# Substitution of Tyrosine for the Proximal Histidine Ligand to the Heme of Prostaglandin Endoperoxide Synthase 2: Implications for the Mechanism of Cyclooxygenase Activation and Catalysis†

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ABSTRACT: Prostaglandin $H_2$ synthesis by prostaglandin endoperoxide synthase (PGHS) requires the heme-dependent activation of the protein's cyclooxygenase activity. The PGHS heme participates in cyclooxygenase activation by accepting an electron from Tyr385 located in the cyclooxygenase active site. Two mechanisms have been proposed for the oxidation of Tyr385 by the heme iron: (1) ferric enzyme oxidizes a hydroperoxide activator and the incipient peroxyl radical oxidizes Tyr385, or (2) ferric enzyme reduces a hydroperoxide activator and the incipient ferryl-oxo heme oxidizes Tyr385. The participation of ferrous PGHS in cyclooxygenase activation was evaluated by determining the reduction potential of PGHS-2. Under all conditions tested, this potential ($<-135$ mV) was well below that required for reactions leading to cyclooxygenase activation. Substitution of the proximal heme ligand, His388, with tyrosine was used as a mechanistic probe of cyclooxygenase activation. His388Tyr PGHS-2, expressed in insect cells and purified to homogeneity, retained cyclooxygenase activity but its peroxidase activity was diminished more than 300-fold. Concordant with this poor peroxidase activity, an extensive lag in His388Tyr cyclooxygenase activity was observed. Addition of hydroperoxides resulted in a concentration-dependent decrease in lag time consistent with each peroxide's ability to act as a His388Tyr peroxidase substrate. However, hydroperoxide treatment had no effect on the maximal rate of arachidonate oxygenation. These data imply that the ferryl-oxo intermediates of peroxidase catalysis, but not the $Fe^{III}/Fe^{II}$ couple of PGHS, are essential for cyclooxygenase activation. In addition, our findings are strongly supportive of a branched-chain mechanism of cyclooxygenase catalysis in which one activation event leads to many cyclooxygenase turnovers.

Prostaglandin endoperoxide synthase (PGHS)[1] is a heme protein with two distinct activities that are required to convert arachidonic acid to prostaglandin $H_2$ ($PGH_2$) (*1*, *2*). The cyclooxygenase activity incorporates two molecules of dioxygen into the substrate to form the hydroperoxide, prostaglandin $G_2$ ($PGG_2$), (*3*, *4*) and the peroxidase activity reduces $PGG_2$ to the corresponding alcohol, $PGH_2$ (*5*, *6*). These two reactions occur at different locations on the protein but are functionally interrelated. This is exemplified by the observations that binding of a sixth ligand to the heme blocks both activities and addition of glutathione/glutathione peroxidase as a hydroperoxide scavenger inhibits both the peroxidase and the cyclooxygenase reactions (*7*, *8*).

The heme prosthetic group is located at the base of a wide opening near the top of the protein that comprises the peroxidase active site (*9*). The cyclooxygenase active site is located beneath one edge of the heme, and a tyrosine residue (Tyr385) is interposed between the heme and this active site (*9*−*11*). Tyr385 is essential for cyclooxygenase activity (*12*) and is oxidized to a radical intermediate during arachidonic acid oxygenation (*13*, *14*). This radical abstracts the 13-*pro-S* hydrogen from the substrate to initiate oxygen incorporation and the eventual formation of $PGG_2$ (*13*, *15*). No tyrosyl radical is detected in resting PGHS. Therefore, this critical oxidant must be generated in order to activate the cyclooxygenase catalytic cycle. Because heme is essential for cyclooxygenase turnover and because modulation of the heme group by a variety of means can inhibit arachidonate oxygenation, it is widely believed that the heme is critical for oxidation of Tyr385 and, hence, for cyclooxygenase activation (*16*).

The exact nature of heme participation in cyclooxygenase catalysis has been the subject of much debate. According to

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[1] Abbreviations: PGHS, prostaglandin endoperoxide synthase; PGHS-1 and -2, prostaglandin endoperoxide synthase isozymes 1 and 2; $PGG_2$, prostaglandin $G_2$; $PGH_2$, prostaglandin $H_2$; $PGE_2$, prostaglandin $E_2$; $PGD_2$, prostaglandin $D_2$; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); $ABTS^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid cation radical; *tert*-butyl-OOH, *tert*-butylhydroperoxide; HpETEs, hydroperoxyeicosatetraenoic acids; 15-HpETE, (5*Z*,8*Z*,11*Z*,-13*E*,15(*S*))-15-hydroperoxyeicosatetraenoic acid; HETEs, hydroxyeicosatetraenoic acids.

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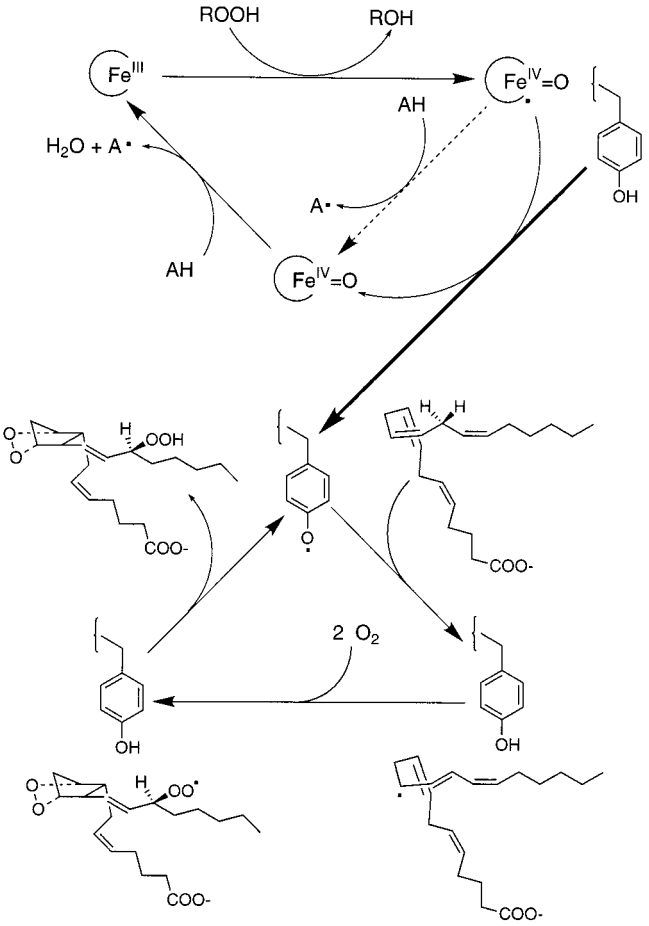


FIGURE 1: Scheme representing the branched-chain mechanism of prostaglandin biosynthesis by PGHS. The intramolecular electron transfer proposed for cyclooxygenase activation is indicated by the bold arrow.

the branched-chain mechanism first proposed by Ruf and co-workers (Figure 1), $Fe^{III}$ PGHS behaves as a peroxidase by reducing a hydroperoxide to the corresponding alcohol (*15*, *17*). In the process, the heme is oxidized to an $Fe^{IV}$═O-[porphyrin•] intermediate (*18*). This intermediate then accepts an electron from Tyr385, resulting in the formation of the critical tyrosyl radical and an $Fe^{IV}$═O heme intermediate. An alternative mechanism has been proposed in which the $Fe^{III}/Fe^{II}$ forms of the enzyme participate in Tyr385 oxidation and cyclooxygenase activation (*19*, *20*).

Disagreement also has arisen over the role of the heme following the initial activation of the cyclooxygenase. According to the branched-chain model, the tyrosyl radical required for the initial oxidation of arachidonic acid is regenerated in the last step of the cyclooxygenase catalytic cycle by the reduction of $PGG_2$ peroxyl radical to the hydroperoxide, $PGG_2$ (Figure 1) (*15*, *21*). Because of this step, the heme is only necessary for the initial oxidation of Tyr385. However, others have proposed that the tyrosyl radical is not regenerated by the $PGG_2$ peroxyl radical but that heme is required to reoxidize Tyr385 at each turn of the cyclooxygenase cycle (*22*, *23*).

Heme prosthetic groups are used by a variety of proteins to accomplish a diverse set of functions. Clearly, the individual proteins modulate the reactivity of the heme group by a variety of interactions, the most obvious of which is through the axial ligands supplied by the protein. Much has been discovered about the catalytic roles of heme prosthetic groups by evaluating naturally occurring and site-directed substitutions of amino acids known to ligate the heme iron. One substitution that has been particularly fruitful is the replacement of histidine ligands with tyrosine in proteins such as hemoglobin, myoglobin, and heme oxygenase (*24*−*30*). One of the primary effects anticipated by such a substitution is in the ability of the mutant protein to stabilize more oxidized forms of the heme (i.e., ferric and ferryl-oxo) in preference to the ferrous form due to the increased anionic character of a phenolate ligand.

Histidine-to-tyrosine ligand substitutions have translated into dramatic changes in protein function. For example, this alteration in hemoglobin renders it unable to carry $O_2$ because the $Fe^{II}$ oxidation state is much less stable in the mutant than in the wild-type protein (*24*). The analogous substitution in heme oxygenase results in an inactive protein because, upon reduction, the heme prosthetic group dissociates from the protein. Interestingly, this modified heme oxygenase also loses its ability to react with peroxide (*30*).

Given the unresolved aspects of heme participation in PGHS catalysis and the wealth of information that can be obtained through manipulation of these amino acids, we substituted the histidine ligand of PGHS-2 (His388) with tyrosine and evaluated the effect of this modification on peroxidase and cyclooxygenase activities. The phenotype of His388Tyr PGHS-2 provides valuable information regarding not only the role of the heme in the mechanisms of cyclooxygenase and peroxidase catalysis but also the nature of the complex interaction between these two spatially and functionally distinct activities in prostaglandin biosynthesis.

## EXPERIMENTAL PROCEDURES

*Materials.* Arachidonic acid (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid) was purchased from Nu-Check-Prep, Inc. (Elysian, MN) and [1-$^{14}$C]arachidonic acid was obtained from NEN Dupont (Boston, MA). (5*Z*,8*Z*,11*Z*,13*E*,15(*S*))-15-Hydroperoxyeicosatetraenoic acid (15(*S*)-HpETE) was purchased from Cayman Chemical (Ann Arbor, MI) or Oxford Biomedical Research (Oxford, MI). Bovine liver catalase, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), xanthine, xanthine oxidase, benzyl viologen, hematin, $H_2O_2$ (30%), and *tert*-butyl hydroperoxide (*tert*-butyl-OOH) (70%) were purchased from Sigma (St. Louis, MO). Indigo carmine and NaCN were obtained from Aldrich (Milwaukee, WI).

*Mutant Construction.* Site-directed mutagenesis was performed on a mCOX-2 pBS+ vector (Stratagene, La Jolla, CA) with the Quick Change site-directed mutagenesis kit (Stratagene). Mutant-containing regions were subcloned into the mCOX-2 pVL1393 baculovirus expression vector (Pharmingen, San Diego, CA) by use of the *Stu*I and *Eco*RI restriction sites in mCOX-2. The subcloned region was fully sequenced to ensure no accidental mutations were incorporated during mutagenesis.

*Protein Expression and Purification.* Wild-type and mutant proteins were expressed by homologous recombination of the mCOX-2-pVL1393 vector with the Baculogold vector (Pharmingen, San Diego, CA) in SF-9 cells (Novagen, Madison, WI). After virus amplification, 4−15 L of uninfected SF-9 cells (>95% viable) were grown in TNM-FH

medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% L-glutamine, 0.1% pluronic, and 4 mL/L antibiotic/antimycotic to a density of $1 \times 10^6$ cells/mL. These cells were then infected with fresh viral stock. Upon reaching 65−70% viability, the total volume was harvested by centrifugation at 2500 rpm in a Sorvall RC-3B centrifuge, washed in ice-cold phosphate-buffered saline, and recentrifuged. The cell pellet was stored at −80 °C.

Purification of wild-type and mutant COX was performed at 4 °C as previously described with minor modifications. Frozen cells were resuspended in 80 mM Tris (pH 7.2), 2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mM diethyl dithiocarbamate. After centrifugation at 160000*g* for 45 min, the pellet was resuspended to a volume of roughly 100 mL in a Dounce homogenizer. PGHS-2 proteins were solublized from the membrane by dropwise addition of CHAPS [11% (w/v)] to give a final detergent concentration of 1%. After 1 h of stirring, the sample was recentrifuged at 160000*g* for 45 min. The supernatant was retained and then diluted 4-fold by the addition of 20 mM Tris (pH 8.0), 0.4% CHAPS, and 0.1 mM EDTA. This mixture was then loaded onto a 50 mL Macro-prep High-Q ion-exchange column preequilibrated with the dilution buffer. PGHS-2 proteins were eluted with a linear gradient of increasing KCl (0−0.3 M). Fractions were tested for cyclooxygenase and/or peroxidase activities by arachidonate-dependent oxygen consumption or $H_2O_2$-dependent ABTS oxidation, respectively. PGHS-2-containing fractions were analyzed by SDS−PAGE (7.5%) to determine the fractions to pool for gel-filtration chromatography. Appropriate fractions were concentrated in an Amicon concentrator (Amicon, Beverly, MA) to a final volume of approximately 6 mL. The sample was then loaded onto a 26 mm × 1000 mm column packed with preequilibrated Superose-12 [20 mM Tris (pH 8.0), 0.4% CHAPS, and 0.15 M NaCl]. Fractions containing PGHS-2 protein, as determined from cyclooxygenase and/or peroxidase activities and SDS−PAGE (as described above), were concentrated and stored at −80 °C. The purity of wild-type and mutant COX-2 protein was estimated by evaluation of Coomassie-stained SDS−7.5% PAGE.

*Determination of PGHS-2 $Fe^{III}/Fe^{II}$ Reduction Potential.* Reduction potentials were determined according to the method described by Massey (*31*). Briefly, PGHS-2 proteins (reconstituted with hematin) were reduced from $Fe^{III}$ to $Fe^{II}$ intermediates with the oxidation of xanthine to urate by xanthine oxidase as a source of electrons. Benzyl viologen was present as an electron-transfer mediator and indigo carmine ($E_{m8} = -158$ mV) was used as a reduction potential indicator (*32*). All reactions contained 4.5 $\mu$M PGHS-2 (wild type or the appropriate mutant), 2 $\mu$M benzyl viologen, 15 $\mu$M indigo carmine, 250 $\mu$M xanthine, and 10 nM xanthine oxidase in 100 mM Tris, pH 8.0 at room temperature. Reactions were made anaerobic by freeze/pump/thaw degassing, and reduction was initiated by mixing xanthine oxidase with all the other components of the solution. Reduction of indigo carmine was monitored at 594 nm, an isosbestic wavelength for $Fe^{III}$ and $Fe^{II}$ PGHS-2. Reduction of PGHS-2 was monitored at 430 nm. The contribution of reduced indigo carmine at 430 nm was subtracted to obtain only the change in absorbance due to PGHS. Reductive titration was carried out until spectral contributions from benzyl viologen semiquinone radical were observed. The potential of the sample at given time during reduction ($E$) was calculated on the basis of the proportion of oxidized and reduced indigo carmine, by use of the Nernst equation:

$$E = E_0 + (RT/nF) \ln ([\text{dye}]_{ox}/[\text{dye}]_{red})$$

*Note on Reconstitution with Hematin.* Reconstitution of wild-type PGHS-2 with hematin resulted in a linear increase in cyclooxygenase activity up to a ratio of 1 heme/subunit. Significant deviations from linearity were detected in His388Tyr cyclooxygenase activity and visible band molar absorptivity above 0.5 heme/subunit. The properties of free or adventitiously bound hematin are such that its presence in excess may have a profound effect on experimental outcomes. Therefore, for each experimental procedure outlined below, a note regarding the rationale for the reconstitution protocol used is provided.

*UV−Visible Spectroscopy and $CN^-$ binding*. All reactions were carried out at room temperature in 100 mM phosphate buffer, pH 7.0, and 10 $\mu$M PGHS-2 (wild type or His388Tyr) reconstituted with 5 $\mu$M hematin. Reconstitution with 0.5 equiv of hematin was performed to minimize spectral contributions from free or adventitiously bound hematin. After the ferric PGHS-2 spectrum was recorded, NaCN was added (2 mM final concentration) and another spectrum was recorded. The $K_D$ of wild type and His388Tyr for $CN^-$ was obtained by titrating NaCN into a cuvette containing 10 $\mu$M PGHS-2 reconstituted with 5 $\mu$M hematin. A spectrum was recorded after each addition.

Extinction coefficients for the Soret bands of ferric wild-type and His388Tyr PGHS-2 were determined by titrating hematin into a cuvette containing 10 $\mu$M PGHS-2 and monitoring the change in absorbance at the $\lambda_{max}$ for each protein. Again, to minimize spectral contributions from free or adventitiously bound hematin, only points obtained with $\leq$ 0.5 equiv of hematin were used for extinction coefficient determination.

*Oxygen Consumption Assays*. Activity was determined for the wild-type and mutant PGHS-2 proteins in the presence of 100 mM Tris, pH 8.0, and 75 $\mu$M arachidonic acid at 37 °C without a reducing substrate. Reconstitution was accomplished by mixing the protein and hematin in 100 $\mu$L of buffer and incubating at 37 °C for not more than 1 min. Oxygen consumption was initiated by adding the reconstituted enzyme to the oxygraph cuvette containing arachidonic acid and buffer. Reconstitution was accomplished with either 0.5 or 10 equiv of hematin/subunit.

Reconstitution with 10 equiv was used to evaluate the maximum activity of His388Tyr cyclooxygenase activity possible with fully reconstituted protein. Because free hematin is known to react with fatty acid hydroperoxides to form the corresponding ketones, reconstitution with 0.5 equiv of hematin was used to minimize consumption of hydroperoxides. The activity profiles obtained with either method were highly similar, except that lag phases were generally longer in the presence of excess hematin. Accordingly, the effect of excess hematin on the lag phase was more pronounced with older preparations of arachidonic acid. The lag phase observed in the presence of excess hematin could be extended indefinitely by the addition of reducing substrates such as ABTS.

*Effect of Hydroperoxide on Activation Time*. The ability of hydroperoxides to affect the lag in cyclooxygenase activity was determined by oxygen consumption as above. Hydroperoxides were placed in the oxygraph cuvette with arachidonate prior to addition of reconstituted His388Tyr. Calculation of the relative activation time was accomplished by determining the time necessary for the enzyme to consume 22.5 $\mu$M oxygen. Maximal rates of $O_2$ consumption were consistently observed at this point in the reaction. The maximum activation time ($a_{max}$) was determined in the absence of any added hydroperoxide, and a theoretical minimum activation time ($a_{min}$) was calculated on the basis of the time required for His388Tyr PGHS-2 to consume 22.5 $\mu$M $O_2$ at its maximal rate. The relative activation time ($t$) with a given hydroperoxide concentration was set on a scale from 1 (maximum activation time) to 0 (minimum activation time) by $(t - a_{min})/(a_{max} - a_{min})$.

*His388Tyr Arachidonate Oxygenation Product Analysis.* Wild-type PGHS-2 or His388Tyr was reconstituted with 0.5 equiv of hematin and reacted with 50 $\mu$M [1-$^{14}$C] arachidonic acid for 1 h at room temperature in 100 mM Tris, pH 8.0. For some His388Tyr reactions, Tyr385Phe PGHS-2 (100 nM final concentration) was added following the 1 h incubation with [1-$^{14}$C] arachidonic acid. Phenol, when present, was added to a concentration of 0.5 mM. All reactions were stopped, and products extracted by the addition of 2 volumes of ether/methanol/acetic acid (85:12:3). The organic phase was collected, dried down, and finally resuspended in methanol for separation by HPLC. Arachidonate oxygenation products were separated on a 5 $\mu$m ODS Ultrasphere C-18 column (4.6 mm × 25 mm) from Beckman Instruments Inc. (Fullerton, CA). Reverse-phase gradient separation (flow rate = 1 mL/min) was achieved by use of a two-solvent system comprising $H_2O$/0.1% acetic acid (solvent A) and acetonitrile/0.1% acetic acid (solvent B) and a gradient similar to that described by Capdevila et al. (*33*). The initial solvent conditions were 70% A/30% B. Following a 5 min isocratic period, the mixture was changed over 30 min to 40% A/60% B and held there for 10 min. Over the following 15 min, the solvent mixture was ramped to 25% A/75% B and from there to 0% A/100% B over the next 10 min. These conditions were maintained for an additional 5 min, giving a total time of 75 min for each run. Arachidonate eluted from the column with a 71 min retention time. $PGE_2$ and $PGD_2$ standards were produced by allowing the spontaneous decomposition of purified $PGH_2$ to take place in 50 mM phosphate buffer (pH 7.4) at room temperature for 30 min (*3, 4*).

As mentioned previously, free hematin is known to react with fatty acid hydroperoxides to form the corresponding ketones (*34*). Thus, we observed that reconstitution of His388Tyr with 10 equiv of hematin resulted in the formation of primarily ketoprostaglandins. Reconstitution of the enzymes with 0.5 equiv of hematin minimized conversion of hydroperoxyprostaglandins to the corresponding ketones.

*Peroxidase Activity Assays*. All reactions were carried out at room temperature. PGHS-2 proteins were reconstituted by the addition of 0.5 equiv of hematin and incubated for 1 min at room temperature in 80 mM Tris, pH 8.0, and 1 mM ABTS. The entire volume containing reconstituted PGHS-2 (≥95% of the total reaction volume) was transferred to a cuvette containing hydroperoxide (≤5% of the total reaction volume). Peroxidase activity was monitored by the oxidation of ABTS to ABTS$^{\bullet+}$ at 417 nm. 15-HpETE and *tert*-butyl-OOH were diluted, when appropriate, with 95% ethanol.

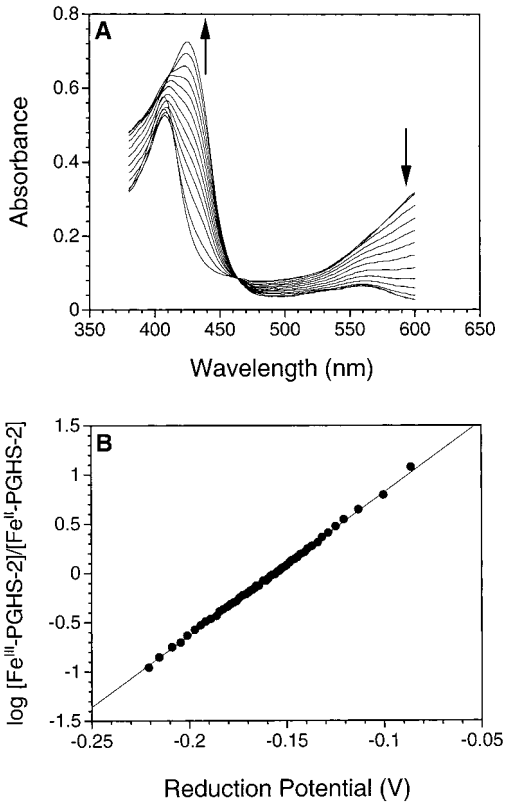


FIGURE 2: Visible absorption spectra (A) and the calculated effect of reduction potential on distribution of $Fe^{III}$ and $Fe^{II}$ forms of wild-type PGHS-2 (B) collected during a typical PGHS-2 midpoint potential determination. Every fifth spectrum collected is shown, and the arrows indicate the direction of absorbance change during reduction. The midpoint potential ($E_m$) was calculated from the *x*-intercept.

Free hematin can act as a pseudoperoxidase by carrying out the reduction of hydroperoxides (especially alkyl and fatty acid hydroperoxides) and oxidizing reducing substrates to the corresponding radicals. Therefore, all peroxidase assays were carried out with PGHS-2 reconstituted with 0.5 equiv of hematin to minimize the pseudoperoxidase activity of free heme.

## RESULTS

*Reduction Potential of Wild-Type and Mutant PGHS-2s*. The possible involvement of $Fe^{II}$ PGHS as an intermediate in cyclooxygenase activation was evaluated by determining the reduction potential of the $Fe^{III}/Fe^{II}$ couple of wild-type PGHS-2 and comparing it to known values for reactions involved in cyclooxygenase activation. Typical spectra obtained during reductive titration of $Fe^{III}$ PGHS in the presence of indigo carmine as well as a typical reduction potential determination are shown in Figure 2. The reduction potential of wild-type PGHS-2 was determined to be −156 ± 1 mV. This was significantly lower than the redox potential reported for sheep PGHS-1 but the differences appear to be due to experimental conditions. We also determined the reduction potential for sheep PGHS-1 in the same buffer as wild-type mouse PGHS-2. The value in the absence of glycerol was −167 mV, which is 115 mV lower

Table 1: Reduction Potentials of Ferric PGHSs and Related Chemical Reactions

| redox couple | $E_m$ (mV) | ref |
|---|---|---|
| $Fe^{III}/Fe^{II}$ wtPGHS-2[a] | $-156 \pm 1$ | this work |
| $Fe^{III}/Fe^{II}$ wtPGHS-2 + indomethacin | $-143 \pm 1$ | this work |
| $Fe^{III}/Fe^{II}$ Tyr385Phe | $-139 \pm 1$ | this work |
| $Fe^{III}/Fe^{II}$ Tyr385Phe + arachidonate | $-138 \pm 1$ | this work |
| $Fe^{III}/Fe^{II}$ Tyr385Phe + indomethacin | $-140 \pm 2$ | this work |
| $Fe^{III}/Fe^{II}$ wtPGHS-1[b] | $-52$ | *42* |
| $Fe^{III}/Fe^{II}$ wtPGHS-1[c] | $-167 \pm 2$ | this work |
| $PUFA^{\cdot}/PUFA$[d] | +600 | *48* |
| $Fe^{III}/Fe^{II}$ lipoxygenase | +600 | *58*, *59* |
| $ROO^{\cdot}/ROOH$ | +1020 to 1110 | *47* |
| tyrosyl•/tyrosine | +880 to 930 | *46* |

[a] All PGHS-2 reduction potentials were obtained at pH 8.0 in the absence of glycerol. [b] Determined in the presence of 30% glycerol. [c] Determined at pH 8.0 in the absence of glycerol; G. P. Hochgesang, unpublished results. [d] All non-PGHS potentials are reported at pH 7.

than the value for PGHS-1 in the presence of glycerol and 11 mV lower than PGHS-2.[2]

It is possible that binding of substrate by PGHS results in a change in the $Fe^{III}/Fe^{II}$ PGHS-2 reducion potential favoring the $Fe^{II}$ intermediate (*19*). This was tested by addition of arachidonic acid to the peroxidase active/cyclooxygenase inactive PGHS-2 mutant, Tyr385Phe (Table 1). The substitution of Tyr385 with Phe produced a +13 mV shift in potential. Addition of arachidonic acid to the Tyr385Phe mutant did not change this potential. Indomethacin, an inhibitor known to bind in the cyclooxygenase active site of PGHS, had little effect on the reduction potential of either wild-type or Tyr385Phe PGHS-2. Also shown in Table 1 are reduction potentials for reactions relevant to cyclooxygenase activation. The reduction of a polyunsaturated fatty acid carbon-centered radical, tyrosyl radical, and a peroxyl radical to the corresponding parent compounds all occur with reduction potentials far more positive than that obtained for any of the PGHS-2 reactions shown in Table 1.

*Spectral Properties of His388Tyr PGHS-2*. We also undertook a structural approach to evaluate the role of $Fe^{II}$ PGHS in cyclooxygenase catalysis. It has been shown that substitution of the proximal histidine ligand of many heme proteins with tyrosine results in significant lowering of the reduction potential of those proteins. This is thought to result from the more anionic character of the phenolate ligand as compared with imidazole. We substituted the known PGHS proximal heme ligand, His388, with a tyrosine by site-directed mutagenesis to evaluate the effect of this substitution on both the peroxidase and cyclooxygenase activities of PGHS and to determine the role of the heme group in cyclooxygenase activation. The mutant protein was expressed in insect cells (SF-9) and purified to near electrophoretic homogeneity.

Visible absorption spectra for wild-type and His388Tyr PGHS-2 are shown in Figure 3. This substitution resulted in a shift in the absorption maximum of the Soret ($\gamma$) band from 407 to 403 nm. A nearly identical shift is reported for metmyoglobin upon substitution of its proximal heme iron ligand (His93) with tyrosine (*26*−*29*). A reduction in the molar absorptivity of the Soret band was detected with His388Tyr (100 $mM^{-1}$ $cm^{-1}$) compared with the wild-type

[2] G. P. Hochgesang, unpublished results.

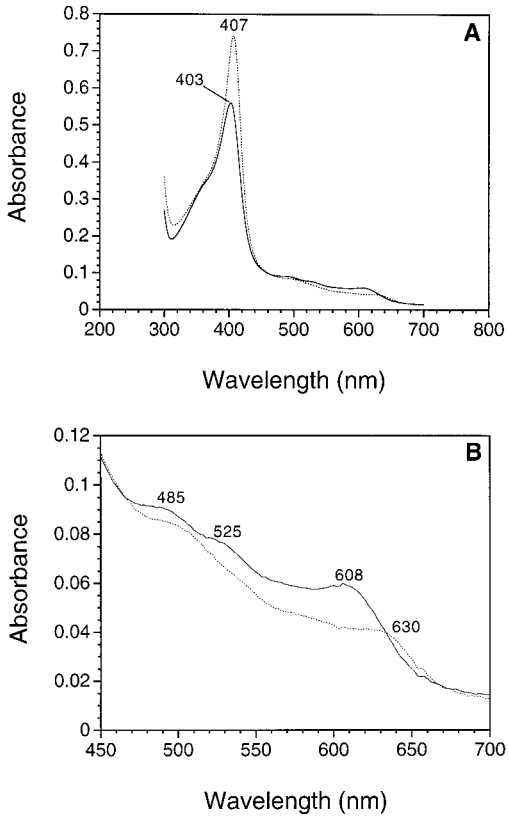


FIGURE 3: Ultraviolet−visible absorption spectra for wild-type and His388Tyr PGHS-2. Panel A indicates the entire scanned range, and panel B is a magnification of the weaker visible bands. Wild-type (dotted line) and His388Tyr (solid line) were placed in 100 mM phosphate buffer, pH 7.0, and reconstituted with 0.5 equiv of hematin (5 $\mu$M reconstituted PGHS-2).

PGHS-2 (129 $mM^{-1}$ $cm^{-1}$). This trend is consistently observed with His → Tyr ligand substitutions in heme proteins, including metmyoglobin, methemoglobin, and heme oxygenase (*26*−*30*).

Shifts in the weaker α, $\beta$, and charge-transfer bands (Figure 2B) also are indicative of His → Tyr heme ligand substitution. A characteristic band for PGHS-2 near 630 nm was lost and replaced by a more intense transition at 608 nm in His388Tyr PGHS-2. Similar shifts are detected in the proximal histidine-to-tyrosine mutants of myoglobin and hemoglobin (*26*−*29*). In wild-type PGHS-2, the α and $\beta$ bands are very weak, and the same is observed in the His388Tyr protein; however, the bands observed near 485 and 525 nm are consistent with a tyrosine ligand to the heme iron.

One of the most profound differences between His388Tyr and wild-type PGHS was in the ability of each protein to bind $CN^-$ as a sixth ligand (Figure 4). In the presence of 2 mM NaCN, a characteristic $Fe^{III}-CN$ complex with absorption maxima at 420 and 535 nm was observed with wild-type PGHS-2. However, little or no change was detected with His388Tyr upon addition of the same NaCN concentration. From titration of wild-type PGHS-2 with NaCN, a $K_D$ of 230 $\mu$M was determined, but titration of His388Tyr resulted in little spectral change at NaCN concentrations up to 5 mM (Figure 4, inset). Addition of 2 mM NaCN to bovine erythrocyte catalase resulted in a spectral transition similar to that of wild-type PGHS-2 (data not shown). Since the

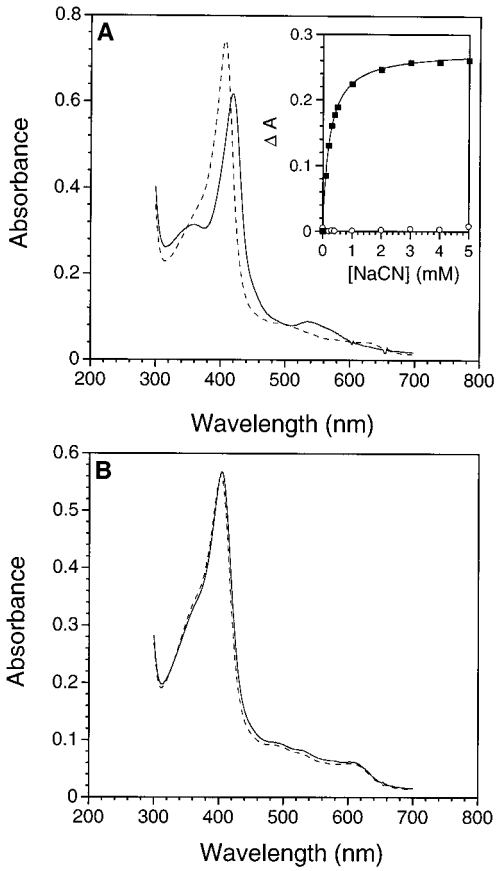


FIGURE 4: Effect of NaCN on the visible absorption spectra of wild-type and His388Tyr PGHS-2. Wild-type (panel A) and His388Tyr (panel B) were reconstituted as in Figure 1 and spectra were recorded for each $Fe^{III}$ form (dashed lines). Following addition of NaCN (2 mM final concentration), spectra were again recorded for each protein (solid lines). The inset indicates the effect of NaCN titration on absorbance at the $Fe^{III}$ maximum for each protein [408 nm for wild-type (■) and 402 nm for His388Tyr (○)].

proximal heme ligand of catalase is tyrosinate, this finding suggests that the substitution of a tyrosinate ligand to the heme iron alone is not sufficient to explain the inability of His388Tyr to bind $CN^-$.

*Catalytic Activities of His388Tyr PGHS-2*. Perhaps the most striking feature of His388Tyr PGHS-2 is that it retains cyclooxygenase activity (Figure 5). Addition of reconstituted His388Tyr to a solution containing arachidonic acid resulted in oxygen uptake, indicative of prostaglandin biosynthesis. The maximal rate of oxygen consumption observed with His388Tyr was somewhat less than that with wild-type PGHS-2 but the extent of arachidonate oxygenation by the two enzymes was identical. The primary difference in the cyclooxygenase activities of the two proteins was the presence of a prolonged lag period in oxygen consumption exhibited by the mutant.

Several studies have indicated that changes in the cyclooxygenase active site of PGHS render the protein unable to produce prostaglandins as the primary product of catalysis (*35*−*38*). Instead, these proteins produce the hydroxyeicosatetraenoic acids (HETEs) as the dominant products. To ensure that the His388Tyr substitution in PGHS-2 did not result in these types of changes, we evaluated the products of arachidonate oxygenation by His388Tyr and compared them with those produced by wild-type PGHS-2 (Figure 6).

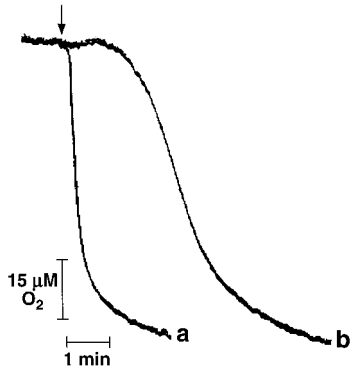


FIGURE 5: Oxygen consumption by wild-type (a) and His388Tyr (b) PGHS-2 in the presence of arachidonic acid. All reactions contained 75 $\mu$M arachidonate, 150 nM PGHS-2, and 100 mM Tris, pH 8.0. Reactions were carried out at 37 °C and initiated at the time indicated by the arrow by addition of reconstituted PGHS-2.

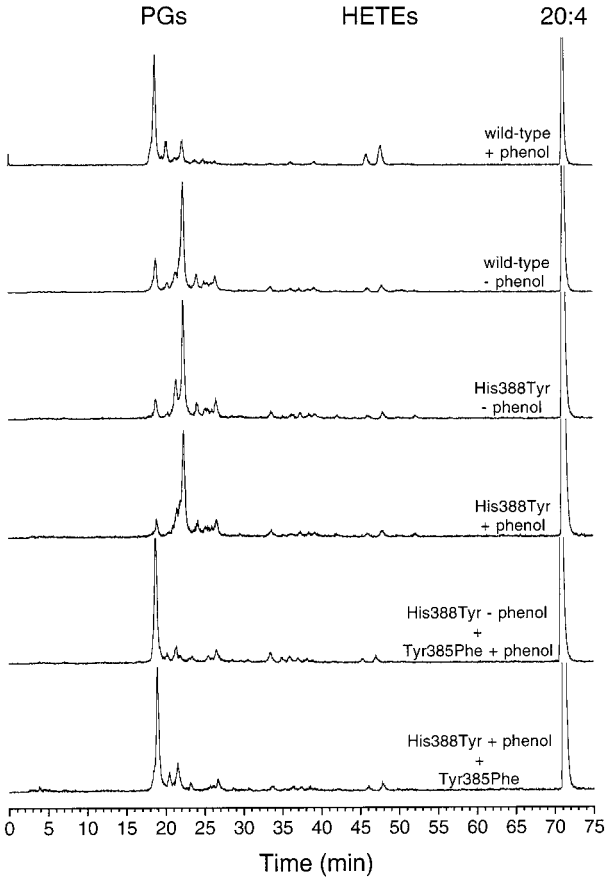


FIGURE 6: HPLC elution profiles of arachidonate oxygenation products from wild-type and His388Tyr PGHS-2. Wild-type or His388Tyr protein was reconstituted with 0.5 equiv of hematin and reacted with 50 $\mu$M [1-$^{14}$C]arachidonic acid in 100 mM Tris, pH 8.0. Tyr385Phe and phenol, when present, were at concentrations of 100 nM and 0.5 mM, respectively. Expected elution times for prostaglandins (PGs), hydroxyeicosatetraenoic acids (HETEs), and arachidonate (20:4) are indicated. $PGE_2$ eluted at 18.9 min and $PGD_2$ at 20.3 min.

Consistent with previous reports, reaction of wild-type PGHS-2 with arachidonate produced a series of prostaglandins (mainly $PGE_2$) along with small amounts of $PGD_2$, 11-HETE, and 15-HETE. When the reaction was carried out in the absence of the peroxidase reducing substrate, phenol, the major prostaglandin products eluted at longer retention times. This suggested the accumulation of hydroperoxyprostaglan-

dins resulting from the inability of wild-type PGHS-2 peroxidase to process the hydroperoxides to the corresponding alcohols in the absence of a peroxidase reducing substrate (*3*, *4*).

Reaction of His388Tyr with arachidonate in the absence of phenol resulted in a product profile nearly identical to that obtained with wild-type enzyme in the absence of phenol. However, the presence of phenol in the incubation mixture did not result in a substantial shift in the prostaglandin products obtained with His388Tyr. This indicated that His388Tyr PGHS-2 can oxygenate arachidonic acid to $PGG_2$ but cannot reduce $PGG_2$ to $PGH_2$ (i.e., it has cyclooxygenase but not peroxidase activity). This was confirmed by the addition of the peroxidase-active/cyclooxygenase-inactive PGHS-2 mutant, Tyr385Phe, to incubations of His388Tyr with arachidonate. The addition of Tyr385Phe (and phenol if necessary) resulted in product profiles nearly identical to those obtained with wild-type PGHS-2 in the presence of phenol.

Consistent with the product profiles above, little or no peroxidase activity was detected when His388Tyr was incubated with the reducing substrate ABTS in a standard peroxidase assay (Figure 7A). Peroxidase activity was detectable but required substantially higher $H_2O_2$ concentrations than typically used with wild-type PGHS-2 (Figure 7B). As with the wild-type protein, His388Tyr peroxidase activity was detected with other hydroperoxide substrates (Figure 7C). The ability of each of the hydroperoxides to support peroxidase activity was highly variable, consistent with previous reports for both PGHS-1 and PGHS-2 (*6*, *39*−*41*). In general, $k_{cat}$ values for reaction of His388Tyr with hydroperoxides were one-tenth those obtained with wild-type PGHS-2. Interestingly, the $k_{cat}$ values for wild-type PGHS-2 or His388Tyr PGHS-2 were independent of peroxide. Kinetic parameters for peroxidase activity supported by *tert*-butyl-OOH, $H_2O_2$, and 15-HpETE for both wild-type and His388Tyr PGHS-2 are given in Table 2. Generally, each of the peroxides tested showed much higher $K_m$ values (>25-fold) and much lower $k_{app}$ values ($10^2$−$10^3$ less) for His388Tyr than for the wild-type PGHS. However, the trend in substrate specificity was the same for both the wild-type and mutant proteins; 15-HpETE was the most reactive followed by $H_2O_2$, and *tert*-butyl-OOH.

*Hydroperoxide-Dependent Activation of His388Tyr PGHS-2*. His388Tyr PGHS-2 has a significantly reduced peroxidase activity and it is clear from the extensive lag phase that the activation of the cyclooxygenase is correspondingly impaired. Since some His388Tyr peroxidase activity can be detected at relatively high peroxide concentrations, we evaluated the ability of the hydroperoxides to enhance cylooxygenase activity by shortening the lag period. The prominent lag phase in oxygen consumption observed with this mutant was shortened in a concentration-dependent manner by the addition of either $H_2O_2$ or 15-HpETE (Figure 8). However, the maximal rate of oxygen consumption was unaffected by the added hydroperoxide. At hydroperoxide concentrations higher than those shown in Figure 8, the extent of $O_2$ consumption decreased due to presumed hydroperoxide-dependent irreversible inactivation of the protein. Addition of *tert*-butyl-OOH also appeared to shorten the lag in cyclooxygenase activity, but it was far less effective than $H_2O_2$. Furthermore, the effect on the lag phase was obscured by inactivation caused by this particular hydroperoxide at concentrations similar to those used for $H_2O_2$. 15-HpETE was a much more effective activator of His388Tyr cyclooxygenase than either $H_2O_2$ or *tert*-butyl-OOH, consistent with its relative peroxidase substrate activity.

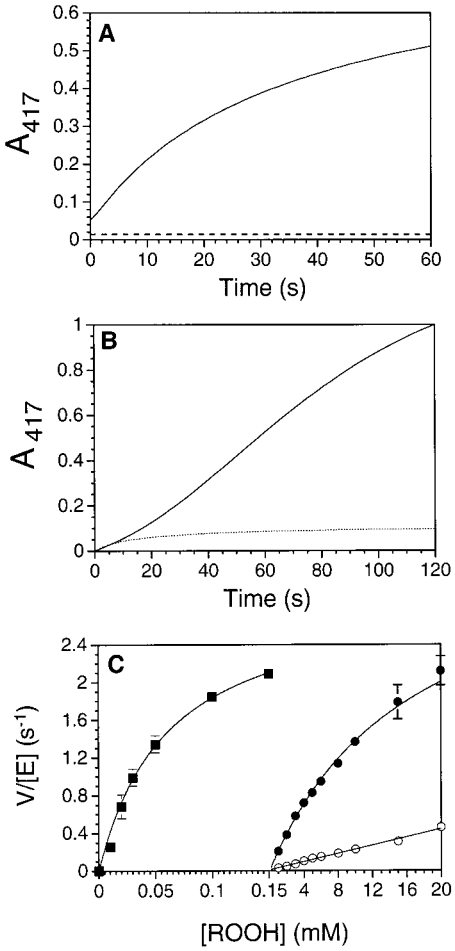


FIGURE 7: Peroxidase activity of His388Tyr PGHS-2. Panel A indicates oxidation of ABTS to the corresponding radical (monitored at 417 nm) by 45 nM wild-type (solid line) and His388Tyr (dashed line) PGHS-2 reconstituted with 22.5 nM hematin. Each reaction contained 500 $\mu$M $H_2O_2$. Panel B indicates ABTS oxidation by His388Tyr (solid line) or hematin (225 nM) (dotted line) in the presence of 10 mM $H_2O_2$. His388Tyr (450 nM) was reconstituted with 225 nM hematin. Panel C indicates peroxidase activity of His388Tyr in the presence of increasing concentrations of 15-HpETE (■), $H_2O_2$ (●), or *tert*-butyl-OOH (○). All reactions contained 1 mM ABTS and were carried out at room temperature in Tris buffer, pH 8.0.

Table 2: Kinetic Parameters for Reaction of Wild-Type and His388Tyr PGHS-2 with Peroxides[a]

| | wild type | | His388Tyr | |
|---|---|---|---|---|
| substrate | $K_m$ (mM) | $k_{app}$ ($M^{-1}$ $s^{-1}$) | $K_m$ (mM) | $k_{app}$ ($M^{-1}$ $s^{-1}$) |
| *t*-butyl-OOH | 1.0 | $3.1 \times 10^4$ | nd | $2.2 \times 10^1$ |
| $H_2O_2$ | 0.36 | $7.8 \times 10^4$ | 18 | $2.2 \times 10^2$ |
| 15-HpETE | <0.005 | $\approx 1 \times 10^7$ | 0.068 | $4.5 \times 10^4$ |

[a] Peroxidase activity was monitored by oxidation of ABTS to $ABTS^{\bullet +}$. Details are provided in Materials and Methods.

## DISCUSSION

*Structural Modifications Induced by His → Tyr Heme Ligand Substitution*. Substitution of the PGHS-2 proximal histidine with a tyrosine fails to abrogate either activity,

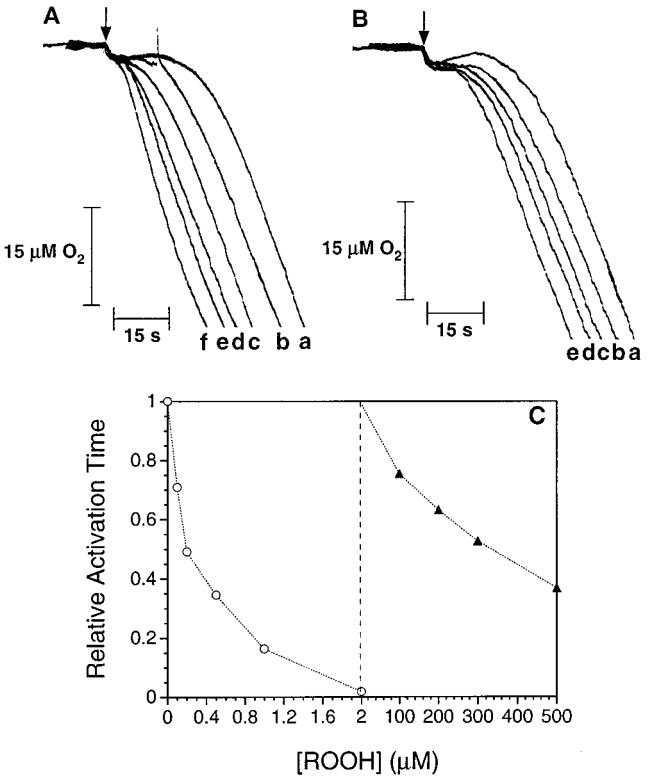


FIGURE 8: Effect of hydroperoxides on the lag in His388Tyr PGHS-2 cyclooxygenase activity. (A) His388Tyr (300 nM final concentration) was reconstituted with 0.5 equiv of hematin (150 nM final concentration) and added to reactions containing 75 $\mu$M arachidonate and 0 nM (a), 100 nM (b), 200 nM (c), 500 nM (d), 1 $\mu$M (e), or 2 $\mu$M (f) 15-HpETE. (B) His388Tyr was reconstituted as in panel A and reacted with arachidonate and 0 $\mu$M (a), 100 $\mu$M (b), 200 $\mu$M (c), 300 $\mu$M (d), or 500 $\mu$M (e) $H_2O_2$. All reactions were carried out at 37 °C in Tris buffer, pH 8.0. (C) Effect of 15-HpETE and $H_2O_2$ on the relative activation time determined as described under Materials and Methods.

although there are substantial differences in their relative activities. His388Tyr retains a surprising amount of cyclooxygenase activity (Figures 5 and 6) and though the maximal rate of turnover is diminished, it catalyzes the same extent of arachidonate oxygenation as wild-type enzyme. The major product of oxygenation is $PGG_2$. Peroxidase activity is also retained in the mutant, but it is dramatically diminished compared to wild-type enzyme. The $k_{cat}$ values for reaction of His388Tyr with hydroperoxides were roughly one-tenth the values determined for wild-type PGHS.

What is most striking is that the loss in His388Tyr peroxidase activity appears to stem as much from steric restrictions imposed by the substitution as the anticipated electronic effects on the heme group. Affinity of His388Tyr for peroxides and $CN^-$ seems to be significantly altered over and above any effects on $k_{cat}$. This raises questions about the nature of the structural changes induced by the substitution. One possibility is that a hexacoordinate heme complex is formed in His388Tyr with Tyr388 and His193 acting as axial ligands. Such an arrangement would bear some structural and electronic similarities to hemoglobin M Saskatoon ($\beta$ chain) (*24*, *25*, *28*) and the equivalent sperm whale myoglobin mutant [His(E7)Tyr] in which the distal histidine has been substituted with a tyrosine (*28*).

The visible spectroscopic characteristics of His388Tyr bear closer resemblance to hemoglobins M Iwate ($\alpha$ chain) and Hyde Park ($\beta$ chain) and His93Tyr myoglobin (*25*−*29*). In all of these proteins, the proximal histidine ligand has been substituted with a tyrosine. Resonance Raman, electron paramagnetic resonance (EPR), and crystallographic data show that these proteins are high-spin pentacoordinate complexes with no contribution from either a distal $H_2O$ or histidine (*25*, *26*, *28*). In contrast, the wild-type forms of these proteins, like PGHS, are suggested to exist as hexacoordinate $Fe^{III}$ species (*26*, *28*, *42*, *43*). In light of the coordination environments of some (but not all) catalases (pentacoordinate ferric hemes) (*44*, *45*), it seems that tyrosine as a proximal ligand tends to disfavor binding of a sixth ligand.

It is possible that structural changes may take place that block the approach of small molecules but still do not allow for formation of a hexacoordinate complex. Such a case has been reported recently for His25Tyr heme oxygenase (*30*). On the basis of high-frequency resonance Raman spectra, Liu et al. (*30*) assign this structure as a high-spin pentacoordinate complex similar to the hemoglobin and myoglobin mutants mentioned above. However, even with an open sixth coordination site, His25Tyr heme oxygenase does not react with $H_2O_2$. This is in stark contrast to the wild-type protein and another heme oxygenase proximal ligand mutant, His25Cys (*30*). These data are similar to our own results with PGHS-2 in which substitution of the proximal ligand yields a protein with limited ability to react with peroxides or ligands such as $CN^-$.

It is interesting to note that although the reactivity of peroxides with His388Tyr is much less than with wild type, the same trend in reactivity is observed with both proteins (i.e., 15-HpETE $\gg$ $H_2O_2$ > *tert*-butyl-OOH). Therefore, fatty acid hydroperoxides are still the preferred substrates for the peroxidase activity despite the His388Tyr substitution. This suggests that structural changes induced by His388Tyr do not eliminate interactions that favor binding of fatty acid hydroperoxides (i.e., long hydrophobic tail, carboxylate group) over that of $H_2O_2$ and small alkyl hydroperoxides. This would seem to indicate that the structural modifications induced by His388Tyr substitution are limited to the immediate vicinity of the heme prosthetic group, an assertion also supported by the ability of the His388Tyr cyclooxygenase to produce prostaglandin products in preference to HETEs or HpETEs.

*Implications of His388Tyr Structure and Activity for PGHS Catalytic Mechanism.* Although PGHS is unique in its ability to catalyze two separate reactions, it is perhaps more interesting to note the complex interaction between the two catalytic cycles. There is general agreement that the radical derivative of Tyr385 is the catalytic oxidant in the cyclooxygenase reaction. Since the resting enzyme does not contain a tyrosyl radical, Tyr385 must be oxidized to activate cyclooxygenase; a derivative of the heme group appears to be the oxidant (*15*, *19*). However, details of the activation mechanism, such as the oxidation state of the heme electron acceptor and the role of the heme following the initial activation event, have been the subject of considerable debate. We believe that the thermodynamic data and characterization of His388Tyr PGHS-2 presented here provide valuable information regarding this unique interaction between PGHS peroxidase and cyclooxygenase activities.

*Heme Oxidation States in Cyclooxygenase Activation*. Proposals have been made that $Fe^{III}$−PGHS or peroxidase intermediates (i.e., $Fe^{IV}$═O and $Fe^{IV}$═O[porphyrin•]) are the oxidants (*19, 20*). Our data seem inconsistent with a role for the $Fe^{III}/Fe^{II}$ in cyclooxygenase activation or catalysis for two main reasons. First, the reduction potential for the $Fe^{III}/Fe^{II}$ transition of PGHS-2 is far too low to participate in cyclooxygenase activation; and second, substitution of a tyrosine for the proximal histidine ligand fails to block cyclooxygenase activity.

The reduction potential for $Fe^{III}/Fe^{II}$ PGHS-2 is well below that necessary for oxidation of a tyrosine, fatty acid hydroperoxide, or arachidonic acid (*46*−*49*). According to all cyclooxygenase activation mechanisms proposed thus far, at least one of these oxidations must be carried out by the PGHS heme. The PGHS-2 reduction potential is similar to that observed with other peroxidases (*50*−*52*) and is consistent with the need of these proteins to stabilize higher oxidation states such as $Fe^{IV}$═O and $Fe^{IV}$═O[porphyrin•] as opposed to the $Fe^{II}$ state. The reduction potentials for the known horseradish peroxidase $Fe^{IV}$═O[porphyrin•]/$Fe^{IV}$═O or $Fe^{IV}$═O/$Fe^{III}$ couples (+950 and +970 mV, respectively) (*53*) are sufficient for oxidation of tyrosine, arachidonate, and a number of hydroperoxides (*46*−*49*). Although reduction potentials for the same couples have not been determined for PGHS, it is clear from the ability of this protein to use phenol and tryptophan as peroxidase reducing substrates (*6, 54, 55*) that the $Fe^{IV}$═O and $Fe^{IV}$═O[porphyrin•] intermediates are sufficiently oxidizing to initiate cyclooxygenase catalysis. The reduction potential of PGHS-2 is also consistent with its isolation in the $Fe^{III}$ state and the apparent inability of the protein to spontaneously convert to the $Fe^{II}$-[Tyr−O•] form. In contrast, lipoxygenase [which is isolated in its $Fe^{II}$ form (*56, 57*)] has an $Fe^{III}/Fe^{II}$ reduction potential (≈+600 mV) close to that required for abstraction of bis-allylic hydrogens from polyunsaturated fatty acids (*48, 58, 59*).

Though no spontaneous conversion of $Fe^{III}$ PGHS to its $Fe^{II}$ form is observed, it is conceivable that a transition in heme potential could take place following major structural changes upon substrate binding. However, our results indicate that binding of molecules in the cylooxygenase active site has little effect on the $Fe^{III}/Fe^{II}$ PGHS reduction potential. Indomethacin binding to wild-type PGHS-2 results in a small positive shift in reduction potential but nothing near that required for cyclooxygenase activation. Similarly, Tyr385Phe PGHS-2 shows no difference in reduction potential in the presence or absence of arachidonate.

The cyclooxygenase activity of His388Tyr PGHS-2 also casts doubt on the involvement of $Fe^{II}$ PGHS in cyclooxygenase catalysis. It is well-documented that substitution of histidine heme ligands with tyrosines results in substantial destabilization of the $Fe^{II}$ state relative to the $Fe^{III}$ state. This has been observed experimentally by either a substantial decrease in reduction potential (*24, 27, 29*) or loss of tyrosine ligation to the heme group upon reduction (*26, 30*). Neither response is compatible with the production of $Fe^{II}$ PGHS during cylooxygenase activation. The inability to form $Fe^{II}$ PGHS would block activation, and it is known from previous work with PGHS-1 His388 mutants that the heme iron must be bound at this position to observe appreciable cyclooxygenase activity (*60*). Our initial studies have not established whether the reduction potential is lowered or the heme dissociates upon reduction; however, dissociation of the heme group seems more likely. PGHS-1 and PGHS-2 bind heme relatively weakly compared with other heme proteins (*9, 61*), and substitution of His388 with tyrosine produces an even weaker interaction with the heme as evidenced by titration of apoprotein with heme. Moreover, upon reduction of His388Tyr, CO readily binds to form the $Fe^{II}$−CO complex. In light of the resistance of the $Fe^{III}$ form to binding of $CN^-$ and other small molecules, it appears that the heme environment is changed markedly upon reduction, which leads us to propose dissociation of the heme from the tyrosinate ligand as the most likely response to reduction.

Taken together, these data do not support a role for reduction of $Fe^{III}$ PGHS in cyclooxygenase activation. The reduction of peroxidase intermediates, on the other hand, is compatible with corresponding oxidations proposed to be involved in the initiation of arachidonate oxygenation to $PGG_2$. The striking effect of the His388Tyr substitution on PGHS-2 peroxidase activity correlates well with the impaired cyclooxygenase activation of the mutant. Moreover, the effect of added hydroperoxides on activation correlates well with the ability of each to act as a substrate for the His388Tyr peroxidase. We suggest that it is highly unlikely that hydroperoxides such as $H_2O_2$ activate His388Tyr by reducing the heme iron.

*Tightly Coupled vs Branched-Chain Mechanism of PGHS Catalysis*. The extent of coupling of the cyclooxygenase to the peroxidase activity of PGHS also has been vigorously debated. The branched-chain mechanism implies minimal coupling because Tyr385 is reoxidized to its radical form with each turnover independent of the heme. Conversely, a tightly coupled mechanism posits that the $PGG_2$ peroxyl radical exits PGHS without regenerating the tyrosyl radical, suggesting that heme-dependent reactivation must take place for oxygenation of each arachidonic acid molecule.

Our results with His388Tyr strongly support the branched-chain mechanism. First, turnover of His388Tyr generates predominantly hydroperoxyprostaglandins, not the corresponding alcohols. This occurs in the presence or absence of an added peroxidase reducing substrate, indicating that peroxides and the peroxidase make a very small contribution to the overall turnover of the cyclooxygenase. The accumulation of hydroperoxides clearly indicates that the continued heme-dependent reoxidation of tyrosine 385 is not required for every turn of the His388Tyr cyclooxygenase cycle because such a model predicts that for each $PGG_2$ molecule produced another $PGG_2$ must be consumed.

According to the tightly coupled mechanism of cyclooxygenase catalysis, a reduced rate of hydroperoxide reduction would reduce the rate of each catalytic cycle. Acceleration of the cyclooxygenase rate with time suggests that the peroxidase is not necessary for continual cyclooxygenase activation following the initial activation event.

At the same time, the lag phase can be dramatically shortened by the addition of exogenous peroxides. The ability of each of these peroxides to stimulate activation corresponds with the ability of each to act as a substrate for the His388Tyr peroxidase. This clearly indicates that although the peroxidase activity is not required for the continued turnover of cyclooxygenase, it is definitely required as an initiator of cyclooxygenase activity.

In conclusion, the relationship between the cyclooxygenase and peroxidase activities of PGHS is complex and much debated. The reliance of both activities on the heme prosthetic group provided us the unique opportunity to probe the interconnection between peroxidase and cyclooxygenase by altering the ligation of the heme. The substitution of the proximal heme ligand, histidine, with tyrosine resulted in a protein with altered properties, allowing us to probe both the peroxidase and cyclooxygenase activities. More importantly, the profound impact of these modifications on PGHS catalysis provides valuable information regarding the intermediates involved in cyclooxygenase activation and the role of these intermediates in the continued synthesis of prostaglandins. Although the contribution of other factors such as peroxidase reducing substrate has not yet been tested with this interesting and informative mutant, we suggest that His388Tyr PGHS and other similar PGHS mutants may provide valuable information regarding the role of these factors in mechanisms of prostaglandin synthesis. Further spectroscopic analysis and structural characterization via EPR, resonance Raman, and other techniques are anticipated to provide additional exciting insight into the mechanism of action of His388Tyr and other axial ligand-substituted mutants of PGHS.

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