutant (H207A), corresponding to the PGHS-2 H193A mutant, has no detectable peroxidase or cyclooxygenase activity (60). In the present study, PGHS-2 H193A exhibits high cyclooxygenase activity but only after a prolonged lag phase. The apparent difference between our results with PGHS-2 H193A and the results with PGHS-1 H207A may reflect a difference in the efficiency of peroxidase turnover and cyclooxygenase activation by the two different enzymes. For example, Smith and co-workers have recently found that the R106Q mutant of PGHS-2 only doubles the $K_m$ of the enzyme for arachidonic acid, whereas the corresponding R120Q mutant of PGHS-1 exhibits a 500-fold higher $K_m$ for arachidonate (61). Alternatively, the differences in biochemical properties of the distal histidine mutants of PGHS-1 and -2 may reflect differences in the purity of the enzyme preparations or the assay conditions. Our study of PGHS-2 H189A employed purified protein isolated from insect cells, whereas the study with PGHS-1 H207A used microsomal fractions from COS cells expressing recombinant protein. Reducing impurities in the microsomal fractions may have prevented the accumulation of hydroperoxides to a level that would accelerate cyclooxygenase activity. The present experiments provide additional insight into the role of individual amino acid residues in peroxidase catalysis and cyclooxygenase activation by PGHS. They also provide a functional test for a role for distal glutamine residues in peroxidase catalysis. Glutamine residues are conserved among all mammalian peroxidases (asparagine is substituted in catalase) and substitute for the arginine residues of plant and fungal peroxidases (2, 62). Based on this substitution and conservation, one might hypothesize that the glutamine residue serves as a functionally equivalent residue to arginine. Our results suggest an important function for a glutamine or asparagine residue at this position in mammalian peroxidases, but the precise definition of this role requires more extensive spectroscopic and mechanistic studies.

REFERENCES


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Mutational Analysis of the Role of the Distal Histidine and Glutamine Residues of Prostaglandin-Endoperoxide Synthase-2 in Peroxidase Catalysis, Hydroperoxide Reduction, and Cyclooxygenase Activation
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