Histidine 386 and Its Role in Cyclooxygenase and Peroxidase Catalysis by Prostaglandin-endoperoxide H Synthases*

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Prostaglandin-endoperoxide H synthases (PGHSs) have a cyclooxygenase that forms prostaglandin (PG) G2 from arachidonic acid (AA) plus oxygen and a peroxidase that reduces the PGG2 to PGH2. The peroxidase activates the cyclooxygenase. This involves an initial oxidation of the peroxidase heme group by hydroperoxide, followed by oxidation of Tyr385 to a tyrosyl radical within the cyclooxygenase site. His386 of PGHS-1 is not formally part of either active site, but lies in an extended helix between Tyr385, which protrudes into the cyclooxygenase site, and His388, the proximal ligand of the peroxidase heme. When His386 was substituted with alanine in PGHS-1, the mutant retained <2.5% of the native peroxidase activity, but >20% of the native cyclooxygenase activity. However, peroxidase activity could be restored (10–20%) by treating H386A PGHS-1 with cyclooxygenase inhibitors or AA, but not with linoleic acid; in contrast, mere occupancy of the cyclooxygenase site of native PGHS-1 had no effect on peroxidase activity. Heme titrations indicated that H386A PGHS-1 binds heme less tightly than does native PGHS-1. The low peroxidase activity and decreased affinity for heme of H386A PGHS-1 imply that His386 helps optimize heme binding. Molecular dynamic simulations suggest that this is accomplished in part by a hydrogen bond between the heme D-ring propionate and the N-δ of Asn326 of the extended helix. The structure of the extended helix is, in turn, strongly supported by stable hydrogen bonding between the N-δ of His386 and the backbone carbonyl oxygens of Asn382 and Gln383. We speculate that the binding of cyclooxygenase inhibitors or AA to the cyclooxygenase site of ovine H386A PGHS-1 reopens the constriction in the cyclooxygenase site between the extended helix and a helix containing Gly526 and Ser530 and restores native-like structure to the extended helix. Being less bulky than AA, linoleic acid is apparently unable to reopen this constriction.

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‡ The abbreviations used are: PGHS, prostaglandin-endoperoxide H synthase; oPGHS, ovine prostaglandin-endoperoxide H synthase; PG, prostaglandin; AA, arachidonic acid; FPLC, fast protein liquid chromatography; MD, molecular dynamic.

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optimal function because forms of PGHS that exhibit relatively low peroxidase activity (1–5% of that of the native enzyme) are capable of cyclooxygenase catalysis (22–25). In earlier studies in which we identified histidine residues critical for cyclooxygenase catalysis, we found two mutants of ovine PGHS (oPGHS)-1 (H386A and H386Q) that lack any detectable peroxidase activity, but retain appreciable cyclooxygenase activity (22). We undertook these studies because a complete lack of PGHS peroxidase function is inconsistent with the prevailing model for cyclooxygenase catalysis (3, 19, 20). During the course of these studies, we observed that the addition of the cyclooxygenase inhibitor flurbiprofen restored significant peroxidase activity to H386A oPGHS-1. Here, we report this result and subsequent biochemical and computational studies to determine the role of His386 in peroxidase and cyclooxygenase catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hemoglobin, hematin, chloroquine, DEAE-dextran, guaiacol, Tween 20, GSH, glutathione peroxidase, N-ethylmaleimide, and dithiothreitol were obtained from Sigma. Ethyl hydrogen peroxide (EIOOH) was purchased from Polysciences. Dalbeco's modified Eagle's medium was purchased from Invitrogen. Calf serum and fetal calf serum were obtained from Hyclone Laboratories. BSA5 (0.45 μL)-nitrocellulose was from Schleicher & Schull. ECL Western blotting reagents were from Amersham Biosciences. TiterMax adjuvant was from CytRx Corp. Oligonucleotides used as primers for mutagenesis were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility. Fatty acids were from Cayman Chemical Co., Inc., and the detergents C12Em and decyl maltoside were from Anistrea (Maine, OH). All other reagents were from common commercial sources.

**PGHS Expression and Purification**—pSVT7 expression plasmids containing the coding regions of native and H386A oPGHS-1 were as reported previously (22) and were used in the productions of COS-1 cells as described in an earlier report (26). The H386A mutant of His6-tagged oPGHS-1 was prepared using protocols described previously (21, 27). Native and H386A His6-oPGHS-1 were produced in Sf21 insect cells following baculovirus infection and purified by nickel affinity chromatography and, in some cases, further purified by FPLC on a 1-ml HiTrap protein A column. Each assay mixture contained 3 ml of 0.1 M Tris-HCl (pH 8.0), 1 mM phenol, 85 μg of hemoglobin, and 100 μg AA (28, 29). One unit of cyclooxygenase activity corresponds to the amount of enzyme that oxygenates 1 nmol of AA/min. Reactions were initiated by adding 10–100 μg of microsomal protein or 1–5 μg of partially purified His6-oPGHS-1, and 0.1 mM hematin, and typically 10–100 μg of microsomal protein or 20–80 μg (0.1–0.4 cyclooxygenase units) of partially purified His6-oPGHS-1 or H386A His6-oPGHS-1. Reactions were initiated by adding peroxide.

**Stopped-flow Analysis**—The molar concentration of the PGHS was calculated using a value of 30,000 cyclooxygenase units/mg of pure enzyme (31, 32). For samples used for stopped-flow or standard peroxidase assays, an equimolar amount of hemin chloride (1 mg in Me2SO) was added typically along with a 10-fold molar excess of flurbiprofen, and the sample was concentrated to 1 ml; the final concentration of Me2SO in this sample was typically ~0.07%. For stopped-flow analyses of peroxidase, ionic concentrations of 20 μM (1 mg/ml) were routinely used. Peroxide solutions were prepared at the same concentration as PGHS in 20 mM Tris-HCl (pH 8.5), 200 mM NaCl, and 0.1% C12Em. The stopped-flow studies were performed at 4 °C using an Olis RSM instrument. Based on differences in molar extinction coefficients between the resting state heme and the reaction intermediates, the rate constants for Compound I (~408 nm for native oPGHS-1) and Intermediate II (~428 nm for native oPGHS-1) formation were determined.

**Western Transfer Blotting**—Microsomal or purified protein samples were subjected to SDS-PAGE (10% gels) and electroblotted onto BA85-nitrocellulose filters as described previously (26, 33, 34). Filters were blocked for 12 h in 3% nonfat dry milk and Tris-buffered saline, followed by incubation with antibody against oPGHS-1 in 1% nonfat dry milk, 0.05% Tween 20, and Tris-buffered saline for 1 h at 25 °C. Filters were washed and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. The filters were then incubated with ECL reagents and exposed to Kodak XAR film for chemiluminescence.

**Kinetic Determinants**—The starting configuration for PGHS-1 was obtained from Protein Data Bank code 1EBV (35). The simulations were performed using the SANDER module of the AMBER Version 7 simulation software package (36). Preparation of the topology and parameter files was done using LEAP (37). We used atom types and parameters for the heme that were developed by Giannoni (38) as modified by C. Bayly. The oxidized state of the heme is set to be ferric (Fe3+), and with the assumption that the propionates are ionized, the overall charge is −1. The ionization states of amino acid residues were set appropriate to pH 7. All histidines were assumed to be neutral with the Nδ-protonated. The proximal histidine (His386) was covalently linked to the heme Fe3+ through Nε. The SHAKE algorithm (39) was used to constrain bond lengths involving hydrogens, permitting a time step of 2 fs. The starting structure was immersed in a rectangular box consisting of ~15,000 explicit water molecules. The temperature was kept at 300 K with the use of a Berendsen thermostat (40) with a coupling constant of 0.2 ps. The particle mesh Ewald method (41) was used to treat the long-range coulombic interactions with the default values of the SANDER performance. The simulations were performed using periodic boundary conditions with the van der Waals non-bonded cutoff at 9.0 Å. Initially, a 2.25-ps constant number, pressure, and temperature simulation at 300 K was performed while restraining the atomic coordinates of the protein with a harmonic potential. This permitted the solvent molecules to relax while allowing the box dimensions to change and bring the whole system to an equilibrated density of ~1.0 g/ml. The protein restraints were then removed, and molecular dynamic (MD) simulations was performed in the constant number, volume, and temperature ensemble for 1.3 ns.

**RESULTS**

**Kinetic Properties of Native oPGHS-1 and Mutants with Substitutions of His368**—H386 and His386 located in the extended helix (378N9FNQNYLHWHHT390) that contains the cyclooxygenase active-site Tyr385 and the proximal ligand to the peroxidase heme group, His388 (Fig. 1). The impetus for the studies reported here was our earlier findings that the H386A and H386Q mutants of oPGHS-1 lack detectable peroxidase activity, but retain oxygenase activity (22), and that this finding is inconsistent with the branched chain model for cyclooxygenase catalysis; this mechanism requires that a peroxide oxidize the...
heme group at the peroxidase site, which, in turn, oxidizes Tyr385 within the cyclooxygenase site (2, 3, 19, 20). Specifically, microsomal preparations of H386A oPGHS-1 expressed in COS-1 cells exhibit 30–35% of the maximal cyclooxygenase activity of native oPGHS-1, but lack detectable peroxidase activity when tested either with H2O2 (Table I) (22) or with the alkyl hydroperoxide 5-phenyl-4-pentenyl-1-hydroperoxide (22). H386A oPGHS-1 was found to have a K_{m} for AA of 4.2 μM under conditions in which the K_{m} for the native enzyme was determined to be 3.1 μM (Table I). As reported previously for other oPGHS-1 mutants (26, 42), Western blot analyses of microsomes prepared from COS-1 cells and used for the kinetic studies summarized in Table I indicated that native and H386A oPGHS-1 were expressed at comparable levels (data not shown). Also shown in Table I are the kinetic values for native and H386A His_{6}-oPGHS-1 purified from baculovirus expression systems. With purified enzymes, H386A His_{8}-oPGHS-1 consistently had 15–20% of the native cyclooxygenase activity and 1.5–2.5% of the native peroxidase activity; this latter level of peroxidase activity is at the lower limit of detection of standard peroxidase assays.

O_{2} electrode tracings of O_{2} consumption observed with equal amounts of native or H386A His_{8}-oPGHS-1 in standard assays with AA as the fatty acid substrate are compared in Fig. 2. A much more pronounced kinetic lag period was observed following addition of H386A oPGHS-1 to assay mixtures containing heme and the two cyclooxygenase substrates, O_{2} and AA. No appreciable lag was apparent with the same amount of native oPGHS-1 protein; lag phases such as the one for H386A oPGHS-1 are common with peroxidase-deficient forms of PGHSs (22–25, 43). In the case of H386A oPGHS-1, the lag phase could be eliminated by adding 3 μM 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (but not 110 μM H_{2}O_{2}) to the assay chamber (data not shown), and the lag phase could be extended by adding GSH plus glutathione peroxidase to the assay mixtures (data not shown). The responses to 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid and glutathione peroxidase are well established properties of native oPGHS-1 (2, 3, 43, 44) and are consistent with a need for peroxide for the activation of the cyclooxygenase activity of H386A oPGHS-1. The lack of response to H_{2}O_{2} was surprising. It suggests that there may be a difference in the oxidant substrate specificities of the peroxidase activities of native and H386A oPGHS-1; H_{2}O_{2} can eliminate the lag phase that can be seen with native PGHS-1 when its peroxidase activity is inhibited with cyanide (43).

**Substrate Specificity and Product Analyses**—To determine whether H386A oPGHS-1 and the homologous H386A hPGHS-2 mutant catalyze bis-oxygenation reactions corresponding to their native enzyme counterparts, microsomes prepared from COS-1 cells expressing oPGHS-1, H386A oPGHS-1, hPGHS-2, or H386A hPGHS-2 were incubated with [1-14C]arachidonic acid. The mobilities of the products upon thin-layer chromatography are compared in Fig. 3. In each case, similar patterns of product formation (PGE_{2}, PGD_{2}, PGE_{2α}, 12-hydroxy-5,8,10-heptadecatrienoic acid, 11-hydroxy-5,8,12,14-eicosatetraenoic acid, and 15-hydroxy-5,8,11,13-eicosatetraenoic acid) were observed. These results indicate that the native and H386A mutant forms of PGHS-1 and PGHS-2 catalyze mechanistically identical bis-oxygenations of AA. However, this is not true for all fatty acid substrates. Linoleic acid is oxygenated by PGHS-1 to a mixture of 9- and 13-hydroxy-octadecadienoic acid at 45% of the rate of AA (45, 46), but linoleic acid was not oxygenated by H386A oPGHS-1.

**Reconstitution of the Peroxidase Activity of H386A oPGHS-1**—In trying to resolve the enigma of microsomal H386A oPGHS-1 lacking detectable peroxidase activity yet retaining appreciable cyclooxygenase activity, we discovered that peroxidase activity could be restored to H386A oPGHS-1 if flurbiprofen, a time-dependent cyclooxygenase inhibitor, was added to the peroxidase assay mixture (Table I). For example, preincubation of purified H386A His_{8}-oPGHS-1 for 2 min with 100 μM flurbiprofen increased the peroxidase activity from barely detectable levels to levels that were 25–35% of the specific activity of native His_{8}-oPGHS-1; as expected (47), flurbiprofen had no effect on the peroxidase activity of the native enzyme. Several other amino acid substitutions of His_{386} were prepared and analyzed for cyclooxygenase and peroxidase activities and restoration of peroxidase activity by flurbiprofen (Table II). oPGHS-1 mutants with relatively large, charged residues at position 386 exhibited both cyclooxygenase and peroxidase activities; however, unlike the case with native oPGHS-1, addition of flurbiprofen to the H386K or H386Y mutant of oPGHS-1 led to a 2-fold increase in peroxidase activity. H386F oPGHS-1 was similar to H386A oPGHS-1 in lacking detectable peroxidase activity but exhibiting flurbiprofen-induced peroxidase activity; however, H386F oPGHS-1 was not characterized further.

Imidazole can sometimes be used to restore functions to hemeproteins in which histidine residues have been replaced with other amino acids (48, 49). Experiments were performed with H386A oPGHS-1 to determine whether imidazole would affect the cyclooxygenase activity or the kinetic lag time. Microsomal preparations of H386A oPGHS-1 were preincubated for 5 min at 37°C with 10 or 100 mM imidazole and then subsequently assayed in the presence of the same concentration of imidazole. With 100 mM imidazole, there was an ~80% inhibition of enzyme activity, perhaps because imidazole binds to the distal position of the heme iron and inhibits peroxidase activity. With 10 mM imidazole, the enzyme retained ~90% of its original activity, and there was no effect on the kinetic lag time.

**Effects of Substrates and Inhibitors on the Peroxidase Activity of H386A oPGHS-1 and H386A hPGHS-2**—As described above, H386A oPGHS-1 exhibited low levels of peroxidase activity that could be stimulated by ~20-fold with flurbiprofen. Preincubation of H386A His_{8}-oPGHS-1 with other cyclooxygenase inhibitors or with 20-carbon substrates also led to expression of latent peroxidase activity (Table III). The amount of peroxidase activity of H386A His_{8}-oPGHS-1 that was restored...
Cyclooxygenase and peroxidase activities were determined using microsomes prepared from COS-1 cells transfected with native or H386A oPGHS-1 constructs or native and mutant enzymes purified following baculovirus expression in SF21 insect cells; in the latter cases, native and H386A His₆-oPGHS-1 were purified by nickel-nitrilotriacetic acid chromatography. Methods for enzyme preparations and measurements of enzyme activities are described under “Experimental Procedures.” Cyclooxygenase measurements were made with AA as substrate; the rates shown for the mutant enzymes indicate the maximal activity following the kinetic lag phase. No cyclooxygenase or peroxidase activities were detected with microsomes from mock-transfected COS-1 cells. The data reported here are from individual experiments in which each entry represents an average of two or three assays, the results of which varied <10%. The experiments were performed a minimum of four times with quantitatively similar results. ND, not determined; FBP, flurbiprofen.

### Table I

| Enzyme                        | Cyclooxygenase | Arachidonate $K_m$ (μM) | Peroxidase |
|-------------------------------|---------------|-------------------------|------------|
| Native oPGHS-1 (COS-1 cells)  | 141           | 3.1                     | 36         |
| H386A oPGHS-1 (COS-1 cells)   | 47            | 4.2                     | 0          |
| His₆-oPGHS-1                  | 12,000        | ND                      | 25,000     |
| H386A His₆-oPGHS-1            | 2,500         | ND                      | 590        |
| H386A His₆-oPGHS-1 with 100 μM FBP | ND         | ND                      | 25,000     |
| H386A His₆-oPGHS-1 with 100 μM FBP | ND         | ND                      | 10,000     |

Microsomes prepared from mock-transfected COS-1 cells and from COS-1 cells expressing (A) oPGHS-1 or H386A oPGHS-1 and (B) hPGHS-2 or H386A hPGHS-2 were incubated for 15 min with 153 μg [1-¹⁴C]arachidonic acid in 200 μl of 0.1 x Tris-HCl (pH 7.4) containing 1 μM phenol and 6.8 μg of hemoglobin. The products were extracted and separated by thin-layer chromatography, and the thin-layer plates were then subjected to autoradiography. First lane, mock transfection (250 μg of protein); second lane, oPGHS-1 (85 μg of protein); third lane, H386A oPGHS-1 (250 μg of protein); fourth lane, H386A hPGHS-2 (250 μg of protein); fifth lane, hPGHS-2 (250 μg of protein). 11-HETE, 11-hydroxy-5,8,12,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid.

Heme Requirements for oPGHS-1 and H386A oPGHS-1—Microsomal preparations of native or H386A oPGHS-1 from COS-1 cells assayed in the absence of an exogenous source of heme exhibited ~20% of the cyclooxygenase activities observed with saturating concentrations of hematin (~5 μM). Titration of microsomal enzyme preparations with hematin (0.05–1.5 μM) indicated that half-maximal cyclooxygenase activities occurred with 0.2 and 0.6 μM hematin for native and H386A oPGHS-1, respectively. Thus, the microsomal forms of both the native and H386A mutant enzymes required heme for maximal activities, but a slightly higher concentration of heme was required for maximal activity with H386A oPGHS-1. With solubilized and purified native His₆-oPGHS-1, maximal peroxidase activity was observed with 0.1 μM hematin; furthermore, the amount of hematin required for maximal activity was the same in the presence or absence of flurbiprofen. In contrast, with solubilized and purified H386A His₆-oPGHS-1, 1.0 μM hematin was varied with different inhibitors and substrates. Flurbiprofen was the most effective agent. The amount of peroxidase activity returned with AA was concentration-dependent; half-maximal restoration of activity occurred with a concentration dependence that approximately paralleled the $K_m$ value (~5 μM) for cyclooxygenase. Eicosapentaenoic acid was somewhat less effective than AA in restoring peroxidase activity; eicosapentaenoic acid is a much less effective substrate for the cyclooxygenase than is AA (45, 46). 18-Carbon fatty acid substrates, including linoleic and α-linolenic acids, could not restore peroxidase activity to H386A His₆-oPGHS-1.

H386A His₆-hPGHS-2 had ~10% of the peroxidase activity of native His₆-hPGHS-2, which was significantly higher than observed in the corresponding cases with oPGHS-1 (Table IV). However, as was the case with the PGHS-1 isozyme, the peroxidase activity of H386A His₆-hPGHS-2 was increased (up to 3-fold) upon incubation with various cyclooxygenase inhibitors and substrates; in contrast, the peroxidase activity of native His₆-hPGHS-2 was relatively unaffected (~15% increase) by cyclooxygenase inhibitors and substrates (Table IV).

**Figure 2.** Time course of O₂ consumption by native and H386A oPGHS-1. Reactions were initiated by adding purified native or H386A His₆-oPGHS-1 (~30 μg, 10 μg of protein) to O₂ electrode chambers containing 3 ml of 0.1 x Tris-HCl (pH 8.0), 1 μM phenol, 5 μM hemin, and 100 μM AA. The specific activities of purified native and H386A His₆-oPGHS-1 were 2500 and 12,000 units/mg.

**Figure 3.** Eicosanoid products formed by oPGHS-1, H386A oPGHS-1, hPGHS-2, and H386A hPGHS-2. Microsomes prepared from mock-transfected COS-1 cells and from COS-1 cells expressing (A) oPGHS-1 or H386A oPGHS-1 and (B) hPGHS-2 or H386A hPGHS-2 were incubated for 15 min with 153 μg [1-¹⁴C]arachidonic acid in 200 μl of 0.1 x Tris-HCl (pH 7.4) containing 1 μM phenol and 6.8 μg of hemoglobin. The products were extracted and separated by thin-layer chromatography, and the thin-layer plates were then subjected to autoradiography. First lane, mock transfection (250 μg of protein); second lane, oPGHS-1 (85 μg of protein); third lane, H386A oPGHS-1 (250 μg of protein); fourth lane, H386A hPGHS-2 (250 μg of protein); fifth lane, hPGHS-2 (250 μg of protein). 11-HETE, 11-hydroxy-5,8,12,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid.
required for maximal peroxidase activity in the presence of flurbiprofen, and 5 μM hematin was needed for maximal activity in its absence. Thus, a higher concentration (3–10-fold) of heme was required to support maximal peroxidase activity with the mutant enzyme. These data suggest that H386A oPGHS-1 binds heme 3–10 times less well than does native oPGHS-1 with the mutant enzyme. These data suggest that H386A His6-oPGHS-1 is expressed in the baculovirus insect cell culture system and purified by FPLC as described under "Experimental Procedures." Purified enzyme was incubated for 2 min with or without the indicated inhibitor, and peroxidase assays were performed. To determine the effects of substrates on peroxidase activities, the substrates were added at the time at which the EtOOH substrate was added to initiate the peroxidase reaction mixture. The data represent an average of duplicate measurements, which varied <5%. This experiment was performed two times using different enzyme preparations and yielded similar results in both cases.

| Enzyme and cyclooxygenase inhibitor or substrate | Peroxidase activity mmol EtOOH/min/mg |
|-----------------------------------------------|--------------------------------------|
| H386A His8-oPGHS-1                           | 720 ± 29                             |
| + Flurbiprofen                                | 9900 ± 310                           |
| + Flufenamic acid                             | 6700 ± 20                            |
| + Meclofenamic acid                           | 7900 ± 190                           |
| + Ibuprofen                                   | 6800 ± 120                           |
| + Indomethacin                                | 6700 ± 1100                          |
| + Arachidonic acid                            | 2000 ± 64                            |
| + Linoleic acid                               | 610 ± 64                             |
| Native His8-oPGHS1                            | 26,000 ± 56                           |
| + Flurbiprofen                                | 26,000 ± 1000                         |

TABLE III

Effects of nonsteroidal anti-inflammatory drugs and fatty acid substrates on the peroxidase activity of H386A His8-oPGHS-1

Native or H386A His8-oPGHS-1 was expressed in the baculovirus insect cell culture system and purified by nickel chromatography as described under "Experimental Procedures." Purified enzyme was incubated for 2 min with or without the indicated inhibitor, and peroxidase assays were performed. To determine the effects of substrates on peroxidase activities, the substrates were added at the time at which the EtOOH substrate was added to initiate the peroxidase reaction mixture. The data represent an average of duplicate measurements, which varied <5%. This experiment was performed three times using different enzyme preparations and yielded similar results in all cases.

| Enzyme and cyclooxygenase inhibitor or substrate | Peroxidase activity mmol EtOOH/min/mg |
|-----------------------------------------------|--------------------------------------|
| Native His8-oPGHS-1                           | 520                                  |
| + Flurbiprofen                                | 1100                                 |
| + Flufenamic acid                             | 1400                                 |
| + Meclofenamic acid                           | 1100                                 |
| + Ibuprofen                                   | 680                                  |
| + Indomethacin                                | 601                                  |
| + Arachidonic acid                            | 700                                  |
| + Linoleic acid                               | 755                                  |
| Native His8-hPGHS-2                           | 5000                                 |
| + Flurbiprofen                                | 5900                                 |

FIG. 4. UV-visible spectra of native and H386A His8-oPGHS-1. Samples of native and H386A His8-oPGHS-1 were purified by FPLC as described under "Experimental Procedures" and concentrated to 7.5 μM based on a molecular mass of 72 kDa. Spectra were recorded on an Olis stopped-flow spectrophotometer.

TABLE IV

Effects of nonsteroidal anti-inflammatory drugs and fatty acid substrates on the peroxidase activity of H386A His8-hPGHS-2

Native or H386A His8-hPGHS-2 was expressed in a baculovirus insect cell culture system and purified by nickel chromatography as described under "Experimental Procedures." Purified enzyme was incubated for 2 min with or without the indicated inhibitor, and peroxidase assays were performed. To determine the effects of substrates on peroxidase activities, the substrates were added at the time at which the EtOOH substrate was added to initiate the peroxidase reaction mixture. The data represent an average of duplicate measurements, which varied <5%. This experiment was performed two times using different enzyme preparations and yielded similar results in both cases.

| Enzyme and cyclooxygenase inhibitor or substrate | Peroxidase activity nmol EtOOH/min/mg |
|-----------------------------------------------|--------------------------------------|
| H386A His8-hPGHS-2                           | 520                                  |
| + Flurbiprofen                                | 1100                                 |
| + Flufenamic acid                             | 1400                                 |
| + Meclofenamic acid                           | 1100                                 |
| + Ibuprofen                                   | 680                                  |
| + Indomethacin                                | 601                                  |
| + Arachidonic acid                            | 700                                  |
| + Linoleic acid                               | 755                                  |
| Native His8-hPGHS-2                           | 5000                                 |
| + Flurbiprofen                                | 5900                                 |
end of the extended helix structure (Fig. 1).

The residues adjacent to His388 in the extended helix are His386 and Trp387. During the MD simulation, both His386 (Fig. 5) and Trp387 (data not shown) hydrogen-bonded to the backbone carbonyl oxygens of other residues comprising the extended helix. The His386 N-δ hydrogen-bonded to both the Asn382 and Gln383 carbonyls, whereas the Trp387 N-ε hydrogen-bonded to the backbone carbonyl of Leu384. The crystal structure (Protein Data Bank code 1EBV) (35) suggests that there is a hydrogen bond between the backbone oxygen of Gln383 and the backbone nitrogen of His386, but during the 1.3-ns MD simulation, the distance between the hydrogen donor and acceptor was, for the most part, >3.0 Å.

The N-δ of Asn382 of the extended helix was also found in MD simulations to hydrogen bond to the D-ring propionate of the heme (Figs. 1 and 6). During the first 750 ps, this hydrogen bonding occurred between the N-δ of Asn382 and the O-δ1 of this propionate, but suddenly switched to the O-δ2 of the same propionate for the rest of the simulation (Fig. 6). During the length of the simulation, the side chain oxygen and the backbone nitrogen of Thr212 also hydrogen-bonded to the D-ring propionate. In contrast, the A-ring propionate was found to interact exclusively with bulk water. Bulk water was found to penetrate into the proximal side of the heme, where some of the waters formed hydrogen bonds with the N-ε of His386. However, the bonding was transitory, with periods of no hydrogen bonding.

**DISCUSSION**

PGHSs are unusual in having an extended helix: in this case, an α-helix encompassing Met379 through Leu390, with, however, His386 and Trp387 having Ramachandran ψ and φ angles well outside the normal α-helical range (Fig. 1) (6–8). The amino acid sequences between positions 380 and 382 and positions 384 and 389 are conserved in all known forms of PGHS-1 and PGHS-2; only at position 383 is there significant sequence variability among isoforms and species (12, 55, 56).

The extended helix is located on the proximal side of the heme. Tyr385 and His388 of this helix play key roles in cyclooxygenase and peroxidase catalysis, respectively (1–3).

Our results show that the extended helical structure persists over a 1.3-ns MD simulation. Our MD and mutagenesis studies suggest that a key interaction that secures the unique architecture of the extended helix is a strong hydrogen bond involving the N-δ of His386 that alternates between the peptide backbone carbonyl groups of Asn382 and Gln383 and is perhaps stabilized by an α-helix/dipole interaction with His386 (Fig. 1).

One way of assessing the stability of interactions involving imidazole groups of histidine residues is to examine the time dependence of the orientations of the imidazole rings. As indicators, we used a dihedral angle around the iron–His386 N-ε2 bond and the side chain C-β–imidazole C-γ bond of His386, His207, and His134. The angles indicate the rotational mobility of the respective imidazole rings. The data are plotted at 50-ps intervals. Simulations were performed as described under “Experimental Procedures.”
The imidazole ring of His386 remained in a fixed orientation, consistent with hydrogen bonding of its N-δ to the backbone oxygens of Asn382 and Gln383. Backbone-side chain hydrogen bonds represent a form of helical capping that stabilizes α-helical conformations (57, 58). For example, a histidine residue placed at the C termini of the two α-helices of barnase confers conformational stability to that helix by a helical capping mechanism (59); in barnase, the integrity of the helix is also supported in part by the helix dipole and its association with the side chain of histidine.

Our results suggest that the extended helix of PGHS is essential for the productive binding of heme at the peroxidase site. Specifically, this unusual helix structure appears to facilitate (a) the binding of Hs388 to the heme iron and (b) hydrogen bonding between Asn382 and the carboxylate group of the D-ring propionate of the heme.

Stabilization of heme binding is particularly important for PGHSs because there is a strong indication that the interaction between the heme iron and the proximal histidine is weak. This is based upon the discovery that the bond between the ferrous iron and the imidazole of the proximal histidine (His388) is weak in comparison with other peroxidases and the assumption that ferric state interactions mirror those of the ferrous state (53). Experimental studies with mutants have demonstrated that the weak interaction between the heme iron and His388 apparently results from the lack of a strong acidic residue in the vicinity of His388 (53, 60). Other peroxidases, including the closely related myeloperoxidase, have an aspartate or another acidic residue hydrogen-bonded to the proximal histidine. A strong iron/His interaction in peroxidases is reflected by a strong iron/His signal in resonance Raman studies. This signal correlates with facile cleavage of the Fe(II)/His bond and is associated with weak or no hydrogen bonding between amino acid side chains and the N-5 proton of the proximal histidine (or heme propionates) (64).

Unlike most other peroxidases, PGHSs also lack another important contributor to a strong heme iron/proximal histidine interaction: a normal α-helix that includes the proximal histidine. A strong iron/His interaction in peroxidases is reflected by the presence of a related high frequency Fe(II)/His signal in resonance Raman spectra (53, 64). For helices whose negative end (i.e., C terminus) terminates at the proximal histidine, as is the case for many peroxidases (65), an effect analogous to having a nearby acidic residue occurs: stabilization of the interaction between the heme iron and the proximal ligand.

The extended helical structure of PGHSs likely diminishes or eliminates this source of heme stabilization. The lack of this type of heme stabilization, coupled with a lack of hydrogen bonding to an acidic residue, is the basis for the weak iron–His388 bond in PGHSs. (We note that modeling of an ideal α-helix in PGHS-1 stretching from Met379 to Leu390 places the imidazole groups of His386 and His388 on opposite sides of the helix, whereas in the extended helix, these side chains are on the same side of the helix and parallel to each other (Fig. 1.).)

Another important contributor to binding of heme to protein is hydrogen bonding of heme propionate groups to amino acid side chains. Successive elimination of this type of hydrogen bond leads to a progressive decrease in the strength of heme binding to proteins (66). In PGHS-1, Thr312 hydrogen bonds through its backbone nitrogen and its side chain oxygen to the carboxylate oxygens of the D-ring propionate. Asn382 also hydrogen bonds to one of the oxygens of this D-ring propionate. Importantly, all three hydrogen bonds persist throughout the 1.3-ns MD simulation. Thus, it may be that the extended helix structure and robust propionate hydrogen bonding may provide factors stabilizing the heme to compensate for a weaker iron/His interaction in PGHS-1.

Mutations of His386 to alanine affect heme binding and peroxidase activity most likely by disrupting the integrity of the extended helix near its C-terminal end, which, in turn, alters the position of His388 and destabilizes the Fe3+/His bond. Mutation of His386 to alanine could also destabilize heme binding by affecting the hydrogen bonding between Asp382 and the D-ring propionate.

The augmentation/restoration of peroxidase activity by the cyclooxygenase inhibitor flurbiprofen seen with four of the His386 mutants (H386A, H386K, H386Y, and H386F) must result from the ability of these inhibitors to restore near-normal architecture to the cyclooxygenase and peroxidase sites. The result would be enhancement of the heme Fe3+/His388 interaction and/or increased hydrogen bonding between the D-ring propionates and Asn382 and Thr312 and perhaps other residues (e.g., Tyr148 stays within 4 Å of the heme ring propionate throughout the MD simulation).

The ability of AA (but not linoleic acid) to restore peroxidase activity (and concomitantly cyclooxygenase activity) to H386A PGHS-1 presumably relates to the manner in which these two substrates bind to the cyclooxygenase site. The crystal structures of Co3+/heme-pGHS-1 complexes with AA versus linoleic acid show subtle differences in protein/fatty acid interactions (46, 67). There is a constriction in the cyclooxygenase channel of native oPGHS-1 in an area between the extended helix in the region containing Trp387 and Tyr385 and an α-helix across the channel containing Gly526 and Ser530; this constriction is observed in all complexes of PGHS-1 with fatty acids (46, 67). Linoleic acid makes relatively fewer contacts with Gly526 than does AA: one versus four contacts, respectively. This is primarily because AA has 20 carbons; C-8 through C-13 of AA are squeezed together in this region of the protein, and thus, AA occupies more space in this constricted area than does linoleic acid (46, 67). We speculate that in the H386A oPGHS-1 mutant, AA can, because of its relative bulk, function more like a rigid nonsteroidal anti-inflammatory drug by pressing against the extended helix at Tyr385 and Trp387 and restoring this helix nearer to its native, extended position. This reasoning is also consistent with the finding that another 20-carbon fatty acid (eicosapentaenoic acid) can partially restore peroxidase activity to H386A oPGHS-1, whereas another 18-carbon fatty acid (α-linolenic acid) cannot.

In summary, comparison of the properties of the native and H386A mutant forms of PGHSs indicates that His386 is important for maintaining the structure of the unique extended helix of PGHSs involving residues 379–390. The extended helix is, in turn, necessary for optimizing heme and the resultant peroxidase and cyclooxygenase activities.

REFERENCES

1. Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kaligutkar, A. S., and Lanzo, C. A. (1999) J. Biol. Chem. 274, 22903–22906
2. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) Annu. Rev. Biochem. 69, 149–182
3. van der Donk, W. A., Tsai, A.-L., and Kulmacz, R. J. (2002) Annu. Rev. Biochem. 71, 315–349
4. Taniura, S., Kamitani, H., Watanabe, T., and Eling, T. E. (2002) J. Biol. Chem. 277, 16823–16830
5. Hwang, D. (2001) FASEB J. 15, 2556–2564
6. Picot, D., Loll, P. J., and Garavito, M. R. (1994) Nature 367, 243–249
7. Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1996) Nature 384, 644–648
8. Luong, C., Miler, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M. F. (1996) Nat. Struct. Biol. 3, 927–933
Histidine 386 and Its Role in Cyclooxygenase and Peroxidase Catalysis by Prostaglandin-endoperoxide H Synthases
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