Structure of Eicosapentaenoic and Linoleic Acids in the Cyclooxygenase Site of Prostaglandin Endoperoxide H Synthase-1*

Prostaglandin endoperoxide H synthases-1 and -2 (PGHSs) can oxygenate 18–22 carbon polyunsaturated fatty acids, albeit with varying efficiencies. Here we report the crystal structures of eicosapentaenoic acid (EPA, 20:5 n-3) and linoleic acid (LA, 18:2 n-6) bound in the cyclooxygenase active site of Co3+/protoporphyrin IX-reconstituted ovine PGHS-1 (Co3+-oPGHS-1) and compare the effects of active site substitutions on the rates of oxygenation of EPA, LA, and arachidonic acid (AA). Both EPA and LA bind in the active site with orientations similar to those seen previously with AA and dihomo-y-linolenic acid (DHLA). For EPA, the presence of an additional double bond (C-17/C-18) causes this substrate to bind in a “strained” conformation in which C-13 is misaligned with respect to Tyr-385, the residue that abstracts hydrogen from substrate fatty acids. Presumably, this misalignment is responsible for the low rate of EPA oxygenation. For LA, the carboxyl half binds in a more extended configuration than AA, which results in repulsion of C-11 next to Tyr-385. Val-349 and Ser-530, recently identified as important determinants for efficient oxygenation of DHLA by PGHS-1, play similar roles in the oxygenation of EPA and LA. Approximately 750- and 175-fold reductions in the oxygenation efficiency of EPA and LA were observed with V349A oPGHS-1, compared with a 2-fold change for AA. Val-349 contacts C-2 and C-3 of EPA and C-4 of LA orienting the carboxyl halves of these substrates so that the α-ends are aligned properly for hydrogen abstraction. An S530T substitution decreases the vmax/Km of EPA and LA by 375- and 140-fold. Ser-530 makes six contacts with EPA and four with LA involving C-8 through C-16; these interactions influence the alignment of the substrate for hydrogen abstraction. Interestingly, replacement of Phe-205 increases the volume of the cyclooxygenase site allowing EPA to be oxygenated more efficiently than with native oPGHS-1.

Prostaglandin endoperoxide H synthase (PGHS)1 converts arachidonic acid (AA) to prostaglandin H2 in the committed step of prostaglandin and thromboxane biosynthesis (1, 2). Two isoforms of PGHS exist in mammalian tissues. PGHS-1 is constitutively expressed and generates prostaglandins in response to hormone stimulation, whereas PGHS-2 is an inducible enzyme that is expressed in response to growth factors, tumor promoters, or cytokines (1–11). Both isoforms are quite similar structurally (12–15) and mechanistically (1, 2, 16), with only subtle kinetic differences in substrate (17, 18) and inhibitor (19, 20) specificities, and hydroperoxide activator requirements (21–23).

Both PGHS-1 and -2 catalyze two different reactions: a cyclooxygenase reaction and a peroxidase reaction. Structural studies indicate that the active sites are spatially distinct from each other and separated by a heme group (12–15). The cyclooxygenase reaction occurs within a hydrophobic channel that extends from the membrane binding domain of the enzyme into the core of the globular domain. The fatty acid substrate is positioned within this site in an extended L-shaped conformation (15, 24). Cyclooxygenase catalysis begins with abstraction of the 13-pro-S hydrogen from AA by a tyrosyl radical centered on Tyr-385 in the rate-determining step to generate an arachidonyl radical (25–27). Two molecules of O2 are then sequentially added to the arachidonoyl radical to form the bicyclic hydroperoxide prostaglandin G2 (PGG2). This intermediate diffuses to the peroxidase active site where the 15-hydroperoxyl group undergoes a two-electron reduction to the alcohol prostaglandin H2. Although the peroxidase activity can function independently of the cyclooxygenase activity (28), activation of the cyclooxygenase requires a functional peroxidase (1, 2, 23). AA is the preferred substrate for PGHS-1 and PGHS-2 (17). Both isoforms will, however, oxygenate a variety of n-3 and n-6 18–22 carbon fatty acids albeit with reduced catalytic efficiencies (17, 29–36). Most of these alternative fatty acid substrates have somewhat higher Km values than AA, but they can compete with AA for binding to the cyclooxygenase active site and thereby inhibit formation of the 2-series prostaglandins that

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‡ Supported by National Institutes of Health National Research Service Award F32 HL10170.

§ To whom correspondence may be addressed: 513 Biochemistry Bldg., Dept. of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824. Tel.: 517-353-9125; Fax: 517-353-9334; E-mail: smithww@pilot.msu.edu.

¶ To whom correspondence may be addressed: 522 Biochemistry Bldg., Dept. of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824. Tel.: 517-353-9125; Fax: 517-353-9334; E-mail: garavito@magma.bch.msu.edu.

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Michael G. Malkowski, Elizabeth D. Thuresson, Karen M. Lakkides, Caroline Jill Rieke, Renée Micielli, William L. Smith§, and R. Michael Garavito

From the Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824
are derived from AA (37–40). Linoleic acid (LA; 18–2 n-6) and eicosapentaenoic acid (EPA; 20:5 n-3) are oxygenated via the cyclooxygenase activity of PGHSs by intact cells both in vitro and in vivo (41–47). Oxygenation of LA produces the monohydroxy acid products 9-hydroxyeicosadienoic acid and 13-hydroxyoctadecadienoic acid. The biological function of these hydroxy acids has not been established. In contrast, EPA, an n-3 essential fatty acid found in relative abundance in fish oil, appears to provide some protection from coronary thrombosis. EPA, like AA, is stored at the sn-2 position of phosphoglycerides and like AA can be released upon activation of phospholipases A₂ (1, 17, 38, 45, 47). For example, in circulating platelets EPA can be converted to the prostanoid PGH₃, which, in turn, can be isomerized to thromboxane A₂ and PGD₂ (1, 7, 38). However, EPA is both a poor substrate for platelet PGHS-1 and a potent competitive inhibitor of AA oxygenation by platelet PGHS-1 (1, 17, 38). Thus, the net effect of increased dietary intake of fish oil is to reduce the production of pro-thrombogenic thromboxane from AA. Low dose aspirin is commonly used to inhibit the platelet PGHS-1. However, understanding how EPA fits into the cyclooxygenase active site of PGHS-1 and competes with AA for binding to this site could lead to the development of more selective anti-thrombogenic agents.

X-ray crystallography and mutational analyses have identified Val-349 and Ser-530 in PGHS-1 as key determinants of specificity for the alternative fatty acid substrate dihomo-γ-linolenic acid (DHHLA; 20:3 ω-6) (24). Here, we extend these findings with additional studies of the cyclooxygenase active site of oPGHS-1 that were designed (a) to determine how two other alternative fatty acid substrates, EPA and LA, bind within the cyclooxygenase active site and (b) to identify active site residues that influence the ability of PGHS-1 to use EPA and LA as substrates.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fatty acids were purchased from Cayman Chemical Co. (Ann Arbor, MI). [1-14C]AA (40–60 mCi/mmol), [1-14C]EPA (50–60 mCi/mmol), and [1-14C]LA (50–60 mCi/mmol) were obtained from New England Nuclear Life Sciences. Restriction enzymes and the manufacturer’s medium were purchased from Life Technologies, Inc., Calf serum and fetal bovine serum were from HyClone Laboratory (Logan, UT). Flurbiprofen was obtained from Sigma. Primary antibodies used for Western blotting were raised in rabbits against purified oPGHS-1 and hPGHS-2 and purified as IgG fractions (48). Goat anti-ribb protein IgG horseradish peroxidase conjugate was purchased from Bio-Rad. Oligonucleotides used as primers for mutagenesis were prepared by the Michigan State University Macromolecular Structure, Synthesis, and Sequencing Facility. All other reagents were obtained from common commercial sources.

**Preparation of oPGHS-1 Mutants**—Mutants were prepared by site-directed mutagenesis of oPGHS-1 in the pSVT7 vector employing the Stratagene QuickChange mutagenesis kit following the manufacturer’s protocol. Oligonucleotides used in the preparation of various mutants were reported previously (24, 35, 49). Plasmids used for transfections were purified by CsCl gradient ultracentrifugation, and mutations were reconfirmed by double-stranded sequencing of the pSVT7 constructs using Sequenase version 2.0 (U. S. Biochemical Corp.) and the protocol described by the manufacturer.

**Transfection of COS-1 Cells with oPGHS-1 Constructs**—COS-1 cells (ATCC CRL-1650) were grown in Dulbecco’s modified Eagle’s medium containing 8% calf serum and 2% fetal bovine serum and transfected with pSVT7 plasmid constructs containing cDNAs encoding native or mutant enzymes using the DEAE-dextran/chloroquine transfection method (35, 49). Forty hours following transfection, cells were harvested and microsomal membrane fractions were prepared as described previously (5). Protein concentrations were determined using the method of Bradford (50) with bovine serum albumin used as the standard. Microsomal preparations were used for Western blotting and for cyclooxygenase and peroxidase assays. All mutant enzymes described were expressed at protein levels similar to those of native enzymes and retained ≥50% of the peroxidase activity of native oPGHS-1 (49).

Cyclooxygenase and Peroxidase Assays—Cyclooxygenase and peroxidase activity were measured as previously described (49). Cyclooxygenase activity was measured by monitoring the initial rate of O₂ uptake using an oxygen electrode (49). Reactions were initiated by adding ~250 μg of microsomal protein in a volume of 20–50 μl to the assay chamber. The oxygenation of all fatty acids by oPGHS-1 enzyme was completely inhibited by the addition of 0.2 mM flurbiprofen to the reaction mixture. To determine the specificity for the alternative fatty acid substrate dihomo-γ-linolenic acid (15S-HPETE, 5 μM), to the reaction mixture was necessary to sustain cyclooxygenase activity with EPA (51). Addition of this peroxide to the reaction mixture had no effect on the conversion rates of other substrates. All data were normalized to the relative levels of PGHS protein expression as determined by Western blotting and densitometry. Fatty acid substrate concentrations of 100 μM were used to estimate the maximal rates. Km values were measured using concentrations of fatty acid substrates between 0.5 and 500 μM as described previously (49). Peroxidase activities were measured spectrophotometrically with Ni,N,N’,N’-tetramethylphenylenediamine as the reducing cosubstrate (52) as reported previously (53). Reactions were initiated by adding 100 μl of 0.3 mM H₂O₂, and the absorbance at 610 nm was monitored with time.

**Western Blot Analysis**—Microsomal samples (~5 μg of protein) were resolved by one-dimensional SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes using a Hoefer Scientific semidyse transfer apparatus. Membranes were blocked for 2 h in 3% nonfat dry milk, 0.1% Tween 20, and Tris-buffered saline, followed by a 2-h incubation with a peptide-directed antibody against oPGHS-1 (48, 54) in 1% dry milk, 0.1% Tween 20, and Tris-buffered saline at room temperature. Membranes were washed and incubated for 1 h with a 1:2000 dilution of goat anti-ribb protein IgG-horseradish peroxidase, after which they were incubated with Amersham Pharmacia Biotech ECL reagents and exposed to film for chemiluminescence.

**Characterization of Fatty Acid Oxidation Products**—A general protocol for product analysis is as follows. Forty hours following transfection, COS-1 cells were collected, sonicated, and resuspended in 0.1 M Tris-HCl, pH 7.5. Aliquots of the cell suspension (100–250 μg of protein) were incubated for 1–10 min at 37 °C in 0.1 M Tris-HCl containing 1 mM phenol and 6.8 μg of bovine hemoglobin in a total volume of 200 μl. Reactions were initiated by adding 1.4°C-labeled fatty acid (35 μM final concentration) and were performed in the presence and absence of 200 μM flurbiprofen and stopped by adding 1.4 ml of CHCl₃-MeOH (1:1 v/v). Insoluble cell debris was removed by centrifugation, and 0.6 ml of CHCl₃ and 0.32 ml of 0.88% formic acid were added to the resulting supernatant. The organic phase was collected, dried under N₂, redisolved in 50 μl of CHCl₃, and spotted on a Silica Gel 60 thin layer chromatography plate; the lipid products were chromatographed for 1 h at room temperature. The plate was air-dried, and the lipids were stained with 15% H₂O₂ in methanol/acetone/CHCl₃ (5:1:4, v/v). Plates were visualized by autoradiography and quantified by liquid scintillation counting. Negative control values from samples incubated with 200 μM flurbiprofen were subtracted from the experimental values observed for each sample in the absence of flurbiprofen.

**Preparation of 15S-HPETE—**15S-HPETE was prepared according to Graff (55) with modifications. Arachidonic acid (50 μmol) was suspended in 250 μl of 0.2 M HCl, 1 M NaCl, and 10 mM ethylenediaminetetraacetic acid (EDTA). This buffer, after which they were incubated with 0.64 M LA complexes were formed by adding soybean lipoxidase (50 mg) and the incubation continued for 5 min at 30 °C. The reaction was quenched by adding enough 0.24 M HCl to bring the pH to 3.0 (20–30 ml) and extracted three times with an equal volume of ice-cold petroleum ether:ethyl acetate (20:1, v/v). The extract was visualized by autoradiography and quantified by liquid scintillation counting. Double bond formation (ε₃₂₃ = 3 × 10⁴ mm⁻¹cm⁻¹) and by measuring peroxide levels using a ferrous sulfate-xylenol orange reagent as described by Gupta (56).

**Purification and Crystallization**—Apo-oPGHS-1 was purified and reconstituted with Co₃⁺—protoporphyrin IX as described previously (57). The Co₃⁺—oPGHS-1-EPA and Co₃⁺—oPGHS-1-LA complexes were formed by the addition of a 10-fold molar excess of EPA or LA prior to crystallization. Crystals were grown in sitting-drops by combining either the Co₃⁺—oPGHS-1-EPA complex or Co₃⁺—oPGHS-1-LA complex with 0.64 mM sodium citrate, 0.3–0.6 M LiCl, 1 M NaN₃, 0.33% (w/v) 2-octyl-β-D-glucopyranoside (β-OG), pH 6.5, and equilibrating over reservoir solutions containing 0.64–0.84 mM sodium citrate, 0.2–0.6 M LiCl, and 1 M NaN₃ to 20 °C. The crystals were harvested by centrifugation and rinsed with 0.19 M sodium citrate, pH 6.5, 1 ml of LiCl, 0.15% (w/v) β-OG, followed by a single-step transfer into the above buffer with 24% (w/v) sucrose added as a cryoprotectant. The crystals were then immediately frozen in liquid propane at −165 °C.

**Data Collection**—Both the Co₃⁺—oPGHS-1-EPA and Co₃⁺—oPGHS-1-LA complexes were collected at −165 °C on beamline 19-ID of the DLS.
Structural Biology Center (Advanced Photon Source, Argonne, IL) at a wavelength of 1.03321 Å. Each data set was processed and scaled using HKL2000 (58) and SCALEPACK (58). Details of the data collection and reduction are summarized in Table I.

### Structural Analysis

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**Table I**  
Summary of data collection and refinement statistics for Co<sup>3+</sup>-oPGHS-1-EPA and Co<sup>3+</sup>-oPGHS-1-LA

| Item     | EPA     | LA     |
|----------|---------|--------|
| Fatty acid chemical formula | 20:5 n-3 | 18:2 n-6 |
| Spacegroup | P6<sub>3</sub>2<sub>2</sub> | P6<sub>3</sub>2<sub>2</sub> |
| a (Å)    | 182.17  | 182.05 |
| b (Å)    | 182.17  | 182.05 |
| c (Å)    | 103.11  | 103.65 |
| Resolution (Å) | 20.1 | 15.2 |
| No. of unique reflections (F > 2σ) | 17814 | 21898 |
| Completeness (%; F > 2σ) | 92 (78) | 93 (76) |
| R<sub>merge</sub> all data (%; I > 2σ) | 8.6 | 9.1 |
| No. of atoms in refinement | 4874 | 4706 |
| R factor (%; reflections > 2σ on F<sup>2</sup>) | 23.0 | 23.5 |
| Free R factor (%; reflections > 2σ on F<sup>2</sup>) | 29.6 | 27.1 |
| Mean positional error (Å) | 0.40 | 0.41 |
| r.m.s.d. in bond distance (Å) | 0.010 | 0.010 |
| r.m.s.d. in bond angle (°) | 1.545 | 1.544 |
| r.m.s.d. in improper angle (°) | 1.058 | 1.023 |
| r.m.s.d. in dihedral angle (°) | 22.021 | 22.084 |

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<sup>a</sup> Values in parentheses represent values in last shell (3.2–3.1 Å and 3.0–2.9 Å for EPA and LA, respectively).

**RESULTS AND DISCUSSION**

**EPA and LA Bound in the Cyclooxygenase Channel**—We have determined the crystal structure complexes of EPA and LA bound in the cyclooxygenase channel of the Co<sup>3+</sup>-oPGHS-1 (57) at 3.1- and 2.9-Å resolution, respectively, using synchrotron radiation. The r.m.s. deviation between the structures of Co<sup>3+</sup>-oPGHS-1-EPA and Co<sup>3+</sup>-oPGHS-1-LA versus Co<sup>3+</sup>-oPGHS-1-AA (15) are 0.20 Å (551 out of 554 Ca atoms) and 0.16 Å (553 out of 554 Ca atoms), respectively, indicating that the epidermal growth factor, membrane binding domain, and catalytic domains are resolvable and virtually unchanged with respect to fatty acid binding in the cyclooxygenase active site (15, 24). In addition, both EPA and LA complexes with Co<sup>3+</sup>-oPGHS-1 reveal interpretable electron density for the Co<sup>3+</sup>-protoporphyrin IX, the carbohydrate moieties linked to Asn-68, Asn-144, and Asn-410, and β-OG detergent molecules bound within the membrane binding domain (data not shown).

Both EPA and LA bind in the cyclooxygenase active site in a configuration generally similar to that observed for AA (15) and DHLA (24). The “L-shaped” configuration positions the carboxylate near the side chain of Arg-120 and Tyr-355 at the opening of the channel (Figs. 1 and 2). The negative charge of the carboxylate is stabilized via a salt bridge with the guanidinium group of Arg-120 (Fig. 3), a required binding determinant of substrates for PGHS-1 (15, 24, 49, 63) and for oxygenation by EPA by PGHS-2 (35). In addition, two hydrogen bonds, each to the phenolic oxygen of Tyr-355 and the guanidinium group of Arg-120, further stabilize the carboxylate in an other-
wise predominantly hydrophobic channel (Fig. 3). The ω-end of the fatty acids bind in a hydrophobic groove above helix 17 (residues 520–535) between Ser-530 and Gly-533 (Figs. 1 and 2). This is accomplished through the weaving of the central part of the carbon chain of each substrate around the side chain of Ser-530, the residue that can be acetylated by aspirin, resulting in the positioning of C-13 below Tyr-385. The position of C-13 in EPA differs by 1.4 Å from those seen with AA (15). This is further evidenced by the r.m.s. deviation of 0.67 Å calculated between carbon positions (C-1 through C-7 only) in LA versus AA. Starting with C-7, the chain takes a more direct route to the apex of the active site to position C-11, analogous to C-13 in AA, below Tyr-385 (Fig. 5); overall, this is a consequence of shortening of the fatty acid substrate chain by two carbons. In contrast, the C-8/C-9 double bond of AA protrudes toward helix 17 where it makes four hydrophobic contacts with Gly-526 before weaving around the side chain of Ser-530 (15). Thus, LA does not contain the "S-shaped" kink that is a structural characteristic observed for binding 20 carbon fatty acid substrates to the cyclooxygenase active site (15, 24). Despite being two carbons shorter and containing two fewer double bonds, LA binds in the cyclooxygenase active site in a conformation very similar to that observed for AA (Fig. 5). The carboxyl half of LA binds such that C-1 interacts with Arg-120, while C-2 through C-7 contact Val-349, Leu-352, Ile-523, Gly-526, and Ala-527 (Fig. 3B); these contacts are all comparable to those seen with AA (15). This is further evidenced by the r.m.s. deviation of 0.67 Å calculated between carbon positions (C-1 through C-7 only) in LA versus AA. Starting with C-7, the chain takes a more direct route to the apex of the active site to position C-11, analogous to C-13 in AA, below Tyr-385 (Fig. 5); overall, this is a consequence of shortening of the fatty acid substrate chain by two carbons. In contrast, the C-8/C-9 double bond of AA protrudes toward helix 17 where it makes four hydrophobic contacts with Gly-526 before weaving around the side chain of Ser-530 (15). Thus, LA does not contain the "S-shaped" kink that is a structural characteristic observed for binding 20 carbon fatty acid substrates to the cyclooxygenase active site (15, 24). Despite the lack of an S-shaped kink in the substrate chain, C-11 is positioned "optimally" below Tyr-385 for hydrogen abstraction and the ω-end of LA is oriented properly in the hydrophobic groove above Ser-530 (Fig. 5). The pro-S

Comparison of EPA, LA, and AA Binding in the Cyclooxygenase Active Site—Although EPA binds in a manner similar to AA, the additional C-17/C-18 double bond at the ω-end decreases the flexibility of this fatty acid substrate in the cyclooxygenase active site resulting in a strained binding orientation. Whereas C-1 is positioned to make the required carboxylate interaction with Arg-120, the rest of the carbon chain is compacted and displaced with respect to the carbon positions in AA (r.m.s. deviation = 1.59 Å; Fig. 4). C-2 through C-10 now pack more closely to helix 17 (residues 520–535), resulting in 24 contacts with active site residues versus 13 for the same carbons in AA (Figs. 3A and 4) (15). Displacement of these carbons is required to accommodate the conformationally inflexible ω-end of EPA in the hydrophobic groove above Ser-530 (Fig. 4). A major consequence of this binding arrangement is the misalignment of C-13 below Tyr-385. The position of C-13 in EPA differs by 1.4 Å with respect to C-13 of AA (Fig. 4A). Furthermore, modeling of the pro-S hydrogen onto C-13 of EPA places it 4.1 Å away from the phenolic oxygen of Tyr-385 and rotated toward the hydroxyl group of Ser-530 (Fig. 4B). Conversely, the 13-pro-S hydrogen modeled onto AA lies directly below Tyr-385, 2.7 Å away from the phenolic oxygen (15).

Despite being two carbons shorter and containing two fewer double bonds, LA binds in the cyclooxygenase active site in a conformation very similar to that observed for AA (Fig. 5). The carboxyl half of LA binds such that C-1 interacts with Arg-120, while C-2 through C-7 contact Val-349, Leu-352, Ile-523, Gly-526, and Ala-527 (Fig. 3B); these contacts are all comparable to those seen with AA (15). This is further evidenced by the r.m.s. deviation of 0.67 Å calculated between carbon positions (C-1 through C-7 only) in LA versus AA. Starting with C-7, the chain takes a more direct route to the apex of the active site to position C-11, analogous to C-13 in AA, below Tyr-385 (Fig. 5); overall, this is a consequence of shortening of the fatty acid substrate chain by two carbons. In contrast, the C-8/C-9 double bond of AA protrudes toward helix 17 where it makes four hydrophobic contacts with Gly-526 before weaving around the side chain of Ser-530 (15). Thus, LA does not contain the "S-shaped" kink that is a structural characteristic observed for binding 20 carbon fatty acid substrates to the cyclooxygenase active site (15, 24). Despite the lack of an S-shaped kink in the substrate chain, C-11 is positioned “optimally” below Tyr-385 for hydrogen abstraction and the ω-end of LA is oriented properly in the hydrophobic groove above Ser-530 (Fig. 5). The pro-S
hydrogen of LA lies 3.0 Å below the phenolic oxygen of Tyr-385 in an orientation similar to that seen for the 13-pro-S hydrogen of AA (2.7 Å) (15) (Fig. 5). C-9 through C-18 in LA are chemically equivalent to C-11 through C-20 in AA, including the location of the double bonds within this segment. Therefore, it is not surprising that these carbons are in correspondingly similar positions (r.m.s. deviation of 0.70 Å) and the contacts made with residues lining the hydrophobic groove are conserved (Figs. 3B and 5) (15).

Oxygenation of EPA and LA by Active Site Mutants—To further define the structural basis for the substrate specificities of the cyclooxygenase activity of PGHS-1, we compared how active site substitutions of groups that contact the substrate affect the rates of oxygenation of EPA, LA, and AA. Replacements of Val-349, Ser-530, and Leu-534 produced relatively dramatic decreases in the oxygenation efficiencies of EPA and LA compared with those seen with AA (Tables II and III). In addition, replacement of Phe-205 with leucine yielded a mutant enzyme that oxygenates EPA better than LA, AA (49), or DHLA (24) when compared with the oxygenation of these substrates by the native enzyme (Table III). All other active site substitutions caused approximately parallel decreases in the rate of oxygenation of EPA and LA when compared with the rates of oxygenation of AA (Table III). The kinetic properties displayed by these mutants are discussed in detail below, and their functions as a determinants of substrate specificity are rationalized based on the crystal structures of AA, EPA, and LA bound in the cyclooxygenase active site of Co<sup>III</sup>-oPGHS-1.

Valine 349—Previous studies have shown that mutation of Val-349 to alanine reduces the rate of oxygenation of AA to 55% that of native PGHS-1, with no significant effect on the $K_m$ value (24, 49). We have extended these studies to show that the V<sub>349A</sub> substitution dramatically reduces the rates of oxygenation of LA and EPA to 0.1% that of native oPGHS-1, with corresponding increases in $K_m$ values between 10- and 100-fold. As a result, there are 175- and 750-fold reductions in the oxygenation efficiencies for LA and EPA, respectively (Table II), compared with less than 2-fold for AA (49). A significant reduction is also observed for the oxygenation of DHLA by V<sub>349A</sub> oPGHS-1, where there is an 833-fold difference in the $V_{\text{max}}/K_m$ value compared with native enzyme (24). Replacement of Val-349 with leucine produces a similar reduction in the oxygenation efficiency. A V<sub>349L</sub> mutation reduces the catalytic efficiency toward AA less than 6-fold, but there are reductions in the $V_{\text{max}}/K_m$ value toward LA and EPA of 350- and 750-fold, respectively (Table II). However, unlike with the V<sub>349A</sub> substitution, the reduced efficiency is due mostly to large increases in the $K_m$ values for EPA (246-fold) and LA (53-fold) (Table II). There is only a modest reduction in the
production of PGG$_2$ (49, 53). Replacement of Val-349 with alanine yields a mutant enzyme that produces 55% 11-HPETE from AA, compared with the formation of only 2.5% 11-HPETE by native oPGHS-1. The V349L substitution shifts the product profile toward the production of 15-HPETE (~20% of the total products) (49, 53). Conversely, analysis of the products formed from the oxygenation of [1-14C]EPA and [1-14C]LA by V349A and V349L oPGHS-1 indicates that the ratios of oxygenated products produced are the same with respect to native oPGHS-1 (data not shown). Similarly, product analysis of [1-14C]DHLA upon oxygenation by V349A and V349L oPGHS-1 again showed that the relative ratio was similar to native oPGHS-1, with only a less than 2-fold increase in monohydroxy acid formation (24). Thus, Val-349 clearly plays a critical role in the positioning of alternative fatty acid substrates for oxygenation, but mutations at this site do not change the distribution of products.

Based on inspection of the structures of AA, EPA, and LA bound in the cyclooxygenase active site of oPGHS-1, one can rationalize why Val-349 is more important for the efficient oxygenation of EPA and LA (Figs. 4 and 5). Val-349 is located in helix 6 (residues 325–353; Ref. 12), which forms one wall of the cyclooxygenase active site (Figs. 4 and 5). The side chain of Val-349 extends into the channel and serves as a structural bumper that positions the carboxyl half of the fatty acid. The C$_1$ atom of Val-349 contacts C-3 and C-4 of AA to favor production of PGG$_2$ (15, 49), and C-5 of DHLA to orient the 13-pro-S hydrogen for abstraction by Tyr-385 (24). Consistent with AA and DHLA, the contacts within this area of the substrate chain are conserved for EPA and LA bound in the active site, where the C$_1$ atom of Val-349 makes two contacts with EPA (C-2 and C-3) and one contact with LA (C-4) (Fig. 3).

Computer modeling indicates that replacement of Val-349 with an alanine creates a large pocket into which the carboxyl half of substrates can move (24, 49, 53). Since the carbon chain of EPA is more compacted toward the carboxyl end of the substrate in order to accommodate the C-17/C-18 double bond in the hydrophobic channel above helix 17 (Fig. 4), the larger opening created by the V349A mutation would allow the substrate to move into the vacated space as a rigid body. As a result, C-13 would be further displaced from Tyr-385 reducing even more the chance for hydrogen abstraction and subsequent product formation (Table II). Furthermore, because the C-1 through C-10 region of EPA makes twice as many contacts as AA with residues Ile-523, Gly-526, and Ala-527 on the wall opposite Val-349 (16 versus 8; Fig. 3A), movement away from helix 17 would serve to destabilize the binding of EPA within the active site (Table II). As LA is more flexible within its carboxyl half of the substrate chain due to a lack of unsaturated bonds, movement into the space vacated by the valine side chain would likely occur with the V349A mutant. The major consequence of this movement would be the altered positioning of C-11 with respect to Tyr-385 (Table II). Moreover, because C-1 through C-8 of LA are stabilized predominantly by contacts with Ile-523, Gly-526, and Ala-527 (Fig. 3B), movement away from these residues would destabilize LA binding in the active site (Table II).

Computer modeling of the V349L mutation shows a constraining of the size of the cyclooxygenase active site near the base of the channel. This reduction in size moderately affects the binding and oxygenation of AA and DHLA, as V349L oPGHS-1 retains relatively similar activities with both substrates, whereas V349A oPGHS-1 is much less active toward DHLA (24). Conversely, the binding of EPA and LA are dramatically affected by the V349L substitution (Table II). The binding of EPA would be significantly altered with a reduction in the oxygenation efficiency of DHLA by V349L oPGHS-1 compared with that observed for EPA and LA (24).

Val-349 in PGHS-1 plays a key role in positioning AA to favor...
Oxygenase activity was measured with AA, EPA, or LA as the substrate using an oxygen electrode as described under “Experimental Procedures.” \( V_{\text{max}} \) values are values for fatty acid turnover and were obtained from measurements of rates of \( O_2 \) uptake and then corrected for the percentage of mono- and bi-oxygenated products formed with the different enzymes and substrates. A \( V_{\text{max}} \) value of 100% is assigned for oxygenase activity of native oPGHS-1 with AA. \( V_{\text{max}} \) and \( K_M \) values represent the mean values from a minimum of four separate determinations with standard deviations within 10% of all values reported. Relative \( V_{\text{max}} \) values reported for mutant enzymes for which \( K_M \) values were not determined represent rate measurements performed using 100 \( \mu \)M fatty acid substrate.

### TABLE II
Oxygenase activity for oPGHS-1 cyclooxygenase active site mutants with AA, EPA, and LA

| oPGHS-1 | AA (20:4 n-6) | EPA (20:5 n-3) | LA (18:2 n-6) |
|---------|--------------|----------------|--------------|
|         | \( V_{\text{max}} \) | \( K_M \) | \( V_{\text{max}}/K_M \) | \( V_{\text{max}} \) | \( K_M \) | \( V_{\text{max}}/K_M \) |
| Native  | 100          | 15             | 44           | 20            | 7.5           | 2.6           |
| V349A   | 55           | 2.0            | 50           | 2.0           | 7.5           | 44            |
| V349L   | 63           | 7.0            | 9.0          | 1.3           | 2.0           | 157           |
| S350A   | 58           | 2.0            | 29           | 58            | 2.0           | 157           |
| S350T   | 17           | 13             | 1.3          | 115           | 0.003         | 7             |

\( a \) From Ref. 49.

### TABLE III
Oxygenase activity for oPGHS-1 cyclooxygenase active site mutants with AA, EPA, and LA

| Enzyme | AA (20:4 n-6) | EPA (20:5 n-3) | LA (18:2 n-6) |
|--------|--------------|----------------|--------------|
| Native oPGHS-1 | 100          | 15             | 44           |
| F205L  | 28           | 7.8            | 3.5          |
| F209A  | 65           | 18             | 22           |
| F381A  | 43           | 5.0            | 9.2          |
| V348/3S352T | 34           | 8.6            | 9.2          |
| S353G  | 61           | 5.1            | 5.7          |
| S353A  | 56           | 5.3            | 15           |
| S353T  | 42           | 11             | 14           |
| Y355F  | 19           | 5.0            | 0            |
| F381L  | 21           | 3.2            | 4.8          |
| W387A  | 0            | 0              | 0            |
| W387L  | 7            | 0.21           | 3.7          |
| L534A  | 44           | 7.1            | 22           |
| IS23A  | 64           | 7.5            | 3.1          |
| IS23V  | 70           | ND             | 12           |
| S350V  | 0            | 0              | 0            |
| G533A  | 0            | ND             | 0            |
| G533A/S533V | 0          | ND             | 0            |
| L534A  | 59           | 7.5            | 18           |
| L534V  | 98           | 1.5            | 0            |

\( a \) All values reported for AA are from Ref. 49 unless otherwise stated.

of space near the carboxyl half of the molecule. The substrate would not be able to negotiate the presence of an additional carbon atom due to its already rigid configuration and compromised binding orientation in the active site. Despite the increased flexibility of LA compared with AA and EPA, the binding of LA is also affected substantially by the reduced size of the active site. Upon inspection, it would seem that the binding of LA would be unaffected by the V349L substitution and in fact, more stabilized by the additional contacts that could be formed with the carboxyl half of this substrate. However, the leucine substitution must perturb C-2 through C-6 such that the substrate cannot extend properly so as to assume an orientation suitable for hydrogen abstraction (Table II).

Ile-523 lies across from Val-349 on the opposite side of the active site channel and interacts with the same region of the substrate chain (Figs. 3–5). The C=O atom of Ile-523 makes three contacts with EPA (C-4, C-5, and C-6) and two contacts with LA (C-3 and C-5) (Fig. 3). Replacement of Ile-523 with an alanine has no effect on the rate of oxygenation of EPA, presumably due to the compensatory interactions made with Leu-352, Ser-353, Gly-526, and Ala-527 (Fig. 3A) and in agreement with results reported previously for AA and DHLA (24, 49). However, the rate of oxygenation of LA is altered significantly with an I523A mutation (Table III). Removal of the C=O atom would destabilize the binding of the carboxyl half of LA, somewhat similar to what occurs with the V349A substitution. Unlike EPA, LA is more extended in this region resulting in fewer compensatory contacts; there are seven fewer contacts with Leu-352, Gly-526, and Ala-527, and LA does not contact the side chain of Ser-353 (Figs. 3B and 5).

Serine 530—The hydroxyl group of Ser-530 is the site of acetylation of oPGHS-1 by aspirin (64, 65). Previous mutational analyses of Ser-530, utilizing AA as the substrate, have shown the following: (a) replacement of Ser-530 with alanine has relatively little effect on the oxygenation of AA (64, 66), (b) replacement of Ser-530 with threonine causes a 38-fold decrease in \( V_{\text{max}}/K_m \) (66), and (c) replacement of Ser-530 with either leucine or asparagine prevents cyclooxygenase catalysis by blocking substrate access to Tyr-385 at the top of the active site channel (66).

Analysis of the product profiles with AA have shown that an abundance of 15-HETE is produced from AA by S530T oPGHS-1 relative to native enzyme, whereas S530A oPGHS-1 produces product ratios similar to that of native oPGHS-1 (67). Replacements at Ser-530 have a greater effect on the oxygenation of DHLA than AA; although the \( V_{\text{max}}/K_m \) for DHLA is only decreased slightly with the S530A mutant due to a 4-fold increase in \( K_m \), there is more than an 800-fold decrease with the S530T mutant (24).

S530A and S530T substitutions also influence the efficiency of oxygenation of AA and LA by oPGHS-1. Replacement of Ser-530 with alanine decreases the \( V_{\text{max}}/K_m \) toward LA by 4.4-fold and EPA by 24-fold (Table II). The magnitude of change in oxygenation efficiency is greater than that observed for the oxygenation of AA by S530T (Table II) (49); however, unlike AA, the change is primarily due to the 2.5- and 24-fold increases in \( K_m \) for LA and EPA, respectively (Table II). Although replacement of Ser-530 with threonine results in an enzyme that is relatively inactive toward all fatty acid substrates (Table II) (24, 49), there are greater than 7-fold increases in \( K_m \) for EPA and LA versus AA. As mentioned above, S530T oPGHS-1 produces an abundance of 15-HETE compared with the native enzyme when AA is used as the substrate. The relative proportions of oxygenated products formed upon incubation of [1-\(^{14}\)C]EPA and [1-\(^{14}\)C]LA with S530T oPGHS-1 are similar to those observed with native oPGHS-1 (data not
shown). Thus, unlike the case with AA, replacement of Ser-530 with either alanine or threonine has a greater effect on the oxygenation efficiencies of EPA and LA than on the product profile. These results from mutagenesis suggest that the Ser-530 side chain plays a key role in positioning EPA and LA for hydrogen abstraction by Tyr-385.

Analysis of the interactions made between AA and cyclooxygenase active site residues shows that Ser-530 makes three van der Waals contacts with AA involving C-10 and C-16 of the substrate (15). The midsection of AA weaves around the side chain of Ser-530 such that C-13 lies below Tyr-385 for hydrogen abstraction; similarly, C-13 lies above the hydroxyl group of Ser-530. The hydroxyl group is not required for cyclooxygenase catalysis (Table II) (49, 64, 66). There are seven contacts made between Ser-530 and EPA involving C-10, C-12, C-13, C-16, C-17, and C-18 (Fig. 3A). Like AA, EPA threads around the side chain of Ser-530 positioning C-13 of EPA below Tyr-385. However, in accommodating the additional n-3 double bond of EPA in the hydrophobic pocket, the midsection of EPA is compacted and displaced toward the side chain of Ser-530 (Fig. 4). Replacement of Ser-530 with alanine widens the area below Tyr-385, allowing C-10 through C-16 of EPA more freedom to rotate for optimal positioning of C-13 for hydrogen abstraction. Indeed, there is virtually no change in the $V_{\text{max}}$ of S530A when compared with the oxygenation of EPA by native oPGHS-1 (Table II). In contrast, replacement of Ser-530 with the larger threonine further restricts the width of the channel between Thr-530 and Tyr-385, and thus the movement of EPA so that C-13 of EPA is misaligned for hydrogen abstraction. As a consequence, the oxygenation efficiency of S530T oPGHS-1 toward EPA is decreased 375-fold compared with the native enzyme (49). C-17 through C-19 of EPA and C-14 through C-17 of LA are also involved in stabilizing contacts with the side chain carbons of Phe-205, Phe-209, and Phe-381 (Fig. 3). Mutation of Phe-205 and Phe-381 to leucine or alanine results in a parallel reduction in the oxygenation rate for AA, EPA, and LA when compared with the ability of native oPGHS-1 to oxygenate these substrates (Table III). Interestingly, replacement of Phe-205 with leucine results in a mutant enzyme with a higher $V_{\text{max}}$ than the native enzyme with EPA (Table III); in contrast, when AA and LA are utilized as substrates, the rates of oxygenation are reduced similarly to levels observed for Phe-209 and Phe-381 replacements (Table III). When Phe-205 is replaced by an alanine, a reduction in the rate of oxygenation is observed for AA, EPA, and LA.

Unlike Phe-209 and Phe-381, the side chain of Phe-205 interacts with fatty acid substrates in an “edge-on” fashion (Figs. 1 and 4) (15, 24). There are two van der Waals contacts between the side chain of Phe-205 and C-19 of EPA (Fig. 3A). Computer modeling of the F205L substitution indicates that the space in the active site above C-18 through C-20 would be enlarged. The additional room could be utilized by EPA if the C-17/C-18 double bond is reoriented via rotation about C-16. As a consequence, the conformation of the ω-end would be aligned such that C-13 is positioned somewhat more optimally below Tyr-385. Despite the loss of three side chain carbons, a leucine at position 205 should be able to maintain the stabilizing contacts with C-18 through C-20 of EPA in the new orientation.

The leucine and alanine substitutions made at Phe-209 and Phe-381 cause similar reductions in oxygenation rates for EPA, LA, and AA (Table III). The side chain of Phe-209 lies above Gly-533 at the end of the hydrophobic groove and makes only one contact with C-19 of EPA (Figs. 3 and 4). Conversely, the side chain of Phe-381 is located adjacent to Tyr-385, where it makes three contacts with the C-17/C-18 double bond of EPA (Figs. 3 and 4). Based on the conformation of the phenolic side chains of Phe-209 and Phe-381 relative to the substrate, the additional space gained through the leucine substitution would not be sufficient to allow EPA the conformational freedom to reorient its ω-end such that C-13 is optimally positioned below Tyr-385. In contrast, replacements at Phe-205, Phe-209, and Phe-381 with alanine would create too large of an opening within the hydrophobic groove resulting in destabilization of the ω-end and misalignment of C-13 for hydrogen abstraction (Table III).

Conclusion—EPA and LA are alternative fatty acid sub-

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2 E. D. Thuresson and W. L. Smith, unpublished results.
stratases for oPGHS-1 that are oxygenated at reduced rates compared with AA (Table II, Table III). Both substrates bind within the cyclooxygenase active site in an L-shaped conformation similar to that observed for AA. However, the structural properties of EPA and LA cause each of these fatty acid substrates to bind in orientations that are suboptimal with respect to hydrogen abstraction and subsequent cyclooxygenase catalysis. Kinetic analyses of mutations of cyclooxygenase active site residues that contact EPA and LA indicate that Val-349, Ser-530, and Leu-534 are particularly important for the efficient oxygenation of these substrates. Finally, replacement of Phe-205 with leucine increases the volume of the cyclooxygenase active site, allowing EPA to be oxygenated somewhat more efficiently.

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Structure of Eicosapentaenoic and Linoleic Acids in the Cyclooxygenase Site of Prostaglandin Endoperoxide H Synthase-1
Michael G. Malkowski, Elizabeth D. Thuresson, Karen M. Lakkides, Caroline Jill Rieke, Renée Micielli, William L. Smith and R. Michael Garavito

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