Human Cyclooxygenase-2 Is a Sequence Homodimer That Functions as a Conformational Heterodimer

Liang Dong*, Alex J. Vecchio+, Narayan P. Sharma*, Brice J. Jurban*, Michael G. Malkowski+, and William L. Smith* ¶

*Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109 USA
+ Hauptman-Woodward Medical Research Institute and Department of Structural Biology, The State University of New York at Buffalo, Buffalo, NY 14203 USA

Running Title: Allosteric Regulation of Cyclooxygenase-2

Key words: COX-1, COX-2, prostaglandin endoperoxide H synthase, NSAID, half-of-sites, arachidonic acid, palmitic acid, diet, fatty acids, coxib

¶ Corresponding author: William L. Smith, Department of Biological Chemistry, University of Michigan Medical School, 5301 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0606. Tel: (734) 647-6180; Fax: (734) 763-4581, E-mail: smithww@umich.edu

Prostaglandin endoperoxide H synthases (PGHSs)-1 and -2, also known as cyclooxygenases (COXs)-1 and -2, convert arachidonic acid (AA) to prostaglandin endoperoxide H2. PGHSs are targets of nonspecific nonsteroidal anti-inflammatory drugs and COX-2 specific inhibitors called coxibs. PGHS-2 is a sequence homodimer. Each monomer has a peroxidase and a COX active site. We find that human PGHS-2 functions as a conformational heterodimer having a catalytic monomer (E\text{cat}) and an allosteric monomer (E\text{allo}). Heme binds tightly only to the peroxidase site of E\text{cat} while substrates, as well as certain inhibitors (e.g. celecoxib), bind the COX site of E\text{cat}. E\text{cat} is regulated by E\text{allo} in a manner dependent on what ligand is bound to E\text{allo}. Substrate and non-substrate fatty acids (FAs) and some COX inhibitors (e.g. naproxen) preferentially bind to the COX site of E\text{allo}. AA can bind to E\text{cat} and E\text{allo}, but the affinity of AA for E\text{allo} is 25 times that for E\text{cat}. Palmitic acid, an efficacious stimulator of huPGHS-2, binds only E\text{allo} in palmitic acid/murine PGHS-2 co-crystals. Non-substrate FAs can potentiate or attenuate actions of COX inhibitors depending on the FA and whether the inhibitor binds E\text{cat} or E\text{allo}. Our studies suggest that the concentration and composition of the free FA pool in the environment in which PGHS-2 functions in cells—the FA tone—is a key factor regulating PGHS-2 activity and its responses to COX inhibitors. We suggest that differences in FA tone occurring with different diets will likely affect both baseline prostanoid synthesis and responses to COX inhibitors.

Prostaglandin endoperoxide H synthases (PGHS\textsuperscript{1}) also known generically as cyclooxygenases (COXs) convert arachidonic acid (AA) to prostaglandin H\textsubscript{2} (PGH\textsubscript{2}) in the committed step of prostanoid biosynthesis (1-6). PGHS-1 and PGHS-2 are products of different genes. In general, PGHS-1 is constitutively expressed while PGHS-2 expression is inducible (4,5,7). The enzymes are embedded in the luminal monolayer of the endoplasmic reticulum and inner membrane of the nuclear envelope (8-11).

PGHSs catalyze two different reactions: a cyclooxygenase (COX) reaction and a peroxidase (POX) reaction. COX catalysis begins with abstraction of the 13-pro-S hydrogen from AA by the enzyme in the rate-determining step to generate an arachidonyl radical (3,6,12,13). Two
molecules of O₂ are then sequentially added to the arachidonyl chain with concomitant rearrangements to form PGG₂. PGG₂ can be reduced to PGH₂ by the POX activity. The purified isoforms are about equally efficient in catalyzing the conversion of AA to PGH₂ (1-6).

PGHSs are homodimers comprised of tightly associated monomers with identical sequences (14). Each monomer comprising a PGHS homodimer has a physically distinct COX and POX active site. Dissociation of the dimers into monomers only occurs upon denaturation (14). Kulmaez and coworkers provided the first evidence that the monomers of PGHS homodimers differ (15,16). They found that maximal COX activity of ovine (ov) PGHS-1 occurred with one heme per dimer (15) and that flurbiprofen and some other nonspecific nonsteroidal anti-inflammatory drugs (nsNSAIDs) inhibit PGHS-1 at a stoichiometry of one nsNSAID per dimer (16).

More recent studies of both PGHS-1 and PGHS-2 using various fatty acids (FAs) and COX inhibitors have indicated that PGHSs function as conformational heterodimers during catalysis and inhibition (17-21). PGHS-2 and probably PGHS-1 exhibits half of sites COX activity with AA; that is, the COX active site of only one monomer of a PGHS-2 dimer can function at any given time (17). There is also evidence that the enzyme functions as an allosteric/catalytic couple in which one COX active site has a catalytic function that is modulated by the nature of the ligand occupying the COX site of the partner, allosteric monomer (17,19,21-23).

In the present study, we examined the functioning of the human (hu) PGHS-2 conformational heterodimer by quantifying interactions involving huPGHS-2 and heme and different FAs and COX inhibitors. The data are consistent with a heme being bound to the POX active site of only one monomer—the catalytic monomer—of the huPGHS-2 dimer. Additional studies with FAs and COX inhibitors indicate that substrate FAs and one subset of COX inhibitors bind to the COX site of the catalytic monomer and that both substrate and non-substrate FAs and a second subset of COX inhibitors bind to the COX site of the allosteric monomer. The results of examining the interplay between FAs and COX inhibitors leads us to speculate that there can be important dietary effects on both baseline PG formation and responses to COX inhibitors. Diets leading to accumulation of different types of FAs in tissue have the potential to either potentiate or attenuate responses to COX inhibitors, depending on the diet and the inhibitor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonic acid (AA), oleic acid (18:1ω9), palmitoleic acid (16:1ω7), elaidic acid (18:1ω9t), 15-hydroxyeicosatetraenoic acid and 12-hydroxyheptadecaenoic acid were purchased from Cayman Chemical Co. Lauric acid (12:0), myristic acid (15:0), pentadecanoic acid (16:0), margaric acid (17:0), stearic acid (18:0), nonadecylic acid (19:0), 11-cis-eicosaenoic acid (20:1ω9), FLAG peptide and FLAG-affinity resin were from Sigma. Hemin was from Frontier Scientific, Logan, UT. Palmitelaidic acid (16:1ω7t) was from MP Biomedicals LLC. Celebrex® (celecoxib) and Vioxx® (rofecoxib) were physician’s samples. Flurbiprofen, indomethacin, aspirin and naproxen were from Sigma. Ibuprofen was from Tocris Bioscience. Co³⁺-protoporphyrin IX was purchased from Frontier Scientific (Logan, UT). Decyl maltoside (C₁₀M), n-octyl β-D-glucopyranoside (β-OG) and C₁₀E₆ were purchased from Anatrace (Maumee, OH). BCA protein reagent was from Pierce. Polycrylic acid (sodium salt) 5100 was from Hampton Research Corp. [1-¹⁴C]-AA (1.85 GBq/mmol) was from American Radiolabeled Chemicals. Hexane, isopropanol, and acetic acid were HPLC grade from Thermo Fisher Scientific, Inc. Complete protease inhibitor was from Roche Applied Science. Restriction enzymes were from New England Biolabs, Inc. Nickel-nitrilotriacetic acid was from Qiagen. All other materials were analytical grade from Sigma.

**Expression, purification and assay of huPGHS-2**—Procedures for the expression of huPGHS-2 in insect cells and purification of the enzyme were essentially the same as those reported previously (17,24,25). The purity of the recombinant huPGHS-2 was determined by SDS-PAGE and by Western blot analysis (24). The average specific activity of purified huPGHS-2 for the preparations used in the experiments reported here was 40 units/mg.
COX activity was measured polarographically at 37°C in glass chambers containing 3 ml of 0.1 M Tris-HCl, pH 8.0, 100 µM AA, 1 mM phenol, and 5 µM hematin. A Yellow Springs Instruments Model 53 oxygen monitor was used to monitor O₂ consumption and kinetic traces recorded using DasyLab (DasyTec) software (24). Reactions were initiated by adding enzyme to the assay chamber unless otherwise indicated. In experiments involving COX inhibitors, the enzyme preparations were typically preincubated with the inhibitor plus or minus a FA and an aliquot added to the reaction chamber. The rates reported are maximal rates occurring after a lag phase. Under all the assay conditions reported here, the maximal rates are linearly related to the enzyme concentration. The lag time is defined as the time required for the COX activity to reach a maximum after initiating the reaction, which under optimal conditions was about 10 sec. One unit of COX activity is defined as 1 µmole of O₂ consumed/min at 37°C in the assay standard mixture. A representative tracing obtained with a low concentration of AA is shown in Supplementary Fig. S1.

COX assays performed at high enzyme/substrate ratios were conducted in a 100 µl reaction mixture containing 1 µM [1-¹⁴C]-AA, 0.10-2.0 µM of huPGHS-2, 5 µM hematin, and 1 mM phenol in 0.1 M Tris-HCl, pH 8.0, at 37°C. The reactions were quenched with 100 µl of stop buffer consisting of ethyl ether/acetic acid (95:5). The resultant samples were centrifuged at room temperature for 5 min at 4000 x g. An aliquot of the organic layer (100 µl) was subjected to HPLC on a Luna C18 (2) column (5 µm, 250 × 4.6 mm, Phenomenex) mounted on a Shimadzu HPLC system equipped with Radio-HPLC detector (IN/US system, ß-RAM model 4). The bound products were eluted in a gradient elution mode eluting with Solution A (acetonitrile:water; 30:70) for five min then with a linear gradient of Solution A and Solution B (acetonitrile:water; 90:10) for 15 min, then Solution B for 8 min and finally Solution A for 4 min; Solutions A and B both contained 0.1% acetic acid (v/v). The flow rate was 1 ml/min. Representative elution times for PGH₂, 17-hydroxyheptadecatrienoic acid and AA are 14, 18 and 30 min, respectively.

Titration of huPGHS-2 with heme. Difference absorption spectra of apo-huPGHS-2 titrated with heme were obtained using a Shimadzu UV-2501 PC scanning spectrophotometer. Aliquots of a 200 µM hematin solution in 20 mM Tris-HCl, pH 8.3 containing 40 mM KCl, 0.1% C₁₀E₆ and 2% DMSO were added to a quartz cuvette containing 200 µl of 5 µM apo-huPGHS-2 in the same buffer solution without DMSO at room temperature (26,27). The increase in absorbance at 408 nm was plotted in a linear form using the equation I₀/α = [K₀/(1-α) + e₀] where I₀ is the concentration of added heme; α, the fraction of enzyme sites occupied by heme, is given by α = Δ/Δmax where Δ is the optical change measured (27); in this plot, the slope is equal to the Kₐ for heme binding and the intercept (e₀) represents the concentration of heme binding sites. Titrations were also performed in the presence of palmitic acid and several COX inhibitors.

Crystallography. Recombinant His₆ N580A murine (mu) PGHS-2 was overexpressed in insect cells and purified as described previously (23). For crystallization, apo His₆ N580A muPGHS-2 was reconstituted with a two-fold molar excess of Co³⁺-protoporphyrin IX, followed by dialysis against 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.6% (w/v) β-OG for 16 hours at 4°C. A ten-fold molar excess of PA was added to the Co³⁺-protoporphyrin IX reconstituted protein just prior to crystallization screening. Crystals were produced utilizing the sitting-drop vapor diffusion technique by combining 3 µL of protein solution with 3 µL of 23-34% polyacrylic acid 5100, 100 mM HEPES, pH 7.5, 20 mM MgCl₂ and 0.6% (w/v) β-OG and equilibrating the resulting drop against reservoir solutions comprised of 23-34% polyacrylic acid 5100, 100 mM HEPES, pH 7.5, 20 mM MgCl₂ and 0.6% (w/v) β-OG supplemented with 10% ethylene glycol for cryoprotection 15-30 min prior to data collection. Data were collected on beamline A1 at the Cornell High Energy Synchrotron Source (Ithaca, NY) using an Area Detector Systems CCD Quantum-210 detector. The data were integrated and scaled using MOSFLM and SCALA, respectively, in the CCP4 suite of programs (28). Details of the data collection statistics are summarized in Table 1.
Protocols implemented during the structure solution of muPGHS-2 complexed with fatty acid substrates (23) were utilized to minimize model bias. Specifically, initial phases for the muPGHS-2/PA co-crystal structure were determined by molecular replacement methods using PHASER (29). The phases were then input into ARP/wARP (30) to build most of the deleted portions of the model. Iterative cycles of manual model building and refinement using COOT (31) and REFMAC5 (32) were then carried out to fit the remaining residues, add waters, PA, and other ligand molecules. The final model includes two monomers in the crystallographic asymmetric unit comprised of protein residues 33-582 in monomer A and residues 33-583 in monomer B, Co<sup>3+</sup>-protoporphyrin IX, N-linked carbohydrates and a β-OG detergent molecule. Minimized coordinates and stereochemical dictionary for PA were generated using the PRODRG server (33). TLS refinement was carried out during the final rounds of refinement (34). Final refinement statistics are summarized in Table 1.

To verify that PA bound with its carboxylate interacting with Arg-120 in only one monomer of the murine PGHS-2/PA co-crystal structure, we performed the following experiments. In the first test, PA was modeled into the COX channel of monomer A such that the carboxylate interacted with Tyr-385 and Ser-530. This conformation is similar to the nonproductive conformations of AA and EPA observed in monomer A of the co-crystal structures of muPGHS-2/AA and muPGHS-2/EPA (23). Cycles of REFMAC5 refinement were performed, followed by calculation of 2F_O-F_C and F_O-F_C electron density maps. Inspection of the resulting electron density maps clearly showed negative difference density around the improperly placed carboxylate of PA at the apex of the channel and excess positive difference density around the ω-end of PA near the side chain of Arg-120. In the second test, we placed PA into the COX channel of monomer B and modeled ethylene glycol into the COX channel of monomer A of the muPGHS-2/PA co-crystal structure. After REFMAC refinement and generation of electron density maps, there were negative density peaks around the carboxylate and ω-end of PA. Similarly, there were excess positive difference density peaks within the channel of monomer A that were not accounted for by the ethylene glycol molecules. Finally, in the third test, we modeled both PA and ethylene glycol molecules into the COX channel of monomer A and assigned occupancies of 50% to each. REFMAC5 refinement followed by electron density map calculations clearly showed positive electron density peaks for the ω-end of PA near the apex of the channel and for carbons 3-5 near the opening of the channel. Hence, these tests support the modeling of PA into monomer A only such that the carboxylate lies near the opening of the cyclooxygenase channel near the side chain of Arg-120.

Structural analysis. van der Waals and hydrogen bond interactions were calculated using the program COOT. The upper limit on distance for consideration as a Van der Waals contact is 4.0 Å. Superposition of coordinates between structures was done using the program LSQAB within the CCP4 suite of programs and the coordinates for all C<sub>α</sub> atoms unless otherwise stated. Simulated annealing omit maps were calculated using CNS (35). Model validation was performed using MOLPROBITY (36). Fig. 6 was created in CCP4MG (37). The coordinates and structure factors for Co<sup>3+</sup>-protoporphyrin IX reconstituted muPGHS-2 in complex with PA have been deposited in the Protein Data Bank (PDB entry 3QH0).

RESULTS

Stoichiometry of heme binding to huPGHS-2. Titration of apo-huPGHS-2 with hematin yielded a major UV/VIS spectral peak with a λ<sub>max</sub> at 408 nm indicative of formation of a heme/PGHS-2 complex (15,38) (Fig. 1A). The data are consistent with one high affinity heme binding site per PGHS-2 dimer (i.e. 0.92 moles heme/mole dimer) with a K<sub>d</sub> = 88 ± 17 nM (n = 6) and a non-saturable lower affinity site(s) (Figs. 1B and 1C). The non-saturable site(s) may represent lower affinity binding to the hexahistidine tag on huPGHS-2 and/or other non-specific hydrophobic sites on the protein.

We presume that at least the higher affinity site represents binding of heme to a POX site of huPGHS-2. Heme titrations of huPGHS-2 were also performed in the presence of 20 μM palmitic...
acid (PA), 25 µM celecoxib, 50 µM naproxen, a combination of 20 µM palmitic acid and 25 µM celecoxib, and 1.0 and 2.5 mM aspirin. The $K_d$ values for heme binding to huPGHS-2 were essentially the same in the presence of PA, celecoxib, naproxen or aspirin (Tables S1 and S2).

In parallel with the spectroscopic titrations, COX assays were performed with huPGHS-2 in the presence of different concentrations of heme (Fig. 1D). Maximal COX activity occurred at a ratio of about one heme per dimer. These titrations, which for practical reasons were performed at $37^\circ C$ and at relatively low enzyme concentrations, predict a $K_d$ value of 5-10 nM. This value is significantly less than the $K_d$ value determined spectrophotometrically (i.e. 88 nM) at 25° in 0-0.15% DMSO with a 200 times higher concentration of enzyme. Nonetheless, the spectroscopic heme titration data indicate that there is only one high affinity heme binding site per dimer, and the COX assay data suggest that huPGHS-2 functions optimally with one heme bound per mole of dimer.

**Effects of FAs on COX activity of huPGHS-2.** We previously reported that the COX activity of huPGHS-2 is stimulated by some non-substrate FAs (24). Fig. 2 compares the concentration dependent effects of several different non-substrate FAs on the COX activities of huPGHS-2 with 5 µM AA as the substrate; the $K_M$ of huPGHS-2 for AA in the absence of other FAs is about 8.5 µM. The rate of AA oxygenation by PGHS-2 was increased by many non-substrate FAs. The most abundant free FA in many mammalian cells (39,40) is palmitic acid (PA), and PA was the most efficacious of the FAs tested in stimulating AA oxygenation. Phytomonic acid (2-hexyl-cyclopropanedecanoic acid), which is produced by *L. arabinosus* and is found in yogurt, also caused a particularly marked increase in the rate of AA oxygenation. FAs stimulated the rate of AA oxygenation in a concentration dependent manner, and with each FA the maximal effects occurred at concentrations of 10-12.5 µM. No inhibition of COX activity occurred at relatively high concentrations of the non-substrate FA concentrations; however, the critical micelle concentration (CMC) of saturated and monounsaturated FAs having 18 or more carbons was determined to be ≤10 µM while the CMC of PA under the conditions of the assay is about 25 µM. When the effects of combinations of non-substrate FAs were tested for their effects on the rate of AA oxygenation the results were approximately additive suggesting that the various FAs compete for the same site with similar affinities (Supplementary Table S3). As reported previously, PA and 20:1o9 decrease the $K_M$ of huPGHS-2 for AA (24), and we presume this to be true for other non-substrate stimulatory FAs as well. The $K_M$ for AA determined in the presence of 25 µM PA was 3.5 µM.

The relationships between the stimulatory effects of saturated and monounsaturated non-substrate FAs and their carbon chain length and degree of unsaturation are shown in Figs. 3A and 3B. These experiments were performed with two different concentrations of AA and non-substrate FAs to minimize potential problems associated with the low CMCs of longer chain FAs. Maximal stimulation was observed with PA (Fig. 3). For saturated FAs with carbon chain lengths from 12-16, an increase in chain length leads to an increase in the stimulatory effect of the FA. For saturated FAs with chain lengths greater than 16 carbon atoms, the stimulatory effect decreases with increasing chain length. Palmitoleic acid stimulates the rate of AA oxygenation, but the stimulatory effect is less than that of PA. In related experiments, some of which are detailed below, HPLC or thin-layer chromatography was used to separate the products formed upon oxygenation of [1-14C]-AA by native huPGHS-2. The presence of non-substrate FAs in the reaction mixture did not change the profile of oxygenated products. PGH2 is the major oxygenation product of AA by huPGHS-2 in the reaction with or without non-substrate FAs, and PA increased the rate of production of 14C-labeled PGH2 and PGH2-derived products by 80% (data not shown).

We also tested the effects of PA on the rate of oxygenation by huPGHS-2 of substrates other than AA (Table 2). As reported earlier PA caused a small increase in the rate of oxygenation of eicosapentaenoic acid but not docosahexaenoic acid (20). PA caused significant increases in the rate of oxygenation of several substrates having an acyl chain with an ω6 double bond including adrenic acid, 2-arachidonoylglycerol ether and dihomog-γ-linolenic acid, but there was no effect on
the oxygenation of eicosadienoic acid. It should be noted that, except in the case of linoleate, PA either increased or had no effect on the rate of oxygenation of COX substrates. This implies that at the maximal concentrations of PA tested (25 µM), it does not compete directly with AA at the site at which AA is oxygenated.

**Oxygenation of AA by huPGHS-2 at high enzyme to substrate ratios.** The results of previous studies (24) and of the experiments described above suggest that FAs can bind the allosteric monomer of huPGHS-2 and modulate the catalytic efficiency of the partner, catalytic monomer toward AA and that non-substrate FAs do not bind the catalytic monomer. We reasoned that all FAs, including AA, must bind with higher affinity to the allosteric monomer than the catalytic monomer, and further, that when a substrate FA like AA is bound to the allosteric monomer, it cannot be oxygenated. To test these concepts, we performed experiments pairing low concentrations of [1-14C]-AA with relatively high concentrations of huPGHS-2 in the presence or absence of non-substrate FAs. As shown in Fig. 4, oxygenation of 1 µM [1-14C]-AA by 1 µM huPGHS-2 plateaued at 4 min with about 6% of the original AA remaining. Simple Michaelis-Menten kinetic values for huPGHS-2 predict that AA should have decreased to undetectable levels in a few seconds.

We also observed that the amount of AA remaining is directly dependent on the amount of huPGHS-2 in the reaction mixture (Table 3). When 1 µM [1-14C]-AA was incubated for 15 sec with 0.1, 0.5, 1.0 or 2.0 µM huCOX-2, approximately 1%, 7%, 11% and 27% of the AA remains. However, when either PA or stearic acid was included in the reaction mixture, the AA was almost completely consumed (Table 4). PA is a more efficacious activator of huPGHS-2 than stearic acid, but both seem to have similar potencies (Fig. 2) and both appear to compete for the same sites (Supplementary Table S3).

Overall, the results are consistent with the idea that both AA and non-substrate FAs bind to the allosteric site of huPGHS-2 and that when added in excess, non-substrate FAs can displace AA from allosteric site. Displaced AA becomes available to the catalytic site where it is oxygenated. This concept is presented diagrammatically in Fig. 5 where Ecat designates the catalytic monomer and Eallo is the allosteric monomer. Using the model in Fig. 5 and the data in Table 4 we can estimate the Kd values for the binding of AA and non-substrate FAs to Ecat and Eallo. At concentrations tested non-substrate FAs do not inhibit AA oxygenation (Figs. 2 and 3). Again, this implies that non-substrate FAs do not bind with appreciable affinities to the catalytic COX site. Accordingly, we estimate that for binding of non-substrate FAs to Ecat the Kd is greater than 50 µM. The data in Fig. 4 and Table 4 suggest that an approximate equilibrium is reached by 4 min and that at that time 4.7% (6.0%-1.3%) of the AA is bound to Eallo (i.e. in the absence of a non-substrate FA). Thus, at 4 min there is 0.047 µM Eallo-AA/Ecat and 0.953 µM Eallo/Ecat and no Eallo-AA/Ecat-AA. We estimate that there is 0.013 µM unbound AA corresponding to the 1.3% AA that remains after adding either 2.5 µM stearic acid or palmitic acid (Table 4). The Kd value calculated from ([Free AA] × [Eallo/Ecat]) / [Eallo-AA/Ecat] is 0.26 µM for binding of AA to Eallo in the absence of other FAs. It can be seen that at a concentration of about 1 µM stearic acid or PA about 50% of the original 6% or about 0.03 µM of unreacted AA remains. Thus, the molar ratio of stearic acid or PA to AA to achieve 50% displacement of AA from Eallo is about thirty. From this we estimate that the Kd for binding of these non-substrates FAs to the Eallo site is about thirty times higher than that for AA or approximately 7.5 µM.

As noted in Fig. 5, the Kd values for AA oxygenation in the absence and presence of 25 µM PA were 8.4 µM and 3.5 µM, respectively (data not shown). We assume that the Kd value for AA to Ecat is 8.4 µM when AA is bound Eallo and that the Kd value for AA binding to Ecat is something less than 3.5 µM when PA is bound to Eallo. We cannot ascertain an accurate value for the Kd for AA when Eallo is completely occupied by PA because the CMC for PA in the assay solution is about 25 µM—conditions under which more than 50% of Eallo would be occupied by AA.

**Binding of PA to murine (mu) PGHS-2 in muPGHS-2/PA co-crystals.** PA stimulated the COX activity of murine (mu) PGHS-2. The potency of PA was similar with muPGHS-2 and huPGHS-2, but maximal stimulation was 43% with muPGHS-2 vs. 76% with huPGHS-2 using 5 µM AA and 25 µM PA. We have elucidated the x-
ray crystal structure of PA bound in the COX channel of murine PGHS-2 to 2.1 Å resolution utilizing synchrotron radiation. There are two monomers in the crystallographic asymmetric unit that comprise the canonical biological dimer. The overall tertiary structure of each monomer, which includes the epidermal growth factor domain, membrane-binding domain, and catalytic domain, is well resolved and conserved with respect to that observed for other murine PGHS-2/fatty acid co-crystal structures (23). The calculated root mean square deviation (rmsd) between monomer A and monomer B is 0.28 Å (for 549 Cα atom pairs) indicating that there are no significant structural differences between these monomers. Interpretable electron density for PA is only observed in monomer A of the muPGHS-2/PA crystal structure. In contrast, two molecules of ethylene glycol, which was utilized as a cryoprotectant during data collection, have been modeled into the COX channel of monomer B as detailed in the Experimental Procedures.

PA is oriented in the COX channel with its carboxylate moiety located near Arg-120 and Tyr-355 at the opening of the channel and its ω-end near Tyr-385 and Ser-530 at the apex of the channel (Fig. 6). PA makes 36 contacts with residues that line the COX channel. Half of these contacts (19 of 36) are made between PA and the side chain atoms of Arg-120, Tyr-355, Tyr-385, and Ser-530 (Supplemental Table S4). Five of these contacts are hydrophilic in nature. Specifically, the carboxylate oxygen atoms of PA form salt bridges to the Nε and Nη2 atoms of Arg-120 and hydrogen bonds to the phenolic oxygen atom of Tyr-355 and the Nε and Nη2 atoms of Arg-120. As PA is only 16 carbons in length, the ω-end of this fatty acid does not fully penetrate the hydrophobic groove above Ser-530 as is typically observed for productive binding of FA substrates to PGHS-1 and -2 (23,41-43) (Fig. 6). Instead, two well-ordered water molecules occupy the groove, which are analogous to the water molecules occupying the groove when AA and EPA bind in their nonproductive conformations to PGHS-2 (23). The side chain of Ser-530 exhibits alternate rotamer conformations, which has also been observed in crystal structures of muPGHS-2 bound with FA substrates (23). As a consequence of not fully extending into the hydrophobic groove, PA does not form interactions with the side chain atoms of Phe-209, Ile-377, Phe-381, Gly-533, and Leu-534. Contacts made between these residues are critical in the stabilization of the ω-end of fatty acid substrates within the hydrophobic groove when they are bound in a productive conformation (23). Hence the increase in hydrophilic interactions observed between the carboxylate oxygen atoms of PA and the side chain atoms of Arg-120 and Tyr-355 serve as compensatory interactions to stabilize PA when it is bound in the COX channel.

Effects of FAs on the inhibition of huPGHS-2 by nsNSAIDs and coxibs. Many time-dependent COX inhibitors, both nsNSAIDs and coxibs, bind to one monomer of PGHSs to cause maximal COX inhibition (16,17,19-21). Naturally occurring non-substrate FAs also appear to bind preferentially to one huPGHS-2 monomer because these FAs do not cause enzyme inhibition (Figs. 2 and 3). Therefore, we investigated the interplay between these two groups of agents. For individual time-dependent inhibitors we first examined the magnitudes of the inhibition following a fixed time of preincubation with different concentrations of an inhibitor (Fig. 7A and 7B). Celecoxib, indomethacin and diclofenac caused complete or near complete loss of activity (Fig. 7A). Naproxen, flurbiprofen and rofecoxib caused incomplete COX inhibition even at relatively high inhibitor concentrations (Fig. 7B). In all of the experiments reported in Fig. 7, we report rates of O2 consumption determined using an O2 electrode. We also examined the product profiles with all combinations of FA and inhibitor, and there were no differences (data not shown). Because the same products were formed in all cases, a direct comparison of rates of O2 consumption across all of the experimental conditions is appropriate.

The lack of complete inhibition by naproxen, flurbiprofen and/or rofecoxib could have resulted from rapid recovery of time-dependent inhibited activity when the enzyme was diluted into the assay chamber to measure COX activity, particularly because there is about a ten second lag before the maximal rate is achieved. To test for this, we measured the rate of recovery of COX activity with time by adding AA substrate to the assay chamber 0, 10, 30 or 90 sec after the enzyme plus inhibitor were added to the assay chamber (Fig. 7C); when diluted into the assay chamber
about 100 fold, the inhibitors are at concentrations that are insufficient to cause time-dependent inhibition. Enzyme treated with rofecoxib recovered sufficient activity relatively rapidly—enough to account for 5% being recovered during the first ten seconds of the assay. Thus, in contrast to what is predicted from the results in Fig. 7B, rofecoxib appears to cause complete time-dependent enzyme inhibition when an appropriate correction is made for recovery in the assay chamber. However, the rates of recovery of activity of huPGHS-2 that had been treated with flurbiprofen or naproxen (Fig. 7C) were too slow to account for the 15% and 30% residual COX activity, respectively, seen with these inhibitors (Fig. 7B).

We next determined the effect of several non-substrate FAs on the time-dependent inhibition by the different inhibitors (Fig. 7D-7H). We found that the effects of celecoxib, rofecoxib and aspirin could be potentiated by at least some FAs; importantly, none of the FAs tested attenuated the effects of any of these inhibitors. However, all of the FAs attenuated the actions of naproxen and flurbiprofen. FAs had no appreciable effects on responses to either diclofenac or indomethacin (data not shown).

Overall, time-dependent COX inhibitors appeared to fall into two broad classes. One class includes aspirin and those inhibitors that effectively cause complete COX inhibition (i.e. celecoxib, diclofenac, indomethacin, rofecoxib); aspirin modifies the COX active site but the acetylated site retains the ability to bind and convert substrate to an unusual product (20). The actions of this class of time-dependent inhibitors are either potentiated or unaffected by FAs depending on the specific FA. The second class of time-dependent inhibitors includes flurbiprofen and naproxen. Neither inhibitor causes complete COX inhibition under standard assay conditions, and the actions of these inhibitors are attenuated by FAs.

Ibuprofen is a time-independent COX inhibitor that binds both monomers of PGHS to cause COX inhibition (18). When ibuprofen was included at low concentrations in a reaction mixture containing PA and huPGHS-2, it caused an immediate drop in activity close to the level found in the absence of PA (Fig. 8). This is consistent with ibuprofen displacing PA from one

COX site but without having an otherwise detectable effect on the other site (18). As the ibuprofen concentration was increased, it appeared that PA caused a minor interference with ibuprofen action by shifting the dose response curve slightly to the right. When several FA were compared for their abilities to affect ibuprofen actions the effects approximately paralleled their abilities to stimulate huPGHS-2 activity.

**DISCUSSION**

We find that huPGHS-2 binds only one heme per dimer with high affinity ($K_d = 88 \text{ nM}$) and that maximal COX activity occurs with a stoichiometry of one heme per subunit. These results conflict with those reported earlier for huPGHS-2 (38) but are consistent with what has been found for ovine (15) and human PGHS-1. We do not have an explanation for the discrepancy between our results and those reported previously for huPGHS-2. Heme binding is relatively unaffected by non-substrate FAs such as PA or by two COX inhibitors celecoxib or naproxen, which, as discussed below, appear to bind the $E_{\text{cat}}$ and $E_{\text{allo}}$ sites, respectively, of huPGHS-2. Adding confusion to the question of the heme stoichiometry have been the results of x-ray studies of PGHS crystals. Crystallizations have generally been performed with approximately 75 $\mu\text{M}$ enzyme and 150 $\mu\text{M}$ heme. These are conditions expected to foster occupancy of both monomers with heme.

An examination of huPGHS-2 heterodimers having a defective POX active site indicated that the COX site of a monomer cannot function unless the POX site of that monomer is functional (25). This finding implies that the monomer of native huPGHS-2 to which heme is bound is the COX catalytic monomer. Accordingly, we have designated this heme-binding monomer as $E_{\text{cat}}$, and the partner monomer—the allosteric monomer—as $E_{\text{allo}}$ (Figs. 5 and 9). It is unclear whether a conformational huPGHS-2 heterodimer with one monomer having a higher affinity for heme exists prior to heme binding or whether heme binding to one monomer of a conformational homodimer induces structural changes that lead to different affinities of the POX sites for the heme (i.e. an $E_{\text{cat}}$ with a higher affinity for heme and an $E_{\text{allo}}$ with a lower affinity for heme). The fact that occupancy
of the $E_{\text{cat}}$ and $E_{\text{allo}}$ sites does not appreciably affect the affinity of huPGHS-2 for heme suggests that apo-huPGHS-2 pre-exists in solution as a conformational heterodimer. Whatever the mechanism, our model (Figs. 5 and 9) is that heme binding defines one monomer as $E_{\text{cat}}$ and the other as $E_{\text{allo}}$.

Another important aspect of the model is that the form of the enzyme ($E_{\text{allo}}$-$\text{FA}/E_{\text{cat}}$) having FA bound to $E_{\text{allo}}$ and heme bound to $E_{\text{cat}}$ is relatively stable. That is, neither FA nor heme dissociate quickly compared to the rates of AA binding to $E_{\text{cat}}$ and subsequent oxygenation. This is consistent with the relative affinities of various FAs for the $E_{\text{allo}}$ and $E_{\text{cat}}$ sites and of the heme for $E_{\text{cat}}$. Having a stable $E_{\text{allo}}$-$\text{FA}/E_{\text{cat}}$ is necessary for half of sites COX activity.

A confusing question that arose from earlier studies of half of sites COX activity was how a heterodimer like G533A/Native huPGHS-2 could have the same activity as the native huPGHS-2 homodimer if there was a rapid equilibrium between the monomers (17). A reasonable explanation in the context of the model in Fig. 5 is that the predominant form of G533A/Native huPGHS-2 in solution is one in which the G533A monomer is $E_{\text{allo}}$ and the native monomer is $E_{\text{cat}}$.

We previously reported that various substrate and non-substrate FAs affect the activities of PGHS-1 and PGHS-2 with AA as substrate (24). The earlier results indicated that non-substrate FAs like PA and 20:1ω9 bind to one monomer, an allosteric monomer $E_{\text{allo}}$, and affect the affinity of AA for the partner, catalytic monomer $E_{\text{cat}}$. Two new lines of evidence provide further support for this concept. First, we observed in kinetic experiments performed with high huPGHS-2 to AA ratios that AA is incompletely oxygenated, but that when non-substrate FAs are added to the reaction mixture, essentially complete oxygenation of AA occurs. These studies have also allowed us to estimate the relative affinities of AA and PA for $E_{\text{cat}}$ and $E_{\text{allo}}$. The results are consistent with the idea that AA binds to $E_{\text{allo}}$ with a significantly higher affinity than to $E_{\text{cat}}$ and that when bound to $E_{\text{allo}}$, AA is not oxygenated. Adding a non-substrate FA like PA displaces AA from $E_{\text{allo}}$, making AA available for oxygenation by $E_{\text{cat}}$. Non-substrate FAs do not bind with appreciable affinities to $E_{\text{cat}}$ at least when a non-substrate FA is bound to $E_{\text{allo}}$, and so non-substrate FAs do not interfere with AA oxygenation by $E_{\text{cat}}$.

Our crystallographic results support the model in Fig. 5 and also provide insights into how non-substrate FAs can bind to $E_{\text{allo}}$. Interestingly, only one monomer of the muPGHS-2 dimer is occupied by PA in the muPGHS-2/PA complex. This finding is consistent with the predicted affinities of PA for $E_{\text{allo}}$ and $E_{\text{cat}}$ that come from the kinetic studies discussed above. In the muPGHS-2/PA structure, the carboxylate group of PA is observed to interact with Arg-120 and Tyr-355 of what we predict is $E_{\text{allo}}$. In a previous structure of AA complexed with muPGHS-2, AA is observed in two different conformations (23). In one, which looks to be a catalytically productive conformation based on studies with ovPGHS-1 (41), AA is bound with its carboxylate group interacting with the hydrogen bonding network involving Arg-120 and Tyr-355 (23). AA is bound in a catalytically nonproductive conformation in the partner monomer with the carboxylate group of AA interacting with the phenolate oxygen of Tyr-385; a related, nonproductive conformation of AA was first suggested by results obtained in examining a complex of AA with apo-muPGHS-2 (44). Mutagenesis studies will be important in evaluating the predictions of the crystallographic studies of the complexes of AA and of PA with muPGHS-2. For example, it would be important to know if AA or PA binding are affected in a native/Y385F huPGHS-2 heterodimer.

We have found that there is a specificity to the actions of non-substrate FAs on huPGHS-2. PA is the most efficacious FA activator of the enzyme while non-substrate FAs having either fewer or greater numbers of double bonds are less effective activators of the enzyme. We hypothesize that prostanooid production in vivo is directly and importantly influenced by the effective concentration and FA composition of the milieu in which PGHSs operate—what we term the FA tone. We presume that every individual establishes a fairly constant FA tone as a consequence of dietary habits and genetic profile (e.g. (45,46)). We imagine that having a FA tone outside some normal range would change prostanooid formation to an extent sufficient to affect the health of an individual. For example, we speculate that having a FA tone that enhances PG formation via PGHS-2
would exacerbate inflammatory conditions believed to underlie many chronic diseases.

At the concentrations tested, the effects of non-substrate FAs are less than two fold. The effects are on the $K_M$ for AA (24) and that $K_M$ is in the range of the expected apparent concentrations of free AA following cell stimulation. While the magnitudes of the FA effects are relatively small, they are in the range of physiologic significance. Studies with hypomorphic PGHS-1 mice and with COX inhibitors in mice and humans have shown that near complete suppression of PG formation is required for effects on PGHS-2 dependent reproductive responses and on PGHS-1 dependent platelet aggregation (47-49). However, incomplete (50-75%) suppression of PG formation correlates with diminution of some PG-mediated inflammatory and analgesic responses (50-52). Thus, seemingly modest changes in PG synthesis can lead to significant clinical changes. One can imagine that a small decrease in PG formation occurring via PGHS-2 in affected joints could diminish the need for anti-inflammatory/analgesic therapy (50-52).

Knowing that there are interactions between PGHSs and non-substrate FAs and between PGHSs and COX inhibitors, it was not unexpected that non-substrate FAs would affect responses to COX inhibitors. The time-independent competitive inhibitor ibuprofen must bind to both COX sites of a PGHS-2 dimer to cause inhibition of AA oxygenation (18). Binding to one site occurs with high affinity but does not affect the rate of oxygenation. Oxygenation of AA is only affected when ibuprofen binds the second COX site. We presume that the latter site corresponds to $E_{cat}$ and the former site to $E_{allo}$ in the model depicted in Fig. 9. Consistent with this idea is that relatively low concentrations of ibuprofen essentially eliminate the stimulatory effect of PA on AA oxygenation. In fact, this form of competition (i.e. for $E_{allo}$) may underlie part of the pharmacologic effect of inhibitors that bind this monomer. Secondly, when the ibuprofen concentration is raised further, there is a dose dependent decrease in AA oxygenation paralleling that seen in the absence of PA.

Unlike time-independent inhibitors, time-dependent inhibitors appear to bind to a single monomer to elicit effects (16,17,19,20). Interactions between non-substrate FAs and time-dependent inhibitors seem to fall into two broad categories depending on whether the inhibitor functions by interacting with $E_{allo}$ or $E_{cat}$ as depicted in Fig. 9. Time-dependent inhibitors that bind to $E_{allo}$ are allosteric regulators and at least in the cases of naproxen and flurbiprofen cause incomplete COX inhibition. Non-substrate FAs, which bind $E_{allo}$ with reasonably high affinities, are observed to attenuate the actions of these inhibitors. We propose that this occurs because non-substrate FAs compete with inhibitors like naproxen and flurbiprofen for binding to $E_{allo}$ (Fig. 9). Naproxen is the one COX inhibitor the use of which appears to be devoid of adverse cardiovascular side effects (53). It would be of interest to determine if this relates to the ability of naproxen to be a modestly efficacious COX-2 inhibitor that functions via $E_{allo}$.

Inhibitors in the second group of time-dependent inhibitors bind to $E_{cat}$ and compete directly with AA (Fig. 9). Not surprisingly, COX inhibitors in this group can cause complete enzyme inhibition. The effects of these inhibitors including celecoxib and rofecoxib are frequently potentiated by the presence of a non-substrate FA. Presumably this involves having the FA bind to $E_{allo}$ and causing an allosteric effect on $E_{cat}$ that facilitates inhibitor binding and/or the time-dependent transformation of huPGHS-2 to an inhibited form (or stabilization in an inhibited form).

We think that aspirin is an exception, unique to PGHS-2, to the case of a time-dependent inhibitor causing complete enzyme inhibition even though aspirin acts on $E_{cat}$. We propose that aspirin acetylates $E_{cat}$ but that this does not completely interfere with AA binding and that bound AA is converted to 15$R$-hydroxyeicosatetraenoic acid instead of the normal product PGG$_2$ (20).

We speculate that both diclofenac and indomethacin bind to $E_{cat}$ to elicit their effects. Binding of one diclofenac molecule per huPGHS-2 (20) or one indomethacin to PGHS-1 (16) causes complete inhibition. The FAs we tested did not potentiate inhibition by these agents. More importantly, non-substrate FAs did not interfere with inhibition as would have been expected were diclofenac or indomethacin binding to $E_{allo}$.

As discussed above FA tone could importantly influence basal prostanooid formation. Similarly, the FA tone would be expected to affect responses
COX inhibitors. These drugs are the most widely used pharmaceutical agents in the U.S., but they have adverse side effects responsible for an estimated 20,000 deaths in the U.S. annually (53). We envisage that some side effects, which are generally of unknown origin, are related to diet/drug interactions and could be ameliorated by altering diet or drug. For example, we speculate that responses of patients to nsNSAIDs or COX-2 specific inhibitors would vary among groups of individuals on typical Western vs. Mediterranean vs. ω3 fish oil-enriched diets. The differences may well be drug specific. Dietary studies need to be performed to address these issues.

ACKNOWLEDGEMENTS

These studies were supported in part by NIH Grants GM 068848 (WLS) and GM 077176 (MGM). This work is also based upon research conducted at the Cornell High Energy Synchrotron Source (CHESS), which is supported by the National Science Foundation under NSF award DMR-0225180, using the Macromolecular Diffraction at CHESS (MacCHESS) facility, which is supported by award RR-01646 from the National Institutes of Health, through its National Center for Research Resources. The atomic coordinates and structure factors for murine PGHS-2/PA (PDB id 3HQ0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

REFERENCES


**FOOTNOTES**

1Abbreviations: AA, arachidonic acid; β-OG, n-octyl β-D-glucopyranoside; C_{10}M, decyl maltoside; CMC, critical micelle concentration; COX, cyclooxygenase; FA, fatty acid; hu, human; mu, murine; nsNSAIDs, nonspecific nonsteroidal anti-inflammatory drugs; ov, ovine; PA, palmitic acid; PG, prostaglandin; PGHS, prostaglandin endoperoxide H synthase; rmsd, root mean square deviation.

2CMC values were determined in 100 mM sodium phosphate, pH 7.6 containing 5 µM 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously (24). CMC values for various FAs tested under these conditions are as follows: lauric acid (12:0), 400 µM; pentadecanoic acid (15:0), 75 µM; palmitic acid (16:0), 30 µM; heptadecanoic acid (17:0), 10 µM; oleic acid (Δ9 cis18:1), ca. 5 µM.

3H. Zou and W.L. Smith, unpublished observations
FIGURE LEGENDS

Fig. 1. Stoichiometry of heme binding to huPGHS-2. A. Difference absorption spectra of apo-huPGHS-2 (5 µM) titrated with heme at room temperature as described under Experimental Procedures. B. Change in absorbance at 408 nm from A. C. Titration data plotted in a linear form using the equation \( I_0 / \alpha = [K_d/(1-\alpha) + e_0] \) where \( I_0 \) is the concentration of added heme; \( \alpha \), the fraction of enzyme sites occupied by heme, is given by \( \Delta / \Delta_{\text{max}} \) where \( \Delta \) is the optical change measured (27). The slope is equal to the \( K_d \) for heme binding and the intercept (\( e_0 \)) represents the concentration of heme binding sites. The data shown in Fig. 1A-C are from one experiment but representative of six separate experiments with different enzyme preparations. The \( K_d \) values varied over a 2-3 fold range among different enzyme preparations (e.g. Fig. S1). D. 22.5 nM huPGHS-2 and 100 µM [1-\(^{14}\)C]-AA was incubated with the indicated concentration of heme at 37° C, and after a 30 sec incubation, the reaction was stopped. The products and remaining AA were separated by HPLC. The amount of PGH\(_2\) formed was calculated based on the percentage of total radioactivity chromatographing with PGH\(_2\). Details of the HPLC separation and analyses are provided under Experimental Procedures. Values in D. are from triplicate determinations. The error bars indicate ± S.E.M.

Fig. 2. Concentration dependent effects of FAs that are not COX substrates on AA oxygenation by huPGHS-2. Results are shown as increases in the percentage of the rate of O\(_2\) consumption from O\(_2\) electrode assays of COX activities of huPGHS-2 with 5 µM AA plus the indicated concentrations of various non-substrate FAs relative to 5 µM AA alone. O\(_2\) consumption was measured using an O\(_2\) electrode as described under Experimental Procedures. The results are from a single experiment involving triplicate determinations. The error bars indicate ± S.E.M.

Fig. 3. Effects of saturated and monounsaturated FAs on AA oxygenation by huPGHS-2. Results are shown as a percentage of the rate of increase for O\(_2\) electrode assays of COX activities of huPGHS-2 with A. FAs (25 µM) in combination with AA (5 µM) vs. 5 µM AA alone and B. FAs (5 µM) in combination with AA (2 µM) vs. 2 µM AA alone. Rates were determined by measuring O\(_2\) consumption using an O\(_2\) electrode as described in Experimental Procedures. The results in panels A. and B. are each from a single experiment involving triplicate determinations. The error bars indicate ± S.D. FA designations and common names: 12:0, lauric acid; 14:0 myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1 Δ9c, palmitoleic acid; 16:1 Δ9t, palmitelaidic acid; 17:0, heptadecanoic acid; 18:0, stearic acid; 18:1 Δ9c, oleic acid; 18:1 Δ9t, elaidic acid; 20:1 Δ11c, eicosaenoic acid.

Fig. 4. Effect of reaction time on oxygenation of [1-\(^{14}\)C]-AA measured by HPLC. [1-\(^{14}\)C]-AA (1 µM) was incubated with 1 µM huPGHS-2 at 37° C and after the indicated incubation times (0, 0.25, 1.0, 4.0 and 16 min), the reactions were stopped and the products and remaining AA were separated and quantified by radio-HPLC as described in Experimental Procedures. Each time point represents the mean value ± S.E.M. for three separate incubations.

Fig. 5. Model for allosteric interactions between monomers of the PGHS-2 homodimer caused by the binding of FAs. E\(_{\text{cat}}\) is the catalytic monomer to which heme is bound while E\(_{\text{allo}}\) is the allosteric monomer that does not bind heme. A. Binding of AA to E\(_{\text{allo}}\) and E\(_{\text{cat}}\) of huPGHS-2 and subsequent oxygenation of AA. The \( K_d \) for E\(_{\text{cat}}\) is the \( K_M \) for AA (8.5 µM) in the absence of other FAs. The \( K_d \) for AA binding to E\(_{\text{allo}}\) (0.26 µM) is calculated from the data in Fig. 4 and Tables 3 and 4 as outlined in the text. B. The binding of AA to E\(_{\text{allo}}\) and E\(_{\text{cat}}\) in the presence of PA. We make the assumptions (a) that the \( K_d \) for binding of AA to the E\(_{\text{cat}}\) site is equal to the \( K_M \) in the presence of PA (i.e. < 3.5 µM) and (b) that the \( K_d \) for PA binding to the E\(_{\text{cat}}\) site is high (≥ 50 µM) because as shown in Fig. 2, PA does not inhibit AA oxygenation by huPGHS-2 even at low concentrations of AA. We estimate that the \( K_d \) values for the
binding of palmitic acid and stearic acid to the E_{allo} site are ~7.5 \mu M based on the concentration of the FAs to cause 50% inhibition of AA binding when the effective concentration of AA is about 0.025 \mu M.

**Fig. 6.** Palmitic acid bound in the cyclooxygenase channel of monomer A of the murine PGHS-2/PA co-crystal structure. Stereo view of PA bound in the COX channel of monomer A. 2Fo-Fc simulated annealing omit map electron density, contoured at 4\sigma, is shown with the final refined model of PA (black carbon and red oxygen atoms). Residues lining the COX channel are labeled accordingly (green, red, and blue for carbon, oxygen, and nitrogen atoms, respectively).

**Fig. 7.** Effects of non-substrate FAs on the responses of huPGHS-2 to different COX inhibitors. **A.** Purified huPGHS-2 (1 \mu M) was pretreated with the indicated concentrations of celecoxib, indomethacin or diclofenac at 37°C for 30 min and then assayed for COX activity with 5 \mu M AA using an O_2 electrode as described in Experimental Procedures. **B.** Purified huPGHS-2 (1 \mu M) was pretreated with the indicated concentrations of naproxen, rofecoxib or flurbiprofen at 37°C for 30 min and then assayed for COX activity with 5 \mu M AA as described in **A.** **C.** Purified huPGHS-2 (1 \mu M) was pretreated with 4 \mu M naproxen, 25 \mu M rofecoxib or 25 \mu M flurbiprofen at 37°C for 30 min, respectively. Pretreated enzyme (40 \mu l) was injected into an O_2 electrode chamber containing 3 ml of assay mixture. AA (10 \mu l; 40 \mu M final concentration in the assay) was injected at the indicated incubation times after the addition of inhibitor-pretreated enzyme and O_2 consumption was measured. **D.** Effect of FAs on inhibition of huPGHS-2 by celecoxib. Purified native huPGHS-2 homodimer (1 \mu M) was incubated at 37°C with or without celecoxib (1 \mu M) for 30 min in the presence or absence of various non-substrate FAs (5 \mu M) and then assayed for COX activity with 5 \mu M AA using an O_2 electrode. **E.** Effect of FAs on inhibition of huPGHS-2 by rofecoxib. The experiments were performed as in **D** except using rofecoxib (1 \mu M) instead of celecoxib. **F.** Effect of FAs on inhibition of huPGHS-2 by flurbiprofen. The experiments were performed as in **D** except using flurbiprofen (1.75 \mu M) instead of celecoxib. **G.** Effect of FAs on inhibition of huPGHS-2 by aspirin. The experiments were performed as in **D** except using aspirin (100 \mu M) instead of celecoxib. Values in each panel are derived from the average of triplicate determinations ± S.D. Similar experiments were performed at least twice with different preparations of enzyme and yielded quantitatively similar results. In panels D-H, the values denoted with an asterisk differ from the corresponding inhibitor treated control without FA as determined using a student t-test (p<0.005).

**Fig. 8.** Effects of Non-substrate FAs on the inhibition of huPGHS-2 by ibuprofen. **A.** Concentration-dependent inhibition of native huPGHS-2 by ibuprofen (IBP). The indicated concentration of ibuprofen (closed circles) or the indicated concentration of ibuprofen plus 25 \mu M palmitic acid (open circles) were present in an O_2 electrode assay chamber along with 5 \mu M AA. Purified huPGHS-2 was added to initiate AA oxygenation. **B.** Effects of non-substrate FAs on the inhibition of huPGHS-2 by ibuprofen (IBP). Purified native huPGHS-2 homodimer was added to O_2 electrode assays containing 5 \mu M AA and either no inhibitor or 50 \mu M ibuprofen and 5 \mu M of the indicated non-substrate FAs and O_2 consumption measured. Values are derived from the average of triplicate determinations ± S.D. In panel A, the values denoted with an asterisk differ from the inhibitor treated control without FA as determined using a student t-test (p<0.005); in panel B, the values denoted with an asterisk differ from the inhibitor treated control without FA as determined using a student t-test (p<0.05).

**Fig. 9.** FA, nsNSAID and coxib modulators of huPGHS-2. As noted, different ligands bind preferentially to either E_{allo} or to E_{cat}. In the illustration E_{allo} is shown binding a non-substrate, activating FA (e.g. PA). E_{allo} with bound FA activates E_{cat} by decreasing the K_M for AA.
Table 1. Data collection and refinement statistics for muPGHS-2/palmitic acid co-crystal.

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<td>Unit cell length (Å)</td>
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<td>b</td>
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<td>RMSD in bond angle (°)</td>
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a The values in parentheses represent the values in the outermost resolution shell.

b $R_{\text{MERGE}}$ and $R_{\text{PIM}}$ as defined in (54).

c Represents reflections with $F > 0$ of $F$, which were used in the refinement.

d 5.0% of the total reflections were used to generate the test set.

e Coordinate error as calculated by Luzatti plot.
Table 2. Rates of oxygenation of various huPGHS-2 substrates in the presence and absence of PA. Results are shown as rates of O$_2$ consumption under standard oxygen electrode assay conditions for measuring COX activity with huPGHS-2. Substrates were present at concentrations of 5 µM either with or without 25 µM PA. Details are provided in the Experimental Procedures. Values are from at least triplicate assays and are shown as the means ± S.D.

<table>
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<tr>
<th>Substrate</th>
<th>COX activity (units/mg)</th>
<th>Relative Rate ($\frac{[(PA + substrate) / (substrate)]}{(%)}$)</th>
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<tr>
<td></td>
<td>Substrate alone</td>
<td>Substrate + 25µM PA</td>
</tr>
<tr>
<td>AA</td>
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<td>28 ± 1.7</td>
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<tr>
<td>Eicosapentaenoic acid$^a$</td>
<td>5.9 ± 0.04</td>
<td>6.3 ± 0.21</td>
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<tr>
<td>Docosahexaenic acid$^a$</td>
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<td>2-Arachidonylglycerol ether</td>
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<td>Eicosadienoic acid</td>
<td>6.4 ± 0.09</td>
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$^a$From Reference (20)

$^b$Significant difference between substrate only and substrate plus PA in student t-test ($p<0.005$).
Table 3. Oxygenation of AA at high huPGHS-2 to AA ratios. [1-\(^{14}\text{C}\)]-AA (1 \(\mu\)M) was incubated with the indicated concentration of huPGHS-2 at 37° C for 15 sec, the reactions stopped and the radioactive products and unreacted AA separated by radio-HPLC and quantified as described in Experimental Procedures. The results show the percentage of the original [1-\(^{14}\text{C}\)]-AA remaining.

<table>
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<th>Reaction Components</th>
<th>Unreacted [1-(^{14}\text{C})]-AA Remaining (%)</th>
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</tr>
<tr>
<td>1 (\mu)M [1-(^{14}\text{C})]-AA + 100 nM huPGHS-2</td>
<td>0.80</td>
</tr>
<tr>
<td>1 (\mu)M [1-(^{14}\text{C})]-AA + 0.5 (\mu)M huPGHS-2</td>
<td>6.27</td>
</tr>
<tr>
<td>1 (\mu)M [1-(^{14}\text{C})]-AA + 1 (\mu)M huPGHS-2</td>
<td>12.0</td>
</tr>
<tr>
<td>1 (\mu)M [1-(^{14}\text{C})]-AA + 2 (\mu)M huPGHS-2</td>
<td>23.3</td>
</tr>
</tbody>
</table>

\(^a\)Significant difference versus control with 100 nM huPGHS-2 in student t-test (p<0.005).
Table 4. Oxygenation of AA at high huPGHS-2 to AA ratios in the presence or absence of palmitic acid or stearic acid. [1-14C]-AA (1 µM) was incubated with huPGHS-2 (1 µM) in the presence and absence of palmitic acid or stearic acid at 37 °C for 15 sec or 4 min. The reactions were stopped and the radioactive products and unreacted AA separated and quantified by radio-HPLC as described in Experimental Procedures. The results are shown as the percentage of unreacted [1-14C]-AA remaining. Values shown are means ± S.D. for triplicate determinations.

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Unreacted AA Remaining after 15 sec (%)</th>
<th>Unreacted AA Remaining after 4 min (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palmitic acid</td>
<td>Stearic acid</td>
</tr>
<tr>
<td>1 µM [1-14C]-AA + 1 µM huPGHS-2 + no additional FA</td>
<td>10.9 ± 1.1</td>
<td>10.9 ± 1.1</td>
</tr>
<tr>
<td>1 µM [1-14C]-AA + 1 µM huPGHS-2 + 1.25 µM PA or SA</td>
<td>3.5 ± 0.5a</td>
<td>6.6 ± 1.5a</td>
</tr>
<tr>
<td>1 µM [1-14C]-AA + 1 µM huPGHS-2 + 2.5 µM PA or SA</td>
<td>1.0 ± 0.4a</td>
<td>5.2 ± 1.1a</td>
</tr>
</tbody>
</table>

*aSignificant difference versus control without palmitic acid or stearic acid in student t-test (p<0.015).*
Figure 1
Figure 2

[Graph showing the increase in rate of O₂ consumption with FA + AA relative to AA alone (%)]

- Phytomononic acid
- Palmitic acid
- Oleic acid
- Δ11 20:1
- Lauric acid
- Stearic acid

[Graph with concentration of non-substrate fatty acid (μM) on the x-axis and increase in rate of O₂ consumption on the y-axis]
Figure 3

A. Increase in rate of $O_2$ consumption with FA + AA relative to AA alone (%)

B. Increase in rate of $O_2$ consumption with FA + AA relative to AA alone (%)
Figure 4
Figure 5A-5B
Figure 6
Figure 7A-7H
Figure 8A-8B
Figure 9
Human cyclooxygenase-2 is a sequence homodimer that functions as a conformational heterodimer
Liang Dong, Alex J. Vecchio, Narayan P. Sharma, Brice J. Jurban, Michael G. Malkowski and William L. Smith

J. Biol. Chem. published online April 5, 2011

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