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Examination of the N-Glycosylation and
Membrane Association of the Prostaglandin
Endoperoxide Synthase Isozymes

presented by

James C. Otto

has been accepted towards fulfillment
of the requirements for

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William L. Smith
Major professor

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EXAMINATION OF THE N-GLYCOSYLATION AND MEMBRANE ASSOCIATION OF THE PROSTAGLANDIN ENDOPEROXIDE SYNTHASE ISOZYMES

By

James C. Otto

A DISSERTATION

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Department of Biochemistry

1994

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ABSTRACT

Using site-directed mutagenesis, we have determined that Asn68, Asn144, and Asn410 of ovine prostaglandin endoperoxide synthase-1 (PGHS-1) are N-glycosylated. A fourth consensus N-glycosylation sequence at Asn104 is not glycosylated. Glycosylation of PGHS-1 at Asn410 and at either Asn68 or Asn144 was required for expression of both the cyclooxygenase and the peroxidase activities of the enzyme. Inactive PGHS-1 glycosylation site mutant proteins do not appear to achieve their native conformations. However, the native enzyme, once in an active conformation, does not appear to require attached carbohydrate for cyclooxygenase or peroxidase activities.

N-glycosylation consensus sequences corresponding to the three glycosylation sites of ovine PGHS-1 are conserved in the deduced amino acid sequences of PGHS-2. Using site-directed mutagenesis, we determined that there is an additional site of N-glycosylation in murine PGHS-2 located at Asn580. This site is N-glycosylated in about 50% of PGHS-2 molecules, resulting in two peptide bands on SDS-PAGE (M_r = 72 and 74 kDa). Glycosylation of PGHS-2 is necessary for expression of cyclooxygenase and peroxidase activity, but glycosylation of PGHS-2 at Asn580 is not required.

N-glycosylated residues are predicted to reside in the lumen of the endoplasmic reticulum (ER). Further analysis of the luminal versus cytoplasmic orientations of several epitopes of PGHS-1 and PGHS-2 was conducted by immunocytofluorescent staining of cells following treatment with membrane-selective permeants. With serum-stimulated, murine NIH/3T3 cells expressing PGHS-2, an anti-peptide antibody directed against a domain near the C-terminus of this isozyme caused staining only after all membranes

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were permeabilized with 0.2% saponin; no staining occurred with 3T3 cells treated with digitonin to permeabilize only the plasma membrane. Similarly, *cos*-1 cells expressing ovine PGHS-1 were stained with anti-peptide antibodies directed against (a) the amino terminus (residues 25-35), (b) a domain containing the tryptic cleavage site at Arg277 (residues 272-284), or (c) a region near the carboxyl terminus (residues 583-594) following permeabilization with saponin but not with digitonin or Streptolysin O. The results obtained with the antibodies against the Arg277-containing domain of PGHS-1 were surprising because the enzyme is susceptible to tryptic cleavage at Arg277 in microsomal preparations. However, enzymatic and immunochemical analyses of microsomes prepared from ovine vesicular glands and *cos*-1 cells indicated that these microsomes are not intact. Accordingly, our results indicate that the trypsin cleavage site (Arg277) as well as the N- and C-termini of ovine PGHS-1 are on the luminal side of the ER. The N-terminus, the Arg277 domain, and the N-glycosylation sites of ovine PGHS-1 are part of a large soluble, globular structure in crystalline ovine PGHS-1 (Picot, D., P.J. Loll, and M. Garavito (1994) *Nature*, 367: 243-249). We conclude that PGHS-1 and, by analogy, the highly homologous PGHS-2 are luminal ER proteins. Assuming that the PGHS-1 and PGHS-2 present in the ER are functional in intact cells, our results indicate that PGH_2 synthesis from arachidonate occurs in the lumen of the ER.

From the crystal structure of ovine PGHS-1, Picot *et al.* predict that PGHS-1, and by analogy PGHS-2, associates with the ER membrane not through transmembrane domains, but rather through a novel monotopic membrane binding domain between residues 74-117 which integrates into a single leaflet of the ER membrane. We have examined this hypothesis using the hydrophobic, photoactivatible reagent 3-

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trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). Ovine PGHS-1 is the major protein labelled in microsomes prepared from ovine seminal vesicles by [^{125}I]TID, consistent with the idea that it is the major integral membrane protein in these microsomes. A peptide comprised of residues 25-166 of ovine PGHS-1 is photolabelled by [^{125}I]TID, suggesting that residues in this region of PGHS-1 associate with the membrane.

ACKNOWLEDGEMENTS

I would like to thank Dr. William Smith for allowing me to work with him in his lab, and for his patience and guidance while serving as my mentor. In working with Dr. Smith, I have learned a great deal about the committment that it takes to be a good scientist, and I hope I will be able to follow his example. The lab has been a great place to work, and a large part of the reason is that Dr. Smith brings such an upbeat attitude to work with him, and this rubs off on everyone who works with him, myself included. I guess sometimes nice guys can finish first. He has taught me, again by his example, the art of sarcasm, something that I'm sure my friends in the future will cherish, and also how to survive as the brunt of all jokes.

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I would like to thank Mom, Dad, Karen, Mike, Kimberly, and Catie for all their support through my years here. They have been a big influence in shaping me into the person I am today, and definitely deserve some credit for my achieving this degree. Perhaps I don't always let them know how important they are to me, but they are a big part of my life. I would like to thank my friends at Michigan State for keeping me sane. Diane, Bear, Mark, Rich, Linda, Nicci (but not Hobbes), Dave, Marty, Odette, Andy, John, Mike, Chuck, you have all been great friends, and I will always remember my years here with you fondly. Thanks also to John, Kirsten, Chuck, Mary and Paul for housing me when I was homeless.

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ABBREVIATIONS

5-LOX	5-lipoxygenase
15-HETE	15*R*-hydroxyeicosatetraenoic acid
10P	10,000 X *g* spin pellet
200P	200,000 x *g* spin pellet, microsomal fraction
200S	200,000 x *g* spin supernatant, cytosol, soluble protein fraction
apoPGHS	heme depleted PGHS
βgal	β-galactosidase
cPLA$_2$	cytosolic, 85 kDa phospholipase A$_2$
EGF	epidermal growth factor
Endo H	endoglycosidase H
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
GlcNAc	N-acetyl glucosamine
HMG CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
IL	interleukin
[^{125}I]TID	3-trifluoromethyl-3-(*m*-[^{125}I]-iodophenyl)diazirine
Luc	luciferase
man	mannose
NSAID	non-steroidal anti-inflammatory drug
PDGF	platelet derived growth factor
PG	prostaglandin
PGHS	prostaglandin endoperoxide synthase
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sPLA$_2$	secretory phospholipase A$_2$
TPA	12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester)
TX	thromboxane

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CHAPTER I

LITERATURE REVIEW

Introduction

Prostaglandin endoperoxide synthase (PGHS), catalyzes the formation of prostaglandin H_2 (PGH_2) from arachidonic acid, the committed step in the formation of prostaglandins and thromboxane (1). The conversion of arachidonate to PGH_2 occurs by two distinct reactions, both catalyzed by PGHS (2, 3): the cyclooxygenase reaction, in which arachidonic acid is bis-oxygenated to form prostaglandin G_2 (PGG_2), and the peroxidase reaction, in which the 15-hydroperoxide group of PGG_2 is reduced to form PGH_2 (Fig. 1). Two isoforms of PGHS are known to exist and referred to here as PGHS-1 and PGHS-2; both enzymes exhibit cyclooxygenase and peroxidase activities, and generate the same products (4). The group of drugs commonly referred to as the non-steroidal anti-inflammatory drugs (NSAIDS) effectively inhibit the formation of prostaglandins by blocking the cyclooxygenase reaction of PGHS isozymes (5, 6).

As an introduction, the mechanisms for the synthesis and action of prostaglandins and thromboxanes will be reviewed. Prostaglandins and thromboxane comprise the group of oxygenated fatty acid hormones collectively called the prostanoids (1). Prostanoid biosynthesis occurs in most mammalian tissues, and the prostanoids play an important role in a number of physiological processes, including vascular homeostasis, kidney function, and inflammation (7). Figure 1 outlines a general scheme for the synthesis of prostanoids

Figure 1
WL. 1992 (19).

Figure 1. Biosynthetic pathway for prostanoid synthesis. Adapted from Smith, WL. 1992 (19).

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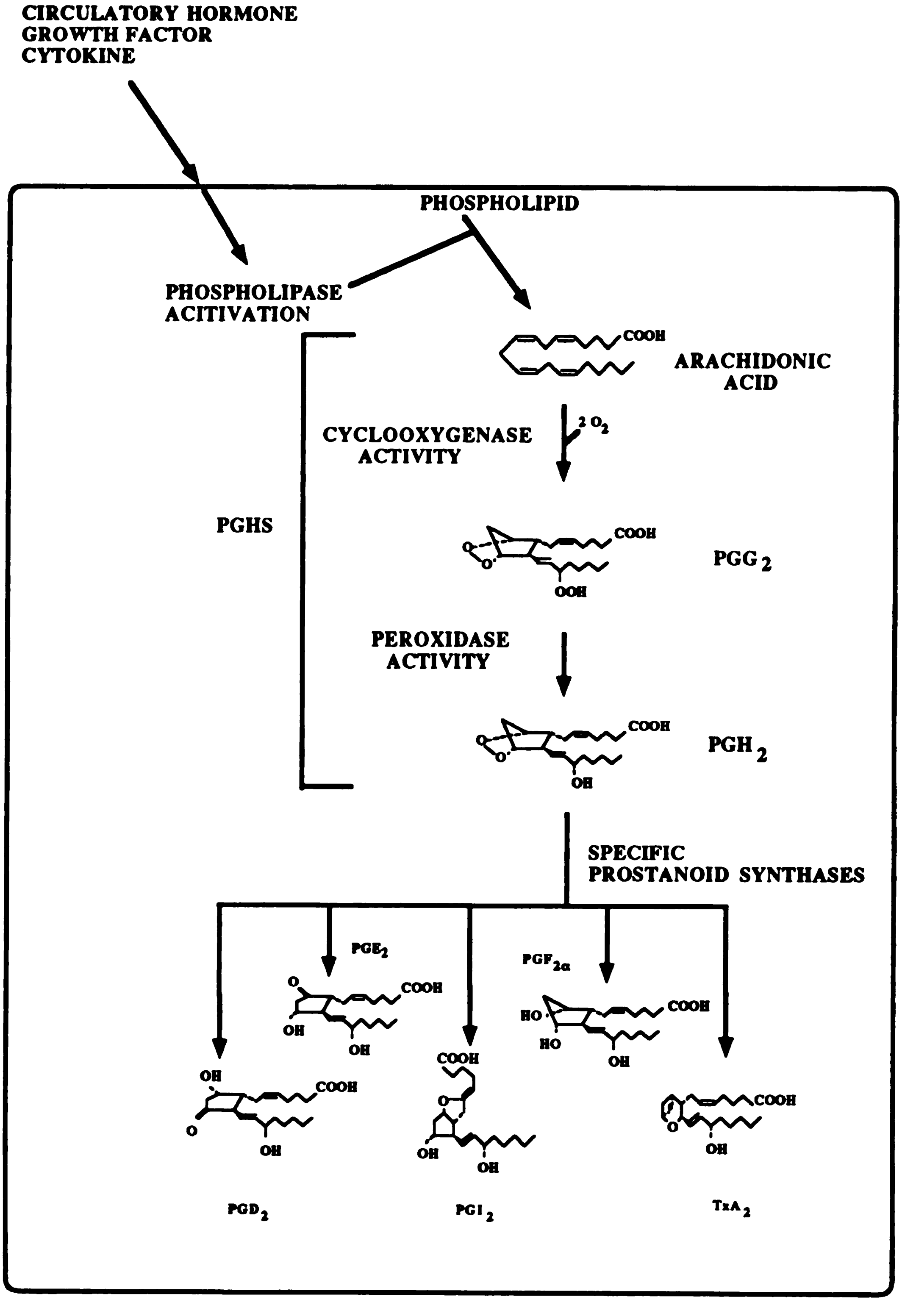
PATHWAY FOR PROSTANOID SYNTHESIS



in a model cell (1). Prostanoid synthesis is initiated by the activation of cytoplasmic or extracellular phospholipases, which liberate arachidonate from phospholipid stores (8). Phospholipase activation is typically the result of cell stimulation by a circulatory hormone or by a cytokine, growth factor or other mitogenic agent. The mobilized arachidonic acid is then converted by a PGHS to PGH_2; other enzymes then convert PGH_2 into the biologically active prostaglandins, which include PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) (9). The biologically active prostaglandins then exit the cell, likely via facilitated diffusion, and activate specific prostanoid G-protein linked receptors in the plasma membrane (10).

Three of the active prostaglandins (PGD_2, PGE_2, and $PGF_{2\alpha}$) are formed spontaneously from PGH_2 *in vitro*; however, *in vivo* the synthesis of the biologically active prostaglandins occurs enzymatically, and a given cell type will only form one of the active prostaglandins. Several enzymes have been identified which can specifically generate PGD_2, PGE_2, or $PGF_{2\alpha}$ from PGH_2 (9). Partly due to the fact that these three active prostaglandins can form spontaneously from PGH_2, it is not clear which, if any, of these enzymes actually serve as prostaglandin isomerases *in vivo*. Prostacyclin and thromboxane A_2, on the other hand, are only formed enzymatically, and prostacyclin and thromboxane synthases have been purified and characterized (11-13), and the thromboxane synthase has been cloned (14, 15). Both enzymes are members of the cytochrome P450 superfamily of proteins, and are integral membrane proteins of the endoplasmic reticulum (16). As cytochrome P450 proteins, the active sites of both of the synthases are presumed to be on the cytoplasmic side of the ER membrane (17, 18).

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and do not survive a single pass through the circulatory system (9). Therefore, the prostanoids are recognized as local hormones, acting at or near their site of synthesis. Upon exiting the cell, prostanoids will stimulate prostanoid receptors on the surface of the synthesizing cell (an autocrine response) and on cells neighboring the producing cell (a paracrine response) (19). While a given cell usually generates only one predominant type of prostanoid, the same cell may express several different prostanoid receptors, enabling the cell to have different responses to different prostanoids (10).

Activation of prostanoid receptors generates a second messenger response which either amplifies or attenuates the response of a group of cells in a tissue to the original stimulus that initiated prostanoid synthesis. In other words, prostanoids coordinate the responses of cells to the original stimulus. For example, platelets stimulated with thrombin generate TXA_2, which causes platelet aggregation and vasoconstriction and leads to the formation of blood clots (20). Balancing this action, the stimulation of endothelial cells with thrombin results in the generation of PGI_2, which acts as a vasorelaxant and inhibits platelet aggregation. Thus, unnecessary clot formation in healthy blood vessels is inhibited (21). Other areas of prostanoid action include inflammation, the regulation of water reabsorption in the kidney, the initiation of ovulation, and the modulation of gastric acid secretion in the stomach (1, 7, 19).

NSAIDS such as aspirin and ibuprofen block prostanoid biosynthesis by inhibiting the cyclooxygenase activity of PGHS-1 and PGHS-2 (5, 6, 22, 23), and are widely used as pharmaceutical agents. Common uses of NSAIDs include a) use as anti-thrombotic agents for the reduction of blood clotting in individuals with a high risk of heart disease; b) use as anti-inflammatory agents for the alleviation of symptoms associated with chronic inflammation such as arthritis; c) use as anti-pyretic agents for fever reduction; and d) use as analgesic agents to alleviate pain (4). Epidemiological studies have also recently associated the use of NSAIDs with a reduced risk of colon cancer. The mechanism for the involvement of prostanoids in cancer is not known, but it is speculated that expression of prostanoids in the colon may be stimulating cell growth, thereby increasing the risk of cancer (24).

One problem with the use of NSAIDs is that they do inhibit both PGHS-1 and PGHS-2, and therefore inhibit the production of prostanoids throughout the body. Chronic administration of NSAIDs for treatment of inflammatory diseases, such as arthritis, can lead to stomach and kidney ailments due to inhibition of prostanoid synthesis in these tissues (7, 25). The relatively recent discovery of PGHS-2 has spurred interest in the creation of a new generation of NSAIDs which inhibit only PGHS-2. The impetus for these efforts is based on evidence which suggests that prostanoid production by PGHS-2 is specifically involved in inflammatory responses (26, 27). On the other hand, prostaglandin production by PGHS-1 appears to be involved in responses to circulatory hormones such as those described for platelets, kidney and stomach (4). This new generation of NSAIDs is predicted to be suitable for chronic anti-inflammatory administration without causing stomach and kidney problems.

Regulation of the expression of PGHS isozymes.

While PGHS-1 mRNA and protein are found in most mammalian tissues, levels of PGHS-2 mRNA in tissues are low, and PGHS-2 protein is not detectible (28, 29). Similarly, in unstimulated cell lines which express the two isozymes, PGHS-1 mRNA and

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protein levels are relatively constant while levels of PGHS-2 mRNA and protein are low. PGHS-2 mRNA and protein levels can be induced in many cell lines with pro-inflammatory cytokines or growth factors; under similar conditions PGHS-1 mRNA and protein levels remain relatively constant (30, 31). This highlights one of the major differences in PGHS-1 and PGHS-2: the regulation their expression.

Although PGHS-1 is expressed in most tissues, only certain cell types in a given tissue actually express the enzyme. In cells which express PGHS-1, protein and mRNA levels remain essentially constant, indicating that the enzyme is expressed constitutively. Cells which produce PGHS-1 are typically highly differentiated (32-35), and it appears that PGHS-1 expression is controlled primarily at the developmental level (36). Because PGHS-1 is always present in a given cell due to its constitutive expression, it is able to form prostaglandins immediately following arachidonate mobilization in response to the stimulation of the cell by hormones such as thrombin or bradykinin. For this reason, it has been predicted that PGHS-1 is responsible for generating prostaglandins for cellular responses to circulatory hormones which require immediate prostaglandin formation (9). These responses are often referred to as "housekeeping" responses.

In contrast to PGHS-1, PGHS-2 protein is not detected in tissues under normal physiological conditions. Low levels of PGHS-2 mRNA have been detected in some tissues by northern blotting (37) and RT-PCR techniques (38), possibly the result of expression of PGHS-2 mRNA by a small number of stimulated cells, such as macrophages. However, it appears that most cells can produce high levels of PGHS-2 mRNA and protein can when provided with the proper stimulus. Growth factors (31), phorbol esters (30), inflammatory cytokines such as IL-1 (39, 40), and lipopolysaccharide

(41) have all been shown to stimulate PGHS-2 formation in cultured cells *in vitro*. Anti-inflammatory glucocorticoids and cytokines such as dexamethasone and IL-10, have been demonstrated to inhibit the expression of PGHS-2 mRNA and protein *in vitro*, while having little effect on PGHS-1 mRNA and protein levels (31, 42). High levels of PGHS-2 protein have been reported *in vivo* in rat tissues used as models of inflammation (29, 43), in synovial joints of individuals with rheumatoid arthritis (43-45), and in rat follicles preceding ovulation (46). Thus, *in vivo* PGHS-2 expression occurs in inflamed tissues or in systems which have inflammation-like responses. These observations have led to the prediction that PGHS-2 is involved in prostanoid production associated with inflammation. Because PGHS-2 protein is not usually present in unstimulated tissues, it would not be available for prostanoid synthesis in response to stimulation by circulatory hormones, and therefore it is not predicted to be involved in prostaglandin production for "housekeeping" responses.

Mobilization of arachidonic acid for prostanoid synthesis.

Within cells, arachidonic acid is found almost exclusively in an esterified form at the *sn*-2 position of membrane phospholipids. Thus, for prostaglandin synthesis to occur, it is thought that free arachidonic acid must be generated from membrane phospholipids. Different patterns of arachidonate release are seen in cultured cells depending on the stimuli. In response to circulatory hormones such as thrombin or vasopressin, a single, transient phase of arachidonate release is seen, occurring primarily within the first 10 min following hormonal stimulation (47). On the other hand, following stimulation of cultured cells with the growth factor PDGF, and presumably other growth factors and

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inflammatory cytokines, two phases of arachidonic acid release are seen. The first phase of release is the same as that seen with circulatory hormones; a transient release occuring primarily in the first 10 min following stimulation. Following the first phase of arachidonic acid release, a second, sustained release occurs over a period of several hours, releasing several times the amount of arachidonic acid as the first phase of release (47). This second phase of arachidonate release is requires *de novo* protein synthesis.

The primary mechanism for the release of arachidonic acid from membrane phospholipids is thought involve phospholipase A_2 enzymes, which hydrolyze arachidonate from the *sn*-2 position of phospholipids (8). Two phospholipases A_2 which have been implicated in arachidonic acid release are a cytoplasmic 85 kDa phospholipase A_2 (cPLA_2) and a non-pancreatic secretory Type II phospholipase A_2 (sPLA_2) (8, 9).

cPLA_2 is activated by elevation of cytoplasmic Ca^{2+} levels. Upon stimulation by intracellular Ca^{2+} increases, cPLA_2 translocates from the cytoplasm to an intracellular membrane, probably the ER membrane (48). The enzyme then selectively releases arachidonate from the *sn*-2 position of phospholipids (49). The phospholipase activity of cPLA_2 can be enhanced by phosphorylation of the enzyme, giving it an additional level of control (50-52). As many circulatory hormones which stimulate prostanoid formation cause a transient Ca^{2+} mobilization in cells, the cPLA_2 is an attractive candidate as a phospholipase involved in arachidonate release in response to circulatory hormones (53).

The second phospholipase A_2 thought to be involved in the generation of arachidonic acid for prostaglandin production, sPLA_2, is associated with the extracellular surface of the plasma membrane. The association of sPLA_2 with the plasma membrane appears to involve the binding of heparin on the cell surface (8, 53, 54). Unlike cPLA_2,

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$sPLA_2$ fails to discriminate between different fatty acids at the *sn*-2 position of phospholipids (8). $sPLA_2$ has been implicated in the sustained arachidonic acid release seen in response growth factors and cytokines (8, 53, 54). Apparently $sPLA_2$ can be upregulated to some extent by growth factors, suggesting that some control over the activation of $sPLA_2$ may be transcriptional (8). Thus, it is possible that the *de novo* protein synthesis which is required for the release of arachidonic acid in response to growth factors (47) involves the synthesis of $sPLA_2$; however, significant levels of $sPLA_2$ are present on the plasma membrane of prostanoid forming cells, so it is likely that other proteins are responsible for the regulation of arachidonate release following stimulation of cells by growth factors.

Arachidonic acid added exogenously to cells can be utilized by both PGHS-1 and PGHS-2 to initiate prostanoid synthesis. However, recent evidence indicates that PGHS-1 cannot utilize arachidonate released by endogenous mechanisms following the stimulation of cells by phorbal esters, factors which induce PGHS-2 formation and stimulate prostaglandin production. This evidence stems from studies in murine fibroblasts which constitutively express PGHS-1, and can be induced to form PGHS-2. Following TPA treatment PGHS-2 protein is induced, arachidonate release occurs and prostaglandin formation was observed. However, when these fibroblasts were transfected with antisense primers which prevent the translation of PGHS-2 mRNA, although arachidonate release was observed in response to TPA stimulation, prostaglandin formation did not occur via the endogenous PGHS-1 (55). This suggests that growth factors and cytokines cause the activation of a phospholipase pathway which channels arachidonic acid specifically to PGHS-2. Possible mechanisms for the channelling of arachidonic acid to PGHS-2 could

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include a specialized localization of the enzyme in the cell, or the presence of accessory proteins in the cell which facilitate channeling of arachidonate to PGHS-2. A channelling pathway does exist for another arachidonic acid metabolizing enzyme, 5-lipoxygenase (5-LOX). *In vivo,* 5-LOX requires a second protein, 5-lipoxygenase activating protein (FLAP), for the synthesis of leukotrienes from arachidonic acid. The role of FLAP in leukotriene biosynthesis is thought to involve the binding of arachidonic acid following release by phospholipases and transferal of the arachidonate to 5-LOX (56-58).

Primary structures of PGHS isozymes.

PGHS-1 was the original cyclooxygenase enzyme first purified and cloned from ovine seminal vesicles (2, 59-61). PGHS-2 was discovered more recently as an inducible gene product in chicken and murine fibroblasts (28, 30). Subsequently, the murine (62), human (63) and rat (64) PGHS-1 and the human (65) and rat (64) PGHS-2 have been cloned (Fig. 2). Within a species, the deduced amino acid sequences of PGHS-1 and PGHS-2 share 60% identity and are 75% similar when conservative amino acid changes are taken into account. All amino acids which have been found to be essential for catalysis in PGHS-1 are conserved in PGHS-2. There are two obvious differences found in the deduced amino acid sequences of the PGHS isozymes. First, the signal peptide at the amino terminus of PGHS-2 lacks a series of hydrophobic amino acids found in the signal peptide of PGHS-1. The second difference in the deduced amino acid sequences of the PGHS isozymes occurs near the carboxyl terminus, where PGHS-2 contains an 18 amino acid insert not found in PGHS-1. The reason for these conserved differences between the amino acid sequences of PGHS-1 and PGHS-2 are not clear. Apparently

Figure 2. Comparison of the deduced amino acid sequences of various PGHS-1 and PGHS-2. Included are ovine PGHS-1 (59-61), murine PGHS-1 (62), human PGHS-1 (63), rat PGHS-1 and PGHS-2 (64), human PGHS-2 (65), murine PGHS-2 (30), and chicken PGHS-2 (28). The numbering corresponds to ovine PGHS-1, starting with Met1 of the deduced amino acid sequence. The signal sequences of ovine PGHS-1 and rat PGHS-2, the cysteines residues of the EGF homology domain (Cys36, Cys41, Cys47, Cys57, Cys59, Cys69 of $PGHS_{ov}$-1), the N-glycosylation consensus sequences (at Asn68, Asn104, Asn144, and Asn410), the proximal and distal heme ligands (His388 and His207), the active site tyrosine (Tyr386), the active site serine acetylated by asspririn (Ser530), and the 18 residue insert at the carboxyl terminus of PGHS-2 are in **bold**. The residues comprising the four helices predicted to form the membrane binding domain are underlined, and labelled Helix A, B, C and D.

```
Chick-2  MLLPC
Human-2  MLAHA
Rat-2    MLFRA
Mouse-2  MLFHA
Mouse-1  MSRRS
Rat-1    MSRRS
Human-1  MSR-S
Sheep-1  MSRQS

Chick-2  LLKP
Human-2  LLKP
Rat-2    PLKP
Mouse-2  LLKP
Mouse-1  SLR
Rat-1    SLR
Human-1  SLR
Sheep-1  TLR

Chick-2  TPW
Human-2  TPL
Rat-2    TPV
Mouse-2  TPV
Mouse-1  TPW
Rat-1    TPV
Human-1  TP
Sheep-1  TP

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Chick-2   MLLPCALLAALL-----------------AAGHAANPCCSLPCQNRGECMTTGFDRYECDCTRTGYYGENCTTPEFFTWLKL
Human-2   MLARALLLCAVL-----------------ALSHTANPCCSHPCQNRGVCMSVGFDQYKCDCTRTGFYGENCSTPEFLTRIKF
Rat-2     MLFRAVLLCACP-----------------GLSHAANPCCSNPCQNRGECMSIGFDQTKCDCTRTGFYGENCTTPRFLTRIKL
Mouse-2   MLFRAVLLCAAL-----------------GLSQAANPCCSNPCQNRGECMSTGFDQYKCDCTRTGFYGENCTTPEFLTRLKL
Mouse-1   MSRRSLSLWFPLLLLLLLPPTPSVLLADPGVPSPVNPCCYYPCQNQGVCVRFGLDNYQCDCTRTGYSGPNCTIPEIWTWLRN
Rat-1     MSRRSLSLQFPLLLLLLLLPPPPVLLTDAGVPSPVIPCCYYPCQNQGVCVRFGLDHYQCDCTRTGYSGPNCTIPEIWTWLRS
Human-1   MSR-SLLLRFLLLLLLL-PPLP-VLLADPGAPTPVNPCCYYPCQHQGICVRFGLDRYQCDCTRTGYSGPNCTIPGLWTWLRN
Sheep-1   MSRQSISLRFPLLLLLL-SPSP-VFSADPGAPAPVNPCCYYPCQHQGICVRFGLDRYQCDCTRTGYSGPNCTIPEIWTWLRT
                  10        20        30        40        50        60        70  Helix A

Chick-2   LIKPTPNTVHYILTHFKGVWNIINNSPFLRDTIMRYVLTSRSHLIDSPPTYNSDYSYKSWEAYSNLSYYTRSLPPVGHDCP
Human-2   LLKPTPNTVHYILTHFKGFWNVVNNIPFLRNAIMSYVLTSRSHLIDSPPTYNADYGYKSWEAFSNLSYYTRALPPVPDDCP
Rat-2     PLKPTPNTVHYILTHFKGVWNIVNNIPFLRIQSMRYVLTSRSHLIDSPPTYNVHYGYKSWEAFSNLSYYTRALPPVADDCP
Mouse-2   LLKPTPNTVHYILTHFKGVWNIVNNIPFLRSLTMKYVLTSRSYLIDSPPTYNVHYGYKSWEAFSNLSYYTRALPPVADDCP
Mouse-1   SLRPSPSFTHFLLTHGYWLWEFVNAT-FIREVLMRLVLTVRSNLIPSPPTYNSAHDYISWESFSNVSYYTRILPSVPKDCP
Rat-1     SLRPSPSFTHFLLTHGYWIWEFVNAT-FIREVLMGWVLTVGAKLIPSPPTYNTAHDYISWESFSNVSYYTRILPSVPKDCP
Human-1   SLRPSPSFTHFLLTHGRWFWEFVNAT-FIREMLMLLVLTVRSNLIPSPPTYNSAHDYISWESFSNVSYYTRILPSVPKDCP
Sheep-1   TLRPSPSFIHFLLTHGRWLWDFVNAT-FIRDTLMRLVLTVRSNLIPSPPTYNIAHDYISWESFSNVSYYTRILPSVPRDCP
               Helix B     Helix C       Helix D 120       130       140       150       160

Chick-2   TPMGVKGKKELPDSKLIVEKFLLRRKFIPDPQGTNVMFTFFAQHFTHQFFKTDHKRGPGFTKAYGHGVDLNHIYGETLER
Human-2   TPLGVKGKKQLPDSNEIVGKLLLRRKFIPDPQGSNMMFAFFAQHFTHQFFKTDHKRGPAFTNGLGHGVDLNHIYGETLAR
Rat-2     TPMGVKGNKELPDSKEVLEKVLLRREFIPDPQGTNMMFAFFAQHFTHQFFKTDQKRGPGFTRGLGHGVDLNHVYGETLDR
Mouse-2   TPMGVKGNKELPDSKEVLEKVLLRREFIPDPQGSNMMFAFFAQHFTHQFFKTDHKRGPGFTRGLGHGVDLNHIYGETLDR
Mouse-1   TPMGTKGKKQLPDVQLLAQQLLLRREFIPAPQGTNILFAFFAQHFTHQFFKTSGKMGPGFTKALGHGVDLGHIYGDNLER
Rat-1     TPMGTKGKKQLPDIHLLAQRLLLRREFIPAPQGTNVLFAFFAQHFTHQFFKTSTKMGPGFTKALGHGVDLGHIYGDSLER
Human-1   TPMGTKGKKQLPDAQLLARRFLLRRKFIPDPQGTNLMFAFFAQHFTHQFFKTSGKMGPGFTKALGHGVDLGHIYGDNLER
Sheep-1   TPMGTKGKKQLPDAEFLSRRFLLRRKFIPDPQGTNLMFAFFAQHFTHQFFKTSGKMGPGFTKALGHGVDLGHIYGDNLER
                 170       180       190       200       210       220       230       240

Chick-2   QLKLRLRKDGKLKYQMIDGEMYPPTVKDTQAEMIYPPHVPEHLQFSVGQEVFGLVPGLMMYATIWLREHNRVCDVLKQEH
Human-2   QRKIRLFKDGKMKYQIIDGEMYPPTVKDTQAEMIYPPQVPEHLRFAVGQEVFGLVPGLMMYATIWLREHNRVCDVLKQEH
Rat-2     QHKLRLFQDGKLKYQVIGGEVYPPTVKDTQVDMIYPPHVPEHLRFAVGQEVFGLVPGLMMYATIWLREHNRVCDILKQEH
Mouse-2   QHKLRLFKDGKLKYQVIGGEVYPPTVKDTQVEMIYPPHIPENLQFAVGQEVFGLVPGLMMYATIWLREHNRVCDILKQEH
Mouse-1   QYHLRLFKDGKLKYQVLDGEVYPPSVEQASVLMRYPPGVPPERQMAVGQEVFGLLPGLMLFSTIWLREHNRVCDLLKEEH
Rat-1     QYHLRLFKDGKLKYQVLDGELYPPSVEQASVKMRYPPGVPPEKQMAVAQEVFGLLPGLMLFSTIWLREHNRVCDLLKEEH
Human-1   QYQLRLFKDGKLKYQVLDGEMYPPSVEEAPVLMHYPRGIPPQSQMAVGQEVFGLLPGLMLYATLWLREHNRVCDLLKAEH
Sheep-1   QYQLRLFKDGKLKYQMLNGEVYPPSVEEAPVLMHYPRGIPPQSQMAVGQEVFGLLPGLMLYATIWLREHNRVCDLLKAEH
                 250       260       270       280       290       300       310       320

Chick-2   PEWDDEQLFQTTRLILIGETIKIVIDDYVQHLSGYHFKLKFDPELLFNQRFQYQNRIAAEFNTLYHWHPLLPDTFQIHNQ
Human-2   PEWGDEQLFQTSRLILIGETIKIVIDDYVQHLSGYHFKLKFDPELLFNKQFQYQNRIAAEFNTLYHWHPLLPDTFQINDQ
Rat-2     PEWDDERLFQTSRLILIGETIKIVIEDYVQHLRGYHFQLKFDPDLLFNQQFQYQNRIASEFKTLYHWHPLLPDTFNIEDQ
Mouse-2   PEWGDEQLFQTSRLILIGETIKIVIDDYVQHLSGYHFKLKFDPELLFNQQFQYQNRIASEFNTLYHWHPLLPDTFNIEDQ
Mouse-1   PTWDDEQLFQTTRLILIGETIKIVIEEYVQHLSGYFLQLKFDPELLFRAQFQYRNRIAMEFNHLYHWHPLMPNSFQVGSQ
Rat-1     PTWDDEQLFQTTRLILIGETIEIIIEEYVQHLSGYFLQLKFDPELLFRAQFQYRNRIAMEFNHLYHWHPFMPDSFQVGSQ
Human-1   PTWGDEQLFQTTRLILIGETIKIVIEEYVQQLSGYFLQLKFDPELLFGVQFQYRNRIATEFNHLYHWHPLMPDSFKVGSQ
Sheep-1   PTWGDEQLFQTARLILIGETIKIVIEEYVQQLSGYFLQLKFDPELLFGAQFQYRNRIAMEFNQLYHWHPLMPDSFRVGPQ
                 330       340       350       360       370       380       390       400

Chick-2   EYTFQQFLYNNSIMLEHGLSHMVKSSKRQIAGRVAGGKNVPAAVQKVAKASIDQSRQMRYQSLNEYRKRFMLKPFKSFEE
Human-2   KYNYQQFIYNNSILLEHGITQFVESFTRQIAGRVAGGRNVPPAVQKVSQASIDQSRQMKYQSFNEYRKRFMLKPYESFEE
Rat-2     EYTFKQFLYNNSILLEHGLAHFVESFTRQIAGRVAGGRNVPIAVQAVAKASIDQSREMKYQSLNEYRKRFSLKPYTSFEE
Mouse-2   EYSFKQFLYNNSILLEHGLTQFVESFTRQIAGRVAGGRNVPIAVQAVAKASIDQSREMKYQSLNEYRKRFSLKPYTSFEE
Mouse-1   EYSYEQFLFNTSMLVDYGVEALVDAFSRQRAGRIGGGRNFDYHVLHVAVDVIKESREMRLQPFNEYRKRFGLKPYTSFQE
Rat-1     EYSYEQFLFNTSMLVDYGVEALVDAFSRQRAGRIGGGRNFDYHVLHVAEDVIKESREMRLQSFNEYRKRFGLKPYTSFQE
Human-1   EYSYEQFLFNTSMLVDYGVEALVDAFSRQIAGRIGGGRNMDHHILHVAVDVIRESREMRLQPFNEYRKRFGMKPYTSFQE
Sheep-1   DYSYEQFLFNTSMLVDYGVEALVDAFSRQPAGRIGGGRNIDHHILHVAVDVIKESRVLRLQPFNEYRKRFGMKPYTSFQE
                 410       420       430       440       450       460       470       480

Chick-2   LTGEKEMAAELEELYGDIDAMELYPGLLVEKPRPGAIFGETMVEIGAPFSLKGLMGNTICSPEYWKPSTFGGKVGFEIIN
Human-2   LTGEKEMSAELEALYGDIDAVELYPALLVEKPRPDAIFGETMVEVGAPFSLKGLMGNVICSPAYWKPSTFGGEVGFQIIN
Rat-2     LTGEKEMAAELKALYHDIDAMELYPALLVEKPRPDAIFGETMVELGAPFSLKGLMGNPICSPQYWKPSTFGGEVGFRIIN
Mouse-2   LTGEKEMAAELKALYSDIDVMELYPALLVEKPRPDAIFGETMVELGAPFSLKGLMGNPICSPQYWKPSTFGGEVGFKIIN
Mouse-1   LTGEKEMAAELEELYGDIDALEFYPGLLLEKCQPNSIFGESMIEMGAPFSLKGLLGNPICSPEYWKPSTFGGDVGFNLVN
Rat-1     FTGEKEMAAELEELYGDIDALEFYPGLMLEKCQPNSLFGESMIEMGAPFSLKGLLGNPICSPEYWKPSTFGGDVGFNIVN
Human-1   LVGEKEMAAELEELYGDIDALEFYPGLLLEKCHPNSIFGESMIEIGAPFSLKGLLGNPICSPEYWKPSTFGGEVGFNIVK
Sheep-1   LTGEKEMAAELEELYGDIDALEFYPGLLLEKCHPNSIFGESMIEMGAPFSLKGLLGNPICSPEYWKASTFGGEVGFNLVK
                 490       500       510       520       530       540       550       560

Chick-2   TASLQSLICNNVKGSPFTAFHVLNPEPTETATINVSTSNFAMEDINPTLLLKEQSAEL
Human-2   TASIQSLICNNVKGCPFTSFSVPDPELIKTVTINASSSRSGLDDINPTVLLKERSTEL
Rat-2     TASIQSLICNNVKGCPFASFNVQDPQATKTATINASASHSRLDDINPTVLIKRRSTEL
Mouse-2   TASIQSLICNNVKGCPFTSFNVQDPQPTKTATINASASHSRLDDINPTVLIKRRSTEL
Mouse-1   TASLKKLVCLNTKTCPYVSFRVPDYPGDDGSVIV------------------RRSTEL
Rat-1     TASLKKLVCLNTKTCPYVSFRVPDYPGDDGSVRV------------------RPSTEL
Human-1   TATLKKLVCLNTKTCPYVSFRVPDASQDDGPAVE------------------RPSTEL
Sheep-1   TATLKKLVCLNTKTCPYVSFHVPDPRQEDRPGVE------------------RPPTEL
                 570       580       590                        600
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the short signal peptide of PGHS is still functional, as PGHS-2 is targeted to the ER (66), and the signal peptide is cleaved in the native enzyme (46). Similarly, although the 18 amino acid insert near the C-terminus of PGHS-2 is highly conserved across species, a function for the insert has not yet been determined.

Physical characteristics and intracellular localization of PGHS isozymes.

While most of the studies on the physical characteristics of the PGHS isozymes have been conducted solely on PGHS-1, preliminary work on the physical characteristics of PGHS-2 have shown that important structural features are conserved between the two enzymes. Because of the abundance of PGHS-1 in ovine seminal vesicles, this tissue has been the source used in most studies of PGHS-1. PGHS-1 has been characterized as a membrane-associated glycoprotein (2) which is localized to the ER and nuclear membranes (66, 67). Ovine PGHS-1 has a molecular weight determined by SDS-PAGE of approximately 72 kDa (2, 3, 68), compared to its theoretical molecular weight of approximately 66 kDa (59-61). The difference in molecular weight has been attributed to the presence of three N-linked high mannose carbohydrate groups (69). In the deduced amino acid sequence of ovine PGHS-1 there are four consensus sequences for N-glycosylation (Asn-X-Ser/Thr), occurring at Asn68, Asn104, Asn144, and Asn410.

Murine PGHS-2 exhibits two bands on SDS-PAGE with molecular weights of 74 and 72 kDa (46), compared to the theoretical molecular mass of about 67 kDa. PGHS-2 contains three of the four consensus sequences for N-glycosylation found in ovine PGHS-1, at positions corresponding to Asn68, Asn144, and Asn410 in ovine PGHS-1. There are also two additional consensus sequences for N-glycosylation in PGHS-2 which are not found in PGHS-1. These two N-glycosylation consensus sequences are located in the 18 amino acid insert near the C-terminus of the enzyme, and occur at Asn580 and Asn592 in murine PGHS-2 (28, 30).

Both PGHS-1 and PGHS-2 are found in microsomal membrane fractions (2, 3, 46, 68). PGHS-1 has been characterized as an integral membrane protein, based on observations that detergents are required to solubilize the enzyme from membrane preparations (2). Consistent with this prediction, purified PGHS-1 can be reconstituted into pure phospholipid liposomes, indicating that the enzyme can associate directly with the phospholipid component of membranes (70).

Intact ovine PGHS-1 is susceptible to proteolytic cleavage by trypsin at Arg277 (71). This property has been utilized in studies of the topology of ovine PGHS-1 in microsomal membranes, and it has been determined that Arg277 is accessible to trypsin in microsomes containing ovine PGHS-1 (71-73). Because the outside of intact, right-side-out microsomes correspond to the cytoplasmic side of intracellular membranes, this evidence led to the prediction that Arg277 was oriented on the cytoplasmic surface of the ER membrane. Several distinct epitopes for monoclonal antibodies were also accessible in microsomal PGHS-1 (72), suggesting that several several regions of ovine PGHS-1 reside in the cytoplasm. Combined with the presence of luminal (N-glycosylated) regions of PGHS, these studies predicted that PGHS-1 contained one or more transmembrane domains. One region of ovine PGHS-1, between residues 285-306, was identified as a potential membrane spanning domain by hydrophobicity analysis (60).

However, the crystal structure of detergent solubilized ovine PGHS-1 has indicated that PGHS-1 is an essentially globular protein, having significant structural homology with two soluble peroxidases, myeloperoxidase and cytochrome c peroxidase (74). There are not any regions in the structure of PGHS-1 corresponding to transmembrane domains; the 285-306 region identified by hydrophobicity analysis as a potential membrane spanning domain lies in the core of the enzyme. Thus, discrepancies exist between the biochemical data on the orientation of ovine PGHS-1 in membranes and the crystal structure of the detergent solubilized enzyme.

The crystal structure of ovine PGHS-1 does contain a domain which might serve as a membrane binding domain. This domain is composed of three amphipathic α-helices, labelled helix A, helix B and helix C, which lie roughly in a triangular plane, and part of a fourth helix D which forms part of the hydrophobic channel which leads into the cyclooxygenase active site (Fig 3). The helices in this domain project hydrophobic residues away from the body of the protein, forming a hydrophobic surface. The domain is predicted to interact with a single leaflet of the lipid bilayer, making PGHS-1 the first protein with a structure predicted to integrate into a single leaflet of the membrane bilayer (74, 75).

Upon solubilization of PGHS-1 from membranes, the enzyme has been demonstrated to exist as a homodimer by crosslinking studies (76), sedimentation analysis (2) and gel filtration. The crystal structure of PGHS-1 agrees with this biochemical data, and has provided information on the regions of PGHS-1 involved in dimerization. Both PGHS isozymes contain an EGF-homology domain at their amino termini, consisting of 6 cysteine residues which form three disulfide bonds. Interestingly, the EGF-homology domains of the two subunits which form the holoenzyme do not associate with one another; rather, each EGF-homology domain interacts with another region of the

Figure 3.
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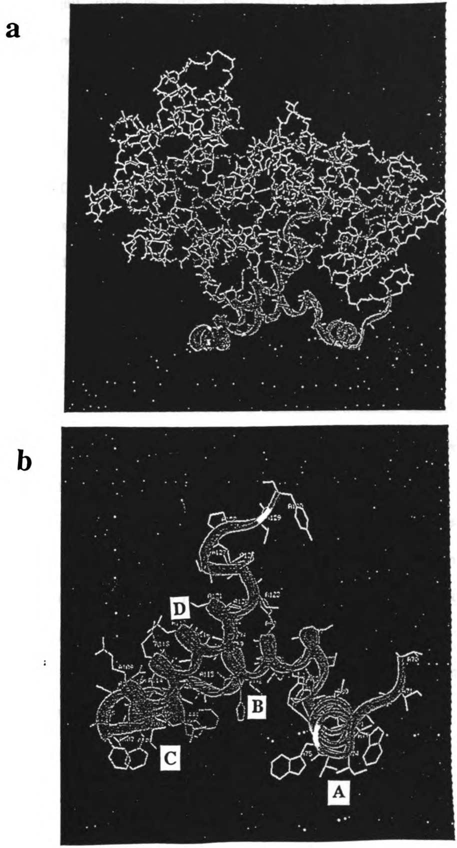
Figure 3. Crystal structure of ovine PGHS-1 and the putative membrane binding domain. Crystal structure coordinates were generously provided by R. Micheal Garavito, Daniel Picot, and Patrick J. Loll of the University of Chicago Department of Biochemisty and Molecular Biology. (a) The crystal structure of the amino acid backbone of one subunit of ovine PGHS-1. Residues 70-130, which contain the putative membrane binding domain, are highlighted with a ribbon. (b) The putative membrane binding domain of ovine PGHS-1. Helices are labelled A, B, C, D.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opposite subunit, giving the dimer a head-to-tail arrangement. It is not known if the PGHS isozymes exist as dimers in membranes. However, because it has not been possible to prepare active monomers of PGHS, and because the crystal structure indicates that there are specific interactions which occur between the subunits of the dimer following solubilization, it is assumed that the PGHS isozymes do function as dimers in membranes. Dimerization could be favorable event for the association of PGHS with the membrane, as dimer formation brings together two membrane association domains, forming a hydrophobic surface along the one face of the enzyme (75).

PGHS-1 and PGHS-2 have similar subcellular localizations when observed by immunofluorescent microscopy. Both isozymes reside in the ER and nuclear membranes. However, PGHS-2 appears to be more heavily concentrated in the nuclear envelope than PGHS-1 (66). Although this difference in localization is subtle, it may be significant. As described earlier, the signal peptide of PGHS-2 lacks several consecutive hydrophobic amino acids found in the signal peptide of PGHS-1. This may result in a difference in the sites of insertion or localization for the protein in the ER and nuclear membranes. Alternatively, the 18 amino acid insert region near the carboxyl terminus of PGHS-2 could be involved in targeting PGHS-2 to the nuclear envelope. The concentration of PGHS-2 on the nuclear envelope may be important for the mechanism by which PGHS-2 can selectively utilize arachidonic acid mobilized following the treatment of cells with growth factors or cytokines (55).

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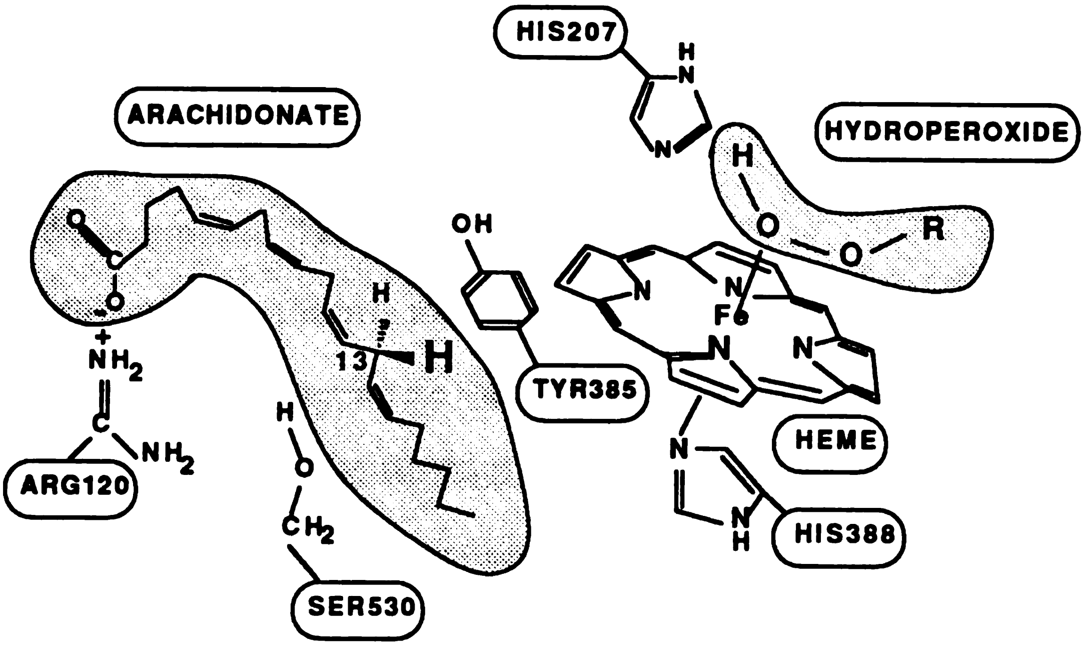
Cyclooxygenase and Peroxidase activities of PGHS isozymes and inhibition by NSAIDS.

PGHS-1 and PGHS-2 generate PGH_2 from arachidonic acid via the same two activities: the cyclooxygenase activity, which catalyzes the bis-oxygenation of arachidonic acid to form the endoperoxide PGG_2, and the peroxidase activity, which reduces the 15-hydroperoxyl group of PGG_2 to form PGH_2 (see Fig. 1). Studies utilizing site directed mutagenesis have identified several residues important for the activity of PGHS-1. The location of these residues in the crystal structure is consistent with their participation in catalysis. Figure 4 illustrates the active site of ovine PGHS-1. Heme is required for both the cyclooxygenase and peroxidase activities of PGHS-1. The heme group is coordinated by a pair of histidine residues, with His388 acting as the proximal heme ligand and His207 acting as the distal heme ligand (74, 77). Both of these residues are conserved in PGHS-2, and it assumed that PGHS-2 binds heme in an identical manner to PGHS-1.

The cyclooxygenase active site of ovine PGHS-1 is thought to be composed of a long hydrophobic channel which extends from the membrane binding domain to near the heme group. This hydrophobic channel has been shown to bind the NSAID flurbiprofen, and it is assumed to bind arachidonate. In ovine PGHS-1, Arg120 lies near the membrane binding domain, and is predicted to bind the carboxylate ion of arachidonate (74). Recent studies involving site-directed mutagenesis of Arg120 are consistent with this prediction (78). Ser530 also lies in this channel. Ser530, a conserved residue in both PGHS isozymes, can be acetylated by aspirin, causing in the elimination of cyclooxygenase activity (22, 62).

Figure 4. Mo
Laneuville, 1994 (9).

Figure 4. Model of the active site for ovine PGHS-1. Adapted from Smith and Laneuville, 1994 (9).


HIS207
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N
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ARACHIDONATE
HYDROPEROXIDE
H
O
O
R
O
C
O
+
NH2
C
NH2
ARG120
OH
H
13
H
N
N
Fe
N
N
TYR385
HEME
H
O
CH2
SER530
N
N
H
HIS388

The properties of the PGHS isozymes following acetylation of the active site serine with aspirin demonstrate that some structural differences do exist between the two enzymes. Acetylation of the active site serine in PGHS-1 with aspirin completely blocks cyclooxygenase activity, while having no effect on peroxidase activity. Through site directed mutagenesis, it was determined that the active site serine is not an essential residue for catalysis; rather, in PGHS-1, acetylation of this serine, or substitution (by site-directed mutagenesis) of the serine residue with a larger amino acid such as asparagine, introduces a steric group into the cyclooxygenase channel which presumably blocks the binding of arachidonic acid (62).

Acetylation of the active site serine in PGHS-2 by aspirin blocks the formation of PGH_2 by this enzyme also. However, in contrast to PGHS-1, acetylation of the active site serine in PGHS-2 does not entirely block the cyclooxygenase active site. This is evident because acetylated PGHS-2 retains the ability to oxygenate arachidonic acid, forming 15-hydroxyeicosatetraenoic acid (15-HETE) (5, 79). Mutations of the active site serine have demonstrated similar results; while replacement of the active site serine of PGHS-1 with asparagine inactivated cyclooxygenase activity, the same mutation in PGHS-2 did not significantly affect cyclooxygenase activity. For inactivation of PGHS-2, mutation to the larger amino acid glutamine was necessary (79). Thus, it appears that the active site of PGHS-2 in the region of the active site serine is somewhat larger than that of PGHS-1. Other NSAIDS act in a similar fashion to aspirin in that they bind in the hydrophobic channel which forms the cyclooxygenase active site of PGHS (74, 80, 81). Because there does appear to be some difference between the sizes of the PGHS isozymes' active sites, it may be possible to discover or design drugs which specifically inhibit PGHS-2.

Following the binding of arachidonic acid in the active site of PGHS, the cyclooxygenase reaction is thought to be initiated by the abstraction of the C-13 *pro-S* hydrogen from arachidonate by a tyrosyl radical present in the enzyme (82-84). Electron paramagnetic resonance studies have demonstrated that a tyrosyl radical is generated by both PGHS-1 and PGHS-2 (85). Tyr385 has been proposed as a source of the tyrosyl radical (84, 86), and it is situated near the cyclooxygenase active site in close proximity to both the heme and the predicted position of C-13 of arachidonic acid.

Because NSAIDS inhibit the cyclooxygenase activity of PGHS-1 and PGHS-2 without affecting the peroxidase activity of the enzymes (87, 88), it was predicted that the enzyme had two distinct active sites, one for the cyclooxygenase reaction and one for the peroxidase reaction. The crystal structure of ovine PGHS-1 is consistent with this prediction. The peroxidase active site appears to be distinct from the cyclooxygenase active site, residing on the opposite side of the heme group. The conformations of the two active sites suggest that PGG_2 produced in the cyclooxygenase reaction is released by the enzyme prior to binding to the peroxidase active site, consistent with biochemical studies.

CHAPTER II

EXAMINATION OF THE N-GLYCOSYLATION OF PGHS-1 AND PGHS-2

Introduction

Approximately 8% of the mass of ovine PGHS-1 is carbohydrate, consisting of three Asn-linked, high mannose oligosaccharides (2, 69). In the deduced amino acid sequence of ovine PGHS-1, there are four consensus sequences for N-glycosylation consisting of the tripeptide Asn-X-Ser/Thr, where X can be any amino acid. These consensus sequences are located at Asn68, Asn104, Asn144, and Asn410 (59-61); these four sites are conserved in human (63) and murine (62) PGHS-1. Three of these four consensus sequences for N-glycosylation, corresponding to Asn68, Asn144, and Asn410 of ovine PGHS-1 are present in murine PGHS-2 (28, 30). There are also two additional consensus sequences for N-glycosylation PGHS-2 which are not found in PGHS-1. These are located at Asn580 and Asn592 in murine PGHS-2. We have used site-directed mutagenesis (a) to determine which of the four N-glycosylation consensus sequences in ovine PGHS-1 are glycosylated, (b) to determine if either of the two additional consensus sequences in murine PGHS-2 is glycosylated; and (c) to investigate the role of N-linked oligosaccharides in enzyme catalysis by the PGHS isozymes.

Methods

Materials. Dulbecco's modified Eagle medium (DMEM) and fetal calf serum were obtained from GIBCO. Calf serum was from Hyclone. Chloroquine, bovine hemoglobin, hematin, guaiacol, trypsin (Type IX), chicken egg white trypsin inhibitor, tunicamycin, DEAE dextran, and nitro blue tetrazolium were from Sigma. Indomethacin was from Merck, Sharp, & Dohme Research Laboratories. Flurbiprofen was provided by the Upjohn Company. pSVT7 was a generous gift from Dr. Joseph Sambrook (University of Texas Southwestern Medical Center). pSVL was from Pharmacia. [α-^{35}S]dATP was purchased from DuPont-New England Nuclear. ^{125}I-labeled Protein A was from ICN Biomedicals. BA85 (0.45 μm) nitrocellulose was from Schleicher & Schuell. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, and endoglycosidase H were from Boehringer Mannheim Biochemicals. Sequenase was from U.S. Biochemical Corp. Purified ovine PGHS-1 was obtained from Oxford Biomedical Research, Inc. Goat anti-rabbit IgG-horseradish peroxidase was from BioRad. ECL western blotting reagents were from Amersham. Oligonucleotides used for preparing mutants of PGHS-1 and PGHS-2 were prepared by the Michigan State University Macromolecular Structure and Sequencing Facility. All other reagents were from common commercial sources.

Preparation of PGHS-1 Mutants. The coding region of ovine PGHS-1 with unique Sal I restriction sites in both the 5′- and 3′-untranslated sequences was as described previously (62, 77, 86). Mutants were prepared starting with M13mp19-PGHS$_{ov}$-1, which contains a 2.3 kb Sal I fragment encoding the native ovine PGHS-1, according to the method of Kunkel *et al.* (89) using a Bio-Rad kit essentially as described previously (62, 77, 86). Table I shows the oligonucleotides used in the preparation of each of the mutants. Phage samples were sequenced using the dideoxy method (90) to identify mutants. A double mutant, N68Q/N144Q, was constructed by a second round of mutagenesis using the N68Q oligonucleotide primer and M13mp19-PGHS$_{ov}$-1 (N144Q) as the original template. The 2.3 kb insert from the replicative form of M13mp19-PGHS$_{ov}$-1 containing the desired mutation was isolated and subcloned into pSVT7 (77, 86). The orientation of the insert was determined by restriction digestion with Pst I (77, 86). Plasmids used for transfections, designated pSVT7-PGHS$_{ov}$-1 (mutant), were purified by CsCl gradient ultracentrifugation. Mutations were reconfirmed by double-stranded sequencing of the pSVT7 constructs using Sequenase (Ver. 2.0) and the protocol described by the manufacturer.

Preparation of PGHS-2 Mutants. The coding region of murine PGHS-2 with the 5′-untranslated region of ovine PGHS-1, no 3′-untranslated region, and unique Sal I restriction sites at the 5′- and 3′-ends was as described previously (91). M13mp19-PGHS$_{mu}$-2, which contains the 1.8 kb Sal I fragment encoding the native murine PGHS-2 described above, was used to prepare mutants essentially as outlined above for PGHS-1. Table I shows the oligonucleotides used in the preparation of each of the mutants. The 1.8 kb Sal I insert fragment containing the mutant PGHS-2 cDNA was isolated from the replicative form of M13mp19-PGHS$_{mu}$-2 and subcloned into the Xho I site of pSVL. Determination of the insert orientation and purification of pSVL-PGHS$_{mu}$-2 constructs were performed as described above for pSVT7-PGHS$_{ov}$-1.

Transfection of Cos-1 Cells with pSVT7 and pSVL Constructs. Cos-1 cells (ATTC CRL-1650) were grown in DMEM containing 8% calf serum and 2% fetal calf

Table I.

Mutatio
N68C T70A
N10
N14 S14
N4 S4 N4 N
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Table I. Oligonucleotides used in site directed mutagenesis.

Mutation	PGHS	Mutagenic Oligonucleotide
N68Q	ov-1	$5'\text{-}^{293}\mathrm{CCGGCCCC}\underline{\mathrm{CAA}}\mathrm{TGCACCATC}^{312}\text{-}3'$
T70A	ov-1	$5'\text{-}^{298}\mathrm{CCCAACTGC}\underline{\mathrm{GCC}}\mathrm{ATCCCGGA}^{317}\text{-}3'$
N104Q	ov-1	$5'\text{-}^{401}\mathrm{ATTTTGTC}\underline{\mathrm{CAG}}\mathrm{GCCACCTTC}^{420}\text{-}3'$
N144Q	ov-1	$5'\text{-}^{516}\mathrm{GGAGTCCTTCTCC}\underline{\mathrm{CAG}}\mathrm{GTGAGCTATTAT}^{543}\text{-}3'$
S146A	ov-1	$5'\text{-}^{523}\mathrm{TTCTCCAATGTG}\underline{\mathrm{GCC}}\mathrm{TATTATACTCGC}^{549}\text{-}3'$
N410Q	ov-1	$5'\text{-}^{1319}\mathrm{TTCTGTTC}\underline{\mathrm{CAA}}\mathrm{ACCTCCATG}^{1338}\text{-}3'$
S412A	ov-1	$5'\text{-}^{1323}\mathrm{GTTCAACACC}\underline{\mathrm{GCC}}\mathrm{ATGCTGGTG}^{1344}\text{-}3'$
N410D	ov-1	$5'\text{-}^{1315}\mathrm{CAGTTTCTGTTC}\underline{\mathrm{GAC}}\mathrm{ACCTCCATG}^{1338}\text{-}3'$
N410S	ov-1	$5'\text{-}^{1315}\mathrm{CAGTTTCTGTTC}\underline{\mathrm{AGC}}\mathrm{ACCTCCATG}^{1338}\text{-}3'$
N580Q	mu-2	$5'\text{-}^{1852}\mathrm{AGCCACCATC}\underline{\mathrm{CAG}}\mathrm{GCAAGTGCC}^{1873}\text{-}3'$
N592Q	mu-2	$5'\text{-}^{1881}\mathrm{CCAGACTAGATGACATT}\underline{\mathrm{CAA}}\mathrm{CCTACAGTAC}^{1910}\text{-}3'$
HelA I74T-W75S-W77S-L78T	ov-1	$5'\text{-}^{312}\mathrm{CCCGGA}\underline{\mathrm{GACATC}}\mathrm{GAC}\underline{\mathrm{CTCGAC}}\mathrm{CCGGACGAC}^{341}\text{-}3'$
HelB F88S-F91S-L92A	ov-1	$5'\text{-}^{353}\mathrm{AGCCCCTCT}\underline{\mathrm{TCC}}\mathrm{ATCCAC}\underline{\mathrm{TCTGCG}}\mathrm{CTGACGCACGGG}^{388}\text{-}3'$
HelC W98S-L99A-F102S	ov-1	$5'\text{-}^{383}\mathrm{CACGGGCGC}\underline{\mathrm{TCGGCT}}\mathrm{TGGGAT}\underline{\mathrm{TCT}}\mathrm{GTCAATGCCACC}^{418}\text{-}3'$
ΔEGF Delete C36-P67	ov-1	$5'\text{-}^{166}\mathrm{GACCCCGGCGCGCCCGCG/CCCAACTGCACCATCCCG}^{315}\text{-}3'$

Numbering of amino acids begins with the Met at the translational start site.
Numbering of nucleotides begins at the transcriptional start site.
Mutagenic codons are underlined.
ΔEGF deletion site marked with a "/".

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serum until near confluence, and the cos-1 cells (ca. $3x10^6$ cells/100 mm dish) were transfected as previously described (77, 86, 91) using a pSVT7 (or pSVL) construct containing the coding region of the native or mutant ovine PGHS-1 (or murine PGHS-2). Microsomal membranes were prepared from transfected cells in 0.1 M Tris-HCl, pH 7.4 (62, 77, 86). Tunicamycin treatments of cos-1 cells transfected with pSVT7-PGHS$_{ov}$-1 or pSVL-PGHS$_{mu}$-2 constructs were performed by adding tunicamycin to the media at a final concentration of 1 µg/ml 20 to 24 hrs posttransfection (92).

Western Transfer Blotting. Solubilized microsomal membranes were resolved by one-dimensional SDS-PAGE and transferred electrophoretically to BA85 nitrocellulose filters (0.45 µM) essentially as described previously (77). For detection of PGHS-1 bands by autoradiography, filters were first incubated overnight with a 1:100 dilution of monospecific rabbit anti-PGHS-1 serum (77). The filters were then washed and incubated for four hours with ^{125}I-Protein A. Following a final wash and air drying, the filters were subjected to autoradiography using Kodak XRP-5 X-ray film.

For detection of PGHS-1 bands by enhanced chemiluminescence, filters were incubated for one to two hours with a 1:20,000 dilution of monospecific rabbit anti-PGHS-1 serum. The filters were washed and incubated for one to two hours with a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase. The filters were again washed and incubated for one minute with Amersham ECL western blotting detection reagents. The filters were immediately blotted dry and exposed to Kodak XRP-5 X-ray film.

PGHS-2 was detected by using a peroxidase stain. The filter was incubated for two hours with a 1:2000 dilution of a monospecific rabbit anti-PGHS-2 serum (66). The filter was then washed and incubated for two hours with a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase. The filter was again washed and stained with the following solution: 50 mM $NaHPO_4$, pH 7.0, 3 mM NADH, 2 µM phenol, 6 µM H_2O_2, and 0.4 mM nitro blue tetrazolium (93). Following staining, the filter was photographed.

Enzymatic Deglycosylation of Denatured PGHS-1 and PGHS-2. Microsomal samples (100 µg protein/25 µl) for analysis by western transfer blotting were incubated with 2% SDS at 100°C for 2 min and then cooled on ice. The solubilized proteins were then diluted in 50 mM NaH_2PO_4, pH 5.7 (100 µg protein/100 µl final concentration), and incubated with 5 mU of endoglycosidase H at 37°C for 12 hrs. SDS-sample buffer was then added, and the samples were analyzed by western transfer blotting.

Tryptic Cleavage of PGHS-1 and Protection with Heme and Indomethacin. Microsomal suspensions (100 µg protein/100 µl) prepared from cos-1 cells transfected with pSVT7 constructs encoding native or mutant PGHS-1 were incubated with 5 µg of trypsin at 25°C for 5 min. The reactions were quenched by adding a 40-fold molar excess of trypsin inhibitor, and the samples were then chilled on ice and incubated for 2 min at 100°C with SDS-sample buffer. The resulting peptide products were analyzed by western transfer blotting.

The ability of heme and indomethacin to protect native and mutant PGHS-1 from cleavage by trypsin was analyzed essentially as described previously (77, 94). Microsomes (100 µg protein/100 µl) were incubated with 32 µM hematin for 15 min. In some experiments, the microsomes were then incubated with 250 µM indomethacin for 15 min. Samples were then incubated with 5 µg trypsin for 10 min at 25°C. The reactions were then quenched, prepared for SDS-PAGE, and analyzed as described above.

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Cyclooxygenase Assays. The cyclooxygenase activities of microsomal preparations were measured by monitoring the initial rate of O_2 uptake at 37°C using an O_2 electrode as described previously (77, 94). The assay mixture contains 3 ml of 0.1 M Tris-HCl, pH 8.0, 1 mM phenol, 85 µg of hemoglobin (as a source of heme), and 100 µM arachidonic acid. Reactions were initiated by adding 5-100 µg of microsomal protein. Cyclooxygenase activities were inhibited by the addition of flurbiprofen (160 µM) to the reaction mixture.

Peroxidase Assays. The peroxidase activities of microsomal preparations were measured spectrophotometrically as described previously (77, 86, 94) using the procedure of Marnett *et al.* (95). The reaction mixture contained 100 mM Tris-HCl, pH 7.2, 5.6 mM guaiacol, 5-100 µg of microsomal protein, and 1 µM hematin (added in DMSO) in a total volume of 0.3 ml. Reactions were initiated by adding 20 µl of 4.5 mM H_2O_2, and the absorbencies measured at 436 nm.

Enzymatic Deglycosylation of Intact PGHS-1. Purified ovine PGHS-1 (100 µg of protein/800 µl) was incubated with 50 mU of endoglycosidase H in 50 mM NaH_2PO_4, pH 5.7, at 37°C. Flufenamate (100 µM) was included to stabilize the enzyme. Alternatively, microsomes from cos-1 cells that had been transfected with a construct encoding native ovine PGHS-1 were solubilized in 1% Tween 20 (final 160 µg of protein/160 µl). The solubilized microsomes were incubated with 50 mU endoglycosidase H in 50 mM NaH_2PO_4, pH 5.7 at 37°C (final volume 300 µl). After different times of incubation, aliquots of the reaction mixtures were assayed for cyclooxygenase and peroxidase activities, and samples were taken for analysis by western transfer blotting.

Protein Determinations. Determinations of protein concentrations were performed by the method of Bradford (96).

Results

Electrophoretic Mobility of PGHS-1 Glycosylation Mutants. PGHS-1 is an N-glycosylated protein with a subunit molecular mass of 72 kDa (2, 3, 97) having three Asn-linked, high mannose oligosaccharides (69). The deduced amino acid sequence of ovine PGHS-1 contains four N-glycosylation consensus sequences (Asn-X-Ser/Thr) with potential glycosylation sites at Asn68, Asn104, Asn144, and Asn410 (59-61). To determine which of these sites are N-glycosylated, we first constructed four mutants of ovine PGHS-1 by replacing the Asn at each potential glycosylation site with a Gln. These mutants were then expressed by transient transfection of cos-1 cells, microsomal membranes were prepared from the transfected cells, and the mobilities of the mutant proteins were examined by western transfer blotting (Fig. 5). Three of the mutant proteins, N68Q, N144Q, and N410Q, each displayed an increased mobility corresponding to a decrease in subunit molecular mass of approximately 2 kDa. The fourth mutant protein, N104Q, exhibited a subunit molecular mass which was the same as that of the native enzyme.

A double mutant, N68Q/N144Q, was subsequently constructed by replacing both Asn68 and Asn144 with Gln residues. In addition, unglycosylated enzyme was produced by expressing native PGHS-1 in cos-1 cells which were cultured in the presence of the N-glycosylation inhibitor, tunicamycin. The N68Q/N144Q double mutant protein exhibited an apparent subunit molecular mass which was 4 kDa less than the native enzyme, and the unglycosylated native PGHS-1 exhibited a 6 kDa decrease (Fig. 5). Treatment with endoglycosidase H reduced the apparent molecular mass of all of the

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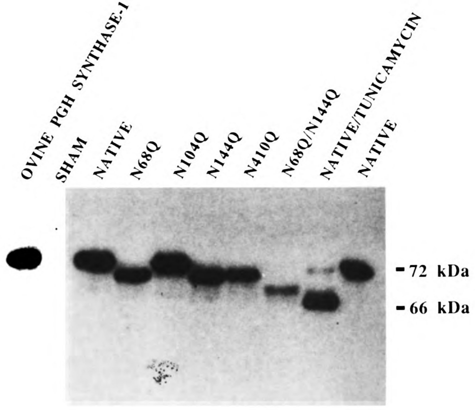
Fig. 5. Electrophoretic mobilities of native PGH synthase-1 and PGH synthase-1 N-glycosylation site mutants. Microsomes were prepared from sham-transfected cos-1 cells, from cos-1 cells transfected with pSVT7 constructs encoding native and mutant PGH synthases-1, and from cos-1 cells transfected with the pSVT7 construct encoding the native PGH synthase-1 and treated with tunicamycin. Samples (20 μg protein) were electrophoresed using a 10% SDS-polyacrylamide gel, and electroeluted onto nitrocellulose. The filter was incubated with a 1:100 dilution of anti-PGH synthase-1 serum and ^{125}I-Protein A as described in Methods. The nitrocellulose was washed with 0.1% Tween 20 in TBS, air dried, and exposed to X-ray film at -80°C. Purified ovine PGH synthase-1 (0.5 μg protein) was electrophoresed as a standard and is noted as OVINE PGH SYNTHASE-1. Microsomal samples were as indicated. Unglycosylated PGH synthase-1 was produced by expression of the native PGH synthase-1 in cos-1 cells to which tunicamycin (1 μg/ml) was added 20 hrs posttransfection, and is noted as NATIVE/TUNICAMYCIN.


OVINE PGH SYNTHASE-1
SHAM
NATIVE
N68Q
N104Q
N144Q
N410Q
N68Q/N144Q
NATIVE/TUNICAMYCIN
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single-site mutant PGHS-1, the native enzyme, and the double mutant (N68Q/N144Q) to 66 kDa (Fig. 6); this latter value was the same as that observed for the unglycosylated native PGHS-1 expressed in tunicamycin-treated cos-1 cells. These results indicate that the changes in apparent molecular masses seen with the mutant proteins are due to changes in the extent of glycosylation of the proteins and not to proteolytic degradation. Taken together, the results shown in Figures 5 and 6 indicate that ovine PGHS-1 is glycosylated at three sites: Asn68, Asn144, and Asn 410. To verify this conclusion, three additional mutants were constructed in which the serine or threonine in the consensus sequences of each of the proposed N-glycosylation sites was substituted with an alanine. When examined by western transfer blotting, each of these three mutant proteins, T70A, S146A, and S412A, exhibited a decrease in apparent subunit molecular mass of 2 kDa (data not shown); this electrophoretic behavior is the same as that observed with the corresponding N68Q, N144Q, and N410Q mutants and is consistent with the N-glycosylation sites being located at Asn68, Asn144, and Asn410.

Digestion of native ovine PGHS-1 with dilute trypsin results in cleavage at Arg277, generating a 38 kDa peptide containing the carboxyl (C-) terminus and a 33 kDa peptide containing the amino (N-) terminus (72). By determining the molecular masses of the peptides derived from tryptic digestion of various glycosylation site mutants, we were able, in some cases, to localize the decreases in molecular mass to the tryptic peptide which contained the mutation (Fig. 7). Following trypsin treatment, the N-terminal peptides for the N68Q and N144Q mutants exhibited decreases in their apparent molecular masses; in contrast, the C-terminal tryptic peptides from native enzyme and the N68Q and the N144Q mutant proteins, as well as the N68Q/N144Q double mutant, each

Fig. 6. Electrophoretic mobilities of native PGH synthase-1 and PGH synthase N-glycosylation site mutants following treatment with endoglycosidase H. Microsomes (100 µg protein) from transfected cos-1 cells were solubilized by boiling in SDS and then incubated with endoglycosidase H (5 mU) at 37°C for 12 hrs at pH 5.7. Following glycosidase treatment, samples (20 µg protein) were analyzed by western transfer blotting utilizing ^{125}I-Protein A as a secondary antibody followed by film exposure by autoradiography. Untreated native and unglycosylated PGH synthase-1 (expressed in tunicamycin treated cos-1 cells) are noted as NATIVE (UNTREATED) and NATIVE/TUNICAMYCIN (UNTREATED). All other samples were treated with endoglycosidase H.

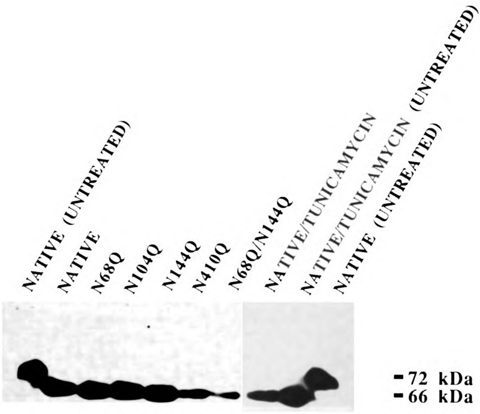
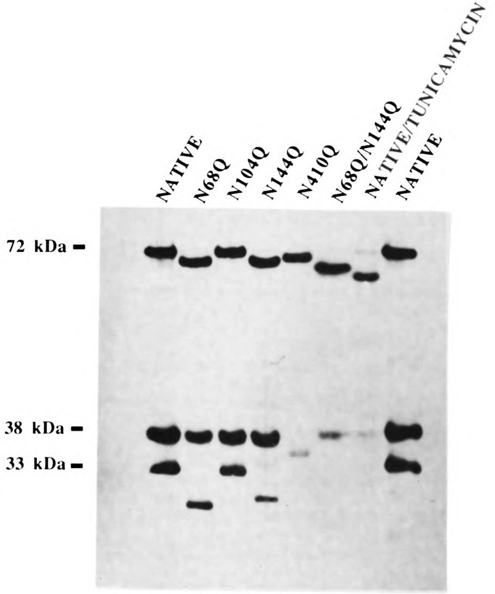

NATIVE (UNTREATED)
NATIVE
N68Q
N104Q
N144Q
N410Q
N68Q/N144Q
NATIVE/TUNICAMYCIN
NATIVE/TUNICAMYCIN (UNTREATED)
NATIVE (UNTREATED)
72 kDa
66 kDa

Fig. 7. Tryptic digestion of native PGH synthase-1 and PGH synthase-1 N-glycosylation site mutants. Microsomes (100 µg protein) prepared from transfected cos-1 cells were incubated for 5 min with trypsin (5 µg) at room temperature. Reactions were stopped by the addition of trypsin inhibitor (40-fold molar excess). Trypsin-treated samples (22 µg protein for the N68Q/N144Q mutant, 8 µg protein for all other samples) were then analyzed by western transfer blotting, using a 1:20,000 dilution of rabbit anti-PGH synthase-1 serum as primary antibody and a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase as the secondary antibody. The filter was then incubated with Amersham's ECL western blotting reagents followed by film exposure by enhanced chemiluminescence.


NATIVE
N68Q
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exhibited the expected apparent molecular mass of 38 kDa. The C-terminal peptide of the N410Q mutant protein exhibited a decreased apparent molecular mass of 36 kDa. These observations are consistent with the concept that there are three glycosylation sites in ovine PGHS-1 (69), and that two of these sites, Asn68 and Asn144, are in the N-terminal peptide and one of the sites, Asn410, is in the C-terminal peptide.

No N-terminal tryptic peptides for the N410Q and N68Q/N144Q mutant proteins or for unglycosylated native enzyme from transfected, tunicamycin treated cells were detected; the reason for this is likely to be an increase in trypsin sensitivity for these proteins. The faint 38 kDa C-terminal tryptic peptide seen in the unglycosylated, native enzyme lane is thought to be due to the cleavage of a small amount of glycosylated PGHS-1 produced in this particular transfection (which is evident by the faint band seen at 72 kDa). In digestions of unglycosylated native enzyme where this 72 kDa band was not present, no 38 kDa tryptic peptide was evident (data not shown).

Cyclooxygenase and Peroxidase Activities of PGHS-1 Mutants. Mutations in the consensus sequences affecting N-glycosylation at either Asn68 or Asn144 decreased both the cyclooxygenase and peroxidase activities of the enzymes considerably but did not abolish either activity (Table II). However, the double mutant, N68Q/N144Q, had no enzyme activity. Moreover, all mutations affecting N-glycosylation at Asn410 were found to eliminate both cyclooxygenase and peroxidase activities. These include the N410Q and S412A mutations, as well as two additional mutations — N410D and N410S. Consistent with these observations, unglycosylated PGHS-1 obtained from expression of the native enzyme in tunicamycin-treated cos-1 cells did not exhibit cyclooxygenase or peroxidase activity. Thus, glycosylation of ovine PGHS-1 at Asn410 and either Asn68 or Asn144

Table II. **Cyclooxygenase and peroxidase activities of PGHS-1 N-glycosylation site mutants.**

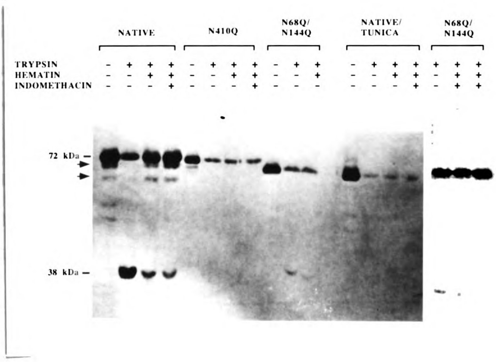
Ovine PGH Synthase-1 Expressed in cos-1 Cells	Cyclooxygenase (% of control)	Peroxidase (% of control)
Native	100	100
Sham transfection	0.8 ± 0.2	0.2 ± 0.2
Native/Tunicamycin	0	0
N68Q	18.1 ± 1.4	11.2 ± 0.8
T70A	39.7 ± 2.5	24.5 ± 4.9
N104Q	48.6 ± 9.7	42.3 ± 1.0
N144Q	6.8 ± 1.3	3.5 ± 0.1
S146A	12.1 ± 3.0	4.8 ± 1.5
N410Q	0.5 ± 0.4	0.2 ± 0.2
N410D	0.6 ± 0.1	0
N410S	0.5 ± 0.2	0
S412A	0.8 ± 0.2	0.8 ± 0.8
N68Q/N144Q	0	0

Activities are expressed as the percent of the cyclooxygenase or peroxidase activity seen for native ovine PGHS-1.

during its synthesis in cos-1 cells is necessary for the enzyme to exhibit measurable cyclooxygenase and peroxidase activities.

Protection of PGHS-1 Mutants from Trypsin Cleavage by Heme and Indomethacin. The binding of heme and the non-steroidal anti-inflammatory agent indomethacin by native ovine apoPGHS-1 protects it from cleavage at Arg277 by dilute trypsin (73, 80). Protection from tryptic digestion by these compounds has been used as a method to determine if enzymatically inactive ovine PGHS-1 mutants do exhibit some native conformation (77). Accordingly, the N410Q and N68Q/N144Q mutant proteins and unglycosylated native PGHS-1 (from expression in the presence of tunicamycin) were treated with trypsin in the presence and absence of indomethacin and/or heme (Fig. 8). Heme alone and heme plus indomethacin protected native PGHS-1 from proteolysis, as evidenced by the dramatic increase in the intensity of the 72 kDa band and also by the decrease in intensity of the 38 kDa C-terminal tryptic peptide (in this particular experiment, the 33 kDa N-terminal peptide was not detected). Neither heme nor heme plus indomethacin appeared to protect either the N410Q mutant protein or unglycosylated PGHS-1 from trypsin cleavage. The N68Q/N144Q mutant protein also does not appear to be protected from tryptic cleavage; however, the faint 38 kDa C-terminal tryptic peptide observed for the N68Q/N144Q double mutant decreased in intensity in the presence of both heme and indomethacin. This may indicate that a small population of the N68Q/N144Q mutant protein can bind heme and indomethacin. However, because of the lack of an increase in the intensity of the intact 68 kDa protein band, compared to that seen for native PGHS-1, we conclude that in general the majority of the double mutant protein N68Q/N144Q was not protected against trypsin cleavage by these

Fig. 8. Protection of native and inactive mutant PGH synthases-1 from tryptic digestion by heme and indomethacin. Microsomes were prepared from cos-1 cells transfected with pSVT7 constructs coding for native PGH synthase-1, the N410Q or N68Q/N144Q mutants of PGH synthase-1, or native PGH synthase-1 in the presence of tunicamycin. Microsomal samples (100 µg protein) were preincubated with or without 32 µM hematin for 20 min and, then, with or without 250 µM indomethacin for 20 min at room temperature prior to addition of trypsin. Microsomes, microsomes incubated with heme, and microsomes incubated with heme and indomethacin were then incubated with trypsin (5 µg) for 10 min at room temperature. Reactions were quenched with trypsin inhibitor, and samples (22 µg protein for N68Q/N144Q samples, 8 µg for all other samples) were analyzed by western transfer blotting using enhanced chemiluminescence detection. Untreated samples of each protein are included to demonstrate levels of protease cleavage. A second series of N68Q/N144Q digestions from a second experiment are included to demonstrate the effect of heme and indomethacin pretreatment on trypsin cleavage, which is missing in the original series of digestions. Electrophoresis time was slightly longer in the second experiment.


NATIVE
N410Q
N68Q/
N144Q
NATIVE/
TUNICA
N68Q/
N144Q
TRYPSIN - + + + - + + + - + + - + + + + + +
HEMATIN - - + + - - + + - - + - - + + - + +
INDOMETHACIN - - - + - - - + - - - - - - + - + +
72 kDa
38 kDa

compounds. From these results, we propose that N-glycosylation of Asn410 and either Asn68 or Asn144 is required for the enzyme to attain and/or maintain a native conformation.

Endoglycosidase H Treatments of PGHS-1. To further determine the role of carbohydrate in PGHS-1 function, the effects of glycolytic cleavage on the enzymatic activities of PGHS-1 were examined. Purified ovine PGHS-1 (from ovine seminal vesicles) or solubilized microsomes from cos-1 cells transfected with a construct encoding native PGHS-1 were incubated with endoglycosidase H. In both cases, the enzymes retained significant cyclooxygenase and peroxidase activities relative to the activities of untreated controls (Table III). Following endoglycosidase H treatment for 36 hr, the purified ovine PGHS-1 displayed an electrophoretic mobility similar to that of unglycosylated native PGHS-1 prepared from tunicamycin-treated cos-1 cells (Fig. 9), suggesting that a significant portion of the N-linked carbohydrate had been removed. Similar results were observed for the native enzyme in solubilized microsomes from cos-1 cells (data not shown).

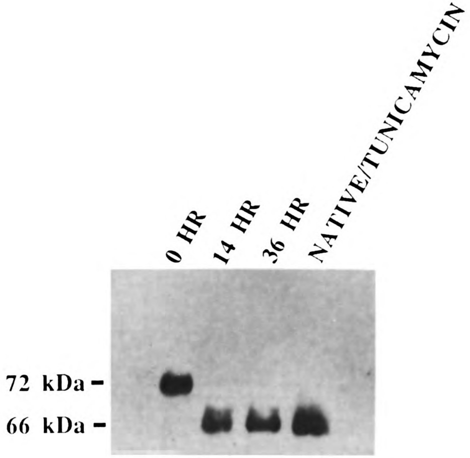
Electrophoretic Mobility and Enzymatic Activity of N-glycosylation Mutants of PGHS-2. Murine PGHS-2 contains three consensus N-glycosylation sites at Asn53, Asn130, and Asn396 which correspond to the three sites (Asn68, Asn144, and Asn410) which we have determined to be N-glycosylated in ovine PGHS-1. The deduced amino acid sequences of PGHS-2 from various species also contain two additional N-glycosylation consensus sequences near the carboxyl terminus (28, 30, 98); in the case of murine PGHS-2, these sites are Asn580 and Asn592. Using site-directed mutagenesis, we constructed two mutants, N580Q and N592Q, in which the Asn at each consensus

TABLE III. Retention of cyclooxygenase and peroxidase activities by ovine PGH synthase-1 following treatment with endoglycosidase H.

	Purified PGH synthase-1		Solubilized PGH synthase-1	
Treatment (hrs)	Cyclooxygenase (% of control)	Peroxidase (% of control)	Cyclooxygenase (% of control)	Peroxidase (% of control)
0	100	100	100	100
14	99	95	97	not determined
36	88	80	84	33

Activities are expressed as the percent of a control incubated in the absence of Endo H.

Fig. 9. Deglycosylation of intact PGH synthase-1 by endoglycosidase H. Purified ovine PGH synthase-1 (100 µg protein) was incubated at 37°C with endoglycosidase H (50 mU) at pH 5.7 in the presence of sodium flufenamate (100 µM). Samples (0.5 µg protein) were taken for analysis by western transfer blotting after 0 hr, 14 hr, and 36 hr of endoglycosidase H-treatment. Detection was by enhanced chemiluminescence. Unglycosylated native PGH synthase-1 from transfected, tunicamycin-treated cos-1 cells is noted as NATIVE/TUNICAMYCIN.


0 HR
14 HR
36 HR
NATIVE/TUNICAMYCIN
72 kDa
66 kDa

sequence was replaced with a Gln. The electrophoretic mobilities of these mutant proteins and unglycosylated PGHS-2 (produced by expression of murine PGHS-2 in cos-1 cells treated with tunicamycin) were compared to native PGHS-2 (Fig. 10). Native PGHS-2 and the N592Q mutant protein each displayed two immunoreactive peptides having apparent molecular masses of 74 kDa and 72 kDa. In contrast, the N580Q mutant protein and unglycosylated PGHS-2 each displayed a single peptide with molecular masses of 72 kDa and 65 kDa, respectively. Upon treatment of native PGHS-2 and the N580Q and N592Q mutant proteins with endoglycosidase H, the apparent molecular masses the proteins were approximately that of unglycosylated PGHS-2 from tunicamycin-treated cells. Taken together, these observations suggest that the two electrophoretically-distinct forms of native murine PGHS-2 are a result of two different N-glycosylation patterns: the 72 kDa peptide is presumed to be N-glycosylated three times at Asn53, Asn130, and Asn396 (sites which correspond to the three glycosylation sites in ovine PGHS-1 at Asn68, Asn144, and Asn410); the 74 kDa peptide is N-glycosylated four times, with the additional site at Asn580. In neither form is Asn592 N-glycosylated.

The N580Q mutant protein retains full cyclooxygenase and peroxidase activities. Unglycosylated PGHS-2 (from expression in cos-1 cells treated with tunicamycin) does not express either activity (Table IV).

Fig. 10. Electrophoretic mobilities of native murine PGH synthase-2 and PGH synthase-2 N-glycosylation site mutants. Microsomes were prepared from sham-transfected cos-1 cells, from cos-1 cells transfected with pSVL constructs encoding native (noted as NATIVE-2) and mutant murine PGH synthases-2, and from cos-1 cells transfected with the pSVL construct encoding the native murine PGH synthase-2 and cultured in the presence of tunicamycin (noted as NATIVE-2/TUNICAMYCIN). Additionally, samples of native PGH synthase-2 (NATIVE-2/ENDO H), the N580Q (N580Q/ENDO H) and N592Q (N592Q/ENDO H) mutant proteins, and unglycosylated native PGH synthase-2 from transfected, tunicamycin treated cells (100 µg microsomal protein) were denatured in SDS and incubated with endoglycosidase H (5 mU). Samples (20 µg protein) were resolved by SDS-PAGE and western transfer blotting was performed using an antibody to PGH synthase-2 with visualization by a peroxidase stain as described in Methods.

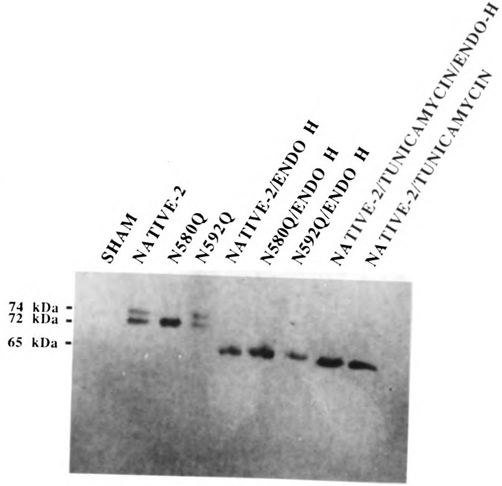

SHAM
NATIVE-2
N580Q
N592Q
NATIVE-2/ENDO H
N580Q/ENDO H
N592Q/ENDO H
NATIVE-2/TUNICAMYCIN/ENDO-H
NATIVE-2/TUNICAMYCIN
74 kDa
72 kDa
65 kDa

TABLE IV. Cyclooxygenase and peroxidase activities of native PGH synthase-2 and PGH synthase-2 N-glycosylation site mutants.

Murine PGH Synthase-2 Expressed in cos-1 Cells	Cyclooxygenase (% of control)	Peroxidase (% of control)
Native	100	100
Sham	9 ± 7	0
Native/Tunicamycin	7 ± 7	0
N580Q	139 ± 33	87 ± 54
N592Q	178 ± 39	126 ± 26

Activities are expressed as the percent of the cyclooxygenase and peroxidase activity seen for native murine PGHS-2.

Discussion

By analyzing mutants of ovine PGHS-1 having changes in each of the four consensus sequences for N-glycosylation, we have deduced that the sites of carbohydrate attachment are Asn68, Asn144, and Asn410, and that a fourth N-glycosylation consensus sequence found at Asn104 is not glycosylated. The Asn104 consensus sequence is located in the membrane binding domain proposed by Picot *et al.* (74). The association of this domain of ovine PGHS-1 with the membrane may explain why Asn104 is not N-glycosylated.

Mutant PGHS-1 which lack N-glycosylation at either residue 68 or 144 have diminished but measurable cyclooxygenase and peroxidase activities. However, PGHS-1 which lack N-glycosylation at both Asn68 and Asn144, and/or at Asn410, have no cyclooxygenase or peroxidase activity. The Asn410 glycosylation site is particularly critical for expression of the catalytic activities of ovine PGHS-1. The failure by heme and indomethacin to protect the various catalytically inactive, glycosylation-deficient forms of PGHS-1 from tryptic digestion suggests that these mutant proteins are not in a conformation which is capable of binding heme or non-steroidal anti-inflammatory drugs. Interestingly, the retention of enzyme activities following partial deglycosylation of PGHS-1 with endoglycosidase H implies that once the enzyme has achieved a mature, active conformation, the attached carbohydrate is not needed to maintain activity. Thus, these observations suggest that the attachment of N-linked oligosaccharides is necessary for PGHS-1 to attain its native conformation, but is not essential for maintaining a catalytically active conformation of the enzyme. Additional evidence for the importance of N-glycosylation for PGHS-1 activity comes from studies of the expression of native PGHS-1 in a baculovirus system (99); while high levels of protein expression of PGHS-1 are achieved in this system, there is a low yield of active enzyme, apparently resulting from inefficient (10-20%) N-glycosylation. Similar observations on the role of N-glycosylation on protein folding have been documented with several cell surface receptors, including the EGF (100), insulin (101), and transferrin (102, 103) receptors.

Site-directed mutagenesis of murine PGHS-2 suggests that the two different forms of PGHS-2 (72 and 74 kDa) result from a variability in the N-glycosylation of Asn580. Consistent with this model, the two polypeptide bands of rat PGHS-2, isolated from preovulatory follicles, have an identical amino terminal sequence (46). The fact that the N580Q mutant PGHS-2 retains both cyclooxygenase and peroxidase activities indicates that both forms of native PGHS-2 are active. The functional significance of variable N-glycosylation of Asn580, or the reason for its occurrence, is not apparent. We have also demonstrated that N-glycosylation of the Asn592 consensus N-glycosylation sequence of PGHS-2 does not occur. This result was not unexpected because Pro593 occupies the second position of the consensus sequence tripeptide Asn-X-Ser/Thr; a proline at this position in a N-glycosylation consensus sequence is generally thought to prevent glycosylation (104).

The three utilized N-glycosylation sites found in ovine PGHS-1 (Asn68, Asn144, and Asn410) are conserved in PGHS-2 (Asn53, Asn130, and Asn396 in the case of murine PGHS-2 (28, 30)). This suggests that each of these sites in PGHS-2 are glycosylated. The 9 kDa difference in the apparent molecular masses of fully glycosylated (74 kDa) and unglycosylated (65 kDa) PGHS-2 is consistent with there being four N-glycosylation sites with high mannose oligosaccharide in murine PGHS-2. The lack of enzyme activities in unglycosylated PGHS-2 (expressed in tunicamycin-treated cos-1 cells) suggests a role for N-glycosylation in PGHS-2 similar to that for PGHS-1.

Both PGHS-1 and PGHS-2 are located in the ER (66, 67). As N-glycosylated residues reside in the lumen of the ER (104), determination that Asn68, Asn144, and Asn410 ovine PGHS-1 and Asn580 in murine PGHS-2 are N-glycosylated demonstrates that these residues reside in the lumen of the ER.

Acknowledgement

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CHAPTER III

ORIENTATION OF PGHS-1 AND PGHS-2 IN THE ER MEMBRANE

Introduction

Earlier examinations of the topology of ovine PGHS-1 in microsomal membranes have indicated that there are regions of PGHS-1 which are on the outside of presumably right-side-out microsomes and therefore on the cytoplasmic side of the ER. One such region includes the Arg277 trypsin cleavage site in ovine PGHS-1 (71-73). Epitopes for several antibodies against PGHS-1 are also exposed in microsomal preparations (71). Thus, cytoplasmic domains containing Arg277 and antibody epitopes and luminal domains containing N-linked carbohydrate have been predicted for PGHS-1. The existence of cytoplasmic and luminal domains would require the presence of membrane spanning domains.

The predictions made by the crystal structure of ovine PGHS-1 for its topology in the membrane are not consistent this biochemical data. The crystal structure depicts an essentially globular domain which does not contain any structures which appear like they could form transmembrane domains, which leads to the prediction that PGHS-1 resides entirely in the lumen of the ER (74).

The determination that Asn68, Asn144, and Asn410 in ovine PGHS-1 and Asn580 in murine PGHS-2 are N-glycosylated demonstrates that these residues reside in the lumen of the ER. We further tested the orientations of regions of PGHS isozymes in the ER membrane, using peptide-directed antibodies in immunocytofluorescent studies. Three antibodies were generated for ovine PGHS-1 against peptides corresponding to the amino terminus (residues 25-35), against the region of the trypsin cleavage site (residues 272-284) and against the carboxyl terminus (residues 583-594). An antibody against a peptide corresponding to the carboxyl terminal insert region of PGHS-2 was also used (66). These antibodies were used in cells which had been treated so only the plasma membrane, and not the ER membrane, had been permeabilized in order to distinguish between cytoplasmic domains and luminal domains. We have also examined the original experiments which predicted cytoplasmic domains for PGHS-1.

METHODS

Materials. Streptolysin O was obtained from GIBCO. Horse myoglobin, saponin, digitonin, rabbit anti-actin, goat anti-rabbit IgG-FITC, goat anti-mouse IgG-FITC and goat anti-rat IgG-FITC were from Sigma. Anti-β-galactosidase and pSVGAL were from Promega. Anti-BiP was the gift of Dr. David Bole (University of Michigan). Peptides were from the Howard Hughes Research Center at Harvard Medical School. Ellman's reagent, Sulfo-link maleimide-activated gel and maleimide-activated coupling kit were from Pierce. TiterMax adjuvant was from CytRx Corp. Frozen ovine seminal vesicles were from Oxford Biomedical.

Generation and purification of peptide-directed antibodies. Table V lists the peptides used in generating peptide-directed antibodies. Peptides were coupled to either maleimide-activated keyhole limpet hemocyanin (KLH) or to maleimide-activated bovine serum albumin (BSA) according to the procedure recommended by the manufacturer. Rabbits were immunized with 100 µg of the coupled peptide-BSA emulsified in 0.2 ml TiterMax adjuvant, and the titer of antiserum was assayed by ELISA using the corresponding peptide coupled to KLH. Rabbits were boosted monthly with 100 µg of peptide-BSA, and immune sera were collected 7 to 10 days following each boost.

Peptide-specific antibodies were purified on peptide affinity columns. Each peptide (10 mg) was coupled to 2 ml of Sulfo-link gel according to the procedure recommended by the manufacturer. Immune serum (5 ml) was diluted 1:1 in phosphate buffered saline (PBS) and passed through the column several times. The column was washed with 20 ml of PBS, and antibodies were eluted in 0.7 ml fractions with 0.1 M

TABLE V. Characteristics of peptide-directed antibodies against ovine PGHS-1 and murine PGHS-2.

PEPTIDE	SOURCE	REGION OF INTEREST	WESTERN BLOT	IMMUNO-FLUORESCENT STAINING
^{25}ADPGAPAVNPC^{35}	PGHS$_{ov}$-1	Amino-terminus	+ ovine-1, human-1 - mouse-1, human-2, mouse-2	+ ovine-1, human-1 - mouse-1
^{272}LMHYPRGIPPQ^{283}C	PGHS$_{ov}$-1	Arg277 trypsin cleavage site	+ ovine-1, human-1 - mouse-1, human-2, mouse-2	+ ovine-1, human-1 - mouse-1
C^{583}PDPRQEDRPGVE^{594}	PGHS$_{ov}$-1	Carboxyl terminus	+ ovine-1 - human-1, mouse-1, human-2, mouse-2	+ ovine-1 - human-1, mouse-1
CY^{584}SHSRLDDINPTVLIK^{598}	PGHS$_{mu}$-2	Carboxyl terminus	+ human-2, mouse-2 + ovine-1, human-1, mouse-1	+ human-2, mouse-2 - mouse-1
^{203}QHFTHQFFKTSGKMGP^{217}C	PGHS$_{ov}$-1	His207 distal heme ligand	+ ovine-1	- ovine-1
C^{568}QDPQPTKTATIN^{580}	PGHS$_{mu}$-2	Carboxyl terminus	+ mouse-2	n.d.
^{2}DEAKNIKKG^{10}	Luciferase	Amino terminus	+ luciferase	- luciferase

glycine, pH 2.2, directly into 0.1 ml of 1 M Na_2PO_4, pH 9.5. Fractions were assayed for protein, and protein-containing fractions were pooled. Final protein concentrations were approximately 0.5 mg/ml.

Cell culture and transfection. *Cos*-1 cells (ATTC CRL-1650) were grown in DMEM containing 8% calf serum and 2% fetal calf serum until near confluence (ca. $3x10^6$ cells/100 mm dish) and were transfected as previously described in the Methods section of Chapter II using expression vectors containing cDNAs coding for either PGHS-1 or -2 or β-galactosidase. The cDNA for ovine PGHS-1 was carried in the vector pSVT7 (77, 86), the cDNAs for murine PGHS-1 and -2 were in pSVL vectors (91), and cDNAs for human PGHS-1 and -2 were in pOSML vectors (6). The construct pSVGAL carried the cDNA for β-galactosidase. Cells were harvested or stained 48 hr post-transfection.

NIH/3T3 cells were grown in DMEM containing 8% calf serum and 2% fetal calf serum until near confluence. The cells were washed with DMEM and serum-starved 24 to 48 hr in DMEM containing 0.2% serum. Cells were stimulated by adding fetal calf serum to the media to a final concentration of 20%. Cells were stained 2 to 4 hr following stimulation (66).

Microsome preparation. Microsomes were prepared from transfected *cos*-1 cells as described in Chapter II. Microsomal proteins were solubilized by adding Tween 20 (1% final concentration) and sonicating for 20 s; supernatants containing solubilized protein were obtained following centrifugation at 200,000 x *g* for 1 hr (2).

Western blotting. Western blotting was performed using chemiluminescent detection as described in Chapter II.

Selective permeabilization of cells using digitonin. Serum-stimulated NIH/3T3 cells and transfected *cos*-1 cells grown on coverslips were washed with PBS and fixed in 2% formaldehyde/PBS. The coverslips were washed with PBS and incubated with a PIPES buffer solution (0.3 M sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM Pipes, pH 6.8) with or without 5 µg digitonin/ml for 15 min at 4°C (105). The coverslips were then washed with PBS and blocked in PBS containing 10% calf serum. Subsequent washings and antibody incubations were conducted in PBS containing 10% calf serum with or without 0.2% saponin. Immunocytofluorescent staining was conducted by incubation with a 1:20 dilution of primary antibody followed by a 1:40 dilution of FITC-labeled secondary antibody.

Selective permeabilization of cells using streptolysin O. Transfected *cos*-1 cells grown on coverslips were washed with PBS and fixed in 2% formaldehyde/PBS. The coverslips were washed with PBS and incubated for 15 min at 4°C with streptolysin O (200 U/ml) which had been preactivated by incubation with 10 mM dithiothreitol in PBS for 10 min at 0°C (106). Unbound streptolysin O was removed by washing, and the coverslips were incubated at 37°C for 20 min in 10 mM dithiothreitol in PBS. The coverslips were washed in PBS and blocked in 1% myoglobin/PBS. Subsequent washings were conducted in PBS, and antibody incubations were conducted in 1% myoglobin/PBS with or without 0.2% saponin. Immunofluorescent staining was conducted as described above.

Immunoprecipitation of microsomal PGHS-1 and -2. Affinity-purified peptide-directed antibodies against ovine PGHS-1 were incubated with attenuated *S. aureus* cells (71) in the presence or absence of the corresponding peptide (10 µM) in 0.1 M Tris-HCl, pH 7.4. Microsomes prepared from ovine seminal vesicles were incubated with the *S. aureus*-antibody complex, and the *S. aureus* cells were collected by centrifugation at 1000 x g. The cyclooxygenase activity of PGHS in the supernatant was assayed using a standard oxygen electrode method (77, 86).

Tryptic cleavage of microsomal PGHS-1 and -2. Microsomal or solubilized protein (100 µg) was incubated with 5 µg of trypsin at 25°C for 5 or 15 min in 0.1 M Tris-HCl, pH 7.4. Reactions were quenched by addition of a 40-fold molar excess of trypsin inhibitor, as described in Chapter II. Tryptic peptides were analyzed by western blotting as described above.

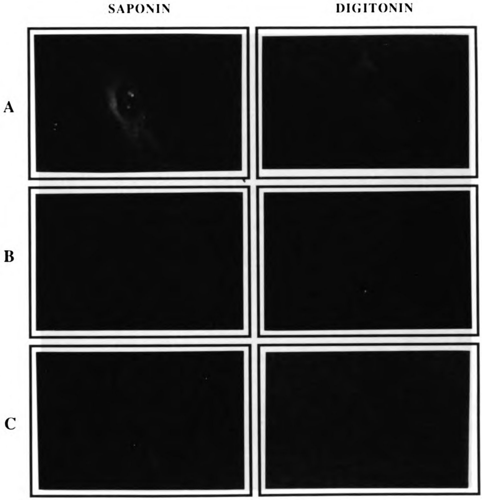
Enzymatic deglycosylation of microsomal PGHS-1 and -2. Microsomal or solubilized protein (100 µg) was incubated for 10 or 60 min with 5 mU of endoglycosidase H (Endo H) at 37°C in 50 mM $NaHPO_4$, pH 6.0. Reactions were stopped by boiling the samples. To completely deglycosylate PGHSs, microsomal protein (100 µg) was denatured by boiling in 1% SDS and then treated for 12 hr with 5 mU of Endo H at 37°C in 50 mM $NaHPO_4$, pH 6.0.

RESULTS

Characterization of peptide-directed antibodies. Peptide-directed antibodies reactive with ovine PGHS-1 were successfully raised against (a) the amino terminus, (b) the domain containing the unique tryptic cleavage site at Arg277, and (c) a region near the carboxyl terminus of the protein; a fourth antibody against an eighteen amino acid cassette located near the carboxyl terminus of murine PGHS-2 and unique to this isozyme was prepared previously (66). The reactivities of each antibody with ovine, human, and murine PGHS-1 and human and murine PGHS-2 as determined by western blotting and immunocytofluorescent staining are summarized in Table V. It should be noted that incubation of each antibody with its corresponding peptide (10 µM) blocked staining in both western blotting and immunocytofluorescence.

Orientation of the carboxyl terminus of murine PGHS-2 in the ER of NIH/3T3 cells. When NIH/3T3 cells were subjected to immunocytofluorescent staining in the absence of detergent, approximately 15% of the cells were stained with an antibody directed against actin; no cells were stained with an antibody either against the luminal ER marker protein BiP (107) or with the antibody against the carboxyl terminal region of PGHS-2. Presumably, the 15% staining occurring with anti-actin antibody represents broken cells. Following selective permeabilization of plasma membranes with digitonin, all of the NIH/3T3 cells could be stained with anti-actin antibody, but again no cells were stained with either the anti-BiP or anti-PGHS-2 antibodies (Fig. 11). However, when NIH/3T3 cells were subjected to immunocytofluorescence in the presence of 0.2% saponin, which nonselectively permeabilizes all cellular membranes, all of the cells were stained with anti-actin, anti-BiP, and anti-PGHS-2 antibodies (Fig. 11). Thus, with

Figure 11. Immunocytofluorescent staining of selectively-permeabilized NIH/3T3 cells. Serum-stimulated NIH/3T3 cells were fixed using 2% formaldehyde/PBS and permeabilized either with saponin (0.2%) or digitonin (5 µg/ml) as indicated in the figure. Cells were then stained by indirect immunofluorescence with either (A) a rabbit polyclonal antibody against actin, (B) a rat monoclonal antibody against BiP, or (C) the affinity-purified antibody against the carboxyl terminus of murine PGHS-2, followed by an appropriate secondary antibody coupled to FITC. Coverslips were mounted using an anti-fade reagent, and microscopy was conducted on a Leitz Wetzlar fluorescence microscope. Magnification is 400X. Photographic exposures times were 2 min, in all cases.


SAPONIN
DIGITONIN
A
B
C

NIH/3T3 cells, the staining patterns observed with anti-BiP and anti-PGHS-2 antibodies are the same and differ from those observed with the anti-actin antibodies. Our previous studies had established that the PGHS-2 antigen is associated with the ER and nuclear membrane of NIH/3T3 cells (24). Because BiP is a luminal ER protein (107), our results indicate that the carboxyl terminal region of PGHS-2 resides in the ER lumen of these cells.

Orientation of ovine PGHS-1 in the ER of transfected *cos*-1 cells. *Cos*-1 cells were transfected with constructs encoding ovine PGHS-1, murine PGHS-2, or β-galactosidase. Following fixation, the transfected cells were subjected to immunocytofluorescent staining with various antibodies under different membrane permeabilization conditions. Anti-actin antibodies were again used as a control for cytoplasmic staining; in addition, an antibody against β-galactosidase was used as a control for the staining of a cytoplasmic protein expressed by transient transfection. The antibody against the carboxyl terminus of murine PGHS-2 described above served as a marker for the ER lumen of *cos*-1 cells because the antibody against BiP failed to stain these cells. Finally, antibodies against the amino terminus, the Arg277 trypsin cleavage site, and the carboxyl terminus of ovine PGHS-1 were used to examine the orientation of this isozyme in the ER.

As expected, *cos*-1 cells treated with either digitonin or streptolysin O to selectively permeabilize plasma membranes were uniformly stained with anti-actin antibodies. A subpopulation of about 30% of the *cos*-1 cells which had been transfected with a cDNA encoding β-galactosidase could be stained using anti-β-galactosidase antibodies when the plasma membranes were permeabilized (Fig. 12 and Fig. 13); this percentage did not increase following permeabilization of all cellular membranes with 0.2% saponin, indicating that the staining percentage reflects the transfection efficiency of the DEAE-dextran/chloroquine protocol. *Cos*-1 cells expressing murine PGHS-2 which had been permeabilized with either digitonin or streptolysin O could not be stained with antibodies to PGHS-2 (Fig. 12 and Fig. 13). However, after permeabilization with 0.2% saponin, approximately 40% of these cells became reactive with the anti-PGHS-2 antibody. This pattern of staining observed with anti-PGHS-2 antibodies in *cos*-1 cells treated with different cell permeants is the same as that observed with these antibodies in staining NIH/3T3 cells. Thus, the orientation in the ER of the carboxyl terminal epitope of PGHS-2 is the same in *cos*-1 cells and murine NIH/3T3 cells. We conclude that this epitope is an appropriate marker for the lumen of the ER of transfected *cos*-1 cells.

Cos-1 cells transfected with PGHS-1 could only be stained with the three antibodies to this protein following permeabilization of all cellular membranes with 0.2% saponin (Fig. 12 and Fig. 13); no staining was observed in cells permeabilized with either digitonin or streptolysin O. Again, because of incomplete transfection, only about 40% of the transfected cells could be stained (Fig. 13). This pattern of staining parallels that observed for PGHS-2, the ER luminal marker in *cos*-1 cells. These results indicate that (a) the amino terminus, (b) the domain containing the unique tryptic cleavage site at Arg277, and (c) a region near the carboxyl terminus of ovine PGHS-1 are all located in the lumen of the ER.

Accessibility of microsomal PGHS-1 and -2 to antibodies and enzymes. Previous studies have indicated that ovine PGHS-1 present in apparently intact, right-side out

Figure 12. Immunocytofluorescent staining of selectively-permeabilized *cos*-1 cells. *Cos*-1 cells were transfected with vectors containing cDNA encoding either ovine PGHS-1 (rows A, D, E, F), β-galactosidase (row B), or murine PGHS-2 (row C). Cells were fixed in 2% formaldehyde/PBS and permeabilized with either saponin (0.2%), digitonin (5 μg/ml), or streptolysin O (100 U/ml) as indicated in the figure. Cells were then stained with either (A) a rabbit polyclonal antiserum against actin, (B) a purified mouse monoclonal antibody against β-galactosidase, (C) affinity-purified antibody against the carboxyl terminus of murine PGHS-2, or affinity-purified antibodies against (D) the amino terminus, (E) the region of the Arg277 trypsin cleavage site, or (F) the carboxyl terminus of ovine PGHS-1.

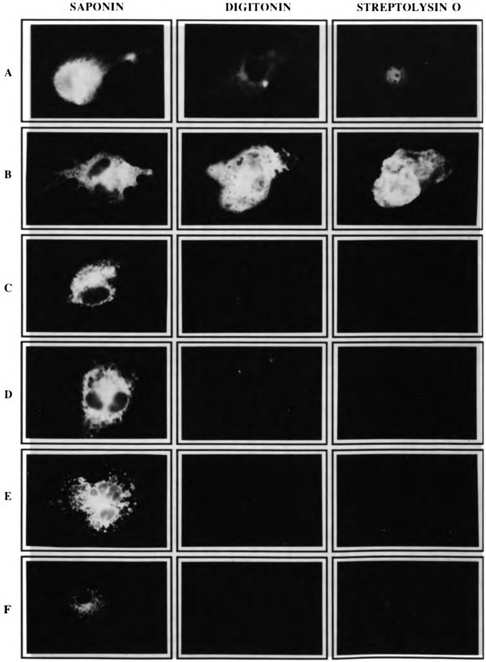
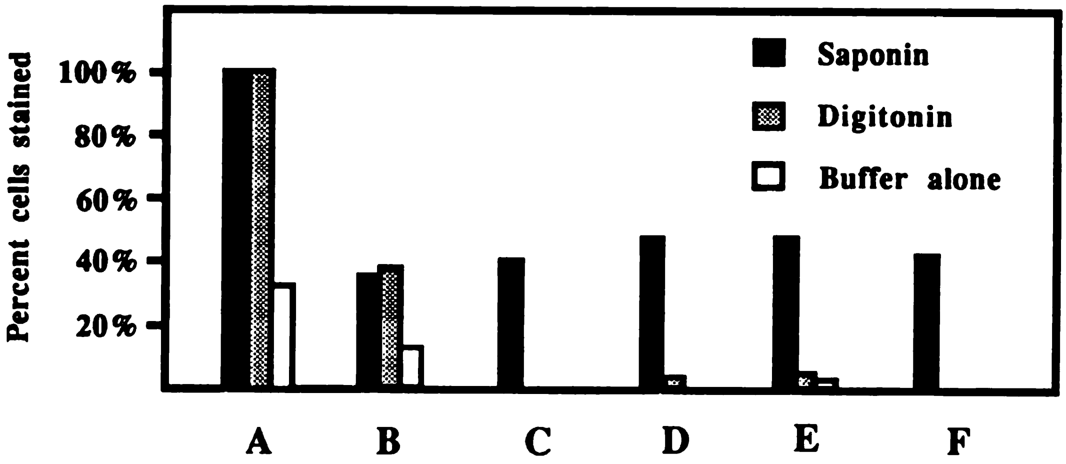
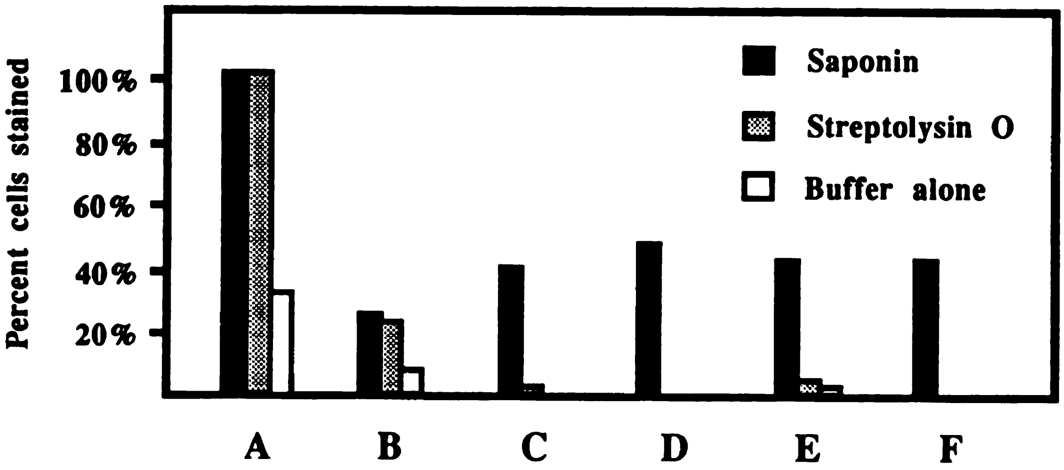

SAPONIN
DIGITONIN
STREPTOLYSIN O
A
B
C
D
E
F

Figure 13. Levels of immunocytofluorescent staining for various antigens in transfected *cos*-1 cells. *Cos*-1 cells that had been transfected with vectors containing cDNAs encoding either ovine PGHS-1, β-galactosidase, or murine PGHS-2 were subjected to immunofluorescent staining. Between 100 and 300 individual cells on each cover slip were counted and examined by both phase microscopy and fluorescence microscopy to determine the percentage of total cells which exhibited immunofluorescence. Figure 13a displays the results for digitonin permeabilization, and Figure 13b displays the results for streptolysin O permeabilization. The antibodies used in each experiment were (A) anti-actin, (B) anti-β-galactosidase, (C) antibody against the carboxyl terminus of murine PGHS-2, and antibodies against the (D) amino terminus, (E) region of the Arg277 trypsin cleavage site, and (F) carboxyl terminus of ovine PGHS-1.

a.


Percent cells stained
100%
80%
60%
40%
20%
Saponin
Digitonin
Buffer alone
A
B
C
D
E
F

b.


Percent cells stained
100%
80%
60%
40%
20%
Saponin
Streptolysin O
Buffer alone
A
B
C
D
E
F

microsomes can be cleaved at Arg277 by trypsin (71-73). This result suggested that the region including Arg277 was located on the cytoplasmic surface of the ER. However, the results of our immunocytofluorescent staining have indicated that Arg277 is located in the lumen of the ER. Therefore, we examined the ovine PGHS-1 present in microsomes prepared using our previous protocols for its accessibility to antibodies and its sensitivity to enzymatic digestion.

The abilities of each of the three antibodies to ovine PGHS-1 to immunoprecipitate PGHS-1 in microsomes prepared from ovine seminal vesicles were examined (Table VI). Each antibody quantitatively immunoprecipitated microsomal cyclooxygenase activity, indicating that the amino and carboxyl termini of ovine PGHS-1, as well as the domain containing of the Arg277 trypsin cleavage site, were accessible to antibody binding in microsomal preparations of ovine PGHS-1. Incubation of each antibody with its corresponding peptide blocked immunoprecipitation of ovine PGHS-1.

Microsomes prepared from ovine seminal vesicles and from *cos*-1 cells expressing murine PGHS-2 were treated with endoglycosidase H (Endo H) (Fig. 14). The incubation times for these treatments were insufficient to completely deglycosylate either microsomal or solubilized PGHS. However, limited deglycosylation did occur in short-term (10 or 60 min) treatments of both preparations of PGHSs, establishing that at least some of the N-linked carbohydrate in ovine PGHS-1 and murine PGHS-2 is accessible to enzymatic deglycosylation in the microsomal preparations.

The ability of trypsin to cleave microsomal and solubilized PGHS-1 and -2 was examined to determine if the trypsin cleavage sites of the enzymes were accessible in our microsomal preparations. Cleavage of ovine PGHS-1 at Arg277 results in a 33 kDa

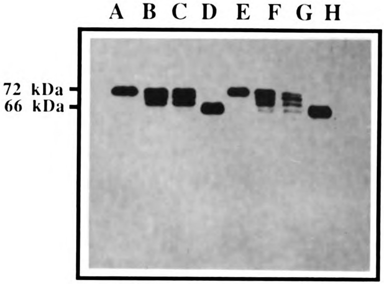
TABLE VI. Immunoprecipitation of microsomal ovine PGHS-1 by peptide-directed antibodies.

	PERCENT CYCLOOXYGENASE ACTIVITY IN THE SUPERNATANT	
ANTIBODY	- 10 µM PEPTIDE	+ 10 µM PEPTIDE
None	100	100
Amino terminus PGHS_{ov}-1	0	110
Arg277 trypsin cleavage site PGHS_{ov}-1	0	102
Carboxyl terminus PGHS_{ov}-1	4	104

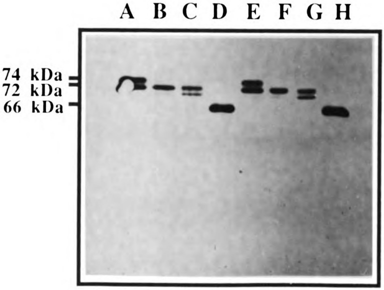
Activity is expressed as the percent cyclooxygenase activity of a control with no antibody added.

Figure 14. Enzymatic deglycosylation microsomal and solubilized PGHS-1 and -2. Microsomal and solubilized PGHSs (100 µg protein) were treated with 5 mU of Endo H for either 10 or 60 min at 37°C in 50 mM $NaHPO_4$, pH 6.0. Reactions were halted by boiling the samples. PGHSs were completely deglycosylated by denaturing the microsomal protein (100 µg) by boiling in 1% SDS followed by a 12 hr incubation with 5 mU Endo H at 37°C in 50 mM $NaHPO_4$, pH 6.0. Proteins were analyzed by SDS-PAGE followed by western blotting. Figure 14a is a western blot of ovine PGHS-1 (5 µg of protein) from sheep seminal vesicles performed using the affinity-purified anti-peptide antibody against the carboxyl terminus of ovine PGHS-1 described in Table VI. Figure 14b is a western blot of murine PGHS-2 (15 µg of protein) from transfected *cos*-1 cells using the affinity-purified anti-peptide antibody against the carboxyl terminus of murine PGHS-2 described in Table VI. The lanes in each blot were (A) untreated microsomal protein, (B) 10 min Endo H treatment of microsomal protein, (C) 60 min Endo H treatment of microsomal protein, (D) and (H) completely deglycosylated protein, (E) untreated solubilized protein, (F) 10 min Endo H treatment of solubilized protein, and (G) 60 min Endo H treatment of solubilized protein.

a



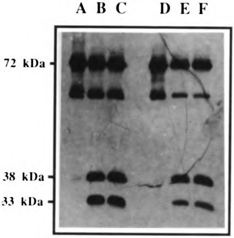
b



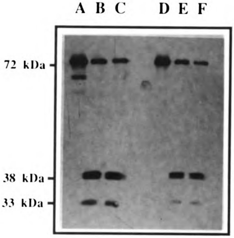
peptide containing the amino terminus and a 38 kDa peptide containing the carboxyl terminus (72, 73). As expected, trypsin was able to cleave ovine PGHS-1 in microsomes prepared from either ovine seminal vesicles or transfected *cos*-1 cells expressing recombinant ovine PGHS-1 (Fig. 15a and 15b). Murine PGHS-2 is partially N-glycosylated (ca. 50%) at Asn580 (Chapter II). This results in the occurrence of two distinct immunoreactive PGHS-2 bands on western transfer blots (M_rs = 72,000 and 74,000 (46)). Trypsin does not cleave murine PGHS-2 at a site analogous to Arg277, but it does cleave approximately 3 kDa from the carboxyl terminus of PGHS-2. The result of trypsin cleavage is to eliminate the N-glycosylation site at Asn580, generating a 69 kDa peptide from both the 72 and 74 kDa forms of the enzyme (66). Trypsin treatment of either solubilized or microsomal murine PGHS-2 prepared from transfected *cos*-1 cells decreased or eliminated the 72 and 74 kDa bands seen in western blots using the antibody against the carboxyl terminus of murine PGHS-2, consistent with the cleavage of the enzyme near the carboxyl terminus (Fig. 15c); moreover, a 69 kDa band was seen in a western blot performed using an anti-peptide antibody raised against residues 568-580 of murine PGHS-2 (Fig. 15d, for antibody see Table V). This result indicates that the site of trypsin cleavage in murine PGHS-2 occurs between residues 568 and 580. Based on the sidechain specificity of trypsin, we predict cleavage occurs at Lys576. This residue neighbors the N-glycosylation site at Asn580 and, thus, is expected to reside in the lumen of the ER. Therefore, the generation of a 69 kDa peptide by tryptic cleavage of murine PGHS-2 at Lys576 indicates that this carboxyl terminal region of the enzyme, which is located in the lumen of the ER, is accessible to trypsin in microsomal preparations.

Figure 15. Tryptic cleavage of microsomal and solubilized PGHS-1 and -2. Microsomes were prepared from sheep seminal vesicles and from *cos*-1 cells transfected with vectors containing cDNAs encoding either ovine PGHS-1 or murine PGHS-2. PGHSs were solubilized from microsomes by addition of Tween 20 at a final concentration of 1%. Microsomal or solubilized PGHSs (100 µg protein) were incubated with 5 µg of trypsin either for 5 and 15 min at 25°C in 0.1 M Tris-HCl, pH 7.4. Reactions were quenched by adding a 40-fold molar excess of trypsin inhibitor, and the resulting peptides were analyzed by SDS-PAGE followed by western blotting. The lanes in each blot are (A) untreated microsomal protein, (B) 5 min trypsin treatment of microsomal protein, (C) 15 min trypsin treatment of microsomal protein, (D) untreated solubilized protein, (E) 5 min trypsin treatment of solubilized protein, and (F) 15 min trypsin treatment of solubilized protein. Figures 15a and 15b are western blots of ovine PGHS-1 (5 µg protein) from sheep seminal vesicles and transfected *cos*-1 cells, respectively. The primary antibody for these blots was antiserum raised in rabbits against purified ovine PGHS-1, used at a 1:20,000 dilution (22-24). Figures 15c and 15d are western blots of murine PGHS-2 (15 µg protein) from transfected *cos*-1 cells. The primary antibody for Figure 15c was the affinity-purified antibody against the carboxyl terminus of PGHS-2 described in Table I, used at a 1:2000 dilution. The primary antibody for 15d was an affinity-purified anti-peptide antibody directed against the murine PGHS-2 sequence C-^{568}QDPQPTKTATIN^{580} (Table V), and was used at a 1:1000 dilution.

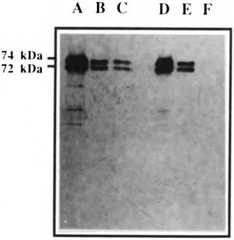
a


A B C D E F
72 kDa
38 kDa
33 kDa

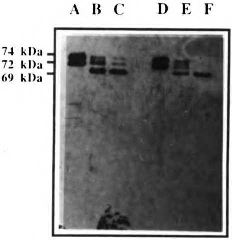
b


A B C D E F
72 kDa
38 kDa
33 kDa

c


A B C D E F
74 kDa
72 kDa

d


A B C D E F
74 kDa
72 kDa
69 kDa

DISCUSSION

The PGHS isozymes are integral membrane proteins (3, 46) located in the ER and nuclear membranes of prostaglandin-forming cells (66, 67). Previous models describing the association of PGHS isozymes with membranes have predicted the existence of one or more transmembrane domains (60, 71). The arguments for the existence of transmembrane domains in PGHS have been predicated on two observations which suggested the existence of both cytoplasmic and luminal domains: first, PGHS is N-glycosylated (3, 69), and N-glycosylated residues groups are found exclusively on the luminal side of the ER (104), and second, microsomal ovine PGHS-1 is susceptible to cleavage by trypsin, which hydrolyzes the enzyme at Arg277 (71-73), suggesting that this residue is on the cytoplasmic face of the ER membrane.

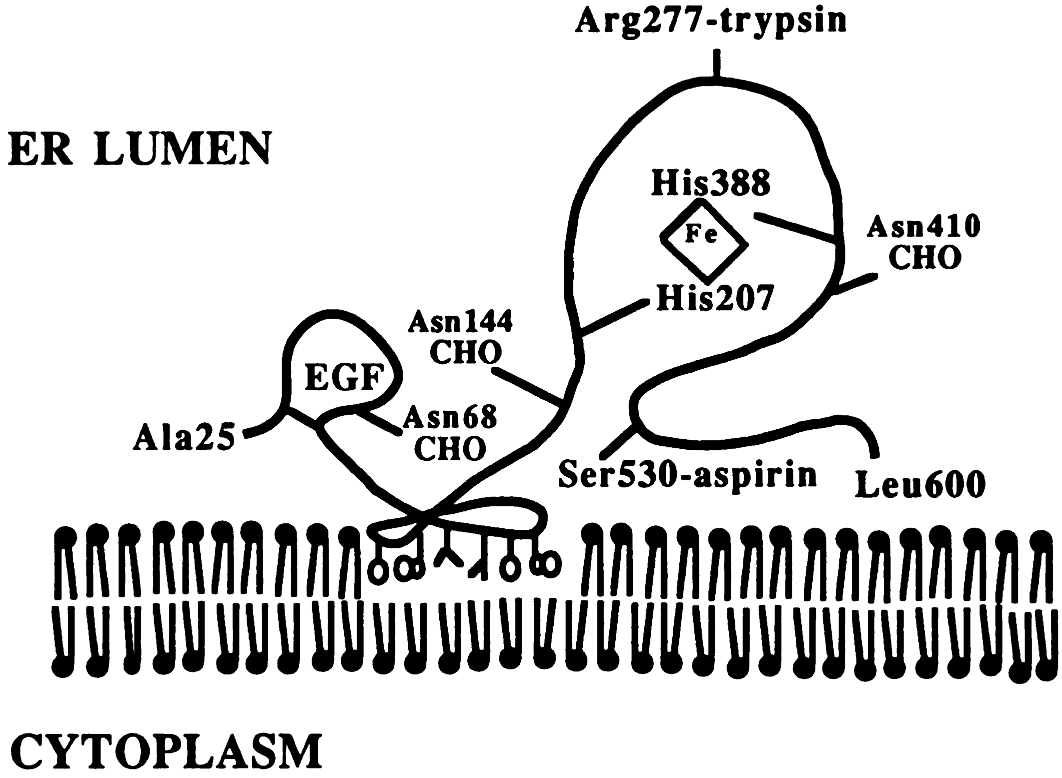
To test the orientation of PGHS-1 in the ER membrane, we prepared antibodies to the domain containing Arg277, as well as to the N- and C-termini, and then performed immunocytofluorescent staining in the presence of detergent treatments which would either expose only the cytoplasmic surface of the ER or both the cytoplasmic and luminal surfaces. The results of these studies have indicated that the domain containing Arg277 and the N- and C-termini of ovine PGHS are only accessible to antibodies following permeabilization of the ER with 0.2% saponin; no staining was observed under conditions found to cause only permeabilization of the plasma membrane. These immunocytochemical results argue that Arg277 is located in the lumen of the ER in cells expressing ovine PGHS-1.

Several additional experiments were performed to determine if microsomes used in earlier studies of tryptic cleavage of the enzyme were intact and right-side-out. The results of these studies have indicated that ovine PGHS-1 in both seminal vesicle and *cos*-1 cell microsomes (a) can interact with antibodies to the N- and C-termini and the Arg277-containing domains of the protein and (b) can be deglycosylated by endoglycosidase H. We have also demonstrated that the luminal side of *cos*-1 microsomes is accessible to trypsin by demonstrating that trypsin cleaves the C-terminus of murine PGHS-2, probably at Lys576. Thus, we conclude that seminal vesicle and *cos*-1 cell microsomes are either wrong-side-out or not intact. In either event, it is clear that the susceptibility of microsomal ovine PGHS-1 to trypsin cleavage at Arg277 cannot be interpreted to mean that Arg277 is present on the cytoplasmic surface of the ER. In preparing microsomes in early studies, ovine seminal vesicles and rat liver had been cohomogenized and cocentrifuged and the mannose-6-phosphatase activity of liver microsomes had been used as a marker for the lumen of the ER (71); this approach was used because there were no known luminal ER markers in ovine seminal vesicles. Mannose-6-phosphatase was not cleaved by trypsin in these experiments, suggesting that the liver microsomes were intact and right-side-out. It is now clear that this interpretation should not have been extended to the ovine vesicular gland microsomes with which the liver microsomes were co-prepared. The simple procedures for preparing intact microsomes from liver clearly are not transferable to ovine seminal vesicles or *cos*-1 cells.

Our current work demonstrates that the N- and C-termini and the Arg277-containing domain of ovine PGHS-1 resides in the lumen of the ER, and that the carboxyl terminus of murine PGHS-2 is also located on the luminal side of the ER. Combined with the presence of N-glycosylation sites at Asn68, Asn144, and Asn410 in ovine PGHS-1, six regions of the enzyme spaced along the length of the amino acid sequence have now been shown to reside in the ER lumen. Because of the close homologies in the amino acid sequences of PGHS-1 and PGHS-2, PGHS-2 is predicted to have an identical orientation in the ER to PGHS-1. The prediction that the amino and carboxyl termini, Arg277, and each of the N-glycosylation sites of ovine PGHS-1 reside on the same side of the ER membrane is in agreement with the recently determined crystal structure of detergent-solubilized ovine PGHS-1 (74). The enzyme has a globular structure similar to that of two soluble peroxidases, myeloperoxidase and cytochrome c peroxidase. No obvious transmembrane regions are apparent in the crystal structure. The one transmembrane domain predicted from the deduced amino acid sequence by hydrophobicity plots (60) is found in the core of the structure. Thus, it appears that the entire PGHS molecule, including both the cyclooxygenase and the peroxidase active sites, resides in the ER. To account for the interaction of ovine PGHS-1 with membranes, Picot *et al.* (74) have proposed that the enzyme associates with the membrane through four amphipathic helices located between amino acids 74 and 117, with each of the helices having a hydrophobic surface which is embedded in one layer of the membrane bilayer. Depicted in Figure 16 is a model for the orientation of ovine PGHS-1 in the ER membrane based on the present evidence.

In serum-starved, quiescent murine NIH/3T3 cells only the PGHS-1 isozyme is expressed. The turnover rate of this enzyme is unknown but appears to be relatively slow because the levels of PGHS-1 remain the same in both serum-starved and serum stimulated cells (31). Thus, the observation that PGHS-1 is present in the ER of various cells can be reasonably interpreted to mean that this isozyme does actually function in the ER. Our present data indicating that PGHS-1 is on the lumen side of the ER raises

Figure 16. Model for the orientation of ovine PGHS-1 in the ER membrane. Ala25 is the amino terminus of the native enzyme following removal of the signal peptide. The epidermal growth factor (EGF) homology domain extends from Cys36 to Cys69. N-glycosylation sites are denoted with CHO and occur at Asn68, Asn144, and Asn410. The putative membrane anchoring domain extends from approximately Ile74 to Val116. His207 and His388 are the distal and axial heme ligands, respectively. Arg277 is the trypsin cleavage site. Ser530 is the active site serine acetylated by aspirin. Symbols attached to the amino acid chain are meant to represent hydrophobic amino acids involved in the association of ovine PGHS-1 with the ER membrane.


Arg277-trypsin
ER LUMEN
His388
Fe
Asn410
CHO
His207
Asn144
CHO
EGF
Asn68
CHO
Ala25
Ser530-aspirin
Leu600
CYTOPLASM

questions about (a) how PGHS-1 acquires free arachidonate for prostaglandin formation and (b) how PGH_2, formed through the action of PGHS-1, is channeled to enzymes downstream in the prostaglandin biosynthetic pathway. Arachidonic acid can diffuse across lipid bilayers (108), and arachidonate added exogenously to cells expressing PGHS-1 is rapidly converted into prostaglandins, indicating that arachidonate can efficiently pass through cell membranes to reach the ER lumen. There are three phospholipases which are potentially involved in supplying arachidonate to PGHS-1 following hormonal stimulation of cells. The first is a cytosolic phospholipase A_2 ($cPLA_2$), which is activated rapidly in response to hormonal stimuli (48, 53). Upon activation, this enzyme translocates to an intracellular membrane, probably the ER (109), and the enzyme would be expected to hydrolyze arachidonoyl groups from phospholipid located on the cytoplasmic side of this membrane (48). Thus, arachidonate would have to cross a single leaflet of the bilayer to reach PGHS. One scenario is that the ω-terminus of arachidonate moves through the center of the four helical membrane anchors of PGHS-1 embedded in the inner bilayer of the ER and on into the hydrophobic channel that forms the core of the cyclooxygenase active site of PGHS-1 (74). The second phospholipase A_2-mediated release involves a secreted nonpancreatic Type II phospholipase A_2 ($sPLA_2$) which is activated in response to mitogenic stimuli (53, 110). Presumably, newly released arachidonate derived from the plasma membrane through the action of $sPLA_2$ could simply diffuse through the plasma and ER membranes to reach PGHS-1 in the ER lumen. Finally, Gross and coworkers have characterized a soluble, Ca^{2+}-independent PLA_2 purportedly involved in hormone-induced prostaglandin formation in pancreatic islets (111) and smooth muscle cells (112). The site of action of this phospholipase A_2 is not known.

Following synthesis of PGH_2 by PGHS-1 in the ER lumen, PGH_2 is converted into active prostanoids by other enzymes. The location of the enzymes which catalyze these conversions may shed light on the mechanism of release of prostanoids from the cell. The synthesis of PGE_2, PGD_2, and $PGF_{2\alpha}$ can be mediated by a variety of different enzymes (19), but it is not clear whether any of these proteins is actually involved in prostaglandin synthesis *in vivo*. In contrast, the synthesis of both prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are mediated by prostacylin and thromboxane synthases, respectively. Both enzymes are members of the cytochrome P450 superfamily, and, as such, are associated with the ER membrane (14-16). The active sites of this family of proteins have been predicted to be located on the cytoplasmic side of the ER (17, 18), suggesting that PGH_2 would need to cross the ER membrane to the cytoplasm for conversion into prostacyclin or thromboxane. Although, in general, prostanoids cannot move freely through membrane bilayers (113), PGH_2 added to cells is rapidly converted to PGI_2 and TXA_2 (113, 114), suggesting that PGH_2 can move through membranes, and thus, generation of PGH_2 in the ER lumen should pose no difficulty for its subsequent enzymatic isomerization occurring on the cytoplasmic face of the ER.

The PGHS-2 antigen is present in the ER and on the nuclear membrane of murine NIH/3T3 cells stimulated to replicate by the addition of serum (66). Because of the rapid synthesis and degradation of PGHS-2 (31, 115), it is not clear whether the PGHS-2 in the ER is in transit to its actual site of action or whether PGHS-2 normally functions in the ER. However, it is clear from our studies that PGHS-2 in the ER is present in the lumen.

Acknowledgements

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The antibody against residues 568-580 of murine PGH synthase-2 was developed by MK Regier and WL Smith.

CHAPTER IV

EXAMINATION OF THE ASSOCIATION OF PGHS-1 WITH THE ER MEMBRANE

Introduction

PGHS-1 was initially characterized as an integral membrane protein because detergents were required to solubilize the enzyme from microsomal membrane preparations (2). Similar conditions are required to solubilize PGHS-2 from microsomal membranes (46). Purified ovine PGHS-1 can be reincorporated into phospholipid liposomes, indicating that the enzyme can associate directly with phospholipid membranes following solubilization (70). As described in Chapter III, it was originally believed that PGHS contained transmembrane domains, and that this was the mechanism by which PGHS was associated with the ER membrane. We have demonstrated that the primary evidence for the existence of cytoplasmic domains for PGHS-1, the cleavage of microsomal PGHS-1 by trypsin, was not valid. In addition, the crystal structure of detergent solubilized ovine PGHS-1 does not contain any regions which would appear to be transmembrane domains; rather, ovine PGHS-1 was shown to be an essentially globular protein (74).

In their description of the crystal structure of ovine PGHS-1, Picot *et al.* proposed that the globular catalytic domain is anchored to the ER membrane by a novel membrane binding domain. This domain consists of three amphipathic helices which essentially lie in a plane, and the amino terminal end of a fourth helix which forms part of the cyclooxygenase active site (helices are labelled A, B, C and D, and the domain is composed of residues 74-117 in ovine PGHS-1 (see Figs. 2 and 3)). These helices project hydrophobic residues away from the body of PGHS-1, forming a hydrophobic surface. The authors predict that this domain associates with a single leaflet of the lipid bilayer of the ER membrane. Although there has been speculation that monotopic integral membrane proteins exist (116), this is the first structural evidence consistent with such a domain (74, 75).

We have taken three approaches to determine if this putative membrane binding domain of ovine PGHS-1 associates with membranes. First, the hydrophobic, photoactivatable reagent 3-trifluoromethyl-3-(*m*-[^{125}I]-iodophenyl)diazirine ([^{125}I]TID) was used to label regions of microsomal ovine PGHS-1. [^{125}I]TID has been used in the past to identify membrane-associated regions of proteins, and non-specific labelling of a region of a protein by [^{125}I]TID is considered to provide evidence for membrane association (117, 118). Second, we have made mutations in helix A, helix B and helix C of ovine PGHS-1, replacing some of the hydrophobic residues in these helices with serine, threonine or alanine in an attempt to disrupt the association of the enzyme with the ER membrane. Finally, we have prepared chimeric proteins in which the putative membrane binding domain of ovine PGHS-1 is fused to the amino terminus of either luciferase and β-galactosidase to determine if addition of this domain to a soluble protein can cause association with membranes.

METHODS

Materials. [^{125}I]TID was from Amersham. pSVgal, pGLuc, Luciferase Assay System, and monoclonal antibody against β-galactosidase were from Promega. Mouse anti-βgal polyclonal antibody, protein A-sepharose 4B, glutathione and o-nitrophenyl-β-D-galactopyranoside were from Sigma. PCR amplification kit was from Boehringer Mannheim Biochemicals. anti-HMG CoA reductase and pSV-HMGal were the gift of Robert D. Simoni (Stanford University).

Photolabelling of ovine PGHS-1 with [^{125}I]TID. Frozen ovine seminal vesicles were sliced into thin strips with a razor and homogenized with a Polytron homogenizer in a HEPES buffer consisting of 20 mM HEPES, 20 mM glutamic acid, 2 mM magnesium acetate, and 200 mM Sucrose, pH 7.5. Homogenized tissue was centrifuged at 10,000 x *g* for 10 min, and microsomes were collected by centrifugation at 200,000 x *g* for 1 h. Microsomes were resuspended in HEPES buffer, homogenized in a dounce homogenizer, and cleared by centrifugation at 10,000 x *g* for 5 min. Microsomes were diluted to a protein concentration of 3 mg/ml and incubated for 10 min at 0°C in the presence or absence of either 20 mM glutathione, 100 µM ibuprofen, or 100 µM sulindac sulfide. [^{125}I]TID was then added to the microsomes at a final concentration of approximately 5 µM, and incubated for 10 min at 0°C. Microsomes were transferred to a 1 cm^2 dish and photolabelled by irradiation at a distance of 5 to 10 cm with a 366 nm wavelength UV illuminator for 10 min at 0°C.

Limited proteolytic digestion of [^{125}I]TID-labelled ovine PGHS-1 by trypsin. Following photolabelling of ovine seminal vesicle microsomes with [^{125}I]TID, microsomes were recollected by centrifugation at 200,000 x *g* for 1 h, and resuspended to a protein concentration of 3 mg/ml. For tryptic digestion, microsomes were incubated with trypsin (10:1 microsomal protein:trypsin) for 15 min at 25°C. A 40 fold excess of trypsin inhibitor was then added, and the microsomal proteins were solubilized by addition of Tween 20 to a final concentration of 1%. Solubilized protein was incubated with affinity purified antibodies against the amino and carboxyl termini of ovine PGHS-1 (Table V), and the antibody-PGHS-1 complexes were precipitated by protein A-sepharose 4B. The protein A-sepharose 4B complex was washed extensively with 0.1 M Tris-HCl/0.1 % Tween 20, pH 8.0, and PGHS-1 was eluted from the complex by boiling in SDS-loading buffer. Samples were resolved on a 15% SDS-PAGE gel, and the gel was silver stained. Photolabelled proteins and peptides in the silver stained gel were visualized by autoradiography.

Complete proteolytic digestion of photolabelled ovine PGHS-1 by endoproteinase Lys C. Following photolabelling, microsomes were solubilized by addition of Tween 20 (final concentration 1%), and ovine PGHS-1 was immunoprecipitated using the monoclonal anti-PGHS-1 antibody *cyo-7* (72) and protein A-sepharose 4B, as described above. PGHS-1 was eluted from the protein A complex by boiling in 0.5% SDS. Samples were diluted to 0.05% SDS in 10 mM Tris-HCl, pH 8.0, and endoproteinase Lys C was added to a final concentration of 300 mU/ml. Samples were incubated overnight at 37°C, and peptides were resolved on a 15% SDS-PAGE gel, and identified by western blotting using chemiluminescent detection as described in Chapter II. After allowing the chemiluminescence to fade, [^{125}I]TID-labelled peptides were identified by autoradiography.

Site directed mutagenesis. Site directed mutagenesis of ovine PGHS-1 was performed as described in Chapter II. Oligonucleotide primers used in site-directed mutagenesis are listed in Table I.

Generation of PGHS-luciferase and PGHS-β-galactosidase fusion proteins. The *BamHI-SalI* cDNA fragment containing the coding region of firefly luciferase was isolated from the vector pGEM-Luc and subcloned into the *BamHI-SalI* sites of pUC19 (pUC19-Luc). The *KpnI-SalI* fragment containing the cDNA for luciferase was then isolated from pUC19-Luc and subcloned into the *KpnI-SalI* sites of the expression vector pOSML (pOSML-Luc).

Similarly, the *BsaI-SalI* cDNA fragment containing the coding region of β-galactosidase (βgal) was isolated from the vector pSVGAL and subcloned into the *SmaI-SalI* sites of pUC19 (puc19-Gal). The *KpnI-SalI* fragment containing the cDNA for β-galactosidase was then isolated from pUC19-Gal and subcloned into the *KpnI-SalI* sites of pOSML (pOSML-Gal). Due to the subcloning procedure, the original translational start site of βgal is removed, and the new translational start site is Met23.

Polymerase chain reaction (PCR) was used to amplify the cDNA of ovine PGHS-1 containing the coding region for either residues 1-35, 1-124, or 1-124 containing a deletion of most of the EGF homology domain, eliminating residues 36-67 (Table I). Primers used in PCR are listed in Table VII. Sense strand primers were designed to incorporate a *PstI* site at the 5'-end of the coding strand in PCR products, and anti-sense strand primers were designed to incorporate a *KpnI* site into the 3'-end of the coding strand in PCR products and to maintain an in frame fusion with either luciferase or βgal. Following amplification, PCR products were treated with *PstI* and *KpnI*, and the ovine PGHS-1 cDNA fragments were purified by electrophoresis on low-melting point agarose gels, and ligated into the *PstI-KpnI* site of pOSML-Luc or pOSML-Gal.

Table VII. PCR oligonucleotide primers for the construction of PGHS$_{ov}$-1-luciferase and PGHS$_{ov}$-1-βgalactosidase fusion proteins.

Primer Description	Oligonucleotide
Coding strand primer	5'-AACTGCAGCCGGAGCTCCCGGGCAGAGTT-3'
35Luc Anti-coding strand primer	5'-GC**GGTACC**TTGGGTTCACTGGCGCGGGCGC-3'
124Luc Anti-coding strand primer	5'-GC**GGTACC**TGATAAGGTTGGAACGCACTGT-3'
35Gal Anti-coding strand primer	5'-GC**GGTACC**TGGGTTCACTGGCGCGGGCGC-3'
124Gal Anti-coding strand primer	5'-GC**GGTACC**GATAAGGTTGGAACGCACTGT-3'

Engineered restriction sites are in bold and coding and anti-coding sequences from ovine PGHS-1 are underlined.

Transient transfection of *cos-1* cells and preparation of membrane fractions. *Cos-1* cells were transfected with expression vectors and harvested as described in the Methods section of Chapter II. For determination of enzyme activities in whole cells, cells were resuspended in 0.1 M Tris-HCL, pH 7.5, and Tween20 was added to a final concentration of 1%. Suspensions were sonicated for 30 s and then homogenized in a dounce homogenizer.

Membrane fractions were prepared by differential centrifugation. Following sonication of the harvested *cos-1* cells, membranes were centrifuged at 10,000 x *g* to obtain a membrane fraction (10P) consisting of cell debris, nuclei, plasma membrane, and mitochondria. The supernatant of the 10,000 x *g* fraction was then centrifuged at 200,000 x *g* to obtain a microsomal fraction (200P) and a cytosolic fraction (200S).

Cyclooxygenase and peroxidase assays. The cyclooxygenase assay utilizing an oxygen electrode was as described in the Methods section for Chapter II. Peroxidase assays were conducted using an assay which relies on oxidation of luminol by the peroxidase activity of PGHS and detection on a luminometer. The assays were conducted in 0.1 M Tris-HCl, pH 8.0, 10% Amersham ECL reagent B, 1 µM hematin with 10-40 µg microsomal protein in a final volume of 100 µl. 50 µl of 0.4 mM H_2O_2 were added to initiate the reaction. Assays were 1 min in length.

Luciferase assay. Luciferase assays were conducted using the Luciferase Assay System kit from Promega. Activity was measured on a luminometer with an assay time of 1 min.

β-galactosidase assay. Endpoint assays were conducted for β-gal activity according to directions from a Promega kit. 20-80 µg protein were added to a solution of 100 mM $NaHPO_4$, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol and 0.67 mg/ml o-nitrophenyl-β-D-galactopyranoside, pH 7.3, with a final volume of 300 µl. Reactions ran 30 min at 25°C, and were terminated by the addition of 1 M $NaCO_3$. Product formation was measured at 420 nm on a spectrophotometer.

Western blotting. Western blotting using chemiluminescent detection was as described in Chapter II. Luciferase and luciferase fusion proteins were blotted using a peptide directed antibody against its amino terminus (Table V). A polyclonal antibody against βgal was used for β-gal, HMGal, and βgal fusion proteins. Fusion proteins were also blotted with an antibody against the amino terminus of ovine PGHS-1 (Table V).

Deglycosylation of proteins was conducted as described in the Methods section of Chapter II.

Results

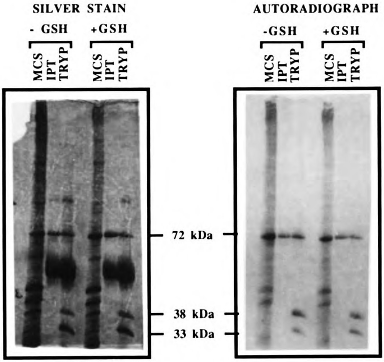
Photolabelling of ovine PGHS-1 by [^{125}I]TID. Microsomes prepared from ovine seminal vesicles were labelled with the photoactivatable, hydrophobic probe [^{125}I]TID. PGHS-1, which makes up approximately 5% of the total microsomal protein of seminal vesicles, was the major protein labelled in these preparations (Fig 17). Tryptic digestion of [^{125}I]TID-photolabelled ovine PGHS-1 demonstrated that both the 33 kDa amino terminal peptide, which contains the putative membrane binding domain, and the 38 kDa carboxyl terminal peptide were photolabelled by [^{125}I]TID. The amino and carboxyl terminal tryptic fragments of PGHS-1 were also photolabelled by [^{125}I]TID in the presence of 20 mM glutathione, which scavenges [^{125}I]TID exposed to the aqueous phase (Fig. 17a). Incubation of microsomes with the non-steroidal anti-inflammatory drugs ibuprofen and sulindac sulfide altered the [^{125}I]TID photolabelling pattern seen for ovine PGHS-1 by diminishing the photolabelling of the 38 kDa carboxyl terminal tryptic peptide (Fig. 17b). This suggests that the photolabelling seen in the carboxyl terminal peptide of ovine PGHS-1 was the result of [^{125}I]TID entering the hydrophobic cyclooxygenase active site, and was not the result of the membrane association of a region of PGHS-1 contained in this peptide.

Exhaustive digestion of ovine PGHS-1 with endoproteinase Lys-C is predicted to yield a peptide containing residues 25-166 and having a theoretical peptide molecular mass of 16.2 kDa; because this peptide contains two N-glycosylation sites at Asn68 and Asn144, the observed molecular mass is predicted to be about 20 kDa. Digestion of [^{125}I]TID-photolabelled PGHS-1 with endoproteinase Lys-C did result in a major peptide with a molecular mass of 20.5 kDa (Fig. 18). This peptide was labelled by [^{125}I]TID, and

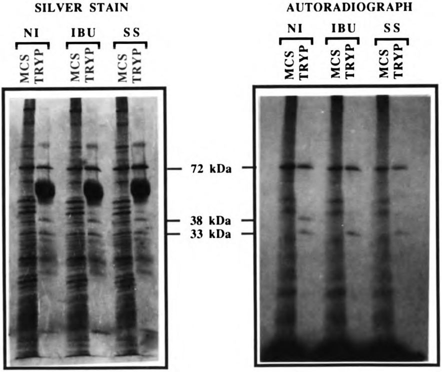
Figure 17. Labelling of PGHS-1 with [^{125}I]TID, and the effects of glutathione, ibuprofen and sulindac sulfide on labelling. a) Microsomes prepared from ovine seminal vesicles were photolabelled with [^{125}I]TID in the presence and absence of 20 mM glutathione (GSH). GSH was removed by repelleting the microsomes by centrifugation at 200,000 x *g*, and some samples were then treated with trypsin. Microsomes and trypsin treated microsomes were then solubilized and PGHS-1 was immunoprecipitated. Photolabelled microsomes (MCS), immunoprecipitated PGHS-1 (IPT), and immunoprecipitated PGHS-1 from trypsin treated microsomes (TRYP) were then resolved by SDS-PAGE and silver stained. Photolabelled products were visualized by autoradiography. The major bands in immunoprecipitated samples not found in microsomes are immunoglobulin heavy and light chains.

b) Microsomes were preincubated with no inhibitor (NI), 100 µM ibuprofen (IBU), or 100 µM sulindac sulfide (SS) prior to photolabelling with [^{125}I]TID. Microsomes were then repelleted, treated with trypsin, and immunoprecipitated as described above prior to SDS-PAGE.

a


SILVER STAIN
- GSH
+GSH
MCS
IPT
TRYP
MCS
IPT
TRYP
AUTORADIOGRAPH
-GSH
+GSH
MCS
IPT
TRYP
MCS
IPT
TRYP
72 kDa
38 kDa
33 kDa

b


SILVER STAIN
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72 kDa
38 kDa
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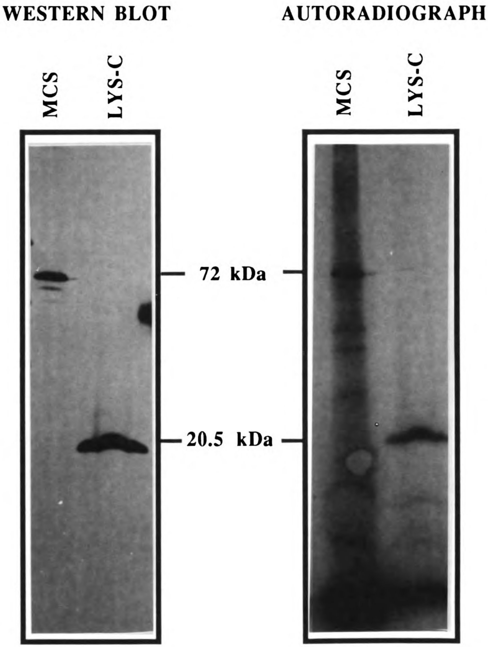
Figure 18. Localization of an [^{125}I]TID-labelled region of PGHS-1 to a proteolytic peptide. Following photolabelling, microsomes prepared from ovine seminal vesicles were solubilized, and PGHS-1 was purified on an immunoaffinity column. Purified PGHS was then incubated with endoproteinase LysC, and proteolytic products were resolved by SDS-PAGE and transferred to a nitrocellulose filters. Western blots were then conducted on the filters to identify products. Shown is a western blot using an antibody against the amino terminus of ovine PGHS-1. After allowing chemilluminescence to disapate, photolabelled peptides on filters were identified by autoradiography.


WESTERN BLOT
AUTORADIOGRAPH
MCS
LYS-C
MCS
LYS-C
72 kDa
20.5 kDa

reacted with an antibody raised against the amino terminus of ovine PGHS-1, but not with an antibody against a peptide corresponding to residues 203-217 of ovine PGHS-1 (Table V, data not shown). Labelling of this peptide is consistent with the concept that the predicted membrane binding domain of ovine PGHS-1 is in fact associated with the ER membrane.

Membrane association of ovine PGHS-1 following site-directed mutagenesis of helices in the putative membrane binding domain of PGHS-1. Three sets of mutations in the putative membrane binding domain were designed in ovine PGHS-1 in an attempt to solubilize an active form of the enzyme by replacing hydrophobic residues predicted to be associated with membranes with small neutral or hydrophilic ones. These mutants have been designated HelA (four mutations: I74T-W75S-W77S-L78A), HelB (three mutations: F88S-F91S-L92A), and HelC (three mutations: W98S-L99A-F102S). These residues were chosen following an examination of the crystal structure; in all cases, residues chosen for mutation were oriented away from the body of the enzyme and thus were ones that were predicted to be interacting with membranes. Other hydrophobic residues in these three helices that were oriented toward the body of the enzyme were not mutated, as we reasoned that they might be important for the conformation of the enzyme. Helix D was not altered, because it is part of the hydrophobic channel involved in binding arachidonic acid. It was anticipated that mutations in this channel probably would result in an inactive protein. If a helix mutation did cause the solubilization of ovine PGHS-1, we expected that the enzyme would be present in the 200S fraction following differential centrifugation of homogenized cells expressing the mutant protein, and would not be retained within microsomes in the 200P fraction. This expectation was based on our results presented in Chapter III which indicate that the luminal surfaces of our microsomal preparations are exposed.

Assays of whole cells revealed that the HelB mutant protein retained low levels of cyclooxygenase and peroxidase activity, while HelA and HelC mutant proteins were inactive (Fig. 19). The distribution of the activity for the HelB fusion protein in the 10P, 200P, and 200S fractions following differential centrifugation was similar to that seen for native ovine PGHS-1. The lack of activities with the HelA and HelC mutants were not the result of degradation or secretion of these proteins, as the levels of these proteins in whole cells were similar to those of the native ovine PGHS-1 (Fig. 20a). In all cases, examination of the distribution of protein in the 10P, 200P, and 200S fractions revealed that each PGHS-1 helix mutant was located in the 10P and 200P fractions, and not in the 200S fraction. This is the same distribution seen for native ovine PGHS-1 (Fig. 20b).

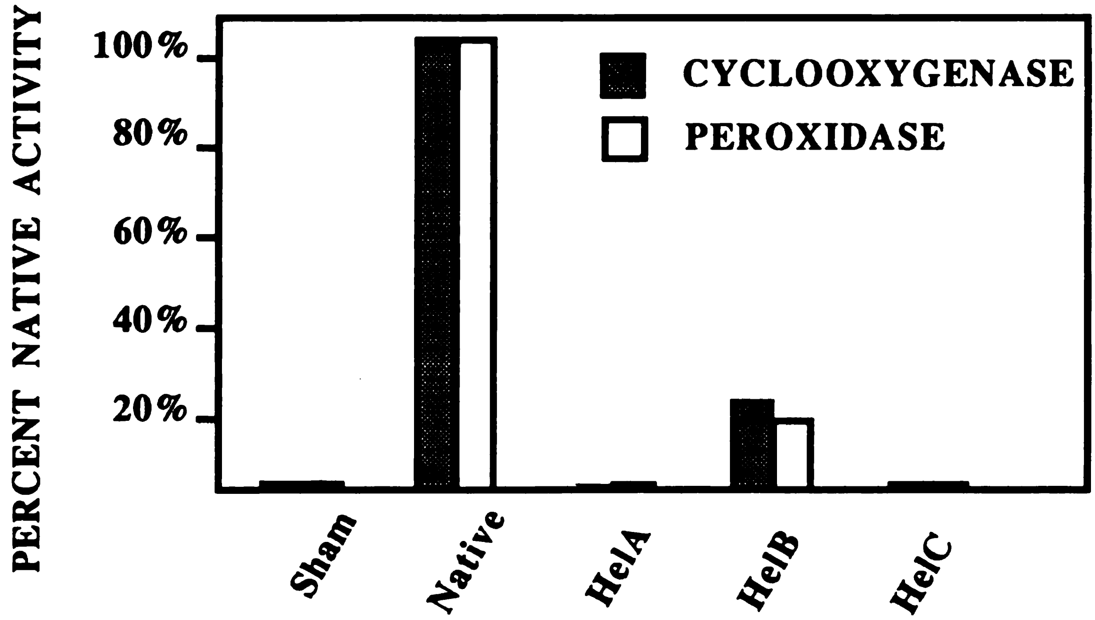
Examination of the electrophoretic mobilities of the HelB and HelC mutant proteins by SDS-PAGE revealed that there was a 74 kDa band in addition to the 72 kDa band typically seen for native ovine PGHS-1. The Asn104 N-glycosylation consensus sequence lies in helix C, and is not N-glycosylated, presumably because the association of this region of the enzyme with the membrane prevents N-glycosylation (Chapter II). Treatment of each of the three mutant proteins with Endo H reduced the molecular weight of each protein to 66 kDa (Fig. 20c), indicating that the 74 kDa band seen for the HelB and HelC mutant proteins was the result of the presence of an additional N-glycosylation site. This result establishes that mutations in helices B and C can cause the Asn104 consensus sequence to become accessible for N-glycosylation.

<u>Membrane association of PGHS-Luc and PGHS-βGal fusion proteins.</u> The

Figure 19. Cyclooxygenase and peroxidase activities of native PGHS-1 and PGHS-1 helix mutant proteins. a) Specific cyclooxygenase and peroxidase activities of PGHS-1 mutant proteins expressed in whole *cos*-1 cells were determined, and are displayed as a percentage of the activity expressed by native PGHS-1 expressed in whole *cos*-1 cells.

b) Total specific activities were determined for native PGHS-1 and the HelB mutant protein by adding together the specific activity seen in the 10P, 200P, and 200S fractions for each of the enzymes. Percentages of the total activity expressed in each fraction for each enzyme were then calculated, and are displayed.

a


PERCENT NATIVE ACTIVITY
100%
80%
60%
40%
20%
CYCLOOXYGENASE
PEROXIDASE
Sham
Native
HelA
HelB
HelC

b

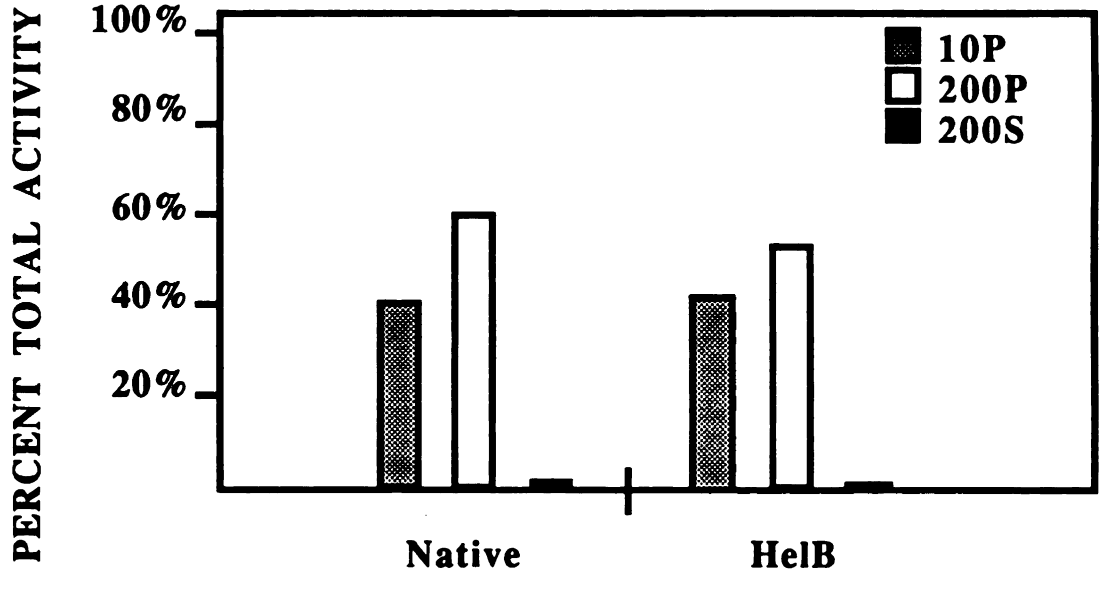

PERCENT TOTAL ACTIVITY
100%
80%
60%
40%
20%
10P
200P
200S
Native
HelB

Figure 20. Western blot analysis of ovine PGHS-1 helix mutant proteins. a) Whole transfected *cos*-1 cells were homogenized and total cellular protein was resolved by SDS-PAGE and transferred to nitrocellulose. Western blots were performed using an antibody against the amino terminus of ovine PGHS-1.

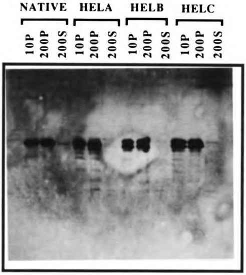
b) Transfected cos-1 cells were homogenized and separated into 10P, 200P, and 200S fractions by differential centrifugation. Proteins in each fraction were western blotted as described above.

c) Microsomes prepared from transfected cos-1 cells boiling in 1% SDS, and proteins were deglycosylated by addition of Endo H. Products were resolved by SDS-PAGE and western blotted as described above.

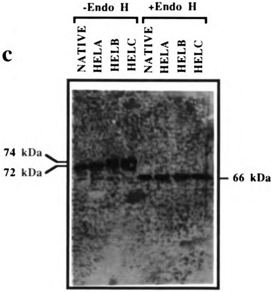
a


SHAM
NATIVE
HELA
HELB
HELC

b


NATIVE
HELA
HELB
HELC
10P
200P
200S
10P
200P
200S
10P
200P
200S
10P
200P
200S

c


-Endo H
+Endo H
NATIVE
HELA
HELB
HELC
NATIVE
HELA
HELB
HELC
74 kDa
72 kDa
66 kDa

following fusion proteins between ovine PGHS-1 and luciferase were constructed: a) 35Luc, which contains the first 35 amino acids of PGHS-1 (including the signal peptide) fused to the amino terminus of luciferase; b) 124Luc, which contains the first 124 amino acids of PGHS-1; and c) 124(ΔEGF)Luc, which contains the first 124 amino acids of PGHS-1 with a deletion residues 36-67, which represent most of the EGF homology domain. The amino acid sequence of luciferase expressed from pOSML-Luc begins at Met1, and the fusion proteins contain the hexapeptide Arg-Tyr-Pro-Gly-Ile-Gln between the carboxyl terminal residue of PGHS-1 and the amino terminal Met1 of luciferase.

Whole cell preparations were assayed to determine the total activity of fusion proteins compared to luciferase (Fig. 21a). The 35Luc, 124Luc and 124(ΔEGF)Luc fusion proteins each exhibited low luciferase activities compared to native luciferase. However, it should be noted that the fusion proteins did exhibit luciferase activity several orders of magnitude higher than background, indicating that the fusion proteins did have a significant level of activity. Protein levels in the cell preparations were compared in western blots using two antibodies: an antibody against residues 25-35 of ovine PGHS-1, which is contained in each of the fusion proteins, and an antibody against the amino terminus of luciferase (Table V). The antibody against PGHS-1 detected 35Luc protein at levels similar to that of PGHS-1; much lower levels of 124Luc protein were detected, and no 124(ΔEGF)Luc protein was detected (Fig. 22a). The anti-luciferase antibody detected similar levels of luciferase and 35Luc protein, but did not detect the 124Luc or 124(ΔEGF)Luc proteins (Fig. 22a). We beleive the discrepency between the two antibodies with respect to the 124Luc protein represents a difference in the relative titer of the two antibodies. No immunoreactive protein was observed in the tissue culture

Figure 21. Luciferase activities of PGHS$_{ov}$-1-luciferase fusion proteins. a) Specific luciferase activities of whole *cos*-1 cells expressing luciferase (Luc) or PGHS-luciferase fusion proteins were determined and are displayed as arbitrary light units/mg. The percentage of native luciferase activity for each mutant has also displayed.

b) Total specific luciferase activity was determined luciferase and for the PGHS-luciferase fusion proteins. The percent of the total activity in 10P, 200P, and 200S fractions was determined and is displayed. The notation for the 124(ΔEGF)Luc fusion protein has been abbreviated to ΔEGFLuc.

a

	Luciferase activity (units/mg)	Percent native activity
Sham	0.5	0.00001
Luc	433345	100
35Luc	19440	4
124Luc	3015	0.7
124(ΔEGF)-Luc	1732	0.4

b

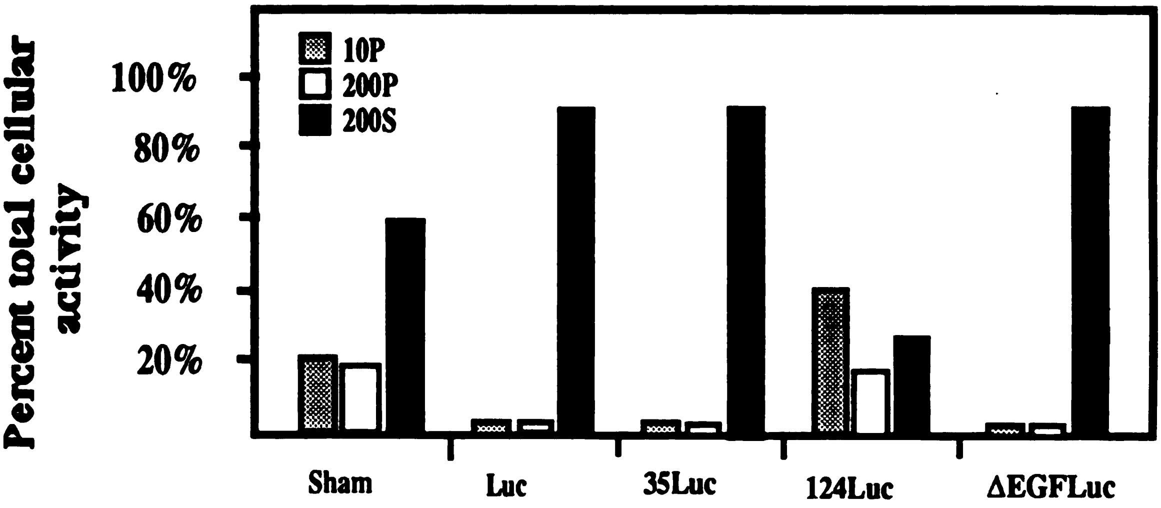
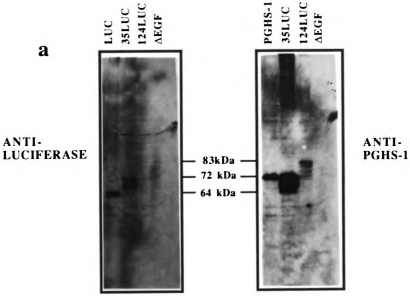


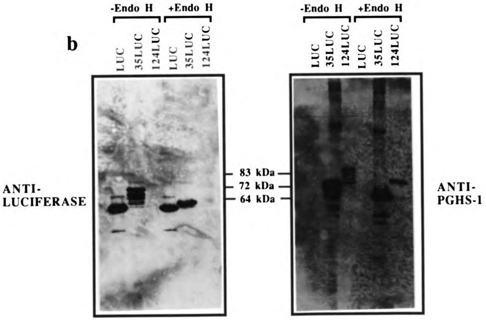
Figure 22. Western blot analysis of PGHS$_{ov}$-1-luciferase fusion proteins. a) Whole *cos*-1 cells expressing luciferase (Luc) or PGHS-luciferase fusion proteins were homogenized, and total cellular protein was resolved by SDS-PAGE and western blotted using either an antibody against luciferase or against the amino terminus of ovine PGHS-1, as indicated. The notation for the 124(ΔEGF)Luc fusion protein has been abbreviated to ΔEGF.

b) 10P fractions prepared from transfected *cos*-1 cells were boiled in 1% SDS and proteins were deglycosylated by addition of Endo H. Proteins were resolved by SDS-PAGE and western blotting was performed as described above.

a


LUC
35LUC
124LUC
ΔEGF
PGHS-1
35LUC
124LUC
ΔEGF
ANTI-
LUCIFERASE
83kDa
72 kDa
64 kDa
ANTI-
PGHS-1

b


-Endo H
+Endo H
LUC
35LUC
124LUC
LUC
35LUC
124LUC
-Endo H
+Endo H
LUC
35LUC
124LUC
LUC
35LUC
124LUC
ANTI-
LUCIFERASE
83 kDa
72 kDa
64 kDa
ANTI-
PGHS-1

media, indicating that the low protein levels of 124Luc and 124(ΔEGF)Luc were not caused by secretion of the protein (data not shown). Therefore, it appears that the lower activities seen with the fusion proteins are the result of low levels of expression.

The 35Luc and 124Luc proteins exhibit multiple bands ranging from 72-64 kDa and 83-73 kDa, respectively, when resolved by SDS-PAGE. Deglycosylation with Endo H caused each set of bands to collapse into a single band, having molecular weights of 64 kDa for the 35Luc protein and 73 kDa for the 124Luc protein (Fig. 22b). As the deduced luciferase amino acid sequence contains three consensus sequences for N-glycosylation (119), and the 124Luc construct introduces a fourth consensus sequence at Asn68 of ovine PGHS-1, we conclude that luciferase is being inserted into the lumen of the ER where it is N-glycosylated.

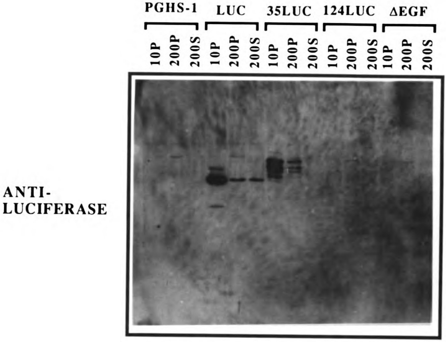
The levels of luciferase activity seen in fractions following differential centrifugation are shown in Figure 21b. A total luciferase activity per mg protein was calculated for each individual fusion protein or for luciferase by totalling the activity per mg protein in each of the fractions. A percent luciferase activity was then calculated for each of the three fractions. For native luciferase, the 35Luc protein and the 124(ΔEGF)Luc protein, approximately 95% of the total activity in the cell was found in the 200S fraction. In the 124Luc protein, however, there was a shift of activity into the membrane fractions, with only 33% of the total activity present in the 200S fraction, suggesting that addition of the first 124 amino acids of PGHS-1 caused the 124Luc protein to associate with membranes.

The distributions of luciferase and PGHS-luciferase fusion proteins in membrane fractions were not in agreement with the luciferase activity data. Western blotting of the fractions revealed that luciferase and the 35Luc protein, as well as the 124Luc protein, were located primarily in the 10P fraction (Fig. 23). Low levels were seen in the supernatant fraction, which had contained 95% of the luciferase activity for luciferase and the 35Luc protein. Similar to the results seen in whole cell preparations, the protein levels were comparable for luciferase and the 35Luc protein, much lower for the 124Luc protein, and undetectable for the 124(ΔEGF)Luc protein.

Fusion proteins were also constructed between ovine PGHS-1 and β-galactosidase (βgal). Similar to the fusion proteins generated between ovine PGHS-1 and luciferase, the βGal fusion proteins were designated 35Gal, 124Gal, and 124(ΔEGF)Gal. The amino acid sequence of βgal expressed from the pOSML-βgal construct begins at Met23. In the fusion proteins, the βgal amino acid sequence begins at Arg17 and the dipeptide Gly-Thr lies between the carboxyl terminal residue of PGHS-1 (either Pro35 or Ile124) and the amino terminal Arg17 of βgal. As a control, a fusion protein between the membrane domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) and βgal, termed HMGal, was used. HMG CoA reductase is a integral membrane protein of the ER, and HMGal displays a similar localization (105).

The 35Gal and 124Gal fusion proteins exhibited very low levels of βgal activity, 2 to 4 fold above background and approximately 1% of that seen for βgal expressed from pOSML-Gal (Fig. 24). The 124(ΔEGF)Gal fusion protein had an activity of 40% of that for the native βgal, while the HMGal protein had 20% of the activity of the native enzyme. When the levels of proteins in whole cells were examined in western blots using an antibody against βgal, levels of the 35Gal protein and the 124Gal protein were low in the cells, while the levels of β-gal, HMGal, and 124(ΔEGF)Gal were similar

Figure 23. Western blot analysis of the distribution of PGHS$_{ov}$-1-luciferase fusion proteins in subcellular fractions. 10P, 200P, and 200S fractions were prepared from *cos*-1 cells expressing with ovine PGHS-1, luciferase or PGHS-luciferase fusion proteins. Proteins were resolved by SDS-PAGE, and western blotting was performed using antibodies against either luciferase or the amino terminus of ovine PGHS-1. The notation for the 124(ΔEGF)Luc fusion protein has been abbreviated to ΔEGF.


PGHS-1
LUC
35LUC
124LUC
ΔEGF
10P
200P
200S
10P
200P
200S
10P
200P
200S
10P
200P
200S
10P
200P
200S
ANTI-
LUCIFERASE

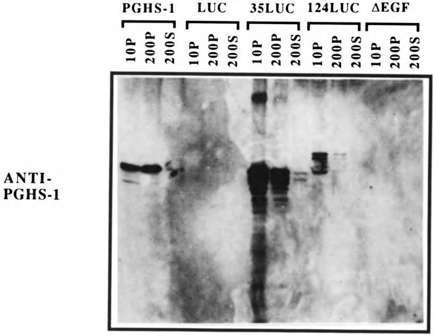
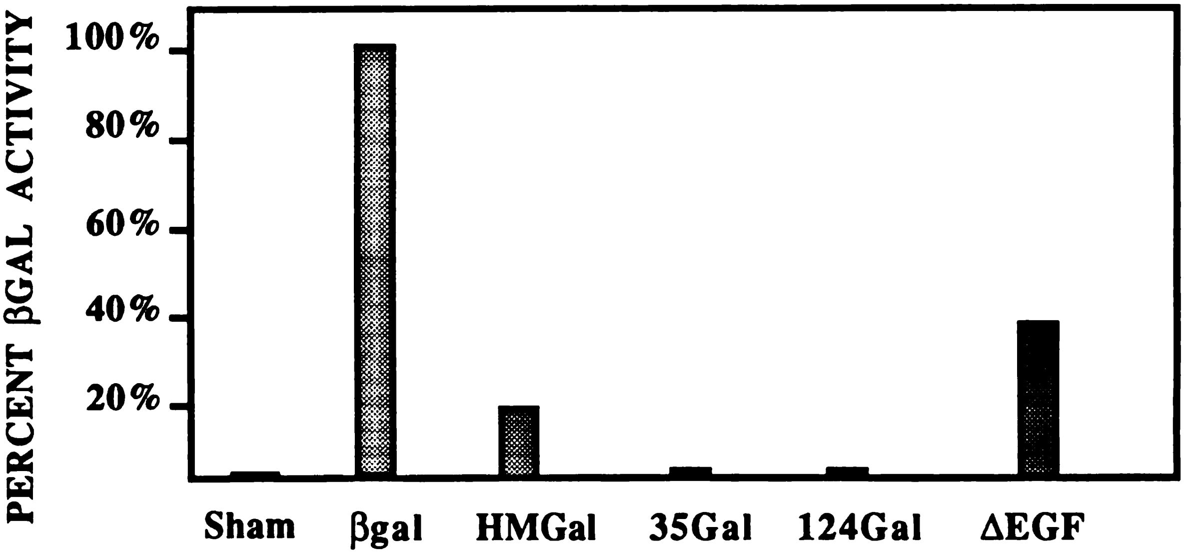

PGHS-1
LUC
35LUC
124LUC
ΔEGF
10P
200P
200S
10P
200P
200S
10P
200P
200S
10P
200P
200S
10P
200P
200S
ANTI-
PGHS-1

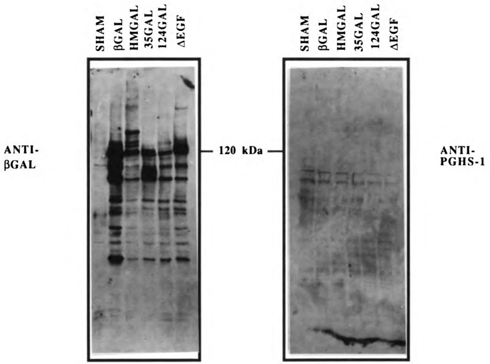
Figure 24. β-galactosidase activity of PGHS$_{ov}$-1-β-galactosidase fusion proteins. Specific β-galactosidase activities were determined for whole *cos*-1 cells expressing β-galactosidase, HMGal, or PGHS-βgal fusion proteins. Activities are expressed as a percentage of the β-galactosidase activity seen for native βgal. The notation for 124(ΔEGF)Gal fusion protein has been abbreviated to ΔEGF.


PERCENT βGAL ACTIVITY
100%
80%
60%
40%
20%
Sham
βgal
HMGal
35Gal
124Gal
ΔEGF

(Fig 25). No protein was observed for 35Gal or 124Gal in the culture medium (not shown).

When the three fusion proteins between PGHS-1 and βgal were blotted with the antibody against the amino terminus of ovine PGHS-1, no staining was observed, suggesting that at least a portion of the PGHS-1 domain in the fusion protein is missing. Because these fusion proteins appeared to lack a portion of the PGHS-1 domain, no further characterization was performed.

Figure 25. Western blot analysis of $\mathrm{PGHS_{ov}}$-1-β-galactosidase fusion proteins. Whole *cos*-1 cells expressing βgal, HMGal, or PGHS-βGal were homogenized, and total cellular protein was resolved by SDS-PAGE. Western blotting was performed using either a polyclonal antibody against βgal or an antibody against the amino terminus of ovine PGHS-1. The notation for the 124(ΔEGF)Gal fusion protein has been abbreviated to ΔEGF.


SHAM
βGAL
HMGAL
35GAL
124GAL
ΔEGF
SHAM
βGAL
HMGAL
35GAL
124GAL
ΔEGF
ANTI-
βGAL
120 kDa
ANTI-
PGHS-1

Discussion

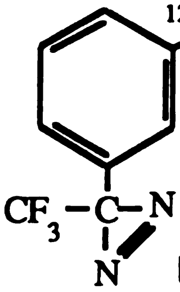
Due to its ability to partition almost completely into the hydrophobic phase of the lipid bilayer, [^{125}I]TID has been used to photolabel regions of proteins which are associated with the lipid phase of membranes (117, 118). Ovine PGHS-1 is one of the major membrane proteins in microsomes prepared from seminal vesicles, comprising approximately 5% of the total microsomal protein, and it was the most prominent microsomal protein photolabelled by [^{125}I]TID. Initially, we were surprised to find that both the amino terminal tryptic peptide and the carboxyl terminal tryptic peptide of PGHS-1 were labelled by [^{125}I]TID, as we had predicted that only the amino terminal peptide, which contains the putative membrane binding domain, was associated with the membrane. The photolabelling of both peptides in the presence of 20 mM glutathione demonstrated that the photolabelling observed was not due to the presence of [^{125}I]TID in the aqueous phase.

An examination of the crystal structure of PGHS-1 did not reveal any obvious region of the protein in the carboxyl terminal tryptic peptide which might be able to associate with the membrane. An alternative explanation for the photolabelling of this peptide is that [^{125}I]TID can occupy and photolabelled the hydrophobic channel which which comprises the cyclooxygenase active site. The channel consists of regions of PGHS-1 found in both the amino and carboxyl terminal tryptic peptides. Studies of the nicotinic acetylcholine receptor have provided a precedence for [^{125}I]TID labelling in both a non-specific manner, consistent with the labelling of regions of proteins associated with membranes, and in a specific manner, which can be competed for with other ligands and antagonists (118, 120). The inhibition of the [^{125}I]TID-labelling of the carboxyl terminal tryptic peptide by the NSAIDs ibuprofen and sulindac sulfide indicates that the labelling of this peptide was the result of a specific interaction between [^{125}I]TID and PGHS-1, and is not consistent with the labelling of a membrane-associated region. It is reasonable to predict that a hydrophobic compound such as [^{125}I]TID could occupy the cyclooxygenase active site; the substrate for PGHS is a fatty acid and many of the inhibitors for PGHS are quite hydrophobic. [^{125}I]TID is also somewhat similar in size and structure to aspirin. Figure 26 compares the structures of [^{125}I]TID and several NSAIDs.

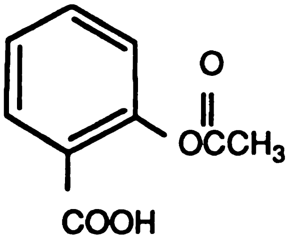
Proteolytic digestion of [^{125}I]TID-labelled PGHS-1 demonstrated that a peptide consisting of residues 25-166 is labelled by [^{125}I]TID. The labelling of this peptide provides the first biochemical evidence consistent with the putative membrane binding domain actually being the region of PGHS-1 that associates with the membrane. Further characterization of smaller peptides will be required establish this point.

The mutation of helices A, B and C did not solubilize PGHS-1. While the HelB protein retained activities, the HelA and HelC proteins lacked both cyclooxygenase and peroxidase activities. It will be of interest to determine if these mutant proteins retain some native structure and are still associated with the membrane, or if they are unfolded aggregates that precipitate with the membrane fractions. If the HelA and HelC proteins are still associated with membranes, it may still be possible to solubilize an inactive form of PGHS-1 by constructing double and triple mutants with the HelA, HelB and HelC mutants. The apparent N-glycosylation of Asn104 in the HelB and HelC helices is interesting because it raises the possibility that these mutations have subtly altered the interaction of PGHS-1 with membranes to the point that N-glycosylation can occur at this residue.

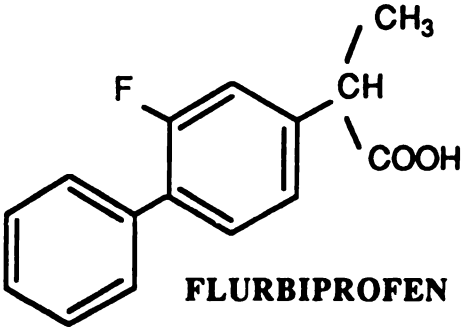
Figure 26. Comparison of the structures of [^{125}I]TID and selected NSAIDs.



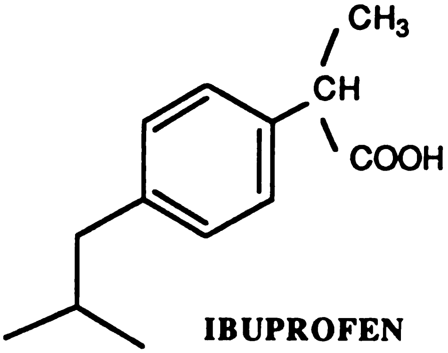
[^{125}I]-TID



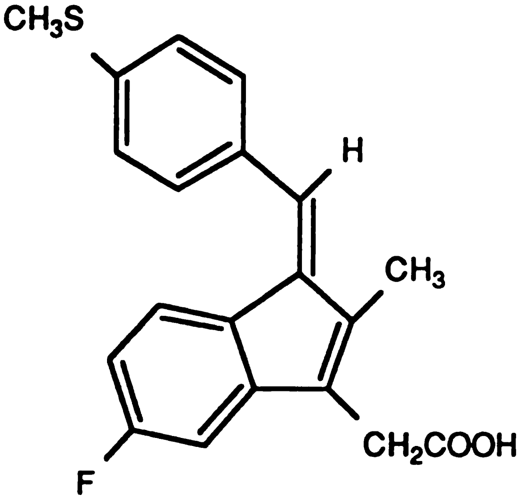
ASPIRIN



FLURBIPROFEN



IBUPROFEN



SULINDAC SULFIDE

Analysis of the fusion proteins between ovine PGHS-1 and luciferase yielded confusing results. While the activity data suggested that addition of the 124 amino terminal residues of PGHS-1 to luciferase resulted in partial association with the membrane, this conclusion was not supported by an examination of the distribution of immunoreactive protein in membrane fractions. While 95% of the total activity was contained in the supernatant fraction for native luciferase and the 35Luc protein, the 10P fraction contained most of the protein. A possible explanation for this observation is that the majority of the protein observed in western blots of the 10P fraction was inactive, aggregated protein (due to the overexpression of protein in *cos-1* cells) that coprecipitates with membranes. Apparently the sensitivity of the luciferase assay is such that the amount of protein needed to generate a signal in an activity assay is much less than the amount of protein necessary to be detected by western blotting. The 124(ΔEGF)Luc protein is an example of this; the luciferase activity of 124(ΔEGF)Luc is approximately 3400 fold higher than background, and yet the mutant protein is not detectable in a western blot. Thus, although it does appear that the first 124 amino acids of ovine PGHS-1 cause a shift in the distribution of active luciferase towards being membrane associated, this cannot be confirmed due to a high background of apparently inactive protein and low levels of expressed fusion protein.

Thus, we were not able to demonstrate that the putative membrane binding domain is in fact associated with the ER membrane. We have, however, provided evidence that the region or regions of PGHS-1 which associate with the membrane are in the first 276 amino acid of the enzyme, and that a region of membrane association resides between residues 25-166. This evidence is consistent with the prediction that the putative membrane binding domain between residues 74-117 is associated with the membrane.

Acknowledgements

We thank R. Micheal Garavito, Daniel Picot, and Patrick J. Loll of the University of Chicago Department of Biochemistry and Molecular Biology for providing assistance and the crystal structure coordinates for ovine PGHS-1. We thank Melvin S. Schindler for assistance with [^{125}I]TID-labelling protocols.

The antibody against residues 203-217 of ovine PGHS-1 was developed by LC Hsi and WL Smith.

BIBLIOGRAPHY

1. Smith, W. L. 1989. The eicosanoids and their biochemical mechanisms of action. *Biochem. J.* 259:315-24

2. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., Van-Dorp, D. A. 1977. Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands. *Biochim. Biophys. Acta* 487:315-31

3. Miyamoto, T., Ogino, N., Yamamoto, S., Hayaishi, O. 1976. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* 251:2629-36

4. Smith, W. L., DeWitt, D. L. 1994. Differential interaction of prostaglandin endoperoxide synthases with nonsteroidal anti-inflammatory drugs. *Current Opinions in Investigatory Drugs* 3:1-20

5. Meade, E. A., Smith, W. L., DeWitt, D. L. 1993. Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* 268:6610-14

6. Laneuville, O., Breuer, D. K., DeWitt, D. L., Hla, T., Funk, C. D., et al. 1994. Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J. Pharm. Exp. Therap.* submitted

7. Vane, J. 1994. Towards a better aspirin. *Nature* 367:215-16

8. Kudo, I., Murakami, M., Hara, S., Inoue, K. 1993. Mammalian non-pancreatic phospholipases A_2. *Biochim. Biophys. Acta* 1170:217-31

9. Smith, W. L., Laneuville, O. 1994. Cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism. In *Eicosanoids and Their Inhibitors in Cancer Immunology and Immunotherapy*, ed. K. M. Anderson, J. Harris, CRC Press, Boca Raton, FL, 1-39.

10. Coleman, R. A., Smith, W. L., Narumiya, S. 1994. Classification of prostanoid receptors: properties, distribution and structure of the receptors and their subtypes. *Pharm. Rev.* 46:205-29

11. DeWitt, D. L., Smith, W. L. 1983. Purification of prostacyclin synthase from bovine aorta by immunoaffinity chromatography. Evidence that the enzyme is a hemoprotein. *J. Biol. Chem.* 258:3285-93

12. Shen, R. F., Tai, H. H. 1986. Immunoaffinity purification and characterization of thromboxane synthase from porcine lung. *J. Biol. Chem.* 261:11592-99

13. Nusing, R., Schneider-Voss, S., Ullrich, V. 1990. Immunoaffinity purification of human thromboxane synthase. *Arch. Biochem. Biophys.* 280:325-30

14. Yokoyama, C., Miyata, A., Ihara, H., Ullrich, V., Tanabe, T. 1991. Molecular cloning of human platelet thromboxane A synthase. *Biochem. Biophys. Res. Commun.* 178:1479-84

15. Ohashi, K., Ruan, K. H., Kulmacz, R. J., Wu, K. K., Wang, L. H. 1992. Primary structure of human thromboxane synthase determined from the cDNA sequence. *J. Biol. Chem.* 267:789-93

16. Smith, W. L., DeWitt, D. L., Allen, M. L. 1983. Bimodal distribution of the prostaglandin I_2 synthase antigen in smooth muscle cells. *J. Biol. Chem.* 258:5922-26

17. Black, S. D. 1992. Membrane topology of the mammalian P450 cytochromes. *FASEB J.* 6:680-85

18. Ruan, K. H., Wang, L. H., Wu, K. K., Kulmacz, R. J. 1993. Amino-terminal topology of thromboxane synthase in the endoplasmic reticulum. *J. Biol. Chem.* 268:19483-90

19. Smith, W. L. 1992. Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.* 263(2 Pt 2):F181-91

20. Arita, H., Nakano, T., Hanasaki, K. 1989. Thromboxane A_2: its generation and role in platelet activation. *Prog. Lipid Res.* 28:273-301

21. Gorman, R. R., Fitzpatrick, F. A., Miller, O. V. 1978. Reciprocal regulation of human platelet cAMP levels by thromboxane A_2 and prostacyclin. *Adv. Cyclic. Nucleotide. Res.* 9:597-609

22. Rome, L. H., Lands, W. E. M. 1975. Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc. Natl. Acad. Sci. USA* 72:4863-65

23. Carty, T. J., Stevens, J. S., Lombardino, J. G., Parry, M. J., Randall, M. J. 1980. Piroxicam, a structurally novel anti-inflammatory compound. Mode of prostaglandin synthesis inhibition. *Prostaglandins* 19:671-82

24. Marnett, L. J. 1992. Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Res.* 52:5575-89

25. Brooks, P. M., Day, R. O. 1991. Nonsteroidal antiinflammatory drugs- differences and similarities. *N. Eng. J. Med.* 324:1716-25

26. Futaki, F., Yoshikawa, K., Hamasaka, Y., Arai, I., Higuchi, s, et al. 1993. NS398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen. Pharm.* 24:105-10

27. Futaki, N., Takahishi, S., Yokoyama, M., Arai, I., Higuchi, S., et al. 1994. NS398, a new anti-inflammatory agent, selectively inhibits a novel prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins* 47:55-59

28. Xie, W., Chipman, J. G., Robertson, D. L., Erikson, R. L., Simmons, D. L. 1991. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. USA* 88:2692-96

29. Kargman, S., Chan, S., Evans, J., Vickers, P., O'Neill, G. 1994. Tissue distribution of prostaglandin G/H synthase-1 and -2 (PGHS-1 and PGHS-2) using specific anti-peptide antibodies. *J. Cell. Biochem.* Supplement 18B:319 (Abstract O109)

30. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., Herschman, H. R. 1991. TIS10, a phorbol ester tumor promoter inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.* 266:12866-72

31. DeWitt, D. L., Meade, E. A. 1993. Serum and glucocorticoid regulation of gene transcription and expression of the prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes. *Arch. Biochem. Biophys.* 306:94-102

32. DeWitt, D. L., Day, J. S., Sonnenburg, W. K., Smith, W. L. 1983. Concentrations of prostaglandin endoperoxide synthase and prostaglandin I_2 synthase in the endothelium and smooth muscle of bovine aorta. *J. Clin. Invest.* 72:1882-88

33. Funk, C. D., Funk, L. B., Kennedy, M. E., Pong, A. S., Fitzgerald, G. A. 1991. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J.* 5:2304-12

34. Smith, W., Bell, T. G. 1978. Immunohistochemical localization of the prostaglandin-forming cyclooxygenase in renal cortex. *Amer. J Physiol.* 235:F451-57

35. Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., et al. 1992. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.* 267:25934-38

36. Brannon, T. S., North, A. J., Wells, L. B., Shaul, P. W. 1994. Prostacyclin synthesis in ovine pumonary artery is developmentally regulated by changes in cycloxygenase-1 gene expression. *J. Clin. Invest.* 93:2230-35

37. Simmons, D. L., Xie, W., Chipman, J. G., Evett, G. E. 1991. Multiple cyclooxygenases: cloning of a mitogen-inducible form. In *Prostaglandins, Leukotrienes, Lipoxins, and PAF*, ed. J. M. Bailey:67-78. New York: Plenum Press

38. O'Neill, G. P., Ford-Hutchinson, A. W. 1993. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. *FEBS Lett.* 330:156-60

39. Maier, J. A. M., Hla, T., Maciag, T. 1990. Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.* 265:10805-8

40. Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T., Hla, T. 1994. Induction of cylooxygenase-2 by interleukin-1α: evidence for post-transcriptional regulation. *J. Biol. Chem.* 269:11769-77

41. O'Sullivan, M. G., Huggins Jr., E. M., Meade, E. A., DeWitt, D. L., McCall, C. E. 1992. Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. *Biochem. Biophys. Res. Commun.* 187:1123-27

42. Kujubu, D. A., Herschman, H. R. 1992. Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase/cyclooxygenase gene. *J. Biol. Chem.* 267:7991-94

43. Sano, H., Hla, T., Maier, J. A. M., Crofford, L. J., Case, J. P., et al. 1992. In vivo cyclooxygenase expression in synovial tissue of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. *J. Clin. Invest.* 89:97-108

44. Hulkower, K. I., Wertheimer, S. J., Levin, W., Coffey, J. W., Anderson, C. M., et al. 1994. Interleukin-1β induces cytosolic phospholipase A_2 and prostaglandin H synthase in rheumatoid synovial fibroblasts: evidence for their roles in the production of prostaglandin E_2. *Arthritis Rheum.* 37:653-61

45. Crofford, L. J., Wilder, R. L., Ristimaki, A. P., Sano, H., Remmers, E. F., et al. 1994. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1β, phorbol ester, and corticosteroids. *J. Clin. Invest.* 93:1095-1101

46. Sirois, J., Richards, J. S. 1992. Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles. *J. Biol. Chem.* 267:6382-88

47. Domin, J., Rozengurt, E. 1993. Platelet-derived growth factor stimulates a biphasic mobilization of arachidonic acid in Swiss 3T3 cells. *J. Biol. Chem.* 268:8927-34

48. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., et al. 1991. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* 65:1043-51

49. Gronich, J. H., Bonventre, J. V., Nemenoff, R. A. 1990. Purification of a high-molecular-mass form of phospholipase A_2 from rat kidney activated at physiological calcium concentrations. *Biochem. J.* 271:37-43

50. Gronich, J. H., Bonventre, J. V., Nemenoff, R. A. 1988. Identification and characterization of a hormonally regulated form of phospholipase A_2 in rat renal mesangial cells. *J. Biol. Chem.* 263:16645-51

51. Lin, L. L., Lin, A. Y., Knopf, J. L. 1992. Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci. U. S. A.* 89:6147-51

52. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., et al. 1993. $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* 72:269-78

53. Murakami, M., Kudo, I., Inoue, K. 1993. Molecular nature of phospholipases A_2 involved in prostaglandin I_2 synthesis in human umbilical vein endothelial cells. Possible participation of cytosolic and extracellular type II phospholipases A_2. *J. Biol. Chem.* 268:839-44

54. Suga, H., Murakami, M., Kudo, I., Inoue, K. 1993. Participation in cellular prostaglandin synthesis of type-II phospholipase A_2 secreted and anchored on cell-surface heparan sulfate proteoglycan. *Eur. J. Biochem.* 218:807-13

55. Reddy, S. T., Herschman, H. R. 1994. Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. *J. Biol. Chem.* 269:15473-80

56. Miller, D. K., Gillard, J. W., Vickers, P. J., Sadowski, S., Leveille, C., et al. 1990. Identification and isolation of a membrane protein necessary for leukotriene production. *Nature* 343:278-81

57. Reid, G. K., Kargman, S., Vickers, P. J., Mancini, J. A., Leveille, C., et al. 1990. Correlation between expression of 5-lipoxygenase-activating protein, 5-lipoxygenase, and cellular leukotriene synthesis. *J. Biol. Chem.* 265:19818-23

58. Dixon, R. A., Diehl, R. E., Opas, E., Rands, E., Vickers, P. J., et al. 1990. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* 343:282-84

59. DeWitt, D. L., Smith, W. L. 1988. Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natl. Acad. Sci. USA* 85:1212-1416

60. Merlie, J. P., Fagan, D., Mudd, J., Needleman, P. 1988. Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). *J. Biol. Chem.* 263:3550-53

61. Yokoyama, C., Takai, T., Tanabe, T. 1988. Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. *FEBS Lett.* 231:347-51

62. DeWitt, D. L., El-Harith, E. A., Kraemer, S. A., Andrews, M. J., Yao, E. F., et al. 1990. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.* 265:5192-98

63. Yokoyama, C., Tanabe, T. 1989. Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. *Biochem. Biophys. Res. Commun.* 165:888-94

64. Feng, L., Sun, W., Xia, Y., Tang, W. W., Chanmugam, P., et al. 1993. Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch. Biochem. Biophys.* 307:361-68

65. Hla, T., Neilson, K. 1992. Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. U. S. A.* 89:7384-88

66. Regier, M. K., DeWitt, D. L., Schindler, M. S., Smith, W. L. 1993. Subcellular localization of prostaglandin endoperoxide synthase-2 in murine 3T3 cells. *Arch. Biochem. Biophys.* 301:439-44

67. Rollins, T. E., Smith, W. L. 1980. Subcellular localization of prostaglandin-forming cyclooxygenase in Swiss mouse 3T3 fibroblasts by electron microscopic immunocytochemistry. *J. Biol. Chem.* 255:4872-75

68. Hemler, M., Lands, W. E. M., Smith, W. L. 1976. Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J. Biol. Chem.* 251:5575-81

69. Mutsaers, J. H., van-Halbeek, H., Kamerling, J. P., Vliegenthart, J. F. 1985. Determination of the structure of the carbohydrate chains of prostaglandin endoperoxide synthase from sheep. *Eur. J. Biochem.* 147:569-74

70. Strittmatter, P., Machuga, E. T., Roth, G. J. 1982. Reduced pyridine nucleotides and cytochrome b5 as electron donors for prostaglandin synthetase reconstituted in dimyristyl phosphatidylcholine vesicles. *J. Biol. Chem.* 257:11883-86

71. Chen, Y. N., Bienkowski, M. J., Marnett, L. J. 1987. Controlled tryptic digestion of prostaglandin H synthase. Characterization of protein fragments and enhanced rate of proteolysis of oxidatively inactivated enzyme. *J. Biol. Chem.* 262:16892-99

72. DeWitt, D. L., Rollins, T. E., Day, J. S., Gauger, J. A., Smith, W. L. 1981. Orientation of the active site and antigenic determinants of prostaglandin endoperoxide synthase in the endoplasmic reticulum. *J. Biol. Chem.* 256:10375-82

73. Kulmacz, R. J., Wu, K. K. 1989. Topographic studies of microsomal and pure prostaglandin H synthase. *Arch. Biochem. Biophys.* 268:502-15

74. Picot, D., Loll, P. J., Garavito, R. M. 1994. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* 367:243-49

75. Picot, D., Garavito, R. M. 1994. Prostaglandin H synthase: Implications for membrane structure. *FEBS Lett.* 346:21-25

76. Roth, G. J., Siok, C. J., Ozols, J. 1980. Structural characteristics of prostaglandin synthetase from sheep vesicular gland. *J. Biol. Chem.* 255:1301-4

77. Shimokawa, T., Smith, W. L. 1991. Essential histidines of prostaglandin endoperoxide synthase. *J. Biol. Chem.* 266:6168-73

78. Lecomte, M., Bhattacharya, D., Smith, W. L. 1994. unpublished result.

79. Lecomte, M., Laneuville, O., Chuan, J., DeWitt, D. L., Smith, W. L. 1994. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J. Biol. Chem.* 269:13207-15

80. Kulmacz, R. J. 1989. Topography of prostaglandin H synthase. Antiinflammatory agents and the protease-sensitive arginine 253 region. *J. Biol. Chem.* 264:14136-44

81. Smith, W. L., DeWitt, D. L., Shimokawa, T., Kraemer, S. A., Meade, E. A. 1990. Molecular basis for the inhibition of prostanoid biosynthesis by nonsteroidal anti-inflammatory agents. *Stroke* 21:IV24-28

82. Hemler, M. E., Graff, G., Lands, W. E. M. 1978. Accelerative autoactivation of prostaglandin biosynthesis by PGG_2. *Biochem. Biophys. Res. Commun.* 85:1325-31

83. Lassmann, G., Odenwaller, R., Curtis, J. F., DeGray, J. A., Mason, R. P., et al. 1991. Electron spin resonance investigation of tyrosyl radicals of prostaglandin H synthase. Relation to enzyme catalysis. *J. Biol. Chem.* 266:20045-55

84. Tsai, A., Hsi, L. C., Kulmacz, R. J., Palmer, G., Smith, W. L. 1994. Characterization of the tyrosyl radicals in ovine prostaglandin H synthase by isotope replacement and site directed mutagenesis. *J. Biol. Chem.* 269:5085-91

85. Hsi, L. C., Hoganson, C. W., Babcock, G. T., Smith, W. L. 1994. Characterization of a tyrosyl radical in prostaglandin endoperoxide synthase-2. *Biochem. Biophys. Res. Commun.* 85:1325-31

86. Shimokawa, T., Kulmacz, R. J., DeWitt, D. L., Smith, W. L. 1990. Tyrosine 385 of prostaglandin endoperoxide synthase is required for cyclooxygenase catalysis. *J. Biol. Chem.* 265:20073-76

87. Mizuno, K., Yamamoto, S., Lands, W. E. 1982. Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. *Prostaglandins* 23:743-57

88. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., Van-Dorp, D. A. 1980. Acetylation of prostaglandin endoperoxide synthetase with acetylsalicylic acid. *Eur. J. Biochem.* 109:1-8

89. Kunkel, T. A., Roberts, J. D., Zakour, R. A. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367-82

90. Sanger, F., Nicklen, S., Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* 74:5463-67

91. Meade, E. A., Smith, W. L., DeWitt, D. L. 1993. Expression of the murine prostaglandin (PGH) synthase-1 and PGH synthase-2 isozymes in cos-1 cells. *J. Lipid Mediators* 6:119-29

92. Rands, E., Candelore, M. R., Cheung, A. H., Hill, W. S., Strader, C. D., et al. 1990. Mutational analysis of β-adrenergic receptor glycosylation. *J. Biol. Chem.* 265:10759-64

93. Taketa, K., Ichikawa, E., Hanada, T. 1986. A tetrazolium method for staining peroxidase labels in blotting assays. *J. Immunol. Methods.* 95:71-77

94. Shimokawa, T., Smith, W. L. 1992. Prostaglandin endoperoxide synthase. The aspirin acetylation region. *J. Biol. Chem.* 267:12387-92

95. Marnett, L. J., Chen, Y. N., Maddipati, K. R., Ple, P., Labeque, R. 1988. Functional differentiation of cyclooxygenase and peroxidase activities of prostaglandin synthase by trypsin treatment. Possible location of a prosthetic heme binding site. *J. Biol. Chem.* 263:16532-35

96. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-54

97. Smith, W. L., Marnett, L. J. 1991. Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta* 1083:1-17

98. Hla, T., Neilson, K. 1992. Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA* 89:7384-88

99. Shimokawa, T., Smith, W. L. 1992. Expression of prostaglandin endoperoxide synthase-1 in a baculovirus system. *Biochem. Biophys. Res. Commun.* 183:975-82

100. Slieker, L. J., Martensen, T. M., Lane, M. D. 1986. Synthesis of epidermal growth factor receptor in human A431 cells. Glycosylation-dependent acquisition of ligand binding activity occurs post-translationally in the endoplasmic reticulum. *J. Biol. Chem.* 261:15233-41

101. Olson, T. S., Lane, M. D. 1987. Post-translational acquisition of insulin binding activity by the insulin proreceptor. Correlation to recognition by autoimmune antibody. *J. Biol. Chem.* 262:6816-22

102. Hunt, R. C., Riegler, R., Davis, A. A. 1989. Changes in glycosylation alter the affinity of the human transferrin receptor for its ligand. *J. Biol. Chem.* 264:9643-48

103. Hoe, M. H., Hunt, R. C. 1992. Loss of one asparagine-linked oligosaccharide from human transferrin receptors results in specific cleavage and association with the endoplasmic reticulum. *J. Biol. Chem.* 267:4916-23

104. Kornfeld, R., Kornfeld, S. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-64

105. Roitelman, J., Olender, E. H., Bar-Nun, S., Dunn, W. A., Jr., Simoni, R. D. 1992. Immunological evidence for eight spans in the membrane domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase: implications for enzyme degradation in the endoplasmic reticulum. *J. Cell Biol.* 117:959-73

106. Gravotta, D., Adesnik, M., Sabatini, D. D. 1990. Transport of influenza HA from the trans-Golgi network to the apical surface of MDCK cells permeabilized in their basolateral plasma membranes: energy dependence and involvement of GTP-binding proteins. *J. Cell Biol.* 111(6 Pt 2):2893-2908

107. Terasaki, M., Reese, T. S. 1992. Characterization of endoplasmic reticulum by co-localization of BiP and dicarbocyanine dyes. *J. Cell. Sci.* 101(Pt 2, Feb):315-22

108. Kamp, F., Hamilton, J. A. 1993. Movement of fatty acids, fatty acid analogues, and bile acids across phospholipid bilayers. *Biochemistry* 32:11074-85

109. Schievella, A., Lin, L. L. 1994. *Personal communication.*

110. Borbour, S. E., Dennis, E. A. 1993. Antisense inhibition of group II phospholipase A_2 expression blocks the production of prostaglandin E_2 by P388D1 cells. *J. Biol. Chem.* 268:21875-82

111. Ramanadham, S., Gross, R. W., Han, X., Turk, J. 1993. Inhibition of arachidonate release by secretagogue-stimulated pancreatic islets suppresses both insulin secretion and the rise in beta-cell cytosolic calcium ion concentration. *Biochemistry* 32:337-46

112. Lehman, J. J., Brown, K. A., Ramanadham, S., Turk, J., Gross, R. W. 1993. Arachidonic acid release from aortic smooth muscle cells induced by [Arg8]vasopressin is largely mediated by calcium-independent phospholipase A_2. *J. Biol. Chem.* 268:20713-16

113. Smith, W. L. 1986. Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. *Annu. Rev. Physiol.* 48:251-62

114. Weksler, B. B., Marcus, A. J., Jaffe, E. A. 1977. Synthesis of prostaglandin I_2 (prostacyclin) by cultured human and bovine endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:3922-26

115. Evett, G. E., Xie, W., Chipman, J. G., Robertson, D. L., Simmons, D. L. 1993. Prostaglandin G/H isoenzyme 2 expression in fibroblasts: regulation by dexamethasone, mitogens, and oncogenes. *Arch. Biochem. Biophys.* 306:169-77

116. Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 77:1496-1500

117. Brunner, J., Semenza, G. 1981. Selective labelling of the hydrophobic core of membranes with 3-(triflouromethyl)-3-(m-[125I]iodophenyl)diazirine, a carbene-generating reagent. *Biochemistry* 20:7174-82

118. Blanton, M. P., Cohen, J. B. 1994. Identifying the lipid-protein interface of the Torpedo nicotinic acetylcholine receptor: secondary structure implications. *Biochemistry* 33:2859-72

119. de-Wet, J. R., Wood, K. V., Helinski, D. R., DeLuca, M. 1985. Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* 82:7870-73

120. Blanton, M. P., Cohen, J. B. 1992. Mapping the lipid-exposed regions in the Torpedo californica nicotinic acetylcholine receptor ;31(25):5951]. *Biochemistry* 31:3738-50