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Studies involving the cyclooxygenase active sites of prostaglandin endoperoxide H synthase-1 and -2

presented by

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William L. Smith

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STUDIES INVOLVING THE CYCLOOXYGENASE ACTIVE SITES OF PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE –1 AND –2

By

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ABSTRACT

STUDIES INVOLVING THE CYCLOOXYGNEASE ACTIVE SITES OF PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-1 AND -2

By

Caroline Jill Rieke

PGHS-1 and -2 catalyze the conversion of arachidonic acid to prostaglandin H_2 via two activities, a cyclooxygenase and a peroxidase activity. Two residues at the mouth of the cyclooxygenase site, Arg120 and Tyr355, are in a position to interact with the substrate, arachidonic acid, and with non-steroidal anti-inflammatory drugs (NSAIDs). The role of these two residues in fatty acid substrate binding and oxygenation and in NSAID interactions was investigated using site-directed mutagenesis.

We determined that Arg120 is necessary in both PGHS-1 and -2 for interaction with carboxylate containing NSAIDs. In addition, we showed that the Arg120 of the two isozymes interacts differently with arachidonic acid. A R120Q mutation of oPGHS-1 greatly affected catalysis while a R120Q mutation in hPGHS-2 had no affect on catalysis. We also determined that Tyr355 plays a role in stereoselectivity of the *S*-isomer of the 2-phenylpropionic acid class of NSAIDs and that it is involved in binding and oxygenation of fatty acid substrates.

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ABBREVIATIONS

20:4 n-6	arachidonic acid (AA)
20:3 n-6	dihomo-γ–linolenic acid
20:5 n-3	eicosapentanoic acid (EPA)
20:2 n-6	eicosadianoic acid
18:2 n-6	.linoleic acid
11-HETE	11-hydroxy-5,8,12,14-eicosatetraenoic acid
15-HETE	15-hydroxy-5,8,11,13-eicosatetraenoic acid
12-HHT	12-hydroxy-5,8,10-heptadecatrienoic acid
15- HPETE	15-hydroperoxy-5,8,11,13-eicosatetraenoic acid
BSA	bovine serum albumin
COX	cyclooxygenase
$cPLA_2$	cytosolic, 85 kDa phospholipase A_2
DMEM	Dulbecco's modifies Eagle medium
EGF	epidermal growth factor
IC_{50}	concentration for 50% inhibition
K_m	Michaelis-Menton constant
NSAID	non-steroidal anti-inflammatory drug
PBS	phosphate-buffered saline
PGHS.	prostaglandin endoperoxide H synthase
oPGHS-1	ovine prostaglandin synthase isozyme 1
hPGHS-1	human prostaglandin synthase isozyme 2
POX	peroxidase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
$sPLA_2$	secretory phospolipase A_2
TBS	tris-buffered saline
TLC	thin layer chromotagraphy
V_{max}	maximum velocity of a reaction

CHAPTER 1

LITERATURE REVIEW

Introduction

Arachidonic acid, a 20 carbon polyunsaturated essential fatty acid, is the precursor for the formation of the eicosanoids. The eicosanoids, which consist of the prostanoids, leukotrienes and epoxyecosatetraenoic acids (EETs), are produced via the actions of the enzymes of the arachidonic acid cascade (Figure 1). There are three major pathways within this cascade, with each being named for the central enzyme of the pathway. The prostanoids, which consist of the prostaglandins, prostacyclins, and thromboxanes are produced via the cyclo-oxygenase pathway. The leukotrienes are produced through the actions of lipoxygenases, and the EETs are produced through the actions of P450 epoxygenases. The lipoxygenase and epoxygenase pathways are reviewed in Ref. 1.

Prostaglandin endoperoxide H synthase (PGHS) is the enzyme which is responsible for catalyzing the committed step in the formation of prostanoid products. This enzyme has two enzymatic functions, a cyclooxygenase (COX) and a peroxidase (POX) activity. These activities convert arachidonic acid to PGH_2 which, in turn, can be further metabolized to the individual prostaglandins, prostacyclins, and thromboxanes by the appropriate synthases (2). These latter prostanoids are then able to act in an autocrine and/or paracrine fashion

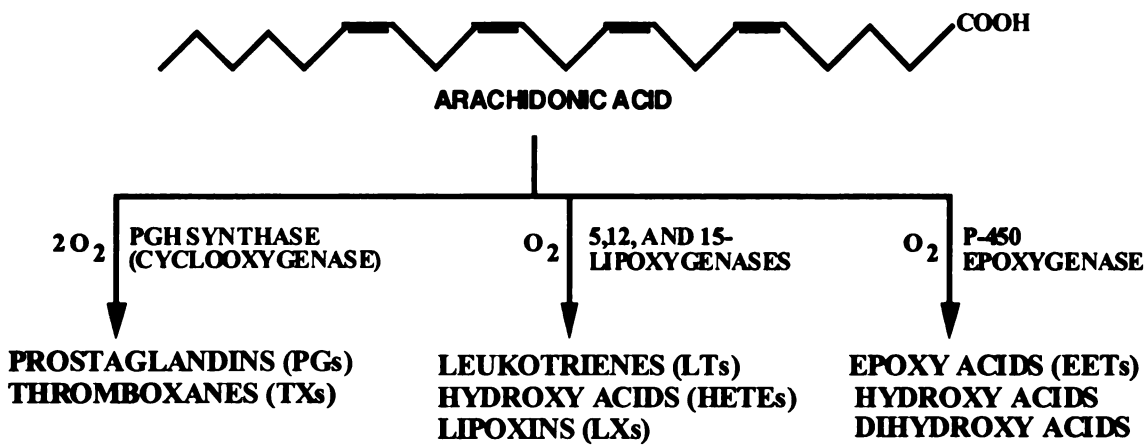


Figure 1. The arachidonate cascade. Arachidonic acid is metabolized by three different enzymes systems: the cyclooxygenase, the lipoxygenase and the P450 epoxygenase pathways

through the actions of G-protein linked receptors with the subsequent generation of second messengers such as cAMP or Ca^{++} in order to mediate their physiologic responses (3,4). Prostaglandins are found in all animal tissues, though not necessarily in all cells of each tissue. They play a broad role in basic physiological events.

The prostanoids have been shown to play a role in vascular homeostasis, platelet aggregation, renal water homeostasis, parturition and ovulation (5,6) and in the inflammatory responses to many different stimuli (2). In addition, they have been proposed to have a role in the development of colon cancer (7,8) and Alzheimer's disease (9).

PGHS is the therapeutic target of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen (2) and as such is the focus of research concerning the mechanisms of catalysis and inhibition.

Prostanoid Biosynthesis

Synthesis of the prostanoid products can be broken down into three steps: 1) Stimulus-activated release of arachidonic acid; 2) conversion of arachidonic acid to PGH_2; 3) cell specific conversion of PGH_2 to a biologically active prostanoid (Figure 2). Following an extracellular stimulus, arachidonic acid is released from membrane phospholipids through the actions of phospholipases. Two classes of phospholipases have been shown to be involved in arachidonic acid release

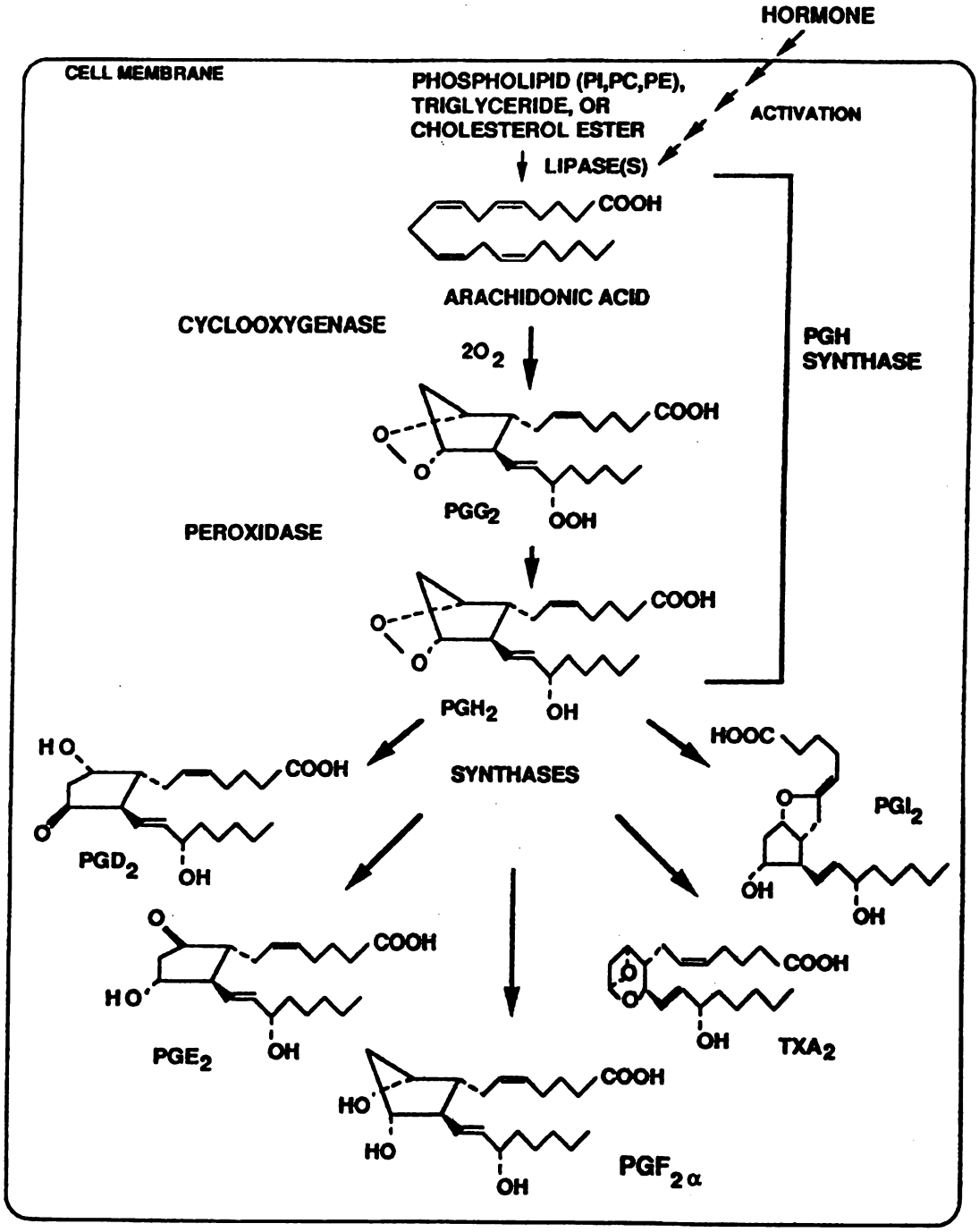


Figure 2. Biosynthetic pathway for prostanoid synthesis

in vitro (10,11,12,13). These are cytoplasmic PLA_2 ($cPLA_2$) and secretory PLA_2 ($sPLA_2$). The 85 kDa $cPLA_2$ is regulated by low concentrations of Ca^{++}(1-10 μM) and phosphorylation and is relatively specific for the arachidonate group located at the sn-2 position of phospholipids (14,15). The 14 kDa $sPLA_2$ requires high concentrations of Ca^{++} (1 mM) and is not specific for any particular phospholipid head group nor for the acyl group at the sn-2 position (16). Once the arachidonic acid is released, it is then converted to PGH_2 through the actions of PGHSs. The conversion of arachidonic acid to PGH_2 is initiated by the removal of the 13 pro-S hydrogen and 2 molecules of oxygen are added at the 11 and 15 positions to give PGG_2. The hydroperoxide at the C-15 position is subsequently reduced to an alcohol to give PGH_2 (17). PGH_2 then can undergo conversion either non-enzymatically or enzymatically to give the individual prostaglandin products (4).

Primary Structure

Two isozymes of PGHS have been described, and are encoded by separate genes. The first isozyme, PGHS-1 was initially purified from sheep vesicular gland (18,19) and the cDNA cloned (20). cDNAs for PGHS-1 have since been cloned from murine (21), human (22) and rat sources (23). There is approximately 90% sequence identity among the PGHS-1 enzymes. In the early 1990s a second isozyme, PGHS-2 ,was discovered as a v-src inducible gene product in chicken fibroblasts (24) and a phorbol ester inducible immediate early gene product in murine 3T3 cells (25). PGHS-2 cDNAs from human (26,28), rat

(23) and ovine (27) sources have also been cloned. Within a species PGHS-1 and PGHS-2 have 60% sequence identity, with all residues identified as important for catalysis being conserved (Figure 3). The most notable differences between the two isozymes are at the amino terminus and at the carboxyl terminus. The signal peptide at the amino terminus of PGHS-1 contains a series of hydrophobic amino acids not present in the signal peptide of PGHS-2, while the carboxyl terminus of PGHS-2 has a unique 18 amino acid "cassette". The function of this "cassette" has not yet been identified, although it has been postulated that it may have a role in targeting, in protein degradation or possibly in protein-protein interactions.

Regulation of PGHSs

The two isozymes of PGHS are regulated differently. PGHS-1 is normally present in many tissues, while PGHS-2 is usually undetectable in most tissues (29).The exceptions to this are brain (30) and kidney (31). Upon stimulation with growth factors, cytokines or mitogens (32,28,33,34), PGHS-2 mRNA and protein levels are dramatically upregulated. In addition, PGHS-2 is down-regulated by dexamethasone (34). Because PGHS-1 is typically present under normal conditions it has been proposed that this isozyme is involved in synthesizing prostanoids involved in "housekeeping" functions such as regulation of stomach acid secretion and renal homeostasis. Inpart because PGHS-2 is expressed by cells of the immune system, this isozyme is believed to be involved in inflam-

Figure 3. Comparison of the deduced amino acid sequences of various PGHSs. The amino acid sequences of various PGH synthases 1 and 2 are aligned for comparison. Numbering corresponds to oPGHS-1, starting with Met 1 of the deduced sequence.

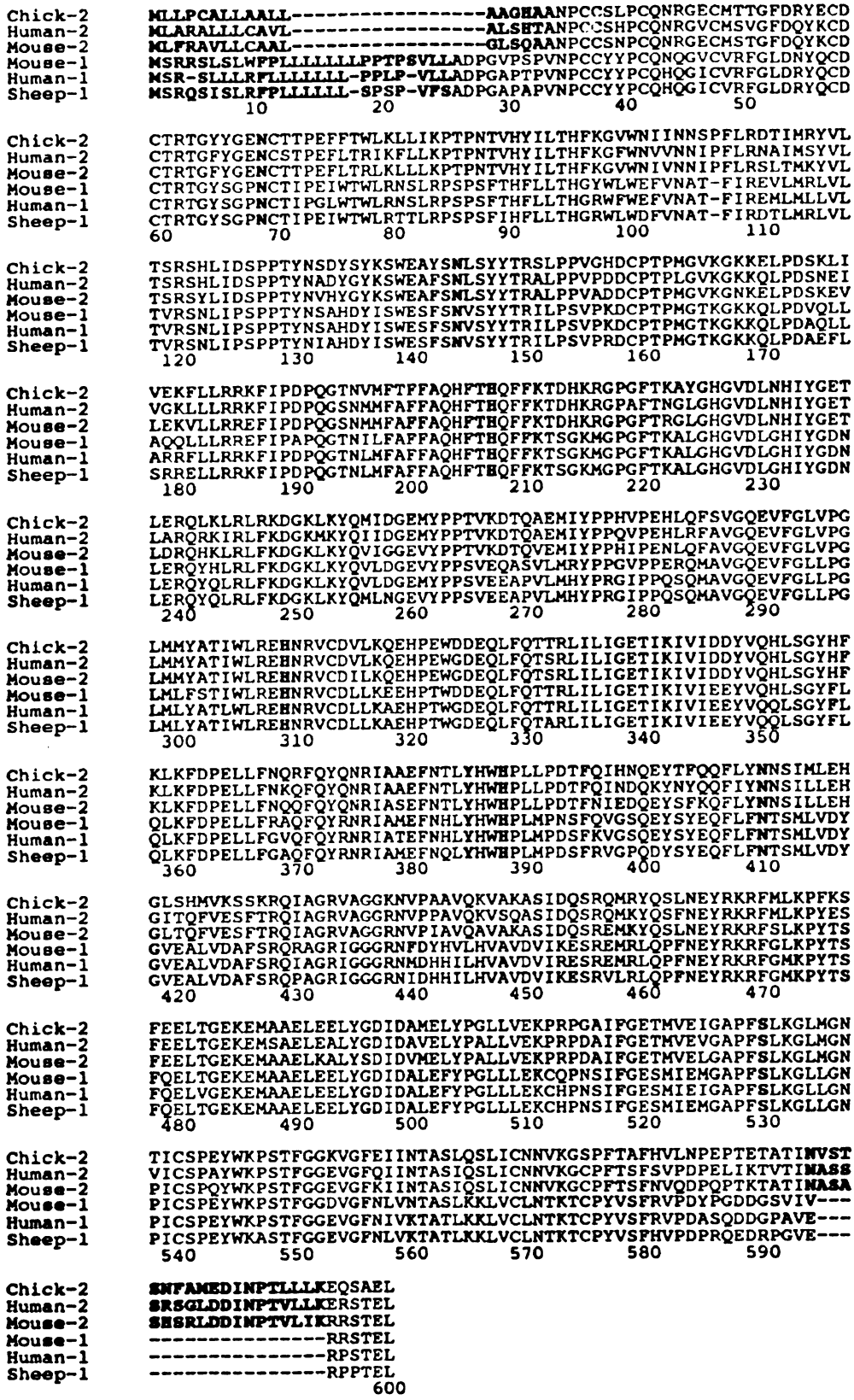

Chick-2
Human-2
Mouse-2
Mouse-1
Human-1
Sheep-1
10 20 30 40 50
60 70 80 90 100 110
120 130 140 150 160 170
180 190 200 210 220 230
240 250 260 270 280 290
300 310 320 330 340 350
420 430 440 450 460 470
480 490 500 510 520 530
540 550 560 570 580 590
600

Figure 3.

mation. Studies with knockout mice have shown that the two knockouts have distinct phenotypes PGHS-1 knockout mice appear to develop normally. These mice have no stomach pathologies and exhibit less indomethacin-induced gastric ulceration than wild type mice (35). PGHS-2 knockout mice exhibit abnormal renal development and die early from renal failure (36).

Biochemical and Structural Properties of PGHSs

PGHSs are classified as integral membrane proteins based on observations that detergents are necessary to solubilize the enzymes from the membrane (19). They are localized to the luminal side of the endoplasmic reticulum and the nuclear envelope (37,38). While it was initially shown, by immunofluorescent staining, that the two isozymes were differentially localized to the two membranes (39), more recent immunogold staining has shown that the two proteins appear to be localized equally between the two membrane systems (40). Initially it was predicted, based on hydrophobicity analysis (41) and trysin cleavage patterns (42), that PGHSs contained one or more transmembrane regions. More recently with the appearance of the crystal structure it has been proposed that the proteins are inserted into one layer of the lipid bilayer via a novel membrane binding domain (43,44). This membrane binding domain consists of four amphipathic helices with the sidechains of the hydrophobic residues inserted into the membrane. Very recently another protein, squalene cyclase, has been shown to contain a similar membrane binding domain (45). PGHS-1 has a MW of 72 kDa on SDS-PAGE (19) while PGHS-2 appears to be a

doublet of 72 and 74 kDa (46). This is in contrast to the theoretical molecular weight of 66 kDa and 67 kDa for the two isozymes, respectively (20,46). This difference is due to the addition of several carbohydrate moieties. Both isozymes contain 3 N- glycosylation sites with PGHS-2 having an additional site, which is glycosylated in approximately 50% of the protein, located at the carboxyl terminal (42).

PGHSs have three distinct folding domains including an EGF homolgy domain, a membrane binding domain and a large globular catalytic domain (Figure 4). Between the two isozymes the EGF domains exhibit 49% identity, the membrane binding domains show 38% identity and the catalytic domains 70% identity.

PGHSs function as homodimers and are heme containing proteins, with one molecule of heme per monomer (18,19). PGHSs have been shown to exist as homodimers by sedimentation analysis (19) and crosslinking studies (46). The crystal structure supports this as the enzyme was crystallized in homodimer form with the dimer interface containing interactions between the EGF domain subunits.

Figure 4. Crystal structures of ovine PGHS-1 and murine PGHS-2 . The three folding domains are colored as listed: EGF domain dark blue, membrane binding domain yellow and catalytic domain green.

A. oPGHS-1

B. mPGHS-2

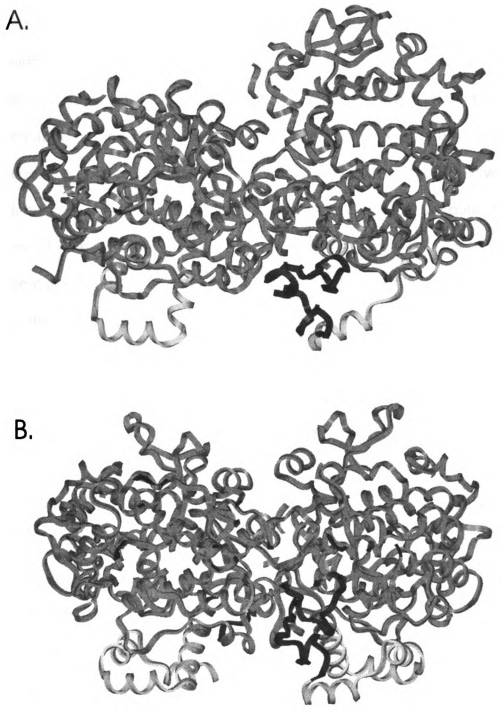


Figure 4

Crystal Structure of PGHSs

Several years ago the crystal structure of oPGHS-1 complexed with the NSAID, flurbiprofen, was solved (43). The enzyme has a large helical content with little B-sheet. As mentioned previously, the enzyme can be divided into three distinct folding regions; an EGF domain, a membrane binding domain and a catalytic domain. The catalytic domain is a large globular structure which contains both cyclooxygenase and peroxidase active sites. The cyclooxygenase active site is a long hydrophobic channel which opens at the outer surface of the membrane binding domain. The peroxidase active site is a shallow cleft containing the Fe- protophorphyrin IX prosthetic group and is structurally related to the peroxidase site of myeloperoxidase.

With the crystal structure it became possible to make hypotheses regarding the function of various active site amino acids. Several amino acids located at the mouth of the cyclooxygenase channel form a hydrogen bonding network involved in binding the carboxylate group of flurbiprofen. These amino acids include Arg120, Tyr355, and Glu524 and it is proposed that in addition to their interaction with NSAIDs they would also interact with the carboxylate group of the substrate.

In 1996 the crystal structure of PGHS-2 was solved by two groups (47,48). This allowed a direct comparison of the structure of the two isozymes (Figure 5). In general, the two structures are superimposible with almost all residues in the active site being conserved. One notable difference is the substitution of Val for Ile at residue 523 in PGHS-2. This results in a slightly larger active site for the

PGHS-2 isozyme and possibly explains some of the differences that the two isozymes exhibit with respect to interaction of the various NSAIDs and substrates. Another difference is the substitution of Arg for His at residue 513. This substitution has been postulated to be involved in an alternative hydrogen bonding network in PGHS-2 and interaction with some PGHS-2 specific inhibitors. Luong *et al* (47) comparison of the crystal structures of hPGHS-2 with two different inhibitors bound in the cyclooxygenase site has provided evidence for flexiblity at the mouth of the active site. In the "closed conformation" Arg120, Tyr355 and Glu524 participate in a hydrogen bonding network at the bottom of the channel similar to that seen in the oPGHS-1 structure, while in the "open conformation" the Arg120 interacts with the backbone carbonyl oxygens of Glu524 and the side chain of Glu524 interacts with Arg513 on the other side of the NSAID binding site.

Kurumball *et al* (48) crystallized the murine PGHS-2 without inhibitor and with several different NSAIDs in the active site, providing evidence for some of the interactions associated with PGHS-2 specific inhibition. Overall they found the structure of the free enzyme to be similar to that of the complexes with inhibitors. They postulated a role for Arg513 involving an interaction with the sulphonamide group of the PGHS-2 specific inhibitor, SC-558.

Figure 5. Kabsch Sander representations of ovine PGHS-1 and murine PGHS-2 showing similarity in secondary structure. Helices are shown in red, B-sheets are shown in yellow.

A. oPGHS-1
B. mPGHS-2

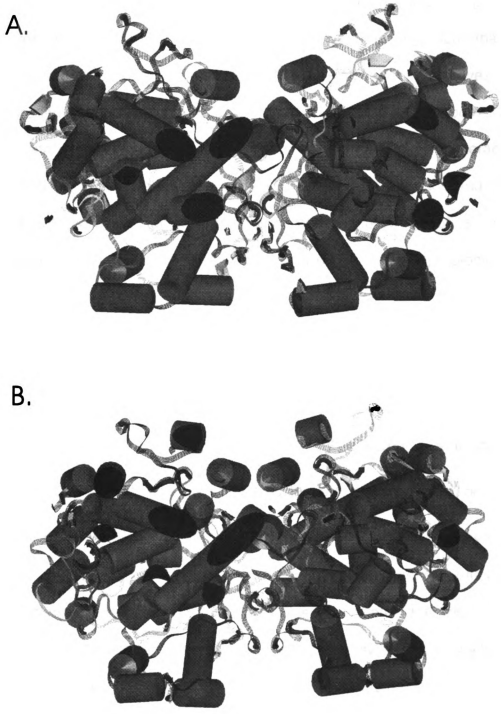


Figure 5

Active Site of PGHSs

As mentioned previously, all residues that have been shown to be important for catalysis are conserved between the two isozymes. Figure 6 is a representation of the active site of oPGHS-1. Tyr385 is the residue that has been postulated to be the source of the tyrosyl radical that removes the pro-S hydrogen from arachidonic acid (49). Arg120 is located at the mouth of the active site and is postulated to be involved in substrate and inhibitor interactions (43). Ser530 is the residue that is acetylated by aspirin (50). His338 and 207 are the proximal and distal ligands of the Fe, respectively (43). Tyr355 is postulated to be involved in stereoselectivity of inhibitors of the 2-phenylpropionic acid class (43).

Reactions of PGHSs

PGHSs have two distinct catalytic activities including a cyclooxygenase component which converts arachidonic acid to the hydroperoxide, PGG_2, and a peroxidase component which catalyzes the two electron reduction of PGG_2 to produce PGH_2. The enzymes have been shown to have an absolute requirement for a hydroperoxide (52). Recently it has been demonstrated that the hydroperoxide requirement for PGHS-2 is lower than that for PGHS-1 (53). These catalytic activies have been shown structurally and functionally to occur at distinct but neighboring sites (54). Initial studies on the mechanistic properties of

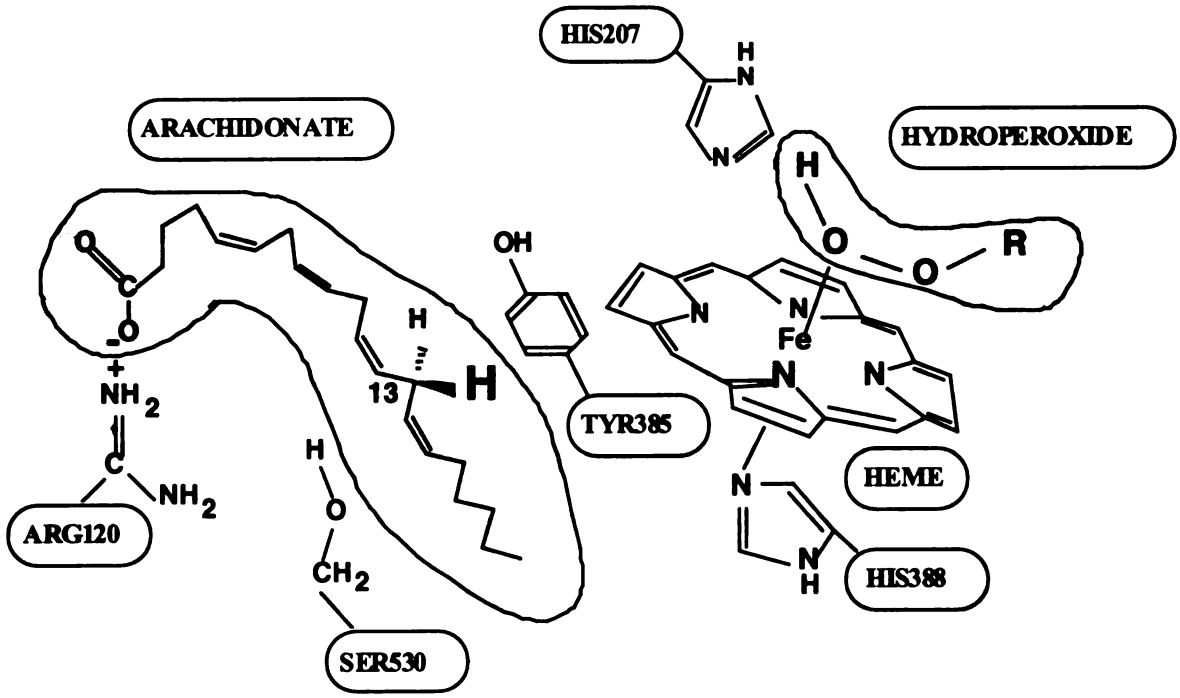


Figure 6. Model of the active site for ovine PGHS-1 Residues shown are conserved between the two isozymes and are described in the text.

the enzyme were performed using oPGHS-1 but subsequent studies have demonstrated that the reaction mechanism and kinetic properties of the two isozymes are similar (54,55). The cyclooxygenase reaction is initiated by the removal of the 13 pro-S hydrogen of arachidonic acid (57). This is the rate limiting step in the reaction and formation of an arachidonyl radical has been demonstrated (58,59). It is proposed that a protein radical is generated that can remove the 13-pro S hydrogen resulting in the formation of the arachidonyl radical. Formation of a tyrosyl radical has been demonstrated using EPR (60). In addition, it has been shown by site directed mutagenesis that Tyr385 is necessary for COX activity (50). This has lead to the proposal that a Tyr385 radical is responsible for the abstraction of the hydrogen from arachidonate. Subsequent rearrangement and addition of two molecules of oxygen leads to bis-oxygenation at carbons 11 and 15. The resulting PGG_2 diffuses from the cyclooxygenase site to the peroxidase site where the two electron reduction of PGG_2 to the corresponding alcohol, PGH_2, takes place.

Ruf and coworkers developed a model to explain the relationship between the cyclooxygenase and peroxidase reactions of PGHSs (60) (Figure 7). During the peroxidase reaction a two electron oxidation of the heme group of PGHS by a hydroperoxide yields a peroxidase spectral intermediate I with an oxyferryl form of iron (Fe IV) and a protoporphorin radical cation. The oxidized heme group oxidizes a neighboring tyrosine residue to yield a spectral Intermediate II having an oxyferryl Fe IV and a tyrosyl radical. This Intermediate II can then cycle back

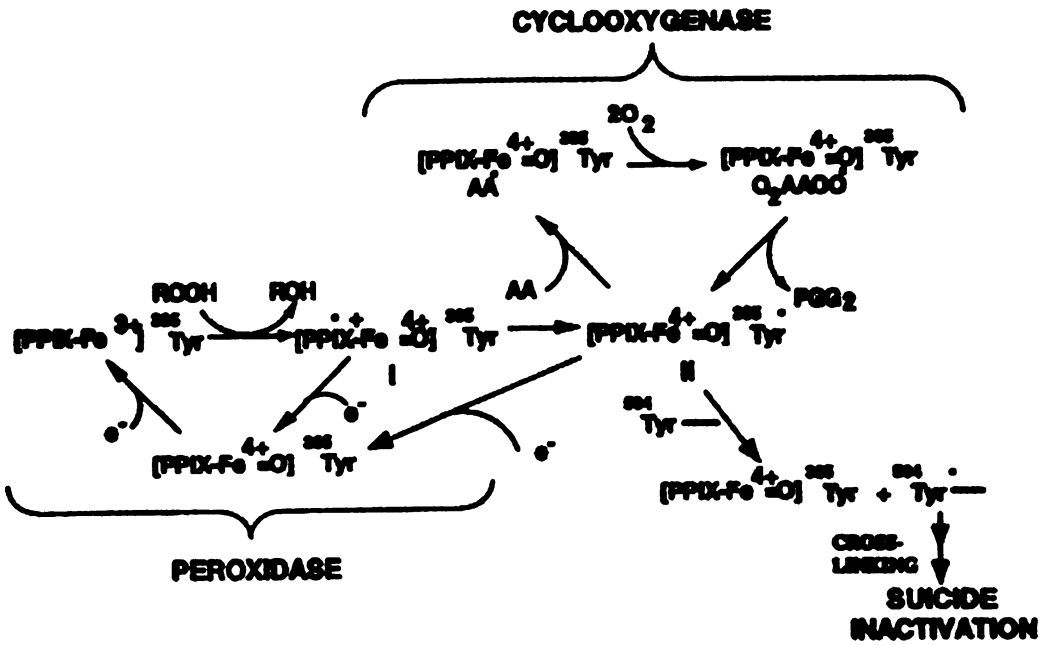


Figure 7. The Ruf Model. Model for peroxide-dependent activation of cyclooxygenase activity of PGH synthase via formation of an intermediate tyrosyl radical (60).

to Intermediate I or undergo suicide inactivation. The catalytic rate of the two enzymes has been shown to be similar and both isozymes of PGHS have been shown to undergo suicide inactivation (56). The mechanism underlying the suicide inactivation has not yet been determined.

Substates of PGHSs

The PGHS isozymes have been shown to be capable of oxygenating a variety of 18- and 20- carbon polyunsaturated fatty acids. Both isozymes show a preference for arachidonate (61). The K_m for arachidonic acid for both enzymes is 5 μM. PGHSs use arachidonic acid as the substate for the formation of the 2-series prostaglandins. The 1-series of prostaglandins are formed from 8,11,14-eicosatetrienoate while the 3-series prostaglandins are formed from 5,8,11,14,17-eicosapentaenoate (EPA). n-3 and n-9 Polyunsaturated fatty acids are poor substrates for the enzymes, but are effective inhibitors of the oxygenation reaction (62, 63). 22:6 n-3 is a competitive inhibitor for both isozymes without being a substrate for either (62). In addition, PGHSs can use the 18 carbon fatty acids (18:2 and 18:3) for the production of hydroxy fatty acids (Figure 8).

Little is known about the roles of the individual residues, within the cyclooxygenase site, in determining substrate specificity for the two isozymes. Laneuville *et al* demonstrated that the two human isozymes show different affinities for the various fatty acid substrates with PGHS-2 being more flexible in the use of the various substrates (61).

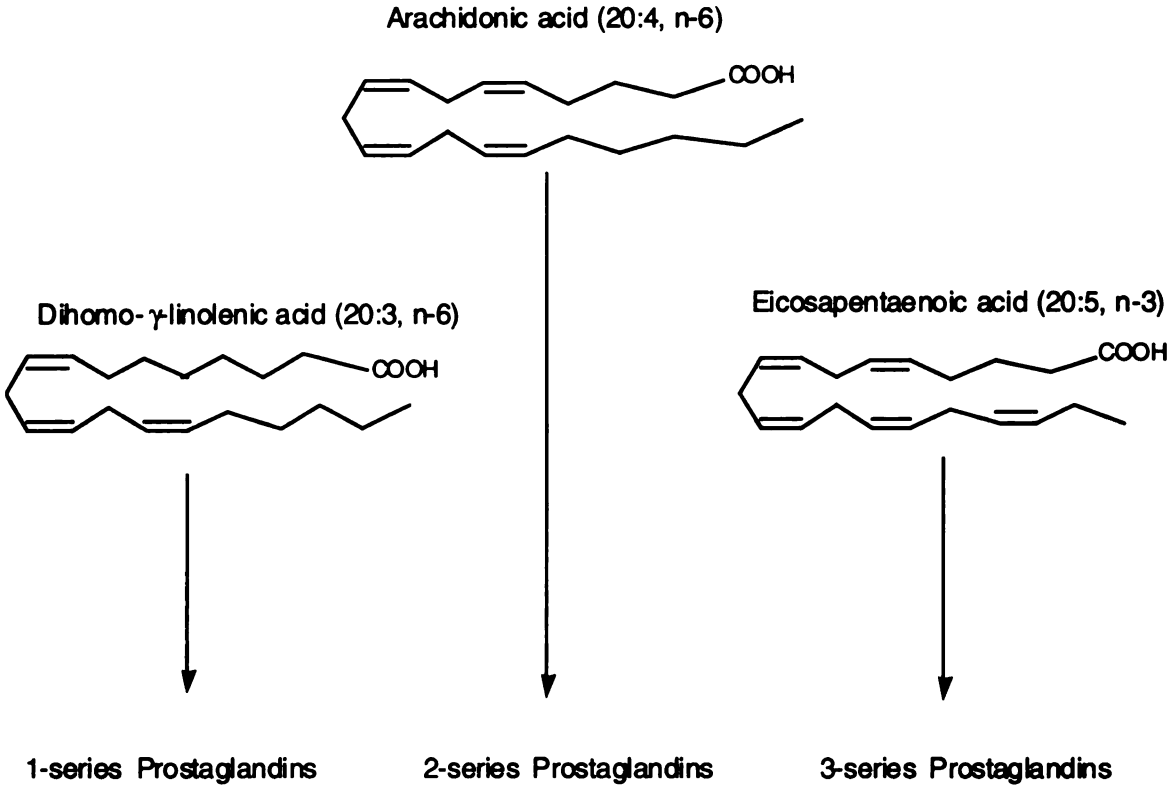


Figure 8. Formation of the 1-, 2- and 3-series prostaglandins
PGHSs use arachidonic acid as the substate for the formation of the 2-series prostaglandins. The 1-series of prostaglandins are formed from 8,11,14-eicosatetrienoate while the 3-series prostaglandins are formed from 5,8,11,14,17-eicosapentaenoate (EPA).

Inhibition of PGHS

Nonsteroidal antiinflammatory drugs (NSAIDs) directly inhibit the cyclooxygenase reaction of PGHSs by competing with arachidonic acid for binding at the cyclooxygenase active site. The interaction of most NSAIDs with PGHSs follow a two-step sequence (64):

$$E + I \longleftrightarrow EI \longleftrightarrow EI^*$$

The first step is rapid and reversible and leads to competitive inhibition of the enzyme. The subsequent step is conversion of the initial enzyme-inhibitor complex to one in which the inhibitor is more tightly bound and functionally irreversible.

NSAIDs can generally be classified into three groups based on their modes of action. Class I NSAIDs, of which ibuprofen and naproxen are examples, show simple competitive inhibition. Class II NSAIDs, flurbiprofen and indomethacin, show a competitive time-dependent slowly reversible interaction with the enyzme. The mechanism for the ability of the class II NSAIDs to form a semistable EI* complex is not yet understood. It has been demonstrated that all of the non-selective, time-dependent inhibitors contain a carboxylic acid moiety and at least one halogen substituent (64). Previously it had been demonstrated that substitution of a methyl ester for the carboxylate group of these inhibitors resulted in a lost of time-dependent inhibition (64). Class III NSAIDs, aspirin and related acylsalicylates, show a competitive, time dependent, irreversible inhibition (covalent modification). Aspirin binds to PGHS with a low affinity and upon

binding transfers its acetyl group from the salicylate to a serine residue (S530) of the enzyme (21).

Generally, all commonly used NSAIDs have been shown to inhibit both PGHS-1 and PGHS-2, although with some differences in affinity as shown by differences in the IC_{50} values between the two isozymes (56,55,65). In addition, it has been shown that while aspirin abolishes the cyclooxygenase activity of PGHS-1, it causes PGHS-2 to produce 15R-HETE upon acetylation of the serine residue (S516) (56,66).

Because of the many side effects associated with the NSAIDs, particularly gastric irritation, much effort has gone into producing compounds specific for the second isozyme of PGHS. Recently several PGHS-2 specific inhibitors have been produced. These inhibitors show a time dependent, slowly reversible inhibition with PGHS-2 but a simple competitive inhibition with PGHS-1 (67,68). In addition, in animal models of inflammation, PGHS-2 specific inhibitors are able to reduce the pain and swelling associated with inflammation without the concomitant gastric irritation (69).

The mechanism underlying this selectivity is not yet understood. All of these inhibitors contain an arylsulfur group rather than a carboxylate group. In addition mutagenesis studies have shown that by exchanging several PGHS-2 residues for those found in PGHS-1 the time dependence can be abolished. In particular, substitution of Ile for Val at residue 523 causes PGHS-2 to exhibit PGHS-1 kinetics with some of the PGHS-2 specific inhibitors (70). Another residue which

has been demonstrated to play a role in determining selectivity for PGHS-2 specific inhibitors is His513 (71).

Based on the crystal structure of PGHS-1 it has been postulated that Arg120 plays a critical role in binding of the substrate, arachidonic acid, and in binding carboxylate-containing NSAIDs such as flurbiprofen. Another hypothesis that was proposed based on the position of Tyr355 was that this latter residue may play a role in the selectivity toward stereoisomers of NSAIDs of the 2-phenyl-propionic acid class. In order to test these hypotheses, mutants of Arg120 and Tyr355 were prepared, and functional studies performed on these mutant PGHSs. In addition, mutations of the homologous Arg120 residue of PGHS-2 (Arg106) were made and experiments performed to compare the role of the Arg120 residue between the two isozymes.

The research presented in this thesis focuses on functional and mechanistic studies involving the residues Arg120 of oPGHS-1 and hPGHS-2 and Tyr355 of oPGHS-1 at the mouth of the active sites of the two PGHS isozymes. Chapter 2 presents investigations involving Arg120, of both oPGHS-1 and hPGHS-2, in interactions with substrate and inhibitors and compares the role of this residue in the two isozymes. Chapter 3 looks at the role of Tyr355 in interaction with substrates and in NSAID stereospecificity.

CHAPTER 2

STUDIES TO DETERMINE THE ROLE OF ARG120 oPGHS-1 AND ARG120 hPGHS-2 IN SUBSTRATE AND INHIBITOR INTERACTIONS

Introduction

Examination of the oPGHS-1 crystal structure complexed with flurbiprofen suggests that Arg120 is positioned at the mouth of the cyclooxygenase active site such that this residue interacts with the carboxylate group of fatty acid substrates and inhibitors (43). To test this hypothesis several mutations were made at this position and the kinetic properties of the mutant enzymes examined. Initial characterization of R120K, R120Q and R120E mutant enzymes supported the hypothesis. Mutations of Arg120 increased the K_m for arachidonic acid up to greater than 500 fold and decreased cyclooxygenase activity (72). Further experiments were performed to examine the effect of the R120Q mutation on time dependent inhibition by flurbiprofen.

It was postulated that the homologous Arg120 in hPGHS-2 (Arg106) may also interact with fatty acid substrates and carboxylic acid containing inhibitors in a similar way. To determine if this is the case two mutations were made at this position and the kinetic properties of the mutant enzymes were examined using a variety of fatty acid substrates. In addition, flurbiprofen and several PGHS-2 specific inhibitors were tested to determine if Arg120 is involved in determining the selectivity of PGHS-2 for specific inhibitors.

Figure 9 shows the active sites of native oPGHS-1 and R120Q oPGHS-1 with arachidonic acid modeled into the cyclooxygenase site. Figure 10 shows mPGHS-2 and R120Q mPGHS-2 with arachidonic acid modeled into the cyclooxygenase site.

Figure 9. Model of active site of oPGHS-1 and R120Q oPGHS-1 with arachidonic acid modeled into the cyclooxygenase site. . Coordinates were obtained from the Protein Data Bank and molecular modeling was performed using InsightII.Arachidonic acid is shown in light blue, the heme is red and residue 120 is dark blue

A. Native oPGHS-1
B. R120Q oPGHS-1

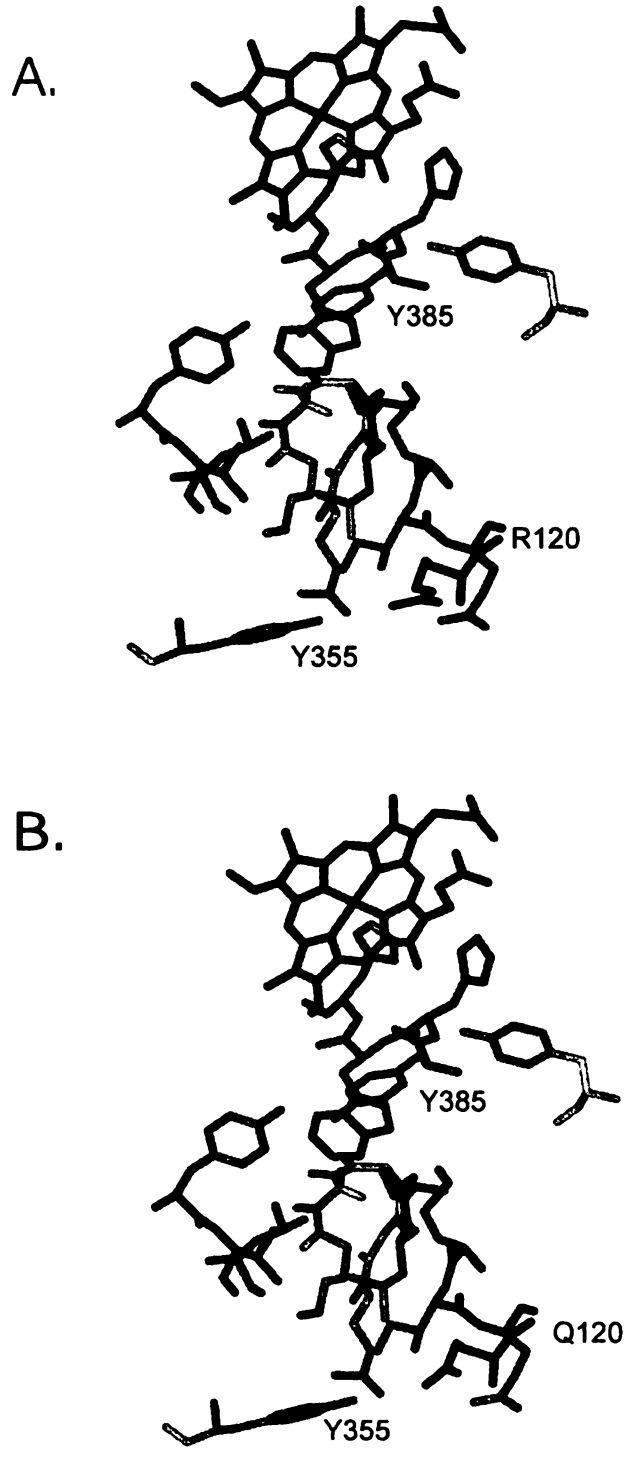
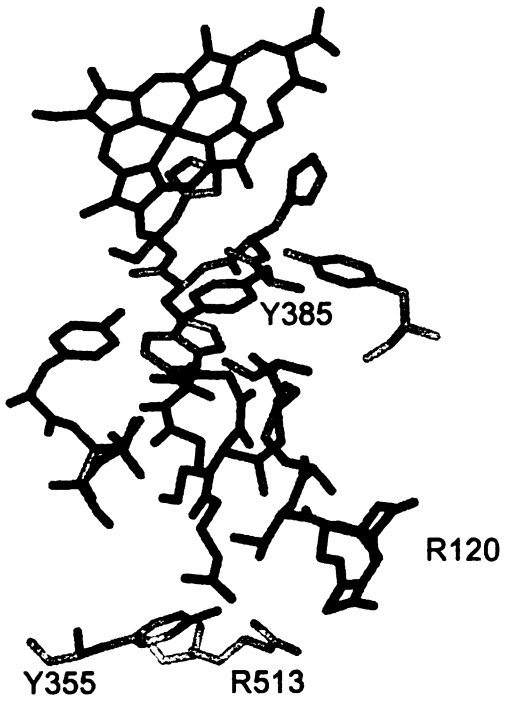


Figure 9.

Figure 10. Model of active site of mPGHS-2 and R120Q mPGHS-2 with arachidonic acid modeled into the cyclooxygenase active site. . Coordinates were obtained from the Protein Data Bank and molecular modeling was performed using InsightII.Arachidonic acid is shown in light blue, the heme is red and residue 120 is dark blue

A. Native mPGHS-2
B. R120Q mPGHS-2

A.



B.

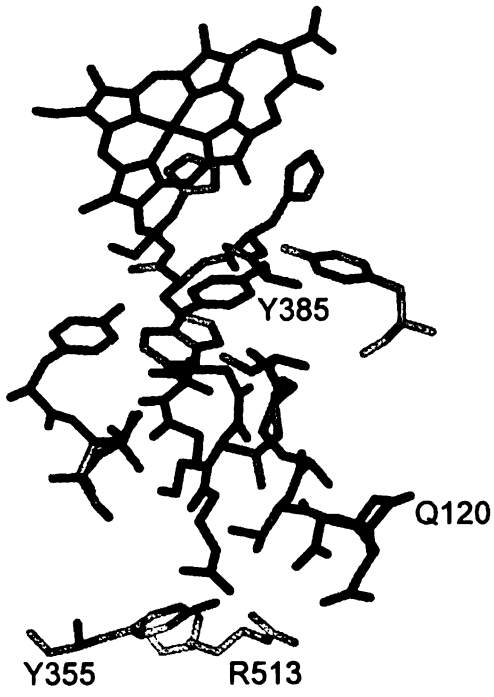


Figure 10

Materials- DMEM was from Life Technologies, Inc. Fetal calf serum and calf serum were from Hyclone. Chloroquine, bovine hemoglobin, DEAE-dextran and penicillin G were from Sigma.CsCl from Boehringer Mannheim. 20:4 n-6, 18:2 n-6 20:5 n-3, 20:2 n-6 and 20:3 n-6 were from Cayman Chemical Co. [1-^{14}C]-arachidonic acid (50-55 mCi/mmole) was from DuPont NEN. All other reagents were purchased from common commercial sources.

Preparation of Mutants by Site Directed Mutagenesis- The R120Q mutant of PGHS-1 was prepared as previously described (72). Briefly, M13mp19-oPGHS-1, which contains the coding region of oPGHS-1, was mutated using a Bio-Rad Muta-Gene kit following manufacterer's instructions. The phage samples were sequenced to identify the mutant. The 2.3 kb insert from the replicative form of M13mp19-oPGHS-1 containing the desired mutation was isolated after digestion with *Sal I* and subcloned into the *Sal I* site of pSVT7. The correct orientation of the insert was determined by restriction digestion with *Pst I*. Plasmids used for transfection were purified by CsCl density gradient ultracentrifugation.

The hPGHS-2 mutants were prepared using a Bio Rad Muta-Gene kit. M13mp19- hPGHS-2, which contains the coding region of PGHS-2, was mutated following manufacterer's instructions. The single stranded M13 phage samples were sequences using the dideoxy method to identify mutants (74). The 1.8 kb insert, from the replicative form of M13mp19-PGHS-2, containing the desired

mutation, was isolated by *Sal I* digestion and subcloned into the *Sal1* site of pOSML (55). The correct orienation was confirmed by restriction digestion with *Pst I*. The double stranded pOSML-hPGHS-2 was sequenced to confirm that the mutation was still present. Plasmids used in transfection were purified by CsCl gradient ultracentrufigation.

The oligonucleotide primers used to prepare the mutants are as listed:

oPGHS-1 R120Q 5'-GTACTCACAGTGGAATCCAACCTTATCCC-3"
hPGHS-2 R120Q 5'-GTGTTGACATCCCAGTCACATTTGATT-3'
hPGHS-2 R120L 5' -GTGTTGACATCCCTATCACATTTG-3'

Transfection- *Cos* -1 cells (ATCC CRL-1650) were grown to near confluency in DMEM containing 8% calf serum and 2% fetal calf serum in a water-saturated 5% CO_2 atmosphere. Approximately 16 hr prior to transfection the cells were subcultured 1:2. The *cos* -1 cells were transfected using the DEAE-dextran/chloroquine method as previously described (21). Forty hours post transfection the cells were harvested by scraping in 3 ml of ice-cold phosphate buffered saline (PBS) with a rubber policeman and collected by centrifugation at 1200 rpm for 5 mins.

Preparation of Microsomes- Pelleted cells from 20-50 plates were resuspended in 4-5 ml of ice-cold 0.1 M Tris-Cl, pH 7.4 and disrupted by sonication The sonicated cells were centrifuged at 10,000 rpm for 10 mins at 4^0 C and the resulting supernatents centrifuged at 45,000 rpm for 50 mins in a Beckman SW50.1 swinging rotor to give a microsomal membrane fraction. Microsomal

membranes were resuspended by homogenation in a volume of 0.1 M Tris-Cl, pH 7.4 sufficient to yield a final protein concentration of about 5 mg/ml. Protein concentrations were determined by a modified Lowrey method, using BSA as a standard (75).

Cyclooxygenase Assays- Cyclooxygenase assays were carried out at 37^0 C using a Yellow Springs Instruments Model 5300 oxygen electrode. A typical assay contained 3 ml 0.1 M Tris-Cl, pH 8.0 with 1 mM phenol, 100 μM arachidonate and 25 μg of hemoglobin (as a source of heme). Reactions were initiated by the addition of enzyme to the chamber and initial rates of oxygen consumption determined.

For instantaneous inhibition, NSAIDs, at the appropriate concentration, were added to the assay chamber prior to the addition of the enzyme. For time-dependent inhibition studies, NSAIDs, at an appropriate concentration were pre-incubated with the enzyme (250 μg) at 37^o C for various times; reactions were then initiated by the addition of the enzyme/inhibitor complex to the assay chamber, and initial rates of oxygen consumption determined. For studies of substrate specificity 100 μM 20:5 n-3, 18:2 n-6, 20:3 n-6, or 20:2 n-6 were used. K_m determinations were performed for 20:4 n-6, 20:5 n-3 and 18:2 n-6 using varying concentrations of these substrate.

Peroxidase assays- Peroxidase assays on microsmal protein were performed spectrophotometrically using a Perkins-Elmer model 552A Double Beam UV/VIS

spectrophotometer, measuring the oxidation of 3,3,3',3'- tetramethyl-phenylenediamine (TMPD) at 611 nm (78).

Western transfer blotting- Microsomal membranes were resolved by one-dimensionsal SDS-PAGE and transfered electrophoretically to 0.45 μm nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked 0.5-12 hr in 3% (w/v) dry milk, 0.1 % (v/v) Tween-20/Tris buffered saline (TBS) followed by an incubation with a 1:3000 dilution of the affinity purified antibody (Dinah) in 1% dry milk, 0.1% Tween-20/TBS for 2 hours at 25^0 C. Membranes were washed and incubated with a 1:1000 dilution of a goat anti-rabbit IgG horseradish peroxidase for 1 hour. The membrane was washed and incubated with Amersham ECL reagents and exposed to Kodak XAR film for chemiluminescence.

Product Analysis by Thin Layer Chromotagraphy- Microsomal membrane fractions (250 μg) were incubated with [1-^{14}C]-arachidonic acid for 10 mins. Reactions were stopped with the addition of 1.4 ml CCl_4:Me (1:1) and centrifuged at 3,000 rpm for 5 min. Supernatants were transfered to clean tubes and 0.6 ml CCl_4 and 0.32 ml 0.88% formic acid added. After centrifugation the organic layer was removed and dried under N_2. The dried samples were redissolved in 50 μl of CCl_4 and spotted on Silca Gel 60 thin layer chromatography plates. Plates were developed twice in benzene: dioxane: acetic acid: formic acid (82:14:1:1) and exposed to XAR-5 film for 48 hours to allow visualization of the products.

Prostaglandin synthesis by hPGHS-2 and the R120 mutant enzymes was quantified by densitometry using a Molecular Dynamics Storm 820 phosphoimager with ImageQuant software.

Results

Native oPGHS-1 and the R120Q mutant (R120Q oPGHS-1) were expressed transiently in *cos* -1 cells and microsomal membrane fractions prepared from these cells were assayed for cyclooxygenase and peroxidase activity. Initial characterization had determined that the native and mutant enzymes were expressed at comparable levels in *cos*-1 cells and that the various prostaglandin products were produced in the same proportions by both enzymes (72). The R120Q mutant enzyme had 3- 5% of the cyclooxygenase activity of the native, with 100 μM arachidonic acid, but retained 60% of the peroxidase activity. The K_m values for archidonic acid were determined for the native oPGHS-1 and the R120Q mutant. The native enzyme had a K_m of 4 μM while the value for the R120Q mutant was approximately 1000 fold greater (3300 μM).

Flurbiprofen causes a time dependent inhibition of oPGHS-1 but the methyl ester of flurbiprofen does not (64). The following experiments were performed to determine if flurbiprofen inhibits R120Q oPGHS-1 time-dependently. First the IC_{50} values for "instantaneous" inhibition was determined. The values were 5 μM and 1 mM for native PGHS-1 and R120Q oPGHS-1 respectively. To determine if the R120Q enzyme undergoes time-dependent inhibition with flurbiprofen the enzyme was incubated with 1 mM flurbiprofen for varying lengths of time. No time dependent inhibition of the mutant enzyme was seen even with incubation periods of up to 20 minutes (Figure 11).

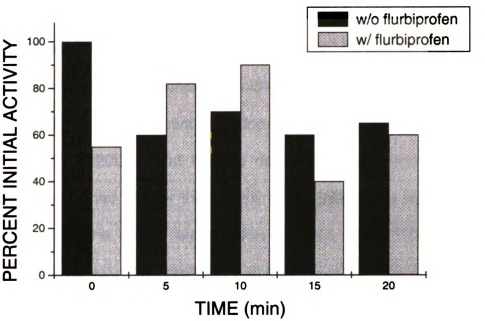


Figure 11. Time course for inhibition of R120Q oPGHS-1 by flurbiprofen
Microsomes prepared from *cos*-1 cells expressing R120Q oPGHS-1 were incubated for the indicated times at 37^0 C with or without 10^{-3} M flurbiprofen and assayed for cyclooxygenase activity using an oxygen electrode. The experiment shown in this figure was performed three times with similar results.

To determine if the R120Q hPGHS-2 enzyme shows similar kinetic properties native hPGHS-2 and R120 hPGHS-2 mutants were transiently expressed in *cos*-1 cells, and microsomal membrane fractions prepared from the transfected cells. Levels of protein expression were determined by Western transfer blotting and quantified by densitometery using Bio-Rad Molecular Analysis software. hPGHS-2, R120L hPGHS-2 and R120Q hPGHS-2 were expressed at similar levels (Figure12). The cyclooxygenase activity, measured using an oxygen electrode and 100 μM arachidonic acid as substrate, indicated that the R120Q mutant had the same amount of cyclooxygenase activity as the native enzyme but that the R120L mutant had no detectable cyclooxygenase activity. The R120Q mutant had greater than 100% of the peroxidase activity of the native enzyme while the R120L enzyme has 17% of native hPGHS-2 peroxidase activity. The K_m value for arachidonic acid for the R120Q mutant was determined to be 10 μM compared to 8 μM for the native enzyme (Table 1). Assays using [1-^{14}C]-arachidonic acid and 1000 μg of membrane protein revealed that the R120L mutant did have approximately 20% of the activity of the native hPGHS-2. Both the R120Q and the R120L mutant enzymes produced the same products as the hPGHS-2 enzyme although in slightly different proportions (Figure 13). The most obvious difference was in the production of monohydroxy fatty acids (HETE's). Both the R120Q and R120L mutants produced twice as much 11-HETE as the native hPGHS-2, 26% vs 13% respectively. The R120Q mutant had a $t_{1/2}$ for suicide inactivation of 22 sec.

Table 1. Kinetic properties of hPGHS-2 and R120 mutant PGH synthases
Cos-1 cells were transfected with plasmid encoding native hPGHS-2 or R120 mutant hPGHSs, and microsomal membrane fractions prepared as described in the text.. Cyclooxygenase activity was determined by oxygen electrode and peroxidase activity determined spectroscopically, as described in the materials and methods section.

Enzyme	Percent COX Activity	Percent POX Activity	K_m AA (μM)
hPGHS-2	100%	100%	8
R120Q	100%	132%	10
R120L	0%	17%	ND

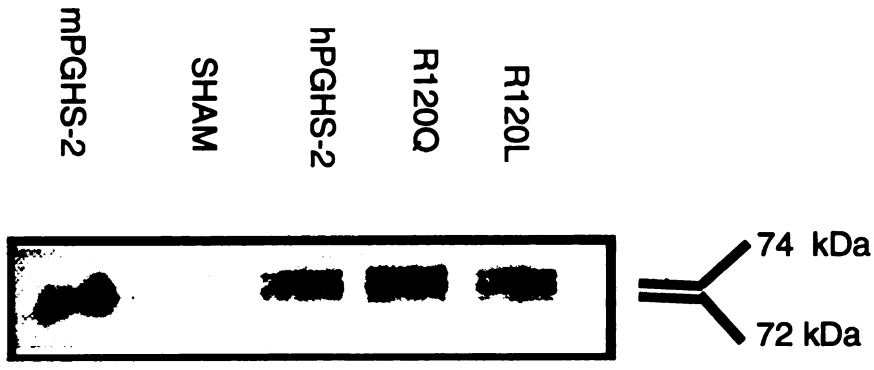


Figure 12. Western blot analysis of hPGHS-2 and R120 mutant PGH synthases. *Cos*-1 cells were sham-transfected or transfected with native hPGHS-2, R120Q hPGHS-2, or R120L hPGHS-2. Microsomal fractions were prepared and 50 μg of protein was subjected to Western transfer blotting as described in the material and methods section.

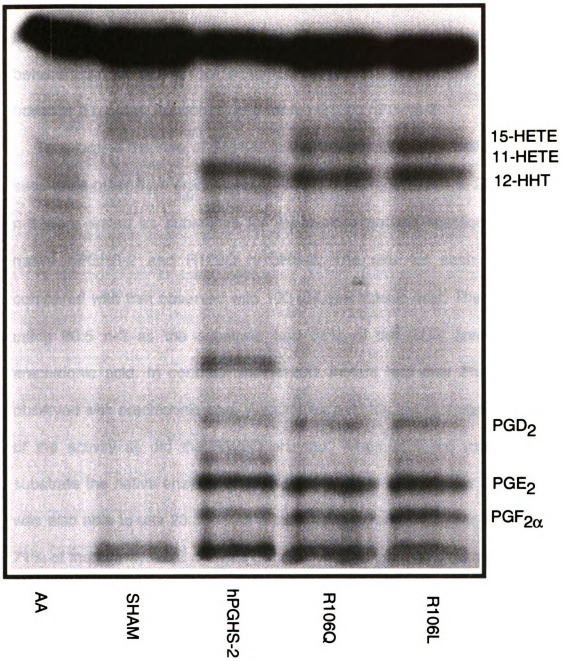


Figure 13. Product characterization of native and R120 mutant PGH synthases Microsomes containing sham, native or mutant hPGHS-2 protein were incubated with [1-^{14}C]-arichadonic acid for 10 minutes and the reaction products separated by thin layer chromatography as described in the text

compared with 15 sec for native hPGHS-2. Using radio thin layer chromatography it was also determined that the R120L enzyme does undergo inactivation, because there was no change in the total product formation between 5 and 10 min for either the native enzyme or the R120L mutant; however a $t_{1/2}$ was not determined for the R120L hPGHS-2.

To examine the role of Arg120 in the binding and oxygenation of fatty acid substrates other than arachidonate 100 μM 20:5 n-3, 20:3 n-6, 20:2 n-6 or 18:2 n-6 were tested as substrates for the cyclooxygenase reaction with both the native hPGHS-2 and R120Q hPGHS-2. The rate for each substrate was compared with that observed with 100 μM arachidonic acid. The native enzyme using 20:5 n-3 as the substrate had 38% of the COX activity found with arachidonic acid. In contrast, the R120Q mutant had only 3% of the activity observed with arachidonic acid. With linoleic acid the native enzyme had 50-60% of the activity as did the R120Q enzyme. When 20:3 n-6 was used as the substrate the native enzyme exhibited 100% of the activity. The R120Q enzyme was also able to use 20:3 n-6 as a substrate, although not quite as well, having 71% of the activity. 20:2 n-6 was not used efficiently by either enzyme; the native enzyme had only 9% activity while the mutant enzyme had no detectable activity. The K_m values for 20:5 n-3 and 18:2 n-6 were determined for native hPGHS-2 and R120Q hPGHS-2. For 20:5 n-3 the values were approximately 2 μM and 2000 μM respectively. With 18:2 n-6 the K_m was approximately 22 μM for the R120Q mutant, which is within the range previously reported for native hPGHS-2 (61). The data are summarized in Table 2. It has been previously demonstrated

that the addition of 15-HPETE to the reaction when 20:5 n-3 is used as the substrate leads to an increase in V_{max}, for PGHS-1 but not for PGHS-2. To determine if this is also true of the R120Q mutant 10 μM 15-HPETE was added to the cyclooxygenase assay mixture; no significant change in V_{max} was observed.

Flurbiprofen causes time-dependent inhibition of native hPGHS-2 similiar to that of oPGHS-1. To determine if the Arg120 in hPGHS-2 plays a similiar role in inhibition as that of Arg120 in oPGHS-1 "instantaneous" and "time-dependent" inhibition experiments were performed. Native hPGHS-2 had an IC_{50} value for flurbiprofen of 1 μM and underwent time-dependent inhibition with a $t_{1/2}$ of less than 1 minute. This is in contrast to the R120Q mutant which had a IC_{50} value of 0.5 mM and did not undergo time-dependent inhibition during a 20 min incubation with 0.5 mM flurbiprofen (Figure 14).

Several PGHS-2 specific inhibitors have been shown to cause time-dependent inhibition with PGHS-2 but simple competitive inhibition of PGHS-1. We examined the role of Arg120 in the interactions with several of these inhibitors. Figure 15 shows the structures of the compounds used. Native hPGHS-2 had an IC_{50} value with NS-398 of 50 μM and exhibited time-dependent inhibition. The R120Q mutant had a 5 fold lower IC_{50} value (10μM) but did not undergo time-dependent inhibition (Figure 16). On the other hand, the mutant enzyme showed an increased IC_{50} value and a decreased $t_{1/2}$ for time-dependent

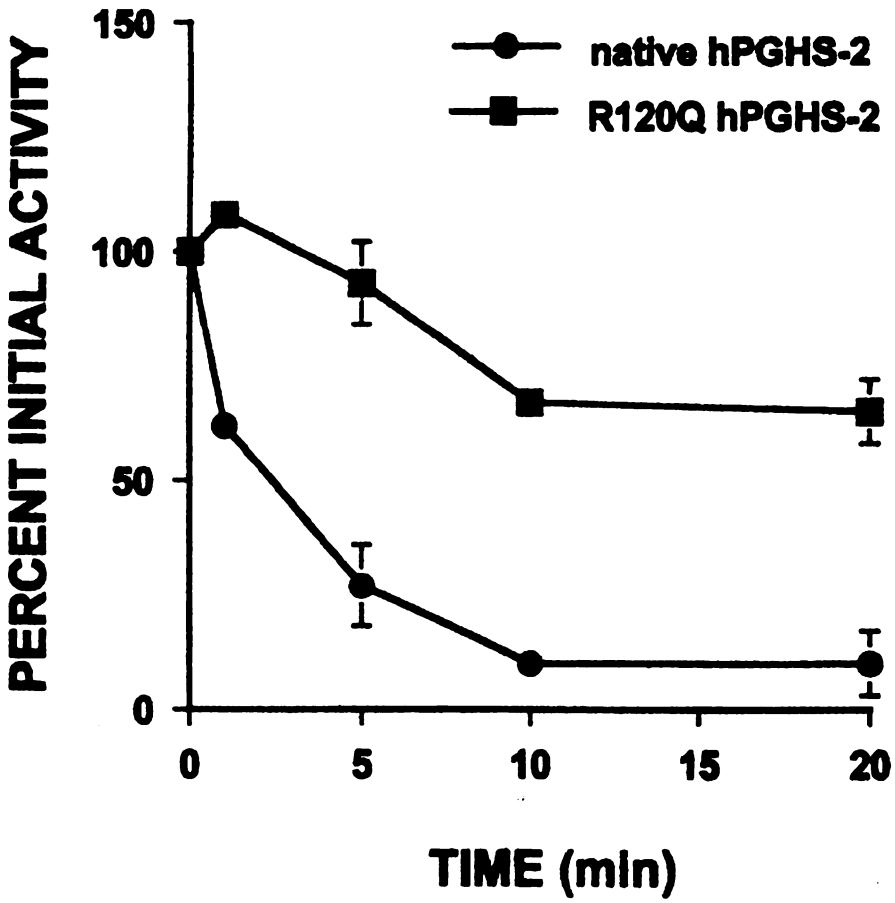
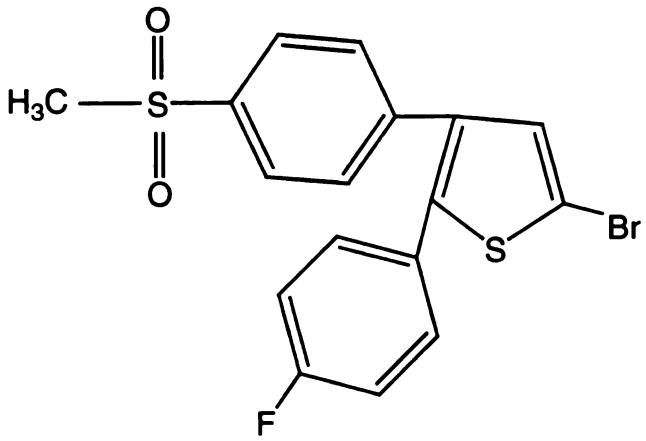


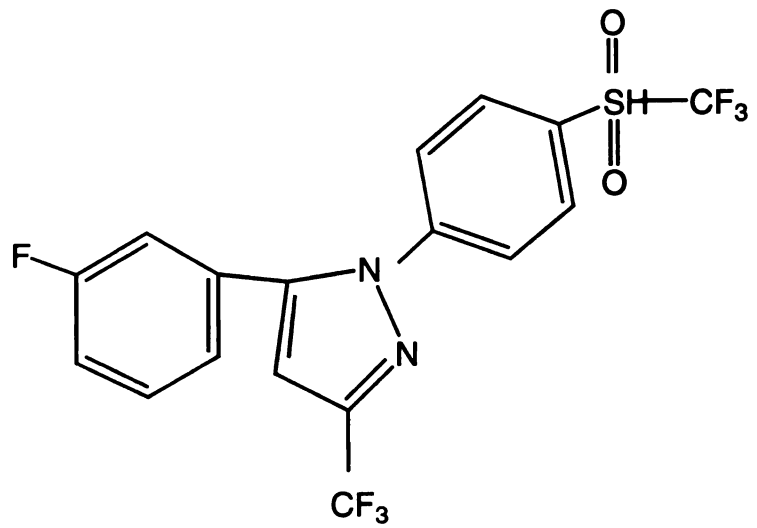
Figure 14. Time-dependent inhibition of hPGHS-2 and R120Q hPGHS-2 by flurbiprofen Microsomes prepared from *cos*-1 cells transfected with hPGHS-2 or R120Q hPGHS-2 were incubated with 500 μM flurbiprofen for the indicated times and assayed for initial cyclooxygenase activity as described. Measurements at each time point were done in duplicate or triplicate and the experiment was repeated several times with similar results.

Table 2. Comparison of Substrate Specificity for hPGHS-2 and R120Q hPGHS-2 V_{max} and K_m values for cyclooxygenase activity were determined by oxygen electrode using varying concentrations of fatty acid substrates as described in the text. Measurements were performed in duplicate or triplicate for each substrate concentration.

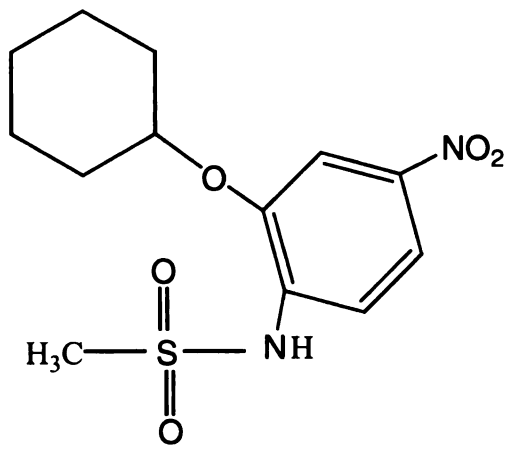
SUBSTRATE	ENZYME							
	hPGHS-2				R120Q			
	K_m (μM)	rate (100μM)	V_{max}	V_{max}/K_m	K_m(μM)	rate (100μM)	V_{max}	V_{max}/K_m
20:4 n-6	8	100%	108%	13	10	100%	110%	11
20:5 n-3	2.5	45%	46%	18.4	3000	4%	124%	0.04
20:3 n-6	ND	97%	ND	ND	ND	71%	ND	ND
20:2 n-6	ND	18%	ND	ND	ND	0%	ND	ND
18:2 n-6	30	60%	78%	2.6	22	60%	73%	3.3

Dup-697





SC-58125



NS-398

Figure 15. Structures of PGHS-2 specific inhibitors tested with hPGHS-2 and R120Q hPGHS-2

inhibition with Dup-697 (Figure 17). SC-58125 has an aryl sulphur group similar to that of Dup-697, and it was anticipated that SC-58125 would exhibit a similiar inhibition profile with the R106Q enzyme. This appeared to be the case. Native hPGHS-2 was not inhibited "instantaneously" by SC-58125 while the R120Q enzyme has a IC_{50} value of 2.5 μM. Both enzymes underwent time-dependent inhibition (Figure 18). The data from the inhibitor studies is summarized in Table 3.

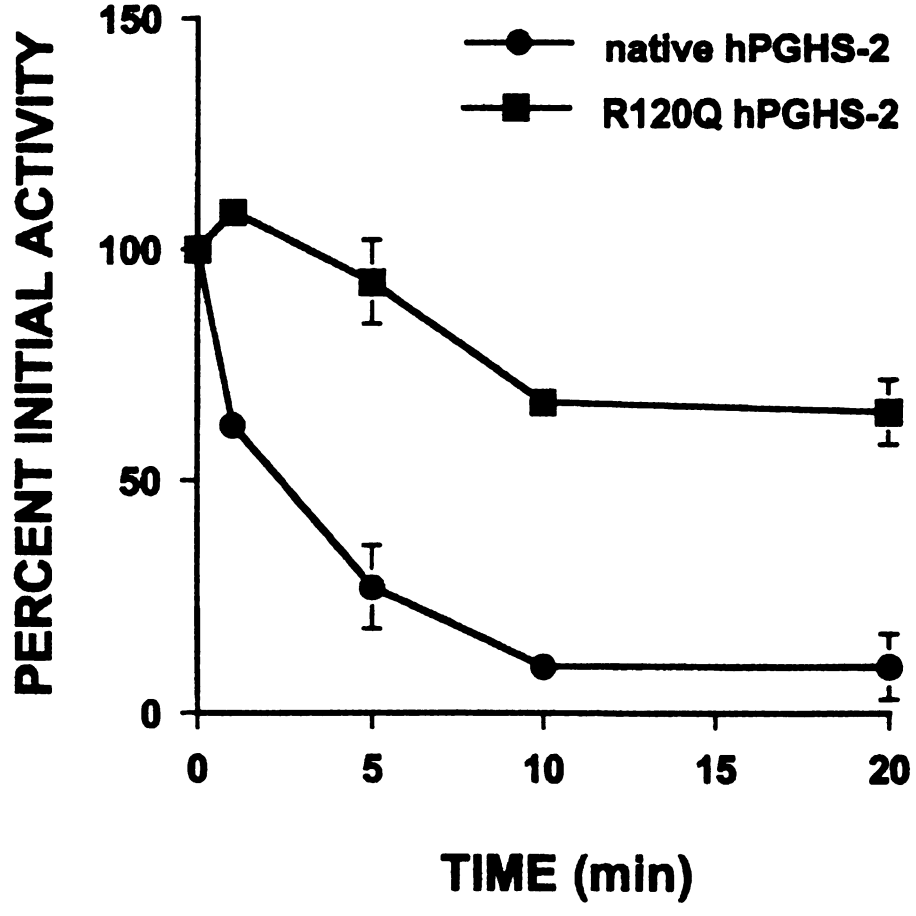


Figure 16. Time-dependent inhibition of hPGHS-2 and R120Q hPGHS-2 by NS-398 Microsomes prepared from *cos*-1 cells transfected with hPGHS-2 or R120Q hPGHS-2 were incubated with 1 μM NS398 for the indicated times and assayed for initial cyclooxygenase activity as described. The experiment was repeated several times with similar results.

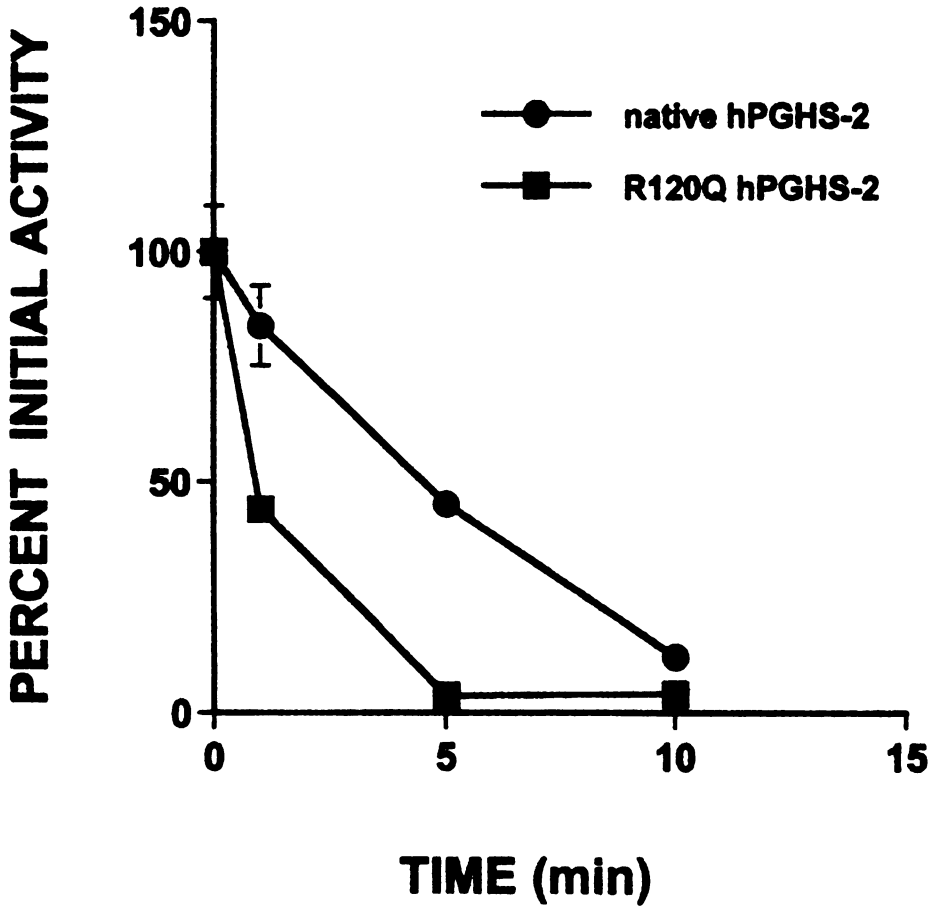


Figure 17. Time-dependent inhibition of hPGHS-2 and R120Q hPGHS-2 by Dup-697 Microsomes prepared from *cos*-1 cells transfected with hPGHS-2 or R120Q hPGHS-2 were incubated with 1μM Dup-697 for the indicated tiimes and assayed for initial cyclooxygenase activity. Measurements for each time point were performed in duplicate and the experiment was performed several times with similar results.

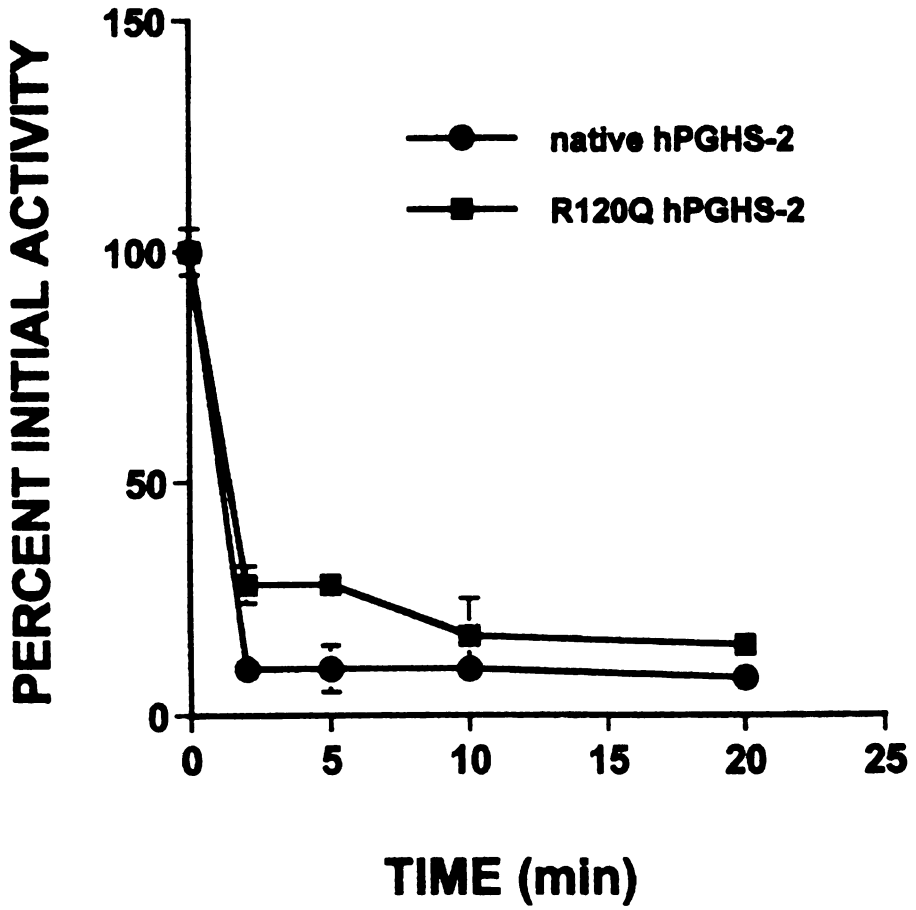


Figure 18. Time-dependent Inhibition of hPGHS-2 and R120Q hPGHS-2 by SC-58125 Microsomes prepared from *cos*-1 cells transfected with hPGHS-2 or R120Q hPGHS-2 were incubated with 10μM SC58125 for the indicated times and assayed for cyclooxygenase activity, The experiment was repeated at least two times with similar results.

Table 3. Instantanous and time-dependent inhibition of native hPGHS-2 and R120Q hPGHS-2. Summary of results of inhibition studies described in detail in the text.

INHIBITOR	ENZYME			
	hPGHS-2		R120Q	
	IC_{50}	$t_{1/2}$	IC_{50}	$t_{1/2}$
flurbiprofen	1 μM	< 1min @ 0.5 mM	0.5 mM	> 20 min @0 .5 mM
NS-398	50 μM	2 min @ 1μM	10 μM	> 20 min @1 μM
Dup-697	0.5 mM	4 min @ 1μM	0.5 μM	1 min @ 1 μM
SC-58125	> 1 mM	< 2min @ 10μM	2.5 μM	< 2 min @ 10 μM

Discussion

In these experiments the role of the Arg120 residue of oPGHS-1 and the Arg120 residue of hPGHS-2, in the binding of fatty acid substrate and nonsteroidal anti-inflammatory drugs (NSAIDs) in the cyclooxygenase active site, was examined. The results support the hypothesis that Arg120 is an important residue in the binding of the carboxylate group of arachidonic acid and inhibitors. Replacing this arginine with a neutral glutamine residue allowed the enzyme to retain a small amount of the cyclooxygenase active and about 60% of the peroxidase activity, but the K_m value for arachidonic acid was increased approximately 1000 fold. This is in contrast to the results obtained with the R120Q hPGHS-2, which had no significant changes in kinetic properties with arachidonic acid as the substrate.

However, replacing this Arg residue with a smaller hydrophobic residue, leucine, resulted in a significant loss of cyclooxygenase activity, suggesting that, in hPGHS-2, while a hydrogen bonding interaction can replace an ionic interaction at this position some interaction is necessary in order for cyclooxygenase activity with arachidonic acid. Previously, it has been demonstrated that there are some differences in substrate specificity, between the two PGHS isozymes, which have been attributed to a larger cyclooxygenase active site in PGHS-2 (61). These results suggest that some of these differences in specificity may result from a need for different stabilizing interactions at the mouth of the cyclooxygenase active site.

With the majority of the other substrates tested (18:2 n-6, 20:3 n-6, 20:2 n-6) it appears that the hydrogen bond can substitute for the ionic interaction. However, when 20:5 n-3 is used as a substrate it appears that the ionic interaction is extremely important as is shown by the dramatic decrease in cyclooxygenase activity and increase in K_m by the R120Q mutant with this substrate. One possible explanation for this could be that with the loss of the ionic interaction the fatty acid is not positioned correctly in the active site. Because the 20:5 n-3 is not in an optimal position to begin with, the loss of this stabilizing interaction could lead to the fatty acid not remaining in the active site in a conformation in which the n-8 hydrogen can be abstracted, as efficiently as by the native enzyme. For arachidonic acid the loss of the ionic interaction does not appear to affect the kinetic factors to any appreciable degree. This probably is due to the carboxylate group of the fatty acid being positioned in such a way that it is still able to form a hydrogen bond interaction with the glutamine. In addition, there may be other interactions with the protein that assist in stabilizing the fatty acid such as one with Arg513. The corresponding residue is a histidine in oPGHS-1. When 20:5 n-3 is the substrate, it is likely that the carboxylate group is not in proximity of the glutamine to form a hydrogen bonding network or that the hydrogen bonding is not sufficient to hold the fatty acid in the active site. Additionally, the fatty acid may be poorly positioned to interact with other potentially stabilizing residues. With the loss of the stabilizing interaction at the mouth of the active site, the fatty acid could then migrate up into the active site

or down through the floor of the active site, so that it is no longer in position for interaction with Tyr385.

There is a difference in the ratio of the products formed with the R120Q; 11-HETE is produced in greater proportions of the products than with the native enzyme. This provides evidence for the position of the arachidonic acid being different in the mutant enzyme. The importance of this type of interaction has also been shown with the lipoxygenase enzyme, where substitution of the the arginine that interacts with the carboxylate group of arachidonic acid by a leucine results in a change in proportion of products (77).

NSAIDs can be grouped into two general catagories based on their ability to cause time-dependent vs simple competitive inhibition. Flurbiprofen causes a time-dependent inhibition, however the methyl ester of flurbiprofen is a simple competitive inhibitor indicating that an ionic interaction with oPGHS-1 is important for time-dependent inhibition. The results with the R120Q mutant are consistant with this concept. R120Q oPGHS-1 did not undergo time-dependent inhibition with flurbiprofen even at high concentrations of the inhibitor. In order for an inhibitor to cause time dependent inhibition the enzyme/inhibitor complex must undergo a secondary conformational rearrangement. It appears that one interaction that is critical for time-dependent inhibition is an ionic interaction involving the carboxylate group of the inhibitor and the guanido group of Arg120.

As with PGHS-1, the arginine group is necessary for the time-dependent inhibition of hPGHS-2 by carboxylic acid inhibitors as demonstrated by the lack of time-dependent inhibition of the R120Q hPGHS-2 mutant with flurbiprofen.

The substitution of glutamine for Arg120 resulted in some changes in inhibitor profiles with PGHS-2 specific inhibitors. Specifically, the R120Q mutant did not undergo time-dependent inhibition by NS-398; however this mutant did undergo time-dependent inhibition with both Dup-697 and SC-58125 although with a reduced $t_{1/2}$.

CHAPTER 3

STUDIES TO DETERMINE THE ROLE OF TYR355 OF oPGHS-1 IN SUBSTRATE AND INHIBITOR INTERACTIONS

Introduction

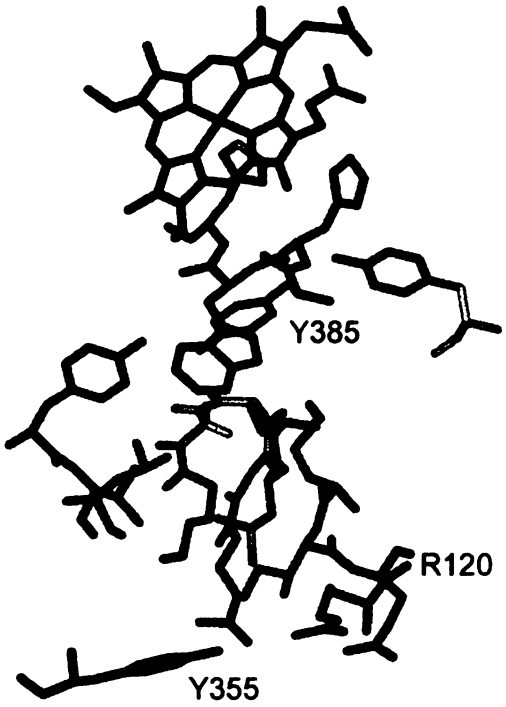
Examination of the crystal structure of oPGHS-1 complexed with flurbiprofen suggests that Tyr355 is involved in the hydrogen bonding network at the mouth of the cyclooxygenase active site that positions flurbiprofen in this site. In addition, the bulky hydroxyl group of this tyrosine residue places a constriction on one side of the cyclooxygenase channel that may determine the stereo-selectivity of the enzyme for the *S* -isomer of 2-phenylpropionic acid inhibitors (43). This hypothesis was tested with the Y355F oPGHS-1 mutant and the mutant enzyme did show a decreased stereoselectivity towards ibuprofen (72). To further determine the interaction of Tyr355 with this class of inhibitors the Y355A and Y355L oPGHS-1 mutants were prepared and the kinetic properties examined. In addition, further characterization of the role of Tyr355 on substrate and inhibitor interactions were performed, to examine the effect of Tyr355 on the binding and oxygenation of various fatty acid substrates and its role in time-dependent inhibition.

Figure 19 shows the active site of native oPGHS-1 and Y355F oPGHS-1 with arachidonic acid modeled into the cyclooxygenase site.

Figure 19. Model of active site of oPGHS-1 and Y355F oPGHS-1 with arachidonic acid modeled into the cyclooxygenase site. Coordinates were obtained from the Protein Data Bank and molecular modeling was performed using InsightII. The arachidonic acid is shown in light blue, the heme in red and residue 355 in green

A. native oPGHS-1
B. Y355F oPGHS-1

A.



B.

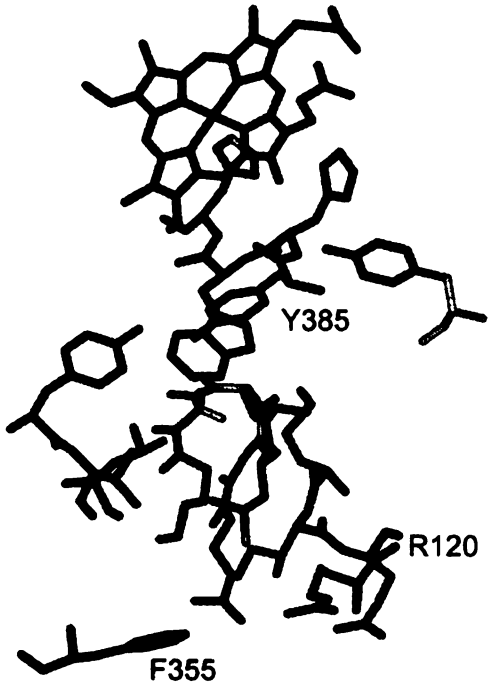


Figure 19

Materials and Methods

Materials- DMEM was from Life Technologies, Inc. Fetal calf serum and calf serum were from Hyclone. Chloroquine, bovine hemoglobin, DEAE-dextran and penicillin G were from Sigma.20:4 n-6, 18:2 n-6, 20:5 n-3, 20:2 n-6, 20:3 n-6 were from Cayman Chemical Co.

[1-^{14}C]-arachidonic acid (50-55 mCi mmole) was from NEN. CsCl was from Boehringer Mannheim. All other reagents were purchased from common commercial sources

Preparation of oPGHS-1 Mutants by Site-directed Mutagenesis- The Y355F mutant was prepared as described previously (77). The Y355A and Y355L mutants were prepared starting with M13mp19-oPGHS-1, which contains the coding region of ovine PGHS-1, using a Bio-Rad kit as described in the manufacterers directions. The oligonucleotide primers used to prepare each mutant are as follows:

Y355L: 5'- CTGAGCGGCCTCTTCCTGCAGCTC- 3'
Y355A: 5'- CTGAGCGGCGCCTTCCTGCACTC - 3'

Phage samples were sequenced using the dideoxy method (74) to identify mutants. The 2.3 kb insert from the replicative form of M13mp19-oPGHS-1 containing the desired mutation was isolated after digestion with *Sal* I and subcloned into the *Sal* I site of pSVT7. The correct orientation of the insert was confirmed by restriction digestion with *Pst* I. Plasmids used for transfection were purified by CsCl density gradient ultracentrifugation.

Transfection- *Cos*-1 cells (ATCC CRL-1650) were grown in DMEM containing 8% calf serum and 2% fetal calf serum until near confluence and transfected with plasmid containing DNA for native oPGHS-1, Y355F oPGHS-1, Y355L oPGHS-1 and Y355A oPGHS-1 as described in Materials and Methods in Chapter 2. Forty hours post transfection the cells were harvested by scraping in 3 ml of ice-cold phosphate buffered saline (PBS) with a rubber policeman and collected by centrifugation at 1200 rpm for 5 min.

Preparation of Microsomes- Pelleted cells from 20-50 plates were resuspended in 4-5 ml of ice-cold 0.1 M Tris-Cl, pH 7.4, and microsmes prepared as described in Material and Methods in Chapter 2. Protein concentrations were determined by a modified Lowrey method, using BSA as a standarad (75).

Western transfer blotting- Microsomal membrane fracions were resolved by 10% SDS-PAGE and transferred electrophorectically to 0.45 μm nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked for 30 min in 3% (w/v) dry milk, 0.1 % (v/v) Tween-20/ Tris buffered saline (TBS) and incubated for 2 hrs with a 1:10,000 dilution of an affinity purified antibody (COX 2). The membranes were washed and incubated with a 1:1000 dilution of a goat anti-rabbit IgG-horseradish peroxidase conjugate for 1 hour. Membranes were washed and incubated for 1 min with Amersham ECL Western Blotting detection

reagents, blot dried and exposed to XAR-5 film. Expression levels were quantified by Bio Rad Molecular Analysis software.

Cyclooxygenase Assays- Cyclooxygenase assays were carried out at 37^0 C using a Yellow Springs Instruments Model 5300 oxygen electrode as described in Materials and Methods in Chapter 2. For determination of the stereoselectivity of the mutant enzymes either *R* or *S*- ibuprofen, at the appropriate concentration, was added to the assay chamber prior to the addition of the enzyme. For time-dependent inhibition studies, flurbiprofen at an appropriate concentration was pre-incubated with the enzyme (250 μg) at 37^o C for various times; reactions were then initiated by the addition of the enzyme/inhibitor complex to the assay chamber, and initial rates of oxygen consumption determined. For substrate studies 100 μM 20:5 n-3, 18:2 n-6, 20:3 n-6, or 20:2 n-6 were used. K_m determinations were done for 20:4 n-6 using varying concentrations of the substrate.

Peroxidase Assays- The peroxidase activity of microsomal membrane preparations was measure spectrophotometrically at 436 nm using guaicol and H_2O_2 as the cosubstrates. The assay mixture contained 0.1 M Tris-Cl, pH7.2, 5.6 mM guaiacol, 1μM hematin and 50-100 μg microsomal protein in a total volume of 0.3 ml. Reactions were initiated by the addition of hydrogen peroxide and the product (3,3'-dimethoxydopheno-4,4'-quinone) formation detected at 436 nm.

Product Analysis by Thin Layer Chromotagraphy- Microsomal membrane fractions (250 μg) were incubated with [1-^{14}C]-arachidonic acid for 10 mins. Reactions were stopped with the addition of 1.4 ml CCl_4:MeOH (1:1) and centrifuged at 3,000 rpm for 5 min. Supernatants were transfered to clean tubes and 0.6 ml CCl_4 and 0.32 ml 0.88% formic acid added. After centrifugation the organic layer was removed and dried under N_2. The dried samples were resuspended in 50 μl of CCl_4 and spotted on Silca Gel 60 thin layer chromatography plates. Plates were developed twice in benzene: dioxane: acetic acid: formic acid (82:14:1:1) and exposed to XAR-5 film for 48 hours to allow visualization of the products.

Results

Native oPGHS-1 and Y355 oPGHS-1 mutants were transiently expressed in *cos*-1 cells and microsomal membrane fractions prepared. Levels of expression were determined by Western transfer blotting and densitometry (Figure 20). The level of expression for the Y355F mutant was previously determined to be similar to that of the native oPGHS-1 (50). The mutant enzymes were expressed at 92-100 % of the native oPGHS-1. Cyclooxygenase and peroxidase activities were measured, and the K_m value for arachidonic acid for each mutant was determined. The Y355F enzyme had 20% of the cyclooxygenase activity of the native oPGHS-1 while the Y355A and Y355L enzymes each had approximately 10 % of native activity. All the mutant enzymes had substantial peroxidase activity compared with the native oPGHS-1. The kinetic data is summarized in Table 4. The Y355L enzyme had a similar K_m value for arachidonic acid as the native enzyme, while both the Y355F and Y355A mutants had values about five fold higher. Product characterization by radio thin layer chromatography revealed that all the enzymes formed the same products in similar proportions (Figure 21).

To examine the role of Tyr 355 in binding of other fatty acid substrates 100 μM 20:5 n-3, 20:3 n-6, 18:2 n-6 and 20:2 n-6 were used as substrates for the cyclooxygenase reaction with the native oPGHS-1 and Y355F oPGHS-1. The Y355F mutant exhibited 20% of the cyclooxygenase activity of the native enzyme with 20:4 n-6. Relative to the native oPGHS-1 the Y355F mutant had 32 %

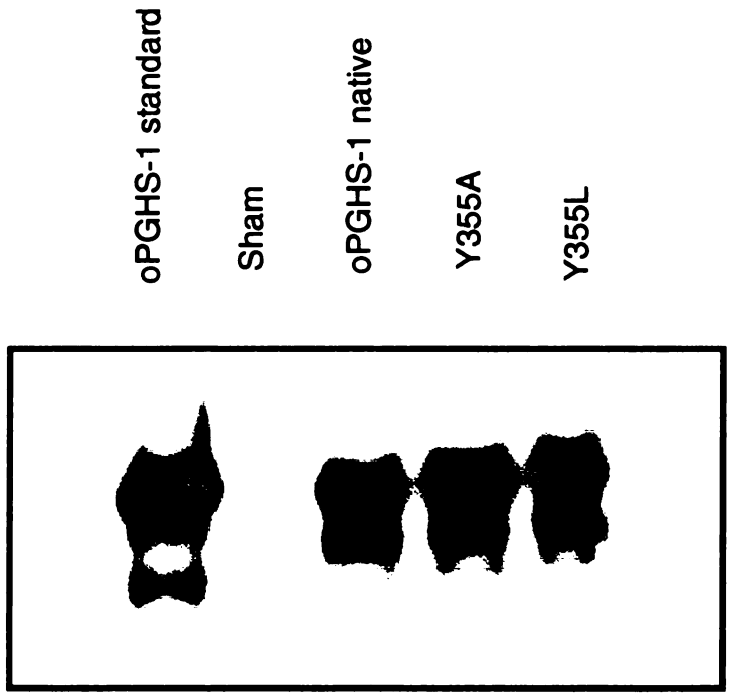


Figure 20. Western blot of oPGHS-1 and Y355A and Y355L mutants PGH synthases *Cos*-1 cells were sham-transfected or transfected with native oPGHS-1, Y355A oPGHS-1 or Y355L oPGHS-1. Microsomal fractions were prepared and 5 μg of protein was subjected to Western transfer blotting as described in the text.

Table 4. Comparison of Kinetic properties of oPGHS-1 and Y355 mutant oPGHSs *Cos*-1 cells were transfected with plasmid encoding native oPGHS-1 or Y355 mutant oPGHS-1s, and microsomal membrane fractions prepared as described in the text.. Cyclooxygenase activity was determined by oxygen electrode and peroxidase activity determined spectroscopically, as described in the text.

ENZYME	% COX ACTIVITY	% POX ACTIVITY	Km AA (mM)
oPGHS-1	100%	100	4.2
Y355F	19 ± 4%	130	18
Y355L	10 ± 3%	82 ± 24%	8
Y355A	10 ± 4%	84 ± 26%	22

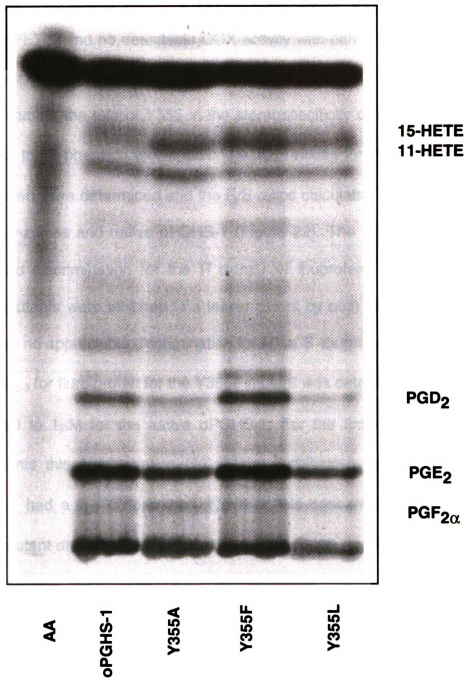


Figure 21. Product characterization of oPGHS-1 and Y355 mutant PGH synthases. Microsomes containing native or Y355 mutant oPGHS-1 protein were incubated with [1-^{14}C]-arachidonic acid and the reaction products separated by thin layer chromatography as described in the text.

cyclooxygenase activity with 20:3 n-6, 32 % with 20:5 n-3 (in the presence of 10 μM 15-HPETE) and no detectable COX activity with either 18:2 n-6 or 20:2 n-6. The data are summarized in Table 5.

To examine the role of Y355 in the stereospecificity of oPGHS-1 for the *S* - isomer of the 2-phenylpropionic inhibitors, IC_{50} values for the two stereoisomers of ibuprofen were determined and the R/S ratios calculated for each of the Y355 mutant enzymes and native oPGHS-1 (Figure 22). The Y355F mutant showed decreased discrimination for the *R* isomer of ibuprofen. The Y355L and the Y355A mutants were inhibited to a lesser extent by both ibuprofen isomers and there was no appreciable discrimination for *R* vs *S* -ibuprofen.

The IC_{50} for flurbiprofen for the Y355F mutant was determined to be 50 μM as compared to 1μM for the native oPGHS-1. For the time-dependent inhibition experiments this concentration (50 μM) of flurbiprofen was used. The native oPGHS-1 had a $t_{1/2}$ of less than 1 min at this concentration, in contrast the Y355F mutant did not lose any activity until after a 10 min incubation, but lost all cyclooxygenase activity during a 20 min incubation.

Table 5. Substrate Specificities of native oPGHS-1 and Y355 mutant oPGHS-1s. V_{max} values for cyclooxygenase activity were determined using 100μM fatty acid substrates, as described in the text. Measurements for each substrate were repeated in duplicate. The experiment was performed at least twice with similar results.

ENZYME	SUBSTRATE (100 μM)	% ACTIVITY (O_2/mol of fatty acid)
oPGHS-1	20:4 n-6	100 ± 2 %
	18:2 n-6	30 ± 5 %
	20:3 n-6	70 ± 3 %
	20:2 n-6	5 ± 2 %
	20:5 n-3 + 10μM HPETE	15%
Y355F	20:4 n-6	100±7 %
	18:2 n-6	0%
	20:3 n-6	113 ± 10%
	20:2 n-6	0%
	20:5 n-3 + 10μM HPETE	24%

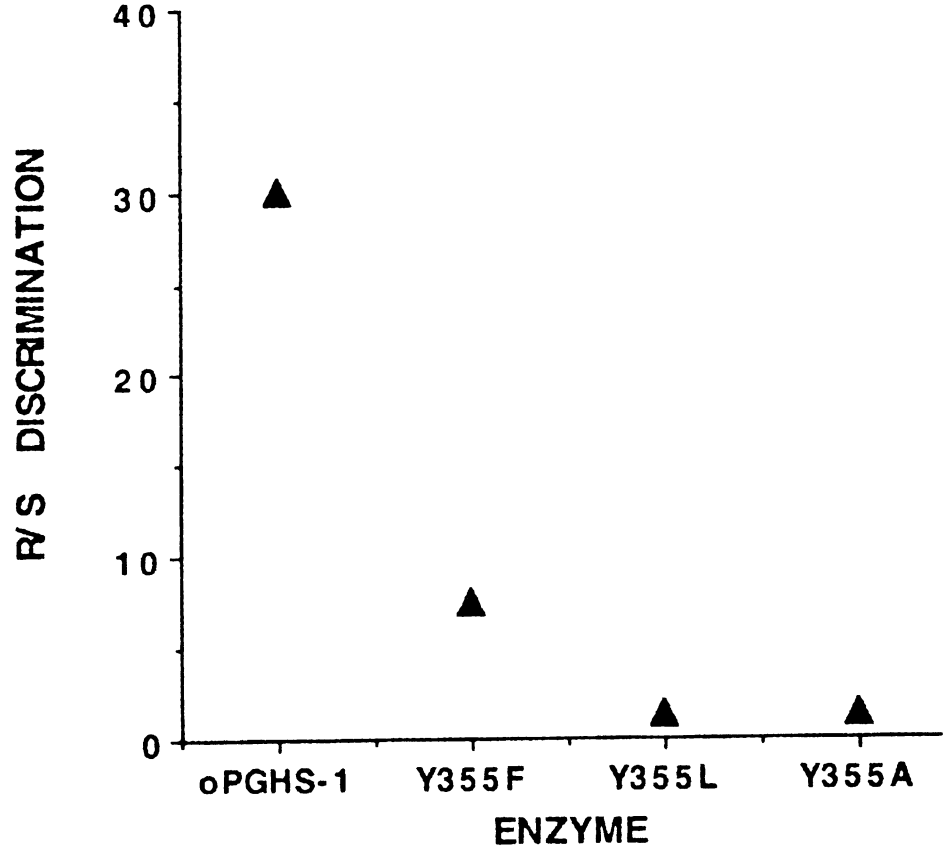


Figure 22. R vs S Ibuprofen discrimination by native oPGHS-1 and Y355 mutant oPGHS-1s IC_{50} values for the two stereoisomers of ibuprofen were determined by oxygen electrode and the R/S ratios calculated for each of the Y355 mutant enzymes and native oPGHS-1.

Discussion

Changing Tyr355 to Phe, Leu, or Ala caused the oPGHS-1 to have a decreased V_{max} and an increased K_m for arachidonic acid. The K_m values for the Y355F and Y355A mutant enzymes were about five fold higher than that of native oPGHS-1 and the cyclooxygenase activities were about five and ten fold lower, respectively. The K_m value for the Y355L mutant was about the same as that of native oPGHS-1 but the cyclooxygenase activity was ten fold lower. These results, interpreted in the context of the oPGHS-1 crystal structure and previous studies of the neighboring Arg120 group, suggest that Tyr355 is involved in an interaction with the carboxylate group of arachidonic acid and elimination of the phenolic hydroxyl group of Tyr355 leads to a decrease in the effeciency of oxygenation of arachidonic acid presumably due to the loss of proper substrate positioning for catalysis. The Y355F mutant was able to utilize both 100 μM 20:3 n-6 and 20:5 n-3 as substrates albeit less efficiently than native oPGHS-1, but it was unable to oxygenate either 18:2 n-6 or 20:2 n-6. Thus, the hydroxyl group of Tyr355 most likely participates in a hydrogen bonding network involving all the carboxylate groups of all fatty acid substrates. In the case of 20:5 n-3 the relative increase in activity as compared with 20:4 n-6 can be explained as loss of a hinderince. By examining stereoviews of the active site it appears that with the removal of the phenolic hydroxyl group the mouth of the active site is less constricted allowing the less flexible fatty acid better access and thus increasing the potential for catalysis. Y355F oPGHS-1 does not oxygenate 18:2 n-6 and 20:2 n-6. Linoleic acid is oxygenated relatively efficiently

by native oPGHS-1. This implies that the phenolic hydroxyl group of Tyr355 is very important for binding and oxygenation for 18:2 n-6. In the case of 20:2 n-6, it appears that the absence of double bonds at C-5 and C-8 cause this substrate to be used relatively poorly by native oPGHS-1; with the loss of the Tyr355 interaction it is not used at all. Further studies will need to be done to examine why these fatty acids are not used as substrates with the Y355F enzyme; if it is decreased affinity for the fatty acid or if it is fatty acid positioning. Experiments will need to be performed to determine if 18:2 n-6 and 20:2 n-6 can inhibit the oxygenation of 20:4 n-6.

Experiments to examine the stereoselectivity of the ibuprofen isomers demonstrate that removal of the hydroxyl group allows the *R* isomer to be a relatively more efficient inhibitor. Examination of stereoviews of the Ala and Leu mutant enzymes show that the substitution of the smaller groups leave a large space at the mouth of the cyclooxygenase active site. While it is possible that some rearrangements takes place among neighboring residues to compensate, our results suggest that it is not sufficient to provide stabilization of the NSAID as evidenced by the decrease in inhibition by the *S* -ibuprofen.

Recently it was shown that the analogous residue (Y341) in hPGHS-2 is involved in time dependent inhibition by flurbiprofen. (76). So *et al* found that the Y355F hPGHS-2 reached maximal inhibition more rapidly than the native PGHS-2. In order to determine if this was also the case with oPGHS-1, the Y355F enzyme was incubated with flurbiprofen for varying amounts of time. The results of two experiments showed the opposite results. With the Y355F oPGHS-

1 enzyme the IC_{50} was 50 μM, a 50 fold increase over that for the native oPGHS-1, but the enzyme had a greatly increased $t_{1/2}$ for time-dependent inhibition. It appears that for oPGHS-1 the interaction of Tyr355 plays a more important role in time-dependent inhibition with carboxylate containing inhibitors than does the corresponding Tyr355 in hPGHS-2. The two notable differences between the active sites of the isomers are Ile 523 to Val and His 513 to Arg.. It would be interesting to look at double mutants of Y355F with either a I523V or a H513R mutant to see if time dependent inhibition by flurbiprofen can be restored.

CONCLUSION

From examination of the crystal structure of oPGHS-1 complexed with flurbiprofen it appears that Arg120 and Tyr355 are in a position to interact with the carboxylate group of fatty acid substrates and non-steroidal antiinflammatory drugs. To determine the role of Arg120 we used site-directed mutagenesis to replace Arg120 with glutamine. The R120Q oPGHS-1 had a decreased catalytic rate and a greatly increased K_m for arachidonic acid. In addition, it was no longer inhibited by flurbiprofen. Thus we conclude that Arg120 is necessary for arachidonic acid binding and oxygenation, and for inhibition by carboxylate containing inhibitors such as flurbiprofen.

Two different isoforms of PGHS, PGHS-1 and PGHS-2, have been identified, and while they have similar primary sequences and overall structures, they demonstrate subtle differences in substrate specificity and inhibition by NSAIDS. The homologous Arg120 residue in PGHS-2 was examined to determine if it interacts with fatty acid substrates and NSAIDs in a similar manner. We used site-directed mutagenesis to replace hPGHS-2 R120 with glutamine and leucine. We determined that Arg120 plays a similar role in binding carboxylate containing inhibitors because a R120Q hPGHS-2 was no longer inhibited by flurbiprofen. However hPGHS-2 R120Q interacts with fatty acid substrates in a different manner than oPGHS-1 Arg120 because the PGHS-2 R120Q mutant retained all of the cyclooxygenase activity and had no change in the K_m for arachidonic acid. Changing the arginine to a leucine did result in a substantial decrease in cyclooxygenase activity. Further experiments to examine the nature of this

interaction will need to be performed. Examination of the role of hPGHS-2 Arg120 in fatty acid binding and oxygenation of fatty acid substrates revealed that the guinido group of this Arg120 is important for binding and oxygenation with 20:5 n-3, because the R120Q hPGHS-2 showed a significant decrease in cyclooxygenase activity and a greatly elevated K_m for 20:5 n-3. Preliminary studies performed to examine if 20:5 n-3 is able to act as an inhibitor for 20:4 n-6 indicate that 20:5 n-3 is able to enter the cyclooxygenase site, as it can inhibit catalysis of 20:4 n-6, but is not able to undergo catalysis as demonstrated by the increase in K_m.

Experiments to examine the role of Arg120 in the specificity for several PGHS-2 selective inhibitors were performed. The R120Q hPGHS-2 lost time dependent inhibition with NS-398, but not with Dup-697 or SC-58125 indicating that the guinido group of arginine is necessary for interaction with the sulphanimide group of NS-398 for time-dependent inhibition.

Site-directed mutagenesis was used to replace Tyr355 with phenylalanine,leucine and alanine to determine the role of Tyr355 in binding and oxygenation of fatty acid substrates and in interactions with inhibitors. PGHSs exhibit selectivity for the *S*-isomer of NSAIDs. From the position of Tyr355 it was postulated that the hydroxyl group of Tyr355 is involved in this selectivity. We found that the Y355F oPGHS-1 showed a decreased selectivity against the *R*-isomer of ibuprofen , but the Y355L oPGHS-1and Y355A oPGHS-1 lost inhibition with both isomers of ibuprofen indicating that some interaction is necessary at this position for inhibition. Additionally, the Y355F oPGHS-1 showed a increased

$t_{1/2}$ for time-dependent inhibition with flurbiprofen indicating that this interaction is important for time-dependent inhibition, but not necessary.

Tyr355 was shown to be involved in fatty acid substrate binding and oxygenation because the Y355F oPGHS-1 demonstrated no detectable cyclooxygenase activity with 18: n-6 and 20:2 n-6. This implies that the phenolic hydroxyl group of Tyr355 is important for positioning the 18:2 n-6 in the cyclooxygenase site. Further studies will need to be done to examine why these fatty acids are not used as substrates with the Y355F enzyme.

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