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Fatty Acid Substrate Interactions with Cyclooxygenase Active Site Residues in Ovine Prostaglandin Endoperoxide H Synthase-1

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Elizabeth Dager Thuresson

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of the requirements for

Ph.D. degree in Biochemistry

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FATTY ACID SUBSTRATE INTERACTIONS WITH CYCLOOXYGENASE ACTIVE SITE RESIDUES IN OVINE PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-1

By

Elizabeth Dager Thuresson

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

2000

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ABSTRACT

FATTY ACID SUBSTRATE INTERACTIONS WITH CYCLOOXYGENASE ACTIVE SITE RESIDUES IN OVINE PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-1

By

Elizabeth Dager Thuresson

Prostaglandin endoperoxide H synthases-1 and –2 catalyze the conversion of arachidonic acid and two molecules of O_2 to prostaglandin G_2. This is the committed step in the formation of prostaglandins and thromboxane A_2. Prostaglandin synthases also exhibit some lipoxygenase activity producing small amounts of the monohydroxy acids, 11*R*-hydroxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid and 15*S/R*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid. Using thin layer chromatography and reverse- and chiral-phase high performance liquid chromatography, we have examined the products formed from AA and a cyclooxygenase active site mutant V349L ovine prostaglandin synthase-1 and have determined the kinetics for the formation of each individual product. With both native and V349L oPGHS-1, the K_M values for prostaglandin G_2 and hydroxy acid formation were different. These results establish that AA can assume at least three catalytically productive arrangements within the cyclooxygenase site of oPGHS-1 leading to prostaglandin G_2, 11*R*- and 15*S*- and/or 15*R*-hydroxyeicosatetraenoic acids, respectively. IC_{50} values for inhibition of formation of the individual products by the competitive inhibitor, ibuprofen, were determined and found to be the same for a given enzyme form, suggesting that the various substrate/enzyme arrangements occur after the binding of substrate in the cyclooxygenase active site.

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We have also performed mutational analyses to determine the roles of residues lining the hydrophobic pocket of the prostaglandin synthase-1 cyclooxygenase active site in (1) arachidonate binding and oxygenation and (2) the oxygenation of fatty acid substrates other than arachidonate. Tyr348, when substituted with phenylalanine, exhibited catalytic properties similar to that of the native enzyme, while a leucine substitution resulted in a complete loss of cyclooxygenase activity. These results suggest that interactions between the phenyl ring of Tyr348 are essential for positioning of C-13 of arachidonate for hydrogen abstraction. Substitutions of Val349 and Leu534, when reacted with arachidonate, resulted in catalytically active enzymes which produced as great as a 20-fold abundance of the monohydroxy acid products, 11*R*- and 15*S*-hydroxyeicosatetraenoic acids, respectively. These results establish that Val349 and Leu534 provide hydrophobic interactions with arachidonate which contribute to positioning the substrate such that when hydrogen abstraction occurs the fatty acid is optimally aligned to yield prostaglandin G_2 rather than monohydroxide products. Val349 and Leu534 were also found to play significant roles in oxygenation of fatty acids other than arachidonate. Conversion of Val349 to alanine caused >800 fold decreases in the efficiencies (V_{max}/K_M) of oxygenation of 20:3n-6 and 20:5n-3 compared with only a 35% decrease in the efficiency of arachidonate oxygenation. Thus, Val349 is particularly critical in positioning the carboxyl half of these alternative substrates for proper alignment of C-13. A L534V mutant oxygenated C_{20} but not C_{18} substrates, suggesting an important role in positioning of the 18-carbon fatty acids for catalysis. The remaining residues comprising the cyclooxygenase active site channel were determined to provide measurable but lesser contributions to optimizing catalysis.

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ACKNOWLEDGMENTS

I would first like to thank my advisor, Bill Smith, for sharing his wealth of knowledge with me and teaching me a tremendous amount about science and scientific thinking. I also want to thank him for opening his lab to me when I knew very little and providing me with a great number of scientific opportunities. He has made my journey through this program and, more importantly, my successful completion of this program, possible. For this, I owe him a debt of gratitude. I have enjoyed working with the members of the Smith and DeWitt labs, past and present, for all of their help and humor and color and friendship during what can sometimes be a very intense experience.

I am grateful to Joseph Leykam of the MSU Macromolecular Structure Facility for his tireless assistance with the HPLCs, to Dr. Honggao Yan for his invaluable instruction in enzyme kinetics and to Mike Malkowski for providing countless crystallographic figures and help with computer modeling, as well as being a very beneficial scientific sounding board. Also to Julie Oesterle who is worth her weight in gold to the graduate students of Biochemistry, thank you for taking some of the stress out of all of our lives. I would like to thank some of my Biochemistry companions for all of their support and friendship through all the years I've been here including Jill, Tonya, Raj, Karen, Colleen and Wayne. Also, I thank my parents for their support and faith, my sister for her sanity and advice and my brother for providing me with the thick skin required for this task. Finally, I never could have done any of this without the love and support of my wonderful husband Magnus and of course, our daughter Anna, who added a sweetness to this part of my life which would have been otherwise absent.

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POX
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sPLA$_2$
NSAID
HPLC
TLC
EGF
MBD
Tx
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PBS
SDS-PAGE
TMPD
Aliphatic and aromatic carbons

LIST OF ABBREVIATIONS

PGHS	prostaglandin endoperoxide H synthase
oPGHS-1	ovine prostaglandin endoperoxide H synthase-1
hPGHS-2	human prostaglandin endoperoxide H synthase-2
COX	cyclooxygenase
AA	arachidonic acid
DHLA	dihomo-γ-linolenic acid
20:4n-6	arachidonic acid
20:3n-6	dihomo-γ-linolenic acid
20:5n-3	eicosapentaenoic acid
18:2n-6	linoleic acid
18:3n-3	α-linolenic acid
11-HETE	11-hydroxy-5,8,12,14-eicosatetraenoic acid
11-HPETE	11-hydroperoxy-5,8,12,14-eicosatetraenoic acid
15-HETE	15-hydroxy-5,8,11,13-eicosatetraenoic acid
15-HPETE	15-hydroperoxy-5,8,11,13-eicosatetraenoic acid
12-HHTre	12-hydroxy-5,8,10-heptadecatrienoic acid
9-HODE	9-hydroxy-10,12-octadecadienoic acid
13-HODE	13-hydroxy-9,11-octadecadienoic acid
V_{max}	maximum velocity of a reaction
K_M	Michaelis-Menton constant
K_I	dissociation constant for enzyme-inhibitor complex
IC_{50}	inhibitor concentration for 50% inhibition
POX	peroxidase
$cPLA_2$	cytosolic 85 kDa phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
NSAID	non-steroidal anti-inflammatory drug
HPLC	high performance liquid chromatography
TLC	thin layer chromatography
EGF	epidermal growth factor
MBD	membrane binding domain
Tx	thromboxane
PG	prostaglandin
DMEM	Dulbecco's modified Eagle medium
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel elecrophoresis
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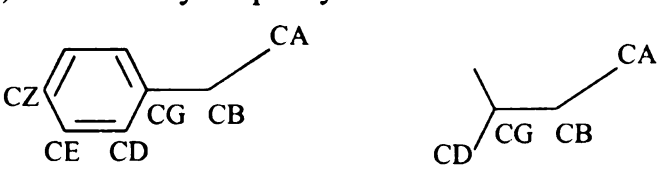
Aliphatic and aromatic carbons	

CHAPTER I

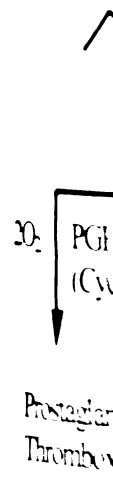
LITERATURE REVIEW

Introduction

Prostaglandin endoperoxide H sythases-1 and –2 (PGHS-1 and –2) or cyclooxygenases (COX-1 and -2) are the key enzymes in the synthesis of prostanoids from arachidonic acid [1-3]. Prostanoids, along with leukotrienes (LTs) and epoxyeicosatetraenoic acids (EETs) belong to a family of oxygenated 20-carbon lipid signaling molecules called eicosanoids (Figure 1). The prostanoids (prostaglandins, prostacyclins and thromboxanes) act in an autocrine or paracrine fashion through the actions of G-protein linked receptors to regulate a broad range of physiological processes including vascular homeostasis, platelet aggregation, renal water homeostasis, inflammation and reproductive functions [4-6]. They have also been implicated in pathologies such as the development of colon cancer [7, 8] and Alzheimer's disease [9].

Prostanoid synthesis is initiated by the interaction of various hormones with their cell surface receptors, resulting in the activation of one or more phospholipase A_2s (PLA_2s). PLA_2s mobilize arachidonic acid from phospholipid stores, after which free arachidonate can then be acted upon by PGHSs and converted to various prostanoid products.

There are two PGHS isozymes, known as PGHS-1 and PGHS–2. Both PGHSs catalyze the first two steps in the biosynthesis of prostaglandins via a cyclooxygenase activity in which arachidonic acid is oxidized to the hydroperoxy endoperoxide PGG_2 and



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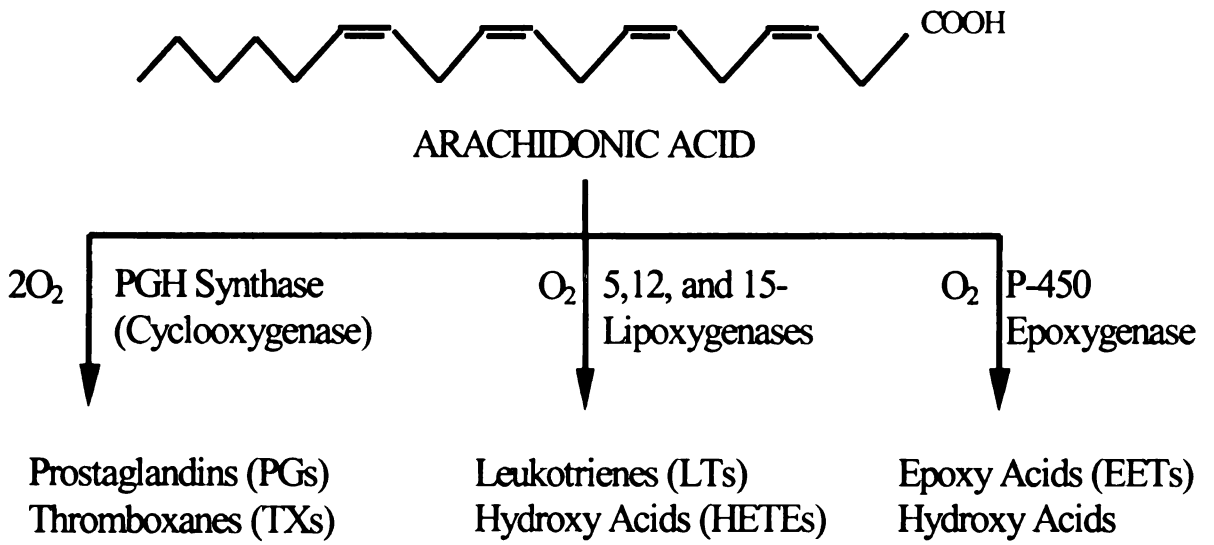

Figure 1. The arachidonate cascade. Arachidonic acid is metabolized by three different enzymes systems: the cyclooxygenase, the lipoxygenase and the P450 epoxygenase pathways

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a peroxidase activity by which PGG_2 is reduced to the hydroxy endoperoxide PGH_2. These two activities occur at distinct albeit interacting sites on the PGHS molecule and both activities require heme [10-13]. PGH_2, the final product of PGHSs, is then transformed by a range of enzymes and nonenzymatic mechanisms into the primary prostanoids, prostaglandins (PG) E_2, $F_{2\alpha}$, D_2, I_2 and thromboxane (Tx)A_2 [14-16] (Figure 2).

PGHS-1 and –2 are considered to be the constitutive and inducible PGHSs, respectively. Despite numerous catalytic and structural similarities, PGHS-1 and –2 exhibit very different patterns of expression in mammalian tissues. PGHS-1 is expressed constitutively in nearly all tissues [17] and forms prostanoids central to several housekeeping functions including water reabsorption in the kidney, vascular homeostasis and platelet aggregation. PGHS-2 is normally undetectable in most cells, but both mRNA and protein can be rapidly induced in many cell types upon treatment with inflammatory cytokines, growth factors and tumor promoters [17]. Thus, prostanoids formed by PGHS-2 have been implicated in the inflammatory response as well as in cell replication and differentiation. Other notable differences between the two isozymes include membrane binding domains of different sequence and an 18-amino acid cassette containing an additional glycosylation site found only near the C-terminus of PGHS-2 [18].

The cyclooxygenase activity of PGHSs is importantly the therapeutic target of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, ibuprofen and naproxen. [19, 20]. These drugs interact with the cyclooxygenase active site competing with arachidonate for binding. In the case of aspirin, the IC_{50} is about 20mM in the

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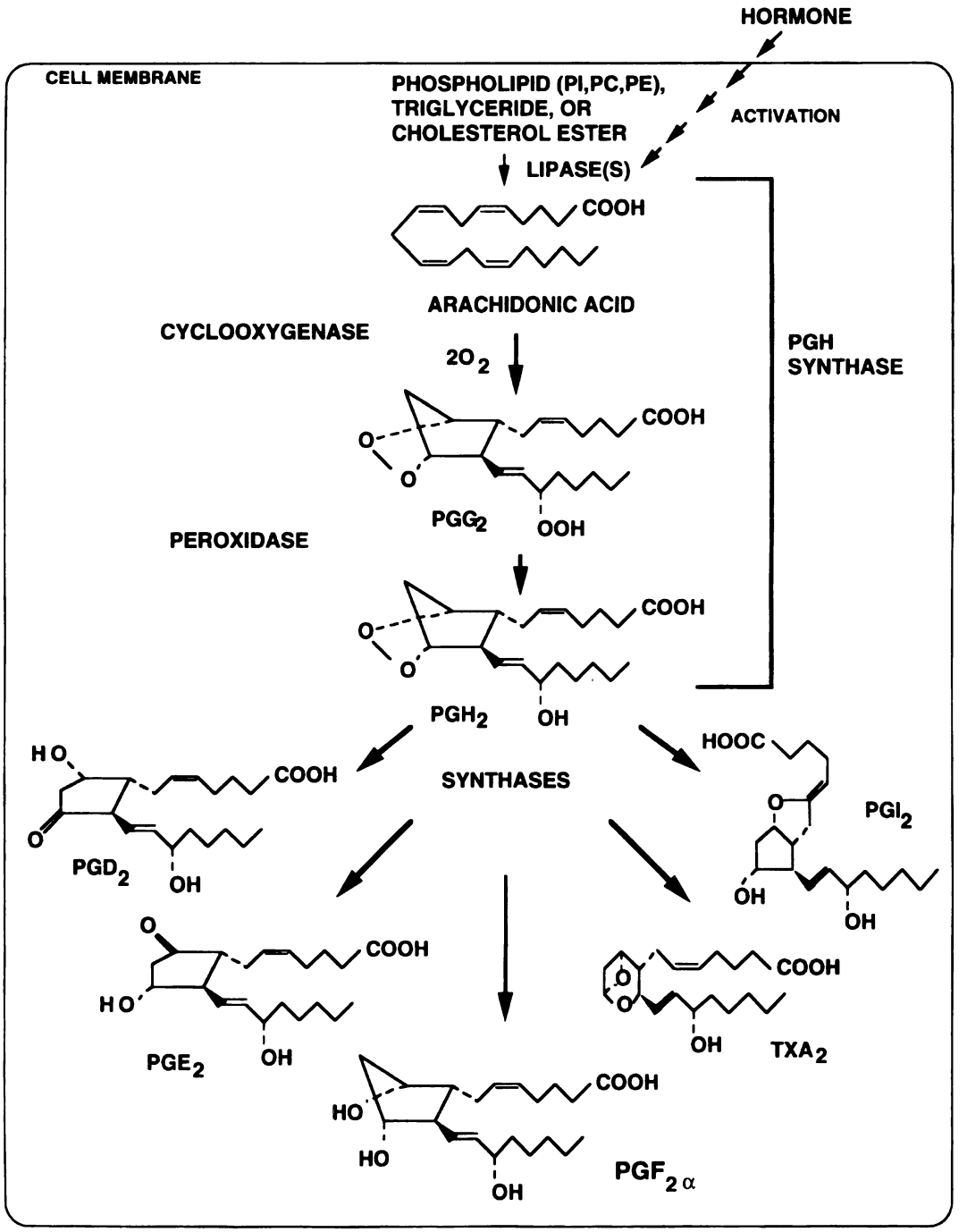

Figure 2. Biosynthetic pathway for the formation of prostanoids derived from arachidonic acid.

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presence of 100μM arachidonate [21]. However, an important time-dependent effect of aspirin is acetylation of the hydroxyl group of Ser530 and concomitant irreversible cyclooxygenase inactivation [22, 23]. Although all residues important for catalysis are conserved between PGHS-1 and PGHS-2, the PGHS-2 cyclooxygenase active site is somewhat larger than that of PGHS-1 and has recently become the target for development of novel drugs such as celecoxib and rofecoxib which selectively inhibit PGHS-2 and appear to lack the gastrointestinal side effects associated with more classical NSAIDs.

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Mobilization of Arachidonic Acid and Prostanoid Biosynthesis

The biosynthesis of prostaglandins requires the release of arachidonic acid from phospholipid stores. Arachidonic acid is preferentially found at the *sn*-2 position of glycerophospholipids and is cleaved by one of a number of phospholipase A_2s (PLA_2s) to generate a lysophospholipid and free arachidonate available to PGHSs and lipoxygenases. The number of identified PLA_2s is growing steadily (there are at least 16) and all are classified according to molecular weight, amino acid sequence, calcium dependence and structural homology [24, 25]. Despite their common function of releasing arachidonic acid from membranes, the physiological roles of different PLA_2s appear to be distinct, and different PLA_2s may be independently coupled to PGHS-1 or PGHS-2 [26].

Cytosolic PLA_2 (called $cPLA_2$ or Group IV PLA_2) is a large (85-100 kDa) enzyme that, when activated, preferentially hydrolyzes arachidonate from the *sn*-2 position of phosphoglycerides. Agonist-induced increases in intracellular calcium levels (μM levels) activate $cPLA_2$ by promoting its association with membranes [27, 28] and $cPLA_2$ has been shown to mediate hormonally-induced arachidonic acid release. Activity of $cPLA_2$ is also potentiated by phosphorylation of Ser-505 by kinases of the mitogen-activated protein kinase cascade [29-31]. $cPLA_2$ can be activated by a wide variety of stimuli including the proinflammatory cytokines interleukin 1 (IL-1) [32, 33], tumor necrosis factor (TNF) [34], thrombin [32, 35] and lipopolysaccharide (LPS), among others. When cells are stimulated by agents that mobilize intracellular Ca^{2+}, $cPLA_2$ translocates from the cytosol to the membrane where it interacts with phospholipid

substrates [36]. This translocation is mediated through the CaLB or C2 domain, a calcium-dependent phospholipid-binding regulatory domain of the phospholipase.

The other major group of PLA_2s comprises the 13-18kDa secretory PLA_2s ($sPLA_2$s or Groups I, IIA, IIC, V and X). These enzymes also require calcium (mM levels) but use it for the catalytic mechanism of arachidonate release rather than for membrane association. The mammalian $sPLA_2$s which have been characterized (Groups IB, IIA, IIC, V and X) are distinguished by their number of disulfide bridges, expression patterns and physiological roles [25]. These enzymes are not specific for arachidonic acid at the *sn*-2 position and will hydrolyze linoleic acid from the *sn*-2 position with equal efficiency.

The calcium independent PLA_2s ($iPLA_2$ or Groups VI, VII and VIII PLA_2s) have most recently been identified, but only the Group VI enzyme has been characterized in detail [37, 38]. It shares some characteristics with both the $sPLA_2$s and others with the $cPLA_2$. Like the $sPLA_2$s, the $iPLA_2$ exhibits no substrate specificity for arachidonate-containing phospholipids and appears not to be subjected to posttranslational covalent modifications, but like the $cPLA_2$ the $iPLA_2$ is large (80-85kDa) and cytosolic [38].

It is unclear as to which group of phospholipases is primarily responsible for providing arachidonate to PGHSs. However, there is a growing body of evidence indicating that $cPLA_2$ acts in concert with various $sPLA_2$s (particularly Groups V and IIA) in generating arachidonic acid for oxygenation by PGHS-1, PGHS-2 or both, depending on the stimulation and cell type [26] [39]. With the PLA_2s as well as the two PGHS isozymes, the contribution to overall prostaglandin synthesis appears to be largely dependent on cell type and stimulus used. Activation of the $cPLA_2$ appears to be the

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principal event [40-42] and may be mediated by several signals such as phosphorylation cascades and intracellular Ca^{2+} levels [28, 43]. The cytosolic phospholipase, upon activation, translocates to the perinuclear and endoplasmic reticular membranes [27, 28], liberating arachidonic acid where PGHSs are co-localized [44]. $cPLA_2$ has been shown to play an important role in immediate eicosanoid biosynthesis occurring within minutes of stimulation as well as in the delayed, PGHS-2-dependent prostanoid generation lasting for hours [45-48]. In cells not expressing $sPLA_2$s, $cPLA_2$ appears to account for the majority of arachidonate mobilized during cellular activation. However, in cells that contain $sPLA_2$, the bulk of arachidonate release appears to be mediated by $sPLA_2$, not the $cPLA_2$ [41, 49, 50], however, $sPLA_2$ action, particularly that of Groups IIA and V, appears to be dependent on an active $cPLA_2$ [41, 45, 49, 50]. Thus $cPLA_2$ is key for arachidonate signaling even when $sPLA_2$ is the major source of arachidonate used for eicosanoid biosynthesis. $sPLA_2$s, once secreted, associate with the outer surface of cells and liberate arachidonate which can be used by surrounding cells to produce eicosanoids [30, 39, 41, 45, 51].

Once the arachidonate is released, it is then converted to PGH_2 through the actions of PGHSs. Release of PGH_2 intracellularly allows for subsequent interaction with relevant synthases for conversion to prostaglandin products. Cell-specific expression of the different prostaglandin synthases dictates the final products formed (Figure 2). Immediately following their synthesis, prostaglandins are released from the cell via at least one known prostaglandin transporter [52, 53] and act in either an autocrine or paracrine fashion on cells via several specific G protein-linked prostanoid receptors on the plasma membrane [54]. These receptors are specific for PGE, PGI, PGD, PGF and

thromboxane (the EP, IP, DP, FP and TP receptors) and are coupled to heterotrimeric G-proteins which, in turn, modulate the activities of effector proteins to control cellular levels of cAMP, calcium and phosphatidyl inositol [53].

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Peroxidase Catalysis by PGHS

PGHSs catalyze both a cyclooxygenase (*bis*-oxygenase) and a peroxidase reaction. The cyclooxygenase reaction involves the addition of two molecules of O_2 to arachidonic acid to produce prostaglandin G_2 (PGG_2). PGG_2 then undergoes a two-electron reduction in the peroxidase reaction to produce PGH_2. These two reactions occur at distinct but structurally and functionally interconnected sites. The peroxidase reaction occurs at a heme-containing active site located near the protein surface. The cyclooxygenase reaction occurs in a hydrophobic channel in the core of the enzyme. In vitro, the peroxidase activity can operate independently of the cyclooxygenase [55, 56]. In contrast, the cyclooxygenase reaction is peroxide-dependent [57] and requires the oxidation of the heme group at the peroxidase site [58].

A combination of electron paramagnetic resonance (EPR) spectroscopy, ^3H-labeling and spectrophotometric studies has led to a branched-chain, mechanistic model for peroxide-dependent activation of the cyclooxygenase activity first proposed by Ruf (Figure 3; [59-62]. This model proposes that the initial reduction of a hydroperoxide results in a two-electron oxidation of the PGHS heme group to produce an oxyferryl group plus a radical-cation protoporphyrin IX (Compound I) and an alcohol derived from the oxidizing peroxide [13, 58, 59, 63]. Compound I next undergoes an intramolecular reduction by a single electron traveling from Tyr385[1] to the proximal heme ligand, His388 and finally, to the heme group. The result is Intermediate II, an oxyferryl group plus a neutral protoporphyrin IX and a Tyr385 tyrosyl radical [13, 59, 64, 65]. This tyrosyl radical is thought to engage in abstraction of the 13-pro*S* hydrogen from

[1] Amino acid numbers correspond to the sequence of ovine PGHS-1.

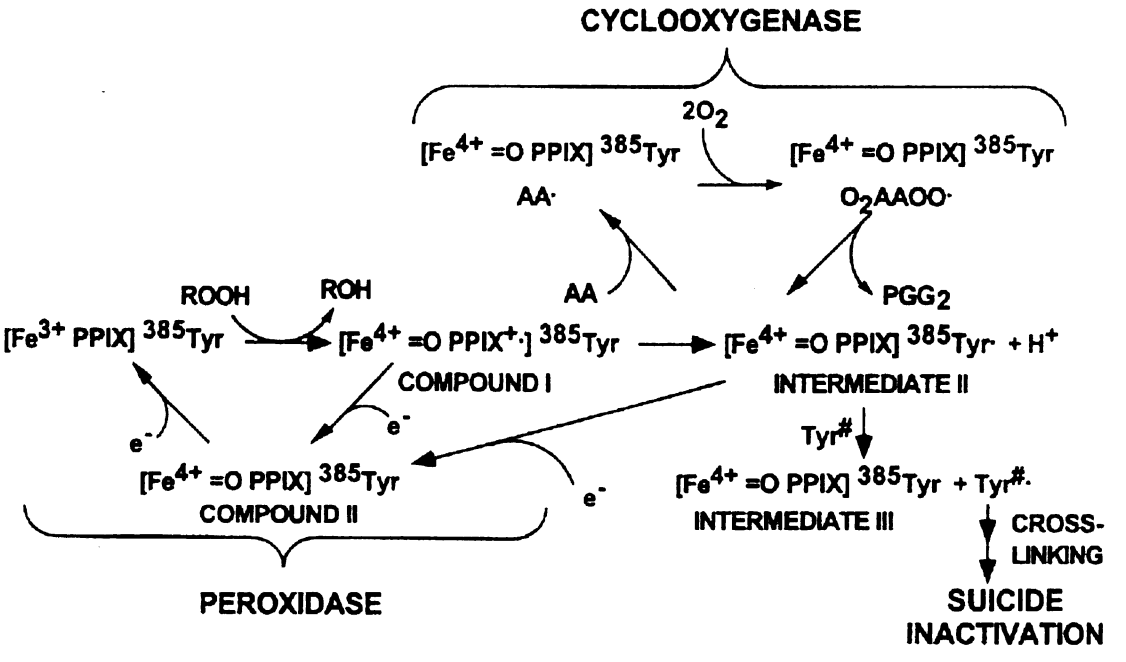

Figure 3. Model for peroxide-dependent activation of cyclooxygenase activity of PGH synthase via formation of an intermediate tyrosyl radical. Fe^{3+} PPIX, ferric iron protoporphyrin IX (heme); ROOH, alkyl hydroperoxide; ROH, alcohol; AA, arachidonic acid; Fe^{4+}=O PPIX, oxyferryl heme. Compound I, an oxyferryl group (Fe(IV)=O) plus a protoporphyrin IX radical cation; Intermediate II, an oxyferryl group plus a neutral protoporphyrin IX plus a Tyr385 tyrosyl radical; Compound II, an oxyferryl group plus a neutral protoporphyrin IX.

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arachidonate, the rate-determining step in cyclooxygenase catalysis. Solution of the Co^{3+}-heme ovine (o)PGHS-1 crystal structure complexed with arachidonate has led to detailed mechanistic implications for the cyclooxygenase reaction presented in the next section [66].

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Cyclooxygenase Catalysis by PGHS

The initial step in the cyclooxygenase reaction is the stereospecific removal of the 13-pro*S* hydrogen from arachidonate [61]. This hydrogen is abstracted as a hydrogen atom [67-70] by a tyrosyl radical at Tyr385, generating an arachidonyl radical at C-11. (Figures 3,4). Examination of the Co^{3+}-heme oPGHS-1/arachidonate crystal structure combined with knowledge derived from previous mechanistic studies have led to the details of the proposed cyclooxygenase mechanism that follow [66]. Following formation of the C-11 arachidonyl radical, molecular oxygen can then migrate from the base of the cyclooxygenase site and add to the substrate from the side opposite hydrogen abstraction to yield an 11*R*-peroxyl radical. This radical then reacts with the double bond at C-9 to form the endoperoxide. After endoperoxide formation, a conformational change must occur in order to position C-8 and C-12 at the appropriate distance for formation of the cyclopentane ring. This movement, in turn, allows the 11*R*-peroxyl group to react with C-9. Formation of the cyclopentane ring results in a repositioning of the ω end of the fatty acid molecule such that C-15 becomes optimally positioned for the addition of a second O_2 molecule coming from the base of the cyclooxygenase site to form PGG_2. Once formed, PGG_2 cycles back into the peroxidase reaction and is quickly reduced at the peroxidase active site to form PGH_2.

Suicide Inactivation

Both PGHS-1 and –2 undergo a poorly understood phenomenon called suicide inactivation. Both the peroxidase and cyclooxygenase activities are inactivated within two minutes of the start of catalysis. On average, each enzyme molecule can turn over

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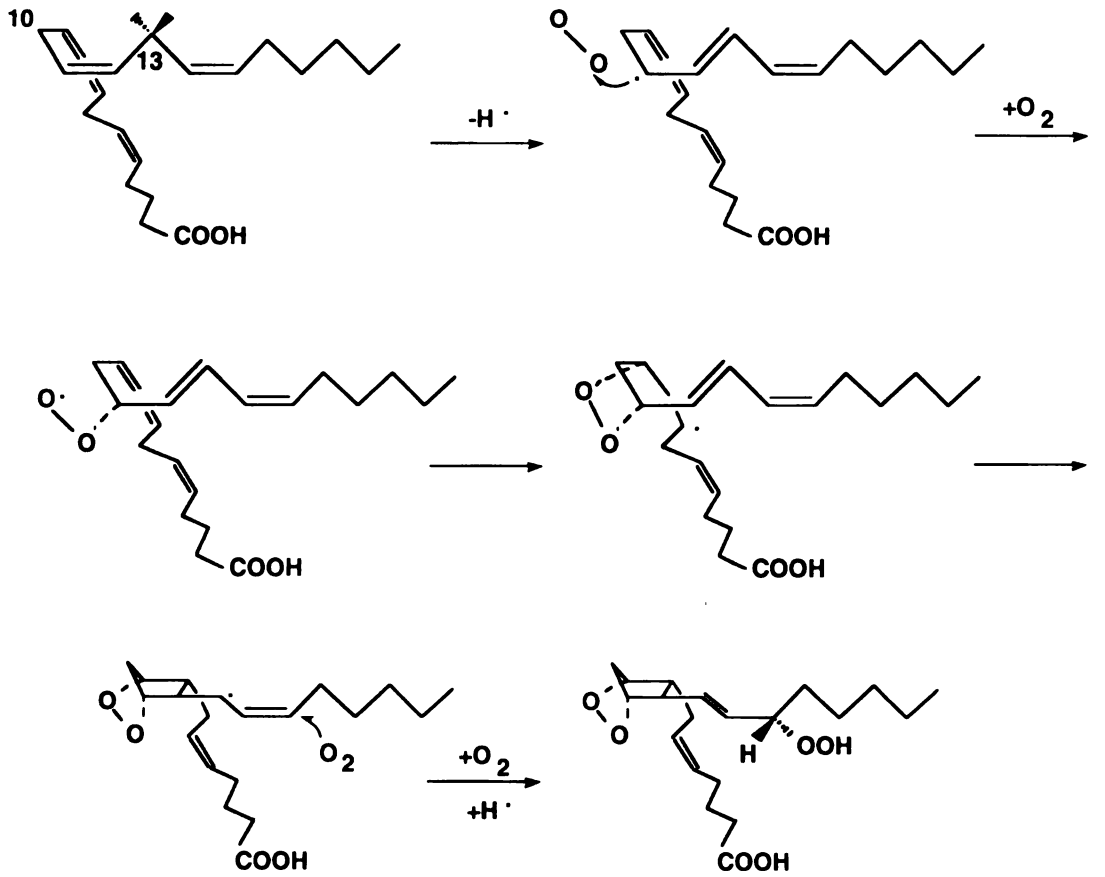

Figure 4. Mechanism model for conversion of arachidonic acid to PGG_2 by the cyclooxygenase activity of PGHSs.

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about 400 times before becoming suicide inactivated. Suicide inactivation originates with a reaction intermediate [71]. This intermediate does not directly involve an arachidonate-derived radical because the rate of fatty acid attachment to PGHS during catalysis is 30 times slower than that of suicide inactivation [72, 73]. As depicted in Figure 3, suicide inactivation likely proceeds from Intermediate II [71] and involves the formation of a tyrosyl radical other than the Tyr385 radical. Protein tyrosyl radicals in oPGHS-1 have been shown to be localized to tyrosines other than Tyr385 [74-76], and an Intermediate III has been detected in association with peroxidase inactivation [71]. Interestingly, one mutation (H386A) results in an enzyme that does not undergo suicide inactivation. The His 386 residue may be involved in the transport of the radical from neighboring Tyr385 that may somehow trigger an aberrant cross linking reaction that kills the enzyme.

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PGHS Structure and Biochemical Properties

The primary structures of PGHSs-1 and –2 from numerous species are known [1]. They are encoded by different genes with PGHS-2 being a small, immediate early gene (8.3kb for human PGHS-2) and PGHS-1 originating from a much larger, 22-kb gene. The proteins, however, have similar molecular weights (72kDa for PGHS-1 and 74kDa for PGHS-2) and share a 60% sequence identity within species and >85% identity among individual isoforms from different species. Additionally, all residues identified as being important for catalysis are conserved between the two isoforms.

PGHSs are integral membrane proteins which have been proposed to interact with membranes through a novel membrane binding domain of about 50 amino acids in which hydrophobic residues of amphipathic helices interdigitate into one leaflet of the lipid bilayer. It is in this region that the major sequence differences occur with only 38% identity between PGHS-1 and -2. Another notable difference between the two isoforms is the absence of a sequence of 17 amino acids from the N terminus and the insertion of a sequence of 18 amino acids at the C terminus of PGHS-2 in comparison to PGHS-1 [77]. The significance of this 18-amino acid insert in PGHS-2 has not been identified, but may be involved in targeting, protein degradation or protein-protein interactions (Figure 5).

PGHSs function as homodimers and are localized to the luminal side of the endoplasmic reticulum and the inner and outer nuclear envelope [78, 79]. These enzymes contain an epidermal growth factor (EGF) homology domain of about 50 amino acids which is present at the dimer interface, neighboring the membrane binding domain. The globular catalytic domain consists of about 460 amino acids [80-82] and is located in the C-terminal of the enzyme.

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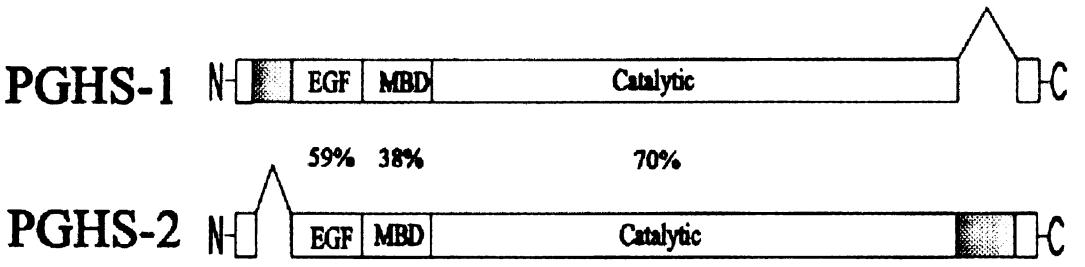

Figure 5. Comparison of the PGHS-1 and PGHS-2 proteins. Percentages denote the sequence identity shared between PGHS-1 and PGHS-2 in the various structural domains. Shaded regions depict short inserts in PGHS-1 and –2 that do not appear in the other isozyme. EGF = endothelial growth factor domain; MBD = putative membrane binding domain; Catalytic = catalytic domain, which includes the cyclooxygenase and peroxidase active sites.

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PGHSs are heme-containing proteins with one molecule of heme per monomer [10, 83] and are glycosylated at three sites (Asn68, 144 and 410). Glycosylation of all three sites is essential for enzyme activity in both isozymes [84]. The 74 kDa mass of PGHS-2 (compared to 72 kDa for PGHS-1) is attributed to an inefficient glycosylation at a unique fourth site, Asn580, whose glycosylation has no effect on the enzymatic activity of PGHS-2 [84].

Kinetic as well as structural properties of both isozymes have been shown to be similar for the primary substrates, arachidonic acid and molecular oxygen. The K_M's for arachidonic acid and O_2 have been determined to be in the range of 1-5 μM and 5 μM, respectively, with cyclooxygenase turnover rates at 3,500 moles of arachidonate/mol of PGHS dimer/minute [85-88].

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PGHS Crystal Structures

The homodimeric crystal structure of ovine (o)PGHS-1 complexed with the NSAID flurbiprofen was solved in 1994 and is shown in Figure 6 [80]. Since then, the structures Co^{3+}-heme oPGHS-1 complexed with arachidonate [66] and of human [81] and murine [82] PGHS-2 complexed with NSAIDs have been solved and were found to be virtually superimposable on the original oPGHS-1 structure.

PGHSs have a large helical content with very little beta sheet and function as homodimers. The reason that dimerization is necessary for catalysis is unknown. The solution of the crystal structures confirmed the implications of the known primary structures, indicating three distinct folding regions for each monomer: an epidermal growth factor (EGF)-like domain at the dimer interface, a large globular catalytic domain and a membrane binding domain [80-82].

The N-terminal EGF homology domain of PGHSs is tethered by three disulfide bridges and may play a role in the dimerization of the monomers. The globular catalytic domains encompass both the cyclooxygenase and peroxidase active sites which neighbor the heme site on opposite sides. The membrane binding domain contains four short, consecutive amphipathic α-helices and creates a hydrophobic patch that interacts with the lipid bilayer. These helices also surround an opening to the cyclooxygenase active site with the fourth helix (Helix D) protruding into the catalytic domain.

The cyclooxygenase active site exists as a long hydrophobic channel which opens at the outer surface of the membrane binding domain and can bind fatty acid substrates and NSAIDs. The peroxidase active site is a shallow cleft containing the Fe-protoporphyrin IX prosthetic group which is coordinated by two histidines. This site is

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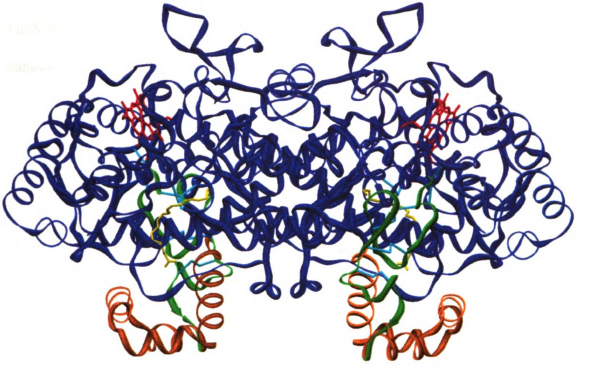

Figure 6. Crystal structure of the oPGHS-1 dimer. The major folding units are shown in green (EGF homology domain), orange (membrane binding domain) and blue (catalytic domain). The heme group is shown in red, bound in the peroxidase active site and arachidonate is shown in yellow, bound in the cyclooxygenase active site. Images in this dissertation are presented in color.

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structurally related to the peroxidase site of myeloperoxidase [80]. PGHS structures also contain several water channels, one of which extends from the cyclooxygenase site near Gly533 to the dimer interface. It is not clear if the water channels play a direct role in catalysis.

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Comparison of PGHS-1 and PGHS-2 Crystal Structures

In 1996, the crystal structure of human PGHS-2 complexed with two different inhibitors, was determined and found to have a tertiary and quaternary structure very similar to that of PGHS-1 [81, 82]. The amino acid sequences of human PGHS-2 and ovine PGHS-1 are 67% identical. The residues that form the substrate binding channel, the catalytic sites and the residues immediately adjacent are all identical between PGHS-1 and PGHS-2 except for three small variations. Isoleucine in PGHS-1 is exchanged for valine in PGHS-2 at positions 434 and 523 and histidine is exchanged for arginine at residue 513. The PGHS-2 structure revealed a second internal pocket extending off the cyclooxygenase site. In PGHS-2, the volume of the cyclooxygenase site is calculated to be 394 Å, whereas the volume of the cyclooxygenase site of PGHS-1 is 316 Å [81]. The 25% increase in the size of the cyclooxygenase site in PGHS-2 is primarily due to the difference at position 523 and possibly explains some of the differences that the two isozymes exhibit with respect to interaction of the various NSAIDs and substrates. This change has also allowed for the development of a number of PGHS-2-specific inhibitors such as celecoxib and rofecoxib [89-91]. The other major structural difference is in the position of the last of the four helices of the membrane binding domain, helix D. In PGHS-2, this helix is tilted toward the body of the enzyme, providing a larger opening in the membrane binding domain.

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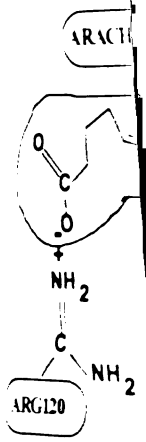
Active Sites of PGHSs

PGHS-1 and –2 each contain a cyclooxygenase active site in the form of a hydrophobic channel into which fatty acid substrates and NSAIDs bind. This channel originates at the membrane binding domain and extends into the core of the globular domain [80-82]. Nineteen residues line the hydrophobic cylooxygenase active site with only one difference between the isozymes – Ile at position 523 in PGHS-1 and Val at position 523 in PGHS-2. Otherwise, all residues important for catalysis are conserved between PGHS-1 and –2. Both PGHS-1 and –2 also contain one iron (FeIII) protoporphyrin IX group per subunit that binds a heme group in the peroxidase active site of the enzyme [83, 92, 93] and is essential for both cyclooxygenase and peroxidase activities [92]. As mentioned earlier, the heme group is coordinated by two histidines, His207 and His388, which were identified by mutational studies. His388 is the proximal heme ligand and is required for both cyclooxygenase and peroxidase activities. His207 acts as the distal histidine and is predicted to be important in deprotonation of the hydroperoxide substrate and subsequent protonation to form the alcohol during generation of Compound I (Figures 3 and 7) [58, 80-82]. Another histidine, His386, was found to be required for peroxidase but not cyclooxygenase activity [94].

Residues critical to PGHS activity were initially identified through a combination of mutational and elecron paramagnetic resonance (EPR) spectroscopy studies. In 1988, Ruf and co-workers used EPR to establish the existence of a tyrosyl radical in PGHS catalysis which was coincident with the formation of Intermediate II [59, 64] (Figure 3). Most of the evidence suggests that Tyr385 is the protein radical species involved in the rate-limiting abstraction of the 13-pro*S* hydrogen of arachidonate. Tyr385 is essential for



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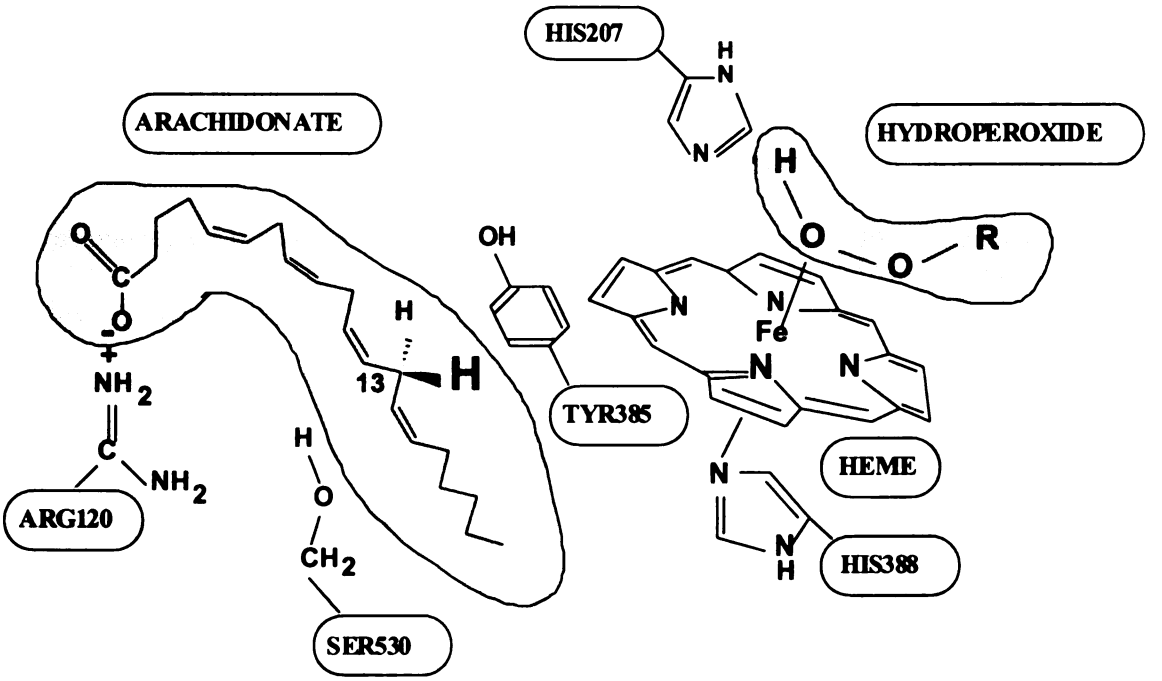

Figure 7. Model of the active site for ovine PGHS-1. Residues shown are conserved between the two isozymes and are described in the text.

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enzyme activity [95] and is found neighboring the heme group and the 13-pro*S* hydrogen of arachidonate bound in the cyclooxygenase active site [66, 80]. The proximity of these peroxidase and cyclooxygenase active site residues to one another and, more importantly, the proximity of Tyr385 to the heme group and peroxidase active site confirmed that the cyclooxygenase and peroxidase activities, although separate, were both functionally and structurally coupled.

Other important residues in the cyclooxygenase active site include Arg120 which resides near the opening of the active site, Tyr355, also near the mouth of the cyclooxygenase active site channel directly across from Arg120, Ser530 residing at the side opposite Tyr385 and Ile523 (Figure 8).

The arginino group of Arg120 has been shown to serve as a counter ion for the carboxylate group of fatty acid substrates and certain NSAIDs such as flurbiprofen and ibuprofen [80, 96]. Replacement of Arg120 with a neutral glutamine causes a 1000 fold increase in the K_M for arachidonate indicating that this residue is essential for high affinity arachidonate binding to the cyclooxygenase site [96, 97] and that this involves an ionic linkage between Arg120 and the carboxylate of arachidonate. In contrast, an ionic interaction between Arg120 and arachidonate is not important with PGHS-2 where an R120Q mutation has no detectable effect on either the V_{max} or the K_M for arachidonate oxygenation although this mutation does cause a small increase in the relative amount of 11-HPETE formed [98].

Tyr355 has been shown to play a role in the effectiveness of both instantaneous and time-dependent PGHS inhibitors and Ser530 is the site of acetylation of PGHSs by aspirin [21, 99]. Lastly, Ile523 which is a valine in PGHS-2 appears to play a role in the

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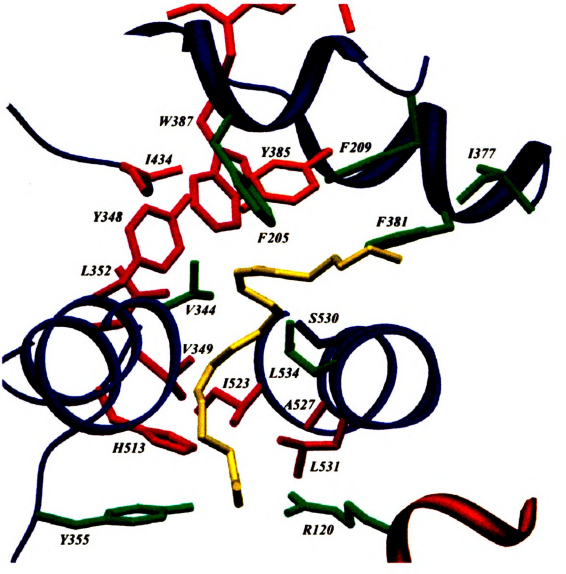

Figure 8. Cyclooxygenase active site from the Co^{3+}-heme oPGHS-1 crystal structure complexed with arachidonic acid [66]. Images in this dissertation are presented in color.

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differences that the two isozymes exhibit with respect to interaction of the various NSAIDs and substrates. These three residues will be discussed in more detail in the discussion of PGHS inhibition.

Recently, the crystal structure of Co^{3+}-heme oPGHS-1 complexed with the substrate, arachidonic acid was solved by Garavito and co-workers [66]. The structure is very similar to the inhibitor-bound complexes and confirms positions and functional properties of active site residues indicated by the earlier structures and mutational studies. More importantly, this structure was able to illuminate the nature of specific substrate-enzyme interactions, allowing for a much more thorough understanding of residue functions and catalysis in the cyclooxygenase active stie. Arachidonic acid is bound in an extended L-shaped conformation and makes a total of 48 hydrophobic contacts (i.e. 2.5-4.0 Å) and two hydrophilic contacts with the protein, involving a total of nineteen different residues (see Figure 8). Additionally, there are several amino acids which are in the first shell of the cyclooxygenase hydrophobic tunnel and contact other first shell amino acids but lie outside of van der Waals distance to arachidonic acid. A large scale mutational analysis of these cyclooxygenase active site area residues is discussed at length in Chapter III.

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Structural Comparison between PGHSs and other Fatty Acid Binding Proteins

There are many proteins known to bind mono- and polyunsaturated fatty acids. Examination of the crystal structures of three of these proteins reveals some fundamental patterns in the protein-substrate interactions also observed in PGHS. The adipocyte lipid-binding protein (ALBP) serves as an intracellular receptor for numerous fatty acid substrates for transport to the appropriate site for use as metabolic fuels and regulatory agents [100]. The cytochrome p450BM-3 is a fatty acid hydroxylase from *Bacillus megaterium* [101] and the soybean and human 15-lipoxygenases catalyze the dioxygenation of polyunsaturated fatty acids [102].

Some common characteristics among these proteins are: (1) folding of the fatty acid substrate is required for accommodation of the entire length of the fatty acid chain in the binding site, (2) all of these proteins, much like PGHS, require at least one stabilizing ionic or hydrogen-bonding interaction between the carboxyl end of the fatty acid and a charged or polar residue and (3) several van der Waals contacts between active site residues and areas along the fatty acid chain serve to stabilize substrate binding.

ALBP has been crystallized in a complex with arachidonic acid [103]. The binding affinity (K_D) for arachidonate in ALBP is 4.4μM, in the same range as that observed for PGHSs (2-5μM). Another similarity is the requirement for an ionic/hydrogen bonding interaction between the carboxylate of arachidonate, two arginines (one of which is linked through a water molecule) and one tyrosine. There are some very major differences between these proteins, however. ALBP is a small protein (130 amino acids) with a β-barrel structure and the substrate binding cavity is water-filled, in contrast to the hydrophobic pocket of PGHSs. Also, the carboxyl end of

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arachidonate is buried in the binding pocket, "upside down" from that observed with PGHS. Although there are a number of other substrate stabilizing forces in the ALBP active site, these include not only hydrophobic interactions, as with PGHS, but also numerous hydrogen bonds between water molecules and other intervening active site residues.

The cytochrome p450BM-3 structure has been solved complexed with the 16-carbon, monounsaturated fatty acid, palmitoleic acid [101]. This protein, similar to PGHS, is a larger heme-containing fatty acid hydroxylase, largely helical in content w/ some β-sheet. Additionally, there is evidence for multiple substrate conformations evidenced by the formation of multiple products (hydroxy acid or epoxide formation), a phenomenon observed for PGHSs [104]. Other similarities include a hydrogen bonding interaction between the arachidonate carboxylate and an active site tyrosine, as well as the probability of an ionic interaction with one arginine, although this interaction may be transient in nature. The substrate is also stabilized by multiple interactions with hydrophobic active site residues. Again, one important difference between PGHS and the cytochrome p450BM-3 structure is that the fatty acid is bound "upside down," compared with PGHS, with the arachidonate carboxyl end buried in the binding pocket.

The soybean 15-lipoxygenase structure has been solved in the absence of any bound substrate and inhibitor [105]. Implications for substrate binding has been determined through docking of arachidonic acid in the soybean lipoxygenase putative binding site and applied to both the soybean and human 15-lipoxygenases [102]. Both soybean and human lipoxygenases catalyze the formation of hydroperoxides from arachidonic acid and, although the plant and mammalian forms share only 28% identity,

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one can assume structural similarity due to their similar activities. Similar to PGHSs, these are iron-containing proteins which are largely helical in structure with some β-sheet. The iron is coordinated by three histidines, an isoleucine and an asparagine and neighbors a solvent-accessible site thought to be the substrate binding site. Arachidonic acid is docked in the putative substrate binding site in close proximity to the catalytic iron with its carboxylate in an ionic linkage with a lysine residue in the soybean enzyme and a corresponding arginine in the human in a manner similar to that of oPGHS-1. Additionally, numerous hydrophobic residues interact with areas along the fatty acid chain, including a Trp, Phe, Ile, Leu, Met and Thr and the K_M for arachidonate binding is, again, similar to that of PGHSs at 7.5 μM.

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Inhibition of PGHSs

The cyclooxygenase activities of both PGHS-1 and PGHS-2 can be inhibited by most common NSAIDs such as aspirin, ibuprofen and naproxen, leaving the peroxidase activity of the enzymes unaffected. NSAIDs such as ibuprofen and naproxen cause reversible inhibition by competing with the substrate for the active site of the enzyme. Flurbiprofen and indomethacin also compete with substrate for the cyclooxygenase site, but produce a time-dependent, slowly reversible inhibition of PGHSs. Both flurbiprofen and indomethacin are carboxylic acid-containing NSAIDs which ion pair with the guanidinium group of Arg120, also known to interact with the carboxylate of arachidonate [82, 99, 106]. This interaction has been shown not only to be essential for high-affinity substrate binding, but also for time-dependent inhibition. This is evidenced by studies in which replacement of Arg120 in PGHS-1 with gluatmine or glutamate yielded an enzyme which was resistant to time-dependent inhibition by these NSAIDs [96, 97].

The inhibition by aspirin is due to the irreversible acetylation of the cyclooxygenase site of PGHS-1 at Ser530. PGHS-2 is also acetylated by aspirin at Ser530, but will still oxidize arachidonic acid to make 15*R*-HETE, whereas similarly acetylated PGHS-1 will not oxidize arachidonic acid at all [107-109]. Additionally, inhibitors will differentiate between PGHS-1 and PGHS-2 and, in general, bind more tightly to PGHS-1 [110].

The biochemical differences between the two PGHS isoforms have been attributed to the larger and more accommodating active site of PGHS-2 resulting from Ile to Val substitutions at positions 523 and 434 and a His to Arg substitution at position 513

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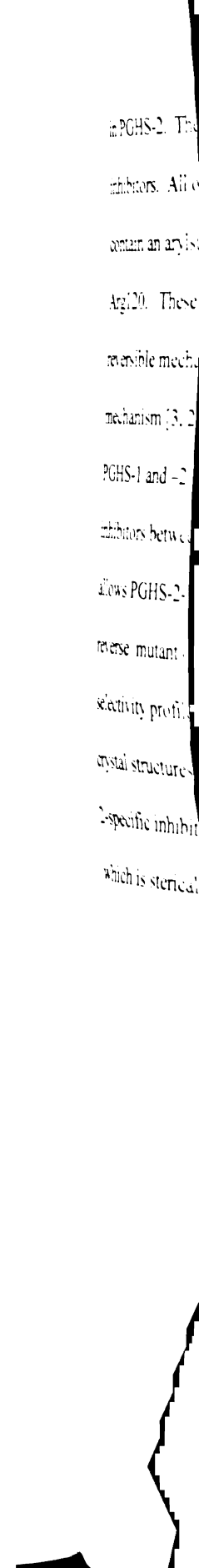

in PGHS-2. These differences are exploited in the development of PGHS-2 specific inhibitors. All of the new PGHS-2 inhibitors are larger than other classical NSAIDs and contain an arylsulfur group rather than a carboxylate group and thus, do not bind to Arg120. These inhibitors selectively inhibit PGHS-2 by a time-dependent, slowly reversible mechanism while they inhibit PGHS-1 by a rapid, competitive and reversible mechanism [3, 20]. Swapping of cyclooxygenase active site area residues unique to PGHS-1 and –2 effectively exchanges the inhibitory pattern of PGHS-2-specific inhibitors between the isoforms. In particular, mutation of Ile 523 to Val in PGHS-1 allows PGHS-2-specific inhibitors to bind and inhibit prostaglandin formation, and the reverse mutant of PGHS-2 in which Val523 is exchanged for Ile shows inhibitor selectivity profiles comparable to native PGHS-1 [111, 112]. Examination of PGHS-2 crystal structures [81, 82] has indicated that the smaller size of Val523 allows the PGHS-2-specific inhibitor access to a side pocket off the main substrate channel in PGHS-2 which is sterically denied by the longer side chain of Ile in PGHS-1.

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Cyclooxygenase Substrates of PGHSs

Arachidonic acid is mobilized by phospholipases A_2 for conversion to the prostanoids by PGHSs. However, only the cytosolic Ca^{2+}-dependent phospholipase A_2 exhibits a marked selectivity for arachidonic acid [113]. Thus, it is likely that under some conditions, PGHS-1 and PGHS-2 will be exposed to a pool of a mixture of different polyunsaturated fatty acids.

PGHSs have been shown to oxygenate a variety of C_{18} and C_{20} n-3 and n-6 fatty acids *in vitro* [114, 115]. The 20-carbon, n-6 fatty acids, arachidonic (20:4) and dihomo-γ-linolenic acids (20:3) are used most efficiently, producing the 2- and 1-series prostaglandins, respectively. Eicosapentaenoic acid (20:5n-3) is converted to the 3-series prostaglandins and the 18-carbon fatty acids are oxidized to monohydroxy acid products by PGHSs. Product characterization from various fatty acid substrates has established that, in most cases oxygenation is initiated following abstraction of hydrogen from the n-8 carbon. One exception is α-linolenate (18:3,n-3) in which the n-5 hydrogen is abstracted [115].

Arachidonic acid (20:4n-6) is converted *in vivo* to the 2-series prostanoids -- the major prostanoids present in plasma, endothelial cells and renal collecting tubule cells [116]. The major prostanoids found in seminal fluid, however, are of the 1-series, indicating that dihomo-γ-linolenic acid (20:3n-6) can also serve as a PGHS substrate *in vivo*. Dihomo-γ-linolenic acid is the primary C_{20} fatty acid found in sheep vesicular gland from which some of the earliest work on prostanoid biosynthesis was performed [61, 117].

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The n-3 and n-9 fatty acids have been shown to act as relatively poor substrates, but effective inhibitors of the oxygenation reaction [114, 115]. Eicosapentaenoic acid (20:5n-3) is converted, albeit in small amounts, to prostanoid products of the 3-series by PGHSs when added exogenously to intact cells [118, 119] or when mobilized from cellular phosphoglycerides [118, 120-123]. Oxygenation of eicosapentaenoic acid by PGHS-1 has been shown to be more sensitive than arachidonate oxygenation to hydroperoxide concentrations and is enhanced by addition of exogenous hydroperoxide or by production of hydroperoxy lipoxygenase products in cells [124, 125]. Curiously, oxygenation of this n-3 substrate by PGHS-2 is not reliant on the same levels of hydroperoxide activator and, although a poor substrate for both isozymes, eicosapentaenoate appears to be a slightly better substrate for PGHS-2 [115]. Eicosapentaenoic acid has been shown to compete effectively with arachidonate for binding to PGHSs and has been shown to inhibit the formation of 2-series prostaglandins when ingested or added to human platelets or endothelial cells [120, 121, 126, 127]. It is through this mode of action that n-3 fatty acids are thought to promote cardiovascular health and protect against thrombosis and atherogenesis. Similar arguments apply to the inhibition of arachidonate oxygenation by docosahexaenoic acid (22:6n-3) which also binds efficiently to the cyclooxygenase site [114], but does not appear to by oxygenated by PGHSs.

The 18-carbon fatty acids are oxygenated to monohydroxy acids by PGHSs. Linoleic acid (18:2n-6) is an essential nutrient because it is converted to arachidonic acid, the primary substrate in formation of the eicosanoids. In addition, a number of tissues synthesize oxygenated products directly from linoleic acid, suggesting that it may have

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other vital functions. PGHSs have been shown to convert 18:2 to a mixture of the monohydroxy acids 9-hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE) with a catalytic efficiency that is 5% that for oxygenation of arachidonate for human PGHS-1 and 15% the efficiency of arachidonate oxygenation by human PGHS-2 [115]. These linoleate metabolites have been shown to be produced under both basal and stimulated conditions from endogenous linoleic acid in alveolar macrophages [123] and human umbilical vein endothelial cells [119], respectively, and were inhibited by the cyclooxygenase inhibitor, indomethacin, suggesting an *in vivo* role for linoleic acid in cyclooxygenase catalysis. Suggested roles for the HODE products include maintainance of the water barrier in the skin [128], inhibition of 5-lipoxygenase activity in leukocytes [129] and modulation of thromboxane A_2 and 12-HETE formation in platelets [130]. α-linolenic acid (18:3n-3) is converted to 12-hydroxy-(9Z,13E/Z,15Z)-octadecatrienoic acids (12-HOTrE) by PGHSs [115]. This n-3 substrate is an exceedingly poor substrate for PGHS-1, but markedly better for PGHS-2, suggesting the possibility that α-linolenate could serve as a substrate for PGHS-2 but not PGHS-1 *in vivo*. Similar to linoleate (18:2n-6), α-linolenate is an important nutrient as the precursor to other n-3 fatty acids such as eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). Other less common fatty acids can also serve as cyclooxygenase substrates including adrenic acid (22:4n-6) [131], the *Mead* acid 5Z,8Z,11Z-eicosatrienoic acid [132], columbinic acid (5E,9Z,12Z-octadecatrienoic acid) [133, 134], and 5,6-oxido-eicosatrienoic acid [132, 135].

As suggested by the preceding discussion, PGHS-2 will accept a wider range of fatty acids as substrates than will PGHS-1. Although both enzymes can use arachidonic

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and dihomo-γ-linolenic acid equally well, PGHS-2 oxygenates other fatty acids substrates such as eicosapentaenoic acid (20:5,n-3), γ- and α-linolenic acids (18:3,n-6 and n-3) and linoleic acid (18:2,n-6) more efficiently than does PGHS-1 [115]. This can most likely be attributed to the larger, more accommodating active site of PGHS-2 compared with PGHS-1 coupled with the fact that PGHS-2 is not dependent on the ionic interaction between Arg120 of the enzyme and substrate carboxylate groups [98].

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CHAPTER II

ARACHIDONIC ACID CONFORMERS IN THE CYCLOOXYGENASE ACTIVE SITE OF OVINE PGHS-1

Introduction

Prostaglandin endoperoxide H synthases-1 and -2 (PGHS-1 and -2) catalyze the conversion of arachidonic acid and O_2 to PGH_2 -- the committed step in the formation of prostanoids (prostaglandins, thromboxane A_2 [1-3, 20, 136, 137]). PGHS-1 (or COX-1) is often referred to as the constitutive enzyme whereas PGHS-2 (COX-2) is known as the inducible isoform [138-146]. Apart from their important biological roles and their functions as targets of nonsteroidal anti-inflammatory drugs [3, 20], PGHSs are of considerable interest in the context of the structural biology and enzymology of membrane proteins. These enzymes are homodimeric (ca. 72 kDa/subunit), heme-containing, glycoproteins with two catalytic sites; moreover, PGHSs represent a prototype of a new class of integral membrane proteins that appear to be anchored to one leaflet of the lipid bilayer through the hydrophobic surfaces of amphipathic helices and not through more typical transmembrane domains [2, 147-149].

PGHSs catalyze two separate reactions: a cyclooxygenase (*bis*-oxygenase) reaction in which arachidonate is converted to PGG_2 and a peroxidase reaction in which PGG_2 undergoes a two electron reduction to PGH_2. PGHS-1 and PGHS-2 have similar cyclooxygenase turnover numbers (ca. 3500 moles of arachidonate/min/mole of dimer; [87, 124, 150]) and K_M values for arachidonate (ca. 5 μM; [86, 87, 115, 150]) and for O_2 (ca. 5 μM; [88, 151]) and exhibit similar fatty acid substrate specificities [115]. The

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cyclooxygenase reaction begins with a rate-limiting abstraction of the 13-pro*S* hydrogen from arachidonate to yield an arachidonyl radical [61, 70]. This is followed by sequential oxygen additions at C-11 and C-15 to yield the prostaglandin endoperoxide PGG_2. Nonsteroidal anti-inflammatory drugs compete directly with arachidonate for binding to the cyclooxygenase site and inhibit cyclooxygenase activity, but have little or no effect on peroxidase catalysis [55, 152, 153].

PGHSs exhibit some lipoxygenase activity producing small amounts of 11-hydroxy-8*Z*,12*E*,14*Z*-eicosatrienoic acid and 15-hydroxy-8*Z*,11*Z*,13*E*-eicosatrienoic acid from 8,11,14-eicosatrienoic acid [61] and the corresponding 11- and 15-HETEs from arachidonic acid [154, 155]. Aspirin-acetylated PGHS-2, which has no cyclooxygenase activity, synthesizes 15*R*-HETE [108, 155]. Studies comparing native and aspirin-acetylated PGHS-2 have raised the possibility that arachidonate can bind in distinct orientations in the PGHS-2 active site to produce either PGG_2 plus 11*R*-HETE or 15*R*-HETE [155]. We have examined the products formed from arachidonate by native ovine (o) PGHS-1 and a cyclooxygenase active site mutant, V349L oPGHS-1, and have determined the kinetics for the formation of each individual product. We have also determined the IC_{50} values for the inhibition of formation of each product by ibuprofen. Collectively, our results indicate that arachidonate can be bound in the cyclooxygenase active site of oPGHS-1 in at least three different, catalytically competent arrangements that lead to PGG_2, 11*R*-HETE, and 15-HETE, respectively, and that these three arrangements of arachidonate occur subsequent to its entry into the cyclooxygenase active site.

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Materials and Methods

Materials. Arachidonic acid was purchased from Cayman Chemical Co., Ann Arbor, MI. [1-^{14}C]arachidonic acid (40-60 mCi/mmol) was purchased from New England Nuclear. Flurbiprofen was from Sigma. Restriction enzymes and Dulbecco's modified Eagle's Medium (DMEM) were purchased from GIBCO. Calf serum and fetal bovine serum were purchased from HyClone. Primary antibodies used for Western blotting were raised in rabbits against purified oPGHS-1 and purified as an IgG fraction [44, 79, 149]; goat anti-rabbit IgG horseradish peroxidase conjugate was purchased from BioRad. $C_{10}E_7$ detergent used for solubilization of oPGHS-1 was from Anatrace, Inc. Oligonucleotides used as primers for mutagenesis were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility. All other reagents were from common commercial sources.

Preparation of V349L oPGHS-1. V349L oPGHS-1 was prepared starting with M13mp19-oPGHS-1, which contains a 2.3 kb *SalI* fragment encoding native ovine PGHS-1 [79, 149], using a BioRad Muta-Gene kit and the manufacturer's protocol. The mutagenic oligonucleotide was 5'-^{1127}AGAGGAGTATCTGCAGCAGCTGA^{1149}-3'. Single-stranded phage samples were sequenced using Sequenase (ver. 2.0, U.S. Biochemical Corp.) and the protocol described by the manufacturer. The replicative form of M13mp19-oPGHS-1 containing the V349L mutation was prepared from phage cultures, digested with *SalI*, isolated by gel electrophoresis and electroluted into dialysis tubing using standard protocols. The resulting 2.3 kb oPGHS-1 cDNA fragment was purified and subcloned into pSVT7, followed by digestion with *PstI* to verify the orientation of the insert [44, 79, 149]. Plasmids used for transfection were purified by

CsCl gradient ultracentrifugation, and the mutation was reconfirmed by double-stranded sequencing of the pSVT7 construct as described above.

Transfection of COS-1 cells with recombinant oPGHS-1. COS-1 cells (ATTC CRL-1650) were grown in DMEM containing 8% calf serum and 2% fetal bovine serum until near confluence (~$3x10^6$ cells/10 cm dish) [44, 79, 149]. Cells were then transfected with a pSVT7 plasmid containing cDNA coding for native or V349L oPGHS-1 using the DEAE dextran/chloroquine transfection method as reported previously [44, 79, 149]. Forty hours following transfection, cells were harvested in cold phosphate-buffered saline (PBS), collected by centrifugation and resuspended in 0.1 M Tris-HCl, pH 7.5. Sham-transfected cells were collected in an identical manner. Microsomal preparations were used for Western blotting and for cyclooxygenase and peroxidase assays.

Purification of oPGHS-1 from seminal vesicles. Microsomes were solubilized with 0.1% $C_{10}E_7$, centrifuged, and the supernatant loaded onto an equilibrated DEAE-cellulose column. Fractions from the DEAE-cellulose column were assayed for peroxidase activity and protein concentration. Desired fractions were pooled, concentrated, and loaded onto an equilibrated S-300 column overnight. Fractions from the DEAE and S-300 columns were used for assays with partially purified enzyme. The specific cyclooxygenase activity was 2-10 units/mg for DEAE fractions and 15-30 units/mg for S300 fractions. One unit of enzyme is defined as that amount of enzyme which will catalyze the oxygenation of 1 μmol of arachidonate/min.

Characterization of arachidonate derived products. Forty hours following transfection, COS-1 cells were collected, sonicated and resuspended in 0.1M Tris-HCl,

pH 7.5. Aliquots of the cell suspension (75 μg of protein) or 5 cyclooxygenase activity units of partially purified oPGHS-1 were incubated for 1 min at 37° C with various concentrations of [1-^{14}C]arachidonic acid with and without 200 μM flurbiprofen. Radioactive products were extracted and separated by thin layer chromatography as described previously [57]. Products were visualized by autoradiography and quantitated 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. Inhibition of product formation by ibuprofen was measured similarly using different concentrations of ibuprofen in reaction mixtures containing 10 μM arachidonate.

Reverse phase (RP)-HPLC. Native or V349L oPGHS-1 (75 μg cell protein) prepared from transfected COS cells or semi-purified oPGHS-1 (5 cyclooxygenase activity units) were incubated with 2-100 μM arachidonic acid for 1 minute at 37° C. The products were extracted as described above and resuspended in HPLC buffers (1/1; v/v). 15- and 11-HETEs were separated by RP-HPLC using a C18 column from Vydac (Hesperia, CA) and detected with a Waters Model 600 HPLC equipped with a 990 photo diode array detector set at 234 nm. The polar component of the mobile phase was 0.1% aqueous acetic acid, and the eluting solvent was acetonitrile. The flow rate was 1 ml/min. The elution profile used was: 0-30 min, 30% acetonitrile; 30-100 min, 50% acetonitrile; 100-125 min, 75% acetonitrile; 125-130 min, 100% acetonitrile. The retention times of 15-HETE and 11-HETE were 36 and 38 minutes, respectively.

Chiral HPLC of methyl 15-HETE and methyl 11-HETE. Material obtained by RP-HPLC was esterified by treatment with excess diazomethane and subjected to chiral

phase HPLC. Diazomethane was prepared from Diazald (N-methyl-N-nitroso-*p*-toluenesulfonamide) (Aldrich Chemical Company) as described in the company's technical bulletin #AL-180. Chiral-phase HPLC was performed on a Chiralcel OC column (250 X 4.6 mm) from Daicel Chemical Industries (Osaka, Japan) using hexane/2-propanol (98/2; v/v) at a flow rate 0.5 ml/min. The retention times of the methyl esters of 15*R*- and 15*S*-HETE were 25 minutes and 27 minutes, respectively. Retention times for the methyl esters of 11*R*- and 11*S*-HETE were 26 minutes and 28 minutes, respectively.

Cyclooxygenase and peroxidase assays. Cyclooxygenase assays were performed at 37°C by monitoring the initial rate of O_2 uptake using an oxygen electrode [98]. Each standard assay mixture contained 3.0 ml of 0.1 M Tris-HCl, pH 8.0, 1 mM phenol, 85 μg of bovine hemoglobin and from 2 to 100 μM arachidonate. Reactions were initiated by adding approximately 250 μg of microsomal protein prepared from COS-1 cells or partially purified oPGHS-1 in a volume of 20-50 μl to the assay mixture. Peroxidase activity was measured spectrophotometrically as described previously [57]. The reaction mixtures contained 0.1 M Tris-HCl, pH 8.0, 0.1 mM 3,3,3',3'-tetramethylphenylenediamine (TMPD), approximately 100 μg of microsomal protein and 1.7 μM hematin in a total volume of 3 ml. Reactions were initiated by adding 100 μl of 0.3 mM H_2O_2, and the absorbance at 610 nm was monitored with time.

Kinetic Schemes and Derivations. As presented later in the Discussion section, two different kinetic schemes were developed to model mechanisms by which oPGHS-1 could produce different products with different kinetic properties. Rate and IC_{50} equations were derived for each scheme using the general rate equations:

$$v = V_{max}[S]/\{K_M + [S]\} \text{ and } v = V_{max}[S]/\{K_M (1 + [I]/K_I) + [S]\}$$

Both schemes were developed for the formation of two separate products to illustrate the principles; however, the schemes are easily expandable to accommodate situations where there are multiple products. Derivations are included in Appendix A of this manuscript.

The relevant rate equations for Scheme 1:

$$v(1)\ \text{no inhibitor} = \frac{k_5\,[E_{tot}][S_0]}{K_1(K_0 + [S_0] + [S_0]/K_2) + [S_0]}$$

$$v(2)\ \text{no inhibitor} = \frac{k_6\,[E_{tot}][S_0]}{K_2(K_0 + [S_0] + [S_0]/K_1) + [S_0]}$$

$$v(1)\ \text{w/ inhibitor} = \frac{k_5\,[E_{tot}][S_0]/\,(K_1 + K_1/K_2 + 1)}{(K_0K_1/\,(K_1 + K_1/K_2 + 1))(\,1 + [I]/K_I) + [S_0]}$$

$$v(2)\ \text{w/ inhibitor} = \frac{k_6\,[E_{tot}][S_0]/\,(K_2 + K_2/K_1 + 1)}{(K_0K_2/\,(K_2 + K_2/K_1 + 1))(\,1 + [I]/K_I) + [S_0]}$$

$$IC_{50}(1) = K_I\,(1 + [S_o]/K_o + [S_o]/K_1K_o + [S_o]/K_2K_o)$$

$$IC_{50}(2) = K_I\,(1 + [S_o]/K_o + [S_o]/K_1K_o + [S_o]/K_2K_o)$$

$$IC_{50}(1) = IC_{50}(2)$$

The relevant rate equations for Scheme 2 are:

$$v(1)\ \text{no inhibitor} = \frac{k_2\,[E_{tot}]\,[S_0]}{K_1(1 + 1/K_0 + [S_0]/\,K_0\,K_2) + [S_0]}$$

$$v(2)\ \text{no inhibitor} = \frac{k_4\,[E_{tot}]\,[S_0]}{K_2(1 + K_0 + K_0[S_0]/\,K_1) + [S_0]}$$

$$v(1)\ \text{w/ inhibitor} = \frac{k_2[E_{tot}][S_0]/(1 + K_1/K_0K_2)}{\dfrac{K_1(1 + [I_0]/\,K_{I(1)} + 1/K_0 + [I_0]/\,K_{I(2)}K_0)}{(1 + K_1/K_0K_2)} + [S_0]}$$

$$v(2)\ \text{w/ inhibitor} = \frac{k_4[E_{tot}][S_0]/(1 + K_0K_2/\,K_1)}{\dfrac{K_2(1 + [I_0]K_0/K_{I(1)} + K_0 + [I_0]/\,K_{I(2)})}{(1 + K_0K_2/\,K_1)} + [S_0]}$$

$$IC_{50}(1) = \frac{1 + 1/K_0 + [S_0](1/K_0K_2 + 1/K_1)}{1/K_{I(1)} + 1/K_{I(2)}K_0}$$

$$IC_{50}(2) = \frac{1 + K_0 + [S_0](K_0/K_1 + 1/K_2)}{1/K_{I(2)} + K_0/K_{I(1)}}$$

$IC_{50}(1) \neq IC_{50}(2)$

Statistical analysis. Results are expressed as the mean +/- S.E.M. for a minimum of four experiments using different preparations of partially purified oPGHS-1 or membrane-associated native or V349L oPGHS-1. Statistical significance was determined using a two-sample t-test assuming equal variances.

Results

Microsomal preparations of oPGHS-1 and V349L oPGHS-1 from COS-1 cells transfected with plasmids encoding native oPGHS-1 or V349L oPGHS-1 as well as partially purified native oPGHS-1 from seminal vesicles were used to determine overall K_M values for the oxygenation of arachidonate using an oxygen electrode assay. The K_M values were 3 μM for both solubilized, partially purified and for membrane-associated, recombinant native oPGHS-1; as expected, this value is in the range (2-8 μM) reported previously for recombinant and purified forms of ovine, murine and human PGHS-1 [86, 87, 115, 150, 156]. The K_M for arachidonate with the V349L oPGHS-1 mutant was 7 μM. Western transfer blotting indicated that V349L oPGHS-1 was expressed in COS-1 cells at the same level as native oPGHS-1 (data not shown) and had 65% of the cyclooxygenase activity and 100% of the peroxidase activity of native oPGHS-1 expressed in parallel transfections.

As illustrated in Figure 9, oPGHS-1 and V349L oPGHS-1 formed the same products from [1-^{14}C] arachidonate including those products derived from PGG_2 (HHTre, PGD_2, PGE_2 and $PGF_2\alpha$) and the monohydroxy acids 11-hydroxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid (11-HETE) and 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HETE) which are derived from reduction of the corresponding hydroperoxides. However, V349L oPGHS-1 produced substantially larger amounts of 15-HETE and relatively less PGG_2 and 11-HETE (Figure 9) than native enzyme. With 2 μM arachidonate and 5 cyclooxygenase units of either partially purified oPGHS-1 or broken cell preparations of COS-1 cells expressing oPGHS-1, 95% of the products were derived from PGG_2, 3% was 11-HETE and 2% was 15-HETE; with 2 μM arachidonate and broken cell preparations of COS-1 cells expressing V349L oPGHS-1, 70% of the product was PGG_2, <0.5% was 11-HETE and 30% was 15-HETE.

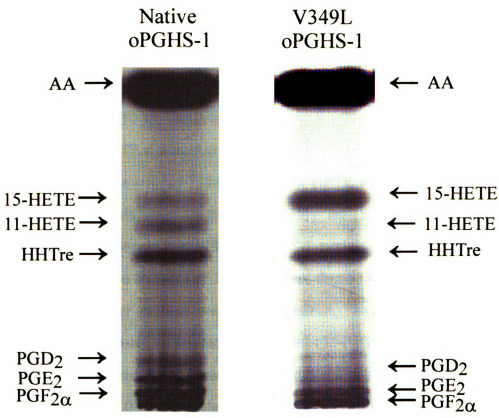

Figure 9. Autoradiogram of thin layer chromatograms of products formed from [1-^{14}C] arachidonate by native and V349L oPGHS-1. Partially purified oPGHS-1 (5 units) or broken cell preparations of COS-1 cells expressing V349L oPGHS-1 (75 μg of cell protein) were incubated for one min with 50 μM [1-^{14}C] arachidonate, and the products were extracted and separated by thin layer chromatography as described in Methods. The locations of the various chromatographic standards are as noted. See Figure 2 for PG structures.

Extracts from incubation mixtures containing partially purified oPGHS-1, recombinant oPGHS-1 or recombinant V349L oPGHS-1 were incubated with different concentrations of arachidonate and then separated by RP-HPLC. 11- and 15-HETE were isolated, converted to their methyl esters and examined by chiral HPLC. This is illustrated in Figure 10 for partially purified native oPGHS-1 incubated with 100 μM arachidonate. 11-HETE was exclusively 11*R*-HETE. 15-HETE was 70% 15*S*-HETE and 30% 15*R*-HETE. The ratio of 15*S*-HETE to 15*R*-HETE was the same for both purified and microsomal, recombinant oPGHS-1. Similarly, the 15-HETE fraction derived from incubation of arachidonate with V349L oPGHS-1 contained 70% 15*S*-HETE and 30% 15*R*-HETE; only trace amounts of 11-HETE were formed by V349L oPGHS-1, and the chirality of this latter product was not determined. Interestingly, the enantiomeric composition of the 15-HETE product was 65-70% 15*S*-HETE and 30-35% 15*R*-HETE at all arachidonate concentrations tested with both native and V349L oPGHS-1 (i.e. when 2, 5, 10, 20, 35, 50 or 100 μM arachidonate was used as the substrate with purified oPGHS-1, when 10 and 100 μM arachidonate was used as the substrate for membrane-associated, recombinant oPGHS-1 or when 2, 5, 10, 20, 35, 50 or 100 μM arachidonate was used as the substrate for membrane preparations of recombinant V349L oPGHS-1) (Figure 11).

We noted in the studies outlined above that there were obvious differences in the relative ratios of the monohydroxy fatty acid and PGG_2-derived products at different arachidonate concentrations. Accordingly, we performed a series of measurements to determine the K_M values for the formation of the different products by native oPGHS-1 (Figure 12) and V349L oPGHS-1 (Figure 13). The results are summarized in Table I. It should be noted that the amounts of enzyme (5 units for partially purified native enzyme or 75 μg of broken COS-1 cell protein for V349L oPGHS-1) used in these assays were adjusted so that <25% of the total substrate was consumed even with the lowest

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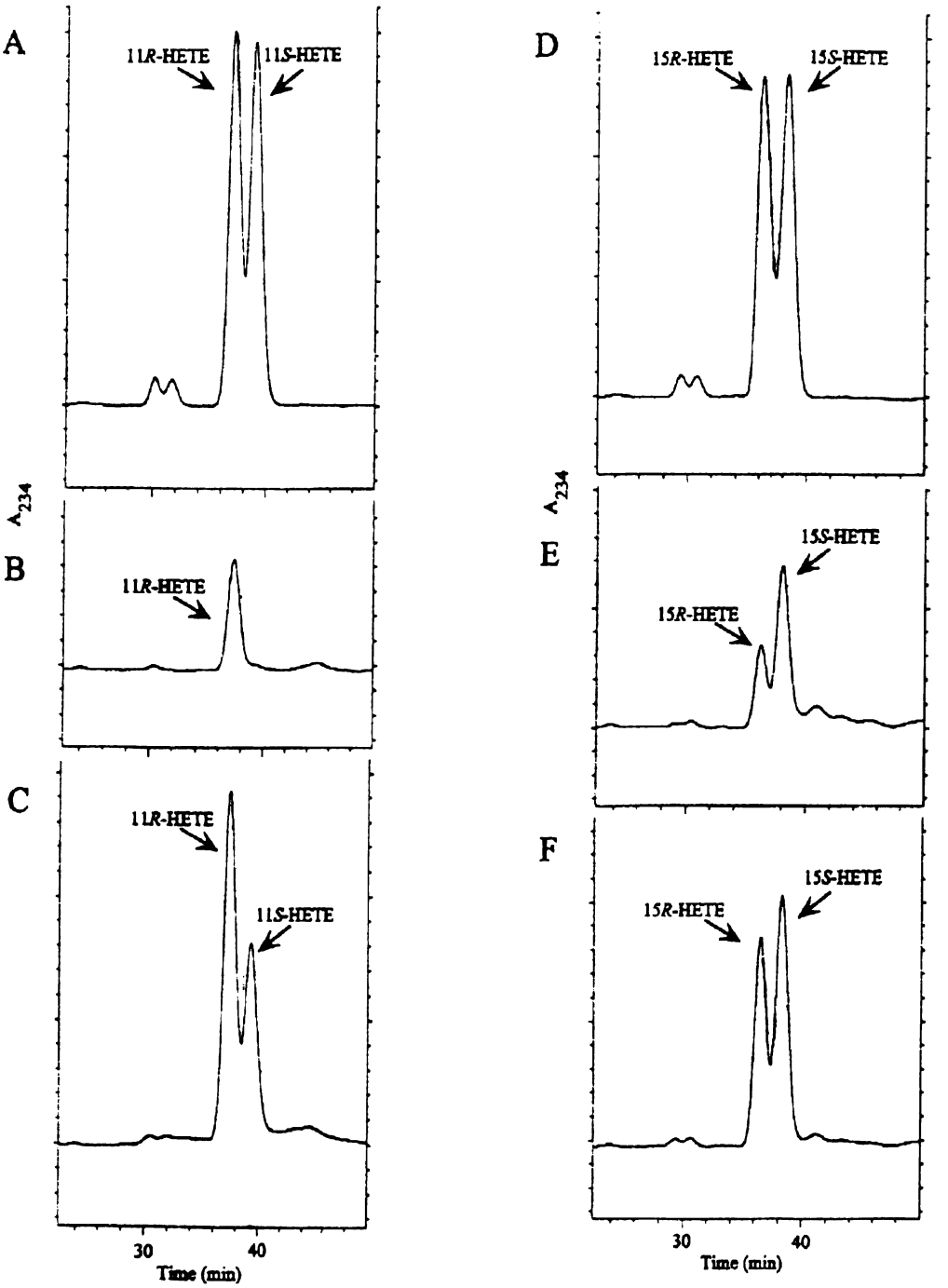
Figure 10. Chiral HPLC of the methyl esters of the 11- and 15-HETEs produced by native oPGHS-1. Arachidonate (100 μM) was incubated with partially purified oPGHS-1 for one min at 37°C. 11- and 15-HETE fractions were separated by RP-HPLC and esterified by treatment with diazomethane. Chiral-phase HPLC was performed as described in Methods using a Chiralcel OC column with hexane/2-propanol (98/2 v/v) as the solvent and a flow rate of 0.5 ml/min. The UV detector was set to monitor absorbance at 234 nm. A. 11(*R/S*)-HETE methyl ester standard; B. 11-HETE methyl ester from incubation of oPGHS-1 with arachidonate; C. Coinjection of 11-HETE methyl ester from incubation of oPGHS-1 with 100 μM arachidonate and 11(*R/S*)-HETE methyl ester standard; D. 15(*R/S*)-HETE methyl ester standard; E. 15-HETE methyl ester from incubation of oPGHS-1 with arachidonate; F. Coinjection of 15-HETE methyl ester from incubation of oPGHS-1 with 100 μM arachidonate and 15(*R/S*)-HETE methyl ester standard.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11R-HETE
11S-HETE
B
11R-HETE
C
11R-HETE
11S-HETE
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40
Time (min)
D
15R-HETE
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15S-HETE
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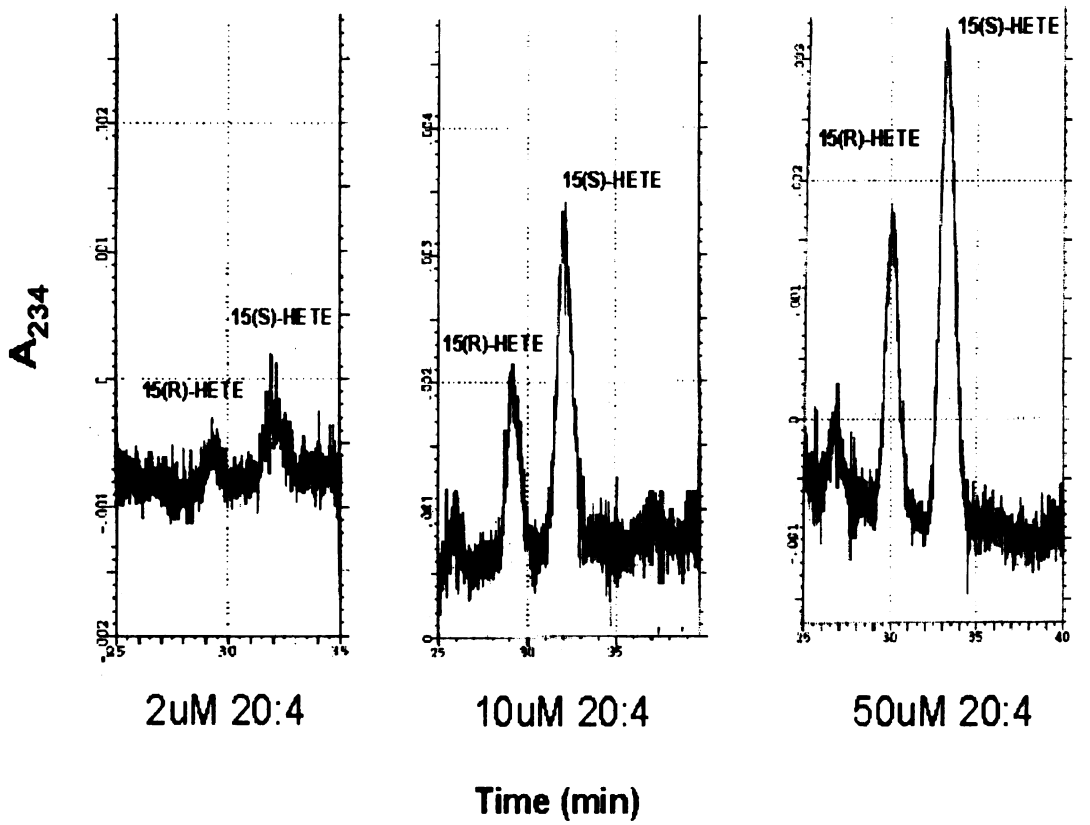
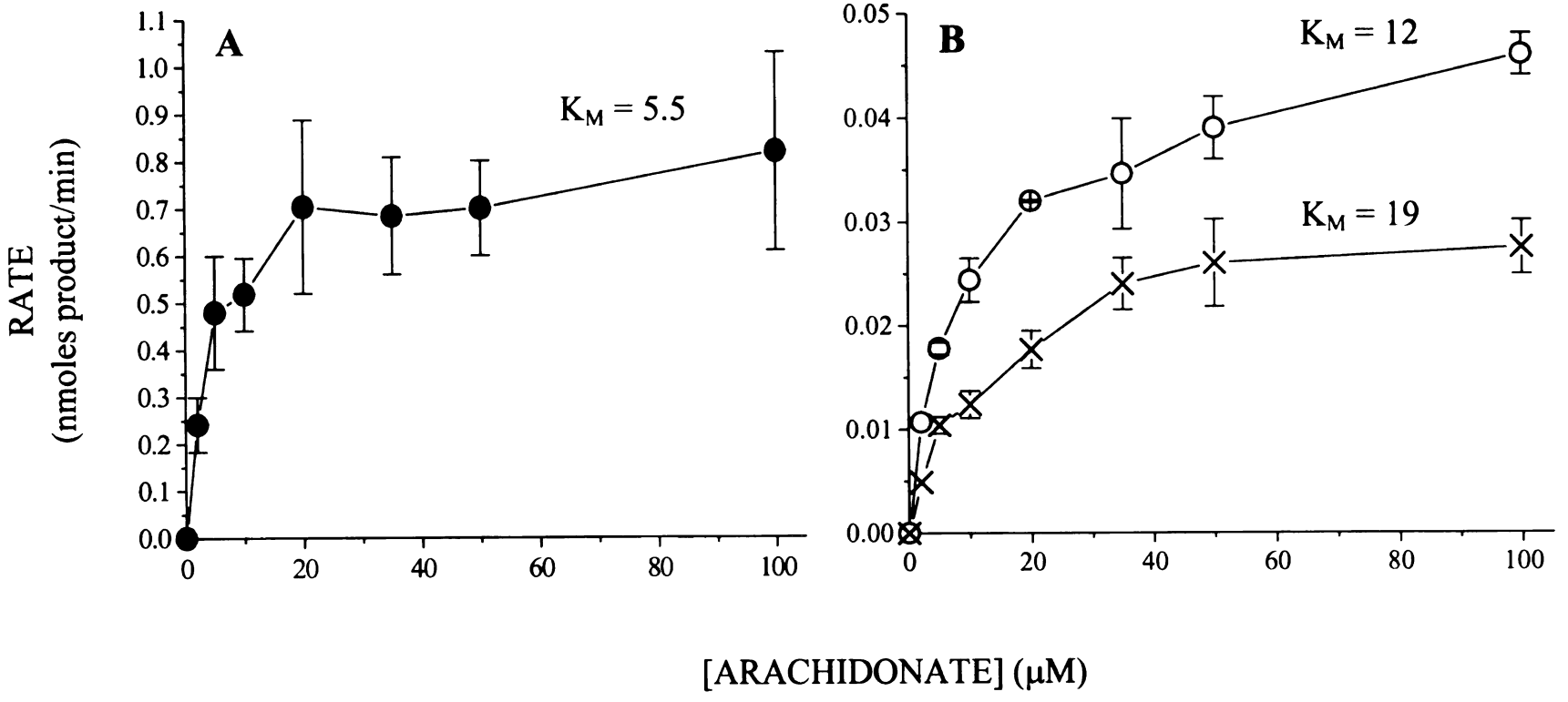


Figure 11. Chiral HPLC of the methyl esters of the 15-HETEs produced by native oPGHS-1 at increasing arachidonate concentrations. Arachidonate (2-100 μM) was incubated with partially purified oPGHS-1 for one min at 37°C. RP- and chiral phase HPLC was carried out as described in Figure 10.

Figure 12. Effect of arachidonic acid concentration on the formation of PGG_2, 11-HETE and 15-HETE by native oPGHS-1. Partially purified oPGHS-1 (5 cyclooxygenase units) was incubated with the indicated concentrations of arachidonate concentrations at 37°C for one min. Products were analyzed by RP-HPLC and/or radio thin layer chromatography as described in Materials and Methods. For HPLC, the amounts of products were calculated using peak areas and comparison with known amounts of PGB_2 standard. Liquid scintillation counting was used to determine the amounts of radioactive products on thin layer chromatograms. Results are from an average of eight separate experiments. A. PGG_2-derived products (●); and B. 11-HETE(○) and 15-HETE(×).


A
B
K_M = 5.5
K_M = 12
K_M = 19
RATE
(nmoles product/min)
[ARACHIDONATE] (μM)
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0.7
0.6
0.5
0.4
0.3
0.2
0.1
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0.05
0.04
0.03
0.02
0.01
0.00
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20
40
60
80
100

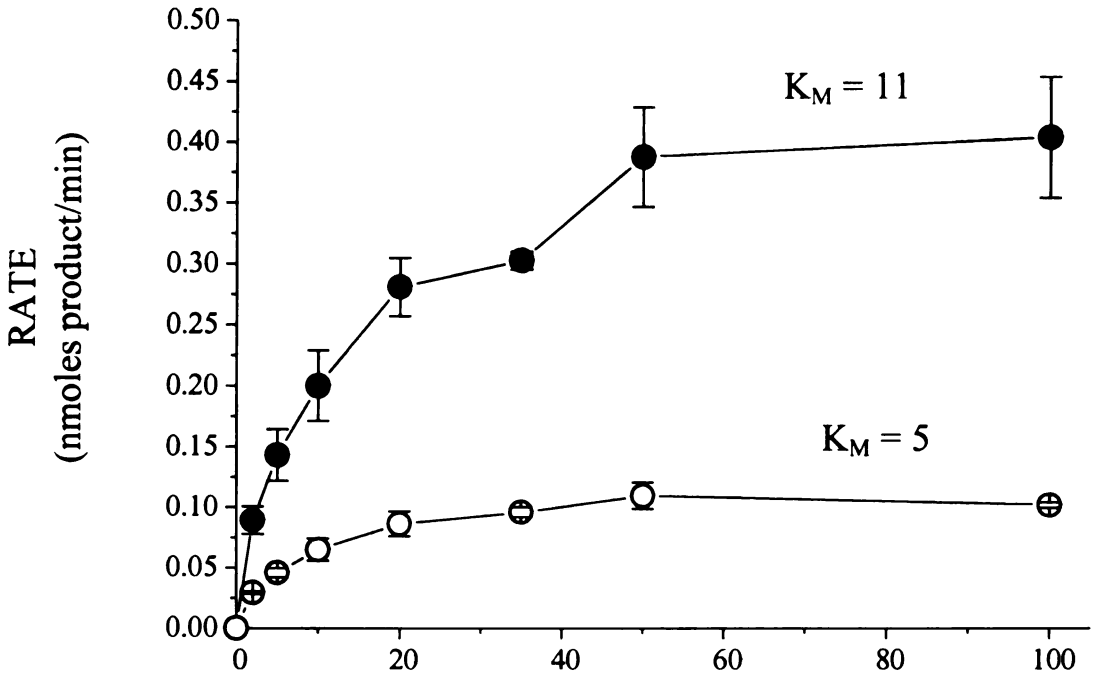


Figure 13. Effect of arachidonic acid concentration on the formation of PGG_2 and 15-HETE by V349L oPGHS-1. V349L oPGHS-1 (75μg protein from transfected COS-1 cells) was incubated with the indicated concentrations of arachidonate at 37°C for one min. Products were analyzed by thin layer chromatography and quantitated by liquid scintillation counting of radioactive products from thin layer chromatograms. Results are an average from four separate transfections. PGG_2-derived products (●) and 15-HETE(○).

concentration of arachidonate tested (2 μM). Product formation was analyzed after 1 min incubations by radio thin layer chromatography and/or by RP-HPLC. The key finding of these experiments is that the K_M values for the formation of PGG_2, 11-HETE and 15-HETE by native oPGHS-1 and the K_M values for PGG_2 and 15-HETE formation by V349L oPGHS-1 were different for each product (Table I).

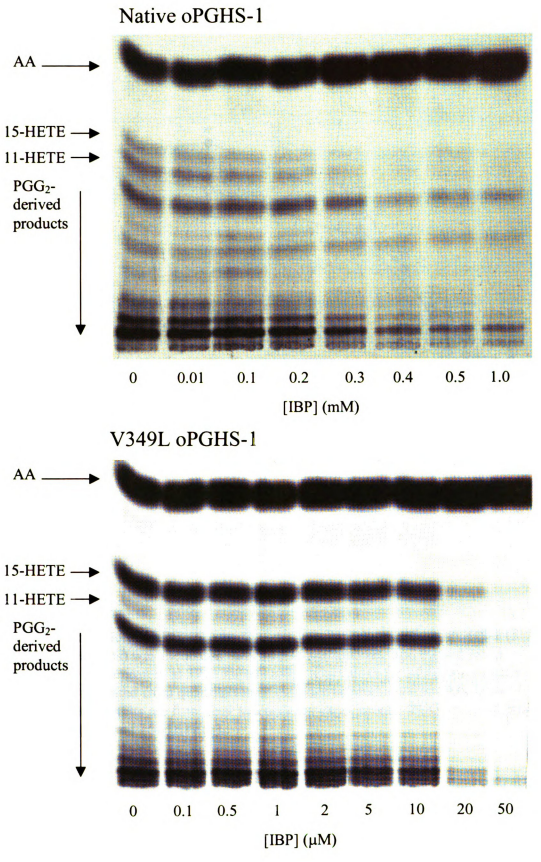
Because the K_M values were different for the formation of each product, it was of interest to determine if the IC_{50} values for the inhibition of PGG_2, 11-HETE and 15-HETE synthesis would be different or the same. Accordingly, we determined IC_{50} values for inhibition by ibuprofen of each of the products formed by partially purified native oPGHS-1 (Figures 14 and 15) and by the V349L oPGHS-1 mutant (Figures 14 and 16). Ibuprofen was chosen because it is a simple competitive, reversible cyclooxygenase inhibitor [157]. The IC_{50} values for inhibition of PGG_2, 11-HETE and 15-HETE were the same (170 μM) for native oPGHS-1; moreover, the IC_{50} values for the inhibition of PGG_2 and 15-HETE formation by V349L oPGHS-1 were also identical (15 μM).

Table I. Catalytic Constants for the Formation of Prostaglandin and Monohydroxy Acid Products from Arachidonate by Native and V349L oPGHS-1.

Partially purified oPGHS-1 (5 cyclooxygenase units) or membrane-associated V349L oPGHS-1 (75 μg of cell protein) was incubated with increasing concentrations of arachidonate at 37°C for one min. K_M and V_{max} values for PGG_2-derived and HETE products were calculated from RP-HPLC and radio thin layer chromatography product analyses as described under Experimental Procedures and illustrated in Figures 12 and 13. Data are from an average of eight and four separate experiments for native and V349L oPGHS-1, respectively and are expressed +/- S.E.M. ND, not determined. [a]p< .01 versus PGG_2 for oPGHS-1; [b]p<.05 versus 11-HETE for oPGHS-1; [c]p<.001 versus PGG_2 for V349L.

Products	oPGHS-1		V349L oPGHS-1	
	K_M (μM)	V_{max} (nmoles product/min)	K_M (μM)	V_{max} (nmoles product/min)
PGG_2-derived products	5.5 +/- 1.7	0.9 +/- .05	11 +/- 0.54	0.43 +/- 0.04
11-HETE	12 +/- 1.4[a]	0.04 +/- .001	ND	ND
15-HETE	19 +/- 4.3[a,b]	0.03 +/- .006	5 +/- 0.7[c]	0.12 +/- 0.04

Figure 14. Autoradiogram of thin layer chromatograms from IC_{50} determinations for inhibition of native (upper) and V349L (lower) oPGHS-1 by ibuprofen. Partially purified oPGHS-1 (5 units) or broken cell preparations of COS-1 cells expressing V349L oPGHS-1 (75 μg of cell protein) were incubated for one min with 10 μM [1-^{14}C] arachidonate in the presence of increasing concentrations of ibuprofen. Products were extracted, separated by thin layer chromatography and quantitated as described in Methods.


Native oPGHS-1
AA
15-HETE
11-HETE
PGG2-derived products
0 0.01 0.1 0.2 0.3 0.4 0.5 1.0
[IBP] (mM)
V349L oPGHS-1
AA
15-HETE
11-HETE
PGG2-derived products
0 0.1 0.5 1 2 5 10 20 50
[IBP] (μM)

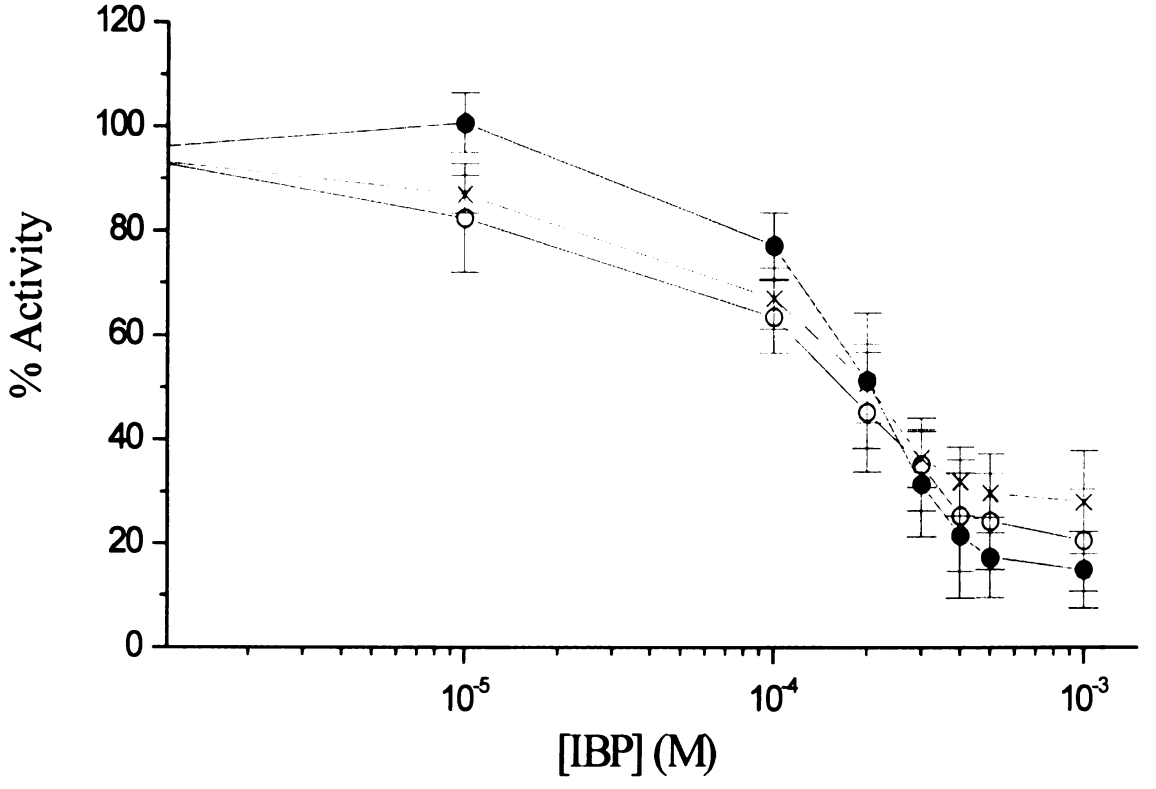


Figure 15. Inhibition of oPGHS-1 by ibuprofen. Partially purified oPGHS-1 (5 cyclooxygenase units) was incubated with 10 μM arachidonate and the indicated concentrations of ibuprofen at 37°C for one min. IC_{50} values for inhibition of each product were calculated from quantitation of radioactive products from thin layer chromatograms. Results are from four independent experiments. PGG_2-derived products (●); 11-HETE(○); and 15-HETE(×).

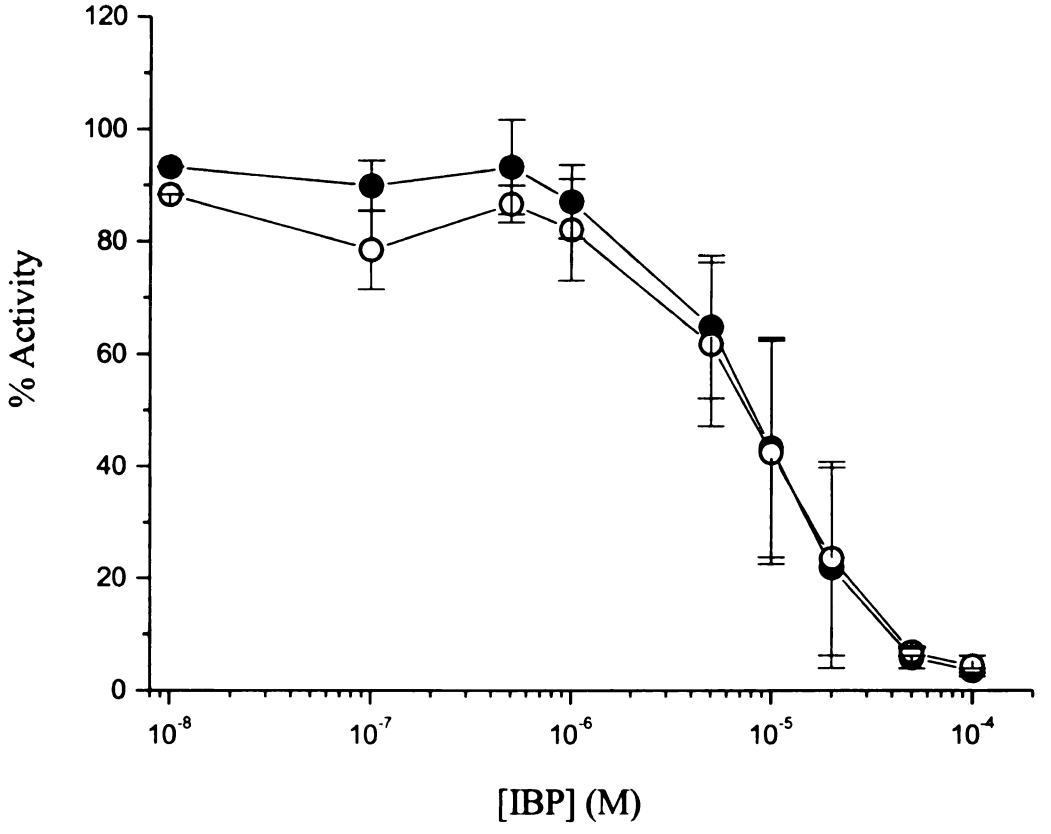


Figure 16. Inhibition of V349L oPGHS-1 by ibuprofen. V349L oPGHS-1 (75μg of protein from transfected COS-1 cells) was incubated with 10 μM arachidonate and the indicated concentrations of ibuprofen at 37°C for one min. IC_{50} values for inhibition of each product were calculated from quantitation of radioactive products from thin layer chromatograms. Results are from four independent experiments. PGG_2-derived products (●) and 15-HETE(○).

Discussion

There are now several x-ray crystallographic structures of PGHS-1 and -2. All of the structures reported to date have nonsteroidal anti-inflammatory drugs bound [80-82]. These crystallographic studies have defined the cyclooxygenase active site as a tunnel lined with the side chains of hydrophobic amino acids that protrudes into the core of a globular catalytic domain; the globular catalytic domain itself closely resembles that of mammalian peroxidases. We are currently addressing the question of how arachidonate itself binds within the cyclooxygenase active site at the time the rate determining step in the reaction, abstraction of the 13-pro*S* hydrogen [61], occurs. These ongoing studies involve a combination of determining the crystal structure of arachidonate bound to a catalytically inactive form of oPGHS-1 with Co^{3+}-heme substituted for Fe^{3+}-heme and analyzing the functional effects of amino acid substitutions of cyclooxygenase active site residues. In establishing a baseline from which to interpret the results of these crystallographic and mutagenic analyses, it was essential to determine precisely which products are formed by oPGHS-1 under different experimental conditions. Accordingly, we characterized the products generated when arachidonic acid was incubated with native oPGHS-1 and an active site mutant, V349L oPGHS-1, and determined kinetic constants for the formation of the different products. Experiments were performed comparing partially purified with recombinant oPGHS-1 to verify that the results are applicable to solubilized and membrane-associated forms of the enzyme.

Our key finding is that the K_M values for the formation of PGG_2, 11*R*-HETE, and 15*S*/*R*-HETE by native oPGHS-1 differ from each other. Similarly, with V349L oPGHS-1, which forms a quantitatively different mix of products, primarily PGG_2 and 15-HETE

but little 11-HETE, the K_M values for formation of these products differed from one another. If the rate determining step in the formation of the various products is, as expected [61, 158], abstraction of the 13-pro*S* hydrogen from arachidonate, differences in K_M values would only occur if there are different enzyme substrate complexes leading to the different products. The simplest interpretation of our findings is that arachidonate can assume up to three catalytically competent "arrangements" in the cyclooxygenase active site of native oPGHS-1 in which the 13-pro*S* hydrogen is removed in the rate determining step, but from which oxygenation and cyclization proceed differently to form PGG_2, 11*R*-HETE or a combination of 15*S*- and 15*R*-HETE depending on the arrangement of arachidonate at the instant of hydrogen abstraction. These arrangements could involve subtle differences in carbon chain conformations, differences in electrostatics along the carbon chain of arachidonate or in active site residues or perhaps, most likely, a combination of these factors. For example, a conformation of arachidonate having an optimal orientation of C-9 with respect to C-11 would result in formation of the C-9 to C-11 endoperoxide and proceed to PGG_2; alternatively, if the orientation between C-9 and C-11 is not optimal--either too great or too near--the incipient 11-peroxyl radical could not proceed to the endoperoxide but would yield the 11-hydroperoxide as the abortive product. Previous studies have shown that 11-hydroperoxide product, 11-HPETE is not converted to PGG_2 by oPGHS-1.

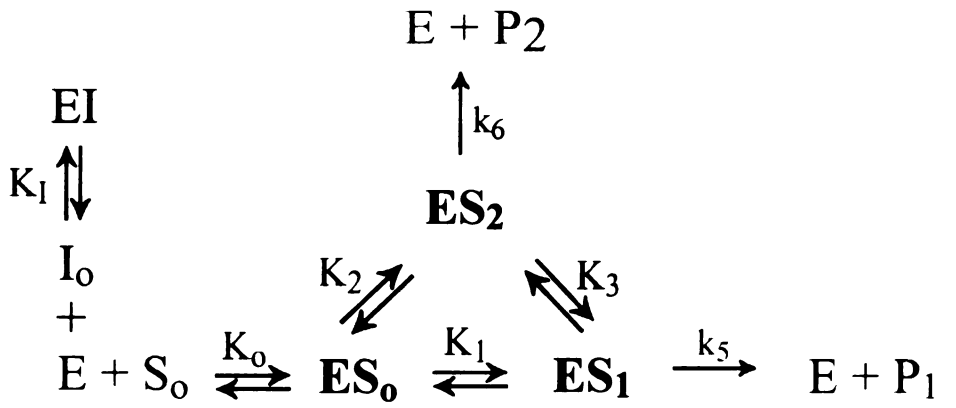
There are two general possibilities which could account for the fact that different products are formed with different K_M and V_{max} (Figure 17, Schemes 1 and 2). One possibility is that there is essentially one form of the enzyme (E) which binds arachidonate and that, secondarily, rearrangements of the substrate (and/or enzyme) occur

after the substrate becomes bound which then proceed to product (Scheme 1). The basic assumption of this scheme is that, changes which occur either in substrate position, enzyme position or a combination of the two, occur only after substrate has entered the cyclooxygenase active site. That is, the enzyme does not undergo any conformations changes in the free form. The other general possibility is that different starting forms of the enzyme occur (E_1 and E_2) which bind substrate differently and yield different products (Scheme 2). The basic assumption of Scheme 2 is that changes are occuring only in the free enzyme form and it is this conformational change in the enzyme which results in variant binding of the substrate.

As a test of these two possibilities, we determined the IC_{50} values for inhibition of the formation of each product by ibuprofen. In Scheme 1, ibuprofen would be expected to inhibit the formation of each product with the same IC_{50}. Indeed, this is the experimental observation with both native oPGHS-1 and V349L oPGHS-1 which leads us to favor the assumptions of Scheme 1 which dictate that conformational changes, whether in substrate alone, enzyme alone, or the substrate/enzyme complex, occur only as a result of substrate binding and not before.

In Scheme 2, one would expect ibuprofen to bind different forms of the enzyme differently and yield different IC_{50} values for each product. The experimental data suggest that this scenario is less likely. Also, structural data which is available from multiple crystal structure determinations of both PGHS-1 and PGHS-2 indicate that the free enzyme conformation is the same, not only among determinations of the same structure, but also between determinations of PGHS-1 compared with PGHS-2. Of course, we cannot rule out the possibility that different forms of the enzyme bind

A



B

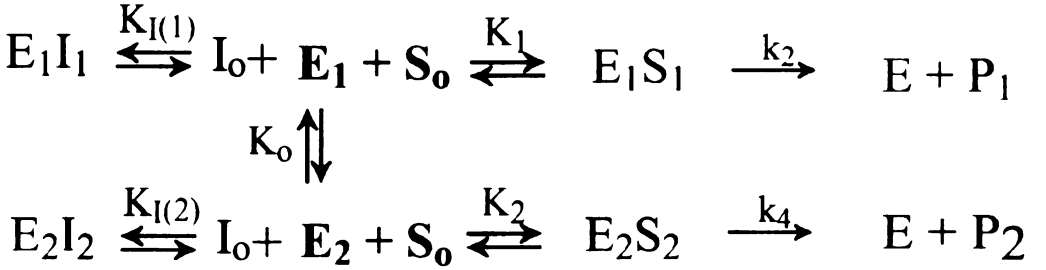


Figure 17. Kinetic schemes for the binding of arachidonate to a single enzyme form and for the binding of arachidonate to a mixture of different enzyme forms. A. Scheme 1: arachidonate enters a static cyclooxygenase active site and then can assume alternative conformations or arrangements leading to different products (e.g. PGG_2, 11-HETE or 15-HETE). This scheme and its equations also apply to situations where arachidonate binding induces changes in the active site. B. Scheme 2: arachidonate binds to different forms of the cyclooxygenase active site which catalyze the formation of different products; a variation of this scheme applies to situations in which different forms of the enzyme bind different forms of the substrate to produce different products.

ibuprofen identically but that the different enzyme forms bind the substrate in different arrangements. Also, we realize the limitations of crystallography, in that certain enzyme forms may very well crystallize more easily than others. Thus, while the results of the experiments with ibuprofen are consistent with Scheme 1, they do not rule out Scheme 2 and there are situations during which IC_{50} values for the inhibition of all products could be equal under the assumptions of Scheme 2.

PGHS-1 and -2 have been shown previously to form small amounts of the monooxygenation products, 11- and 15-HETEs [61, 154, 155]. We found that oPGHS-1 forms an additional product, 15*S*-HETE, which is apparently not produced from arachidonate by PGHS-2 [155]. In fact, 15*S*-HETE is the predominant 15-HETE enantiomer formed by oPGHS-1 whereas both PGHS-2 and aspirin-acetylated PGHS-2 form almost exclusively 15*R*-HETE [108, 155].

Both 15*S*-HETE and 15*R*-HETE would be expected to be formed under conditions favoring a combination of formation of 15-arachidonyl radical and O_2 access to C-15. Formation of 15*R*-HETE by aspirin-acetylated PGHS-2 involves abstraction of the 13-pro*S* hydrogen as does formation of PGG_2 (and presumably 11*R*-HETE and 15*S*-HETE) [61, 154]. We assume, therefore, that the 15*R*-HETE formed in our experiments involves abstraction of this same prochiral hydrogen. The 15*S*-/15*R*-HETE ratio remained constant for 15-HETE synthesized by oPGHS-1 at arachidonate concentrations between 2 and 100 μM arachidonate (Figure 11). This was also true with the V349L oPGHS-1 mutant. Collectively, this information suggests that 15*S*- and 15*R*-HETE derive from the same arrangement of arachidonate in the cyclooxygenase site and that the 15*S*-/15*R*-HETE ratio simply reflects positional access of O_2 to a 15-arachidonyl radical

formed by removal of the 13-pro*S* hydrogen. Although antarafacial O_2 addition is observed in most lipoxygenase reactions, there is recent precedent for a suprafacial O_2 addition with a fungal lipoxygenase [159].

The sidechain of Val349 protrudes into the cyclooxygenase active site approximately opposite the sidechain of Ser530, the site of aspirin acetylation [21, 80, 99]. Our results indicate that replacing Val349 with the larger leucine has the overall effects of decreasing the amounts of products formed as a result of O_2 insertion at C-11 (PGG_2 and 11-HETE) and increasing the amount of product involving O_2 insertion at C-15 (15-HETE). That is, with the V349L mutant, arrangements of arachidonate in the active site which lead to O_2 insertion at C-11 become relatively less favorable than in native oPGHS-1. The K_M for PGG_2 formation is increased and PGG_2 and 11-HETE formation occurs to a significantly decreased extent with V349L oPGHS-1. In contrast, O_2 access to C-15 is relatively enhanced with a relative increase in the rate of monooxygenation at C-15 and a relative decrease in the K_M for 15-HETE production.

We have emphasized in this report the fact that oPGHS-1 forms products other than PGG_2. However, in concluding we point out that the K_M and V_{max} values favor the synthesis of this endoperoxide over monohydroxy acids by native oPGHS-1. At arachidonate concentrations ($\leq$2 μM) expected to be most relevant physiologically, PGG_2 represents >95% of the total oxygenated product.

Acknowledgment

This work was previously published in the *Journal of Biological Chemistry* as Thuresson, ED, Lakkides, KL, and Smith WL (2000) "Different catalytically competent arrangements of arachidonic acid within the cyclooxygenase active site of prostaglandin endoperoxide H synthase-1 lead to the formation of different oxygenated products." *J.Biol.Chem.*, 275, 8501-8507., and is reprinted with permission.

CHAPTER III

THE FUNCTIONS OF CYCLOOXYGENASE ACTIVE SITE RESIDUES IN THE BINDING, POSITIONING AND OXYGENATION OF ARACHIDONIC ACID

Introduction

Prostaglandin endoperoxide H synthases-1 and -2 (PGHS-1 and -2) catalyze the conversion of arachidonic acid (AA), two molecules of O_2 and two electrons to PGH_2. This is the committed step in the formation of prostaglandins and thromboxane A_2 [2, 3, 137]. PGHS-1 (or COX-1 (for cyclooxygenase-1)) is a constitutive enzyme while PGHS-2 (COX-2) is the inducible isoform [2, 3, 137] [138-140, 142-146, 160].

PGHSs catalyze two separate reactions including a cyclooxygenase (*bis*-oxygenase) reaction in which AA is converted to PGG_2 and a peroxidase reaction in which PGG_2 undergoes a two- electron reduction to PGH_2 [2, 3, 137]. These reactions occur at physically distinct but interactive sites within the cyclooxygenase structure. The cyclooxygenase reaction begins with a rate-limiting abstraction of the 13-pro*S* hydrogen from AA to yield an arachidonyl radical [61, 70]. This is followed by sequential oxygen additions at C-11 and C-15 producing PGG_2. NSAIDs compete directly with AA for binding to the cyclooxygenase site [80, 82]thereby inhibiting cyclooxygenase activity (but not peroxidase activity; [55, 152, 153]).

Crystallographic studies of enzyme-inhibitor complexes have suggested that the cyclooxygenase active site exists in the form of a hydrophobic channel that protrudes into the body of the major globular domain of the protein [80-82]. More recently, the structure of AA bound within the cyclooxygenase active site of ovine (o) PGHS-1 was

determined [66]. AA is bound in an extended L-shaped conformation and makes a total of 48 hydrophobic contacts (i.e. 2.5-4.0 Å) and two hydrophilic contacts with the protein, involving a total of nineteen different residues (Figure 18). Additionally, there are several amino acids which are in the first shell of the cyclooxygenase hydrophobic tunnel and contact other first shell amino acids but lie outside of van der Waals distance to AA. Although AA can assume some 10^7 low energy conformations [161], only three of these conformations are catalytically competent [104]; one conformation leads to PGG_2, one, to 11*R*-HPETE and another to 15*R*- plus 15*S*-HPETE. Previous mutational studies have demonstrated that the guanidinium group of Arg120 is important for high affinity binding of AA to PGHS-1 [96, 97](but not PGHS-2; [98, 162]), that Tyr385 is involved as a tyrosyl radical in abstracting the 13-pro*S*-hydrogen from AA [74, 95], and that Ser530 and Ile523 are determinants of inhibitor specificity [21, 111, 112, 156, 163]. Other than for Arg120 and Tyr385 relatively little is known about the functions of residues located in the core of the hydrophobic cyclooxygenase tunnel [95, 164]. In the study reported here we have performed mutational analyses of a number of the residues that line the hydrophobic active site channel to determine their functional importance in arachidonate binding and oxygenation. Our results suggest that individually and collectively these residues function primarily to position arachidonate in a specific conformation that optimizes its conversion to PGG_2.

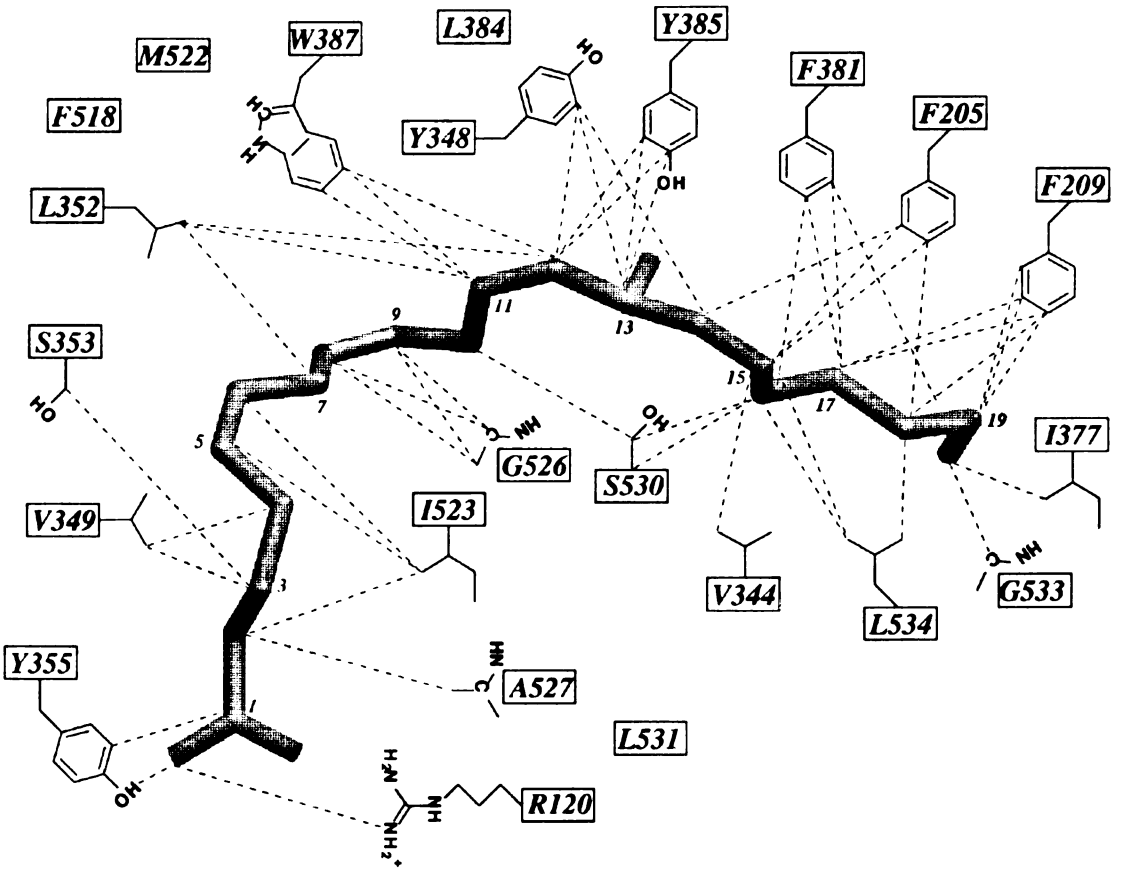


Figure 18. Schematic representation of interactions between arachidonic acid and amino acid residues lining the cyclooxygenase active site channel. All dashed lines represent interactions of $\leq$ 4.0 Å between specific side chain atoms of the protein and carbon or oxygen atoms of AA [66]. The distances between various carbons of AA and interacting sidechain residues are listed in Tables III and IV.

Materials and Methods

Materials. Fatty acids were purchased from Cayman Chemical Co., Ann Arbor, MI. [1-^{14}C] arachidonic acid (40-60 mCi/mmol) was purchased from New England Nuclear. Flurbiprofen was purchased from Sigma. Diazald (N-methyl-N-nitroso-*p*-toluenesulfonamide) was from Aldrich Chemical Company. Restriction enzymes and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO. Calf serum and fetal bovine serum were purchased from HyClone. Primary antibodies used for Western blotting were raised in rabbits against purified oPGHS-1 and purified as an IgG fraction [149] and goat anti-rabbit IgG horseradish peroxidase conjugate was purchased from BioRad. Oligonucleotides used as primers for mutagenesis were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility. All other reagents were from common commercial sources.

Preparation of oPGHS-1 mutants. Mutants were prepared either starting with M13mp19-PGHS$_{ov}$ which contains a 2.3-kilobase *SalI* fragment coding for native oPGHS-1 and employing the BioRad Muta-Gene kit and the protocol as described by the manufacturer [149] or by site-directed mutagenesis of oPGHS-1 in the pSVT7 vector [95] employing the Strategene QuikChange mutagenesis kit and the protocol of the manufacturer. Oligonucleotides used in the preparation of various mutants are summarized in Table II. Plasmids used for transfections were purified by CsCl gradient ultracentrifugation and mutations were reconfirmed by double-stranded sequencing of the pSVT7 constructs using Sequenase (ver. 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 (ATTC CRL-1650) were grown in DMEM containing 8% calf serum and 2% fetal bovine serum until near confluence (~$3x10^6$ cells/10 cm dish). Cells were then transfected with pSVT7 plasmids containing cDNAs coding for native oPGHS-1 or mutant oPGHS-1 using the DEAE dextran/chloroquine transfection method as reported previously [149]. Forty hours following transfection, cells were harvested in ice cold phosphate-buffered saline (PBS), collected by centrifugation and resuspended in 0.1 M Tris-HCl, pH 7.5. The cells were disrupted by sonication and microsomal membrane fractions were prepared at 0-4°C, as described previously [149]. Membranes were isolated from sham-transfected cells in an identical manner. Protein concentrations were determined using the method of Bradford [165] with bovine serum albumin as the standard. Microsomal preparations were used for Western blotting and for cyclooxygenase and peroxidase assays.

Cyclooxygenase and peroxidase assays. Cyclooxygenase assays were performed at 37°C by monitoring the initial rate of O_2 uptake using an oxygen electrode [94, 96]. Each standard assay mixture contained 3.0 ml of 0.1 M Tris-HCl, pH 8.0, 1 mM phenol, 85 μg of bovine hemoglobin and 100 μM arachidonic acid. Reactions were initiated by adding approximately 250 μg of microsomal protein in a volume of 20-50 μl to the assay chamber. K_M values were measured using 0.5-500 μM arachidonate. Inhibition of cyclooxygenase activity was measured by adding aliquots of microsomal suspensions to assay mixtures containing 100 μM arachidonate and 200μM flurbiprofen. Peroxidase activity was measured spectrophotometrically with N,N,N',N'-tetramethylphenylenediamine (TMPD) as the reducing cosubstrate [166]. The reaction mixture contained 0.1 M Tris-HCl, pH 8.0, 0.1 mM TMPD, approximately 100 μg of

microsomal protein and 1.7 μM hematin in a total volume of 3 ml. Reactions were initiated by adding 100 ml of 0.3 mM H_2O_2 and the absorbance at 610 nm was monitored with time.

Inhibition of cyclooxygenase activity. IC_{50} values were determined using cyclooxygenase assays as described above and rate measurements of oPGHS-1 enzymes with 50μM arachidonate and increasing concentrations of inhibitors.

Western blot analysis. Microsomal samples (ca. 5 μg of protein) were resolved by one-dimensional SDS-PAGE and transferred electrophoretically to nitrocellulose membranes using a Hoeffer Scientific Semi-Dry Transfer apparatus. Membranes were blocked for 12 hr in 3% non-fat, dry milk, 0.1% Tween20 and Tris-buffered saline, followed by a two-hour incubation with a peptide-directed antibody against oPGHS-1 [84] in 1% dry milk, 0.1% Tween-20 and Tris-buffered saline at room temperature. Membranes were washed and incubated for one hr with a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase, after which they were incubated with Amersham ECL reagents and exposed to film for chemiluminescence.

Characterization of arachidonic acid-derived products. A general protocol for product analysis is as follows. Forty hr 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, pH 7.5, containing 1 mM phenol and 6.8 μg bovine hemoglobin in a total volume of 200 μl. Reactions were initiated with 35 μM [l-^{14}C] arachidonic acid and were performed with or without 200 μM flurbiprofen and stopped by adding 1.4 ml of $CHCl_3$:MeOH (1:1; v/v). Insoluble cell debris was removed by centrifugation and 0.6 ml of $CHCl_3$ and 0.32

ml of 0.88% formic acid were added to the resulting supernatant. The organic phase was collected, dried under N_2, redissolved in 50 µl of $CHCl_3$ and spotted on a Silica Gel 60 thin layer chromatography plate; the lipid products were chromatographed for 1 h in benzene:dioxane:formic acid:acetic acid (82:14:1:1; v/v/v/v). Products 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.

For RP-HPLC analyses of products, native or mutant oPGHS-1 (1 mg of microsomal protein) was reacted with 100 µM arachidonic acid for 30 minutes at 37° C, and products were collected as described previously. Products were dried under N_2 and resuspended in HPLC buffers (1:1; v/v). 15- and 11-HETEs were separated by reverse phase HPLC using a C18 column (Vydac); the Waters Model 600 HPLC was equipped with a 990 photo diode array detector set to 200 and 234 nm. The strong component of the mobile phase was 0.1% acetic acid and the eluting solvent was acetonitrile. The flow rate was 1 ml/min. The following elution profile was used: Initial conditions were 30% acetonitrile, increased linearly over 30 min to 50% acetonitrile, then increased linearly from 30-100 min to 70% acetonitrile, then increased linearly from 100-125 min to 100% acetonitrile and held for 5 min before returning to initial conditions. The retention times of 15-HETE and 11-HETE averaged 36 and 38 min, respectively. Material obtained by RP-HPLC was esterified by treatment with excess diazomethane and subjected to chiral-phase HPLC. Chiral-phase HPLC separations of the methyl esters of 11- and 15-HETEs were performed with a Chiralcel OC column (250 X 4.6 mm; Daicel Chemical Industries, Osaka) using hexane/2-propanol (90:10; v/v) as the solvent and a flow rate of 0.5 ml/min.

Diazomethane was prepared from Diazald and distilled in ether per the Aldrich Chemical Company Technical Bulletin #AL-180.

Molecular modeling . Mutations were modeled and analyzed in the program CHAIN [167] using the coordinates from the crystal structure of Co^{3+}-oPGHS-1 complexed with AA (PDB entry 1DIY) and Fe^{3+}-oPGHS-1 complexed with flurbiprofen (PDB entry 1CQE).

Table II. Oligonucleotide Primers Used for Preparing oPGHS-1 Mutants

Mutants were prepared using two different procedures. For those made using the BioRad Mutagene kit, short single-stranded oligonucleotide primers were used. For PCR mutagenesis, double standed primers were used; in the latter cases, designed with an asterisk (*), only a single strand is shown. Mutation sites are underlined. The numbering refers to the location in cDNA for oPGHS-1 [22].

Mutation	Oligonucleotide Primer
F205A*	5'-^{687}CCTTCTTTGCTCAGCACGCCACCCATCAGTTCTTCAAAACTTCC-3'
F205L*	5'-^{687}CCTTCTTTGCTCAGCACCTCACCCATCAGTTCTTCAAAACTTCC-3'
F209A*	5'-^{687}CCTTCTTTGCTCAGCACTTCACCCATCAGGCCTTCAAAACTTCC-3'
F209L*	5'-^{687}CCTTCTTTGCTCAGCACTTCACCCATCAGCTCTTCAAAACTTCC-3'
Y348L	5'-^{1123}TGATAGAGGAGCTTGTGCAGCAG-3'
Y348W	5'-^{1123}GATAGAGGAGTGGGTGCAGCAGC-3'
V349A	5'-^{1127} AGAGGAGTATGCGCAGCAGCTGA-3'
V349L	5'-^{1127}AGAGGAGTATCTGCAGCAGCTGA-3'
V349S	5'-^{1127}AGAGGAGTATTCGCAGCAGCTGA-3'
V349T	5'-^{1127}AGAGGAGTATACGCAGCAGCTGA-3'
S353A*	5'- ^{1139}GCAGCAGCTGGCCGGCTACTTCCTGCAGC-3'
S353G*	5'-^{1139}GCAGCAGCTGGGCGGCTACTTCCTGCAGC-3'
S353T*	5'-^{1139}GCAGCAGCTGACCGGCTACTTCCTGCAGC-3'
I377V*	5'-^{1205}CCAGTACCGCAACCGCGTCGCGATGGAGTTCAACC-3'
F381A*	5'-^{1219}GCATCGCCATGGAGCCAACCAGCTGTACC-3'
F381L*	5'-^{1219}GCATCGCCATGGACTCAACCAGCTGTACC-3'
I523A*	5'-^{1645}CCATCTTTGGGGAGAGTATGGTAGAAATGGGGGCTC-3"
S530V	5'-^{1670}GGCTCCTTTTGTCCTTAAGGGCC-3'
L531A	5'-^{1673}TCCTTTTTCCGCTAAGGGCCTCT-3'
L531V	5'-^{1673}TCCTTTTTCCGTTAAGGGCCTCT-3'
L534A*	5'-^{1682}CCTTAAGGGCGCCCTTGGAAACCCCATCTGTTCTCC-3'
L534V*	5'-^{1682}CCTTAAGGGCGTCCTTGGAAACCCCATCTGTTCTCC-3'

Table III. Contacts between Arachidonic Acid (AA) and Cyclooxygenase Active Site Residues

Van der Waals and hydrogen bond interactions were calculated using CHAIN [167]. Van der Waals contacts within 4Å are listed. See list of abbreviations for carbon designations.

Residue	Atom	Residue	Atom	Distance (Å)
Phe 205	CE2	AA	C14	4.0
	CE2		C15	3.4
	CZ		C15	3.9
	CZ		C18	3.9
Phe 209	CD1	AA	C19	3.9
	CE1		C19	3.6
	CE2		C17	3.9
	CZ		C17	3.7
	CZ		C18	3.9
	CZ		C19	3.7
Tyr 348	CE2	AA	C12	3.2
	CE2		C13	3.9
	CE2		C14	3.6
Val 349	CG1	AA	C3	3.4
	CG1		C4	3.1
Ser 353	CB	AA	C3	4.0
Ile 377	CD1	AA	C20	3.7
Phe 381	CE2	AA	C17	3.8
	CE2		C20	3.9
	CZ		C16	3.9
	CZ		C17	3.5
Ile 523	CG2	AA	C2	3.8
	CG2		C5	3.3
	CG2		C6	3.4
Ser 530	CA	AA	C16	3.7
	CB		C10	3.5
	CB		C16	3.8
Leu 534	CD1	AA	C18	3.9
	CD2		C15	3.5
	CD2		C16	3.6

Table IV. Interactions Between Leu531 and other Cyclooxygenase Active Site Area Residues

Leu531 does not contact arachidonic acid, but is involved in a number of hydrogen bonding interactions with other active site area residues. Hydrogen bonds were calculated using CHAIN [167]. Hydrogen bonding was assigned for O-H···N, C=O···H and H-N-C bond angles > 90° and distances not exceeding 3.6Å.

Residue	Atom	Residue	Atom	Distance (Å)	Comments
Leu 531	N	Ala 527	O	2.8	main chain H-bond
	O	Gly 533	N	3.1	main chain H-bond
	O	Leu 534	N	3.1	main chain H-bond
	O	Leu 535	N	2.9	main chain H-bond

Table V. Kinetic Properties and Product Analyses for oPGHS-1 Cyclooxygenase Active Site Mutants

Peroxidase activity was measured spectrophotometrically using 0.2 mM H_2O_2 and 100 μM TMPD as substrates and oxygenase activity was measured with an oxygen electrode as described in the text. Values are calculated for arachidonic acid turnover and are corrected for the percentage of mono- and bis-oxygenated products formed with the mutants. A value of 100% is assigned for peroxidase and oxygenase activity of native oPGHS-1. V_{max} and K_M values represent the means from a minimum of four separate determinations with standard deviations within 10% of all values reported. Relative V_{max} values reported for mutant enzymes for which K_M values were not determined represent rate measurements taken using 100 μM arachidonate. ND, not determined.

ENZYME	OXYGENASE			PEROXIDASE (% of Control)	11-HETE (% of Total Products)	15-HETE (% of Total Products)
	V_{max} (%)	K_M (μM)	V_{max}/K_M			
NATIVE	100	2	50	100	2.5 (11*R*)	2.5 (15*S*)
SHAM	0	--	--	0	--	--
F205A	28	3	9.3	90	11	5
F205L	65	ND	ND	106	5	2
F209A	15	10	1.5	55	13	3
F209L	43	ND	ND	104	5	2
Y348L	0	--	--	9	--	--
Y348F [74,75,95]	83	2.9	29	76	2.1	2.2
Y348W	0	--	--	0	--	--
V349A	55	1.7	32	52	53 (11*R*)	0
V349A/ S353T	3	ND	ND	49	27	12
V349S	43	14	3.2	94	41 (11*R*)	0
V349T	39	13	3.0	136	5.5 (11*R*)	0.6
V349L	63	7.1	8.9	117	0	13(15*S*), 11(15*R*)
S353G	61	2	30.5	87	4	2
S353A	56	ND	ND	60	3	3
S353T	42	ND	ND	46	14	12
I377V	72	1.1	65	110	10	2

Table V (cont'd)

F381A	4	6	0.7	101	9	4
F381L	21	ND	ND	74	7	4
I523A	64	ND	ND	112	12	3
I523V	70	ND	ND	57	2.5	2.5
S530A [21, 156]	58	2	29	68	2.5	2.5
S530T [21, 156]	17	13	1.3	30	0	10 (15*R*)
S530V	0	--	--	94	--	--
L531A	4.6	54	.08	100	2.5	2.5
L531D [156]	13	2	6.5	26	2.5	2.5
L531N [156]	7	3	2.3	9	2.5	2.5
L531V	7.7	1.6	4.8	75	2.5	2.5
L531I [156]	19	.4	48	74	ND	ND
L531K [156]	0	--	--	0	--	--
L534A	59	8	7.4	72	2	56 (95 *S*, 5*R*)
L534V	98	ND	ND	102	8	47 (96 *S*, 4*R*)

Results and Discussion

Overview. As illustrated in Figure 18, arachidonic acid (AA) is bound in an extended L-conformation in the AA/Co^{3+}-heme oPGHS-1 co-crystal structure [66]. The carboxylate group of AA interacts with Arg120, the ω end abuts Ile 377 and Gly533, the 13-pro*S* hydrogen is appropriately aligned with Tyr385, and there is ample space for the first O_2 insertion at C-11 and facile bridging of the incipient 11-hydroperoxyl radical to C-9 to form the endoperoxide. Formation of the cyclopentane ring is proposed to involve rotation about the C-10/C-11 bond and consequent movement of the ω terminus so that C-12 can react with the C-8 radical that is produced upon formation of the endoperoxide group; this movement, in turn, positions C-15 adjacent to Tyr385 for a second antarafacial O_2 insertion and a one electron reduction of the 15-hydroperoxyl radical by Tyr385 to regenerate the Tyr385 radical [66].

In the AA/Co^{3+}-heme PGHS-1 co-crystal structure AA makes two hydrophilic and 48 hydrophobic contacts involving a total of 19 residues in the first shell of the cyclooxygenase active site (Figure 18, Table III; [66]). These studies involve mutations of 10 of the residues that are putatively involved in direct interactions with AA as well as one other residue (Leu531) which is in the first shell of the active site but does not directly contact the substrate (Figure 18; Tables III and IV). In analyzing the various mutants, we identified the AA oxygenation products, determined kinetic constants for AA oxygenation and measured peroxidase activity (Table V). Cyclooxygenase and peroxidase activities for native and all mutant enzymes were normalized to levels of protein expression determined from western blot analysis and densitometric quantitation. The results of western transfer blotting indicated that the native enzyme as well as all

mutants tested were expressed at similar levels by COS-1 cells as shown in a representative figure (Figure 19). A discussion of the implications of the results are presented below for individual residues.

These studies identify residues which fall into three functional groups: (a) residues essential for positioning C-13 of AA for hydrogen abstraction (Tyr348); (b) residues critical for conforming AA such that when hydrogen abstraction does occur the AA is appropriately arranged to yield PGG_2 (Val349 and L534); and (c) various other active site residues, which individually make lesser but measurable contributions to optimizing catalytic efficiency. The simplifying assumptions that serve as the basis for this categorization are as follows. First, residues, which when altered yield mutant enzymes without cyclooxygenase activity are considered to be critical for positioning C-13 for hydrogen abstraction by Tyr385. Second, AA can exist in three distinct, catalytically competent conformations in native oPGHS-1 which yield PGG_2 (95%), 11-HPETE (2.5%) and 15*R/S*-HPETE (2.5%), respectively [104]; as a first approximation, amino acids residues, mutation of which causes a major change in the composition of the oxygenation products, are considered to be those critical for positioning AA in the conformation that yields PGG_2. These categories are meant to draw attention to those residues that exhibit the most dramatic effects on catalytic constants and product profiles. Mutations of other residues have similar effects, but to lesser degrees, and are discussed in subsequent sections of the text. We also emphasize that in interpreting the results of the mutational analyses, we have in most instances made the simplifying assumption that amino acid substitutions alter only interactions with the AA chain and have largely ignored potential effects on interactions among adjoining residues. This is clearly an

oversimplification which can only be justified broadly by pointing out that most of the mutant enzymes retain both peroxidase or cyclooxygenase activities; however, subtle changes in inter-residue interactions brought about by various mutations certainly may have important effects on kinetic properties and product compositions. Future studies on the crystal structures of AA complexed with mutant forms of oPGHS-1 will be necessary to help resolve the relative influences of mutations on interactions with AA versus those with neighboring amino acid residues.

Residues essential for positioning C-13 of AA for hydrogen abstraction (Tyr348).

Tyrosine 348. Examination of the crystal structures of oPGHS-1 complexed with nonsteroidal anti-inflammatory drugs [80, 99] and the Co^{3+}-heme oPGHS-1/AA complex (Figure 18; [66]) suggests that Tyr348 may be involved in the appropriate positioning of Tyr385 and/or AA. The distance between the phenolic oxygens of Tyr348 and Tyr385 suggest that there is a hydrogen bond between these two atoms that is important for positioning Tyr385; however, there are no hydrophobic contacts between Tyr348 and Ty385. The CE2 phenyl ring carbon of Tyr348 is within van der Waals distance of C-12, C-13 and C-14 of AA suggesting that hydrophobic interactions between Tyr348 and substrate could be important in positioning C-13 of AA. Our results indicate that these latter contacts between Tyr348 and AA are essential in positioning C-13 of AA but that there is no functionally important hydrogen bonding between Tyr348 and Tyr385.

Results which argue against a significant hydrogen bonding interaction between Tyr348 and Tyr385 are as follows. Previous studies have established that substitution of Tyr348 with phenylalanine has little effect on either the cyclooxygenase or peroxidase activity of the enzyme [74, 75, 95]. A more detailed kinetic analysis of Y348F oPGHS-1

indicates that the V_{max}/K_M is 60% of that of native oPGHS-1 and that there are no significant differences in product formation between native and Y348F oPGHS-1 (Table V). Additionally, Y348F oPGHS-1 forms a tyrosyl radical with properties similar to that of native oPGHS-1 [74,75].

The concept that Tyr348 is essential for positioning AA comes from comparing results obtained with native oPGHS-1 and the Y348F and Y348L mutants (Table V). Y348F oPGHS-1 has properties similar to those of native enzyme. In contrast, Y348L oPGHS-1 lacks cyclooxygenase activity while retaining 9% native peroxidase activity; no cyclooxygenase products were detected with Y348L oPGHS-1 even with a sensitive radio thin layer chromatographic assay using [1-^{14}C] arachidonic acid as the substrate. Examination of the Co^{3+}-heme oPGHS-1/AA complex (Figure 18; [66]) suggests that substituting leucine at position 348 would permit C-13 of AA to move away from Tyr385 increasing the distance between Tyr385 and C-13 such that hydrogen abstraction could not occur. There may also be significant pi bond interactions between the phenyl group of Tyr348 and the substrate which are eliminated in the substitution with a leucine residue. The drop in peroxidase activity for this mutant also suggests the possibility of structural changes affecting the peroxidase as well as the cyclooxygenase active site. Tyr348 lies directly below Tyr385 which resides in one of the helices contacting the heme group of the peroxidase active site. Substitution of Tyr348 with a leucine could create a space which might allow movement of this helix and perturbation of the peroxidase active site and could eliminate critical pi bond interactions holding the substrate in a catalytically favorable configuration. Thus, we conclude that the phenyl

ring of Tyr348 is essential in positioning C-13 of AA with respect to Tyr385 but could play a role in the preservation of the structural integrity of the peroxidase site as well.

A Y348W mutation disrupted both peroxidase and cyclooxygenase functions; addition of 15*S*-HPETE to a reaction mixture containing AA and Y348W oPGHS-1 failed to activate cyclooxygenase activity. The lack of peroxidase activity observed with the Y348W mutant suggests that this substitution has an effect on the peroxidase active site. We speculate that when a tryptophan group is inserted at position 348, it causes a change in the position of Trp387 and/or Tyr385 which, in turn, leads to movement of His388, the proximal heme ligand, and results in a change in the binding of the heme group so that peroxidase catalysis does not occur.

Residues critical for conforming AA such that when hydrogen abstraction does occur the AA is appropriately arranged to yield PGG_2 (Val349, Leu534).

Valine 349. One of the methyl groups (CG1) of Val349 lies within van der Waals distance of both C-3 (3.36 Å) and C-4 (3.14 Å) of AA in the AA/Co^{3+}-heme oPGHS-1 crystal structure (Figure 18; [66]). To assess the role of Val349 in cyclooxygenase catalysis, we substituted this residue with alanine, serine, threonine and leucine and evaluated these mutants. All of these mutant proteins were expressed at comparable levels in COS-1 cells (Figure 19). All of the mutants retained oxygenase and peroxidase activity. The catalytic efficiencies (V_{max}/K_M values) for the oxygenase reaction ranged from 6-60% of native oPGHS-1 (Table V).

Lipid soluble products synthesized from [1-^{14}C] arachidonic acid by sham-transfected COS-1 cells and COS-1 cells transfected with native oPGHS-1 or various oPGHS-1 mutants were separated by thin layer chromatography (Figure 20) or RP-HPLC

and the amounts of each product quantified (Table V). Mutants in which Val349 was replaced with a smaller residue (i.e. V349A, V349S and V349T oPGHS-1) all produced an abundance of 11-HETE versus PGG_2-derived products and little or no detectable 15-HETE; the 11-HETE/PGG_2 ratio for these mutants increased as the size of the group at position 349 was decreased. In contrast, as reported previously [104], replacing Val349 with a larger leucine residue (V349L oPGHS-1) led to the generation of a relative abundance of 15-HETE and only trace amounts of 11-HETE. Sham-transfected COS-1 cells did not transform [1-^{14}C]arachidonic acid to products.

Chiral HPLC analysis of 11-HETEs formed from arachidonic acid by V349A, V349S and V349T oPGHS-1 mutants established that the product was exclusively 11*R*-HETE. A representative chromatographic profile for the chiral analysis of the 11-HETE formed by the V349A oPGHS-1 mutant is presented in Figure 21. As reported previously, the V349L oPGHS-1 mutant formed a 65/35 mixture of 15*S*/*R*-HETE, the same enantiomeric profile obtained with native oPGHS-1 [104].

We also examined the interaction of various cyclooxygenase inhibitors with the V349A and V349L mutants. IC_{50} values for cyclooxygenase inhibition were determined for docosahexaenoic acid (22:n-3), flurbiprofen and flufenamic acid. Inhibition was studied using the O_2 electrode assay with 50 μM AA as substrate. The V349A mutant was more sensitive than native oPGHS-1 to inhibition by both flurbiprofen (IC_{50} values of 0.28 μM and 2.9 μM, respectively) and flufenamic acid (IC_{50} values of 0.9 μM and 6.0 μM, respectively); the substrate analogue, docosahexaenoic acid was a less potent inhibitor of the V349A mutant than the native enzyme (IC_{50} values of 350 μM vs. 100 μM, respectively).

Flurbiprofen and flufenamate have similar structures, including two phenyl rings and a carboxylate moiety. The carboxyl groups of flurbiprofen and other carboxylate-containing NSAIDs such as iodosuprofen and iodoindomethacin all form salt bridges with Arg120 at the mouth of the cyclooxygenase active site [80, 96], and the structurally similar flufenamate is thought to be bound in the same manner. Examination of the flurbiprofen-bound crystal structure of oPGHS- shows extensive van der Waals contacts between the CG1 of Val349 and the lower phenyl ring of flurbiprofen [80]. Substitution of Val349 with an alanine results in more potent inhibition by both flurbiprofen and flufenamate, suggesting that the contacts made by Val349 are preventing the binding of these inhibitors in the most thermodynamically stable conformation. Thus, additional space created by V349A seems to create a cyclooxygenase active site which can better accommodate the extra bulk of the 2-phenyl inhibitor compounds. In contrast, doxosahexaenoic acid (22:6n-3) is structurally similar to the substrate, arachidonic acid, and was a somewhat less potent inhibitor of the V349A oPGHS-1 mutant compared with the native enzyme. 22:6 is a relatively poor inhibitor of native oPGHS-1 (IC_{50} = 100μM) and an even less potent inhibitor of PGHS-2 which is known to have a slightly larger and more accommodating active site [98]. Because it is elongated and contains two extra double bonds compared with arachidonic acid, 22:6 may be too long and rigid to thread all the way into the cyclooxygenase active site, making it a poor binder. Val349 makes multiple contacts at the carboxyl end of arachidonate (Figure 18, Table III) and would be expected to contact the same region of 22:6. Stabilization of 22:6 apparently relies somewhat on Val349 contacts, removal of which creates a larger active site which further reduces the already poor binding affinity of this inhibitor.

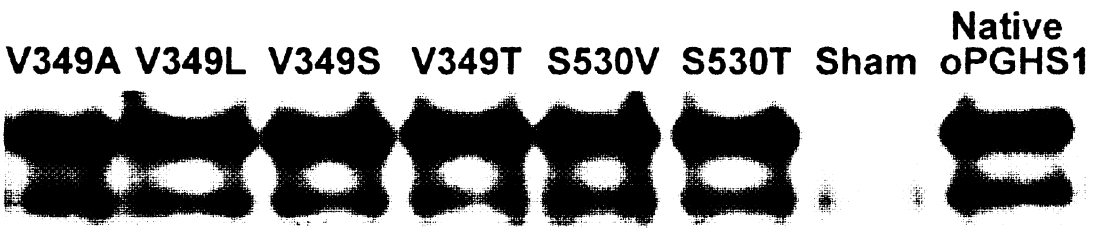


Figure 19. Western blot analysis of Val349 oPGHS-1 mutant proteins. Cell protein (5 μg) from COS-1 cells transfected with native or mutant oPGHSs-1 was resolved by SDS-PAGE, transferred to nitrocellulose and visualized using a polyclonal antibody raised against oPGHSs-1 as described in the text under Methods. Densitometric analysis was performed to determine relative levels of protein expression.

We reported previously that AA can assume at least three catalytically competent arrangements in the cyclooxygenase active site of oPGHS-1 [104]. These arrangements, occurring at the time of hydrogen abstraction, lead to different products--PGG_2, 11*R*-HPETE or 15*R/S*-HPETE. With native oPGHS-1 the kinetically most favorable arrangement of AA is that which yields PGG_2. With V349A oPGHS-1 the arrangements yielding PGG_2 and 11-HPETE appear to be equally favorable. 11-HPETE would be expected to be formed from an arrangement of AA in which C-9 and C-11 are slightly misoriented such that the endoperoxide bridge between these carbons cannot be formed. The kinetics of both PGG_2 and 11-HPETE formation were found to be similar to one another with the V349A mutant (Figure 22), and the V_{max}/K_M ratios are similar for V349A and native oPGHS-1 (Table V). These observations suggest that the valine to alanine substitution does not appreciably influence the positioning of C-13 with respect to Tyr385; moreover, formation of both PGG_2 and 11-HPETE presumably proceeds in the same way via abstraction of the 13pro*S* hydrogen and formation of an 11-hydroperoxyl radical [168]. Accordingly, we suggest that Val349 plays a major role in positioning the carboxyl half of AA and especially in positioning C-9 without appreciably affecting the location of the ω half of AA within the cyclooxygenase site. Obviously, the effect of Val349 on the position of C-9 must occur indirectly because Val349 contacts only C-3 and C-4 of the substrate (Figure 18). Placing smaller amino acids at position 349 apparently permits the carboxyl half of AA greater flexibility, which in turn, translates into a small shift in the orientation of C-9 with respect to C-11.

Leucine 534. The CD1 and CD2 methyl groups of Leu534 are within van der Waals distance of C-15 (3.54 Å), C-16 (3.6 Å) and C-18 (3.91 Å) of AA in the

AA/Co^{3+}-heme oPGHS-1 crystal structure [66]. Interestingly, substitution of Leu534 with either alanine or valine yields mutant enzymes which produce large amounts of 15-HETE (Table V) and the 15-HETE which is produced is almost exclusively (≥95%) 15*S*-HETE (Figure 23); in contrast, with native oPGHS-1 15*S*-HETE comprises 65% of the total 15-HETE. Apparently, having a hydrophobic residue smaller than leucine at position 534 enlarges the cyclooxygenase active site near C-15 providing for relatively greater access of O_2 antarafacial to the site of hydrogen abstraction. Thus, Leu534 is important in facilitating formation of PGG_2 versus 15-HPETE. 15*S*-HETE is the only 15-HETE formed by native human PGHS-2 [155], and the cyclooxygenase site of PGHS-2 is somewhat larger and more accommodating than that of PGHS-1 [137].

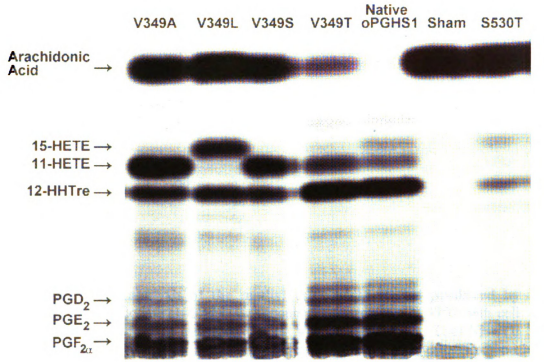
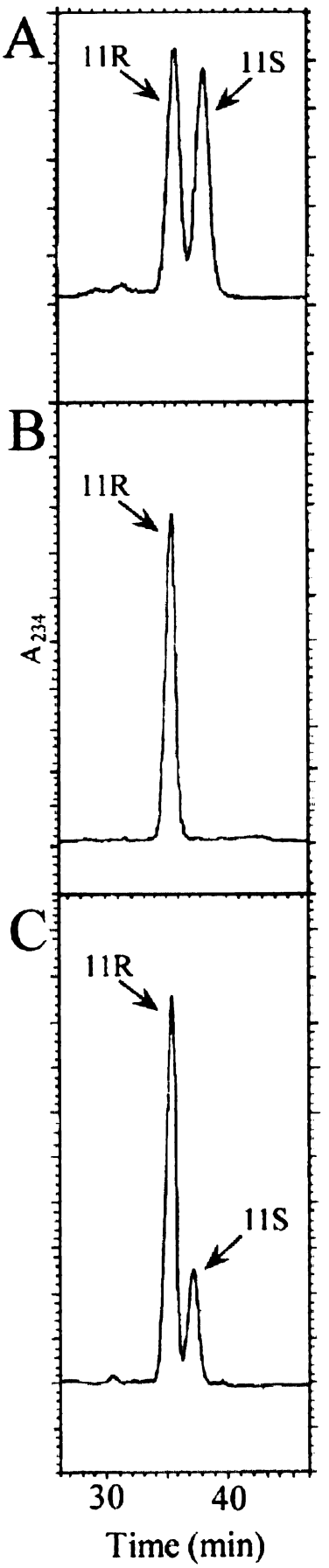


Figure 20. Thin layer chromatogram of products formed from [1-^{14}C]arachidonic acid by native and mutant oPGHS-1 enzymes. Cell protein (250 μg) from COS-1 cells transfected with native or mutant oPGHSs-1 was incubated for 10 min with 35 μM [1-^{14}C]arachidonic acid, and the products were extracted, separated by thin layer chromatography and visualized by autoradiography as described in Methods. The appearance of variability of various product levels between samples shown in the figure is not representative of relative V_{MAX} values among enzymes (determined by oxygen electrode measurements (Table V)) because of the variability in extraction efficiencies among the samples. The locations of the various chromatographic standards are as noted.

Figure 21. Chiral HPLC analysis of the methyl ester of 11-HETE produced by V349A oPGHS-1. Arachidonate (100 μM) was incubated for ten min at 37°C with cell protein (1 mg) from COS-1 cells transfected with V349A oPGHS-1. The11-HETE fraction was isolated by RP-HPLC and esterified by treatment with diazomethane. Chiral-phase HPLC of the methyl ester was performed as described in Materials and Methods using a Chiralcel OC column with hexane/2-propanol (98:2; v/v) as the solvent and a flow rate of 0.5 ml/min. The UV detector was set to monitor absorbance at 234 nm. A. 11(*R/S*)-HETE methyl ester standard; B. 11-HETE methyl ester derived from incubation of V349A oPGHS-1 with arachidonate; C. coinjection of the 11(*R/S*)-HETE methyl ester standard and the 11-HETE methyl ester from incubation of V349A oPGHS-1 with 100 μM arachidonate.


A
11R
11S
B
11R
A234
C
11R
11S
30
40
Time (min)

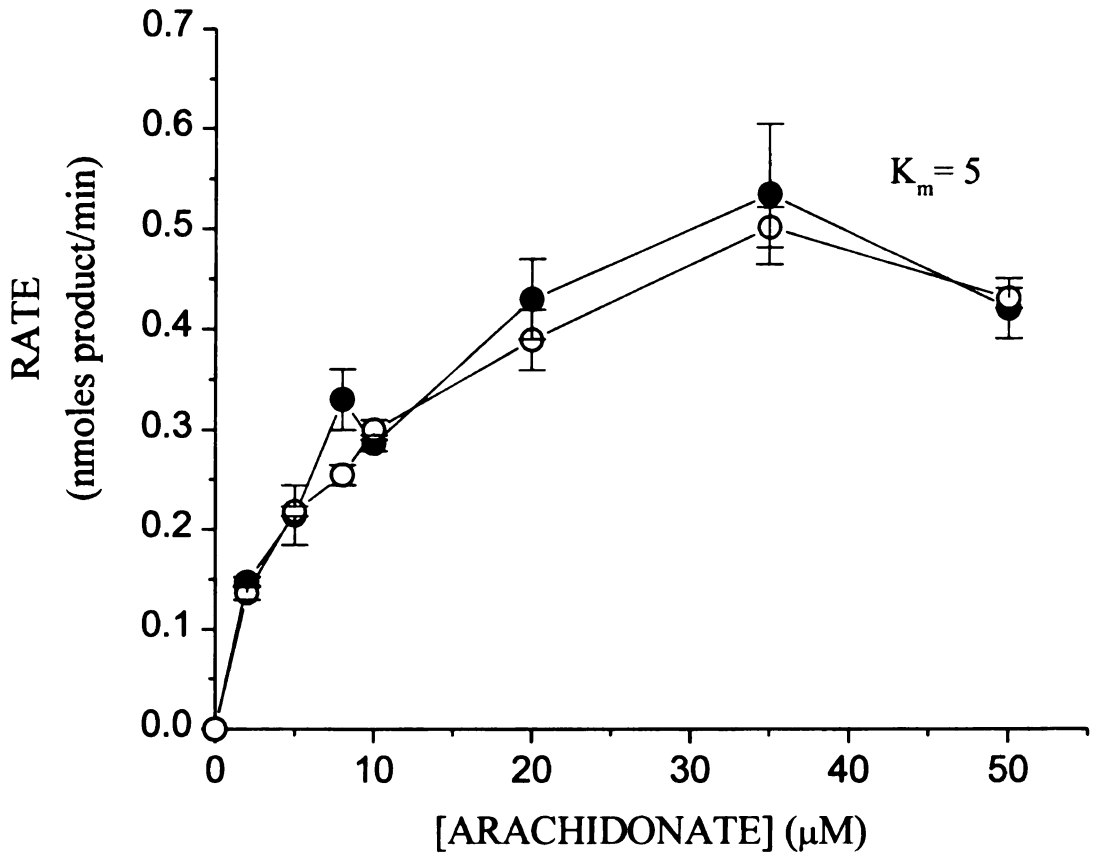
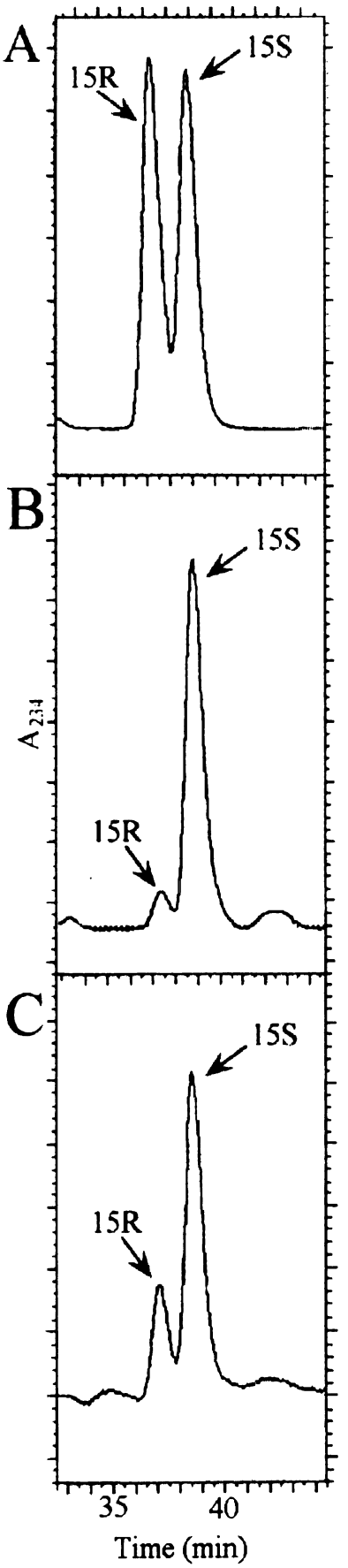


Figure 22. Effect of arachidonic acid concentration on the formation of PGG_2 and 11-HETE by V349A oPGHS-1. V349L oPGHS-1 (75μg of protein from transfected COS-1 cells) was incubated with the indicated concentrations of arachidonic acid at 37°C for one min. Products were analyzed by radio thin layer chromatography as described in Methods. Radioactive products were scraped from TLC plates, quantitated using liquid scintillation counting and normalized for the relative extraction efficiencies among samples. Results are from an average of two separate experiments. K_m values determined from 1 minute incubations with arachidonate and TLC quantitation were the same, within experimental error, as those determined repeatedly from rates calculated from O_2 electrode measurements; however, because of the phenomenon of suicide inactivation, the oxygenase rates reported here are somewhat less than those which would be obtained by measurements of O_2 consumption. PGG_2-derived products (●) and 11-HETE(○).

Residues which optimize cyclooxygenase catalysis (Phe205, Phe209, Phe381, Ser353, Ile377, Ser530 and Leu531).

Phenylalanine 205, Phenylalanine 209, and Phenylalanine 381. Various phenyl ring carbons of Phe205, Phe209 and Phe381 lie within van der Waals distance of C-14 through C-20 of AA (Figure 18, Table III; [66]). Leucine substitutions of Phe209, Phe205 and Phe381 reduce the V_{max} for AA oxygenation somewhat but have little effect on the K_M for AA or on the product distribution (Table V). Alanine substitutions at positions 205, 209 and 381 also have relatively little impact on K_M values but reduce the V_{max} for AA oxygenation to a greater degree than observed for leucine substitutions, particularly for F381A which has a V_{max} of only 4% of the native enzyme, and further, cause a modest change in the product profile (i.e. relatively small increases in 11-HPETE formation). Examination of the crystal structure indicates that the edge of the phenyl ring of Phe205 makes four van der Waals contacts with C-14, C-15 and C-18 and that one face of the phenyl ring of Phe209 makes six contacts with C-17, C-18 and C-19. However, removal of these contacts has only modest effects on PGHS catalysis (Table III). The crystal structure predicts that C-14, C-15, C-17 and C-18 of AA are each contacted by at least one other active site residue (Val344, Tyr348 and/or Leu534 (Table III)). Apparently, these latter interactions are (a) relatively more important functionally than the contacts made by either Phe205 or Phe209 and/or (b) compensate for the loss of these contacts in the mutant proteins containing substitutions of either Phe205 or Phe209. For instance, as described above, Tyr348 makes van der Waals contacts with C-14 of AA, and elimination of this contact with a leucine substitution results in a complete loss of cyclooxygenase activity. Thus, the Tyr348 contact with C-14 appears to be a major

Figure 23. Chiral HPLC analysis of the methyl ester of the 15-HETE produced by L534A oPGHS-1. The15-HETE fraction from L534A was analyzed as described in Figure 21. A. 15(*R/S*)-HETE methyl ester standard; B. 15-HETE methyl esters from incubation of L534A oPGHS-1 with arachidonate; C. coinjection of the 15(*R/S*)-HETE methyl ester standard and the 15-HETE methyl esters from the incubation of L534A oPGHS-1 with 100 μM arachidonate.


A
15R
15S
B
15S
A_{234}
15R
C
15S
15R
35
40
Time (min)

contributor to substrate positioning and stabilization at this point in the AA molecule whereas the C-14 contact made by Phe205 is relatively unimportant. Also, there are 34 contacts between active site residues and the methyl half of AA compared with 16 contacts with the carboxyl end; thus, eliminating a contact with the methyl end of AA may be relatively less important than eliminating a contact with the carboxyl half of AA. The positioning of AA seems to be most critically dependent on having enough space at the methyl end of the substrate (e.g. Gly533) to permit the correct positioning of the 13-pro*S* hydrogen with respect to Tyr385.

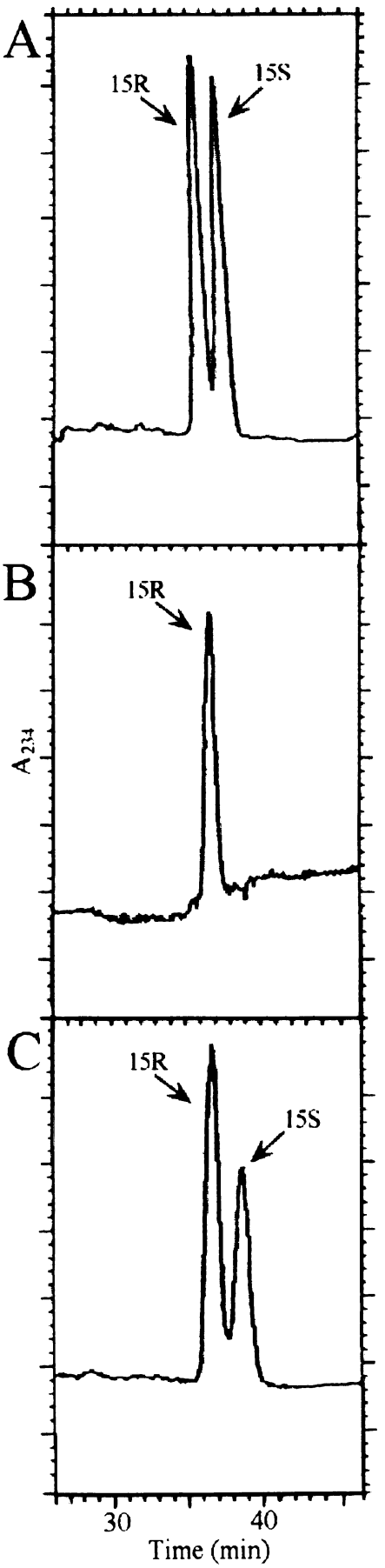
Serine 353. The Cβ carbon of Ser353 makes a van der Waals contact with C-3 of AA at the side opposite the C-3 and C-4 contacts made by Val349 (Figure 18, Table III; [66]). When this residue was mutated to glycine, alanine or threonine, all of the mutants retained substantial cyclooxygenase activity. The products formed by the S353G and S353A oPGHS-1 mutants were the same as those formed by native oPGHS-1; S353T oPGHS-1 formed larger amounts of both 11- and 15-HETE. A V349A/S353T double mutant, designed to remove all contacts between C-3 and C-4, had only 3% of the cyclooxygenase activity of the native enzyme compared with the single mutants which had 55% (V349A) and 42% (S353T) of native activity. This suggests that a C-3 contact is particularly important, albeit indirectly, in positioning C-13 with respect to Tyr385.

Isoleucine 377. Ile377 lies at the distal end of the cyclooxygenase active site channel with one of its delta carbons within 3.7 Å of C-20 of AA (Figure 18, Table III; [66])). Substitution of Ile377 with a smaller valine residue yields a mutant enzyme with kinetic properties very similar to native oPGHS-1. Substitution of a valine at this position will result in the removal of the delta carbons and increase the distance between

this residue and C-20 of AA minimally to 5.2 Å, a distance too great to support van der Waals interactions. This indicates that the contact made by Ile377 makes little contribution to either substrate binding or positioning of C-13 with respect to Tyr385, but does not address the possible necessity for a space-filling residue at this position. I377V oPGHS-1 forms somewhat more 11-HETE than native oPGHS-1 (Table III) suggesting that this residue does play a small role in orienting C-11 with respect to C-9.

Serine 530. Ser530 is the site of acetylation of oPGHS-1 by aspirin [21, 99], whereby cyclooxygenase activity is completely eliminated. In the AA/Co^{3+}-heme oPGHS-1 crystal structure the Cα and Cβ carbons of Ser530 make van der Waals contacts with C-10 and C-16 (Figure 18; [66]); these contacts are on the side of AA directly opposite that Tyr385. Earlier studies had shown that substitution of Ser530 with alanine has relatively little effect on AA oxygenation, that replacement of Ser530 with threonine causes a 95% drop in catalytic efficiency, and that replacement of Ser530 with glutamine prevents catalysis apparently by blocking substrate access to the Tyr385 radical [21, 156]. Here we have extended these findings showing (a) that a S530V mutant is catalytically inactive (Table V) and (b) that the S530T mutant forms significant amounts of 15*R*-HETE but not 15*S*-HETE (Figure 24). Recent studies have indicated that aspirin-acetylated PGHS-2 forms exclusively 15*R*-HPETE [108, 155] and that formation of this product involves removal of the 13-pro*S* hydrogen [168]. Assuming that 15*R*-HPETE synthesis by S530T oPGHS-1 occurs in a similar manner, this would imply that 15*R*-HPETE formation involves suprafacial addition of O_2 to C-15. Observation of Figure 18 suggests that a residue slightly larger than serine at position 530 might be expected to block antarafacial O_2 addition without preventing abstraction of

Figure 24. Chiral HPLC analysis of the methyl ester of 15-HETE produced by S530T oPGHS-1. The 15-HETE fraction produced by S530T was analyzed as described in Figure 21. A. 15(*R/S*)-HETE methyl ester standard; B. 15-HETE methyl ester from incubation of S530T oPGHS-1 with arachidonate; C. coinjection of the 15(*R/S*)-HETE methyl ester standard and the 15-HETE methyl ester from incubation of S530T oPGHS-1 with 100 μM arachidonate.


A
15R
15S
B
15R
A_{234}
C
15R
15S
30
40
Time (min)

the 13-pro*S* hydrogen. Moreover, a slightly larger valine residue would be expected to narrow the cyclooxygenase channel so that C-13 would be mispositioned with respect to Tyr385 such that hydrogen abstraction could not occur.

Leucine 531. Leu531 is present in the first shell of the cyclooxygenase site in close proximity to Arg 120 on the side opposite the carboxylate group of AA and outside of van der Waals contact distance from the carbon skeleton of AA (Figure 18). Substitutions of Leu531 with similarly sized, hydrophilic residues (Asp and Asn) decreased the V_{max} values for AA but with little effect on the K_M's [156]. In contrast, replacement of Leu531 with alanine caused a 25 fold increase in the K_M. The relative amounts of oxygenated products formed with Leu531 mutants are similar to those formed with the native enzyme (Table V). Apparently, replacement of Leu531 with hydrophilic residues results in stable, however, improper liganding of the substrate carboxylate to the enzyme, resulting in a decreased V_{max} and slight mispositioning of C-13 of arachidonate with respect to Tyr385. Reduction of the residue size at position 531, however, is damaging to high affinity substrate binding of the substrate carboxylate to the enzyme, supporting the notion that proper AA binding to Arg120 is critical for PGHS-1 activity and even modest strutural changes in the immediate environment are not tolerated.

Summary. Nineteen residues in the cyclooxygenase channel of oPGHS-1 are predicted to make contacts with arachidonic acid (Figure 18, Table III; [66]). Three of the residues critical in permitting oPGHS-1 to convert arachidonic acid to PGG_2 are discussed in this section. Tyr348 is involved in hydrophobic interactions with AA that are necessary to position C-13 appropriately with respect to Tyr385. Without the interaction between the Tyr348 phenyl ring and AA, catalysis cannot occur at all. Val349 and

Leu534 provide hydrophobic interactions with AA which contribute, not primarily to initiation of catalysis, but to positioning AA such that when hydrogen abstraction occurs the fatty acid molecule is optimally aligned to yield PGG_2 rather than monohydroperoxide products. Val349 stabilizes the carboxyl half of AA to promote proper positioning of C-9 with respect to C-11 for efficient endoperoxide formation and Leu534 provides steric hindrance which blocks premature oxygenation at C-15. The remaining residues comprising the cyclooxygenase active site channel provide measurable but lesser contributions to optimizing catalysis.

CHAPTER IV

THE FUNCTIONS OF CYCLOOXYGENASE ACTIVE SITE RESIDUES IN SUBSTRATE SPECIFICITY OF PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-1

Introduction

Prostaglandin endoperoxide H synthases (PGHSs) catalyze the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) [2, 3, 137]. This is the committed step in the biosynthesis of prostaglandins and thromboxanes. These compounds are local hormones that act at or near their sites of synthesis to coordinate intercellular responses evoked by circulating hormones and perhaps intracellular events associated with cell replication and differentiation [137]. There are two PGHS isozymes designated PGHS-1 and PGHS-2 (or cyclooxygenase-1 and -2 (COX-1 and -2)). PGHS-1 and -2 are often referred to as the constitutive and inducible forms of the enzyme, respectively.

These isozymes are closely related structurally [66, 80-82] and mechanistically [3, 137, 169] although there are some subtle kinetic differences between the two isoforms with respect to hydroperoxide activator requirements [170, 171] and substrate [115] and inhibitor [20, 172] specificities. Both PGHS isozymes catalyze two different reactions–a cyclooxygenase reaction in which arachidonate is converted to an intermediate prostaglandin endoperoxide PGG_2 and a peroxidase reaction in which the 15-hydroperoxyl group of PGG_2 is reduced to an alcohol yielding PGH_2 [2, 3, 137]. Although the peroxidase activity can function independently of the cyclooxygenase activity [56], activation of the cyclooxygenase requires a functional peroxidase.

X-ray crystallographic studies indicate that the cyclooxygenase reaction occurs in a hydrophobic channel that protrudes from the membrane binding domain of the enzyme into the core of the globular domain [66]. The fatty acid substrate is positioned in this site in an extended L-shaped conformation. Cyclooxygenase catalysis begins with abstraction of the 13-pro*S* hydrogen from arachidonate in the rate determining step to generate an arachidonate radical [61, 70]. A tyrosyl radical positioned on Tyr385 abstracts this hydrogen from the substrate fatty acid [3, 70, 95, 137]. The tyrosyl radical is formed as a result of oxidation of the heme group at the peroxidase site on the enzyme [3, 137, 169].

Most studies of the cyclooxygenase have utilized arachidonate as the substrate. Although arachidonate is the best substrate, both enzymes will oxygenate n-3 and n-6 and C_{18} and C_{20} fatty acids *in vitro* with catalytic efficiencies in the range of 0.05-0.7 of that of arachidonate [173]. These substrates including 9,12-octadecadienoate (18:2n-6) , 8,11,14-eicosatrienoate (20:3n-6) and 5,8,11,14,17-eicosapentaenoate (20:5n-3) are also oxygenated via cyclooxygenase activity when added exogenously to intact cells [118, 119] or when mobilized from cellular phosphoglycerides [120-123, 174] (Figure 25). In tissues such as vesicular gland which have low levels of Δ^5 desaturase activity and thus low levels of arachidonate, 20:3n-6 is converted efficiently to the 1-series prostanoid products found in semen [121]. Other less common fatty acids can also serve as cyclooxygenase substrates including adrenic acid (22:4n-6) [131], the *Mead* acid 5Z,8Z,11Z_eicosatrienoic acid [158], columbinic acid (5E,9Z,12Z_octadecatrienoic acid) [133, 134], and 5,6-oxido-eicosatrienoic acid [132, 135].

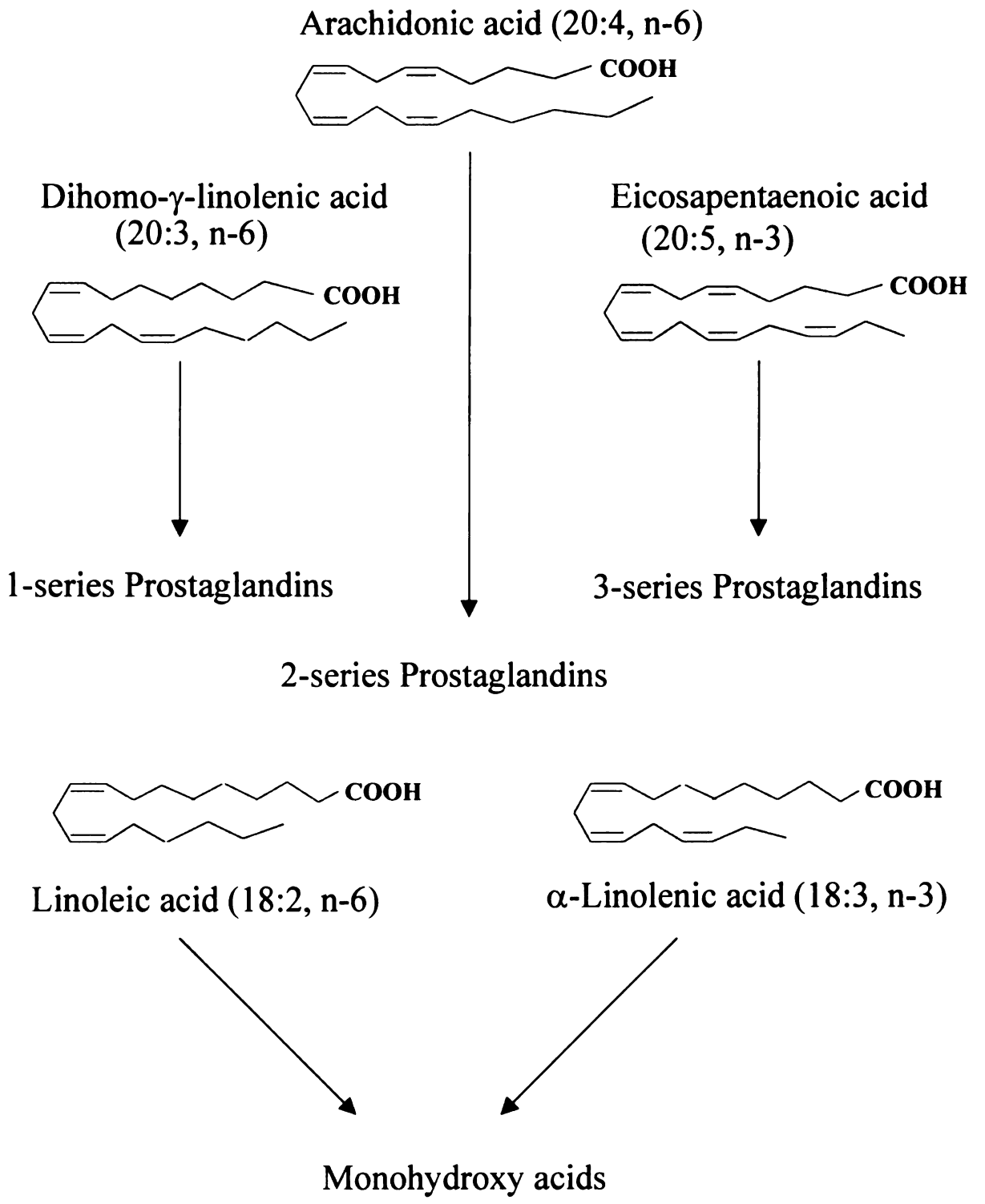


Figure 25. Structures of and products formed from various n-6 and n-3 fatty acid substrates of PGHSs

Substrates other than arachidonate typically have K_M values comparable to those of arachidonate and compete with arachidonate for the cyclooxygenase active site thereby inhibiting arachidonate oxygenation. This was first documented by Lands and coworkers *in vitro* [114] but this form of inhibition appears to occur *in vivo* as well [126, 127, 175]. Here we report studies of PGHS-1 that were designed to identify active site residues which are determinants of cyclooxygenase fatty acid substrate specificity.

Materials and Methods

Materials. Fatty acids were purchased from Cayman Chemical Co., Ann Arbor, MI. [l-^{14}C] arachidonic acid (40-60 mCi/mmol), [l-^{14}C]homo-γ-linolenic (8,11,14-eicosatrienoic acid) (50-60 mCi/mmol), [l-^{14}C]linoleic acid (50-60 mCi/mmol) and [1-^{14}C]eicosapentaenoic acid (50-60 mCi/mmol) were from New England Nuclear. Flurbiprofen and soybean lipoxygenase-1 were from Sigma Chemical Company. Restriction enzymes and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO. Calf serum and fetal bovine serum were from HyClone. Primary antibodies used for Western blotting were raised in rabbits against purified oPGHS-1 and purified as an IgG fraction [149] and goat anti-rabbit IgG horseradish peroxidase conjugate was purchased from BioRad. Oligonucleotides used as primers for mutagenesis were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility. All other reagents were 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 Strategene QuikChange mutagenesis kit and the protocol of the manufacturer [149]. Oligonucleotides used in the preparation of various mutants are found in Table II and as follows:

I434V	5'-^{1375}GCCAGCCTGCAGGCCGGGTTGGTGGGGGTAGG-3'
H513R	5'-^{1617}CTTGAGAAGTGTCGACCGAACTCCATCTTTGG-3'
L352V	5'-^{1136}ATGTGCAGCAGGTGAGCGGCTAC 3'

Plasmids used for transfections were purified by CsCl gradient ultracentrifugation and mutations were reconfirmed by double-stranded sequencing of the pSVT7 constructs using Sequenase (ver. 2.0, U.S. Biochemical Corp.) and the protocol described by the manufacturer as described previously.

Transfection of COS-1 cells with oPGHS-1 constructs. COS-1 cells (ATTC CRL-1650) were grown in DMEM containing 8% calf serum and 2% fetal bovine serum, transfected with pSVT7 plasmid constructs containing cDNAs coding for native oPGHS-1 or mutant oPGHS-1 using the DEAE dextran/chloroquine transfection method. Forty hours following transfection, cells were harvested and microsomal membrane fractions prepared as described previously [149]. Protein concentrations were determined using the method of Bradford [165] with bovine serum albumin as the standard. Microsomal preparations were used for Western blotting and for cyclooxygenase and peroxidase assays.

Cyclooxygenase and peroxidase assays. Cyclooxygenase assays were performed at 37°C by monitoring the initial rate of O_2 uptake using an oxygen electrode. Reactions were initiated by adding approximately 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 enzymes was completely inhibited by the addition of 0.2 mM flurbiprofen to the reaction mixture. The addition of exogenous peroxide (5 μM 15*S*-HPETE) to the reaction mixture was necessary to sustain cyclooxygenase activity with eicosapentaenoic acid (EPA) [124]. 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 V_{max} values. K_M values for the different fatty acids were measured using concentrations of fatty acid substrates between 0.5 and 500 μM. Peroxidase activities were measured spectrophotometrically with N,N,N',N'-tetramethylphenylenediamine (TMPD) as the reducing cosubstrate [166] as

reported previously [104]. Reactions were initiated by adding 100 ml of 0.3 mM H_2O_2 and the absorbance at 610 nm was monitored with time.

Western blot analysis. Microsomal samples (ca. 5 μg of protein) were resolved by one-dimensional SDS-PAGE and transferred electrophoretically to nitrocellulose membranes using a Hoeffer Scientific Semi-Dry Transfer apparatus. Membranes were blocked for 12 hr in 3% non-fat, dry milk, 0.1% Tween20 and Tris-buffered saline, followed by a two-hour incubation with a peptide-directed antibody against oPGHS-1 [84] in 1% dry milk, 0.1% Tween-20 and Tris-buffered saline at room temperature. Membranes were washed and incubated for one hr with a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase, after which they were incubated with Amersham ECL reagents and exposed to film for chemiluminescence.

Characterization of fatty acid oxygenation products. A general protocol for product analysis is as follows. Forty hr 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, pH 7.5, containing 1 mM phenol and 6.8 μg bovine hemoglobin in a total volume of 200 μl. Reactions were initiated by adding 1-^{14}C-labelled fatty acid (35 μM final concentration) and were performed with or without 200 μM flurbiprofen and stopped by adding 1.4 ml of $CHCl_3$:MeOH (1:1; v/v). Insoluble cell debris was removed by centrifugation and 0.6 ml of $CHCl_3$ and 0.32 ml of 0.88% formic acid were added to the resulting supernatant. The organic phase was collected, dried under N_2, redissolved in 50 μl of $CHCl_3$ and spotted on a Silica Gel 60 thin layer chromatography plate; the lipid products were chromatographed for 1 h in benzene:dioxane:formic acid:acetic acid

(82:14:1:1; v/v/v/v). Products 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. 15*S*-HPETE was prepared according to Graff [176] with modifications. Arachidonic acid (50 μmol) was suspended in 100 ml of 0.2 M borate buffer, pH 9.0. The reaction was initiated by adding soybean lipoxygenase (50 mg) and the incubation continued for five min at 30°C. The reaction was quenched by adding enough 0.24 M HCl to bring the pH to 3.0 (ca. 20 ml) and extracted three times with an equal volume of ice-cold petroleum ether:ethyl acetate (1:1). Extracts were pooled, dried under a nitrogen stream and resuspended in 1 ml of ethanol. The concentration of 15-HPETE was determined spectrophotometrically by measuring cis-trans conjugated double bond formation (ϵ_{234} = 3 x 10^4 mM^{-1} cm^{-1}) and by measuring peroxide levels using a ferrous sulfate-xylenol orange reagent as described by Gupta [177].

Results and Discussion

Overview. As discussed in the Introduction, both PGHS-1 and PGHS-2 can oxygenate a variety of C_{18} and C_{20} polyunsaturated fatty acids substrates. Arachidonic acid is the preferred substrate for the cyclooxygenase activity of both isozymes (Table VII; [173, 178]). Crystallographic [66] and mutagenic analyses of the interaction of arachidonic acid within the cyclooxygenase active site of ovine (o) PGHS-1 has led to the assignment of active site residues to five functional categories [179]: (a) residues directly involved in abstraction of the 13 pro*S* hydrogen (Tyr385); (b) residues involved in positioning C-13 for hydrogen abstraction (Tyr348 and Gly533); (b) residues essential for high affinity binding of arachidonate (Arg120) and (d) residues critical for positioning arachidonate such that following hydrogen abstraction the arachidonyl radical is converted to PGG_2 (Val349, Trp387 and L534).

In the following paragraphs we describe the effect of active site substitutions on the oxygenation of fatty acids other than arachidonate. Substitutions of most active site residues caused approximately parallel changes in the oxygenation of all fatty acids. However, there were two significant exceptions--Val349 and Leu534.

Val349 interacts with the C-3 and C-4 of arachidonate. Substitution of Val349 with alanine caused 175-1000 fold decreases in the efficiencies (V_{max}/K_M) of oxygenation of 18:2n-6, 20:3n-6, and 20:5n-3 with only a 35% decrease in the efficiency of arachidonate oxygenation; kinetic analyses indicate that the large decrease in V_{max}/K_M values for 20:3n-6 with the Val349Ala mutant results from mispositioning of C-13 for hydrogen abstraction. The crystal structure of a 20:3n-6/Co^{3+}-heme oPGHS-1 complex

has been determined and compared to that of the 20:4n-6/Co^{3+}-heme oPGHS-1 complex[2]. The regions involving C-3 to C-7 are differently positioned for arachidonate versus eicosatrienoate and suggest that Val349, which interacts with both substrates in this region of the carbon chain, is particularly critical in positioning the carboxyl half of eicosatrienoate in a way that provides for proper alignment of C-13 for hydrogen abstraction by the Tyr385 radical. A L534V oPGHS-1 mutant oxygenated C_{20} but not C_{18} substrates; Leu534 interacts with the ω end of C_{20} substrates (i.e. C-15, C-16 and C-18 of arachidonic acid [66] and dihomo-γ-linolenic acid) and thus this residue appears to be particularly important for the positioning and oxygenation of C_{18} substrates. All mutant enzymes were expressed at protein levels similar to that of the native enzyme and retained >50% the peroxidase activity of native oPGHS-1.

Valine 349. Val349 plays an important role in positioning arachidonate for PGG_2 formation [104]; substitutions with smaller residues lead to substantial increases in the formation of 11-HPETE versus PGG_2 whereas substitution of Val349 with a larger leucine residues leads in increases in 15-HPETE synthesis. For example, V349A oPGHS-1 forms 55% 11-HPETE from arachidonate versus 2.5% 11-HPETE by native oPGHS-1. In contrast substitutions of Val349 with either alanine or leucine caused less than two-fold increases in hydroperoxy acid formation from [1-^{14}C]dihomo-γ-linolenic acid and produced oxygenated products in the same ratios as native enzyme when tested with [1-^{14}C]eicosapentaenoic acid or [1-^{14}C]linoleic acid (data not shown). However, Val349 appears to be critical for the oxygenation of substrates other than arachidonate. As shown in Table VII the oxygenation of linoleic (18:2n-6), eicosadienoic (20:2n-6),

[2] Personal communication, M.G. Malkowski and R.M. Garavito

dihomo-γ-linolenic (20:3n-6), eicosapentaenoic (20:5n-3) α-linolenic (18:3n-3) acids by native oPGHS-1 occurs with 0.6-50% of the efficiencies (V_{max}/K_M values) observed with arachidonate. Substitutions of Val349 led to dramatic decreases in the V_{max}/K_M values for substrates other than arachidonate. For example, while V349L oPGHS-1 was six fold less efficient than native oPGHS-1 in the oxygenation of arachidonate, this mutant showed a 350 fold decline in the V_{max}/K_M value with 18:2n-6 as the substrate. V349A oPGHS-1 oxygenates arachidonate with a catalytic efficiency (V_{max}/K_M) that is 65% that of native oPGHS-1. However, the V_{max}/K_M value for the V349A oPGHS-1 mutant was nearly 200 fold less when tested with 18:2n-6, 1000 fold less when tested with 20:5n-3, and >800 fold less when tested with 20:3n-6 (Table VII). The only difference between 20:3n-6 and 20:4n-6 is the Δ^5 double bond. Val349 has been shown previously to make van der Waals contacts with C-3 and C-4 of the arachidonate molecule [66]. Removal of these contacts by substitution of an alanine at position 349 does not significantly decrease the enzyme's catalytic efficiency in the oxygenation of arachidonate (20:4n-6). The crystal structure of oPGHS-1 bound with dihomo–γ-linolenic acid (20:3n-6) shows a structure in which the carboxyl half of the substrate is stretched due to the loss of the C-5--C-6 double bond. This is the area which is in direct contact with Val349 at C-5 (Table VI). Val349 and Ile523 both make contacts at C-5, but from opposites sides of the substrate. Removal of the C-5 contact made by Ile523 by replacement with an alanine has relatively little effect on the oxygenation of 20:3 (V_{max} is ~ 60% that of native enzyme) while removal of the C-5 contact made by Val349 decreases 20:3 oxygenation dramatically (V_{max} is only 4% that of native enzyme and the V_{max}/K_M is reduced by >800 fold) (Table VII). With the removal of the CG1 Val349 contact, the lower portion of

Table VI. Contacts between Dihomo-γ-linolenic acid (DHLA) and Cyclooxygenase Active Site Residues*

Van der Waals and hydrogen bond interactions were calculated using CHAIN [167]. Van der Waals contacts within 4Å are listed. See list of abbreviations for carbon designations.

Residue	Atom	Residue	Atom	Distance (Å)
Phe 205	CE2	DHLA	C15	3.6
	CZ		C18	3.9
Phe 209	CE1	DHLA	C19	3.9
	CE2		C19	3.9
	CZ		C17	3.9
	CZ		C18	3.9
	CZ		C19	3.6
Tyr 348	CD2	DHLA	C14	3.9
	CE2		C12	3.6
	CE2		C13	4.0
	CE2		C14	3.4
Val 349	CG1	DHLA	C5	3.4
Leu 352	CD2	DHLA	C6	3.8
	CD2		C7	3.2
	CD2		C11	3.3
	CD2		C12	3.9
Phe 381	CE2	DHLA	C17	3.8
	CZ		C16	3.9
	CZ		C17	3.5
Ile 523	CA	DHLA	C8	3.9
	CG2		C3	3.3
	CG2		C4	3.3
	CG2		C5	3.7
	CG2		C6	3.2
	CG2		C7	3.9
	CG2		C8	3.9
Ser 530	CA	DHLA	C16	3.3
	CA		C17	3.9
	CB		C10	3.6
	CB		C13	3.6
	CB		C15	4.0
	CB		C16	3.1
Leu 534	CD1	DHLA	C18	3.6
	CD2		C15	3.8
	CD2		C16	3.7
	CD2		C18	3.9

*Personal communication from M.G. Malkowski and R.M. Garavito.

Table VII. Kinetic Properties for Oxygenation of Various Fatty Acids by Native and Mutant PGHS-1 and PGHS-2

Oxygenase activity was measured with an oxygen electrode as described in the text. Values are calculated for fatty acid turnover and are corrected for the percentage of mono- and bis-oxygenated products formed. A value of 100% is assigned for oxygenase activity of native PGHS-1 and -2 with arachidonic acid. V_{max} and K_M values represent the means from a minimum of four separate determinations with standard deviations within 10% of all values reported. Relative V_{max} values reported for mutant enzymes for which K_M values were not determined represent rate measurements performed using 100 μM fatty acid substrate. V_{max} (%) for native oPGHS-1 with 20:2 (n-6) was found to be 15%. ND, not determined.

	Arachidonic Acid 20:4 n-6			**Dihomo-γ-linolenic Acid 20:3 n-6**			**Eicosapentaenoic Acid 20:5 n-3**			**Linoleic Acid 18:2 n-6**			**α-Linolenic Acid 18:3 n-3**		
ENZYME	V_{max} (%)	K_M (μM)	V_{max}/K_M	V_{max} (%)	K_M (μM)	V_{max}/K_M	V_{max} (%)	K_M (μM)	V_{max}/K_M	V_{max} (%)	K_M (μM)	V_{max}/K_M	V_{max} (%)	K_M (μM)	V_{max}/K_M
NATIVE	100	2	50	81	3.3	25	15	2	7.5	44	6.3	7	29	57	0.5
V349A	55	1.7	32	3.2	102 (K_I = 2)	0.03	1.5	203	0.007	3	67	.04	2	ND	ND
V349A/I523V	56	ND	ND	4.9	ND	ND	2	ND	ND	4	ND	ND	0.9	ND	ND
V349A/I523V/ H513R/I434V	50	ND	ND	4.9	ND	ND	1.5	ND	ND	4	ND	ND	0	ND	ND
V349L	63	7	9	28	21	1.3	4.7	492	.01	6.2	336	.02	2.9	ND	ND
hPGHS-2*	100	5.6	18	115	30	3.8	46	2.5	18.4	76	27	2.8	74	48	1.5
V349A hPGHS-2*	59	6	9.8	51	35	1.5	5	ND	ND	30	ND	ND	ND	ND	ND
V523I hPGHS-2*	85	ND	ND	60	ND	ND	ND	ND	ND	20	ND	ND	ND	ND	ND

*Personal communication from C.J. Rieke and W.L. Smith.

20:3 (C-3 through C-5) is supported solely by van der Waals interactions on the opposite side of the substrate with Ala527, Gly526 and Ile523. Thus, Val349 may provide the one critical interaction with the carboxyl end of 20:3 responsible for proper positioning of the 13-pro*S* hydrogen for abstraction by Tyr385. Although there is not currently an oPGHS-1 crystal structure bound with 18:2n-6 or 20:5n-3 available, it is likely that the carboxyl end of 18:2 is bound similarly to that of 20:3, causing it to be reliant on similar, if not the same, carboxyl end interactions with Val349, particularly at C-5.

As shown in Table VII, V349A, oxygenates arachidonate (20:4) with 65% the efficiency of the native enzyme but oxygenates the similar substrate, 20:3, with only 0.1% native efficiency although it is able to bind 20:3 relatively easily (K_I = 2μM). This suggests that dihomo-γ-linolenic acid can bind to the V349A oPGHS-1 mutant with about the same affinity as binding to native oPGHS-1, but that V349A oPGHS-1 binds dihomo-γ-linolenate in a nonproductive conformation such that abstraction of the 13pro*S* hydrogen cannot occur. Thus, we conclude that Val349 is a key determinant of the cyclooxygenase substrate specificity of oPGHS-1.

Although Val349 is an important determinant of the substrate specificity for PGHS-1, it is not an important determinant for PGHS-2. The corresponding mutation in PGHS-2 results not only in efficient oxygenation of arachidonate but also in efficient oxygenation of 20:3 (61% the efficiency of native enzyme) (Table VII). This discrepancy between substrate specificities of identical active site mutants (V349A) in PGHS-1 and PGHS-2 has led to the construction of two oPGHS-1 enzymes containing multiple mutations. PGHS-2 has been shown to accommodate a wider range of substrates than PGHS-1 [115], possibly because of its slightly larger cyclooxygenase site

[82]. The cyclooxygenase active site differences between PGHS-1 and -2 are the result of three residue substitutions in PGHS-2. Ile523 is a valine in PGHS-2 and is the only residue in the hydrophobic lining of the cyclooxygenase active site which is different between PGHS-1 and -2. Two other residues immediately outside the cyclooxygenase active site, in the outer shell, are His513, an arginine in PGHS-2 and Ile434, a valine in PGHS-2. V349A/I523V and V349A/I523V/H513R/I434V oPGHS-1 were constructed in order to make the area surrounding the oPGHS-1 cyclooxygenase active site identical to that of PGHS-2 and possibly restore the ability of V349A to oxygenate 20:3 efficiently. V349A has been shown to produce an abundance of 11*R*-HPETE when reacted with arachidonic acid. It should be noted that both the double and quadruple V349A mutants exhibited the same pattern of oxygenated product distribution as the single V349A mutant (data not shown). When tested with 100μM substrate, the double mutant showed a slight increase in oxygenation of all substrates tested. The quadruple mutant showed a further slight increase in oxygenation of 20:3, but had little effect on other substrates and was unable to oxygenate 18:3n-3 at all. In general, the effects of these changes were not largely significant, with a maximal increase in V_{max} of 1.5 fold. By making changes of this nature, we have not succeeded in mimicking the activity of V349A PGHS-2 with 20:3. This may be due in part to the difference between the necessity of the ionic bond between Arg120 and the carboxyl group of fatty acid substrates for oPGHS-1 activity and the lack of this necessity in PGHS-2 [98]. This difference could potentially result in significant deviations between the positioning of substrates in each active site (oPGHS-1 and PGHS-2) and the substrate-stabilizing influences, particularly at the carboxyl end of the fatty acid molecules.

Residues involved in stabilization of n-6 vs. n-3 fatty acid substrates (Phe381, Phe205 and Leu531).

Phenylalanine 381. Phe381 makes four contacts with three carbons (C-16, C-17 and C-20) at the methyl end of arachidonic acid [66] and three contacts with two carbons (C-16 and C-17) at the methyl end of dihomo-γ-linolenic acid, both n-6 substrates (Tables III and VI). F381A was not able to oxygenate any of the substrates well, but had the most detrimental effect on the n-6 fatty acids, 20:4, 20:3 and 18:2, using these substrates only 2-4% as well as the native enzyme, while using the n-3 fatty acid, 20:5 with 17% the activity of native oPGHS-1 (Table VIII). The contacts made by the phenylalanine are probably somewhat maintained by the leucine mutation, but nearly nonexistent when changed to the smaller alanine. These contacts, apparently, are very important in keeping the 13 pro*S* hydrogen aligned for abstraction by Tyr385, thus having a significant effect on the oxygenation of all substrates. The n-6 substrates will be much more acutely affected by mutations made near the methyl end of fatty acids because of the lack of rigidity at the methyl end compared with n-3 substrates, thus making contacts at that end more important for substrate stabilization. In particular, n-6 fatty acids with fewer double bonds such as 20:3 and 18:2 will be more destabilized by the removal of three methyl end contacts at C-16 and C-17, which, in the case of 20:3 and 18:2, result in a 98% decrease in the enzyme's ability to oxygenate either of these substrates. These fatty acids are not only more flexible than n-3 substrates, but also more flexible than arachidonate.

Phenylalanine 205. Phe205 contacts C-14, C-15 and C-18 of arachidonate [66] and C-15 and C-18 of dihomo-γ-linolenic acid (Tables III and VI). F205A showed a

Table VIII

Oxygenase Activity for oPGHS-1 Cyclooxygenase Active Site Mutants with Various Fatty Acid Substrates

Oxygenase activity was measured with an oxygen electrode as described in the text. Values are calculated for fatty acid turnover and a value of 100% is assigned for oxygenase activity of native oPGHS-1. V_{max} and K_M values represent the means from a minimum of four separate determinations with standard deviations within 10% of all values reported. Relative V_{max} values reported for mutant enzymes for which K_M values were not determined represent rate measurements performed using 100 μM fatty acid substrate. ND, not determined.

ENZYME	20:4 (n-6)	20:2 (n-6)	20:3 (n-6)	20:5 (n-3)	18:2 (n-2)	18:3 (n-3)
SHAM	0	0	0	0	0	0
F205A	28	ND	17	52	8	29
F205L	65	ND	32	118	49	115
F209A	15	ND	6	2	4	3
F209L	43	ND	39	33	21	27
Y348L	0	ND	0	0	0	0
V349A/S353T	3	ND	0	0	0	0
L352V	34	ND	30	57	21	ND
S353G	61	ND	60	34	13	28
S353A	56	ND	43	35	33	96
S353T	42	ND	26	73	32	19
I377V	72	ND	52	60	56	37
F381A	4	ND	2	17	2	6
F381L	21	ND	14	21	11	5
I523A	64	ND	58	50	7	33
I523V	70	ND	ND	ND	28	11
S530A [21, 156]	70 (Km = 2)	ND	109 (Km 8.6)	97 (Km = 48)	56 (Km = 16)	62
S530T [21, 156]	17 (Km =13)	ND	3.3 (Km = 100)	2 (Km = 115)	16 (Km = 305)	~1
S530V	0	0	0	0	0	0
L531A	4.6 (Km = 54)	20	7	46	11	36
L531V	7.7 (Km = 1.6)	18	16 (Km = 8.4)	63	15	18
L534A	59	ND	43	50	41	14
L534V	98	ND	34	10	0	0

similar pattern to F381A, using 20:5n-3, 52% as well as native, while retaining only 8-28% the activity with the n-6, 20-carbon fatty acids. This mutant also retained 30% native activity with the n-3 fatty acid, 18:3, while retaining only 8% activity with 18:2,n-6. F205L, while quite active with all substrates (32-118% native activity) highly favored the n-3's, using both 20:5 and 18:3 with greater than 100% native activity (Table VIII). Similar to Phe381, the contacts made by Phe205 are also near C-13 of both arachidonate (C-14, C-15 and C-18) and dihomo-γ-linolenante (C-15 and C-18), thus the reasoning is similar to that for Phe381. F205L actually oxygenates the n-3 fatty acids, 20:5 and 18:3 more efficiently than native. PGHS-2 has been shown to oxygenate both 20:5 and 18:3 significantly more efficiently than PGHS-1 [115]. In an inhibitor-bound crystal structure of murine PGHS-2, the cyclooxygenase pocket has been shown to be slightly larger than that of oPGHS-1 [82]. This extra space created by changing Phe205 to the smaller leucine may provide just enough extra space to provide the n-3 double bond with enough flexibility so that the ω8 hydrogen in the case of 20:5 and the ω5 hydrogen in the case of 18:3 can be better aligned for abstraction.

Leucine 531. Leu531 is present in the first shell of the cyclooxygenase site in close proximity to both the carboxyl and methyl ends of arachidonate and dihomo-γ-linolenate, but outside of van der Waals contact distance from the carbon skeleton of either substrate (Table IV). Again, when Leu531 is mutated to the smaller residues (L531V or L531A), oxygenation of n-3 substrates remains relatively unaffected, while oxygenation of n-6 substrates decreases considerably (Table VIII). For instance, L531A oxygenates the n-3 substrates 20:5 and 18:3 with 46 and 36% the efficiency of the native enzyme while oxygenating n-6 substrates with only 5-11%. Leu531 makes four main

chain hydrogen bonding interactions with active site residues, two of which are involved in interactions with the methyl ends of both arachidonate and dihomo-γ-linolenate, Gly533 and Leu534. Changes in Leu531 could adversely affect the interactions between these adjoining active site residues and the methyl ends of substrates, resulting in decreased stabilization of the more flexible n-6 versus n-3 substrates.

Residues selectively involved in stabilization of 18:2n-6 (Ser353, Ile523)

Serine 353. The Cβ of Ser353 makes one contact with arachidonic acid [66] at C- 3 but is greater than 4Å from C-3 of dihomo-γ-linolenate. However, shortening of this residue by replacement with a glycine results in an enzyme which no longer oxygenates 18:2n-6 efficiently at 13% the rate of the native, compared with 61% the rate of the native enzyme with arachidonate (Table VIII). Because this mutant retains high cyclooxygenase activity and 87% the native peroxidase activity, we can assume that we have not perturbed the structural integrity of the enzyme overall. There is not currently a structure of oPGHS-1 bound with 18:2, but these results suggest the possibility of a Ser353 contact with the 18:2 substrate, which appears to play a role in stabilization of the carboxyl end of this 18-carbon substrate for proper positioning and efficient hydrogen abstraction.

Isoleucine 523. One of the gamma carbons of Ile523 makes van der Waals contacts with C2, C5 and C6 of arachidonate [66], but makes six contacts with C-3 through C-8 of dihomo-γ-linolenate as well as an additional contact between its Cα and C-8 of 20:3. This isoleucine is a valine in PGHS-2. Not surprisingly, substitution of Ile523 with a valine has very little effect on the oxygenation of all fatty acid substrates tested. Substitution with an alanine eliminates the gamma carbon contacts made between

Ile523 and multiple carboxyl end carbons of 20:4 and 20:3, but still has relatively little effect on the oxygenation of 20:4, 20:3 or the n-3 fatty acid 20:5 (V_{max} values are 64%, 58% and 50% that of native enzyme, respectively). As mentioned earlier, the carboxyl end of 20:3 is more flexible than that of 20:4 and most likely relies more heavily on carboxyl end interactions, however, there are numerous other carboxyl end contacts with the same side of the 20:3 substrate as Ile523 including contacts at C-3 and C-4 made by Ala527, a contact at Gly526 at C-8 and the one Cα contact at C-8 retained by I523A which could compensate for the loss of Ile523 interactions. In contrast, removal of one C-5 contact made by Val349 on the side of the substrate opposite Ile523 results in almost a complete loss of 20:3 oxygenation, most likely because of the lack of compensatory interactions on that side. The I523A substitution did, however, exhibit a significantly decreased ability to oxygenate 18:2n-6 (Table VIII). Again, 18:2 is likely to be more heavily reliant on stabilizing van der Waals interactions at its carboxyl end due to the absence of the C-5 and C-8 double bonds, thus interactions made by Ile523 are more significant for the stabilization of 18:2 versus 20:3, 20:4 or 20:5.

Residues involved in stabilization of n-3 vs. n-6 fatty acid substrates (Phe209, Ser530)

Phenylalanine 209. Phe209 resides near the methyl end of arachidonate and dihomo-γ-linolenate and makes numerous contacts with the C-17 through C-19 regions of both substrates [66]. Mutation of Phe209 to an alanine resulted in significantly decreased oxygenation of all substrates tested, while the leucine mutant was much more active and showed little selectivity among substrates. This is most likely because some of the original phenylalanine contacts were maintained by the leucine substitution. The n-3

substrates were somewhat more negatively affected by the F209A change than their n-6 counterparts. Phe209 is the only residue near the methyl end of substrates for which this is true, suggesting that the methyl end contacts made by Phe209 may be the primary contributors to stabilization of n-3 fatty acids at the methyl ends of these substrates.

Serine 530. Ser530 is the site of acetylation of oPGHS-1 by aspirin [21, 99]. Van der Waals contacts between Ser530 and arachidonic acid and dihomo-γ-linolenic acid occur at C-10 and C-16 for arachidonate and at C-10, C-13, C-15, C-16 and C-17 for dihomo-γ-linolenate (Table VI) [66]. In both cases, these contacts are on the side of the substrate directly opposite Tyr385. Earlier studies had shown that substitution of Ser530 with alanine has relatively little effect on arachidonate oxygenation, that replacement of Ser530 with threonine causes a 95% drop in catalytic efficiency, and that replacement of Ser530 with glutamine or valine prevents catalysis apparently by blocking substrate access to the Tyr385 radical [21, 156]. Here we have extended these findings showing that S530A has very little effect on oxygenation of substrates other than arachidonate while S530T exhibits poor catalytic efficiency when tested with all substrates (Table VIII), confirming that having a small residue at position 530 is important for optimal positioning of all fatty acids for hydrogen abstraction by Tyr385. Also, S530T seems to have a more negative effect on the n-3 versus the n-6 substrates, with a V_{max} which is ~17% that of native oPGHS-1 for oxygenation of 20:4n-6 and 18:2n-6 and only a V_{max} of 2 and 1% for oxygenation of 20:5n-3 and 18:3n-3, respectively. Positioning of Ser530 near the mid-chain carbons of fatty acids combined with the methyl end contacts made by Phe209 appear to play a significant role in situating the n-3 substrates optimally for hydrogen abstraction by Tyr385.

Residues involved in stabilization of 18-carbon vs. 20-carbon fatty acid substrates (Leu534)

Leucine 534. Leu534 makes van der Waals contacts between one or more of its delta carbons and the C-15 through C-18 regions of both arachidonate and dihomo-γ-linolenate (Table VI) [66]. Although active with all 20-carbon fatty acids tested, a L534V mutant is unable to oxygenate either of the 18-carbon fatty acids tested, 18:2n-6 or 18:3n-3. Interestingly, the L534A mutant is able to oxygenate both 18:2 and 18:3 with 41 and 14% the rate of native oPGHS-1, respectively. Computer modeling of these mutants with 18:2 bound in the cyclooxygenase active site suggests that this 18-carbon substrate may be bound differently at the methyl end compared with both 20:3 and 20:4, possibly in closer proximity to Leu534 rather than passing over Gly533 as is observed for 20:3 and 20:4. Although interpretation of these data is based solely on a computer model, one possibility may be that the gamma carbons of the L534V are configured differently than those of the leucine and could possibly engage in an aberrant contact which is detrimental to a catalytically favorable orientation of the 18-carbon substrates.

Residues equally involved in stabilization of all substrates tested (Tyr348)

Tyrosine 348. Phenyl ring carbons of Tyr348 are within van der Waals distance of C-12, C-13 and C-14 of both arachidonic and dihomo-γ-linolenic acids (Table VI) [66]. These interactions have been shown to be important in positioning C-13 of arachidonate as evidenced by a Y348L oPGHS-1 mutant which lacks cyclooxygenase activity while retaining significant peroxidase activity as discussed in Chapter III. Here we show that this mutant is also unable to oxygenate any fatty acid substrates tested (Table VIII), indicating that substitution of leucine at position 348 permits the

mispositioning of C-13 of most n-6 and n-3 substrates with respect to Tyr385 such that hydrogen abstraction cannot occur.

Residues which oxygenate substrates with a pattern similar to native oPGHS-1 (Leu352, Ile377)

All of the residues in and around the cyclooxygenase active site, when mutated, lessen the ability of oPGHS-1 to efficiently oxygenate arachidonate and other substrates to some extent. Leu352 and Ile377, when mutated, result in enzymes which negatively affect the oxygenation of all substrates tested to roughly the same degree. One delta carbon of Leu352 makes multiple contacts with the upper carboxyl to mid region of both arachidonate (C-7, C-11 and C-12) and dihomo-γ-linolenate (C-6, C-7, C-11 and C-12). Substitution of this residue with a valine should, in all likelihood, eliminate these contacts. L352V, however, retains 21-57% the V_{max} of the native enzyme when tested with 20:4, 20:3, 20:5 and 18:2. The mid regions of both arachidonate and dihomo-γ-linolenate are supported by numerous van der Waals interactions, both on the side of Leu352 and the side opposite. Some of the residues contributing in these interactions are Ile523, Phe518, Gly526, Ala527, Ser530, Trp387 and Tyr348. The interactions made by Leu352 appear to be complementary to the many others in place in this region of the substrate

One delta carbon of Ile377 contacts C-20 of arachidonate but is out of van der Waals range of dihomo-γ-linolenate [66]. Again, mutation of this residue to a valine should, in all likelihood, eliminate this one contact. I377V oxygenates 20:4, 20:3, 20:5, 18:2 and 18:3 with 40-70% the rate of the native enzyme and does not appear to be selectively involved in the stabilization of certain substrates over others (Table VIII).

The best known substrate for ovine Prostaglandin Endoperoxide H Synthase is arachidonic acid [115]. However, both dihomo-γ-linolenic acid (20:3n-6) and linoleic acid (18:2n-6) have also been shown to be substrates for PGHSs *in vivo* [61, 117,123,119]. Here we have determined that two of the most significant determinants of substrate specificity for oPGHS-1 are Val349 in the case of 20:3 and Leu534 in the case of 18:2. Val349 plays a crucial role in stabilization of the carboxyl ends of substrates other than arachidonate, particularly in the case of substrates lacking one or more double bonds toward the carboxyl end of the molecule, while Leu534 appears to play a more significant role in the methyl-end stabilization of 18-carbon fatty acids such as 18:2. Ongoing crystallographic and mutational analyses with PGHS-1 and PGHS-2 will help to further illuminate the key determinants in substrate usage by both PGHSs.

APPENDIX

APPENDIX A

Derivations for Kinetic Equations

Scheme 1 Derivations (no inhibitor):

Term definitions

$v(1) = k_5[ES_1]$ $\quad K_0 = [E][S_0]/[ES_0]$ $\quad K_2 = [ES_0]/[ES_2]$

$v(2) = k_6[ES_2]$ $\quad K_1 = [ES_0]/[ES_1]$

$[S_{tot}] = [S_0]$

$[E_{tot}] = [E] + [ES_0] + [ES_1] + [ES_2]$

$[ES_0] = [E][S_0]$ $\quad [ES_2] = [ES_0]/K_2$ $\quad [ES_2]/[ES_1] = K_2/K_1$

$[ES_1] = [ES_0]/K_1$

Formation of Product 1:

$$\frac{[ES_1]}{[E_{tot}]} = \frac{[E][S_0]/K_0K_1}{[E] + [ES_0] + [ES_1] + [ES_2]} = \frac{[E][S_0]/K_0K_1}{[E] + [E][S_0]/K_0 + [E][S_0]/K_0K_1 + [E][S_0]/K_0K_2}$$

$$= \frac{[S_0]/K_0K_1}{1 + [S_0]/K_0 + [S_0]/K_0K_1 + [S_0]/K_0K_2} * (K_0K_1/K_0K_1)$$

$$= \frac{[S_0]}{K_0K_1 + [S_0]K_1 + [S_0] + [S_0]K_1/K_2}$$

Therefore:

$$[ES_1] = \frac{[E_{tot}][S_0]}{K_1(K_0 + [S_0] + [S_0]/K_2) + [S_0]}$$

Since $v(1) = k_5[ES_1]$ and $v(1) = V_{max}(1)[S]/K_M(1) + [S]$,

$$\mathbf{v(1) = \frac{k_5[E_{tot}][S_0]}{K_1(K_0 + [S_0] + [S_0]/K_2) + [S_0]}} \quad ; \quad \mathbf{V_{max}(1) = k_5[E_{tot}]} \text{ and } \mathbf{K_M(1) = K_1(K_0 + [S_0] + [S_0]/K_2)}$$

Formation of Product 2:

$$\frac{[ES_2]}{[E_{tot}]} = \frac{[E][S_0]/K_0K_2}{[E] + [E][S_0]/K_0 + [E][S_0]/K_0K_1 + [E][S_0]/K_0K_2} * (K_0K_2/K_0K_2)$$

$$= \frac{[S_0]}{K_2(K_0 + [S_0] + [S_0]/K_1) + [S_0]}$$ Therefore:

$$\mathbf{v(2) = \frac{k_6 [E_{tot}][S_0]}{K_2(K_0 + [S_0] + [S_0]/K_1) + [S_0]}} \quad ; \quad \mathbf{V_{max}(2) = k_6 [E_{tot}]} \text{ and } \mathbf{K_M(2) = K_2(K_0 + [S_0] + [S_0]/K_1)}$$

Scheme 1 Derivations (with inhibitor):

Term Definitions

Same as above with the following additions and changes:

$K_I = [E][I]/[EI]$
$[E_{tot}] = [E] + [ES_0] + [ES_1] + [ES_2] + [EI]$
$[EI] = [E][I]/K_I$

Formation of Product 1 in the presence of inhibitor:

$$\frac{[ES_1]}{[E_{tot}]} = \frac{[E][S_0]/K_0K_1}{[E] + [E][S_0]/K_0 + [E][S_0]/K_0K_1 + [E][S_0]/K_0K_2 + [E][I]/K_I}$$

$$= \frac{[S_0]/K_0K_1}{1 + [S_0]/K_0 + [S_0]/K_0K_1 + [S_0]/K_0K_2 + [I]/K_I} * (K_0K_1/K_0K_1)$$

$$= \frac{[S_0]}{K_0K_1 + [S_0]K_1 + [S_0] + [S_0]K_1/K_2 + [I]K_0K_1/K_I}$$ Therefore:

$$[ES_1] = \frac{[E_{tot}][S_0]}{K_0K_1 + [S_0]K_1 + [S_0] + [S_0]K_1/K_2 + [I]K_0K_1/K_I}$$

Since $v(1) = k_5[ES_1]$ and

$$v(1) = \frac{V_{max}(1)[S]}{K_M(1)(1 + [I]/K_I) + [S]}$$; then

$$v(1) = \frac{k_5 [E_{tot}][S_0]}{K_0K_1(1 + [I]/K_I) + [S_0](K_1 + K_1/K_2 + 1)} * \frac{(1/K_1 + K_1/K_2 + 1)}{(1/K_1 + K_1/K_2 + 1)}$$

Rearranges to: $$\mathbf{v(1) = \frac{k_5 [E_{tot}][S_0]/ (K_1 + K_1 /K_2 + 1)}{(K_0K_1/ (K_1 + K_1 /K_2 + 1))(1 + [I]/K_I) + [S_0]}}$$

$$\mathbf{V_{max}(1) = k_5 [E_{tot}]/ K_1 + K_1 /K_2 + 1 \text{ and } K_M(1) = K_0K_1/ (K_1 + K_1 /K_2 + 1)}$$

Formation of Product 2 in the presence of inhibitor:

$$\frac{[ES_2]}{[E_{tot}]} = \frac{[E][S_0]/ K_0K_2}{[E] + [E][S_0]/K_0 + [E][S_0]/K_0K_1 + [E][S_0]/K_0K_2 + [E][I]/K_I}$$

$$= \frac{[S_0]/ K_0K_2}{1 + [S_0]/ K_0 + [S_0]/ K_0K_1 + [S_0]/ K_0K_2 + [I]/K_I} * (K_0K_2/ K_0K_2)$$

$$= \frac{[S_0]}{K_0K_2 + [S_0]K_2 + [S_0] + [S_0]K_2 /K_1 + [I] K_0K_2/ K_I}$$ Therefore:

$$[ES_2] = \frac{[E_{tot}][S_0]}{K_0K_2 + [S_0]K_2 + [S_0] + [S_0] K_2 /K_1 + [I] K_0K_2/ K_I}$$

Since $v(2) = k_6[ES_2]$ and

$$v(2) = \frac{V_{max}(2)[S]}{K_M(2)(1 + [I]/K_I) + [S]}$$; then

$$v(2) = \frac{k_6 [E_{tot}][S_0]}{K_0K_2 (1 + [I]/K_I) + [S_0](K_2 + K_2 /K_1 + 1)} * \frac{(1/ K_2 + K_2 /K_1 + 1)}{(1/ K_2 + K_2 /K_1 + 1)}$$

Rearranges to: $$\mathbf{v(2) = \frac{k_6 [E_{tot}][S_0]/ (K_2 + K_2 /K_1 + 1)}{(K_0K_2/ (K_2 + K_2 /K_1 + 1))(1 + [I]/K_I) + [S_0]}}$$

$$\mathbf{V_{max}(2) = k_6 [E_{tot}]/ K_2 + K_2 /K_1 + 1 \text{ and } K_M(2) = K_0K_2/ (K_2 + K_2 /K_1 + 1)}$$

Relationship of K_I to IC_{50} values for Scheme 1:

v(1) + inhibitor = 0.5v(1) – inhibitor gives the relationship:

$$K_I = \frac{K_M\,(IC_{50})}{K_M + [S]}$$

Inhibition of Product 1 by a competitive inhibitor:

$$K_I = \frac{K_1K_0\,(IC_{50}(1))/\,(K_1 + K_1/K_2 + 1)}{(K_1K_0/\,(K_1 + K_1/K_2 + 1)) + [S_0]}$$

$$K_IK_1K_0 + K_IK_1[S_0] + K_I[S_0] + K_I[S_0]\,K_1/K_2 = K_1K_0\,(IC_{50}(1))$$

$$IC_{50}(1) = K_I + K_I[S_0]/\,K_0 + K_I[S_0]/\,K_1K_0 + K_I[S_0]/\,K_2\,K_0$$

$$\mathbf{IC_{50}(1) = K_I(1 + [S_0]/\,K_0 + [S_0]/\,K_1K_0 + [S_0]/\,K_2\,K_0)}$$

Inhibition of Product 2 by a competitive inhibitor:

$$K_I = \frac{K_2\,K_0\,(IC_{50}(2))/\,(K_2 + K_2/K_1 + 1)}{(K_2\,K_0/\,(K_2 + K_2/K_1 + 1)) + [S_0]}$$

$$K_IK_2K_0 + K_IK_2[S_0] + K_I[S_0] + K_I[S_0]\,K_2/K_1 = K_2K_0\,(IC_{50}(2))$$

$$IC_{50}(2) = K_I + K_I[S_0]/\,K_0 + K_I[S_0]/\,K_2K_0 + K_I[S_0]/\,K_1\,K_0$$

$$\mathbf{IC_{50}(2) = K_I(1 + [S_0]/\,K_0 + [S_0]/\,K_1K_0 + [S_0]/\,K_2\,K_0)}$$

$$\mathbf{IC_{50}(1) = IC_{50}(2)}$$

Scheme 2 Derivations (no inhibitor):

Term definitions

$v(1) = k_2[E_1S_1]$ $\quad K_0 = [E_1]/\,[E_2]$ $\quad K_2 = [E_2][S_0]/\,[E_2S_2]$

$v(2) = k_4[E_2S_2]$ $\quad K_1 = [E_1][S_0]/\,[E_1S_1]$

$[S_{tot}] = [S_0]$

$[E_{tot}] = [E_1] + [E_2] + [E_1S_1] + [E_2S_2]$

$[E_1] = [E_2]K_0$ $\quad [E_1S_1] = [E_1][S_0]/\,K_1$

$[E_2] = [E_1]/K_0$ $\quad [E_2S_2] = [E_2][S_0]/\,K_2$

Formation of Product 1

$$\frac{[E_1S_1]}{[E_{tot}]} = \frac{[E_1][S_0]/\,K_1}{[E_1] + [E_1]/K_0 + [E_1][S_0]/\,K_1 + [E_1][S_0]/\,K_0\,K_2}$$

$$= \frac{[S_0]/ K_1}{1 + 1/K_0 + [S_0]/ K_1 + [S_0]/ K_0 K_2} * (K_1/ K_1)$$

$$= \frac{[S_0]}{K_1 + K_1/K_0 + [S_0] + [S_0] K_1 / K_0 K_2}$$

Since $v(1) = k_2[E_1S_1]$ and $v(1) = V_{max}(1)[S]/ K_M(1) + [S]$,

$$\mathbf{v(1) = \frac{k_2 [E_{tot}] [S_0]}{K_1(1 + 1/K_0 + [S_0]/ K_0 K_2) + [S_0]}} \quad ; \quad \mathbf{V_{max}(1) = k_2 [E_{tot}]}, \quad \mathbf{K_M(1) = K_1(1 + 1/K_0 + [S_0]/ K_0 K_2)}$$

Formation of Product 2

$$\frac{[E_2S_2]}{[E_{tot}]} = \frac{[E_2][S_0]/ K_2}{[E_2] + [E_2]K_0 + [E_2][S_0] K_0/ K_1 + [E_2][S_0]/ K_2}$$

$$= \frac{[S_0]/ K_2}{1 + K_0 + K_0 [S_0]/ K_1 + [S_0]/ K_2} * (K_2/ K_2)$$

$$= \frac{[S_0]}{K_2 + K_2K_0 + K_0K_2[S_0]/ K_1 + [S_0]}$$

Since $v(2) = k_4[E_2S_2]$ and $v(2) = V_{max}(2)[S]/ K_M(2) + [S]$,

$$\mathbf{v(2) = \frac{k_4 [E_{tot}] [S_0]}{K_2(1 + K_0 + K_0[S_0]/ K_1) + [S_0]}} \quad ; \quad \mathbf{V_{max}(2) = k_4 [E_{tot}]}, \quad \mathbf{K_M(2) = K_2(1 + K_0 + K_0[S_0]/ K_1)}$$

Scheme 2 Derivations (with inhibitor):

Term Definitions

Same as above with the following additions and changes:

$K_{I(1)} = [E_1][I_0]/ [E_1I_1]$
$K_{I(2)} = [E_2][I_0]/ [E_2I_2]$
$[E_{tot}] = [E_1] + [E_2] + [E_1S_1] + [E_2S_2] + [E_1I_1] + [E_2I_2]$
$[E_1I_1] = [E_1][I_0]/ K_{I(1)}$
$[E_2I_2] = [E_2][I_0]/ K_{I(2)} = [E_1][I_0]/ K_0K_{I(2)}$

Formation of Product 1 in the presence of inhibitor

$$\frac{[E_1S_1]}{[E_{tot}]} = \frac{[E_1][S_0]/K_1}{[E_1] + [E_1]/K_0 + [E_1][S_0]/K_1 + [E_1][S_0]/K_0K_2 + [E_1][I_0]/K_{I(1)} + [E_1][I_0]/K_0K_{I(2)}}$$

$$= \frac{[S_0]/K_1}{1 + 1/K_0 + [S_0]/K_1 + [S_0]/K_0K_2 + [I_0]/K_{I(1)} + [I_0]/K_0K_{I(2)}} * (K_1/K_1)$$

$$= \frac{[S_0]}{K_1 + K_1/K_0 + [S_0] + [S_0]K_1/K_0K_2 + [I_0]K_1/K_{I(1)} + [I_0]K_1/K_0K_{I(2)}} \text{ ; Therefore:}$$

$$v(1) = \frac{k_2[E_{tot}][S_0]}{K_1(1 + [I_0]/K_{I(1)} + 1/K_0 + [I_0]/K_0K_{I(2)}) + [S_0](1 + K_1/K_0K_2)} * \frac{1/(1 + K_1/K_0K_2)}{1/(1 + K_1/K_0K_2)}$$

$$\mathbf{v(1) = \frac{k_2[E_{tot}][S_0]/(1 + K_1/K_0K_2)}{\frac{K_1(1 + [I_0]/K_{I(1)} + 1/K_0 + [I_0]/K_{I(2)}K_0)}{(1 + K_1/K_0K_2)} + [S_0]}}$$

$$\mathbf{K_M(1) = \frac{K_1(1 + [I_0]/K_{I(1)} + 1/K_0 + [I_0]/K_{I(2)}K_0)}{(1 + K_1/K_0K_2)}}$$

$$\mathbf{V_{max}(1) = k_2[E_{tot}]/(1 + K_1/K_0K_2)}$$

Formation of Product 2 in the presence of inhibitor

$$\frac{[E_2S_2]}{[E_{tot}]} = \frac{[E_2][S_0]/K_2}{[E_2] + [E_2]K_0 + [E_2]K_0[S_0]/K_1 + [E_2][S_0]/K_2 + [E_2][I_0]/K_{I(2)} + [E_2]K_0[I_0]/K_{I(1)}}$$

$$= \frac{[S_0]}{K_2 + K_0K_2 + K_0K_2[S_0]/K_1 + [S_0] + [I_0]K_2/K_{I(2)} + [I_0]K_0K_2/K_{I(1)}} \text{ ; Therefore:}$$

$$v(2) = \frac{k_4[E_{tot}][S_0]}{K_2(1 + [I_0]K_0/K_{I(1)} + K_0 + [I_0]/K_{I(2)}) + [S_0](1 + K_0K_2/K_1)} * \frac{1/(1 + K_0K_2/K_1)}{1/(1 + K_0K_2/K_1)}$$

$$\mathbf{v(2) = \frac{k_4[E_{tot}][S_0]/(1 + K_0K_2/K_1)}{\frac{K_2(1 + [I_0]K_0/K_{I(1)} + K_0 + [I_0]/K_{I(2)})}{(1 + K_0K_2/K_1)} + [S_0]}}$$

$$K_M(2) = \frac{K_2(1 + [I_0]K_0/K_{I(1)} + K_0 + [I_0]/K_{I(2)})}{(1 + K_0K_2/K_1)}$$

$$V_{max}(2) = k_4[E_{tot}]/(1 + K_0K_2/K_1)$$

Relationship of K_I to IC_{50} values for Scheme 2:

v(1) + inhibitor = 0.5v(1) – inhibitor

Inhibition of Product 1 by a competitive inhibitor

$$\frac{k_2[E_{tot}][S_0]/(1 + K_1/K_0K_2)}{\frac{K_1(1 + [I_0]/K_{I(1)} + 1/K_0 + [I_0]/K_{I(2)}K_0)}{(1 + K_1/K_0K_2)} + [S_0]} = \frac{0.5\, k_2[E_{tot}][S_0]}{K_1(1 + 1/K_0 + [S_0]/K_0K_2) + [S_0]}$$

Multiply left side by $(1 + K_1/K_0K_2)/(1 + K_1/K_0K_2)$, rearrange and multiply both sides by 2 to give:

$$2K_1+2K_1/K_0+2K_1[S_0]/K_0K_2+2[S_0] = K_1+K_1[I_0]/K_{I(1)}+K_1/K_0+K_1[I_0]/K_0K_{I(2)}+[S_0]+[S_0]K_1/K_0K_2$$

Subtract K_1, K_1/K_0, $[S_0]$ and $[S_0]K_1/K_0K_2$ from both sides, divide by K_1 and rearrange to give:

$$[I_{50}(1)] = \frac{1 + 1/K_0 + [S_0](1/K_0K_2 + 1/K_1)}{1/K_{I(1)} + 1/K_{I(2)}K_0}$$

Inhibition of Product 2 by a competitive inhibitor

$$\frac{k_4[E_{tot}][S_0]/(1 + K_0K_2/K_1)}{\frac{K_2(1 + [I_0]/K_{I(2)} + K_0 + K_0[I_0]/K_{I(1)})}{(1 + K_0K_2/K_1)} + [S_0]} = \frac{0.5\, k_4[E_{tot}][S_0]}{K_2(1 + K_0 + K_0[S_0]/K_1) + [S_0]}$$

Multiply left side by $(1 + K_0K_2/K_1)/(1 + K_0K_2/K_1)$, rearrange and multiply both sides by 2 to give:

$$2K_2+2K_0K_2+2[S_0]K_0K_2/K_1+2[S_0] = K_2+K_0K_2+K_2[I_0]/K_{I(2)}+K_0K_2[I_0]/K_{I(1)}+[S_0]+[S_0]K_0K_2/K_1$$

Subtract K_2, K_0K_2, $[S_0]$ and $[S_0]K_0K_2/K_1$ from both sides, divide by K_2 and rearrange to give:

$$[I_{50}(2)] = \frac{1 + K_0 + [S_0](K_0/K_1 + 1/K_2)}{1/K_{I(2)} + K_0/K_{I(1)}}$$

$IC_{50}(1) \neq IC_{50}(2)$

The only situation in which $IC_{50}(1) = IC_{50}(2)$ is that in which $K_0 = 1$

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