Role of Asn-382 and Thr-383 in Activation and Inactivation of Human Prostaglandin H Synthase Cyclooxygenase Catalysis*

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Cyclooxygenase catalysis by prostaglandin H synthase-1 and -2 (PGHS-1 and -2) requires activation of the normally latent enzyme by peroxide-dependent generation of a free radical at Tyr-385 (PGHS-1 numbering) in the cyclooxygenase active site; the Tyr-385 radical has also been linked to self-inactivation processes that impose an ultimate limit on cyclooxygenase catalysis. Cyclooxygenase activation is more resistant to suppression by cytosolic glutathione peroxidase in PGHS-2 than in PGHS-1. This differential response to peroxide scavenging enzymes provides a basis for the differential catalytic regulation of the two PGHS isoforms observed in vivo. We sought to identify structural differences between the isoforms, which could account for the differential cyclooxygenase activation, and used site-directed mutagenesis of recombinant human PGHS-2 to focus on one heme-vicinity residue that diverges between the two isoforms, Thr-383, and an adjacent residue that is conserved between the isoforms, Asn-382. Substitutions of Thr-383 (histidine in most PGHS-1) with histidine or aspartate decreased cyclooxygenase activation efficiency by about 40%, with little effect on cyclooxygenase specific activity or self-inactivation. Substitutions of Asn-382 with alanine, aspartate, or leucine had little effect on the cyclooxygenase specific activity or self-inactivation. Asn-382 and Thr-383 mutations did not appreciably alter the Km value for arachidonate, the cyclooxygenase product profile, or the Tyr-385 radical spectroscopic characteristics, confirming the structural integrity of the cyclooxygenase site. The side chain structures of Asn-382 and Thr-383 in PGHS-2 thus selectively influence two important aspects of cyclooxygenase catalytic regulation: activation by peroxide and self-inactivation.

Prostaglandin H synthase (PGHS, EC 1.14.99.1) catalyzes the first committed step in biosynthesis of prostanooid lipid mediators (1). PGHS has two distinct enzymatic activities, a heme-dependent peroxidase activity and a cyclooxygenase activity. The two reactions are mechanistically connected in that a free radical generated on Tyr-385 during peroxidase catalysis is required for cyclooxygenase catalysis (2). Once activated, cyclooxygenase catalysis in one PGHS molecule produces peroxide, PGG2, which can diffuse to activate the cyclooxygenase in previously latent molecules of PGHS; this results in a powerful feedback activation loop (3). The Tyr-385 radical also participates in self-inactivation processes, which impose an ultimate upper limit on prostanooid production (4, 5).

There are two PGHS isoforms: PGHS-1 is generally considered a housekeeping form whereas PGHS-2 is usually detectable only after induction by cytokines or mitogens (6). Differential cyclooxygenase catalytic control has been observed in many cell types, with “constitutive” PGHS-1 remaining latent despite the presence of abundant substrate, while “inducible” PGHS-2 produces a burst of prostanooid (6). Decreasing cellular peroxide levels by introducing glutathione peroxidase or non-enzymatic peroxide scavengers decreased PGHS-2 cyclooxygenase activity; increasing peroxide levels in the same cells by addition of glutathione-depleting reagents increased PGHS-2 cyclooxygenase activity (7, 8). This established that PGHS-2 cyclooxygenase catalysis could be modulated by peroxide levels in cells where PGHS-1 cyclooxygenase remained latent. In intact fibroblasts, PGHS-1 cyclooxygenase catalysis was found to accelerate more slowly than PGHS-2 cyclooxygenase catalysis, and addition of peroxide-generating reagents increased cyclooxygenase catalysis (9), indicating that the cellular peroxide level can be a key element in differential control of cyclooxygenase catalysis by the two isoforms in vivo. A variety of glutathione peroxidase isozymes, including cytosolic glutathione peroxidase (cGPx), contribute to controlling cellular peroxide levels (10), and titration with cGPx has proven a useful biomimetic approach for quantitative characterization of the regulation of cyclooxygenase activity by peroxide (11, 12). PGHS-1 cyclooxygenase activity is much more readily suppressed by cGPx than is PGHS-2 cyclooxygenase (12, 13), consistent with the observation of active PGHS-2 cyclooxygenase catalysis in many cells where the PGHS-1 cyclooxygenase remains latent (6).

We sought to identify structural differences between the two PGHS isoforms, which could account for the difference in cyclooxygenase activation efficiency, and focused attention on residues in the vicinity of heme and Tyr-385 (Fig. 1). Amino acids in this region are quite highly conserved between the two isoforms, but residue 383 is an exception, appearing uniformly as threonine in archived PGHS-2 sequences and as histidine in PGHS-1 from most species (glutamine in ovine PGHS-1). To examine the effects of side chain structure at residue 383, we
have expressed recombinant human PGHS-2 with various mutations of Thr-383 and characterized the cytochrome P450 and peroxidase kinetics and the sensitivity of the cyclooxygenase to suppression by cGMP. For comparison, we analyzed the effects of mutations in Asn-382, an adjacent residue that is conserved in both PGHS isoforms. The results indicate that structural perturbation of Thr-383 decreases cyclooxygenase activation efficiency in PGHS-2 by about 40%, accounting for almost half the difference with PGHS-1, whereas perturbation of Asn-382 did not change cyclooxygenase activation efficiency but led to a doubling of prostanoid production before self-inactivation.

**EXPERIMENTAL PROCEDURES**

Materials—Arachidonic acid was purchased from NuChek Preps, Inc. Heme, phenol, TMPD, glutathione, glutathione reductase, and cGMP were from Sigma. Tween 20 was purchased from Pierce Chemical Co. Recombinant wild-type and mutant PGHS-2 were expressed in Sf9 cells using a baculovirus vector as described below and reconstituted with excess heme before use (12). PGHS-1 was purified to homogeneity from ovine seminal vesicles as the apoenzyme (14) and reconstituted with excess heme before use (1-19). Arachidonic acid was purchased from Amersham Biosciences.

**Expression of PGHS-2 with Mutations at Asn-382 or Thr-383**—The QuickChange site-directed mutagenesis kit (Stratagene) was used with a template of human PGHS-2 cDNA (a gift from Dr. T. Hla, University of Connecticut Health Center) inserted in pSG5 or pVL1393 plasmids and the following primer pairs: N382A-F, 5′-GTC-TGAATTTAAC-3′; N382D-F, 5′-GGC-TCTATCAC-3′; N382L-F, 5′-GTCATAGAGGGT-3′; N382R-F, 5′-GCTGAAATTGCCACCCTCATAC-3′; N382R-A, 5′-GTGATAGGGGTCAATACTACG-3′; N382D-F, 5′-GTGATAGGGGGTCAATACTACG-3′; N382L-F, 5′-GCTGAAATTGCCACCCTCATAC-3′; N382R-F, 5′-GTGATAGGAGGTGAAATTTAAC-3′; T383D-F, 5′-GTCATAGAGGGTCAATACTACG-3′; T383D-R, 5′-GTCATAGAGGGTCAATACTACG-3′; T383H-F, 5′-GCTGAAATTGCCACCCTCATAC-3′; T383H-R, 5′-GTCATAGAGGGTCAATACTACG-3′. The mutations at Asn-382 or Thr-383 were confirmed by sequencing the purified plasmids. Sequencing the wild-type plasmid revealed some unexpected differences from the published human PGHS-2 nucleotide sequence (15) at four places in the coding region: 4C→G (L2V change in cleaved signal peptide); 20GC→T (P86L change in membrane anchor Helix A); 494G→T (G179E change; this residue is conserved as Glu in most mammalian PGHS-2); and 1227T→C (no change in protein). The cDNA coding for wild-type PGHS-2 or the mutants was then ligated into a pVL1393 transfer vector and used to co-transfect Sf9 cells along with a Baculovirus DNA (BD PharMingen) according to the manufacturer’s recommendations. Sf9 cells were propagated in a spinner flask at 27 °C with Grace’s supplemented medium containing 10% fetal bovine serum. Cells (2 × 10^6) were plated in 60-mm dishes for cotransfection. Working virus stocks were generated by three rounds of amplification of the resulting recombinant virus particles.

**Expression of Recombinant Protein**—Sf9 cells were grown in suspension culture to a density of 1.2–1.7 × 10^6 cells/ml and then infected with high titer recombinant virus at a multiplicity of infection of 2–5. Sf9 cells expressing recombinant PGHS-2 were harvested by centrifugation 3 days after infection and stored at −70 °C. For preparation of the membrane fraction, cell pellets were thawed, suspended in 3 volumes of 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM diethyldithiocarbamate, 1 mM phenol, and homogenized by sonication (five pulses at 20 W with a microprobe tip). The membrane fraction was pelleted by centrifugation at 150,000 × g for 1 h at 4 °C and resuspended with a Dounce homogenizer in 50 mM Tris-HCl, pH 8.0, 0.1 mM NaClO4, 1 mM EDTA, 1 mM phenol. The washed membranes were collected by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenol. Recombinant PGHS-2 was purified by adding 15% (v/v) of saturated sodium sulfate to the supernatant liquid containing solubilized PGHS-2, clarified by passage through a 0.2-μm filter and divided into aliquots for storage at −70 °C. This procedure results in at least a 10-fold purification of the cyclooxygenase activity from the Sf9 cell homogenate and provides a suitably pure preparation of recombinant PGHS-2 suitable for reliable characterization of enzymatic activity, susceptibility to suppression by cGMP and, after buffer exchange to lower the phenol concentration, EPR spectroscopy.

**Protein Characterization**—Protein concentrations were determined using a modified Lowry method (16). For immunoblot analysis, solubilized PGHS-2 samples were separated by electrophoresis under denaturing conditions on a 10% polyacrylamide gel (17) and then transferred onto nitrocellulose membranes in CAPS buffer, pH 11.00, at 4 °C. Nitrocellulose membranes were probed with polyclonal antibody raised against the PGHS-2 C-terminal peptide (Cayman Chemical), and immunoreactive bands were visualized with Opti-4CN reagent (BioRad) following the manufacturer’s protocol. The concentration of recombinant PGHS-2 protein in each extract was determined by dot blot. For this, 1.0-μl aliquots of serial dilutions of the extracts were spotted directly on a nitrocellulose membrane, and the membrane was processed with Opti-4CN reagent as described above. Dot intensities were measured by densitometry using Alpha Image software (Alpha Innotech) and the quantities of recombinant PGHS protein in each dot were calculated by reference to a standard curve constructed with purified PGHS-2 standard samples applied to the same membrane.

**Cyclooxygenase Assay**—Oxygen consumption during the cyclooxygenase reaction was monitored with a Model 53 oxygen electrode system (Yellow Springs Instruments) connected to either a Model ADC-1 A/D converter or a Bird Remote Measurement Systems model, connected to a minitosh computer or to a personalDAQ A/D converter (IOtech) linked to a Gateway Solo laptop (18). The standard reaction buffer (3 ml of 0.1 M potassium phosphate, pH 7.2) contained 100 μM arachidonate, 1 mM phenol, and 1 mM heme and was thermostatted at 30 °C. Reactions were initiated by injection of enzyme. One unit of cyclooxygenase has an optimal velocity of 1 nmol of O_2/min. Cyclooxygenase Km values were determined by measuring activity at 1–60 μM arachidonate and fitting the values to the Michaelis-Menten equation using a nonlinear least squares algorithm and Kaleidagraph software (Synergy Software). Cyclooxygenase self-inactivation rates during catalysis were estimated from plots of the logarithm of oxygenase velocity as a function of time during the deceleration phase of the reaction (19).

**Peroxidase Assay**—Peroxidase activity was routinely measured at room temperature (23 °C) in stirred spectrophotometer cuvettes containing 3 ml of 100 mM Tris-HCl, pH 8.5, 50 mM TMPD, and 1 μM heme. Enzyme was preincubated in the cuvette for about 20 s before injection of H_2O_2 (final concentration of 0.8 mM) to start the reaction. Oxidation of TMPD was monitored at 611 nm using an extinction coefficient of 13.5 (mM oxidized TMPD)−1 cm−1, assuming a stoichiometry of 2.0 mol of TMPD oxidized per mol of hydroperoxide reduced (20). Peroxidase Km values were determined by measuring activity with various peroxide levels and fitting the rates to the Michaelis-Menten equation as described above. Peroxidase Km values were determined by measuring activity at 1.0 M heme in a reaction initiated by injection of 0.5 mM hydrogen peroxide. For each experiment, Km values were determined by measuring activity with various peroxide levels and fitting the rates to the Michaelis-Menten equation as described above.

**Analyses of Oxygenated Arachidonate Products**—The reaction mixture (0.1 μl) contained 100 mM potassium phosphate, pH 7.2, 0.02% Tween 20, 1 μM heme, 1 mM phenol, and 11 μM [1-14C] arachidonate (150 nCi). Aliquots of solubilized recombinant wild-type or mutant PGHS-2 (40 units) were added and after 30 s the reaction was stopped by addition of 0.75 ml of ice-cold ethyl ether-methanol-2% citric acid (30:4:1). After vortexing, the layers were allowed to separate, and the lipid products were recovered in the upper layer; some anhydrous sodium sulfate was added as desiccant. Each extract was treated with 0.5 mM triphenyl phosphate for 30 min at room temperature to convert peroxides to alcohols and then stored at −20 °C. For HPLC analysis, 20 μl of each extract (2000–5000 cpm) was injected on a 0.46 × 15 cm Varian Microsorb-MV-
Asn-382 and Thr-383 Roles in PGHS-2 Catalysis

Computer Simulations of PGHS Reaction Kinetics

Table I
Summary of Kinetic Parameters

| Recombinant protein | Cox sp. act. | Cox Kₐ | Pox sp. act. | Pox Kₐ | µM AA | µM ROOH | µM AA | µM ROOH |
|---------------------|-------------|---------|-------------|--------|-------|---------|-------|---------|
| PGHS-2 wild type    | 6.3         | 2.1 ± 0.4 | 5.2        | 16.3 ± 2.1 | 10.0 ± 2.1 | 1.3 ± 0.3 | 0.08 ± 0.01 |
| N382A               | 5.9         | 4.1 ± 0.6 | 4.0        | ND     | ND    | ND      | ND    | ND      |
| N382D               | 4.8         | 2.9 ± 0.4 | 3.4        | 15.8 ± 3.5 | 55.4 ± 28.8 | >100   | 0.29 ± 0.07 |
| T382D               | 6.0         | 2.1 ± 0.2 | 2.4        | 6.5 ± 0.01 | 5 ± 1.0 | 5 ± 1.0 | 0.9 ± 0.6 |
| T383D               | 7.0         | 2.2 ± 0.3 | 14.6       | 10.0 ± 1.2 | 10.0 ± 1.2 | ND     | ND      |
| T383H               | 7.3         | 3.7 ± 0.6 | 10.1       | 8.3 ± 1.4 | 8.3 ± 1.4 | ND     | ND      |

* With 57 µM guaicol.
* With 2.2 mM HOOH.
* ND, not determined.

[Table I continued...]

RESULTS
Expression of Recombinant Wild-type and Mutant PGHS-2 Proteins—Immunoblot analysis was performed on detergent-solubilized proteins from the membrane fraction of SF9 cells expressing either wild-type human PGHS-2, PGHS-2 with a substitution at Asn-382 (leucine, aspartate, or alanine) or PGHS-2 with a substitutions at Thr-383 (histidine or aspartate). In each case, the major immunoreactive species was a multiplet with an average Mₐ of about 73 kDa (data not shown), similar to previous results (12), thus confirming expression of the full-length recombinant proteins in detergent-extractable form.

Analysis of General Cyclooxygenase and Peroxidase Kinetics in Asn-382 and Thr-383 Mutant Proteins—Cyclooxygenase specific activity values were determined for wild-type PGHS-2 and each of the mutants to assess the effects of the side chain substitutions on overall cyclooxygenase function (Table I). In each case, the cyclooxygenase activity was normalized to the detergent solubilization stage. This value for partially purified PGHS-2 is in the range of 6–42 kilounits/mg protein reported for purified PGHS-2 (6 kilounits/mg (25); 34 kilounits/mg (26); 20–42 kilounits/mg (27); 10–12 kilouns/mg (28); 30 kilouns/mg (29)). For comparison, the average cyclooxygenase specific activity (with 100 µM arachidonate) for the three Asn-382 mutants was 5.7 kilouns/mg, essentially the same as the wild-type, whereas the average of the two Thr-383 mutants was slightly higher, at 7.1 kilouns/mg (Table I). Thus, the cyclooxygenase catalytic rate appears largely insensitive to structural changes at residues 382 and 383.

Cyclooxygenase Kₐ values for arachidonate were determined for the recombinant proteins as a way of detecting perturbations of cyclooxygenase site interactions with substrate fatty acid. Wild-type PGHS-2 cyclooxygenase had a Kₐ value of 2.1 µM arachidonate, essentially the same as the mutations at Thr-383 and the N382L mutant (Table I). Two of the substitutions at Asn-382 (N328A and N328D) did show slight elevations in cyclooxygenase Kₐ value, but these are probably not significant. Thus, side chain substitutions at residues 382 and 383 had little or no effect on cyclooxygenase site substrate interactions.

The peroxygenase specific activity of wild-type PGHS-2 was 5.2 kilouns/mg (Table I). The three Asn-382 mutants peroxygenase activities were somewhat lower, averaging 3.3 kilouns/mg,
whereas the peroxidase activities of the two Thr-383 mutants were noticeably higher than wild-type, averaging 12.3 kilounits/mg. These results point to a significant effect of structural changes at both residues 382 and 383 on peroxidase activity. This is perhaps not surprising, given the proximity of these residues to the heme. The higher peroxidase/cyclooxygenase ratio observed in the Thr-383 mutants (Table I) could conceivably have a suppressive effect on the cyclooxygenase activity by increasing the rate of peroxide removal relative to the rate of peroxide generation. However, the 2-fold increase in endogenous peroxide-scavenging activity observed here are modest compared with the 10-fold excess of aspirin-treated PGHS-1 peroxidase scavenger needed to inhibit PGHS-1 cyclooxygenase activity (30) and is therefore unlikely to have a significant effect on the PGHS-2 cyclooxygenase.

Peroxidase $K_m$ values with PPHP as substrate for the mutants were comparable to those of wild-type PGHS-2 except in the case of N382L, where the $K_m$ values was about 5-fold higher (Table I). Elevated $K_m$ values were also obtained with two other substrates, HOOH and 15-HPETE. The elevated $K_m$ values may explain the lower specific activity of the N382L mutant, because specific activity measurements were done at a fixed peroxide level considerably less than saturating for that particular mutant. $K_m$ values in steady-state PGHS peroxidase reactions reflect the efficiency of interactions with both substrate peroxide and with reducing cosubstrate, with increases in the substrate level increasing the cosubstrate $K_m$ value and vice versa (31). The observation that $K_m$ values were comparably increased for both a small water soluble peroxide and hydrophobic peroxides indicates that the N382L substitution led to an enhanced reaction with cosubstrate, requiring more peroxide to reach saturation. This was confirmed by measurements of the peroxidase $K_m$ for cosubstrate, which showed a 4-fold higher $K_m$ value for guaiacol with the N382L mutant than with wild-type PGHS-2 (Table I).

Analysis of Cyclooxygenase Products in Recombinant Asn-382 and Thr-383 Mutant Proteins—The cyclooxygenase product profile is sensitive to structural changes in the cyclooxygenase site, with both side chain substitutions and chemical modifications in the cyclooxygenase pocket capable of shifting fatty acid oxygenation products of both PGHS-1 and -2 from prostaglandins to non-cyclized hydroperoxy fatty acids (32, 33). To further assess the possibility that substitutions at Asn-382 and Thr-383 have long distance effects on cyclooxygenase pocket structure, we examined the product profiles of wild-type PGHS-2 with the Asn-382 and Thr-383 mutants (Fig. 2). Two major arachidonate metabolite peaks were observed when wild-type PGHS-2 was reacted with arachidonate. The first major peak, at 3.7 min, co-eluted with standard prostaglandins. The second major peak, at 14.5 min, co-eluted with 12-hydroxyeicosatetraenoic acid, which forms by PGHS-catalyzed breakdown of prostaglandin endoperoxides (13). Unreacted arachidonate eluted at 30.8 min. A very similar pattern of metabolites was observed for each of the Asn-382 and Thr-383 mutants, although the extent of decomposition to 12-hydroxyeicosatetraenoic acid varied (Fig. 2). In particular, there was no indication of significant increases in formation of 11-HETE or 15-HETE by any of the mutants, indicating that none of these mutations perturbed the structure of the cyclooxygenase pocket.

Effects of Asn-382 and Thr-383 Mutations on Cyclooxygenase Activation Efficiency—Cyclooxygenase activation efficiency of the wild-type and mutant PGHS-2 was assessed in titrations with a peroxide scavenger enzyme, cGPx. In these titrations, the cyclooxygenase velocity produced by a fixed number of cyclooxygenase units is determined in the presence of a variable amount of cGPx. Reactions with a higher cGPx/cyclooxygenase ratio have more complete interception of activator peroxide (PGG2) keeping a larger fraction of the cyclooxygenase in the latent state (3).

For wild-type PGHS-2, the observed cyclooxygenase activity declined linearly as the cGPx/cyclooxygenase ratio was increased, with the line extrapolating to a ratio of about 500 for complete suppression of the cyclooxygenase activity (Fig. 4). In experiments with the same batch of cGPx, the cyclooxygenase activity of PGHS-1 was much more easily suppressed, with all of the cyclooxygenase remaining in the latent state at a cGPx/cyclooxygenase ratio of only 65 (Fig. 4). The higher resistance of PGHS-2 cyclooxygenase to suppression by cGPx compared with
PGHS-2 cyclooxygenase confirms earlier observations and is a consequence of the greater cyclooxygenase activation efficiency in PGHS-2 (12, 13).

To simplify quantitative comparisons of cyclooxygenase activation efficiency among PGHS-2 and the several mutants, the slope of the line fitted to the cGPx titration data for each PGHS-2 construct being tested was divided into the corresponding slope obtained with PGHS-1, producing a “cGPx Resistance Index” value (Table III). For wild-type PGHS-2, the value of the index was 6.2. For the two Thr-383 mutants, the cyclooxygenase was about 40% less resistant to suppression by cGPx (Fig. 4), with index values of 3.6 and 3.8 (Table III). Thus, almost half of the difference between PGHS-2 and -1 in terms of their regulation by the peroxide scavenger was reversed by mutation of Thr-383, a residue that diverges between the isoforms. In contrast, all three constructs with substitution at the adjacent conserved residue, Asn-382, responded much like wild-type PGHS-2, with index values of 7.1–10.0 (Table III).

Effects of Asn-382 and Thr-383 Mutations on Peroxide-induced Radicals—A peroxide-induced Tyr-385 radical has key roles in PGHS catalysis and self-inactivation (2). Intense tyrosyl radical EPR signals were observed when wild-type PGHS-2 and the N382L and T383H mutants were reacted with ethyl peroxide (Fig. 5). In each case the radical was of the “wide singlet” variety previously reported for wild-type PGHS-2 (2), centered at $g = 2.003$, with overall peak-trough widths of 29 G. These results indicate that mutation at either Asn-382 or Thr-383 had a negligible effect on the structure of the Tyr-385 radical in the cyclooxygenase active site.

**TABLE III**

| Recombinant protein | cGPx resistance index | cGPx resistance index |
|---------------------|-----------------------|-----------------------|
| PGHS-2 wild type    | 6.2                   |                       |
| N382A               | 7.1                   |                       |
| N382D               | 7.1                   |                       |
| N382L               | 10.0                  |                       |
| T383D               | 3.8                   |                       |
| T383H               | 3.6                   |                       |

* Slope of line fitted to cGPx titration data for PGHS-1 standard divided by corresponding slope for wild-type or mutant PGHS-2.

**DISCUSSION**

**Understanding Differential Catalytic Regulation at the Structural Level**—Differential control of PGHS-1 and -2 cyclooxygenase catalysis has emerged as a key feature of regulation of eicosanoid synthesis in cells having both isoforms (6, 34, 35). One biochemical explanation for this differential catalytic control is the more efficient cyclooxygenase activation by peroxide in PGHS-2, which makes cyclooxygenase catalysis by PGHS-2 much more resistant to peroxide scavenging enzymes such as cGPx (12, 13). The kinetic basis for more efficient cyclooxygenase activation in PGHS-2 has been identified as the more rapid formation and slower dissipation of the Tyr-385 radical which initiates oxygenation of fatty acid substrate (22, 36). The present results indicate that the side chain structure at residue 383, which diverges between the isoforms, accounts for a large portion of the differential cyclooxygenase activation, thus extending understanding of the catalytic regulatory mechanism to a structural level. It is interesting that residue 383 is conserved as a threonine in all data base sequences for PGHS-2, whereas in PGHS-1 the residue appears as histidine or glutamine. This suggests that the constraints on side chain structure at residue 383 are more rapid formation and slower dissipation of the Tyr-385 radical which initiates oxygenation of fatty acid substrate (22, 36). The present results indicate that the side chain structure at residue 383, which diverges between the isoforms, accounts for a large portion of the differential cyclooxygenase activation, thus extending understanding of the catalytic regulatory mechanism to a structural level. 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The values in parentheses are the radical intensities in spin/wild-type PGHS-2 and PGHS-2 with N382L and T383H mutants (Table I), indicating that the cyclooxygenase activation by peroxide can also be impaired by cutting the rate of the cyclooxygenase reaction itself (k₄ in Fig. 6), as can be seen when eicosapentaenoic acid is used in place of arachidonate for reactions with PGHS-1 (39). This is not the case with the present PGHS-2 Thr-383 mutants, which have minimal effects on the cyclooxygenase specific activity and Kₘ for arachidonate (Table I) and on the product profile (Fig. 2). The observed minimal effect of Thr-383 mutations on arachidonate Kₘ is consistent with the lack of contacts between this side chain and fatty acid in the PGHS-2 crystal structure (40). The present Thr-383 mutations are thus the first instances of a single substitution leading to a marked change in the regulation of cyclooxygenase catalysis by peroxide without drastic changes in cyclooxygenase or peroxidase behavior under V₉₉₉₉ conditions.

Asn-382 Structure Affects Cyclooxygenase Self-inactivation, Not Activation—Results from substitutions of Asn-382, which unlike the neighboring Thr-383 is conserved between the two PGHS isoforms, were both surprising and informative. The lack of effect of Asn-382 mutations on susceptibility to GPx indicate that the side chain structure had no effect on the efficiency of tyrosyl radical formation and, as noted above, argues against electron transfer from Tyr-385 to heme via the 385–382 polypeptide backbone and the 382 side chain. However, the near doubling of overall cyclooxygenase catalytic capacity seen with the Asn-382 mutants indicates that the 382 side chain structure does have considerable influence on deconstructive side reactions associated with self-inactivation.

Schematic Representation of the Effects of Thr-383 Mutations on the Catalytic Rate of PGHS-2

The k₂ step is an intramolecular redox reaction involving electron transfer from Tyr-385 to the oxidized heme (Fig. 6), and efficient electron transfer may require conformational changes in the protein to optimally align components of the transfer pathway. Mutations at Thr-383 might thus affect k₂ either directly (if Thr-383 is a component of the transfer pathway) or indirectly by impeding a necessary conformational shift. Modification of side chain structure can alter electron transfer rates along the backbone by a factor of two in peptide models (37), and so modification of the Thr-383 side chain in PGHS-2 might conceivably slow down electron transfer from Tyr-385 along the backbone to Asn-382 and the heme (Fig. 1). However, mutation of Asn-382 to a larger leucine, to an isosteric but positively charged aspartate, or to a smaller alanine had minimal effects on the PGHS-2 cyclooxygenase activation efficiency (Table III), indicating that the side chain structure of Asn-382 has little effect on Tyr-385 radical formation. The underlying mechanism for any effect of Thr-383 side chain structure on the k₂ step thus remains unclear.

The k₄ step is a bimolecular reaction between the Tyr-385 radical and reducing cosubstrate. This step is part of a potential peroxidase catalytic cycle (comprising steps 1, 2, 6, and 4). The increased peroxidase specific activity in both Thr-383 mutants (Table I) is thus consistent with an increase in the k₄ rate, although an accompanying increase in the peroxidase Kₘ value was not observed.

Overall, the effects of the Thr-383 mutations on cyclooxygenase activation efficiency seem most likely to be due to indirect effects either decreasing the efficiency of electron transfer from Tyr-385 to the heme (k₂ in Fig. 6) along another path not involving Thr-383 itself or increasing the rate of electron transfer from reducing cosubstrates to the Tyr-385 radical (k₄ in Fig. 6).

Other Mechanisms for Decreasing Cyclooxygenase Activation Efficiency—Two other PGHS-2 mutants, H207A, and H388Y, have decreased cyclooxygenase activation efficiency, manifested by prolonged lag phases in cyclooxygenase kinetics (28, 38). Both of these mutants also show greatly decreased peroxidase activity, suggesting a defect in their initial reaction with peroxide (k₁ in Fig. 6) is responsible for the weaker cyclooxygenase activation. The Thr-383 mutants examined here, in contrast, have somewhat higher peroxidase activities than wild-type PGHS-2 (Table I), indicating that the cyclooxygenase activation is being affected at a step other than k₁. Cyclooxygenase activation by peroxide can also be impaired by cutting the rate of the cyclooxygenase reaction itself (k₄ in Fig. 6), as can be seen when eicosapentaenoic acid is used in place of arachidonate for reactions with PGHS-1 (39). This is not the case with the present PGHS-2 Thr-383 mutants, which have minimal effects on the cyclooxygenase specific activity and Kₘ for arachidonate (Table I) and on the product profile (Fig. 2). The observed minimal effect of Thr-383 mutations on arachidonate Kₘ is consistent with the lack of contacts between this side chain and fatty acid in the PGHS-2 crystal structure (40). The present Thr-383 mutations are thus the first instances of a single substitution leading to a marked change in the regulation of cyclooxygenase catalysis by peroxide without drastic changes in cyclooxygenase or peroxidase behavior under V₉₉₉₉ conditions.
cyclooxygenase site damage can occur independently of peroxi-
dase site damage during cyclooxygenase catalysis. The two
distinct modes of damage proposed are represented by the $k_7$
(cyclooxygenase site damage) and $k_8$ (peroxidase site damage)
steps in the mechanism in Fig. 6. The observed ability of
peroxidase activity to survive cyclooxygenase inactivation is
recognized in the mechanism shown in Fig. 6 by the postulated

\[ k_7 \text{ (cyclooxygenase site damage) and } k_8 \text{ (peroxidase site damage) } \]

\[
\begin{array}{c}
\text{E(IV)/PPIX/Tyr} \\
\text{E(III)/PPIX/Tyr} \\
\text{EIV/PPIX*/Tyr} \\
\text{E(III)/PPIX/Tyr*} \\
\text{E(IV)/PPIX/Tyr} \\
\end{array}
\]

\[
\begin{array}{c}
k_f \text{AH} \\
k_g \text{ROOH} \\
k_d \text{AH} \\
k_3 \text{ROOH} \\
k_2 \text{AH} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Plastyl} \\
\text{Ferriyl} \\
\text{Ferryl} \\
\text{Perferryl} \\
\text{Ferric} \\
\end{array}
\]

Fig. 6. Proposed mechanism for peroxidase and cyclooxygenase catalysis by PGHS. The mechanism has been adapted from earlier versions (18, 22) to specify damage to the cyclooxygenase via $k_7$ and to the peroxidase via $k_8$. Details are described under “Experimental Procedures.”

| $k_7$ | cGPx titration slope | Cycloox velocity | Cycloox extent* |
|-------|----------------------|------------------|-----------------|
| 3500  | 2.8 x 10^{-2}        | 0.56             | 10.04           |
| 1750  | 4.8 x 10^{-2}        | 0.56             | 10.03           |
| 875   | 6.7 x 10^{-2}        | 0.56             | 10.01           |
| 350   | 17.2 x 10^{-2}       | 0.55             | 9.97            |

* Predicted arachidonate consumption for 10 nM PGHS after self-
inactivation of the cyclooxygenase activity.

| $k_8$ | cGPx titration slope | Cycloox velocity | Cycloox extent* |
|-------|----------------------|------------------|-----------------|
| 50    | 2.9 x 10^{-3}        | 0.56             | 10.04           |
| 100   | 3.6 x 10^{-3}        | 0.55             | 10.02           |
| 200   | 5.2 x 10^{-3}        | 0.55             | 9.99            |
| 500   | 9.3 x 10^{-3}        | 0.54             | 9.91            |
| 1000  | 15.4 x 10^{-3}       | 0.55             | 9.76            |

* Predicted arachidonate consumption for 10 nM PGHS after self-
inactivation of the cyclooxygenase activity.
peroxidase catalytic cycle involving only the heme in protein with damage to Tyr-385 or its vicinity. Peroxide (PGG₃) is generated during reaction of PGHS with arachidonic acid, but its accumulation is transient and can be expected to peak only near 1 µM when cosubstrate is present (20); these levels are below those used for examination of peroxide-driven inactivation (5). Another possible factor decreasing peroxidase inactivation during reaction with arachidonate and reducing cosubstrate is that both fatty acid and cosubstrate decrease accumulation of Intermediate II, thereby decreasing the rate of peroxidase inactivation from this species.

Possible Mechanisms for Decreased Self-inactivation in Asn-382 Mutants—All three of the Asn-382 mutants showed a marked decrease in the rate of cyclooxygenase self-inactivation (Table II). The slower self-inactivation in these mutations was not accompanied by substantial changes in the cyclooxygenase acceleration (Fig. 3), suggesting that the rate of Tyr-385 radical formation (k₇ in Fig. 6) is similar to that in wild-type PGHS-2. Indeed, computer simulations predict that the cyclooxygenase reaction extent (reflecting self-inactivation) is insensitive to changes in the magnitude of k₂ (Table IV). The reaction extent is also predicted to be rather insensitive to changes in the value of k₇ (Table V) and k₉ (data not shown). As expected, changes in the value of k₇ are predicted to dramatically affect the cyclooxygenase self-inactivation kinetics (Table VI). A k₇ value of 0.05 s⁻¹ predicts an inactivation rate of 3.8 min⁻¹, similar to the value of 3.2 min⁻¹ observed for wild-type PGHS-2, and decreasing the k₇ value to 0.01 s⁻¹ is sufficient to account for the slower cyclooxygenase inactivation in the N382L mutant (Tables II and VI). Alterations in the k₇ value in this range are predicted to have little effect on the cyclooxygenase velocity (Table VI), in accord with observations with the Asn-382 mutants (Table I). Interestingly, decreasing the k₇ value is predicted to decrease the slope in a cGPx titration (Table VI), indicating an increased resistance to cGPx inhibition, and the Asn-382 mutants (especially N382L) did show higher cGPx resistance indices than did wild-type PGHS-2 (Table III). This analysis suggests that mutations at Asn-382 slow down the rate of cyclooxygenase site damage during catalysis without perturbing the generation or reduction of the Tyr-385 radical.

Other PGHS Mutations Affecting Cyclooxygenase Self-inactivation—Cyclooxygenase self-inactivation can be decreased by mutations at residues other than Asn382. These include a H386A mutation in PGHS-1 (46) and H207A and H388Y mutants in PGHS-2 (28, 38). As mentioned above, each of these mutants also shows greatly diminished peroxidase activity and signs of impaired cyclooxygenase activation by peroxide (prolonged cyclooxygenase kinetic lags), neither of which is evident in any of the Asn-382 mutants (Fig. 3 and Table I). Modification of the Asn-382 side chain structure thus perturbs a very different aspect of cyclooxygenase self-inactivation than the earlier mutants. The Asn-382 mutants thus should be useful probes in further characterization of the cyclooxygenase inactivation process.

Summary—The results identify the Thr-383 side chain structure as a selective determinant of cyclooxygenase feedforward activation efficiency and identify the wild-type structure of Asn-382 as a selective determinant of cyclooxygenase self-inactivation characteristics. Both residues are in the vicinity of the key redox centers in the protein, namely heme and Tyr-385. The decreased cyclooxygenase activation efficiency when Thr-383 in PGHS-2 is modified indicates that the structural divergence at this residue can account for a large part of the difference in cyclooxygenase catalytic regulation between the two PGHS isoforms.

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