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

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Prostaglandin endoperoxide H synthases 1 and 2, also known as cyclooxygenases (COXs) 1 and 2, convert arachidonic acid (AA) to prostaglandin endoperoxide H₂. Prostaglandin endoperoxide H synthases 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\textsubscript{cat}) and an allosteric monomer (E\textsubscript{allo}). E\textsubscript{cat} binds only to the peroxidase site of E\textsubscript{cat}, whereas substrates, as well as certain inhibitors (e.g. celecoxib), bind the COX site of E\textsubscript{cat}. E\textsubscript{cat} is regulated by E\textsubscript{allo} in a manner dependent on what ligand is bound to E\textsubscript{allo}. Substrate and nonsubstrate fatty acids (FAs) and some COX inhibitors (e.g. naproxen) preferentially bind to the COX site of E\textsubscript{allo}. AA can bind to both E\textsubscript{cat} and E\textsubscript{allo}, but the affinity of AA for E\textsubscript{allo} is 25 times that for E\textsubscript{cat}. Palmitic acid, an efficacious stimulator of human PGHS-2, binds only E\textsubscript{allo} in palmitic acid/murine PGHS-2 co-crystals. Nonsubstrate FAs can potentiate or attenuate actions of COX inhibitors depending on the FA and whether the inhibitor binds E\textsubscript{cat} or E\textsubscript{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 syntheses (PGHSs), also known generically as cyclooxygenases (COXs), convert arachidonic acid (AA) to prostaglandin H₂ (PGH₂) 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, whereas 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 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 PGGs. 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 composed 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). Kulmacz and Lands (15, 16) provided the first evidence that the monomers of PGHS homodimers differ. They found that maximal COX activity of ovine 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 exhibit half of sites’ COX activity sites with AA, i.e. 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 this 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, i.e. the catalytic monomer, of the huPGHS-2 dimer. Additional studies...
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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 nonsubstrate 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 lead us to speculate that there can be important dietary effects on both base-line 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-hydroxyheptadecaeanoic acid were purchased from Cayman Chemical Co. Lauric acid (12:0), myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), margaric acid (17:0), stearic acid (18:0), nonadecylic acid (19:0), 11-cis-eicosanoic acid (20:1ω9), FAD, and FLAG peptide were from Frontier Scientific (Logan, UT). Decyl maltoside, reported here was 40 units/mg.

blot analysis (24). The average specific activity of purified huPGHS-2 was determined by SDS-PAGE and by Western

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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 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 acetate/ acetic acid (95:5). The resultant samples were centrifuged at room temperature for 5 min at 4000 × 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, β-RAM model 4). The bound products were eluted in a gradient elution mode eluting with Solution A (acetoni trile/water; 30:70) for 5 min and then with a linear gradient of Solutions A and B (acetoni trile/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 PGE2, 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% C12E6, 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

\[
\frac{\text{d}A}{\text{d}t} = -k \cdot A
\]

where

\[
A = \frac{I_{\infty} - I_0}{I_{\infty} - I_0}
\]

and

\[
I_0 = I_{\infty} \cdot \alpha
\]

where \( I_{\infty} \) is the concentration of added heme; \( \alpha \), the fraction of enzyme sites occupied by heme, is given by

\[
\Delta = \Delta_{max},
\]

where Λ is the optical change measured (27); in this plot, the slope is equal to the \( K_f \) for heme binding, and the intercept (\( e_0 \)) represents the concentration of heme-binding sites. Tittrations 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, murPGHS-2 were reconstituted with a 2-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 h at 4 °C. A 10-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% polyacryl 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% polyacryl acid 5100, 100 mM HEPES, pH 7.5, and 20 mM MgCl2. The crystals were transferred to a solution of 30% polyacryl acid 5100, 100 mM HEPES, pH 7.5, 20 mM MgCl2, and 0.6% (w/v) β-OG supplemented with 10% ethylene

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 s. One unit of COX activity is defined as 1 μmol of O2 consumed per min at 37 °C in the assay standard mixture. A representative tracing obtained with a low concentration of AA is shown in supplemental Fig. S1.
TABLE 1

| Crystallographic parameter | muPGHS-2/PA |
|---------------------------|-------------|
| Space group               | I222        |
| No. in asymmetric units   | 2           |
| Unit cell length          |             |
| a                         | 121.91 Å    |
| b                         | 130.89 Å    |
| c                         | 179.97 Å    |
| α = β = γ                 | 90°         |
| Wavelength                | 0.9769 Å    |
| Resolution                | 20.0 to 2.10 Å |
| Highest resolution shell  | 2.21 to 2.10 Å |
| Rmerge                    | 9.6 (56.2)  |
| Rpim                      | 4.6 (26.0)  |
| Total observations        | 457,344 (63,157) |
| Total unique              | 82,053 (11,717) |
| I/σ(I)                    | 13.5 (2.9)  |
| Completeness              | 98.0% (96.8%) |
| Multiplicity              | 5.6 (5.4)   |
| Wilson B factor           | 24.9 Å      |
| No. of atoms in refinement| 10,038      |
| Rwork                     | 0.159 (0.211) |
| Rsigma                    | 0.199 (0.276) |
| Average B factor, protein | 27.1 Å^2    |
| Average B factor, solvent | 28.1 Å^2    |
| Average B factor, palmitate| 50.6 Å^2   |
| Mean positional error     | 0.257 Å     |
| r.m.s.d. in bond length   | 0.01 Å      |
| r.m.s.d. in bond angle    | 1.32°       |

^The values in parentheses represent the values in the outermost resolution shell.
^Rmerge and Rpim as defined in Ref. 54.
^Data represent reflections with F > 0 σF, which were used in the refinement.
^5.0% of the total reflections was used to generate the test set.
^Coordinate error was calculated by Luzatti plot.

Results of AA and eicosapentaenoic acid observed in monomer A of the co-crystal structures of muPGHS-2/AA and muPGHS-2/eicosapentaenoic acid (23). Cycles of REFMAC5 refinement were performed, followed by calculation of 2Fo – Fc and Fo – Fc, 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 REFMAC5 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 Ca 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 Co3+-protoporphyrin IX reconstituted muPGHS-2 in complex with PA have been deposited in the Protein Data Bank, code 3QH0.

RESULTS

Stoichiometry of Heme Binding to huPGHS-2—Titration of apo-huPGHS-2 with hematin yielded a major UV-visible spectral peak with a λmax at 408 nm indicative of formation of a heme-huPGHS-2 complex (Fig. 1A) (15, 38). The data are consistent with one high affinity heme-binding site per PGHS-2 dimer (i.e. 0.92 mol of heme/mol of dimer) with a Kd = 88 ± 17 nM (n = 6) and a nonsaturable lower affinity site(s) (Fig. 1, B and C). The nonsaturable 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 Kd values for heme binding to huPGHS-2 were essentially the same in the presence of PA, celecoxib, naproxen, or aspirin (supplemental 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 °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 °C 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 mol 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 nonsubstrate FAs (24). Fig. 2 compares the concentration-dependent effects of several different nonsubstrate FAs on the COX activities of huPGHS-2 with 5 μM AA as the substrate; the $K_m$ value 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 nonsubstrate FAs. The most abundant free FA in many mammalian cells (39, 40) is PA, and PA was the most efficacious of the FAs tested in stimulating 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 nonsubstrate FAs in the reaction maximal effects occurred at concentrations of 10–12.5 μM. No inhibition of COX activity occurred at relatively high concentrations of the nonsubstrate FA concentrations; however, the critical micelle concentration (CMC) of saturated and monounsaturated FAs having 18 or more carbons was determined to be ≈ 10 μM, although the CMC of PA under the conditions of the assay is about 25 μM.3 When the effects of combinations of nonsubstrate 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 (supplemental Table S3). As reported previously, PA and 20:1 o9 decrease the $K_m$ value of huPGHS-2 for AA (24), and we presume this to be true for other nonsubstrate stimulatory FAs as well. The $K_m$ value for AA determined in the presence of 25 μM PA was 3.5 μM.

The relationships between the stimulatory effects of saturated and monounsaturated nonsubstrate FAs and their carbon chain length and degree of unsaturation are shown in Fig. 3, A and B. These experiments were performed with two different concentrations of AA and nonsubstrate 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 to 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 described 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 nonsubstrate FAs in the reaction

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3 CMC 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), −5 μM.
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 nonsubstrate 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 arachidonic acid, 2-arachidonoylglycerol ether, and dihomo-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.

| Substance                    | COX activity (substrate) | COX activity (substrate + 25 μM PA) | Relative rate (25 μM PA/substrate) |
|------------------------------|-------------------------|------------------------------------|-----------------------------------|
| AA                           | 16 ± 0.73               | 28 ± 1.7              | 178%                              |
| Eicosapentaenoic acid        | 5.9 ± 0.04              | 6.3 ± 0.21             | 107%                              |
| Docosahexaenoic acid         | 2.0 ± 0.08              | 2.0 ± 0.11             | 100%                              |
| 2-Arachidonoylglycerol ether | 10 ± 0.17               | 14 ± 0.42              | 137%                              |
| Adrenic acid                 | 8.1 ± 0.44              | 12 ± 0.06              | 153%                              |
| Dihomo-γ-linolenic acid      | 18 ± 0.97               | 23 ± 1.2               | 127%                              |
| Linoleic acid                | 2.6 ± 0.18              | 1.6 ± 0.16             | 62%                               |
| Eicosadienoic acid           | 6.4 ± 0.09              | 6.5 ± 0.49             | 101%                              |

TABLE 2: Rates of oxygenation of various huPGHS-2 substrates in the presence and absence of PA.

Results are shown as COX activity 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 under “Experimental Procedures.” Values are from at least triplicate assays and are shown as the means ± S.D.

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 nonsubstrate 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, furthermore, 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 nonsubstrate 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.
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**TABLE 3**

Oxygenation of AA at high huPGHS-2 to AA ratios

| Reaction components | Unreacted [1-14C]AA remaining | % | Average ± S.D. |
|---------------------|-------------------------------|---|---------------|
| 1 µM [1-14C]AA + 100 nM huPGHS-2 | 0.80 | 1.38 | 0.12 | 0.77 ± 0.63 |
| 1 µM [1-14C]AA + 0.5 µM huPGHS-2 | 6.27 | 9.74 | 7.11 | 7.7 ± 1.8* |
| 1 µM [1-14C]AA + 1 µM huPGHS-2 | 12.0 | 9.85 | 10.9 | 10.9 ± 1.1* |
| 1 µM [1-14C]AA + 2 µM huPGHS-2 | 23.3 | 30.5 | 25.6 | 26.5 ± 3.6* |

* Significant difference versus control was with 100 nM huPGHS-2 in Student’s 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

| Reaction components | Unreacted AA remaining after 15 s | Palmitic acid | Stearic acid | % | Palmitic acid | Stearic acid | % |
|---------------------|----------------------------------|--------------|--------------|---|--------------|--------------|---|
| 1 µM [1-14C]AA + 1 µM huPGHS-2 + no additional FA | 10.9 ± 1.1 | 10.9 ± 1.1 | 6.0 ± 0.8 | 6.0 ± 0.8 |
| 1 µM [1-14C]AA + 1 µM huPGHS-2 + 1.25 µM PA or SA | 3.5 ± 0.5* | 6.6 ± 1.5* | 2.9 ± 0.5* | 3.1 ± 0.4* |
| 1 µM [1-14C]AA + 1 µM huPGHS-2 + 2.5 µM PA or SA | 1.0 ± 0.4* | 5.2 ± 1.1* | 1.3 ± 0.8* | 1.3 ± 0.8* |

* Significant difference versus control was without palmitic acid or stearic acid in Student’s t test (p < 0.015).

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 s with 0.1, 0.5, 1.0, or 2.0 µM huCOX-2, ~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 (supplemental Table S3).

Overall, the results are consistent with the idea that both AA and nonsubstrate FAs bind to the allosteric site of huPGHS-2 and that when added in excess nonsubstrate FAs can displace AA from the allosteric site. Displaced AA becomes available to the catalytic site where it is oxygenated. This concept is presented diagrammatically in Fig. 5, where $E_{cat}$ designates the catalytic monomer and $E_{allo}$ is the allosteric monomer. Using the model in Fig. 5 and the data in Table 4, we can estimate the $K_d$ values for the binding of AA and nonsubstrate FAs to $E_{cat}$ and $E_{allo}$. At concentrations tested, nonsubstrate FAs do not inhibit AA oxygenation (Figs. 2 and 3). Again, this implies that nonsubstrate FAs do not bind with appreciable affinities to the catalytic COX site. Accordingly, we estimate that for binding of nonsubstrate FAs to $E_{cat}$ the $K_d$ 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 to 1.3%) of the AA is bound to $E_{allo}$ (i.e. in the absence of a nonsubstrate FA). Thus, at 4 min there is 0.047 µM $E_{allo}$.AA/$E_{cat}$ and 0.953 µM $E_{allo}/E_{cat}$ and no $E_{allo}$-$AA$/$E_{cat}$.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 $K_d$ value calculated from $([\text{free AA}] \times [E_{allo}/E_{cat}] + (E_{allo} - \text{AA}/E_{cat})$ is 0.26 µM for binding of AA to $E_{allo}$ 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 0.036 µM of unreacted AA remains. Thus, the molar ratio of stearic acid or PA to AA to achieve 50% displacement of AA from $E_{allo}$ is about 30. From this, we estimate that the $K_d$ value for binding of these nonsubstrates FAs to $E_{allo}$ site is about 30 times higher than that for AA or ~7.5 µM.

As noted in Fig. 5, the $K_m$ values for AA oxygenation in the absence and presence of 2.5 µM PA were 8.4 and 3.5 µM, respectively (data not shown). We assume that the $K_d$ value for AA to $E_{cat}$ is 8.4 µM when AA is bound to $E_{allo}$ and that the $K_d$ value for AA binding to $E_{cat}$ is something less than 3.5 µM when PA is bound to $E_{allo}$. We cannot ascertain an accurate value for the $K_d$...
for AA when $E_{\text{allo}}$ is completely occupied by PA because the CMC for PA in the assay solution is about 25 $\mu$M, conditions under which more than 50% of $E_{\text{allo}}$ would be occupied by AA.

**Binding of PA to muPGHS-2 in muPGHS-2/PA Co-crystals**—PA stimulated the COX activity of muPGHS-2. The potency of PA was similar with muPGHS-2 and huPGHS-2, but maximal stimulation was 43% with muPGHS-2 versus 76% with huPGHS-2 using 5 $\mu$M AA and 25 $\mu$M 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 include 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 (r.m.s.d.) between monomer A and monomer B is 0.28 Å (for 549 C atoms) 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 under “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 $\omega$-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 Ne and N$\eta2$ atoms of Arg-120 and hydrogen bonds to the phenolic oxygen atom of Tyr-355 and the Ne and N$\eta2$ atoms of Arg-120. As PA is only 16 carbons in length, the $\omega$-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 (Fig. 6) (23, 41–43). Instead, two well ordered water molecules occupy the groove, which are analogous to the water molecules occupying the groove when AA and eicosapentaenoic acid 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 $\omega$-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 nonsubstrate 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. 7, A and B). Celecoxib, indomethacin, and diclofenac caused complete or near com-

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**FIGURE 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. $2F_o - F$, simulated annealing omit map electron density, contoured at 4σ, 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).
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 O$_2$ consumption determined using an O$_2$ 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 O$_2$...
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 10-s 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 s 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 10 s 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 nonsubstrate FAs on the time-dependent inhibition by the different inhibitors (Fig. 7, D–H). 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, and 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 PGH2 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 FAs 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 ($K_d = 88$ 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 nonsubstrate FAs such as PA or by two COX inhibitors celecoxib or naproxen, which, as discussed below, appear to bind the $E_{cat}$ and $E_{allo}$ sites, respectively, of huPGHS-2. Adding confusion to the question of the heme stoichiometry are the results of x-ray studies of PGHS crystals. Crystallizations have generally been performed with $75$ μM enzyme and $150$ μM 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 $E_{cat}$ and the partner monomer, i.e. the allosteric monomer, as $E_{allo}$ (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 $E_{cat}$ with a higher affinity for heme and an $E_{allo}$ with a lower affinity for heme).

The fact that occupancy of the $E_{cat}$ and $E_{allo}$ 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 $E_{cat}$ and the other as $E_{allo}$.

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

A confusing question that arose from earlier studies of half sites of 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 $E_{allo}$ and the native monomer is $E_{cat}$.

We previously reported that various substrate and nonsubstrate FAs affect the activities of PGHS-1 and PGHS-2 with AA as substrate (24). The earlier results indicated that nonsubstrate FAs like PA and $20:1:09$ bind to one monomer, an allosteric monomer $E_{allo}$, and affect the affinity of AA for the partner, catalytic monomer $E_{cat}$. Several new lines of evidence provide further support for this concept. For example, we observed in kinetic experiments performed with high huPGHS-2 to AA ratios that AA is incompletely oxygenated, but when nonsubstrate 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 $E_{cat}$ and $E_{allo}$. The results are consistent with the idea that AA binds to $E_{allo}$, with a significantly higher affinity than to $E_{cat}$ and when bound to $E_{allo}$, AA is not oxygenated. Adding a nonsubstrate FA like PA displaces AA from $E_{allo}$ making AA available for oxygenation by $E_{cat}$. Nonsubstrate FAs do not bind with appreciable affinities to $E_{cat}$ at least when a nonsubstrate FA is bound to $E_{allo}$, and so nonsubstrate FAs do not interfere with AA oxygenation by $E_{cat}$.

Our crystallographic results also provide support for the model in Fig. 5 as well as insights into how nonsubstrate FAs can bind to $E_{allo}$. 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 $E_{allo}$ and $E_{cat}$, 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 $E_{allo}$. 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 ovine PGHS-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.

4 H. Zou and W. L. Smith, unpublished observations.
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 the AA or PA binding is affected in a native Y385F huPGHS-2 heterodimer.

We have found that there is a specificity to the actions of nonsubstrate FAs on huPGHS-2. PA is the most efficacious FA activator of the enzyme, although nonsubstrate FAs having either fewer or greater numbers of double bonds are less effective activators of the enzyme. We hypothesize that prostanoid 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 (45, 46). We imagine that having a FA tone outside some normal range would change prostanoid 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 nonsubstrate FAs are less than 2-fold. The effects are on the $K_m$ value for AA (24) and that the $K_m$ value is in the range of the expected apparent concentrations of free AA following cell stimulation. Although the magnitudes of the FA effects are relatively small, they are in the range of physiological 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 nonsubstrate FAs and between PGHSs and COX inhibitors, it was not unexpected that nonsubstrate 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 pharmacological effect of inhibitors that bind this monomer. Secondarily, 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 nonsubstrate FAs and time-dep-
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. Nonsubstrate FAs, which bind $E_{allo}$ with reasonably high affinities, are observed to attenuate the actions of these inhibitors. We propose that this occurs because nonsubstrate 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 whether 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 nonsubstrate 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 (15R)-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, nonsubstrate 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 prostanoid formation. Similarly, the FA tone would be expected to affect responses to COX inhibitors. These drugs are the most widely used pharmaceutical agents in the United States, but they have adverse side effects responsible for an estimated 20,000 deaths in the United States 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 nSSAIIds or COX-2-specific inhibitors would vary among groups of individuals on typical Western versus Mediterranean versus $\omega$3 fish oil-enriched diets. The differences may well be drug-specific. Dietary studies need to be performed to address these issues.
Allosteric Regulation of Cyclooxygenase-2

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