Cyclooxygenase Allosterism, Fatty Acid-mediated Cross-talk between Monomers of Cyclooxygenase Homodimers*  

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Prostaglandin endoperoxide H synthases (PGHSs) 1 and 2, also known as cyclooxygenases (COXs), catalyze the oxygenation of arachidonic acid (AA) in the committed step in prostaglandin (PG) biosynthesis. PGHSs are homodimers that display half of sites COX activity with AA; thus, PGHSs function as conformational heterodimers. Here we show that, during catalysis, fatty acids (FAs) are bound at both COX sites of a PGHS-2 dimer. Initially, an FA binds with high affinity to one COX site of an unoccupied homodimer. This monomer becomes an allosteric monomer, and it causes the partner monomer to become the catalytic monomer that oxygenates AA. A variety of FAs can bind with high affinity to the COX site of the monomer that becomes the allosteric monomer. Importantly, the efficiency of AA oxygenation is determined by the nature of the FA bound to the allosteric monomer. When tested with low concentrations of saturated and monounsaturated FAs (e.g. oleic acid), the rates of AA oxygenation are typically 1.5–2 times higher with PGHS-2 than with PGHS-1. These different kinetic behaviors of PGHSs may account for the ability of PGHS-2 but not PGHS-1 to efficiently oxygenate AA in intact cells when AA is a small fraction of the FA pool such as during “late phase” PG synthesis.

Prostaglandin endoperoxide H synthases (PGHSs)² also known generically as cyclooxygenases (COXs) convert arachidonic acid (AA), two O₂ molecules, and two electrons from one or more unknown reductants to prostaglandin H₂ (PGH₂) (1–5). The conversion involves two steps. First, the cyclooxygenase activity of PGHSs catalyzes the introduction of two O₂ molecules into the AA backbone forming prostaglandin G₂. Prostaglandin G₂ can be reduced to PGH₂ by the peroxidase (POX) activity of the enzyme. There is a functional interplay between the COX and POX sites in that the heme group at the POX site needs to be oxidized by a peroxide to oxidize Tyr-385 in the active COX site to a tyrosyl radical. The Tyr-385 radical abstracts the 13-proS hydrogen from AA to initiate the COX reaction in the rate-determining step.

PGHSs are homodimers with monomer molecular masses of ~72 kDa (1). Previous studies have indicated that there is cross-talk between the COX activities of the partner monomers and that the COX function exhibits half of sites activity with AA (6). That is, only one monomer of a dimer catalyzes AA oxygenation at any given time. Furthermore, the binding of inhibitors of the 2-phenylpropionic acid class such as flurbiprofen (FBP) or ibuprofen to one COX site is sufficient to inhibit all COX activity of a dimer (6, 7). The existence of half of sites activity establishes that PGHSs are conformational heterodimers when the rate-determining step in catalysis occurs.

Despite exhibiting half of sites activity, there has been little evidence for cooperativity between the two COX sites during substrate turnover, and no physiological importance has been attached to the half of sites activity. However, we recently found in testing PGHS-2 with AA and another COX substrate eicosapentaenoic acid (EPA) that the enzyme showed a marked preference for AA (8). This was not predicted from the kinetic parameters determined for AA and EPA individually with PGHS-2. Also surprising was the observation that PGHS-1 differed from PGHS-2 in that PGHS-1 exhibits no apparent selectivity toward AA in the presence of EPA.

To investigate the mechanistic basis for half of sites COX activity and the basis for the selectivity of PGHS-2 for AA, we sought to identify segments of the interface between the monomers that become altered during ligand binding. We approached this by inserting cysteine residues at positions that appeared to be across from one another in the crystal structures. We determined what substitutions led to cross-linking and then determined which FAs and inhibitors would affect the cross-linking between cysteine residues located on partner monomers of dimers. In the process, we found that fatty acids (FAs) can bind to both COX sites of a dimer at the same time and that binding of FAs to one COX site can alter the activity of the enzyme at the COX site of the partner monomer. These biochemical findings led us to speculate that the cross-talk between monomers serves as a way for PGHS-2 to exhibit selectivity toward AA even when AA is a minor component of the available FA pool. In contrast, PGHS-1 does not exhibit selectivity toward AA, so during metabolic conditions in which AA is a small fraction of available FAs, PGHS-1 is effectively latent.
**EXPERIMENTAL PROCEDURES**

**Materials**—All fatty acids and their derivatives except 20:1ω9 were purchased from Cayman Chemical Co. Diclofenac, meclofenamic acid, 1,10-phenanthroline, FLAG peptide, FLAG-affinity resin, hemin, 20:1ω9, and FBP were from Sigma. Co3+-protoporphyrin IX was from Frontier Scientific. Horse-radish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were from Bio-Rad. The detergent C10E4 was from Anachem. BCA protein reagent was from Pierce. [1-14C]AA was from American Radiolabeled Chemicals. Hexanes, isopropanol, and acetic acid were HPLC grade from Fisher. 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.

**Synthesis of 5Z,11Z,14Z- and 5Z,8Z,14Z- Eicosatrienoic Acids**—In the first step of the synthesis AA was partially reduced with hydrazine 8.0 g of methyl ester of AA (25 mmol) in 200 ml of ethyl alcohol was mixed with 22 ml of hydrazine monohydrate (0.44 mole, 17.5 eq), and 30 ml of 50% hydrogen peroxide (0.44 mole, 17.5 eq) was added. The reaction mixture was stirred, and its temperature was kept at +15 to +20 °C. After 75 min, 300 ml of distilled water was added to the reaction mixture, which was then acidified to pH 2 with 2 N HCl and extracted with ethyl acetate (2 × 400 ml). The combined organic layers were washed sequentially with distilled water (3 × 300 ml), saturated NaCl (2 × 200 ml) and dried over 50 g of anhydrous Na2SO4 with stirring for 30 min. The resulting extract was evaporated under vacuum, and the dry residue was purified by column chromatography on 100 g of silica gel (40–63 μm) eluting with 5% ethyl acetate in 95% hexane (v/v). Preparative reversed-phase HPLC was used to separate the fatty acid methyl esters (7.34 g) present in the resulting colorless oil mixture of fatty acid methyl esters; the HPLC conditions were as follows: column Phenomenex Luna-C18(2) (22 × 250 mm), 5 μm with a mobile phase of acetonitrile-water-acetic acid (65:35:0.1, v/v) and a flow rate of 30 ml/min. The methyl esters were then saponified with KOH in ethanol-water to generate the free fatty acid as described recently (9). The elution order of the product fatty acids, determined by analytical HPLC on a C18 reversed-phase column (Phenomenex Luna-C18(2), 4.6 × 250 mm, 5 μm), with a mobile phase of acetonitrile-water-acetic acid (65:35:0.1, v/v) and flow rate of 1.5 ml/min, was: dithomo-γ-linolenic acid, 5Z,11Z,14Z-eicosatrienoic acid (k’ = 6.3 min), 5Z,8Z,14Z- isomer (k’ = 6.6 min), and then Mead acid.

The structure assignments of the isolated fatty acids were based on the following observations. Neither isomer exhibited any UV-absorption above 210 nm indicating that the double bonds are not conjugated and in their Fourier transform-IR spectra (film) no absorption band for a trans-double bond was detected (800–1000 cm⁻1). 1H and 13C NMR analyses did not permit us to distinguish between the isomers. Both the isomers produced only individual single peaks with different retention times when examined by capillary gas-liquid chromatography analysis on three different stationary phases with various polarities: Restec Rxi-1 (90 m, 0.25 mm inner diameter, 0.25 μm); Agilent HP-Innowax (30 m, 0.32 mm inner diameter, 0.25 μm), and Restec RT-2560 (100 m, 0.25 mm inner diameter, 0.2 μm). The positions of the double bonds in the isomers were located by gas chromatography/mass spectrometry analysis of the pyrrolidines derived from them according to the procedure described previously (10). Thus, in the mass spectrum of the pyrrolidine of 5Z,11Z,14Z-eicosatrienoic acid, we observed a relatively strong signal for the molecular ion at the predicted m/z of 359 (5%) and a set of signals of diagnostic fragmentations at m/z (1%) of 181 (4%), 195 (3%), and 208 (3%); these data establish that there is no double bond at the Δ8 position of this isomer. In contrast, we observed in the mass spectrum of pyrrolidine of 5Z,8Z,14Z-eicosatrienoic acid a set of signals of diagnostic fragmentations at m/z (1%) of 180 (4%), 192 (2%), and 206 (5%) that clearly demonstrate the absence of a double bond at the Δ11 position of this isomer (presence of the molecular ion at the predicted m/z of 359 (5%) proves the structure assigned).

**Synthesis of 14Z-Eicosenoic Acid**—14Z-Eicosenoic acid was prepared by Wittig coupling between the methyl ester of 14-oxotetradecanoic acid and commercially available n-hexyltriphenylphosphonium bromide using sodium hexamethyldisilazide as a base. The methyl ester of 14-oxotetradecanoic acid was synthesized from commercially available 12-bromo-1-dodecanol by C2-elongation using the technique reported earlier (11) and subsequent oxidation of the resulting methyl ester of 14-hydroxytetradecanoic acid to the target compound with pyridinium chlorochromate in dichloromethane (12).

14Z-Eicosenoic acid was synthesized from 14-oxotetradecanoic acid as follows: to a suspension of 9.2 g (21 mmol, 1.1 eq) of n-hexyltriphenylphosphonium bromide in 200 ml of dry tetrahydrofuran at −30 °C (acetone-dry ice bath), 12.2 ml of a 2 M solution of sodium hexamethyldisilazide (24 mmol, 1.25 eq) in tetrahydrofuran was added with stirring under dry nitrogen. The resulting orange solution was stirred for 30 min, and after that the flask was cooled to −78 °C and 5.0 g of the methyl ester of 14-oxotetradecanoic acid (19.5 mmol) in 25 ml of tetrahydrofuran was added dropwise. The reaction mixture was stirred at −78 °C for 45 min after which the bath was removed and the reaction mixture was stirred for additional 45 min, evaporated under vacuum and the residue was extracted with hexane (4 × 100 ml). The combined hexane layers were evaporated under vacuum and purified by column chromatography on 50 g of silica gel (40–63 μm) eluting with 3% ethyl acetate in 95% hexane (v/v). The fraction containing methyl ester of the target 14Z-eicosenoic acid were combined and evaporated under vacuum to yield 3.32 g (52%) of a mixture of methyl 14Z-eicosenoate (94% by gas-liquid chromatography) and methyl 14Z-eicosanoate (5% by gas-liquid chromatography).

The methyl esters were saponified with KOH in ethanol-water as described previously for polysaturated fatty acids (9) and separated by preparative HPLC on a C18-reverse phase column (22 × 250 mm, 5 μm) with a mobile phase of acetonitrile-water-acetic acid (85:15:0.1, v/v) and a flow rate 30 ml/min to yield 98% of the target fatty acid as a colorless wax.

**Construction of pFastBac PGHS-2 Mutations or pFastBac Dual for Heterodimer Expression**—All human (hu) PGHS-2 mutations were performed using a modified pFastBac vector (Invitrogen). A QuikChange site-directed mutagenesis kit (Stratagene) was used to create mutations, which were con-
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firmed by sequencing the University of Michigan DNA Sequencing Core. The construction of expression vectors encoding PGHS-2 heterodimers was performed in two steps (6). In the first step the desired DNA fragments with hexahistidine (His$_6$) tags were harvested from pFastBac using Stul-KpnI and subcloned into the PpH$_{10}$ site of pFastBacDual, which had been digested with SmaI-KpnI. In the second step, the other DNA fragment with a FLAG epitope tag also derived from pFastBac by treatment with EcoRI-HindIII was subcloned into the PpH$_{11}$ site of pFastBacDual, which had been cleaved with the same restriction enzymes. The correct constructs were confirmed by restriction digestion.

PGHS-2 Expression and Purification—Protein expression from pFastBac or pFastBacDual expression vectors was performed according to the instructions of the manufacturer for the Invitrogen Bac-to-Bac expression system. SF21 cells at a density of 1.2–1.5 × 10$^6$ cells/ml were infected with recombinant virus at a multiplicity of infection of ~0.1. After expression for 3–4 days, the cells were harvested, washed with phosphate-buffered saline, and stored at −80 °C.

The following steps were all performed at 4 °C. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, with 100 mM KCl) containing Complete EDTA-free Protease Inhibitor and disrupted by sonication. C$_{10}$E$_6$ was added (0.8% v/v), and solubilization was performed with gentle shaking at 4 °C for at least 1 h. Insoluble material was removed by centrifugation at 10,000 × g for 10 min, and the resulting supernatant was centrifuged at 100,000 × g for 1 h. The resulting supernatant was adjusted to 300 mM KCl, 2 mM imidazole, and 5% glycerol and incubated with fast-flow nickel-nitrilotriacetic acid resin pre-equilibrated with Buffer A (20 mM Tris-HCl, pH 8.0, containing 300 mM KCl, 5 mM imidazole, 5% glycerol, and 0.1% C$_{10}$E$_6$) for 1–12 h at 4 °C with gently shaking. The protein-resin slurry was then poured onto a column and washed sequentially with Buffer B (20 mM Tris-HCl, pH 8.0, containing 1M KCl, 20 mM galactose, 20 mM imidazole, 5% glycerol and 0.1% C$_{10}$E$_6$) and Buffer C (20 mM Tris-HCl, pH 8.0, containing 40 mM KCl, 40 mM imidazole, 5% glycerol, and 0.1% C$_{10}$E$_6$). Finally, the proteins were eluted with Buffer D (20 mM Tris-HCl, pH 8.0, containing 40 mM KCl, 250 mM imidazole, 5% glycerol, and 0.1% C$_{10}$E$_6$) and buffer-exchanged with buffer E (20 mM Tris-HCl, pH 8.0, containing 40 mM KCl and 0.1% C$_{10}$E$_6$).

For purification of heterodimers, solubilized supernatants were prepared as described above for the purification of homodimers. Subsequently, a two-step process was used to purify the heterodimers. First, the solubilized supernatant was purified using chromatography on fast-flow nickel-nitrilotriacetic acid resin. Second, the nickel-nitrilotriacetic acid elution fraction, which contained doubly His$_6$-tagged homodimers and heterodimers that were both FLAG- and His$_6$-tagged, was subjected to buffer-exchange with Buffer F (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl) and further purified using FLAG affinity resin following the protocol of the manufacturer. PGHS-2 heterodimers containing both FLAG and His$_6$ tags were eluted with FLAG peptide (100 µg/ml) in Buffer F. All protein eluants were subjected to buffer-exchange with buffer E and kept at 4 °C. Protein purity was estimated visually by SDS-PAGE and staining with Coomassie Brilliant Blue G-250. Protein concentrations were determined using BCA reagent with bovine serum albumin as a standard.

Disulfide Cross-linking between Monomers of PGHS-2—Before cross-linking all purified PGHS-2 variants containing cysteine substitutions (1 µM of monomer) were reconstituted with a 1.5 molar excess of Co$_{3}$$^{3+}$-protoporphyrin IX. Cross-linking to form disulfide bonds was initiated by making the solution to 250 µM Cu(II)/(1,10-phenanthroline)$_2$ (i.e. 250 µM CuSO$_4$ plus 625 µM 1,10-phenanthroline) at room temperature for various times. To test the effects of various ligands (FAs or inhibitors) on cross-linking, the proteins were preincubated with different ligands for 10 min. The reactions were stopped by adding SDS-loading buffer (Invitrogen) containing 20 mM N-ethylmaleimide and 20 mM EDTA (and no reductants). The reaction mixtures were subjected to SDS-PAGE using 7% or 10% gels, and immunoblotting was performed with a rabbit polyclonal antibody reactive with against huPGHS-2 (13).

Determination of Critical Micelle Concentrations—CMC values for AA and several other FAs were determined using a fluorescent dye and/or isothermal titration calorimetry. Using a fluorescent dye, a series of concentrations of AA were prepared in 100 mM sodium phosphate, pH 7.6, containing 5 µM 1,6-diphenyl-1,3,5-hexatriene. The samples were incubated in the dark at room temperature for 30 min before fluorescence measurements were performed. The excitation wavelength for 1,6-diphenyl-1,3,5-hexatriene was 358 nm, and the intensities at 430 nm were recorded. CMC values were estimated as described by Chattopadhyay and London (14). Microcalorimetry for determination of CMC values was performed on a VP-Isothermal Titration Calorimeter (MicroCal, Amherst, MA) with a comput- er-controlled stirrer syringe. Typically, 8-µl aliquots of a FA suspension in 100 mM sodium phosphate, pH 7.6, were injected into a 1.42-ml reaction chamber containing the same buffer at 37 °C. The dissociation of FAs from the injected micelles was monitored as the heat of de-micellization. At a concentration unique for each FA, the dissociation into individual FAs from the injected micelles no longer occurs and CMC values can be calculated.

COX and Peroxidase Activity Measurements—Oxygen electrode assays for COX activity were performed as detailed in previous reports (6, 15, 16). COX assays of purified enzymes utilizing radio-TLC assays of PGH$_2$ formation were performed using [1-$_{14}$C]AA as described previously (16). POX activity was measured spectrophotometrically using 0.1 mM H$_2$O$_2$ and 4 mM guaiacol as substrates (6).

RESULTS

Effects of FAs on COX Activities of PGHSs—Tables 1 and 2 compare the effects of various FAs, some COX substrates and some not, on the COX activities of purified ovPGHS-1 and huPGHS-2 with AA. In Table 1, a combination of 100 µM FA and 20 µM AA was assayed for COX activity polarographically with an O$_2$ electrode and, in some cases, by radio-TLC by meas- uring the conversion of [1-$_{14}$C]AA to oxygenated, radioactive products. The data in Table 2 are from O$_2$ electrode measurements only and were performed with lower concentrations of FAs (0.5–1.0 µM) and AA (2 µM). The polarographic assay is accurate but does discriminate between oxygenation of AA versus the comparator FA, if the comparator FA is also a COX
Effects of high concentrations of various FAs on AA oxygenation by PGHS-1 versus PGHS-2

Results are shown as percentages of rates for O₂ electrode assays of COX activities of PGHSs with FAs alone (100 μM) and FAs (100 μM) in combination with AA (20 μM) except in the case of Δ⁵,8,11,14,17-20:5, which was tested at a concentration of 40 μM. Results from radio-TLC assays are also shown for certain FAs (100 μM) in combination with [1-14C]AA (20 μM). Details of the experiments are provided under “Experimental Procedures.” The average specific activities of preparations used in the experiments of purified ovPGHS-1 and huPGHS-2 with AA averaged 40 and 42 nmol of O₂ consumed per min per mg of protein, respectively, under standard assay conditions. Values are from at least triplicate assays, except for Mead acid and Δ⁵,8,11,14,17-20:5, which was assayed in duplicate, using different preparations of purified proteins.

An unpaired Student’s t-test, and standard errors were calculated using Microsoft Excel. ND, not determined.

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### TABLE 1

Effects of low concentrations of nonsubstrate FAs on AA oxygenation by PGHS-1 versus PGHS-2

Table presents the average specific activities of preparations used in the experiments of purified ovPGHS-1 and huPGHS-2 with AA averaged 40 and 42 nmol of O₂ consumed per min per mg of protein, respectively, under standard assay conditions. Values are from at least triplicate assays, except for Mead acid and Δ⁵,8,11,14,17-20:5, which was assayed in duplicate, using different preparations of purified proteins.

An unpaired Student’s t-test, and standard errors were calculated using Microsoft Excel. ND, not determined.

### TABLE 2

Effects of low concentrations of nonsubstrate FAs on AA oxygenation by PGHS-1 versus PGHS-2

Table presents the average specific activities of preparations used in the experiments of purified ovPGHS-1 and huPGHS-2 with AA averaged 40 and 42 nmol of O₂ consumed per min per mg of protein, respectively, under standard assay conditions. Values are from at least triplicate assays, except for Mead acid and Δ⁵,8,11,14,17-20:5, which was assayed in duplicate, using different preparations of purified proteins.

An unpaired Student’s t-test, and standard errors were calculated using Microsoft Excel. ND, not determined.
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FIGURE 1. Concentration dependence of the effect of 20:1ω9 on the oxygenation of AA by huPGHS-2. The cyclooxygenase activity of purified huPGHS-2 was assayed using a standard O2 electrode assay as detailed under “Experimental Procedures” with or without 10 μM 20:1ω9 at the indicated concentrations of AA. This experiment was performed three times with similar results. Error bars indicate average ± S.E. for the results from a single experiment involving triplicate determinations.

TABLE 3

Cross-linking between PGHS-2 monomers having cysteine residues substituted into the dimer interface

The indicated mutant proteins were expressed in baculovirus, purified, and assayed for COX specific activities as described under “Experimental Procedures.” Cross-linking (i.e. disulfide bond formation) between monomers was monitored by the appearance of higher molecular mass species following treatment with the oxidant Cu2+/o-phenanthroline and SDS-PAGE in the absence of a reducing agent as detailed under “Experimental Procedures.” Values for specific activities are averages of two determinations that varied by <10%. Mutants for which cross-linking was not observed or cross-linking was not inhibited by FBP were examined only one time. +, cross-linking occurred; −, cross-linking did not occur.

| huPGHS-2 variant | Starting COX specific activity | Cross-linking | Inhibited by FBP |
|------------------|--------------------------------|---------------|------------------|
| Native           | 100                            | −             | Yes              |
| C313S C540S (ΔC) | 100                            | +             | ND               |
| V50C E322C ΔC    | 63                             | +             | ND               |
| S138C L334C ΔC  | ND*                            | −             | ND               |
| S126C ΔC         | 91                             | +             | Yes              |
| A543C ΔC         | 88                             | −             | ND               |
| S126C A543C ΔC  | 65                             | +             | Yes              |
| P127C ΔC         | 80                             | −             | ND               |
| S541C ΔC         | 100                            | +             | ND               |
| P127C S541C ΔC  | 56                             | +             | Yes              |

* ND, not determined.

changed this profile of radioactive products. We emphasize this because it has been reported (19) that very high (i.e. mM) concentrations of non-substrate FAs in combination with AA cause a relative increase in the formation of hydroxyeicosatetraenoic acids from AA via PGHS-1.

Overall, the data in Tables 1 and 2 suggest that PGHS-2 oxygenates AA more efficiently than does PGHS-1 when common FAs other than adrenic acid are present. ovPGHS-1 was compared with huPGHS-2 because of the ease of handling of these particular enzyme forms. The FA substrate specificity of mesosomal huPGHS-1 when tested with AA, dihomo-γ-linolenic acid, EPA, linoleic acid, and α-linolenic acid exhibits relative Vmax values (20) that are very similar to those found here for ovPGHS-1 (Table 1).

To learn more about the effects of FAs on AA oxygenation by PGHS-2 we tested the effects of 20:1ω9. The CMC for 20:1ω9 was determined to be between 1 and 10 μM (data not shown), and values in this range would also be anticipated for palmitic acid and oleic acid. Both 1 μM (e.g. Table 2) and 10 μM 20:1ω9 (Fig. 1) had similar effects on the oxygenation of AA by PGHS-2 consistently causing a 2- to 3-fold decrease in the Km for AA with and without 10 μM 20:1ω9 calculated from the data in Fig. 1 was 3.6 μM versus 9.2 μM, respectively. The stimulatory effect of 20:1ω9 on PGHS-2 was overcome as the concentration of AA was increased. A smaller (<20%) and non-significant decrease in the Km for AA was observed when 20:1ω9 was tested with PGHS-1 (e.g. Tables 1 and 2).

PGHSs undergo a substrate turnover-dependent, reaction-based suicide inactivation (15). In principle, performing a reaction with AA in the presence of another FA like 20:1ω9 could diminish the rate of suicide inactivation and have the effect of increasing the initial rate of AA oxygenation. However, there was no significant difference between the first order rates of inactivation of huPGHS-2 with 20 μM AA in the presence or absence of 100 μM 20:1ω9: average t1/2 values of 17 s and 19 s, respectively.

Previous studies of PGHS dimers have indicated that the enzymes exhibit half of sites catalytic activity (6, 7) and that the monomers are tightly associated (21). Thus, PGHSs must be conformational heterodimers during catalysis and inhibition and cross-talk must occur between partner monomers. Viewed in this context, our kinetic data suggest that the effects of FAs on PGHSs result from the binding of these FAs to one COX active site of a PGHS dimer to affect the catalytic efficiency of the partner monomer with AA. This concept is supported by the results of experiments described below in which we characterized cross-talk between PGHS-2 monomers.

Effects of Ligands on the Cross-linking of PGHS-2 Monomers—To delineate the location(s) at the dimer interface where cross-talk occurs, we first prepared a C313S C540S PGHS-2 mutant (ΔC-huPGHS-2) that lacks free cysteine residues, then substituted pairs of cysteines at five different positions along the interface between monomers and finally examined disulfide bond formation between monomers promoted by an oxidant Cu²⁺/o-phenanthroline (CuP) (Table 3). The five locations where cysteines were introduced were chosen on the basis of PGHS crystal structures (22, 23) where it appeared that cross-linking might occur. The cysteine-less mutant ΔC-huPGHS-2 expressed well and had the same specific COX activity as native
FIGURE 2. FBP attenuates cross-linking between monomers of P127C S541C ΔC-huPGHS-2. P127C S541C ΔC-huPGHS-2 was expressed in a baculovirus system, purified, and subjected to cross-linking with Cu2+/o-phenanthroline for the indicated times in the presence or absence of 100 μm FBP. Samples were subjected to SDS-PAGE in the absence of a disulfide reducing agent (i.e. dithiothreitol), and Western blotting was performed using an anti-PGHS-2 antibody. Additional experimental details are provided under “Experimental Procedures.” The nature of the cross-linked forms is indicated diagrammatically, and assignments of the immunoreactive bands are based on experimental data presented in Fig. 3. Staining intensities for the uppermost, middle, and lowest bands relative to one another within each lane were estimated using ImageJ open source software (National Institutes of Health).

FIGURE 3. Cross-linking between monomers of S126C A543C ΔC-huPGHS-2 and between variants of P127C S541C ΔC-huPGHS-2 in which P127C and S541C substitutions are present in either one or both monomers. A, S126C A543C ΔC-huPGHS-2 was expressed in a baculovirus system, purified, and subjected to cross-linking with Cu2+/o-phenanthroline for 2 h. The samples were subjected to one of the following treatments prior to performing SDS-PAGE in the absence of a disulfide reducing agent: Lane 1, no treatment control; lane 2, 6 M urea; lane 3, 100 μm dithiothreitol; and lane 4, 200 μm 2-mercaptoethanol. B, P127C S541C ΔC-huPGHS-2 variants were treated with or without Cu2+/o-phenanthroline for 2 h and then subjected to SDS-PAGE without a disulfide reducing agent. M, protein molecular weight standards; a, native huPGHS-2; b, P127C S541C ΔC-huPGHS-2 (all substitutions in both monomers); c, P127C ΔC-/S541C ΔC-huPGHS-2 (P127C substitution in one monomer and S541C substitution in the partner monomer); A, native huPGHS-2 following cross-linking; B, P127C S541C ΔC-huPGHS-2 following cross-linking; and C, P127C ΔC-/S541C ΔC-huPGHS-2 following cross-linking. Samples were visualized by staining with Coomassie Brilliant Blue G-250.

enzyme. Mutants designed to explore cross-linking between two of the five regions, including T60C S546C ΔC-huPGHS-2 and Q370C Q372C ΔC-huPGHS-2 did not express well in a baculovirus expression system and were not examined further.

An S138C L334C ΔC-huPGHS-2 was expressed, but did not undergo cross-linking in response to CuP. Mutants such as V50C E322C ΔC-PGHS-2 exhibited ~60% of native PGHS-2 activity and underwent CuP-induced cross-linking, but the cross-linking was not affected by FBP. Importantly, substituting cysteine residues at positions 126 or 127 and positions 541 or 543 of each monomer of ΔC-huPGHS-2 led to the formation of dimers, and cross-linking was attenuated by FBP (Table 3 and Fig. 2). As shown in Fig. 2 for P127C S541C ΔC-huPGHS-2, dimers were formed in a time-dependent manner upon treatment with CuP. P127C S541C ΔC-huPGHS-2 had <60% of the activity of native enzyme (Table 3), but the specific activity of P127C S541C ΔC-huPGHS-2 was not affected by CuP-induced cross-linking.

Three immunoreactive bands are observed with P127C S541C ΔC-huPGHS-2 even before treatment with CuP (Fig. 2). This is because some spontaneous cross-linking occurs between monomers during expression and isolation of the enzyme. The three immunoreactive bands observed in the Western blot panels in Fig. 2 correspond, in order of greatest mobility, to the PGHS-2 monomer and singly and doubly cross-linked PGHS-2 partner monomers. The experimental evidence for this is presented in Fig. 3 and is as follows. The most rapidly migrating band has an apparent molecular mass of 72 kDa corresponding to that expected for a PGHS-2 monomer. The two slower migrating bands found with S126C A543C ΔC-huPGHS-2 collapse into a single 72-kDa band when the protein is treated with 100 μm dithiothreitol or 200 μM 2-mercaptoethanol, but not upon treatment with 6 M urea (Fig. 3A). This indicates that the two more slowly migrating, upper bands in the panels in Figs. 2 and 3A are cross-linked via disulfide bond(s). The more rapidly migrating cross-linked species appears to have a single disulfide bond, because it has the same mobility as a cross-linked ΔC-huPGHS-2 heterodimer prepared such that one monomer contains only the P127C mutation, and the partner has only the S541C mutation (designated P127C ΔC-/S541C ΔC-huPGHS-2 in Fig. 3B). Moreover, following extended treatment with CuP virtually all the monomers of the P127C S541C ΔC-huPGHS-2 homodimer are converted to doubly cross-linked dimers (Figs. 2 and 3B). In the case of the P127C S541C ΔC-PGHS-2, the activity was ~55% of native enzyme, and treatment with CuP did not reduce activity; with S126C A543C ΔC-PGHS-2, the activity was 65% that of native enzyme and cross-linking caused a 60% decrease in activity. Importantly, AA oxygenation by P127C S541C ΔC-PGHS-2 was stimulated in a similar though less robust
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FIGURE 4. Concentration dependence of the effect of 20:1ω9 on the oxygenation of AA by P127C SS41C ΔC-huPGHS-2. The cyclooxygenase activity of purified P127C SS41C ΔC-huPGHS-2 was assayed using a standard O2 electrode assay as in Fig. 1 with or without 10 μM 20:1ω9 at the indicated concentrations of AA. The experiments were performed three times with similar results. Error bars indicate average ± S.E. for the results from a single experiment involving triplicate determinations.

FIGURE 5. Relative specific COX and POX activities of native/native, native/G533A, and G533A/G533A P127C SS41C ΔC-huPGHS-2 dimers. The indicated homo- and heterodimeric versions of P127C SS41C ΔC-huPGHS-2 were expressed and isolated as described under “Experimental Procedures.” COX activity was measured using an oxygen electrode with 100 μM AA as the substrate, and POX activity was measured spectrophotometrically using 0.1 mM H2O2 and 4 mM guaiacol as substrates. The error bars indicate the average ± S.E. for the results from a single experiment involving triplicate determinations.

manner as native huPGHS-2 by 20:1ω9 (Fig. 4). The Kₘ values for P127C SS41C ΔC-huPGHS-2 with AA were 9.1 μM and 14 μM with and without 20:1ω9, respectively; we suspect that the lesser kinetic effect of 20:1ω9 on the P127C SS41C ΔC-huPGHS-2 mutant is because a fraction of this mutant (∼30–50%) has already become cross-linked during its isolation. Importantly, a P127C SS41C ΔC-/G533A P127C SS41C ΔC-huPGHS-2 heterodimer that has only one COX-active subunit, had >80% of the COX activity as the P127C SS41C ΔC-huPGHS-2 homodimer (Fig. 5). This indicates that P127C SS41C ΔC-huPGHS-2, like native huPGHS-2 exhibits half of sites COX activity with AA (6). Because of its kinetic similarity to native huPGHS-2, P127C SS41C ΔC-huPGHS-2 was used for most of our subsequent cross-linking studies.

Certain COX-2 substrates, including AA and EPA, attenuated cross-linking at concentrations at or near their Kₘ values; however, linoleic acid and docosahexaenoic acid were ineffective (Fig. 6A). Oleic acid, which is not a COX substrate, also was without any effect on cross-linking. Aspirin (2 mM) had relatively little effect on cross-linking, but all other common nonsteroidal anti-inflammatory drugs (NSAIDs), which are competitive cyclooxygenase inhibitors, did inhibit Cu²⁺/o-phenanthroline for 2 h in the presence or absence of a FA at the indicated concentration (μM) (A) or an NSAID at the indicated concentration (μM) (B) except for aspirin (2 mM). Samples were subjected to SDS-PAGE in the absence of a disulfide reducing agent, and Western blotting was performed using an anti-PGHS-2 antibody as described under “Experimental Procedures.” AA, arachidonic acid; LA, linoleic acid; OA, oleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. The negative control for A also applies to B as all samples were run in parallel. Staining intensities for the uppermost, middle, and lowest bands relative to one another within each lane were estimated using ImageJ open source software.
FIGURE 7. Δ\textsuperscript{11,14}-20:1 interferes with the ability of AA to attenuate oxidant-induced cross-linking of between monomers of P127C S541C ΔC huPGHS-2. P127C S541C ΔC huPGHS-2 was expressed in a baculovirus system, purified, and subjected to cross-linking by Cu\textsuperscript{2+}/\textit{o}-phenanthroline for 2 h as indicated in the absence of no FA, 5 μM AA, 50 μM 20:1ω9, or 5 μM AA plus 50 μM 20:1ω9. Samples were subjected to SDS-PAGE in the absence of a disulfide reducing agent, and Western blotting was performed using an anti-huPGHS-2 antibody as described under “Experimental Procedures.” Staining intensities for the uppermost, middle, and lowest bands relative to one another within a lane were estimated using ImageJ open source software.

Table 4

| FA                  | Cross-linking in presence of 50 μM FA | Cross-linking in presence of 50 μM FA and 5 μM AA |
|---------------------|--------------------------------------|-----------------------------------------------|
| No FA               | +                                    | ND                                            |
| 16:0                | ND                                   | +                                             |
| 18:0                | ND                                   | +                                             |
| 18:1ω9              | +                                    | ND                                            |
| 18:2ω6 (LA)         | +                                    | ND                                            |
| 18:3ω3              | +                                    | ND                                            |
| 18:3ω6              | –                                    | NA                                            |
| 18:4ω3              | –                                    | NA                                            |
| 20:1ω6              | +                                    | +                                             |
| 20:2ω6              | +                                    | ND                                            |
| 20:3ω3              | +                                    | ND                                            |
| 20:3ω6              | –                                    | NA                                            |
| 20:3ω9              | –                                    | NA                                            |
| Δ\textsuperscript{11,14}-20:3 | –                                | NA                                            |
| Δ\textsuperscript{11,14}-20:3 | –                                | NA                                            |
| 20:4ω3              | –                                    | ND                                            |
| 20:4ω6 (AA)         | –                                    | ND                                            |
| 20:5ω3 (EPA)        | –                                    | NA                                            |
| 22:4ω6              | ND                                   | ND                                            |
| 22:6ω3 (DHA)        | +                                    | ND                                            |
| 2-Arachidonylethanolamide | +                                | ND                                            |
| Methyl arachidonate | +                                    | ND                                            |

* NA, not applicable.  
\* ND, not determined.

cross-linking by NSAIDs and substrates were the same whether the cross-linking reactions were performed in the presence or absence of Co\textsuperscript{3+}-heme; Co\textsuperscript{3+}-heme can substitute for native heme in binding to the POX site of PGHSs, but the enzyme is inert because Co\textsuperscript{3+}-heme cannot undergo the oxidation reaction necessary to activate the enzyme (24).

Several FAs that are not COX substrates, including 20:1ω9, failed to inhibit cross-linking when tested alone but interfered with the ability of AA to prevent cross-linking (Fig. 7 and Table 4). Under conditions in which 20:1ω9 interferes with the ability of AA to prevent cross-linking (Fig. 7), 20:1ω9 stimulates AA oxidant-induced oxygenation (Figs. 1 and 4). These findings imply that AA is able to prevent cross-linking only if bound to both COX sites of PGHS-2 and that during catalysis AA is bound at both COX sites, although it is only being oxygenated at one site at any given time; moreover, when 20:1ω9 and AA are incubated together with PGHS-2 at the appropriate concentrations, AA is oxygenated at the COX site of one PGHS-2 monomer while 20:1ω9 is bound to the COX site of the partner monomer.

A number of free FAs as well as esters and amides of AA were tested both alone and in combination with AA for their abilities to block Cu\textsuperscript{2+}-induced cross-linking of P127C S541C ΔC-PGHS-2 in experiments like the one depicted in Fig. 7. The data are summarized in Table 4. Most straight-chain FAs having three or more double bonds and 18–22 carbons inhibited cross-linking when tested alone. Conversely, FAs having only one or two double bonds did not by themselves inhibit cross-linking. However, most of these latter FAs, including palmitic acid and oleic acid, did prevent AA from inhibiting cross-linking. Esters, ethers, and amides of AA by themselves failed to inhibit cross-linking at concentrations as high as 100 μM. The lack of protection from cross-linking observed with 2-AG suggests that there are differences in the ways that 2-AG and AA, 20:1ω9 and AA and huPGHS-2 interact. Collectively, the cross-linking data in Table 4 suggest that at concentrations of 50 μM all straight-chain FAs having 16–22 carbons can bind to at least one, and in some cases both, COX sites of a PGHS-2 homodimer.

**DISCUSSION**

There is a single high affinity binding site on the PGHS-2 dimer for NSAIDs of the 2-phenylpropionate class, including FBP and ibuprofen (6), and most NSAIDs inhibit COX activity upon binding one COX site of a PGHS-1 homodimer (7). Based on these observations and because PGHS-2 exhibits half of sites occupied by FAs during AA oxygenation (Fig. 8), there is a single high affinity binding site on the PGHS-2 dimer for NSAIDs of the 2-phenylpropionate class, including FBP and ibuprofen (6), and most NSAIDs inhibit COX activity upon binding one COX site of a PGHS-1 homodimer (7). Based on these observations and because PGHS-2 exhibits half of sites occupied by FAs during AA oxygenation (Fig. 8), one monomer binds a FA with relatively high affinity and acts as an “allosteric” monomer by modulating the catalytic efficiency of its partner “catalytic” monomer.

Our most well developed evidence for the model depicted in Fig. 8 comes from the combination of kinetic and cross-linking studies of PGHS-2 employing 20:1ω9 and AA. 20:1ω9 reduces the $K_m$ for AA in a manner that is most easily explained as 20:1ω9 binding one COX site of a monomer and facilitating AA binding and oxygenation at the other site. Studies of cross-linking between monomers of P127C S541C...
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DeltaC-huPGHS-2 homodimer are also most simply interpreted by having both COX sites occupied by FAs. In the absence of 20:1ω9, AA, at concentrations near its \( K_m \) value with PGHS-2, must bind both COX sites of a dimer simultaneously to prevent cross-linking.

Common FAs that have not heretofore been associated with eicosanoid metabolism, including palmitic acid, stearic acid, and oleic acid, are among the FAs that bind to the allosteric monomer and stimulate AA oxygenation in the catalytic monomer of PGHS-1 and/or PGHS-2 (Tables 1 and 2). The effects of palmitic and oleic acids on the kinetics of AA oxygenation and on cross-linking of P127C S541C DeltaC-huPGHS-2 homodimer are similar to those observed with 20:1ω9 and are readily explained by the model depicted in Fig. 8. Although we have not investigated this in detail, we speculate, based on measuring the effects of FAs on [1-14C]PGH₂ formation from [1-14C]AA that 16:0, 18:1ω9, 20:1ω9, and AA act allosterically to promote the use of any 20- and 22-carbon \( \omega 6 \) FA substrate, including AA, 20:3ω6, and 22:4ω6.

We have not resolved whether (a) FAs that have no apparent effect on AA oxygenation (e.g. EPA) or (b) \( \omega 6 \) FAs that are substrates even in the presence of AA (Table 1) compete effectively with AA for binding to the allosteric site. It is also unclear how certain FAs inhibit PGHS activity. They may inhibit AA oxygenation by binding to the allosteric site or by competing with AA for the catalytic site or both.

The affinity of AA for the allosteric site must be at least somewhat lower than for the catalytic site in order for PGHS-2 to display what appears to be a conventional hyperbolic kinetic curve with AA. Kinetic analysis with 1 and 10 \( \mu \)M 20:1ω9 indicate that this FA has a higher affinity than AA for the allosteric site, but that once bound to the allosteric site, 20:1ω9 does not compete effectively with AA for the catalytic site of PGHS-2.

Although we have uncovered what appears to be a general phenomenon regarding the regulation of PGHS-2 by FAs, we have no clear idea about what structural features of FAs underlie their functions as allosteric regulators nor which protein residues mediate the interplay between the monomers. All PGHS x-ray structures reported to date have been performed with crystals grown at high enzyme (\( \sim 75 \mu \)M) and excess ligand (\( \sim 500 \mu \)M) concentrations (22–28). Ligands are seen to occupy both monomers, and there are no obvious structural differences between the monomers. It is not known if this is because the resolutions are inadequate and the differences between monomers subtle or because the individual monomers represent average structures of two different monomers. There is also no high resolution structure of a ligand-free PGHS useful for comparison with ligand-occupied PGHSs. Thus, we can only speculate about the structural changes that occur upon binding of a FA to the allosteric monomer. It is clear that Pro-127 is located in a loop emanating from a helix involving Arg-120, a residue known to interact with FA substrates and many inhibitors of PGHSs. One can imagine a ligand-induced change in the position of Arg-120 that could, in turn, alter the position of Pro-127 and neighboring residues. This structural change is presumably transmitted to the partner, catalytic monomer so that Tyr-385 and the 13-pro-S-hydrogen of AA become optimally juxtaposed for efficient hydrogen abstraction.

Unlike the situation with PGHS-2, examining cross-talk between monomers of PGHS-1 is technically challenging; for example, a cysteine-less version of this protein that in principle could be employed for cross-linking studies lacks activity (29). We have observed that in the presence of most other common and naturally occurring FAs (i.e. 16:0, 18:0, 18:1ω9, 18:2ω9, and 20:5ω3) PGHS-1 has less activity with AA than does PGHS-2. Kinetic studies with 20:1ω9 and PGHS-1 indicate that 20:1ω9 stimulates PGHS-1 COX activity. Accordingly, we speculate that FAs do bind to an “allosteric” monomer of PGHS-1 but that this binding does not foster the use of 20- and 22-carbon \( \omega 6 \) FA substrates.

We have not established whether allosteric regulation of PGHS-1 and/or PGHS-2 by FAs is physiologically significant. An intriguing possibility is that the differences in the efficiency with which PGHS-1 and PGHS-2 oxygenate AA when AA is present in mixtures of FAs having different compositions may be of physiological importance. For example, PGHS-1 can oxygenate AA efficiently in so-called “early phase” cellular PG synthesis but not in “late phase” synthesis (30–33). In early phase synthesis, only PGHS-1 is present in cells. A bolus of AA is

FIGURE 8. Model for allosteric interactions between monomers of the PGHS-2 homodimer brought about by the binding of substrate and non-substrate fatty acids. The circles depict the two equivalent monomers of PGHS-2 lacking bound FA. Binding of at least certain FAs (e.g. 16:0, 18:0, 18:1ω9, 20:1ω9, or AA) to one monomer causes this monomer to become the allosteric monomer shown as a hexagon. The allosteric monomer causes a change in the structure of the second monomer, which becomes the catalytic monomer shown as a rectangle. The catalytic monomer is able to efficiently oxygenate AA. The model is consistent with results of kinetic and cross-linking studies for the 16:0, 18:0, 18:1ω9, 20:1ω9, and AA; we suggest that EPA behaves similarly in the presence of AA, because it is not a substrate when AA is present. In contrast to 16:0, 18:0, 18:1ω9, 20:1ω9, and EPA, most \( \omega 6 \) FAs served as COX substrates and interfered with AA oxygenation.
mobilized by cytosolic phospholipase A₂, and because of the FA specificity of cytosolic phospholipase A₂, AA probably represents a large fraction of the free FAs available to PGHS-1 in this early phase. However, in late phase synthesis both PGHS-1 and PGHS-2 are present and AA is mobilized in part through the action(s) of nonspecific, secretory phospholipase A₂(s). Under these conditions AA presumably comprises a much smaller part of the free FAs available to PGHS-1 in this phase. However, in late phase synthesis both PGHS-1 and PGHS-2 are present and AA is mobilized in part through the action(s) of nonspecific, secretory phospholipase A₂(s). Under these conditions AA presumably comprises a much smaller part of the free FAs available to PGHS-1 in this early phase but be inhibited by other, competing FAs in the late phase. In contrast, PGHS-2 could catalyze AA oxygenation in the late phase, because PGHS-2 can catalyze AA efficiently on AA in the early phase but be inhibited by other, competing FAs in the late phase. In contrast, PGHS-2 could catalyze AA oxygenation in the late phase, because PGHS-2 can catalyzeAA efficiently on AA in the early phase but be inhibited by other, competing FAs in the late phase. In contrast, PGHS-2 could catalyze AA oxygenation in the late phase, because PGHS-2 can oxygenate AA even in the presence of relatively high concentrations of other FAs (5). Studies with cultured cells under conditions of early and late phase PG will be important in testing these concepts.

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