Crystal Structure of Arachidonic Acid Bound to a Mutant of Prostaglandin Endoperoxide H Synthase-1 That Forms Predominantly 11-Hydroperoxyeicosatetraenoic Acid

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Kinetic studies and analysis of the products formed by native and mutant forms of ovine prostaglandin endoperoxide H synthase-1 (oPGHS-1) have suggested that arachidonic acid (AA) can exist in the cyclooxygenase active site of the enzyme in three different, catalytically competent conformations that lead to prostaglandin G_2 (PGG_2), 11R-hydroperoxyeicosatetraenoic acid (HPETE), and 15R,S-HPETE, respectively. We have identified an oPGHS-1 mutant (V349A/W387F) that forms predominantly 11R-HPETE. Thus, the preferred catalytically competent arrangement of AA in the cyclooxygenase site of this double mutant must be one that leads to 11-HPETE. The crystal structure of Co^3+-protoporphyrin IX V349A/W387F oPGHS-1 in complex with AA was determined to 3.1 Å. Significant differences are observed in the positions of atoms C-3, C-4, C-5, C-6, C-10, C-11, and C-12 of bound AA between native and V349A/W387F oPGHS-1; in comparison, the positions of the side chains of cyclooxygenase active site residues are unchanged. The structure of the double mutant presented here provides structural insight as to how Val^{349} and Trp^{387} help position C-9 and C-11 of AA so that the incipient 11-peroxyl radical intermediate is able to add to C-9 to form the 9,11 endoperoxide group of PGG_2. In the V349A/W387F oPGHS-1-AA complex the locations of C-9 and C-11 of AA with respect to one another make it difficult to form the endoperoxide group from the 11-hydroperoxy radical. Therefore, the reaction apparently aborts yielding 11R-HPETE instead of PGG_2. In addition, the observed differences in the positions of carbon atoms of AA bound to this mutant provides indirect support for the concept that the conformer of AA shown previously to be bound within the cyclooxygenase active site of native oPGHS-1 is the one that leads to PGG_2.

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The atomic coordinates and structure factors (code 1U67) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡The abbreviations used are: PGHS, prostaglandin endoperoxide H synthase; FG, prostaglandin; oPGHS, ovine PGHS; AA, arachidonic acid; 11-HPETE, 11-hydroperoxyeicosatetraenoic acid; PG, prostaglandin; oPGHS, ovine PGHS; AA, arachidonic acid (AA) to prostaglandin (PG) H_2 (Fig. 1), the immediate precursor to prostaglandins, thromboxanes, and prostacyclin. In mammalian tissues, there are two isozymes of PGHS designated PGHS-1 (or cyclooxygenase-1 (COX-1)) and PGHS-2 (or COX-2) (1, 2). PGHS-1 is constitutively expressed and produces prostaglandins in response to hormone stimulation mainly for regulating housekeeping functions. PGHS-2, the inducible form, is expressed in response to mitogens, growth factors, tumor promoters, and/or cytokines and produces prostaglandins associated with pain, fever, and inflammation (1, 2).

Despite the differences in patterns of expression, PGHS-1 and PGHS-2 are quite similar both structurally (3–5) and mechanistically (6). Both isozymes function as dimers with each monomer having an epidermal growth factor domain, a membrane-binding domain, and a large catalytic domain. The catalytic domain of PGHS possesses two distinct but functionally connected active sites (3–5, 7). These two sites include the COX site, which exists as a long hydrophobic channel within the core of the protein and binds AA (8) and nonsteroidal anti-inflammatory drugs (3–5), and a more solvent-exposed peroxidase site containing a heme moiety, which is involved in reducing PGG_2 formed at the COX site to PGH_2, the final enzymatic product.

The crystal structure of native Co^3+-protoporphyrin IX oPGHS-1 in a complex with AA shows that AA binds within the COX active site having the carboxyate group anchored at the mouth of the hydrophobic channel via electrostatic interactions with Arg^{210} and Tyr^{255} (8). The carbons of the AA chain weave into the COX active site, making forty-eight hydrophobic contacts with the residues lining this channel. Based on the crystal structure and knowledge of the stereochemical requirements of the COX reaction (9), a structural sequence of catalytic events was proposed (8). The COX reaction begins with the abstraction of the 13proS hydrogen of AA by a tyrosyl radical formed at Tyr^{255}, creating an arachidonyl radical centered at C-13 (6) (Fig. 1). Following a rearrangement centering the radical on C-11, an attack of molecular oxygen occurs to form an 11R-hydroperoxy radical (Fig. 1). At this point it is proposed that rotation about the C-10–C-11 bond moves the 11R-hydroperoxy radical in close proximity to C-9 to form the 9,11-endoperoxide group; concurrently, rotation about the C-10–C-11 bond additionally brings C-8 near to C-12 to form the cyclopentane ring. Repositioning of C-12 closer to C-8 for ring formation also acid, 11-HETE, 11-hydroxy-5c,8c,12t,14c-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5c,8c,11c,13t-eicosatetraenoic acid; 11-HPETE, 11-hydroxy-5c,8c,12t,14c-eicosatetraenoic acid; 15-HPETE, 15-hydroxy-5c,8c,11c,13t-eicosatetraenoic acid; COX, cyclooxygenase; PBS, phosphate-buffered saline; β-OG, n-octyl-β-D-glucopyranoside; Ni-NTA, nickel-nitrilotriacetic acid.

The number of residues in oPGHS-1 is based on numbering the N-terminal methionine of the signal peptide as residue number 1.
repositions atoms C-13 to C-20. The result is an optimal positioning of C-15 for a second attack by molecular oxygen. Additionally, with the 15-hydroperoxyl radical now positioned in proximity to Tyr385, Tyr385 is poised to donate a hydrogen to form the product of the COX site, PGG₂, thus simultaneously reforming the Tyr385 radical and regenerating the enzyme for another round of catalysis.

Both PGHS isoforms exhibit some lipoxygenase activity. Small amounts of 11-hydroperoxy-8Z,12E,14Z,-eicosatrienoic acid and 15-hydroperoxy-8Z,11Z,13E-eicosatrienoic are formed from dihomo-/H9253\-linolenic acid (9), and the corresponding 11- and 15-HPETEs are produced from AA (10–12). Thus, the native enzyme is not 100% efficient in converting AA to PGG₂. Additionally, with native enzyme the Km values for AA in forming 11-HPETE, 15-HPETEs, and PGG2 are different for each product (11). This suggests that AA can exist in the COX site in at least three catalytically competent arrangements at the time of hydrogen abstraction to yield either PGG₂, 11R-HPETE, or 15R,S-HETE (11).

Extensive kinetic and product analysis of numerous mutants of the COX active site have identified several residues that influence the relative proportions of PGG₂ and monohydroperoxide products formed from AA (11–13). Two single amino acid substitutions, V349A and W387F, were of particular interest to us because relatively more 11R-HPETE is formed (35–50% of total products) without substantially altering the kinetics of oxygenation (12). Because each single mutation favors the formation of 11R-HPETE –50% of the time, we constructed and analyzed a V349A/W387F oPGHS-1 mutant. As described in this report, this double mutant produces predominantly 11R-HPETE. To investigate why this double mutant produces predominantly 11R-HPETE from AA, we determined the crystal structure of Co₃\-protoporphyrin V349A/W387F oPGHS-1 with AA bound in the COX active site. Significant differences in the conformations of AA bound to native oPGHS-1 versus V349A/W387F oPGHS-1 mutant enzyme were observed. Our results provide insight into how Val349 and Trp387 influence the conformation of AA and how these residues are involved in directing the formation of the endoperoxide group.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonic acid, 11R-HETE, and 15S-HETE were purchased from Cayman Chemical Co. (Ann Arbor, MI). [1-14C]Arachidonic acid (40–60 mCi/mmol) was from PerkinElmer Life Sciences. SF21 insect cells and 100× antibiotic-antimycotic were purchased from Invitrogen. Fetal bovine serum and HyQ-SFX insect medium used for cell growth and protein expression were purchased from HyClone (Logan, UT). Ni-NTA superfloresin was purchased from Qiagen, Tween 20 used for solubilization and purification was from Pierce, and n-octyl-\(\beta\)-D-glucopyranoside (\(\beta\)-OG) used for crystallization was from Anatrace (Maumee, OH). Flurbiprofen and hemin were purchased from Sigma, and Co\(^{3+}\)-protoporphyrin IX was from Porphyrin Products (Provo, UT). Oligonucleotides used as primers for mutagenesis and for sequencing were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility.

**Preparation of V349A/W387F oPGHS-1**—Site-directed mutants were prepared with a pFastbac vector containing a cDNA encoding native oPGHS-1 with a hexahistidine tag (14) and mutagenic oligonucleotides using the Stratagene QuickChange kit (Stratagene, La Jolla, CA). Mutations were confirmed with sequence analysis performed by the DNA Sequencing Facility at Michigan State University.

**Generation of Recombinant Baculovirus and Expression of oPGHS-1 Mutants**—DH10Bac cells were transformed with pFastbac containing cDNA for either V349A oPGHS-1 or V349A/W387F oPGHS-1 to obtain baculovirus. The recombinant baculovirus was amplified, and supernatants of amplifications were used as inoculum stocks. SF21 insect cells grown in a 17-liter bioreactor were infected with recombinant baculovirus at a multiplicity of infection of 0.01 at 27 °C. Three to four days after infection, the cells were harvested by centrifugation.

**Purification of PGHS-1 Mutants from SF21 Insect Cells**—Cell pellets from three bioreactors were resuspended in 1× phosphate-buffered saline (PBS), pH 8.0, containing 20 mM imidazole and were lysed by...
sonication. To solubilize PGHS from the membranes, Tween 20 was added to the lysate up to 1% (v/v) and was then incubated at 4°C for 5 min with gentle agitation. Solubilized lysate was centrifuged at 10,000 rpm at 4°C for 20 min, followed by ultracentrifugation at 45,000 rpm at 4°C for 90 min. The supernatant was carefully removed and incubated for at least 3 h at 4°C with Ni-NTA resin equilibrated in 1× PBS, pH 8.0, 20 mM imidazole, and 0.1% Tween 20. After this incubation period, the mixture of Ni-NTA resin plus supernatant was poured into a column that was washed with four column volumes each of 1× PBS, pH 8.0, 50 mM imidazole, 0.1% Tween 20 and 1× PBS, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.1% Tween 20. Hexahistidine-tagged PGHS was eluted with 1× PBS, pH 8.0, 200 mM imidazole, and 0.1% Tween 20. Fractions with the highest specific peroxidase activity (8) were pooled and concentrated using a MilliPore Ultrafree-15 spin concentrator (30-kDa molecular mass cut-off) to a protein concentration of 8–10 mg/ml. Detergent exchange and desalting of the concentrated protein was performed by spinning protein over 5 ml of packed G-25 Sephadex (Amersham Biosciences) equilibrated in 20 mM HEPES, pH 7.0, 20 mM NaCl, 1 mM Na3PO4, and 0.5% β-OG. The protein concentrations were determined using BCA protein assays (Pierce).

Analysis of Products Formed from Arachidonic Acid—Aliquots (equivalent to 150 COX units, where one unit is defined as 1 nmol of O2 consumed per min) of purified oPGHS-1 native and/or mutants were incubated for 1 min at 37°C with 35 μM [1-14C]arachidonic acid with and without 200 μM flurbiprofen in a reaction volume of 200 μl. The radioactive products were extracted and separated by thin layer chromatography as previously described (12). The products were visualized by autoradiography and quantified by liquid scintillation counting. Negative controls from samples containing flurbiprofen were subtracted from values measured from the corresponding samples not containing flurbiprofen.

Characterization of V349A/W387F oPGHS-1—Kinetic parameters for V349A/W387F oPGHS-1 were measured with a cytochrome oxidase assay by monitoring the initial rate of O2 uptake at 37°C using an oxygen electrode (12). A typical assay consisted of 3 ml of 100 mM Tris-HCl, pH 8.0, 1 mM phenol, 1 mM hemin, and 2–100 μM AA. The reactions were initiated by adding a volume of enzyme equivalent to 150 units of Ni-NTA-purified V349A/W387F oPGHS-1.

Crystalization and Data Collection—Purified apo-V349A/W387F oPGHS-1 (~8 mg/ml (0.11 mg/ml) was reconstituted with a 2:9-fold molar excess of Co2+-protoporphyrin IX. Prior to setting up sitting drop crystallization experiments, the protein was incubated in the presence of AA at a 5:fold molar excess over the protein concentration. The protein was mixed 1:1 with buffer containing 0.68 M sodium citrate, 0.6–0.9 M LiCl, 1 mM NaN3, and 0.3% β-OG and equilibrated within a reservoir containing 0.68–0.9 M sodium citrate, 0.60–0.90 M LiCl, and 1 mM NaN3. Crystals appeared after 4 weeks to several months. Crystals were harvested with a nylon loop and transferred briefly into a cryo solution containing 0.9 M sodium citrate, 1.0 M LiCl, 0.15% β-OG, 1 mM NaN3, and 24% (v/w) sucrose. The crystals were immediately flash frozen in liquid propane at −165°C. The data were collected at beamline 19ID of the Structural Biology Center (Advance Photon Source, Argonne IL). The data from a single crystal were indexed and integrated using HKL2000 (15) and then scaled together using SCALEPACK (15) (Table I).

Structure Determination and Refinement—The crystals of V349A/W387F oPGHS-1 with AA were essentially isomorphous with crystals of oPGHS-1 complexed with AA. Thus, the structure of V349A/W387F oPGHS-1 was determined by using the protein atoms of native oPGHS-1:AA (Protein Data Bank code IDIY) as a phasing model for simple rigid body refinement utilizing CNS version 1.1 (16), to correct for small changes in lattice parameters. After an initial round of simulated annealing and positional and group B-factor refinement, the resulting R and Rmerge factors were 25.4 and 31.2%. Inspection of the initial 2Fo – Fc (contoured at 1σ) and Fc – Fc (contoured at 2.5σ) electron density maps using the program CHAIN (17) allowed the placement of Co2+-protoporphyrin IX, seven carbohydrate residues, and three β-OG detergent molecules. Strong electron density was observed for AA starting from the carbohydrate group up to carbon C-4. With subsequent rounds of refinement, the improved phases led to the appearance of further electron density, which allowed the additional placement of atoms C-5–C-12 of AA. No further electron density was observed for AA. Although no density was observed for C-13, the position of C-13 was modeled based on the limited orientations it can adopt because of the stereochemical restrictions of the C-11–C-12 double bond and was included in the refinement; thus C-1 (including the carbohydrate) to C-13 of AA was included in the refinement of the model. Further refinement included the addition of 22 water molecules at positions that were within 2.4–3.6 Å of a hydrogen bond donor/acceptor and an electron density peak of 3σ or greater. The R and Rmerge values of the final model are at 23.2 and 30.8% (Table I). Evaluation with PROCHECK (18) showed that all non-Pro and non-Gly residues lay within the most favored or allowed regions of the Ramachandran plot.

Because medium resolution diffraction data were used in this analysis, the following two tests were performed to verify that changes observed in the AA conformation between the structures of native and V349A/W387F oPGHS-1 were significant. In the first test, Fc – Fc difference electron density maps were calculated using the observed structure factors and modified phase sets. Because the crystals of native and mutant oPGHS-1 were isomorphous, a calculated phase set was created by combining the Co2+-heme V349A/W387F oPGHS-1, excluding the observed AA atoms, with atoms from the AA conformer observed in the refined model of native Co2+-heme oPGHS-1:AA complex (Protein Data Bank code IDIY). A similar method has been utilized previously to verify observed differences between AA and several other fatty acid substrates bound within the COX site (19, 20). In areas where the carbon positions differ between the two observed conformations of AA, we would expect to observe the appropriate positive and negative difference density peaks, indicating a shift in atom positions.

The second test addressed the possibility that other conformations of
Crystal Structure of AA bound to Mutant PGHS-1

**Comparison of Products Formed from Arachidonic Acid by Native and Mutant oPGHS-1**—There are considerable differences in the proportions of oxygenated products formed from [1-14C]arachidonic acid by native oPGHS-1, V349A oPGHS-1, and V349A/W387F oPGHS-1 (Fig. 2). Native enzyme produces predominantly products derived from PGG2 (~95% including 12-hydroxy-5c,8c,10t-heptadecatrienoic acid and various prostaglandins) and small amounts of 11-HETE (~2.5%) derived from 11-HPETE and 15-HETE (~2.5%) from 15-HPETE. In comparison, the V349A/W387F double mutant produces predominantly 5,11-HETE (~84%) and correspondingly less PGG2-derived products (~12%) than native enzyme. V349A oPGHS-1 forms about 50% 11-HETE (12).

**Kinetic Analysis of V349A/W387F oPGHS-1**—To determine whether AA is utilized with similar efficiencies by native and V349A/W387F oPGHS-1, the Km value for the oxygenation of AA was determined using a cyclooxygenase assay. The Km for AA of V349A/W387F oPGHS-1 was determined to be 5 μM, a value very similar to that previously reported for native enzyme under similar assay conditions (11, 12).

**Arachidonic Acid Bound in the COX Site of Co3+-heme V349A/W387F oPGHS-1**—The electron density of the Co3+-heme V349A/W387F oPGHS-1:AA complex was of good quality for building in considerable amounts of nonprotein substructure (i.e., the Co3+-porphyrin IX, the carbohydrate groups linked to residues Asn68, Asn144, and Asn410, and three β-GD detergent molecules bound within the membrane binding domain). The overall structure, which includes the epidermal growth factor and the membrane-binding and the catalytic domains of the Co3+-heme V349A/W387F oPGHS-1:AA complex, is virtually identical to that of Co3+-heme oPGHS-1:AA complex having a root mean square deviation of 0.3 Å over all Co atoms. This indicates that the overall structure is relatively unchanged by the two mutations (V349A and W387F) in the COX active site.

The Fo − Fc electron density map allowed the placement of carbons C-1–C-12 into the electron density with some degree of confidence (Fig. 3), although no electron density was observed for carbons C-13–C-20. Given the broad extent of the electron density observed at this medium resolution, it was essential to verify that the observed differences in conformation of bound AA between native and V349A/W387F oPGHS-1 were significant. In a test using a Fo − Fc difference electron density map analysis (see “Experimental Procedures”), inspection of the electron density maps calculated with AA from the native oPGHS-1:AA complex within the V349A/W387F oPGHS-1 active site showed a significant positive peak contoured at 3 σ representing the correct position of atoms C-3–C-7 of AA bound within the mutant active site (data not shown). In addition, a smaller negative peak contoured at 3 σ was observed representing the misplaced positions of C-2–C-4 of the conformation of AA observed in the oPGHS-1:AA complex (Protein Data Bank entry 1DIY). The results of this test indicate that the data are of good quality at this limited resolution and show that conformation of AA bound within the V349A/W387F oPGHS-1 mutant differs from that seen in the native enzyme.

A second validity test, using a series of simulated annealing refinement (see “Experimental Procedures”), generated a family of AA conformers that were indistinguishable from the conformation of AA bound within the V349A/W387F mutant. All conformers from this refinement series fit the observed density well. The conformer that best fit the electron density is shown in Fig. 3, along with the conformer of AA obtained by model building. Both conformers are bound in the COX channel of the mutant enzyme with the carboxylate group oriented and stabilized at the mouth of the channel by two ionic interactions with the guanidinium group of Arg120 and one with the phenolic oxygen of Tyr265 (Fig. 3). From the carboxylate at C-1, the carbons of AA weave through the COX active site making hydrophobic contacts with various side chains along the path to Tyr265. Continuous density was observed from the carboxylate of AA to C-12; however, no density was observed past C12 (Fig. 3). The placement of C-13, the site of initiation of catalysis via hydrogen abstraction, was based on the stereochemical constraints imposed by C-12 and the C-11–C-12 double bond. Although there are minor shifts in the position of the C-8–C-9 and C-11–C-12 double bonds, both conformers are strikingly...
similar particularly when comparing the carbon positions of the carboxylate group up to C-7.

The family of conformers, including the conformer shown in Fig. 3 obtained from the simulated annealing experiments described under “Experimental Procedures,” is shown in Fig. 4. Aside from minor differences in atom positions, the four conformations obtained from the simulated annealing refinement starting from the AA bound to native enzyme are all markedly similar to that obtained by model building (Fig. 4A). The simulated annealing experiments show that the carbon chain weaves up into the active site with the same trajectory for all eight conformers. The results of simulated annealing tests strongly suggest that the X-ray data contain a sufficient amount of information to derive a unique family of AA conformers that differ in conformation from that seen in the native oPGHS-1:AA complex.

The conformers in Fig. 4A are shown with their ω ends (C-13–C-20). Although there is no observable density for this portion of the substrate, the positioning of these atoms from simulated annealing refinement was entirely guided by the nonbonded energy terms of the molecular dynamics. Despite the lack of experimental data to validate the positions of these atoms, the conformation of the AA in this region is constrained by energy-minimized, nonbonded contacts. Thus, the ω ends of the AA conformers are in stereochemically reasonable conformations. While having a limited level of confidence, it is interesting to note that the positions of the proS hydrogens are generally found to cluster in a group near to that of AA found in native enzyme (Fig. 4A).

**Structural Comparison of Arachidonic Acid Bound to Native and V349A/W387F oPGHS-1.**—To verify that the two mutations V349A/W387F did not alter the overall integrity of the active site, all atoms of 17 side chains (excluding residues 349 and 387) within the COX active site of native and double mutant were superimposed giving an root mean square deviation of 0.4 Å. This indicates that the positions of the active site residues in the crystal structure of V349A/W387F oPGHS-1 were not significantly perturbed by the presence of the two mutations. The overall binding of AA within the COX active sites of both the native and the double mutant is similar with respect to many of the key ionic and hydrophobic interactions (Table II).

Although the structure of AA bound to the V349A/W387F mutant is grossly similar to that of AA bound to native enzyme, there are several significant differences. Atoms C-3–C-6 of AA are shifted into the space created by the V349A substitution (Fig. 5). In the native structure, Val-349 contacts C-3 and C-4 (Table II). In the mutant, these interactions are lost, and C-3 and C-4 rotate and move into the space created by this mutation. Rotations in this portion of the AA molecule result in large shifts of more rigid regions of AA as evidenced by the significant downward shift of the C-5–C-6 double bond (Fig. 5). The differences in the positions of carbons between AA in the native enzyme versus V349A/W387F oPGHS-1 structures are as great as 3.0 Å when taking into account coordinate error of 0.43 Å.
There is also a significant difference in the location of the C-11–C-12 double bond of AA, which is moved via rotation about the C-9–C-10 bond toward the space created by the W387F mutation. In the native structure, Trp 387 makes two contacts with C-11 and one with C-12 (Table II). The loss of these contacts in the double mutant contributes to displacement of the C-11–C-12 bond.

**DISCUSSION**

Native PGHS-1 forms small amounts of 11R-HPETE and 15R,S-HPETE in addition to the major product PGG₂ (11, 12, 21). The monohydroperoxides have little or no biological activity and are considered to be abortive products that result from a failure of the enzyme to form the endoperoxide. In earlier studies, we provided kinetic evidence that the nature of the oxygenated product formed by native PGHS-1 is determined by the conformation of AA when the rate determining step in cyclooxygenase catalysis, abstraction of the 13-proS hydrogen (9), occurs (11, 12). We performed the studies reported here as a structural test of this concept. We developed a V349A/W387F mutant of PGHS-1 that forms primarily 11-HPETE, determined the structure of AA in the COX active site of the mutant, and finally compared the structures of AA in the native and mutant oPGHS-1.

| Atom of AA | Native oPGHS-1 (residue/atom) | Distance | V349A/W387F oPGHS-1 (residue/atom) | Distance |
|------------|-------------------------------|----------|-----------------------------------|----------|
| C-3        | Val³⁴⁹/CG1                     | 3.4      | Ala³⁴⁹/CG1                         | >4       |
| C-4        | Val³⁴⁹/CG1                     | 3.1      | Ala³⁴⁹/CB                          | >4       |
| C-5        | Ile³⁵²/CG2                     | 3.3      | Ile³⁵²/CG2                         | >4       |
| C-6        | Val³⁴⁹/CB                       | >4       | Ala³⁴⁹/CB                          | 3.0      |
| C-7        | Leu³⁵³/CD2                     | 3.9      | Leu³⁵³/CD2                         | >4       |
| C-8        | Trp³⁵⁷/CD2                     | 3.4      | Leu³⁵³/CD2                         | 3.8      |
| C-9        | Trp³⁵⁷/CH2                     | 3.4      | NA                                 | NA       |
| C-10       | NA                             | NA       | NA                                 | NA       |
| C-11       | Leu³⁵³/CD2                     | 3.4      | Leu³⁵³/CD2                         | 3.8      |
| C-12       | Leu³⁵³/CD2                     | 3.9      | Leu³⁵³/CD2                         | >4       |
| C-12       | Tyr³⁵⁶/CEZ                     | 3.2      | Tyr³⁵⁶/CEZ                         | >4       |
| C-12       | Trp³⁵⁷/CH2                     | 3.7      | NA                                 | NA       |
| C-12       | NA                             | NA       | Phe³⁵⁷/CE2                         | 3.9      |
| C-12       | NA                             | NA       | Phe³⁵⁷/CE2                         | 3.7      |

There is also a significant difference in the location of the C-11–C-12 double bond of AA, which is moved via rotation about the C-9–C-10 bond toward the space created by the W387F mutation. In the native structure, Trp³⁵⁷ makes two contacts with C-11 and one with C-12 (Table II). The loss of these contacts in the double mutant contributes to displacement of the C-11–C-12 bond.

**DISCUSSION**

Native PGHS-1 forms small amounts of 11R-HPETE and 15R,S-HPETE in addition to the major product PGG₂ (11, 12, 21). The monohydroperoxides have little or no biological activity and are considered to be abortive products that result from a failure of the enzyme to form the endoperoxide. In earlier studies, we provided kinetic evidence that the nature of the oxygenated product formed by native PGHS-1 is determined by the conformation of AA when the rate determining step in cyclooxygenase catalysis, abstraction of the 13-proS hydrogen (9), occurs (11, 12). We performed the studies reported here as a structural test of this concept. We developed a V349A/W387F mutant of PGHS-1 that forms primarily 11-HPETE, determined the structure of AA in the COX active site of the mutant, and finally compared the structures of AA in the native and mutant oPGHS-1.

Previous studies identified Val³⁴⁹ and Trp³⁵⁷ as playing par-
Crystal Structure of AA bound to Mutant PGHS-1

particularly significant roles in guiding formation of the 9,11-endoperoxide group (11–13). Substitutions at either site yield mutant enzymes that produce 30–55% 11R-HPETE (12). The fact that the V349A/W387F mutant described here forms mainly 11R-HPETE suggests that in native PGHS-1 Val349 and Trp387 function coordinately to facilitate PGG2 formation.

The structure of AA in the cyclooxygenase site of V349A/W387F oPGHS-1 was observed to be significantly different from that of AA in native PGHS-1. Both AA structures appear to be catalytically competent in that the distances between the phenolic oxygen of Tyr385, and the 13proS hydrogen of AA are estimated to be within about 3 Å in both structures; however, it should be noted that the 13proS hydrogen modeled into the AA structure in V349A/W387F oPGHS-1 is more distant than that in the native oPGHS-1:AA complex. Because native and V349A/W387F oPGHS-1 form primarily PGG2 and 11R-HPETE, respectively, the results of our structural studies are consistent with the overall model that the product formed by PGHS-1 is determined by the structure of AA in the cyclooxygenase site when the rate-limiting step in the reaction occurs.

Comparisons of the AA structures observed in the native and mutant enzymes and modeling the structure of a putative 11-hydroperoxyl radical into the COX sites suggest how Trp387 and Val349 might participate in forming the endoperoxide group. In the native enzyme there are interactions between Trp387 and both C-11 and C-12 that are eliminated when Trp387 is mutated to phenylalanine. The interactions between Trp387 and C-11 and C-12 may (a) constrain the orientation of the C-11–C-12 bond, thereby positioning C-11 optimally with respect to C-9, and (b) after oxygen insertion at C-11, guide rotations of the 11R-hydroperoxyl group and the C-10–C-11 bond to move the hydroperoxyl group toward C-9 for an R side attack. Mutation of Trp387 to phenylalanine eliminates the steric interactions involving C-11 and C-12 that direct formation of the endoperoxide group. We suggest that with the mutant enzyme, changes in the rotational freedom about both the C-10–C-11 bond and the bond between C-11 and the peroxyl oxygen allow the 11-hydroperoxyl intermediate to assume unproductive conformations, where the attack by the 11-hydroperoxyl group on C-9 is unfavorable and no endoperoxide can be formed.

The V349A mutation further contributes to the altered orientation of the C-8–C-13 portion of AA in the V349A/W387F mutant. Past studies (11–13, 19, 20) have documented the influence of Val349 on product formation and the stereochemistry of O2 insertion. The role of Val349 in supporting formation of the endoperoxide group is interesting because the effect is exerted over a relatively long distance. In native oPGHS-1, Val349 protrudes into the COX channel, and the C61 atom of Val349 contacts C-3 and C-4 of AA (Table II), restricting movement of the flexible C-1–C-5 region at the carboxyl end of AA (8, 12). When Val349 is mutated to alanine, C-3 and C-4 of AA rotate into the space created by the mutation, and the C-5–C-6 double bond moves into the newly available space. These results are consistent with the idea that in the native enzyme, Val349 acts as a structural guide to position the carboxyl end of AA (12). The shifts in the positions of atoms C-3–C-6 of AA in the V349A/W387F result in a significant change in the final orientation of the C-11–C-12 bond that, in turn, permits movement of atoms C-9–C-12 of AA into the extra space created by the W387F mutation (Fig. 5). Thus, in the native enzyme the positioning the carboxyl end of AA by Val459 must facilitate proper positioning of the atoms C-8–C-13 for hydrogen abstraction and endoperoxide formation.

In summary, residues Val349 and Trp387 in native oPGHS-1 are crucial for ensuring that AA adopts the proper conformation so that the reaction proceeds on course to produce PGG2 (12). We have found that V349A/W387F human PGHS-2 also produces predominantly 11R-HETE, suggesting that these two residues play similar roles in PGHS-2. The structural evidence provided by our present study supports the hypothesis that the nature of the products formed in the COX reaction depends on the conformation of AA at the instant of abstraction of the 13proS hydrogen. The conformation of AA in the COX site of native oPGHS-1 differs significantly from the conformation of AA in the COX site of V349A/W387F oPGHS-1. This additional, albeit indirect evidence supports the conclusion that the conformation of AA observed in the native enzyme (8, 12) is that which leads to PGG2 formation.

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