ISOLATION AND CHARACTERIZATION OF A PGH-PGE ISOMERASE FROM SHEEP VESICULAR GLAND MICROSOMES

By

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF A PGH-PGE ISOMERASE FROM SHEEP VESICULAR GLAND MICROSOMES

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PGH-PGE isomerase (EC 5.3.99.3) catalyzes the glutathione (GSH)-dependent formation of prostaglandin E_2 (PGE_2) from the prostaglandin endoperoxide, PGH_2. Sheep vesicular gland (SVG) is a tissue with a very abundant supply of this microsomal membrane enzyme. PGH-PGE isomerase has been only partially purified and characterized, because the enzyme activity is very labile. We, therefore, prepared anti-PGH-PGE isomerase monoclonal antibodies, so that we could purify and characterize this enzyme.

Two monoclonal antibodies that precipitated PGH-PGE isomerase activity from solubilized SVG microsomes were identified (IgG_1(*hei*-7) and IgG_1(*hei*-26)). IgG_1(*hei*-7) precipitated a maximum of 45%, and IgG_1(*hei*-26) 22%, of the total PGH-PGE isomerase activity from solubilized microsomal proteins. These precipitated activities were additive, had different kinetic parameters, and had different cellular distributions. The proteins reactive with IgG_1(*hei*-7) and IgG_1(*hei*-26) were identified, and have subunit molecular weights of 17,500 and 180,000 daltons, respectively.

Two factors were found to be essential for stabilizing the solubilized microsomal PGH-PGE isomerase activity: solubilization of the microsomal proteins using CHAPS,

and the maintenance of fresh GSH in the solubilized proteins. A procedure was developed in which the enzyme reactive with IgG_1(*hei*-7) could be separated from other microsomal proteins using IgG_1(*hei*-7) which was immobilized on Protein A-agarose. This immobilized PGH-PGE isomerase was active, relatively stable, showed linear enzyme kinetic behavior, and was useful for studying the role of the cofactor, GSH, in the reaction. The immobilized enzyme had a pH optima of 7.0 to 8.1. It was partially inhibited by flurbiprofen, ten different PGH_2 analogs, and sulfhydryl modifying reagents, but not by S-alkyl GSH's. The Km for GSH at pH 8.0 was 40 uM at 48 uM PGH_2, and was 170 uM at 640 uM PGH_2. The Km for PGH_2 was 387 uM (pH 8.0, 0.5 mM GSH). There was no time-dependent formation of PGE_2 in the absence of GSH. GSH was specifically required by the immobilized PGH-PGE isomerase for both activity and stability. GSH was also not oxidized stoichiometrically as PGE_2 was formed during the PGH-PGE isomerase reaction. Thus, it appears that GSH acts as a cofactor in the PGH-PGE isomerase reaction.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AA, arachidonic acid; BSA, bovine serum albumin; cAMP, adenosine-3',5'-cyclic monophosphate; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle's medium; GSH, glutathione; HRP, horseradish peroxidase; IgG_1, immunoglobulin G_1; MPB, microsomal preparation buffer; MSB, microsomal solubilization buffer; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PG, prostaglandin; Tx, thromboxane; TBS, Tris-buffered saline; Tris, tris(hydroxymethyl)-aminomethane; TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SVG, sheep vesicular gland.

INTRODUCTION

Prostaglandin E_2 (PGE_2) is synthesized from arachidonic acid by the sequential action of two microsomal membrane enzymes, PGH synthase and PGH-PGE isomerase. PGH synthase catalyzes the conversion of arachidonic acid to the prostaglandin endoperoxide PGH_2. PGH-PGE isomerase then catalyzes the GSH-dependent formation of PGE_2 from PGH_2. PGH synthase has been purified and characterized (1,2). PGH-PGE isomerase activity in solubilized microsomal membranes from bovine and ovine vesicular gland has been only partially purified and characterized (3,4,5). Significant purification has not been achieved due to the instability of the enzyme.

Our laboratory is involved in the study of prostaglandin metabolism in renal collecting tubules. PGE_2 is the major prostaglandin released from these cells (6,7,8), and acts to inhibit arginine vasopressin-induced water resorption in the canine cortical collecting tubule (8). PGE_2 also modulates other renal tubular functions, and thus we were interested in studying the regulation of renal PGH-PGE isomerase. Anti-PGH-PGE isomerase monoclonal antibodies were prepared, so that we could purify and characterize this enzyme.

Monoclonal antibodies that precipitated PGH-PGE isomerase activity from solubilized sheep vesicular gland (SVG) microsomes were prepared by Dr. Yasuhito Tanaka, a former postdoctoral associate in our laboratory. Mice were immunized with a partially purified, solubilized SVG microsomal enzyme fraction, and the spleen cells were subsequently fused with SP2/O-Ag14 myeloma cells. Three hybridoma cell lines (*hei*-2, *hei*-7, and *hei*-26) which secreted antibodies that precipitated PGH-PGE isomerase activity were cloned. All three antibodies were of the mouse IgG_1 subclass, and are thus

designated IgG_1(*hei*-2), IgG_1(*hei*-7), and IgG_1(*hei*-26). The maximum amount of activity precipitated by each antibody out of the total solubilized PGH-PGE isomerase activity was: 10% by IgG_1(*hei*-2), 45% by IgG_1(*hei*-7), and 22% by IgG_1(*hei*-26) (5,9). The isomerase activities precipitated by IgG_1(*hei*-7) and IgG_1(*hei*-26) were additive.

Dr. Tanaka also determined some kinetic parameters of the immunoprecipitated PGH-PGE isomerase activities, as well as their cellular and subcellular distributions. The K_m for PGH_2 of the activities precipitated by IgG_1(*hei*-7), IgG_1(*hei*-2), and IgG_1(*hei*-26) were 40, 44, and 150 uM, respectively. Each of the immunoprecipated PGH-PGE isomerase activities required GSH for activity, but none of the precipitates showed GSH-S-transferase activity. Immunocytochemical staining of tissue sections revealed no co-localization of PGH-PGE isomerase and PGH synthase in the kidney or vasculature, but the enzymes were located together in the glandular epithelium of SVG. Furthermore, the time courses for PGE_2 formation of the activities precipitated by the three antibodies were different, and the cellular distribution of antigens reactive with the three antibodies was also different as determined by immunocytochemical staining of tissue sections. Immunoprecipitations using intact microsomes rather than solubilized microsomal proteins were used to determine the membrane orientation of the PGH-PGE isomerases. IgG_1(*hei*-2) and IgG_1(*hei*-7), like anti-PGH synthase, precipitated their respective enzyme activities from intact microsomes, indicating that the activities reactive with these antibodies are associated with the cytoplasmic surface of microsomal membranes. The isomerase activity associated with IgG_1(*hei*-26), however, was not precipitated when intact microsomes were used, indicating that the epitope reactive with IgG_1(*hei*-26) is not accessible to the antibody in intact microsomes.

The above data suggested that each of the three anti-PGH-PGE isomerase antibodies was directed against a different antigen. But further proof was needed to establish that the different antibodies were reacting with different proteins. Chapter II of this dissertation describes the characterization of the antigens reactive with IgG_1(*hei*-7) and

IgG_1(*hei*-26), and confirms that there are at least two distinct PGH-PGE isomerases in SVG microsomes. Chapter III describes some factors important for the stabilization of PGH-PGE isomerase activity in solubilized SVG microsomes, and Chapters IV and V deal with the isolation and characterization of the PGH-PGE isomerase reactive with IgG_1(*hei*-7). The role of GSH in the PGH-PGE isomerase reaction was examined using this enzyme.

CHAPTER I

LITERATURE REVIEW

Prostaglandins comprise a group of 20-carbon, oxygenated, unsaturated fatty acids that are synthesized in all mammalian tissues, and in many cells (10). They exert their biological effects through modulation of hormone action to affect various cellular functions. Prostaglandins are released from cells almost as rapidly as they are synthesized (11), but only very low concentrations of prostaglandins are found in the blood due to their rapid catabolism in the circulation (12,13). Therefore, prostaglandins are called autacoids, or local hormones, because they exert their physiological effects near their cells of origin.

Prostaglandins are formed when free fatty acids are released from cell membrane phosphoglycerides by cellular phospholipases. Phospholipase A_2 has an important role in the release of these fatty acids (14,15), and in some cells phospholipase C is also involved (16,17). The exact biochemical mechanism of arachidonate release is rather complex, and may vary with the type of cell or with the type of stimuli. The most common lipid precursor of prostaglandins in mammalian cells is all *cis*-5,8,11,14-eicosatetraenoic acid, more commonly known as arachidonic acid (AA), which is the precursor for the 2-series prostaglandins (PGE_2, PGD_2, PGI_2, $PGF_{2\text{-alpha}}$) and thromboxane (TxA_2). The subscript indicates the number of unsaturated double bonds in the prostaglandin side chains, and the letters indicate different substituents on the 5-membered ring. Arachidonic acid is released from its most common location, esterified at the *sn*-2 position of phosphoglycerides (14), when the cell

membrane is perturbed by a variety of hormonal (18,19,20), inflammatory or immunological (21,22), chemical (23), or physical (24) stimuli. Free arachidonate can be converted into prostaglandins and/or leukotrienes, or can be metabolized by cytochrome P-450 enzymes. The fate of free arachidonate is probably dictated by the original stimuli and the cell type.

The first step in the biosynthesis of prostaglandins involves the conversion of free arachidonic acid into prostaglandin H_2 (PGH_2) by PGH synthase (prostaglandin endoperoxide synthetase (EC 1.14.99.1)). PGH synthase contains two separate enzyme activities, a dioxygenase (cyclooxygenase) activity and a peroxidase activity (for review see 25,26) (Figure 1). The cyclooxygenase activity catalyzes the addition of molecular oxygen at C-11 and C-15 of arachidonic acid to form prostaglandin G_2 (PGG_2). The hydroperoxidase activity then catalyzes a two electron reduction at the 15-hydroperoxy group of PGG_2 to form the 15-hydroxy compound prostaglandin H_2 (PGH_2). PGH synthase is a membrane-bound protein, and has been purified and characterized (1,2). It requires one molecule of heme per subunit (M_r = 72,000) for maximal activity (1). The cyclooxygenase activity is inhibited by nonsteroidal anti-inflammatory drugs such as aspirin (27), thereby regulating subsequent prostaglandin synthesis.

The intermediates in prostaglandin biosynthesis, PGG_2 and PGH_2, were first isolated in 1973 (28,29). These endoperoxides are relatively unstable in aqueous solution, where the half-life for PGH_2 is only 5 min at pH 7.4 and 37°. PGG_2 and PGH_2 are themselves potent biological compounds, but when added to cells or tissues elicit the response characteristic of the prostaglandin formed by that tissue (30).

A particular cell type usually forms primarily one kind of prostaglandin from PGH_2, determined by which prostaglandin-forming enzyme is present in that cell (69). The enzymes that utilize PGH_2 as a substrate for prostaglandin synthesis are PGI_2 synthase, TxA_2 synthase, PGH-PGE isomerase, PGH-PGD isomerase, and $PGF_{2\text{-alpha}}$ reductase. All of these prostaglandin-forming enzymes are membrane-bound proteins

Figure 1. Arachidonic acid cascade in prostaglandin biosynthesis

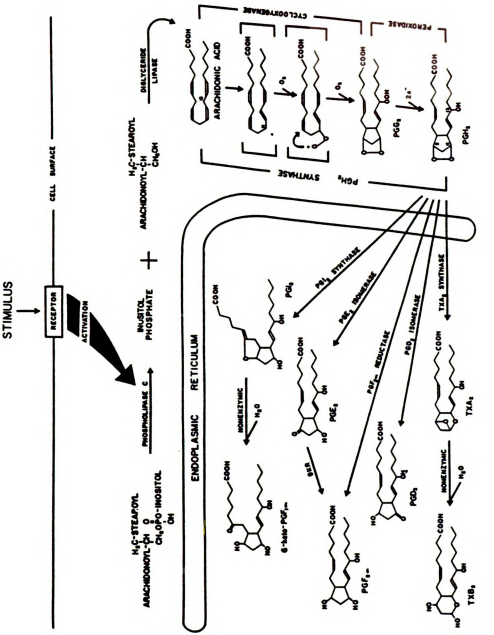

STIMULUS
RECEPTOR
ACTIVATION
CELL SURFACE
ENDOPLASMIC RETICULUM
PHOSPHOLIPASE C
INOSITOL PHOSPHATE
DIGLYCERIDE LIPASE
ARACHIDONIC ACID
CYCLOOXYGENASE
PEROXIDASE
PGH SYNTHASE
HOMEOSTHIC
6-keto-PGF1α
PGF SYNTHASE
PGD ISOMERASE
PGE ISOMERASE
PGI SYNTHASE
TXA SYNTHASE

Figure 1

with the exception of PGH-PGD isomerase, and possibly $PGF_{2\text{-alpha}}$ reductase; and all have been purified and characterized with the exception of PGH-PGE isomerase. PGI_2, or prostacyclin, is formed predominantly by endothelial cells and smooth muscle cells, and is a potent vasodilator and inhibitor of platelet aggregation in the vasculature (25). PGI_2 has been purified from bovine and porcine aorta (31,32). PGI_2 synthase requires heme for activity, and has a subunit molecular weight of 50,000 daltons. Thromboxane A_2 (TxA_2), the principal prostaglandin metabolite in platelets, is a potent vasoconstrictor, and causes platelet aggregation (25). TxA synthase has been purified from porcine platelets, and is also a hemoprotein (33). PGH-PGD isomerase purified from rat brain requires reduced GSH for activity, and has a subunit molecular weight of 80-85,000 daltons (34). PGH-PGD isomerase purified from rat spleen has no cofactor requirement, and has a subunit molecular weight of 26-34,000 daltons (35). Serum albumin also catalyzes the formation of PGD_2 from PGH_2 (36). PGH-PGE isomerase from bovine and ovine vesicular gland have been only partially purified, and show a requirement for reduced glutathione (GSH) for activity (3,4). Enzymatic formation of PGE_2 from $PGF_{2\text{-alpha}}$ (37), and $PGF_{2\text{-alpha}}$ from PGH_2 and PGD_2 (38) have been described. In addition to their enzymatic formation, PGE_2, PGD_2, and $PGF_{2\text{-alpha}}$ can be generated nonenzymatically from PGH_2. The endoperoxide can also be cleaved either enzymatically or nonenzymatically into malondialdehyde and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (39).

Thus there are three levels of enzymes involved in prostaglandin synthesis: 1) the phospholipases and other proteins involved in arachidonate release, 2) PGH synthase, and 3) the enzymes that form the different prostaglandins from PGH_2. Because of the very small amounts of nonesterified arachidonate found in cells, the release of arachidonic acid from cellular phospholipids is probably the major rate-limiting step in prostaglandin biosynthesis (14). However, the next step in the pathway may also play an important role in the regulation of prostaglandin production. The cyclooxygenase activity

has a continuous requirement for activator hydroperoxide which may provide another point of regulation (40,41). Both the cyclooxygenase and the hydroperoxidase activities of PGH synthase undergo self-catalyzed inactivation (42,43), a phenomena which may also be important in the regulation of prostaglandin synthesis *in vivo*. The prostaglandin-forming enzymes are usually thought of as constitutive enzymes. An increase or decrease in the formation of a particular prostaglandin can usually be traced back to divergence of PGH_2 through another pathway (44,45,100).

The biological effects of prostaglandins in many cases are mediated through increased cAMP in the cells (8,46,47). Prostaglandin receptors are coupled to activation of adenylate cyclase in many cells (48,49), but some prostaglandin actions are elicted independent of cAMP (50,51). Cellular receptors for many of the prostaglandins have been identified. Recently, a PGE receptor-N protein complex was isolated, suggesting that prostaglandin receptors may, in general, act through coupling to guanine nucleotide regulatory proteins (52). Prostaglandin receptors may provide another point of regulation in the cyclooxygenase pathway. There is evidence for the hormonal regulation of a variety of prostaglandin receptors (8,53,54).

Prostaglandins are rapidly metabolized to biologically inactive metabolites by both enzymatic and nonenzymatic prosesses. PGI_2 and TxA_2 are hydrolyzed nonenzymatically to the inactive compounds 6-keto-$PGF_{1\text{-alpha}}$ and TXB_2, respectively. The half-life of PGI_2 in aqueous solution at pH 7.4 and 24^o is 10 min (55), and the half-life of TxA_2 in aqueous solution at pH 7.4 and 37^o is 30 sec (25). Prostaglandins can be subjected to three types of modification by catabolizing enzymes. Prostaglandins that enter the circulation are rapidly inactivated by the action of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which converts the 15-hydroxyl function to a 15-keto group, followed by reduction of the 13,14 double bond by 15-ketoprostaglandin Δ^{13} reductase (13-PGR) (26,56). 15-PGDH and 13-PGR are found together in the lung and kidney. More than 90% of PGE and PGF in theblood can be converted to the

13,14-dihydro-15-keto product on a single passage through the lungs (56). 9-Hydroxyprostaglandin dehydrogenase can further modify these essentially inactive 13,14-dihydro-15-keto prostaglandins (57). Two other kinds of modifications of prostaglandins are β-oxidation which removes C1-C2 and C3-C4 of the side chain, and w-oxidation at C-19 or C-20 (56).

Prostaglandin E2. Prostaglandin E_2 (PGE_2) was one of the first prostaglandins identified and characterized. PGE_2 is synthesized from PGH_2 by the GSH-dependent action of PGH-PGE isomerase. PGE_2 is the major prostaglandin product of many tissues and cells. A wide variety of physiologically important functions are modulated by PGE_2. A few examples will be described.

Activated monocytes and macrophages secrete PGE_2 (58), which has immunomodulatory effects (59,60). A recent report on the effect of PGE_2 on *in vitro* human T-cell proliferation has demonstrated that PGE_2 supressed T-cell proliferation in a dose-dependent manner through a direct inhibitory effect on an early step of T-cell activation (61).

PGE_2 is the major prostaglandin formed by endothelial cells in the microvasculature (62). PGE_2 usually acts as a vasodilator in the vasculature (63), but can also act as a vasoconstrictor in some vascular beds, or at some concentrations. For example, 10^{-10} to 10^{-7} M PGE_2 caused relaxation in baboon cerebral artery strips, but caused contraction of the same tissue when the concentration of PGE_2 was greater than 10^{-7} M (64).

In the kidney, PGE_2 has a modulatory effect on various renal tubular functions when formed in response to a variety of hormonal stimuli, which include angiotensin II (65), kinins (66), and arginine-vasopressin (AVP) (67,68). Renal prostaglandin metabolism is compartmentalized so that prostaglandins can exert their various effects independently (69). PGE_2 is the major prostaglandin product formed in all three regions of the collecting tubule (6,7,8). PGE_2 is also formed by medullary interstitial cells

(18,70), thin descending limb cells (71), glomerular mesangial and epithelial cells (72), and by vascular endothelial cells and smooth muscle (62). Effects of PGE_2 in the kidney include: stimulation of renin release (69,73), inhibition of AVP-induced water resorption in the cortical collecting tubule (8,53), and inhibition of Na^+ resorption by collecting tubules, and thick ascending limb (69). PGE_2 exerts many of its effects in the kidney by affecting cAMP levels in the cells (8,46,47,69). Recently, receptors with different affinities for PGE_2 have been identified in the kidney (53). These PGE receptors may provide another level of regulation of prostaglandin metabolism in the kidney.

Enzymatic Formation of PGE2. There are two different kinds of proteins that can catalyze the formation of PGE_2 from PGH_2: 1) membrane-bound PGH-PGE isomerases that catalyzes the GSH-dependent conversion of PGH_2 into PGE_2; and 2) soluble glutathione-S-transferases that catalyze the formation of PGE_2 from PGH_2 in the presence of GSH, with or without the formation of other prostaglandins.

In the presence of GSH, GSH-S-transferases convert PGH_2 into $PGF_{2\text{-alpha}}$, PGD_2, and/or PGE_2, depending upon which GSH-S-transferase is used. Rat liver transferases B and D+E form PGE_2 predominately, and also some $PGF_{2\text{-alpha}}$ (74,75). At least some of the enzymatic formation of PGE_2 in bovine and pig lung is also thought to be due to the action of GSH-S-transferases (75). A soluble PGH-PGE isomerase from human brain was purified, and also shown to be a GSH-S-transferase (76). All of these transferase activities had several features in common. They were all soluble enzymes, they were all dimeric proteins with subunit molecular weights of 23,000 to 25,000, they all required GSH for activity, and they were all inhibited by 1-chloro-2,4-dinitrobenzene, a substrate for GSH-S-transferase.

When PGH_2 and GSH were incubated together for 15 min, and then acidified and extracted at 0^o with ether, all of the prostaglandins were found in the ether phase, indicating that no prostaglandin-GSH adduct was formed, or that it was too unstable to

be isolated (74,75).

A microsomal membrane activity has also been described that catalyzes the GSH-dependent conversion of PGH_2 into PGE_2 (3,4,77,78,79). This enzyme has been designated prostaglandin endoperoxide-E isomerase or PGH-PGE isomerase (EC 5.3.99.3). Reduced GSH was recognized as a cofactor required for optimal PGE synthesis in vesicular gland tissues as far back as 1967 (80,81). Nugteren *et al.* (82), and Yoshimoto *et al.* (83) reported that GSH oxidation was associated with PGE synthesis in microsomal preparations. However, microsomal PGH-PGE isomerase was not described as an enzyme activity distinct from PGH synthase until 1973 by Nugteren *et al.* (77), and in 1974 by Miyamoto *et al.* (78). Due to its extreme instability PGH-PGE isomerase has not yet been purified to homogeneity. Sheep vesicular glands (SVG) are probably the most abundant source of PGH-PGE isomerase, but the enzyme has also been identified in rabbit renal cortex (79), and perhaps in bovine coronary microsomes (84). Ogorochi *et al.* reported that most of the PGH-PGE isomerase activity in human brain homogenate was located in the particulate fraction (76), indicating perhaps another organ containing a microsomal PGH-PGE isomerase.

PGH-PGE isomerase activity in solubilized microsomal membranes from bovine and ovine vesicular gland has been partially purified and characterized (3,4,5). Glutathione has been designated as a coenzyme or cofactor in the isomerization of PGH by PGH-PGE isomerase (3,77,85). Ogino *et al.* performed a series of experiments using partially purified microsomal PGH-PGE isomerase from bovine vesicular gland (3). Their purification involved several chromatographic procedures using Tween 20-solubilized microsomal proteins to yield a 26-fold purification and a specific activity of 1.9 umol/min/mg. Most of their experiments were performed using PGH_1 as the substrate and measuring the formation of PGE_1, but they showed that the enzyme was equally capable of converting PGH_2 to PGE_2, and that PGE was essentially the only product of the enzymatic reaction. They reported a half-life of 30 min at 25^{o} for bovine

PGH-PGE isomerase activity in potassium phosphate buffer without any thiol, at pH 8.0. The addition of various thiol compounds, including GSH, partially protected PGH-PGE isomerase activity from inactivation during a 2 hr incubation at 25°, while the addition of oxidized thiols or the use of anaerobic conditions had no effect on the time course of inactivation. The addition of GSH did not restore lost enzyme activity. Ogino *et al.* also showed that GSH stimulated the enzymatic synthesis of PGE_1 in a dose-dependent manner, and that the addition of thiol compounds other than GSH would not support the enzymatic conversion of PGH_1 to PGE_1. They incubated the GSH-free enzyme with substrate for different amounts of time before adding GSH to initiate the reaction, and found that the reaction rates were identical. Thus the enzyme was not inactivated by interaction with the substrate, and the role of GSH was not merely to protect the enzyme. Tanaka reported that the SVG PGH-PGE isomerase reactive with IgG_1(*hei*-7) was inactived by a 30 sec preincubation with PGH_2 (86). Contrary to previous reports (82,83), Ogino *et al.* detected no oxidation of GSH during the course of the PGH-PGE isomerase reaction. Oxidation of GSH by the hydroperoxidase activity of PGH synthase was recently reported (87), and may account for the earlier observations of GSH oxidation during PGE formation when tissue homogenates were used. The addition of sulfhydryl reagents destroyed PGH-PGE isomerase activity even when 10 to 20-fold excess GSH was included in the activity assays (3). Inactivation of PGH-PGE isomerase activity by sulfhydryl reagents had also been reported by other investigators (75,79).

Moonen *et al.* attempted to purify a Triton X-100-solubilized microsomal PGH-PGE isomerase from sheep vesicular gland (4). They reported the half-life of the PGH-PGE isomerase activity to be about 20 hrs at 0°. Since their purification procedure involved several lengthy chromatographic procedures, the specific activity of the final preparation (1.4 umol/min/mg) was only slightly greater than the specific activity in the initial homogenate (1.0 umol/min/mg). They reported that their protein specifically required

GSH for activity, and the pH optimum for activity was 7.0. Analysis of their final preparation by SDS-PAGE showed the presence of two protein bands between 60,000 and 67,000 daltons, and some proteins between 10,000 and 30,000 daltons.

Sheep Vesicular Gland as a Source of PGH-PGE Isomerase. The discovery of prostaglandins can be traced back to observations by Kurzrok and Lieb that human semen could cause uterine contractions or relaxation in women (56). Treatment of strips of uterine tissue *in vitro* with semen caused the same responses. At about the same time Goldblatt (88), and von Euler (89) found that a lipid-soluble material in human seminal fluid and in the vesicular gland of sheep not only stimulated the contraction of smooth muscle, but also had the effect of lowering blood pressure when injected into animals. Von Euler named this lipid-soluble factor prostaglandin when he discovered that it was also present in the prostate gland (90). Prostaglandin research did not progress much further until better analytical techniques were developed for the isolation and identification of the compounds. In 1960, PGE_1 and $PGF_{1\text{-alpha}}$ were isolated from sheep vesicular glands (SVG) (91), and in 1963, prostaglandins E_1, E_2, E_3, $F_{1\text{-alpha}}$, and $F_{2\text{-alpha}}$ were identified in human seminal fluid (92). As recently as 1986, two novel PGE metabolites were identified in human seminal fluid (93). Sheep vesicular gland tissue (or ram seminal vesicles) is still considered to be the most bountiful source of PGH synthase and PGH-PGE isomerase.

Today, not much more is known about the physiological role of prostaglandins in seminal fluid. Human seminal vesicle secretions comprise about 60% of the total volume of semen (94). The high concentrations of prostaglandins in semen come from the seminal vesicles, but their function(s) is not known. Other components of semen that are contributed by the seminal vesicles are fructose, phosphorylcholine, ergothioneine, ascorbic acid, and flavins.

The Role of GSH in the PGH-PGE Isomerase Reaction. Eling *et al.* have investigated the effect of addition of GSH to sheep vesicular gland microsomes incubated with ^{3}H-arachidonic acid (87). Only PGE_2 was formed in the presence of 5 mM GSH. At GSH concentrations of <1 mM, both PGE_2 and 15-hydroperoxy-PGE_2 were observed. In the absence of GSH, 15-hydroperoxy-PGE_2 was the major reaction product, with some PGH_2, PGE_2, and PGD_2. 15-hydroperoxy-PGE_2 is the nonenzymatic decomposition product of PGG_2. They also reported that 15-hydroperoxy-PGE_2 is a good substrate for the hydroperoxidase activity of PGH synthase, so that *in vivo* PGE_2 may be obtained from the reduction of 15-hydroperoxy-PGE_2. Eling *et al.* also found that >1 mM GSH can serve as a reducing cofactor for the hydroperoxidase activity, while this same concentration of GSH significantly inhibits cyclooxygenase activity. Therefore, since intracellular GSH concentrations are usually in the mM range, cells containing PGH-PGE isomerase will form predominately PGE_2.

Glutathione exists in two forms: reduced, G-SH, and oxidized, GS-SG. Glutathione is a ubiquitous compound in cells, and is found at relatively high concentrations (0.4 to 12 mM) (95). The primary role of GSH in cells is probably to protect thiol groups in proteins. Glutathione also functions as a specific coenzyme in a small number of reactions. GSH is the coenzyme of the reaction catalyzed by Glyoxalase I (96,97). GSH forms an adduct nonenzymatically with methylglyoxal, and the conversion of this hemimercaptal into a thioester is catalyzed by Glyoxalase I. Glyoxalase II then catalyzes the hydrolysis of the thioester into D-lactic acid and GSH. GSH is also the coenzyme of formaldehyde dehydrogenase (98). A hemimercaptal, again, is the substrate of formaldehyde dehydrogenase, and a thioester is formed, which undergoes subsequent hydrolysis to produce formic acid and GSH. A third type of reaction in which GSH acts as a coenzyme are the *cis-trans* isomerizations catalyzed by maleylacetoacetate and maleylpyruvate isomerase in which GSH adds to the ethylene

group of the substrate (95,99). Reduced GSH has also been proposed to be the cofactor in several other reactions (121,122).

A mechanism to describe the role of GSH in the isomerization of PGH_2 to PGE_2 was proposed by Lands *et al.* (26,100) as being analogous to the role of GSH in the glyoxalase I reaction (Figure 2). In the glyoxalase I reaction GSH catalyzes an intramolecular 1,2-shift of a hydride ion to an adjacent carbonium ion, while in the PGH-PGE isomerase reaction the hydride shift would be to an oxonium ion.

Figure 2. Proposed mechanism for the role of GSH in the PGH-PGE isomerase reaction.

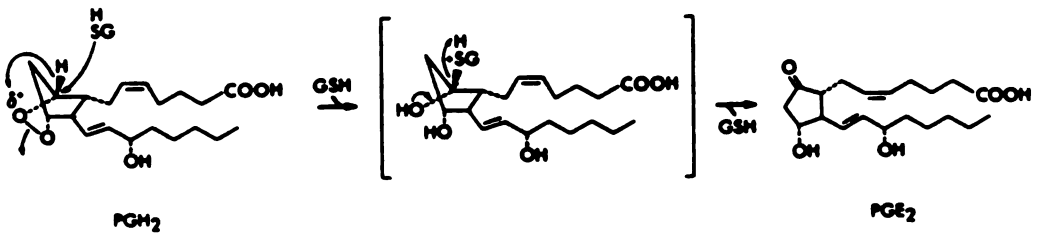


Figure 2

CHAPTER II

IDENTIFICATION OF AT LEAST TWO DIFFERENT PGH-PGE ISOMERASES FROM SHEEP VESICULAR GLAND MICROSOMES

Dr. Yasuhito Tanaka had isolated three monoclonal antibodies that precipitated PGH-PGE isomerase activity from solubilized sheep vesicular gland microsomes. These anti-PGH-PGE isomerase antibodies were designated IgG_1(*hei*-2), IgG_1(*hei*-7), and IgG_1(*hei*-26). Immunochemical and kinetic studies of the PGH-PGE isomerase activities precipitated by the three different monoclonal antibodies suggested that different enzymes were being precipitated by the different antibodies (5,9). The introduction to this dissertation summarizes the results of Dr. Tanaka that led to this conclusion. This chapter describes the identification of the protein antigens reactive with two of the monoclonal antibodies, IgG_1(*hei*-7) and IgG_1(*hei*-26). The data confirms that there are at least two distinct proteins with PGH-PGE isomerase activity in sheep vesicular gland microsomes.

MATERIALS AND METHODS

Materials. Sheep vesicular glands were obtained from a local slaughterhouse and stored at -80°. $Na^{125}I$ (100 mCi/ml) was purchased from Amersham. Bio Gel P-4, SDS-PAGE standards, gelatin, Trans Blot nitrocellulose paper, horseradish peroxidase (HRP)-labelled goat anti-mouse IgG, and 4-chloro-1-napthol were obtained from Bio-Rad. Rabbit anti-mouse IgG was obtained from Miles Laboratories, Inc. Acrylamide and N,N'-methylenebisacrylamide were from Aldrich Chemical Co., Inc. Triton X-100, leupeptin, and Bolton-Hunter Reagent (3-(*p*-hydroxy-phenyl)propionic acid N-hydroxysuccinimidyl ester) were from Calbiochem-Behring. Normal horse serum was from Flow Laboratories. Fetal bovine serum, Dulbecco's modified Eagle's medium containing D-glucose (DMEM), gentamycin, and L-glutamine were purchased from Grand Island Biological Company. NCTC 109 medium was from M.A. Bioproducts. Protein A-Sepharose CL-4B was from Pharmacia Fine Chemicals. Hypoxanthine, thymidine, reduced glutathione, dithiothreitol, Tween 20, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (Fraction V), and chloramine-T were obtained from Sigma Chemical Co. XRP-5 X-ray film and Comassie brilliant blue G250 were purchased from Eastman Kodak Company. The Cronex Lightning-Plus ^{125}I-intensifying screen was from Dupont.

Production of Monoclonal Antibodies. Hybridoma cell lines that secreted the desired monoclonal antibodies had been frozen in sealed vials in N_2. These cells were thawed and cultured in HT medium (450 ml DMEM (pH 7.4) containing 3.9 mg/l thymidine, 13.6 mg/l hypoxanthine, 2.0 ml/l gentamycin, 2 mM L-glutamine, 50 ml horse

serum, and 50 ml fetal bovine serum (FBS)). The cells were transferred to the same media containing IgG-free FBS and no horse serum for 8-10 days for the production of media containing monoclonal antibody.

IgG was removed to produce IgG-free FBS by chromatography on Protein-A Sepharose CL-4B. Serum was passed over the column maintained at 4°. The column was rejuvenated by washing with 0.1 M citric acid (pH 2.5), and the column was reequilibrated with 0.1 M sodium phosphate (pH 8.1). This procedure was repeated until no IgG could be eluted from the Protein A column in the pH 2.5 wash buffer.

Monoclonal antibodies were purified from the cell culture media by chromatography on Protein A-Sepharose CL-4B. Cell culture media (300-500 ml) was filtered (0.45 um), and diluted 5:1 with 0.5 M sodium phosphate (pH 8.2) containing 0.75 M NaCl. The media was then passed several times over a 5 ml column of Protein A-Sepharose which had been preequilibrated at 4° with 0.1 M sodium phosphate (pH 8.2). The column was then washed with 0.1 M sodium phosphate (pH 8.1) containing 0.15 M NaCl until the absorbance at 280 nm was less than 0.05. IgG_1 was eluted from the Protein A with 0.1 M citric acid (pH 4.0). Fractions of 3.0 ml were collected into 1.0 ml of 1 M Tris (pH 8.0), and the fractions containing antibody were detected by measuring the absorbance at 280 nm. Fractions containing antibody were pooled and neutralized immediately, and dialyzed against 0.05
M sodium phosphate (pH 8.0) at 4°. Aliquots were then freeze-dried and stored at -20°. A typical yield of antibody (depending upon the particular hybridoma) was 30-50 ug/ml cell culture media.

Preparation of Solubilized Sheep Vesicular Gland Microsomes. Frozen sheep vesicular glands (SVG) (24 g) were slightly thawed and diced with a razor blade. The tissue was added to 60 ml of ice-cold 0.05 M Tris-chloride (pH 8.0) containing 0.5 mM EDTA, 0.5 mM GSH, 0.05 mM DTT, and 20% (v/v) ethylene glycol, and then

homogenized using a Brinkman Polytron homogenizer. All steps during the microsome preparation were carried out on ice or at 2-4°. The homogenate was centrifuged at 12,000 x g for 15 min, and the supernatant filtered through eight layers of cheesecloth. This supernatant was then centrifuged at 200,000 x g for 30 min. The supernatants were discarded, and the microsomal pellets resuspended using a glass-teflon homogenizer in 63 ml of the above buffer containing 1.5% (w/v) Triton X-100. This solution was incubated on ice for 30 min. The solubilized microsomal proteins were then obtained as the supernatant after centrifugation at 200,000 x g for 40 min.

Protein Assays. Protein concentrations of the Triton X-100-solubilized microsomes were determined using the micro method of Bradford (101), and were typically 3-4 mg/ml.

Iodination of Triton X-100-Solubilized Microsomes. SVG microsomes were prepared as described above, but solubilized with 1.5% Triton X-100 in 0.05 M sodium phosphate (pH 8.0), instead of Tris buffer. Bolton-Hunter reagent was labelled with $Na^{125}I$ as described previously (102). Solubilized microsomes (80 ug in 67 ul) were added to a tube containing 0.8 mCi Bolton-Hunter reagent, mixed, and incubated on ice for 20 min. The reaction was quenched by the addition of 0.2 M glycine, and the ^{125}I-labelled proteins separated by chromatography on Bio-Gel P4 (1 x 4 cm) in 0.05 M sodium phosphate (pH 7.5) containing 1 mM GSH, 0.5% Triton X-100, and 0.25% gelatin.

Immunoprecipitation of Iodinated Microsomal Proteins. All procedures were performed at room temperature. ^{125}I-labelled microsomes (100 ul, 15 ug, 4.5×10^5 cpm/ug) were incubated with 25 ul (100 ug) of purified monoclonal antibody for 30 min. Rabbit anti-mouse IgG (550 ug, 100 ul) was added, and the tubes were incubated again for

30 min. The final buffer composition of these incubations was 0.05 M sodium phosphate (pH 8.0) containing 1 mM GSH, 0.5% Triton X-100, and 0.25% gelatin. The precipitates were collected by centrifugation, and washed two times with the above buffer. The pellets were resuspended, transferred to new tubes, and washed three more times with buffer. The pellets were resuspended in SDS-PAGE sample buffer (0.125 M Tris (pH 6.8) containing 4% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 0.003% bromphenol blue), and heated at 100° for 5 min. One-half of each sample was subjected to electrophoresis on a discontinuous 15% SDS-polyacrylamide gel according to the procedure of Laemmli (103). Proteins on the gel were visualized using the silver stain method of Wray *et al.* (104). An autoradiogram was obtained by incubation of the gel with Kodak XRP-5 X-ray film, and a Dupont Lightning-Plus intensifying screen for 7 hrs at -80°.

Western Transfer/Immunoblotting of Solubilized Microsomal Proteins. The method described here is a modification of that of Towbin, *et al.* (105). All procedures were performed at room temperature. Solubilized SVG microsomes were prepared as described above, but with the addition of 1.0 mM PMSF and 1 ug/ml leupeptin. Solubilized microsomes were incubated for 40 min in SDS-PAGE sample buffer (1:1), and then 250 ug protein/lane was subjected to electrophoresis on a discontinuous 9% SDS-polyacrylamide mini-gel (1.5 mm thick) using a "Mighty Small" Vertical Slab Gel Unit from Hoefer Scientific Instruments. Proteins on the gel were transferred electrophoretically at 400 mA for 100 min to 0.45 um nitrocellulose paper. The transfer buffer consisted of 25 mM Tris base containing 0.192 M glycine, and 20% methanol. Molecular weight standards were visualized on the nitrocellulose by staining with a 5% solution of India ink in PBS (pH 7.2) containing 0.5% Tween 20. The nitrocellulose was blocked by incubation with 5% BSA in Tris-buffered saline (TBS) (20 mM Tris-chloride (pH 7.5) containing 500 mM NaCl) for 12 hrs. Strips of nitrocellulose containing the

microsomal proteins were incubated for 5 hrs in cell culture media containing the monoclonal antibodies. These media had been adjusted to pH 7.3 by a 5:1 dilution with 0.5 M HEPES (pH 7.3) containing 2.5 M NaCl. The nitrocellulose strips were washed 4 times, 15 min each, with 0.01% Tween 20 in TBS, and then incubated with HRP-labelled, affinity purified goat anti-mouse IgG (1/2500 in TBS) for 1 hr. The nitrocellulose strips were then washed as before, followed by one additional wash in TBS alone. The location of the second antibody on the nitrocellulose was visualized by incubation for 10-20 min in 0.05% 4-chloro-1-naphthol in TBS containing 0.015% H_2O_2.

In the second immunoblot experiment, 50 ul of Protein A-agarose was incubated for 35 min with 0.75 mg of purified monoclonal antibody in 0.05 M sodium phosphate (pH 8.1). The pellets were then washed 4 times with 1 ml of 0.05 M sodium phosphate (pH 8.1). A volume of Protein A-agarose containing 0.5 mg of antibody was incubated with 1.1 mg of solubilized SVG microsomal protein (4.45 mg/ml) for 40 min at 4^{o} with shaking. Proteins in 25 ul aliquots of the supernatants were separated on a 9% SDS-polyacrylamide mini-gel, transferred to nitrocellulose, and blotted as described above.

RESULTS

Characterization of the SVG Microsomal Protein Antigen Reactive with IgG1(*hei*-7). The antigen reactive with IgG_1(*hei*-7) was identified as a protein with M_r = 17,500. In this experiment the different monoclonal antibodies were incubated with ^{125}I-labelled, solubilized SVG microsomes, and the conjugates were precipitated by the addition of an anti-mouse IgG second antibody. The immunoprecipitate from IgG_1(*hei*-7) contained a unique protein with M_r = 17,500, when analyzed by SDS-PAGE and autoradiography (Figure 3, lanes 2 and 3). The immunoprecipitate using an antibody against PGH synthase, IgG_1(*cyo*-3), contained only one protein with M_r = 72,500 (lane 4), as expected from its previous characterization (2). A control antibody, IgG_1(*day*-1), which does not react with any SVG protein, precipitated no radioactive proteins. A faint band with M_r = 47,200 was present in all the immunoprecipitates, and can probably be attributed to free ^{125}I that was bound to the heavy chain of IgG. Using the same method, immunoprecipitations with IgG_1(*hei*-2) and IgG_1(*hei*-26) usually failed to precipitate any ^{125}I-labelled proteins.

Characterization of the SVG Microsomal Protein Antigen Reactive with IgG1(*hei*-26). The antigen reactive with IgG_1(*hei*-26) was identified as a protein with M_r = 180,000 by a Western transfer/immunoblot procedure. Solubilized SVG microsomal proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose. Nitrocellulose strips were then incubated with the various antibodies, and binding of antibody to an antigen on the nitrocellulose was visualized using

horesradish-peroxidase anti-mouse IgG. The nitrocellulose strip incubated with IgG_1(*hei*-26) contained a unique protein band with M_r = 180,000 (Figure 4, lane 2). No protein bands were observed when using the control antibody IgG_1(*day*-1) (lane 1), IgG_1(*hei*-2), IgG_1(*hei*-7), or IgG_1(*cyo*-3).

Further Verification that IgG1(*hei*-7) and IgG1(*hei*-26) are Directed Against Unique Antigens. Aliquots of solubilized microsomal proteins were precipitated with excess IgG_1(*hei*-2), IgG_1(*hei*-7), or IgG_1(*hei*-26) that was immobilized on Protein A-agarose, and the resulting supernatants were analyzed by Western transfer/immunoblotting for reactivity with IgG_1(*hei*-26). When the microsomes had been precipitated with IgG_1(*hei*-26), the previously identified band with M_r = 180,000 was no longer observed; however, it was still present when the microsomes had been precipitated first with either IgG_1(*hei*-2) or IgG_1(*hei*-7). This experiment further supports the conclusion that IgG_1(*hei*-7) and IgG_1(*hei*-26) are directed against unique antigens. The data is not shown here. There were light bands of background staining in all the lanes containing microsomal proteins that had been incubated with antibodies immobilized on Protein A-agarose. The background bands may have been due to elution of some Staph A proteins from the Protein A-agarose. The IgG_1(*hei*-26)-reactive protein (M_r = 180,000) always stained much more intensely than these background bands.

IgG_1(*hei*-2) appeared to react specifically with more than one protein according to results obtained with both immunoprecipitation and immunoaffinity chromatography followed by SDS-PAGE. Since this antibody reacts with no more than 10% of the total PGH-PGE isomerase activity of solubilized SVG microsomes, further characterization of the antigen(s) reactive with IgG_1(*hei*-2) was discontinued.

Figure 3. Immunoprecipitation of iodinated, solubilized SVG microsomes with IgG1(*hei* -7). Iodinated microsomes (lane 1) (15 ug, 4.5 x 10^5 cpm/ug) were incubated with 100 ug of IgG1(*hei* -7) (lanes 2 and 3), or IgG1(*cyo*-3) (lane 4), and the complexes precipitated by the addition of 550 ug of anti-mouse IgG. Labelled proteins were separated by SDS-PAGE on a 15% acrylamide gel from which the autoradiogram was obtained.

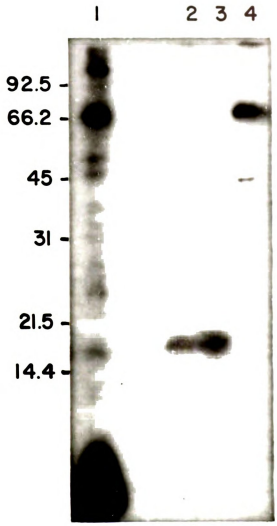


Figure 3

Figure 4. Western transfer blotting of solubilized SVG microsomes with IgG1(*hei* -26). Microsomal proteins were separated on a 9% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose. Nitrocellulose strips were incubated with a control antibody IgG1(*day* -1) (lane 1), or with IgG1(*hei* -26) (lane 2) for 5 hrs at 24°. Location of the antigen-antibody complex was visualized by incubation with HRP-goat anti-mouse IgG. Details of the methodology are presented in the text.

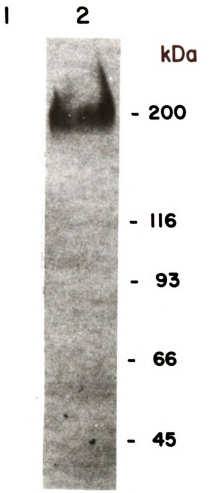


Figure 4

DISCUSSION

The data presented in this chapter identifies the antigens that react with the anti-PGH-PGE isomerase antibodies, IgG_1(*hei*-7) and IgG_1(*hei*-26), as proteins having subunit molecular weights of 17,500 and 180,000 daltons, respectively. The protein antigen of IgG_1(*hei*-7) was identified by immunoprecipitation of ^{125}I-labelled, solubilized SVG microsomal proteins, followed by SDS-PAGE and autoradiography of the precipitated proteins. The protein antigen of IgG_1(*hei*-26) could not be identified by this method, but was identified by Western transfer/immunoblotting. Bolton-Hunter reagent reacts with primary amino groups of proteins, with most of the ^{125}I being incorporated into lysine residues. Some polypeptides do not react as well as others with Bolton-Hunter reagent, perhaps due to hindered or inaccessible lysine residues in the proteins (107). The hydrophobic, membrane-bound IgG_1(*hei*-26)-reactive protein may be buried in the Triton X-100 micelles, and relatively inaccessible to the iodination procedure. Alternatively, it may not have been immunoprecipitated in these experiments. The antigen reactive with IgG_1(*hei*-26) was identified by Western transfer/immunoblotting, whereas IgG_1(*hei*-7) failed to react with any proteins using this procedure. Some monoclonal antibodies react only with their nondenatured antigen, and therefore cannot be used for Western blotting. Thus, two different methods were needed to identify the two different antigens.

This evidence that IgG_1(*hei*-7) and IgG_1(*hei*-26) react with unique antigens, coupled with the kinetic and antigen localization data obtained by Dr. Tanaka indicate that there are at least two different proteins in SVG microsomes that catalyze the GSH-dependent formation of PGE_2 from PGH_2. These two PGH-PGE isomerases are the proteins reactive with IgG_1(*hei*-7) and IgG_1(*hei*-26). Since the two antibodies together

precipitate only 60-70% of the total PGH-PGE isomerase activity from SVG microsomes, at least one more PGH-PGE isomerase may exist.

The kinetic and antigen localization data obtained by Dr. Tanaka also suggested that the PGH-PGE isomerase that reacts with IgG_1(*hei*-7) has the biological properties necessary for an important PGH-PGE isomerase *in vivo*. This protein is responsible for at least 40% of the total PGH-PGE isomerase activity in solubilized SVG microsomal proteins. The Km of about 50 uM for PGH_2 of the IgG_1(*hei*-7)-reactive PGH-PGE isomerase is similar to that of other prostaglandin-forming enzymes, andwould therefore favor PGE_2 formation in vivo. The facts that the protein reactive with IgG_1(*hei*-7) is a membrane-bound protein with a subunit molecular weight of 17,500 daltons, and that no GSH-S-transferase activity could be measured using 1-chloro-2,4-dinitrophenol, indicate that the protein precipitated by IgG_1(*hei*-7) is not a GSH-S-transferase. Also, the PGH-PGE isomerase reactive with IgG_1(*hei*-7) is co-localized both cellularly and subcellularly with PGH synthase, the enzyme that forms the substrate, PGH_2, for PGH-PGE isomerase.

The data presented in the remainder of this dissertation describes my progress toward the purification and characterization of the PGH-PGE isomerase reactive with IgG_1(*hei*-7).

CHAPTER III

SOLUBILIZATION AND STABILIZATION OF PGH-PGE ISOMERASE ACTIVITY IN SHEEP VESICULAR GLAND MICROSOMES

Microsomal PGH-PGE isomerase was initially solubilized with 1.5% Triton X-100 in Tris buffer containing EDTA and GSH (5,9). PGH-PGE isomerase activity prepared in this manner was very unstable, and almost all of the isomerase activity was lost after a three hour incubation on ice. In this chapter, the factors important for the stabilization of PGH-PGE isomerase activity in solubilized sheep vesicular gland microsomes are examined.

Several pieces of evidence initially suggested that cyclooxygenase might be copurifying with the IgG_1(*hei*-7)-reactive PGH-PGE isomerase in 10 mM CHAPS-solubilized microsomes. Sequential antibody precipitations of the solubilized microsomal proteins showed that this was not the case.

Purification of PGH-PGE isomerase by immunoaffinity chromatography has not been successful.

MATERIALS AND METHODS

Materials. CHAPS, Triton X-100, and ethylene glycol were from Pierce Chemical Co. Arachidonic acid, PGE_2, $PGF_{2\text{-alpha}}$, TxB_2, 6-keto-$PGF_{1\text{-alpha}}$, and PGD_2 were purchased from Cayman Chemical Co. Silica gel 60 thin-layer chromatography plates were from E.Merck. [5,6,8,9,11,12,14,15-^{3}H]-Arachidonic acid (100 Ci/mmol) was from Amersham. Protein A-AffiGel 10 (Protein A-agarose), AffiGel 10, and the Protein A-MAPS II kit were obtained from Bio-Rad. Reduced glutathione, dithiothreitol, 2-mercaptoethanol, and acetylsalicylic acid (aspirin) were obtained from Sigma Chemical Co. Flufenamic acid was purchased from Aldrich Chemical Company, Inc. Nutridoma-SP was purchased from Boehringer Mannheim Biochemicals. Flurbiprofen was a gift from Dr. Udo Axen of The Upjohn Company. All other reagents were described in Chapter II, or were from common commercial sources.

Monoclonal Antibody Production. The procedures were as described in Chapter II with two exceptions. Hybridoma cells were cultured in serum-free medium containing 1% Nutridoma-SP for monoclonal antibody production. Monoclonal antibodies were purified by chromatography on Protein A-agarose using the Bio-Rad Protein A-MAPS II buffers.

Preparation of Solubilized SVG Microsomes. The preparation of microsomes solubilized with Triton X-100 or CHAPS was as described in Chapter II, except for the buffer composition. For these experiments, SVG tissue was homogenized in microsomal preparation buffer (MPB) (0.05 mM Tris-chloride (pH 8.0) containing 1 mM GSH, 1 mM

EDTA, 0.5 mM PMSF, and 1 ug/ml leupeptin). The 3 x 10^6 g x min microsomal pellets were solubilized in MPB containing either 1.5% Triton X-100 or 10-20 mM CHAPS. MPB containing 10 mM CHAPS has been designated microsomal solubilization buffer (MSB). Solubilized microsomes were stored in small aliquots at -80°, and used in experiments over a period of several months. The protein concentration of the solubilized microsomes was typically 3 - 4.5 mg/ml for the Triton X-100-solubilized microsomes, and 2 - 3 mg/ml for microsomes solubilized using 10 mM CHAPS.

Assay of PGH-PGE Isomerase Activity. Tubes containing usually 4-12 ug of microsomal protein were brought to 50 ul with ice cold MSB. Assay buffer (50 ul; 0.2 M Tris-chloride (pH 8.0) containing 2 mM GSH, and 1.4% Triton X-100) was added to a tube, the tube vortexed, and then incubated for two minutes in a room temperature water bath. The reaction was initiated by the addition of 3-5 ul of ^{3}H-PGH_2 (0.27 or 0.30 nmol/ul in acetone), and the tube continuously mixed for the reaction time (15-30 sec). The reaction was stopped by the addition of an amount of ice-cold 0.2 M citric acid sufficient to bring the solution to pH 3.5. The acidified solution was then extracted two times with 2 volumes of ice-cold ethyl acetate. The pooled ethyl acetate extracts were evaporated under nitrogen, the sample resuspended in 40 ul of acetone, and 25 ul was applied to a 2 cm-wide lane on a Silica Gel 60 thin-layer chromatography (TLC) plate. PGE_2 standard (2 ul in acetone, 10 mg/ml) was applied to each sample lane, so that the location of ^{3}H-PGE_2 could be determined. TLC plates were developed two times at room temperature in 100 ml of the solvent system: chloroform/ ethyl acetate/ methanol/ citric acid/ water (140/60/16/2/1, v/v/v/v/v), and the plates were placed in a tank of iodine vapor to visualize the location of the PGE_2 standards. Each TLC plate lane was divided into three sections: the section containing the PGE_2 standard, and the sections above and below the PGE_2 standards. Each section was scraped into a scintillation vial, 14 ml of Safety Solve scintillation fluid added, and the radioactivity was determined. The percent of the

total radioactivity corresponding to PGE_2 was calculated, and converted into nmol PGE_2 formed by multiplying by the nmol ^3H-PGH_2 added to the reaction. PGE_2 formation in a tube containing everything except enzyme was subtracted from each enzymatic reaction, and was typically 4-7% of the total radioactivity added to the reaction.

Assay of Cyclooxygenase Activity. Cyclooxygenase activity was measured as nmol O_2 consumed/min using a Gilson oxygen electrode as described previously (106).

Immunoprecipitations Using Antibody Bound to Protein A-Agarose. Aliquots of Protein A-agarose (100-125 ul) were placed in 5 ml polypropylene columns and washed with 8-10 volumes of 0.05 M Tris-chloride (pH 8.2) containing 0.15 M NaCl (Tris/NaCl buffer). Antibody was bound to the Protein A by adding 0-200 ug of antibody per 100 ul of gel (0.5 mg/ml in Tris/NaCl buffer), and incubating at room temperature for 30 min with shaking. The columns were washed with 15 volumes of Tris/NaCl buffer, and then with 3 volumes of Tris/NaCl buffer containing 5% BSA, and then again with 15 volumes of Tris/NaCl buffer. At this point, the columns were transferred to the cold room (4^{o}), where all subsequent operations were performed. The Protein A-antibody columns were washed with 10 volumes of microsomal solubilization buffer containing 0.15 M NaCl (MSB+NaCl). Solubilized sheep vesicular gland microsomal proteins (0.4 mg/ml in MSB, 80 ug added per 100 ul gel) were incubated with the antibody-Protein A-agarose for 30 min, with shaking every 5 min. For nonenzymatic controls, buffer was added to the antibody-Protein A instead of microsomal protein. The columns were washed with 15 volumes of MSB+NaCl, and then with 8 volumes of buffer A or B. Samples for the cyclooxygenase activity assay (75-90 ul gel/assay) were washed and resuspended in buffer A (0.1 M Tris-chloride (pH 8.05) containing 1 mM phenol). Samples for the PGH-PGE isomerase activity assay (15-25 ul gel/assay) were washed and resuspended in buffer B (0.1 M Tris-chloride (pH 8.05) containing 2 mM GSH).

Preparation of ^{3}H-PGH2. ^{3}H-PGH$_2$ (84,700 cpm/nmol/3.3 ul, n=3) was synthesized from 250 uCi [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (100 Ci/mmol) and 1 mg arachidonic acid as described previously (32). The purity as analyzed by TLC was always greater than 90%, and the yields were 25-35%.

Immunoaffinity Chromatography. IgG$_1$(*hei*-7) and other antibodies were coupled to AffiGel 10 (1 to 15 mg antibody/ml gel) according to the manufacturers instructions. Antibody-AffiGel 10 was stored at 4^o in PBS containing 0.05% sodium azide until used in the experiments. For the purification of PGH-PGE isomerase, columns of antibody-AffiGel 10 were washed with 10-15 volumes of MPB. Triton X-100 or CHAPS were included in the buffers as indicated in the Results. Solubilized microsomal proteins were then incubated with antibody-AffiGel 10 for 40 min at 4^o with shaking. The gels were washed with 15-20 volumes of MPB containing 0.15 M NaCl, and then either resuspended and aliquots removed for enzyme activity assays; or the columns were eluted with 0.2 M citric acid (pH 2.6) containing 0.15 M NaCl and Triton X-100 or CHAPS, and the eluants were analyzed by SDS-PAGE.

RESULTS

Modifications Made in the PGH-PGE Isomerase Activity Assay. The PGH-PGE isomerase activity assay described in the Methods section of this chapter is a modification of the assay described by Tanaka *et al.*(5). Problems encountered with that assay included carry-over of some of the aqueous phase or detergent into the organic phase, and streaking of the samples and PGE_2 standards on the TLC plates. Extraction of the acidified samples with ethyl acetate instead of diethyl ether/methanol (100/3.2, v/v) resolved the problem, and also improved the recovery of ^3H-PGE_2 standard from 74% to 84% (from aqueous buffer containing 13 ug of microsomal protein). Ethylene glycol in the microsomal buffers did not cause a problem with TLC as long as it comprised <10% of the aqueous phase.

Elimination of $FeCl_2$ from the stop solution also increased the recovery of ^3H-compounds extracted from the aqueous solutions. It had been proposed that $FeCl_2$ degraded PGH_2, and would therefore stop reactions in which PGH_2 was the substrate. An experiment to test this proposal showed that PGH-PGE isomerase activity assays stopped by the addition of $FeCl_2$ contained only slightly less ^3H migrating with PGH_2 than a reaction stopped by acidification alone (Figure 5). In this experiment, the location of ^3H-PGH_2 on the TLC plate was estimated by the migration PGA_2, and corresponds to the area containing the largest % of radioactivity in the lane in which ^3H-PGH_2 was analyzed. The addition of $FeCl_2$ slightly increased the amount of ^3H migrating with PGE_2 and at the origin. An alternative explanation would be that $FeCl_2$ caused an increase in the nonenzymatic formation of $PGF_{2\text{-alpha}}$, and a decrease in the nonenzymatic formation of PGD_2. Regardless, PGH-PGE isomerase activity assays are stopped by acidification using only ice-cold citric

Figure 5. Analysis of the different methods for stopping PGH-PGE isomerase activity assays. A-D each represent a lane of a TLC plate divided into sections 1-8. Section 1 was at the origin, section 3 contained PGE2 standard, and section 5 contained PGA2 which migrates with PGH2 (PGF2-alpha would be found in section 2, and PGD2 in section 4). Different samples were separated on lanes A-D, and the percent of the total radioactivity migrating in each section of that lane was calculated. The samples were: A) 5 ul ^{3}H-PGH2; B) Microsomal reaction products from a reaction stopped by acidification; C) Microsomal reaction products from a reaction stopped the addition of 6 mg/ml FeCl2 and then acidified; and D) Microsomal reaction products from a reaction stopped by the simultaneous addition of 6 mg/ml FeCl2 and acidification.

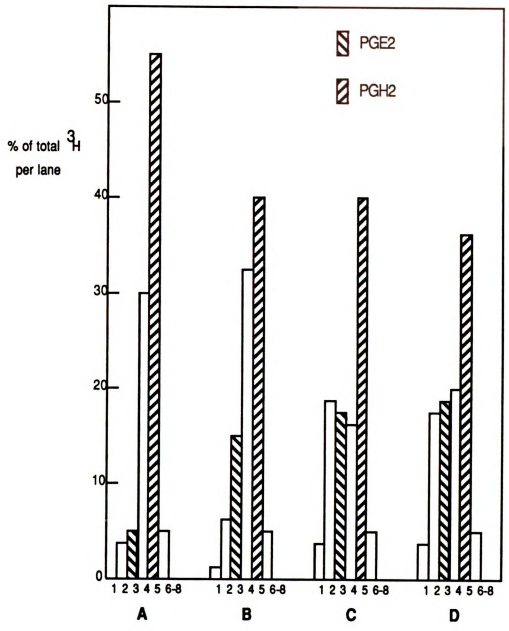


Figure 5

ice-cold ethyl acetate.

It was verified that PGE_2 was completely separated from other prostaglandins by TLC using the solvent system chloroform/ ethyl acetate/ methanol/ acetic acid/ water (140/60/16/2/1, v/v/v/v/v). Better separation of prostaglandin standards was achieved when the TLC tanks were not preequilibrated, when no paper was used to line the tanks, and when the plates were developed two times to the top in the same solvent.

Flurbiprofen (FBP) was originally included in the PGH-PGE isomerase activity assay buffer to inhibit endogenous microsomal formation of PGH_2. However, it was discovered that PGE_2 formation by microsomes assayed in the presence of 0.1 mM FBP was decreased by 47%, so FBP was eliminated from the PGH-PGE isomerase activity assay buffer. Microsomal proteins used in these activity assays are the solubilized fraction only, with most of the lipid removed at several steps during the microsome preparation, and so endogenous formation of PGH_2 is probably insignificant.

CHAPS cannot replace Triton X-100 in the PGH-PGE isomerase activity assay buffer. Enzyme activity is greatly reduced, and product formation does not increase with the amount of enzyme when 10 mM CHAPS is substituted for Triton X-100 in the PGH-PGE isomerase activity assay buffer.

Stabilization of PGH-PGE Isomerase Activity in Solubilized SVG Microsomes. Microsomes were prepared in 0.05 M Tris-chloride buffer (pH 8.0) containing 0.5 mM GSH, 1.0 mM EDTA, and 20% ethylene glycol, and then solubilized in the same buffer containing 1.5% Triton X-100. PGH-PGE isomerase activity in these Triton X-100-solubilized microsomes was very unstable. Less than 25% of the initial isomerase activity remained after a 3 hr incubation of the solubilized microsomal proteins on ice. All of the buffer components were tested for their effect on the activity and stability of PGH-PGE isomerase. Substitution of the original Triton X-100 and ethylene glycol with peroxide-free Triton X-100 and ultra-pure ethylene glycol did not alter PGH-PGE

isomerase activity or stability. Addition of protease inhibitors, 0.5 mM PMSF and 1 ug/ml leupeptin, to the microsomal preparation buffers did not inhibit PGH-PGE isomerase activity nor alter the amount of activity solubilized, so they have been included in all subsequent microsomal preparations. Deionized water had already been used for buffer preparation and to rinse all glassware, and EDTA had been included in the buffers. The addition of 5 mM EDTA to a microsomal sample inhibited PGH-PGE isomerase activity by 48%, so the amount of EDTA added to the microsomal preparation buffers was reduced from 1 to 0.5 mM. EDTA was still added to the buffers not just to protect the enzyme, but also to slow down the oxidation of GSH. If the loss in activity caused by EDTA were due to removal of a metal from the enzyme, DTT would probably also have the same effect. The addition of up to 10 mM dithiothreitol (DTT) and 25 mM 2-mercaptoethanol (2-ME) to solubilized microsomes did not cause any decrease in PGE_2 formation over a 5 hr incubation of the enzyme on ice. There was, however, the formation of an additional unknown product that migrated below PGE_2 on TLC plates in reactions containing DTT or 2-ME. In the case of DTT, the additional product may have been $PGF_{2\partial}$, which has been shown to form nonenzymatically from PGH_2 in the presence of dithiols and Cu^{+2} (100). Since DTT and 2-ME do not enhance the activity or stability of PGH-PGE isomerase, and do cause formation of this unknown compound, they were not added to the enzyme buffers. To test the effect of Tris buffer on enzyme activity, microsomes were prepared and solubilized in four different buffers: Tris-chloride, tricine, HEPES, and glycylglycine. PGH-PGE isomerase activity in microsomes prepared in tricine buffer was 72%, in HEPES buffer 137%, and in glycylglycine 181% relative to the activity in microsomes prepared in Tris buffer. Thus, the enzyme activity in Tris buffer is not extremely different from that in other buffers. None of the buffer components seemed to be responsible for the observed instability of PGH-PGE isomerase activity in the SVG microsomes solubilized with 1.5% Triton X-100.

Solubilization of PGH-PGE isomerase activity from SVG microsomes had already

been compared using Triton X-100, Lubrol, and Tween 20 detergents (5). However, the enzyme had never been solubilized in a zwitterionic detergent, like CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate) (CMC = 8 mM), which has been shown to solubilize some membrane-bound proteins without denaturation (108,109). Identical microsomal pellets were solubilized in either 1.5% Triton X-100, or 12 or 20 mM CHAPS, and PGH-PGE isomerase activity and stability in these preparations was compared (Table 1). Essentially no PGH-PGE isomerase activity remained after a 6.5 hr incubation on ice in the microsomes solubilized with Triton X-100, while both CHAPS-solubilized preparations retained at least 30% of their initial isomerase activity. A greater amount of PGH-PGE isomerase activity was solubilized using 20 mM CHAPS, but the isomerase activity solubilized in 12 mM CHAPS was more stable during an overnight incubation of the solubilized proteins on ice. A refreeze/thaw step inactivated all of the PGH-PGE isomerase activity in the Triton X-100 microsomes, but some activity remained in the CHAPS-solubilized microsomes. PGH-PGE isomerase activity solubilized with 10 mM CHAPS was found to be even more stable than that solubilized with 12 mM CHAPS. In fact, up to 100% of the isomerase activity remained in an overnight incubation of 10 mM CHAPS-solubilized microsomes on ice when fresh GSH and PMSF were added at zero and 10 hrs. The percent of the total PGH-PGE isomerase activity precipitated by IgG_1(*hei*-7) was about 40-45% when either Triton X-100 or 10 mM CHAPS were used to solubilized the microsomal proteins.

Up to this point, all the microsomal preparations had contained 20% ethylene glycol which was added to increase the stability of PGH-PGE isomerase. However, ethylene glycol appeared to inhibit the binding of PGH-PGE isomerase to immunoaffinity columns (IgG_1(*hei*-7)-AffiGel 10) (see page 58). The effect on the stability of PGH-PGE isomerase activity of eliminating ethylene glycol and/or GSH from the 10 mM CHAPS-solubilized microsomes, and the effect of adding IgG_1(*hei*-7) was determined Table 2). Ethylene glycol had little effect on PGH-PGE isomerase activity in freshly

Table 1. Relative stabilities of PGH-PGE isomerase activities solubilized with Triton X-100 and CHAPS.

Solubilization Buffer	% of initial activity 0 hrs	6.5 hrs	21 hrs	Thaw/ Refreeze
1.5% Tx-100	93	3	4	0
12 mM CHAPS	100*	33	29	40
20 mM CHAPS	120	32	17	--
10 mM CHAPS+			~100	80

* Assigned value.
+ Separate experiment, where fresh PMSF and GSH were added at t=0, 10 hrs.
Microsomes prepared in 0.05 M Tris (pH8.0), 0.5 mM GSH, 0.5 mM EDTA, 20% ethylene glycol, 1 ug/ml leupeptin, 0.5 mM PMSF, and 0.05 mM DTT.
Specific activities at t=0:

Tx-100	143 nmol/min/mg
12 mM CHAPS	154 nmol/min/mg
20 mM CHAPS	186 nmol/min/mg

thawed microsomes, but did have a significant stabilizing effect when the microsomes were incubated on ice for 3.5 hrs. Addition of 4 ug of IgG_1(*hei*-7) per 10 ug of microsomal protein decreased the total PGH-PGE isomerase activity by only 7-11%. On other occasions, addition of IgG_1(*hei*-7) to microsomal proteins had no effect or even slightly increased PGH-PGE isomerase activity. In the presence of IgG_1(*hei*-7), PGH-PGE isomerase activity in the absence of ethylene glycol was stabilized to almost the same extent as in microsomes prepared with ethylene glycol. The specific PGH-PGE isomerase activity of microsomes prepared in GSH-free buffer was less than one-half of that observed in microsomes prepared in buffer containing 1 mM GSH. GSH was removed by resuspending the microsomal pellet in GSH-free buffer, and solubilized proteins could therefore contain a small amount of GSH. Also, the assay buffer contained GSH, so the isomerase activity lost here represents activity lost during preparation and storage of the microsomes.

PGH-PGE isomerase activity in microsomes prepared without ethylene glycol was stabilized to almost the same degree as isomerase activity in microsomes prepared with ethylene glycol by the addition of fresh 1 mM GSH to the microsomes immediately after they were thawed (Table 3). Therefore, microsomes for the isolation and characterization of PGH-PGE isomerase were routinely prepared in 0.05 M Tris-Chloride (pH 8.0), containing 1 mM GSH, 0.5 mM EDTA, 0.5 mM PMSF, and 1 ug/ml leupeptin, and solubilized in the same buffer containing 10 mM CHAPS. Immediately upon thawing, the microsomes were diluted with buffer containing fresh GSH so that an additional 1 mM GSH was added.

Solubilization of PGH-PGE Isomerase from SVG Microsomes. PGH-PGE isomerase activities solubilized from SVG microsomes with Triton X-100 and 10 mM CHAPS are compared in Table 4. The specific PGH-PGE isomerase activity in four preparations of 10 mM CHAPS-solubilized microsomes was 395 to 645 nmol of PGE_2

Table 2. Effect of ethylene glycol, GSH, and IgG1(*hei*-7) on the stability of PGH-PGE isomerase activity solubilized with 10 mM CHAPS.

		% Initial Activity			
		- IgG1(*hei*-7)		+ IgG1(*hei*-7)	
		0 hrs	3.5 hrs	0 hrs	3.5 hrs
+GSH	+ ethylene glycol	100*	93	93	81
	- ethylene glycol	105	39	94	71
-GSH	+ ethylene glycol	47	30	-	-
	- ethylene glycol	36	10	-	-

* Assigned value.
Specific activity at t=0: 161 nmol/min/mg.
Microsomes prepared in 0.05 M Tris (pH 8.0), 1 mM EDTA, 1 ug/ml leupeptin, 0.5 mM PMSF, +/- 1 mM GSH, and +/- 20% ethylene glycol.

Table 3. Fresh GSH added to microsomes increases the stability of the PGH-PGE isomerase activity.

Initial activity when thawed	100%*
Activity after 4 hrs on ice	30-65%
Activity after 4 hrs on ice - 1 mM GSH added at t=0	80-95%

* Assigned value.
Microsomes were prepared in 0.05 M Tris (pH 8.0), 1 mM GSH, 1 mM EDTA, 1 ug/ml leupeptin, and 0.5 mM PMSF (without ethylene glycol), and solubilized with 10 mM CHAPS. Results show a range of values obtained using three different microsome preparations.
Microsomal proteins stored at -80° were thawed and assayed for PGH-PGE isomerase activity. 1 mM GSH was added to an aliquot of the thawed microsomes, and microsomes both with and without fresh GSH were incubated on ice for 4 hrs.

Table 4. Solubilization of PGH-PGE isomerase activity using 1.5% Triton X-100 or 10 mM CHAPS.

	1.5% Triton X-100	10 mM CHAPS
Specific Activity	350 nmol PGE2 formed/ min/mg (24°, pH 7.0, 50 uM PGH2)	395-645 nmol PGE2/ min/mg (24°, pH 8.0, 34 uM PGH2)
% Isomerase Activity Solubilized	about 50%	44%
% Total Isomerase Activity ppt'ed by IgG1(*hei* -7)	about 40 %	40-45%
Stability (+GSH)	almost no activity after 3 hrs on ice	80-95% of initial activity after 4 hrs on ice

microsomes solubilized with 10 mM CHAPS was precipitated by IgG_1(*hei*-7). In one preparation 44% of the total PGH-PGE isomerase activity present in the microsomal pellet was solubilized using 10 mM CHAPS (Table 5). The addition of 0.15 M NaCl to the solubilization buffer had no significant effect on the amount of PGH-PGE isomerase activity solubilized with 10 mM CHAPS.

Gel filtration chromatography was used to determine whether the PGH-PGE isomerase activity solubilized from SVG microsomes with 10 mM CHAPS was in a soluble form. Solubilized microsomal proteins were applied to a Sepharose 6B column which had been equilibrated and was eluted with MSB. Fractions were collected and assayed for protein, PGH-PGE isomerase activity, and cyclooxygenase activity. All of the cyclooxygenase activity, and more than 85% of the PGH-PGE isomerase activity were included in the column volume (Figure 6). Cyclooxygenase activity eluted as one large, symmetric peak, and PGH-PGE isomerase activity eluted as three overlapping peaks. When the concentration of CHAPS in the equilibration and elution buffers was reduced to 1 mM, all of the cyclooxygenase and PGH-PGE isomerase activities eluted in the void volume.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) Inhibit PGH-PGE Isomerase Activity in Solubilized SVG Microsomes. Flurbiprofen (FBP) at a final concentration of 0.1 mM had inhibited PGH-PGE isomerase activity in Triton X-100-solubilized microsomes by 48% (see above). To determine whether other NSAIDs would also inhibit PGH-PGE isomerase, assay buffers containing FBP, flufenamic acid (FA), and aspirin (ASA) were prepared so that their final concentration in the reactions would be 1 mM. Each assay buffer was added to 8.9 ug of 20 mM CHAPS-solubilized microsomes, and ^{3}H-PGH_2 was added to initiate the reactions. ASA inhibited PGH-PGE isomerase activity by 15%, FBP by 56%, and FA inhibited 81% of the total isomerase activity. Since more than one PGH-PGE isomerase is present in the solubilized microsomes, no further

Table 5. PGH-PGE isomerase activity and protein measurements during the preparation of SVG microsomes solubilized using 10 mM CHAPS.

Fraction	~ Total vol(ml)	Protein (mg/ml)	Total mg	Total Units (nmol/min)	SA (U/mg)
Crude Homog.	60	2.66	160	35,530*	222*
Post 10K Super	40	2.13	85	21,890*	257*
1st 50K Super	56	0.76	42	12,660	299
Solub. Pellet	10	4.76	48	4821	101
Solub. Pellet +0.1 M NaCl	10	4.76	48	5292	111
2nd 50K Super+	10	1.60	[a]15	[b]2139	141
2nd 50K Super +0.1 M NaCl	10	1.60	15	1947	128

* Probably less than actual value due to high activity in nonlinear range of activity assay.

\+ 2nd 50K supernatant = solubilized microsomal proteins.

a 32% of protein in microsomal pellet was solubilized.

b 44% of PGH-PGE isomerase activity in microsomal pellet was solubilized.

Figure 6. Gel filtration chromatography on Sepharose 6B of microsomes solubilized in 10 mM CHAPS. One mg of protein was applied to a 0.95 x 30 cm column which was equilibrated and eluted with MSB+NaCl. The flow rate was 2.2 ml/hr, and fractions of 0.5 ml were collected. 75 ul of each fraction was assayed for PGH-PGE isomerase activity, and 200 ul for cyclooxygenase activity. The protein concentration of each fraction was determined by the method of Bradford.

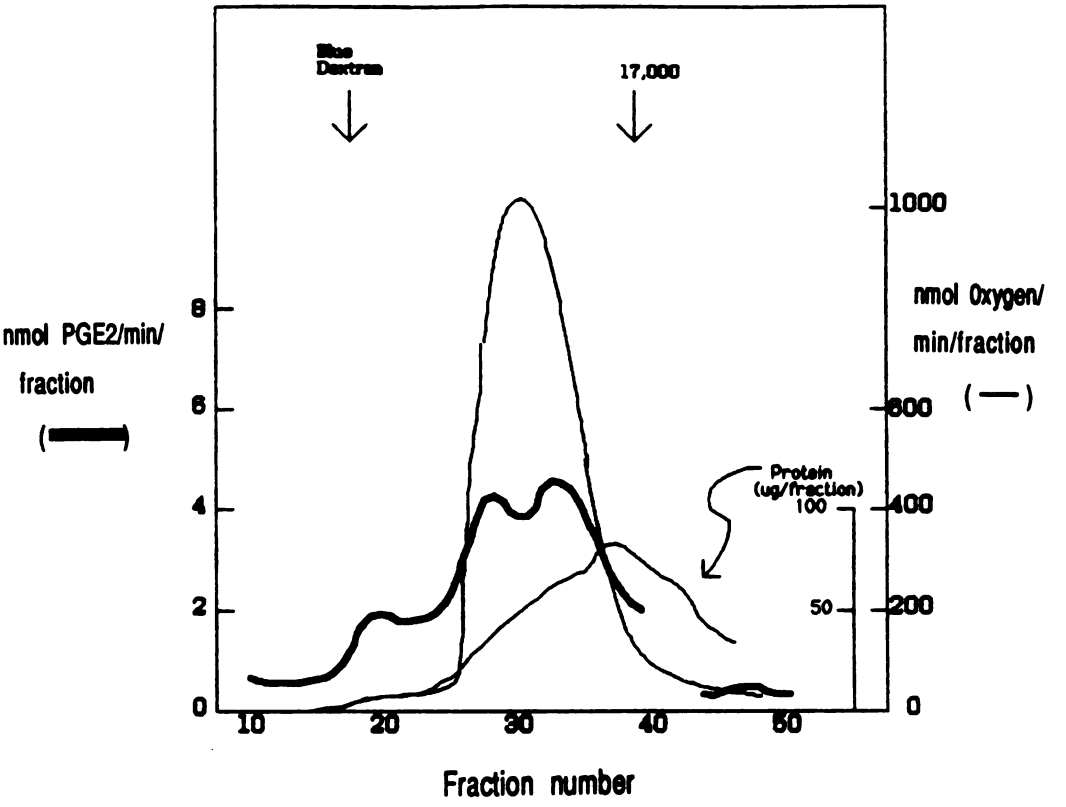

Blue Dextran
17,000
nmol PGE2/min/ fraction
nmol Oxygen/ min/fraction
Protein (ug/fraction)
100
50
0
2
4
6
8
200
400
600
800
1000
10
20
30
40
50
Fraction number

Figure 6

characterization of the inhibitors was attempted at this time.

Lack of Association Between PGH-PGE Isomerase and Cyclooxygenase. There was evidence to indicate that some cyclooxygenase might be co-purifying with the PGH-PGE isomerase that reacted with IgG_1(*hei*-7) from microsomes that had been solubilized with 10 mM CHAPS. Eluants from IgG_1(*hei*-7) immunoaffinity columns contained a protein band of the same subunit molecular weight as cyclooxygenase. Also, anti-cyclooxygenase antibodies used as controls in immunoaffinity chromatography experiments had bound more PGH-PGE isomerase activity than other control antibodies. Anti-cyclooxygenase antibodies had precipitated no PGH-PGE isomerase activity from Triton X-100-solubilized microsomes. In addition, one of the peaks of PGH-PGE isomerase activity from the above gel filtration analysis of CHAPS-solubilized microsomes co-eluted with the peak of cyclooxygenase activity (Figure 6).

Titration curves were generated using IgG_1(*hei*-7); an anti-cyclooxygenase antibody, IgG_1(*cyo*-3); and a control antibody, IgG_1(II-C7) (Figure 7). Increasing amounts of these antibodies coupled to Protein A-agarose were used to precipitate both cyclooxygenase and PGH-PGE isomerase activities from microsomes solubilized with 10 mM CHAPS. The control antibody precipitated neither of the enzyme activities. IgG_1(*hei*-7) precipitated PGH-PGE isomerase activity in a concentration-dependent manner, but appeared to react only nonspecifically with cyclooxygenase. The anti-cyclooxygenase antibody, however, appeared to specifically precipitate both cyclooxygenase and PGH-PGE isomerase activities. Precipitation of PGH-PGE isomerase activity by anti-cyclooxygenase antibodies had not occurred when using microsomes solubilized with Triton X-100.

An experiment was performed to determine whether the PGH-PGE isomerase activity precipitated by IgG_1(*cyo*-3) was identical to the activity precipitated by IgG_1(*hei*-7). Solubilized microsomes were precipitated sequentially with excess amounts

Figure 7. Titration curves showing cyclooxygenase and PGH-PGE isomerase activities precipitated by increasing amounts of A, IgG1(*hei* -7), B, IgG1(*cyo*-3), and C, IgG1(IIC7). Minicolumns containing antibody coupled to Protein A-agarose were incubated with 120 ug of solubilized microsomal protein per 100 ul of gel at 4^{o} for 35 min. The columns were washed with 20 volumes of solubilization buffer, and aliquots of the Protein A-agarose were assayed for both PGH-PGE isomerase activity (——), and cyclooxygenase activity (----).

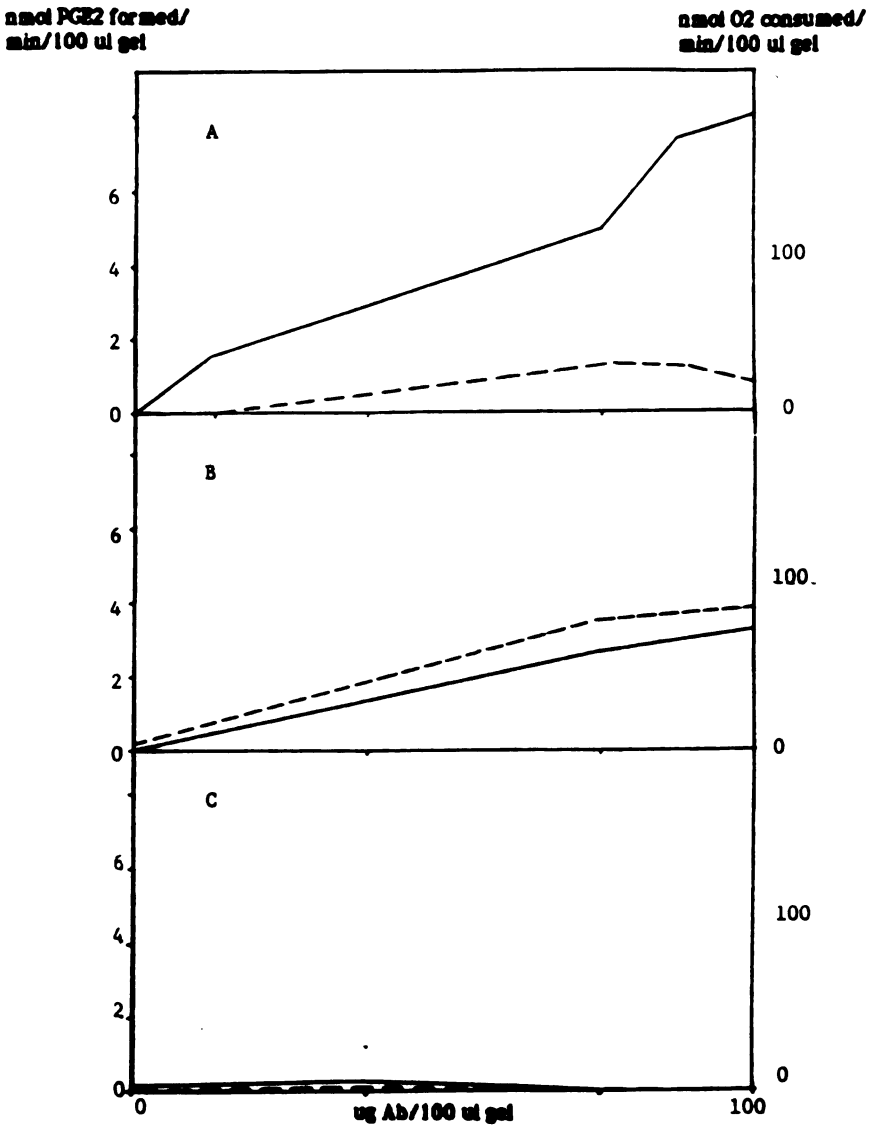

nmol PGE2 formed/
min/100 ul gel
nmol O2 consumed/
min/100 ul gel
A
B
C
6
4
2
0
100
0
ug Ab/100 ul gel
100

Figure 7

of anti-cyclooxygenase antibody and then with IgG_1(*hei*-7), or vice versa (Table 6). As usual, 40% of the total PGH-PGE isomerase activity present in the solubilized microsomes was precipitated by IgG_1(*hei*-7) (line 5). A second precipitation with IgG_1(*hei*-7) contained no isomerase activity, indicating that under the experimental conditions all the IgG_1(*hei*-7)-reactive PGH-PGE isomerase was removed from the microsomes by one precipitation with IgG_1(*hei*-7). The anti-cyclooxygenase antibodies, IgG_1(*cyo*-3) and IgG_1(*cyo*-5), precipitated 24-28% of the total PGH-PGE isomerase activity from solubilized microsomes (lines 1 and 2). When the same microsomes were precipitated again using IgG_1(*hei*-7), about the same amount of PGH-PGE isomerase activity was precipitated as when no prior precipitation had occurred (lines 1 and 2), indicating that the PGH-PGE isomerase that binds to the *cyo* antibodies is not the same as that which binds to IgG_1(*hei*-7). Conversely, when the microsomes were first precipitated by IgG_1(*hei*-7), the anti-cyclooxygenase antibodies could still precipitate up to 20% of the total PGH-PGE isomerase activity from the solubilized microsomes (lines 3 and 4).

Immunoaffinity Chromatography using IgG1(*hei*-7). IgG_1(*hei*-7) was covalently attached to AffiGel 10 (1 to 15 mg antibody/ml gel). AffiGel 10 is an N-hydroxysuccinimide ester of a derivatized cross-linked agarose gel which forms a stable amide bond with primary amino groups of the ligand.

In initial experiments with Triton X-100-solubilized microsomes, IgG_1(*hei*-7) immunoaffinity columns were eluted with a pH gradient from pH 7.0 to 2.6 using 0.2 N acetic acid containing 0.15 M NaCl, and 0.2% Triton X-100. No isomerase activity was recovered from the eluants, and none was expected below pH 4.5. About 15% of the PGH-PGE isomerase activity was lost from the microsomes when they were passed over the AffiGel 10-IgG_1(*hei*-7) column, indicating that some activity had bound to the column. Analysis of the column eluants by SDS-PAGE showed some proteins that eluted between pH 5.5 to 2.6, but a protein band corresponding to the known subunit molecular weight of

Table 6. PGH-PGE isomerase activity precipitated from solubilized microsomes using sequential antibody-Protein A minicolumns.

	Ab on 1st Column	% Total Activity Bound	Ab on 2nd Column	% Total Activity Bound
1.	IgG1(cyo-3)	28.0	IgG1(hei-7)	35.9
2.	IgG1(cyo-5)	24.3	IgG1(hei-7)	49.0
3.	IgG1(hei-7)	40.3	IgG1(cyo-3)	12-20
4.	IgG1(hei-7)	40.3	IgG1(cyo-5)	16-20
5.	IgG1(hei-7)	40.3	IgG1(hei-7)	0
6.	IgG1(II-C7)	5.2	IgG1(hei-7)	40.7

Microsomes prepared in 0.05 M Tris (pH 8.0), 1 mM GSH, 1 mM EDTA, 1 ug/ml leupeptin, and 0.5 mM PMSF; solubilized with 10 mM CHAPS.
Antibody-Protein A-agarose prepared as described under Methods was used to precipitate PGH-PGE isomerase from solubilized microsomes. PGH-PGE isomerase activity remaining in the microsomes was then measured.

the protein that reacts with IgG_1(*hei*-7) was observed only in the pH 2.6 eluant.

Conditions for the elution of PGH-PGE isomerase activity from IgG_1(*hei*-7) immunoaffinity columns were examined. The pH range over which PGH-PGE isomerase activity in 10 mM CHAPS-solubilized microsomes was stable was determined (Figure 8). Microsomal proteins were incubated with 3 volumes of buffer (pH 2.7 to 9.8) on ice for 3 or 8 min. The solutions were then adjusted to pH 8.0 with 1.0 M Tris, and 12 ug of microsomal protein was assayed for PGH-PGE isomerase activity. The isomerase activity was most stable when microsomal proteins were incubated with buffers between pH 5.3 and 8.8. Isomerase activity decreased 15-20% in microsomes incubated with buffers between pH 9 and 10, but decreased much more abruptly below pH 5. A 3 min incubation at or below pH 3.6 caused a loss of all PGH-PGE isomerase activity. The microsomes used in these experiments contained 20% ethylene glycol which could enhance the stability of the isomerase, but the data is relevant since ethylene glycol may be included in immunoaffinity elution buffers (see below).

In subsequent experiments it became obvious that the AffiGel 10-IgG_1(*hei*-7) columns were not binding PGH-PGE isomerase activity very efficiently. Ethylene glycol has been added in immunoaffinity elution buffers by other investigators to decrease the hydrophobic interactions between antigen and antibody (110). Microsomes used thus far for immunoaffinity chromatography had contained 20% ethylene glycol. In one experiment, more than four times as much PGH-PGE isomerase activity was bound to the IgG1(hei-7)-column when ethylene glycol was omitted from the microsomes and from the column buffers. Therefore, buffers containing ethylene glycol could possibly be used to elute PGH-PGE isomerase activity from IgG_1(*hei*-7)-affinity columns.

The stability of PGH-PGE isomerase activity in some possible elution buffers containing ethylene glycol was determined. The initial PGH-PGE isomerase activity retained in a 3 min incubation of microsomal proteins in 0.1 M Tris-chloride (pH 7.1) containing 1 mM GSH, 1 M NaCl, 20% ethylene glycol, and 10 mM CHAPS was 84%.

Figure 8. Stability of PGH-PGE isomerase activity at different pH's. Solubilized microsomes (12 ug, solubilized in 10 mM CHAPS and containing 20% ethylene glycol) were incubated on ice in the different buffers for 3 min, and then neutralized to pH 8.0. PGH-PGE isomerase activity was assayed as usual.

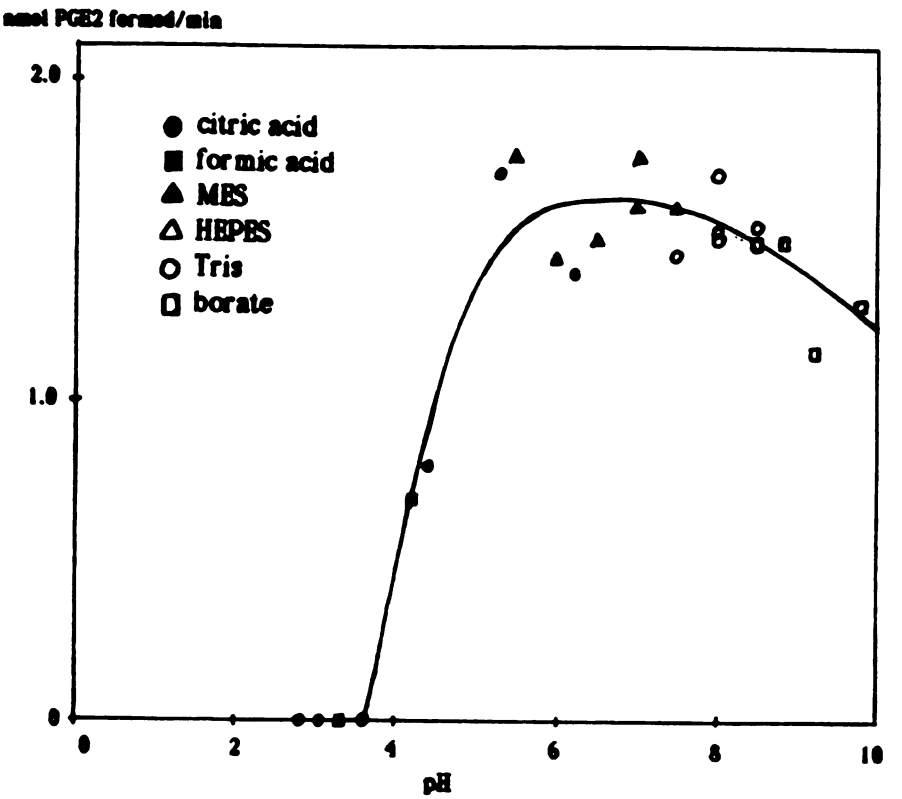

nmol PGE2 formed/min
2.0
1.0
0
citric acid
formic acid
MES
HEPES
Tris
borate
0
2
4
6
8
10
pH

Figure 8

Only 62% of the initial activity remained after incubation in the same buffer with 0.2 M MES (pH 5.4) instead of Tris-chloride, and substitution of the buffer with 3 M $MgCl_2$ (pH 7.1) or 0.2 N citric acid (pH 2.6) caused a loss of all PGH-PGE isomerase activity.

A concentration of 10 mM CHAPS must be maintained in the equilibration and wash buffers to prevent enzyme aggreagation and nonspecific adsorption to the immunoaffinity columns. An experiment using buffers without detergent resulted in control antibody columns adsorbing almost as much PGH-PGE isomerase activity as the IgG_1(*hei*-7)-AffiGel 10 column. When only 2 mM CHAPS was added to the column buffers, the control columns retained about 30% as much PGH-PGE isomerase activity as was specifically bound. It appears that 10 mM CHAPS is required in the column buffers to keep the microsomes in a soluble form and thus prevent nonspecific adsorption. However, in an identical experiment using buffers containing 10 mM CHAPS no PGH-PGE isomerase activity was bound to the affinity columns.

The poor binding of PGH-PGE isomerase activity to IgG_1(*hei*-7)-AffiGel 10 has continued to be a problem, even after the elimination of ethylene glycol from the buffers. This is not due to poor binding of antigen to antibody in general, or due to the presence of 10 mM CHAPS, since IgG_1(*hei*-7) can precipitate all of its antigen from 10 mM CHAPS-solubilized microsomes when used in double-antibody immunoprecipitations, or when bound to Protein A-agarose. Something could be happening to the antibody when it is bound to AffiGel 10 that reduces its ability to bind to the antigen. The standard coupling procedure for antibody to AffiGel 10 is carried out for four hours at 4°C. An experiment was performed to determine whether the coupling time for antibody to AffiGel 10 would have any effect on the ability of IgG_1(*hei*-7) to bind PGH-PGE isomerase activity. First, an IgG_{2b} was coupled to AffiGel 10 (1 and 5 mg antibody/ml gel) for 5, 10, 20, 30, and 60 min. By 5 min 70% of the antibody was already bound to the gel, and by 60 min 90% of the antibody was coupled to the AffiGel 10. Then, AffiGel 10 was coupled to IgG_1(*hei*-7) for 3, 5, 15, 45, and 180 minutes using 1 mg and 5 mg of antibody per ml of

gel. These gels were incubated with microsomal proteins, and the amount of PGH-PGE isomerase activity bound to the gels was measured (Figure 9). AffiGel 10 coupled with 1 mg antibody per ml gel for only 3-5 minutes precipitated the most PGH-PGE isomerase activity. The amount of isomerase activity bound to the AffiGel 10 columns was still less than 30% of the activity bound to an equivalent amount of Protein A-IgG_1(*hei*-7). The binding would have to be increased even further to obtain enough antigen for purification. Also, conditions for the elution of active PGH-PGE isomerase from the immunoaffinity columns still need to be defined. At this time, the immunoaffinity purification of PGH-PGE isomerase was discontinued, and the Protein A-agarose immobilized enzyme was used to examine the role of GSH in the reaction.

Figure 9. Binding of PGH-PGE isomerase activity to IgG1(*hei*-7) and AffiGel 10 that were reacted with each other for different amounts of time. 10 mM CHAPS-solubilized microsomes (without ethylene glycol) were incubated with the different aliquots of antibody-AffiGel 10 for 40 min, the gels were washed, and 17 ul aliquots of the gels were assayed for PGH-PGE isomerase activity.

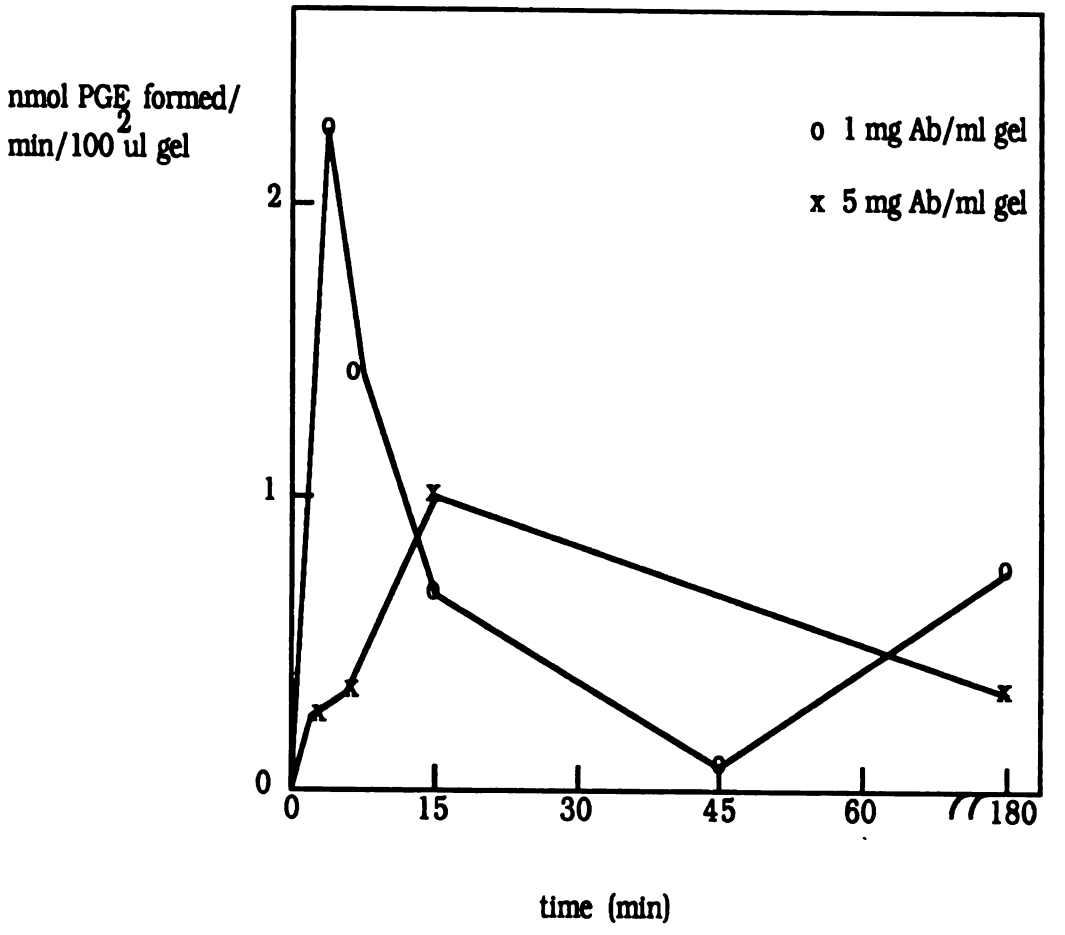

nmol PGE2 formed/
min/100 ul gel
o 1 mg Ab/ml gel
x 5 mg Ab/ml gel
2
1
0
0
15
30
45
60
180
time (min)

Figure 9

DISCUSSION

Changes made in the solvent used to extract the samples, and in the method by which the reactions were stopped resulted in increased recovery of ^{3}H-PGE$_2$ and greater resolution on the TLC plates in the PGH-PGE isomerase activity assays. Therefore the accuracy of the assays was improved. Flurbiprofen was eliminated from the activity assay buffer because it inhibited the enzyme.

PGH-PGE isomerase activity solubilized from sheep vesicular gland microsomes using 10 mM CHAPS was much more stable than the activity solubilized using 1.5% Triton X-100. The inclusion of 20% ethylene glycol in the buffers had a stabilizing effect on the solubilized PGH-PGE isomerase activity, but it was necessary to prepare the solubilized microsomal proteins without ethylene glycol for immunoaffinity chromatography. The addition of IgG$_1$(*hei*-7) and/or fresh GSH enhanced the stability of PGH-PGE isomerase activity in microsomes prepared without ethylene glycol. When 1 mM fresh GSH was added to freshly thawed and previously solubilized microsomal proteins, up to 95% of the initial PGH-PGE isomerase activity remained over a 4 hour incubation of the microsomes on ice.

PGH-PGE isomerase and cyclooxygenase were in a soluble form in the 10 mM CHAPS-solubilized microsomes as judged by their inclusion in a gel filtration column. Some PGH-PGE isomerase activity was precipitated by anti-cyclooxygenase antibodies from the 10 mM CHAPS-solubilized microsomes (a phenomena that was never observed in the Triton X-100-solubilized microsomes), but it was not identical to the isomerase activity precipitated by IgG$_1$(*hei*-7). Several explanations are possible to explain the binding of PGH-PGE isomerase activity by the cyclooxygenase antibodies. Cyclooxygenase could be associated

with one of the other proteins in SVG microsomes that has PGH-PGE isomerase activity, or the association could represent nonspecific binding (unlikely, or it would also be seen with the control antibodies). Alternatively, the PGH-PGE isomerase activity immunoprecipitated along with cyclooxygenase could represent a small portion of the IgG_1(*hei*-7)-PGH-PGE isomerase, or some other protein, that copurifies with cyclooxygenase only when the microsomes are solubilized in CHAPS, and thus has never been observed before. If this activity that coprecipitates with cyclooxygenase is the IgG_1(*hei*-7)-PGH-PGE isomerase, the antigenic site is not available for binding to IgG_1(*hei*-7) when associated with cyclooxygenase. At the time these experiments were performed no method was known to distinguish between these possibilities. If a second antibody to the PGH-PGE isomerase that reacts with IgG_1(*hei*-7) was available, an ELISA assay could be performed to determine whether this was the PGH-PGE isomerase activity bound to the cyclooxygenase antibody. Subsequent to these experiments, the protein bound to IgG_1(*hei*-7)-Protein A was identified using SDS-PAGE (Chapter IV). This method could also be used to identify the proteins bound to IgG_1(*cyo*-3).

Three non-steroidal anti-inflammatory drugs partially inhibited PGH-PGE isomerase activity in solubilized SVG microsomes. The NSAIDs are known to competitively inhibit cyclooxygenase (26), but have never been described as inhibitors of PGH-PGE isomerase. Flufenamic acid inhibited 81% of the total PGH-PGE isomerase activity. One other effect of flufenamic acid was reported that could not be attributed to inhibition of cyclooxygenase activity. Flufenamic acid was shown to compete with ^{3}H-PGE_2 for binding sites on rabbit uterus *in vitro* (118). Since more than one protein with PGH-PGE isomerase activity is present in SVG, further characterization of their inhibition by NSAIDs will await the purification of the enzymes.

The binding of PGH-PGE isomerase activity to IgG_1(*hei*-7)-AffiGel 10 was enhanced by the elimination of ethylene glycol from the buffers, and by reduction of the coupling time between antibody and AffiGel 10. However, the amount of PGH-PGE isomerase activity

bound was still less than 30% of the activity bound by an equivalent amount of IgG_1(*hei*-7)-Protein A. To purify the PGH-PGE isomerase by immunoaffinity chromatography the binding would need to be improved further, nondenaturing conditions for elution of the enzyme would have to be determined, and the dilute enzyme would have to be concentrated. All of these steps may or may not be possible. Since a significant amount of time had already been invested in the immunoaffinity purification, I decided to proceed to use the enzyme immobilized on IgG_1(*hei*-7)-Protein A-agarose to examine the role of GSH in the PGH-PGE isomerase reaction.

CHAPTER IV

CHARACTERIZATION OF THE PGH-PGE ISOMERASE REACTIVE WITH IgG_1(*hei*-7)

A microsomal PGH-PGE isomerase has never been significantly purified in an active form. Monoclonal antibodies were prepared to SVG microsomal PGH-PGE isomerase so that the enzyme could be purified by immunoaffinity chromatography. However, attempts to use IgG_1(*hei*-7) coupled to Affi-Gel 10 for the immunoaffinity purification of PGH-PGE isomerase have been unsuccessful (Chapter III). A system was devised, however, in which the IgG_1(*hei*-7)-reactive PGH-PGE isomerase could be separated from all other microsomal proteins by binding the enzyme to IgG_1(*hei*-7) which was immobilized on Protein A-agarose. Below pH 7 the antibody, since it is an IgG_1, will detach from Protein A, and PGH-PGE isomerase could be obtained bound only to the antibody. However, I found that the enzyme immobilized on the Protein A-agarose gel was active, showed linear enzyme kinetic behavior, and was useful for studying the role of the cofactor, GSH, in the reaction. GSH could be separated from the immobilized enzyme very quickly, thereby permitting me to examine the activity in the presence and absence of cofactor. This chapter describes some characteristics of the PGH-PGE isomerase immobilized on IgG_1(*hei*-7)-Protein A-agarose.

MATERIALS AND METHODS

Materials. S-methyl GSH, S-hexyl GSH, and S-*p*-chloro-phenacyl GSH were obtained from Sigma Chemical Company. AffiGel 10-Protein A (Protein A-agarose), polypropylene Econo-Columns (5 ml), and the SDS-PAGE Silver Stain Kit were from Bio-Rad. Other SDS-PAGE reagents were as described in Chapter II. The PGH^2 analogs (U-61621, U-45861E, U-46619, U-48271, U-51570, U-53720, U-56513, U-61156, U-51605, and U-68492) were a gift from Dr. John Pike of The Upjohn Company. U-44069 was purchased from Cayman Chemical Co.

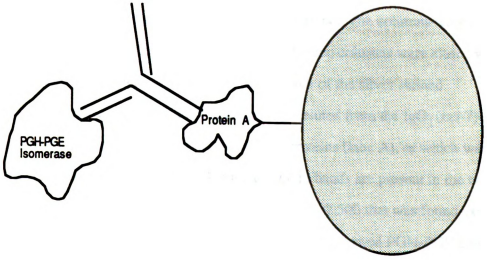
Isolation of PGH-PGE Isomerase. Aliquots of Protein A-agarose (100-300 ul) were placed in 5 ml polypropylene columns and washed with 20 volumes of 0.05 M Tris-chloride (pH 8.2) containing 0.15 M NaCl (Tris/NaCl buffer). Antibody was bound to the Protein A by adding 50 ug of IgG_1(*hei*-7) per 100 ul of gel (0.5 mg/ml in Tris/NaCl buffer), and incubating at room temperature for 35 min with shaking every 5 min. The columns were then washed with 5 volumes of Tris/NaCl buffer. To prevent nonspecific adsorption of microsomal proteins to the gel, the columns were washed with 3 volumes of Tris/NaCl buffer containing 5% BSA, and then again with 5 volumes of Tris/NaCl buffer. At this point, the columns were transferred to the cold room (4^{o}), where all subsequent operations were performed. The Protein A-antibody columns were washed with 10 volumes of microsomal solubilization buffer (MSB) (0.05 M Tris-chloride (pH 8.1) containing 1 mM GSH, 0.5 mM EDTA, 0.5 mM PMSF, 1 ug/ml leupeptin, and 10 mM CHAPS) containing 0.15 M NaCl (MSB+NaCl). Solubilized sheep vesicular gland microsomal proteins (1 mg/ml in MSB, 180 ug added per 100 ul gel) were incubated with

the IgG_1(*hei*-7)-Protein A-agarose for 40 min with shaking (for nonenzymatic controls, buffer was added instead of solubilized microsomes). The columns were washed with 15 volumes of MSB+NaCl, and then with 5 volumes of MSB. Gels were resuspended in 2 volumes of MSB, and aliquots of usually 51 ul (17 ul gel) were removed for the enzyme assays. Thus the immobilized enzyme consists of PGH-PGE isomerase bound to IgG_1(*hei*-7) which is bound to Protein A-agarose (Figure 10).

PGH-PGE Isomerase Activity Assays. Aliquots of IgG_1(*hei*-7)-Protein A-agarose both with and without immobilized PGH-PGE isomerase were distributed into silanized 10x75 cm glass test tubes on ice. The volume of each tube was brought to 50 ul with MSB. Each sample was assayed by adding 50 ul of assay buffer (0.2 M Tris (pH 8.0) containing 1 mM GSH, and 1.4% Triton X-100), the tube vortexed, and then incubated in a room temperature water bath for 3 min. The reaction was initiated by the addition of the substrate, ^{3}H-PGH_2 (usually 4 ul, 6.438 nmol, in acetone), and the tube continuously mixed for the reaction time (usually 20 sec). The reaction was stopped by acidification, extracted two times with ethyl acetate, and the products separated by TLC as described in Chapter II.

SDS-Polyacrylamide Gel Electrophoresis. IgG_1(*hei*-7)-Protein A-agarose both with and without bound PGH-PGE isomerase was prepared as described above. All buffer was removed, and the gels were incubated with 50 ul of SDS-PAGE sample buffer (0.125 M Tris-HCl (pH 6.8) containing 5.6% SDS (w/v), 20% glycerol (v/v), 0.002% bromphenol blue (v/v), 10% 2-ME (v/v), and 5% H_2O (v/v)) for 3 hrs at room temperature, and then for 20 min at 60°. The sample buffers were separated from the gels by centrifugation, and 30 ul of the resulting supernatants were separated on a 12% SDS-polyacrylamide gel. The electrophoresis procedures used here were as described in Chapter II.

Figure 10. Immobilized PGH-PGE isomerase.



Immobilized Enzyme System =

PGH-PGE Isomerase - IgG1(hel-7) - Protein A - Agarose

Figure 10

RESULTS

Identification of Proteins Bound to IgG1(*hei*-7)-Protein A-Agarose. Two columns containing IgG_1(*hei*-7)-Protein A-agarose were prepared and incubated either with solubilized microsomal proteins or with buffer alone. The columns were washed with 20 volumes of buffer, and proteins bound to the two columns were eluted with SDS-PAGE sample buffer. Figure 11 is a photograph of the silver stained SDS-polyacrylamide gel lanes showing the proteins eluted from the IgG_1(*hei*-7)-Protein A-agarose which was incubated with microsomal proteins (lane A), or which was incubated with only buffer (lane B). Identical protein bands are present in the two lanes, except for the presence of one protein band with M_r = 17,500 that was found exclusively in lane A. The gel with this protein of M_r = 17,500 also showed PGH-PGE isomerase activity. This unique protein in Lane A has the same subunit molecular weight as was previously determined for the PGH-PGE isomerase reactive with IgG_1(*hei*-7) by immunoprecipitation of ^{125}I-labelled, solubilized SVG microsomal proteins (see Chapter II). These results demonstrate that the PGH-PGE isomerase immobilized on the IgG_1(*hei*-7)-Protein A-agarose was probably free of contamination with other microsomal proteins. Thus, the only protein present in the enzymatic reactions, that was not also present in the nonenzymatic reactions, appears to be the protein with M_r = 17,500.

Kinetic Properties of the Immobilized PGH-PGE Isomerase. PGE_2 formation by immobilized PGH-PGE isomerase was shown to be linear both over time, and with the amount of enzyme in the reaction (Figure 12). Product formation was linear from 0 to 90 seconds when assaying 12 ul of gel with bound enzyme using 49.2 uM PGH_2. Product

Figure 11. Identification of a unique protein bound to IgG1(*hei*-7)-Protein A-agarose. The photograph of the silver stained SDS-polyacrylamide gel lanes shows the proteins eluted from IgG1(*hei*-7)-Protein A-agarose which had been incubated with solubilized microsomal proteins (lane A), or which had been incubated with buffer alone (lane B).

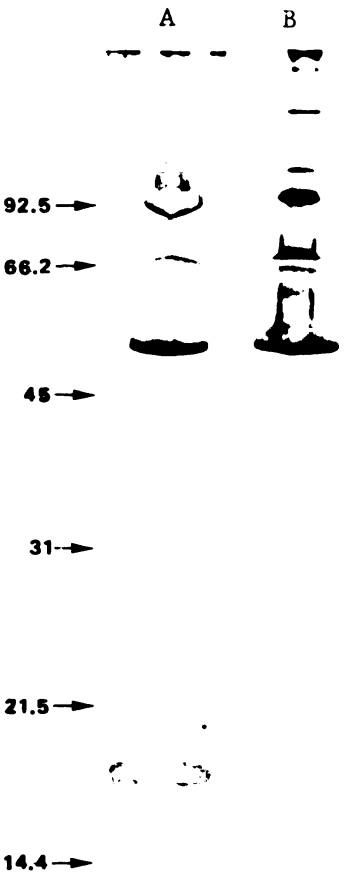
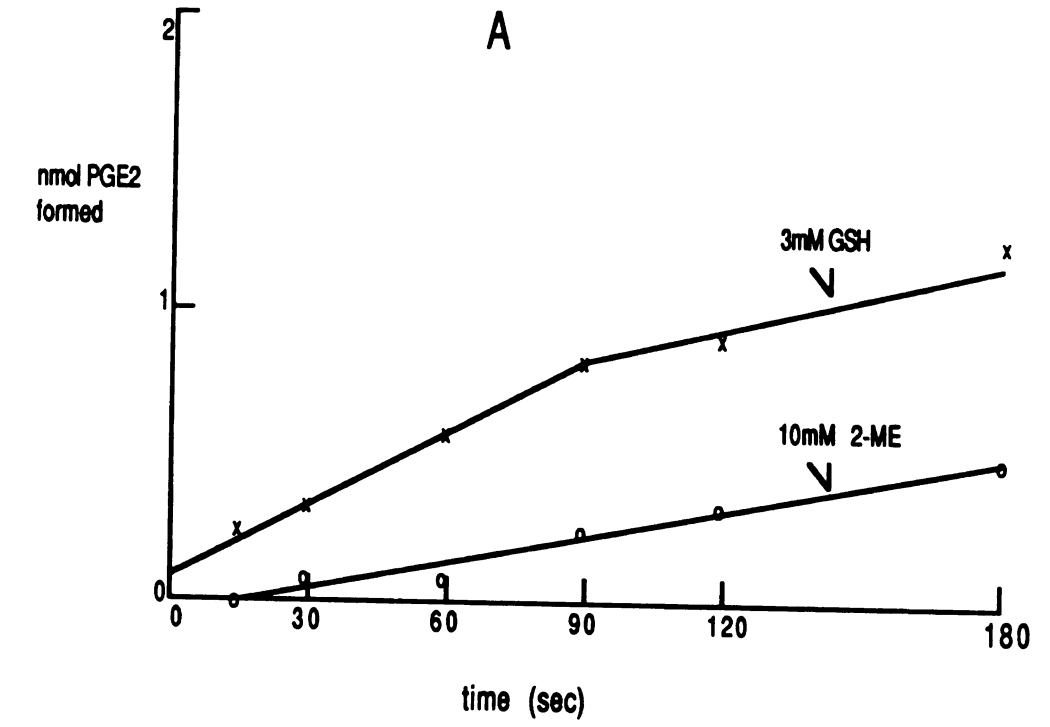


Figure 11

Figure 12. PGE2 formation by immobilized PGH-PGE isomerase is linear with time (A), and with the amount of enzyme in the reaction (B). In A, PGE2 formation was measured at the indicated times using 12 ul of gel with bound enzyme and 49.2 uM PGH2. In B, PGE2 formation was measured using 15 sec reaction times, the indicated amount of gel with bound enzyme, and 42.4 uM PGH2. PGE2 formation in nonenzymatic reactions was subtracted before the values were plotted. In plot A, the lower line represents PGH-PGE isomerase activity isolated from microsomes that had been prepared in buffer containing 10 mM 2-mercaptoethanol instead of GSH.


A
nmol PGE2
formed
2
1
0
3mM GSH
10mM 2-ME
0
30
60
90
120
180
time (sec)

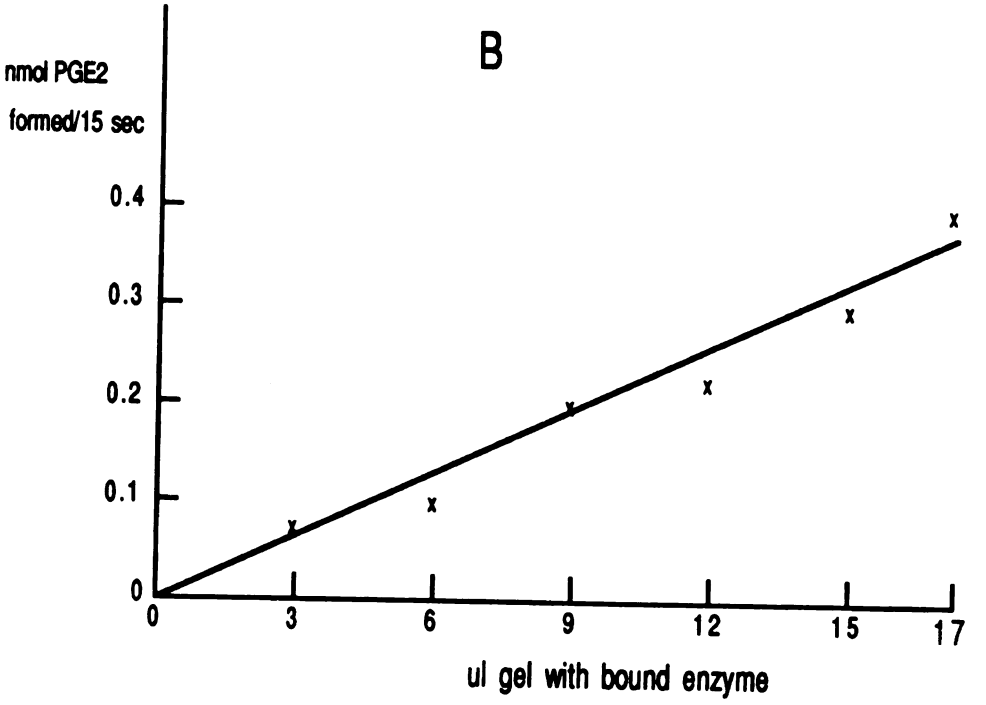

B
nmol PGE2
formed/15 sec
0.4
0.3
0.2
0.1
0
0
3
6
9
12
15
17
ul gel with bound enzyme

Figure 12

formation was also linear when assaying from 3 to 17 ul of gel with bound enzyme, using 15 sec reaction times and 42.4 uM PGH_2. The bottom line in plot A shows that the PGH-PGE isomerase activity isolated from microsomes prepared using 10 mM 2-mercaptoethanol instead of GSH is significantly reduced.

Crude estimates of the Km of PGH-PGE isomerase for PGH_2 and GSH were determined from Lineweaver-Burke plots of the reaction velocity at three different concentrations of PGH_2 or GSH (Figures 16 and 17; Chapter V). The Km for PGH_2 at pH 8.0 and 0.5 mM GSH was 387 uM. The Km for GSH at pH 8.0 and 48 uM PGH_2 was 40 uM, and was 170 uM at 640 uM PGH_2.

pH Activity Profile. The pH activity profile of immobilized PGH-PGE isomerase appears as a broad bell-shaped curve, with optimal isomerase activity occurring between pH 7.0 and 8.1 (Figure 13). All buffers used in this determination were within 1 pH unit of their pKa value, and the pH range in which a buffer was used was overlapped with the next buffer, so that any effects of a particular buffer on the enzyme activity could be observed. The only "buffer effect" was observed when using borate buffer. PGE_2 formation at pH 8.8 was greater with enzyme in Tris-chloride buffer than in borate buffer. In the reactions with borate buffer, both with and without enzyme, a much larger percentage of the radioactivity than usual was found at the origin of the TLC plates. This might be due to borate complexing with PGH_2 or with one of its degradation products. Below pH 7.0 PGH-PGE isomerase activity decreased rapidly, and no activity could be measured below pH 4.5 (observed in previous experiments). The activity decreased gradually between pH 8.4 to 9.2.

Stability of Immobilized PGH-PGE Isomerase. PGH-PGE isomerase activity, both in the presence and absence of 1 mM GSH, was measured at several times during a two hour incubation of the enzyme on ice (Figure 14). Immobilized PGH-PGE isomerase

Figure 13. pH activity profile of immobilized PGH-PGE isomerase. 17 ul of gel in a final volume of 51 ul MSB was added to 50 ul of the different buffers: (+)borate, (o)Tris, (*), HEPES, (x)MES. The final pH of the reactions is indicated in the figure. Reactions were initiated by the addition of 6.44 nmol PGH2, and were carried out for 25 sec.

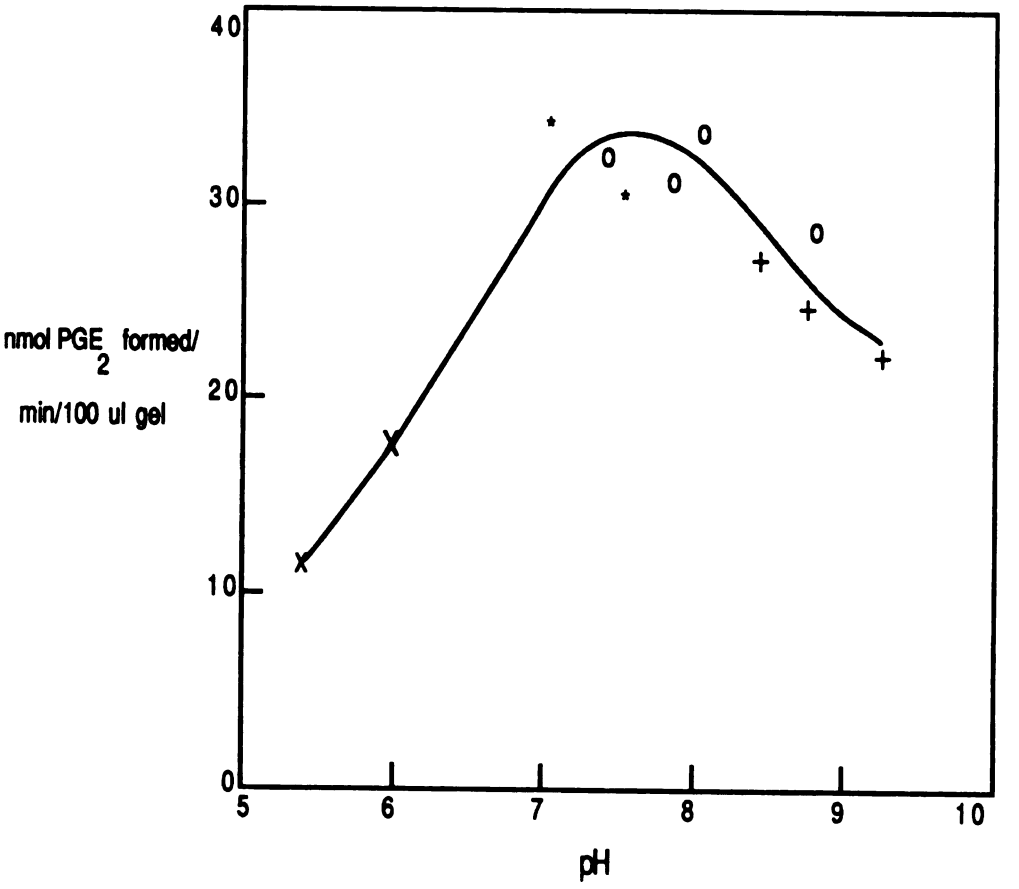


Figure 13

was very stable over the two hour time period when incubated in the presence of GSH, showing only a 7% loss in activity. However, in the absence of GSH the enzyme lost one-half of its initial activity. In a similar experiment, immobilized PGH-PGE isomerase retained 91% of its initial activity over a 4 hr incubation on ice, when incubated in the presence of 1 mM GSH, but lost 72% of its activity in a 24 hr incubation. Addition of fresh GSH during the 24 hr incubation might stabilize the immobilized isomerase activity, as it did with the solubilized enzyme (Chapter III), but that was not tested.

Inhibitors of PGH-PGE Isomerase. Three types of compounds were tested for their effect on the activity of immobilized PGH-PGE isomerase: a non-steroidal antiinflammatory drug, three different GSH analogs, and various substrate analogs synthesized by The Upjohn Company.

Non-steroidal antiinflammatory drugs, which are competitive inhibitors of cyclooxygenase, also partially inhibited PGH-PGE isomerase activity in solubilized microsomes (Chapter III). Flurbiprofen (FBP) at 10^{-3} and 10^{-4} M inhibited about 50% of the total PGH-PGE isomerase activity in solubilized microsomal proteins. Since the enzyme immobilized on IgG_1(*hei*-7)-Protein A-agarose comprises at least 40% of the total PGH-PGE isomerase activity of solubilized microsomes, it was possible that FBP was exclusively inhibiting this enzyme. However, when tested on the immobilized PGH-PGE isomerase, 1 mM FBP in reactions containing 34 uM PGH_2 and 1 mM GSH inhibited PGH-PGE isomerase activity by only 47% (Figure 15).

Three S-blocked GSH analogs, S-methyl GSH, S-hexyl GSH, and S-*p*-chloro-phenacyl-GSH, showed no inhibition of PGH-PGE isomerase activity when using 0.5 to 2.5 mM GSH analog (also 7.5 mM S-methyl GSH) and 0.5 mM GSH in the reactions.

Ten different substrate analogs and one PGE_2 analog were also tested as inhibitors of immobilized PGH-PGE isomerase. In this experiment, the PGH_2 analog stock

Figure 14. Stability of immobilized PGH-PGE isomerase activity incubated in the presence of 1 mM GSH (*), or in the absence of GSH (x). Immobilized PGH-PGE isomerase was prepared as usual in two columns. Both columns were washed with 10 vol of MSB+NaCl and 6 vol of MSB(-GSH). One column was then washed with 6 vol MSB containing 1 mM GSH, and the other with 6 vol MSB without GSH. The columns were incubated at 4^{o}, and aliquots of the gels were assayed for PGH-PGE isomerase activity at the indicated times. PGE2 formation in identical reactions without enzyme were subtracted from the enzymatic reactions before the data was plotted.

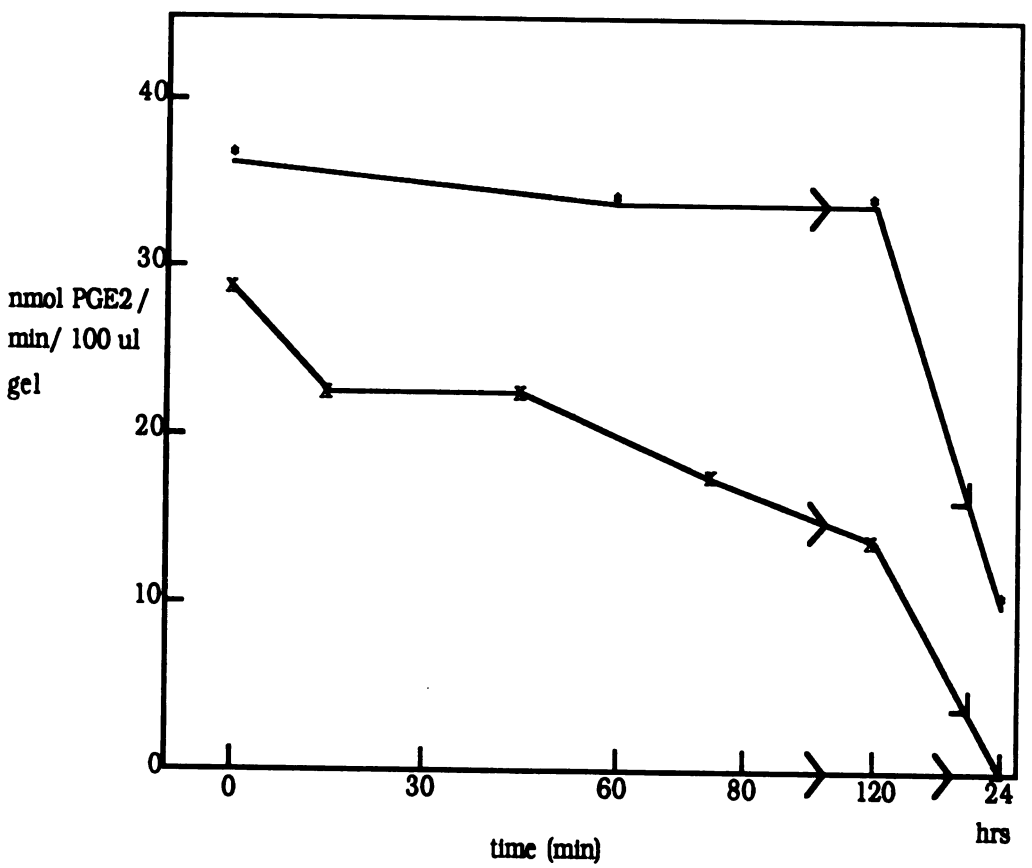

40
30
20
10
0
nmol PGE2 /
min/ 100 ul
gel
0
30
60
80
120
24
hrs
time (min)

Figure 14

Figure 15. Partial inhibition of immobilized PGH-PGE isomerase activity by flurbiprofen. Increasing amounts of FBP were added to 17 ul aliquots of gel (with or without immobilized PGH-PGE isomerase), and the reactions were initiated by the addition of 3.4 nmol PGH2. PGE2 formation in the nonenzymatic reactions was subtracted from the enzymatic reaction results.

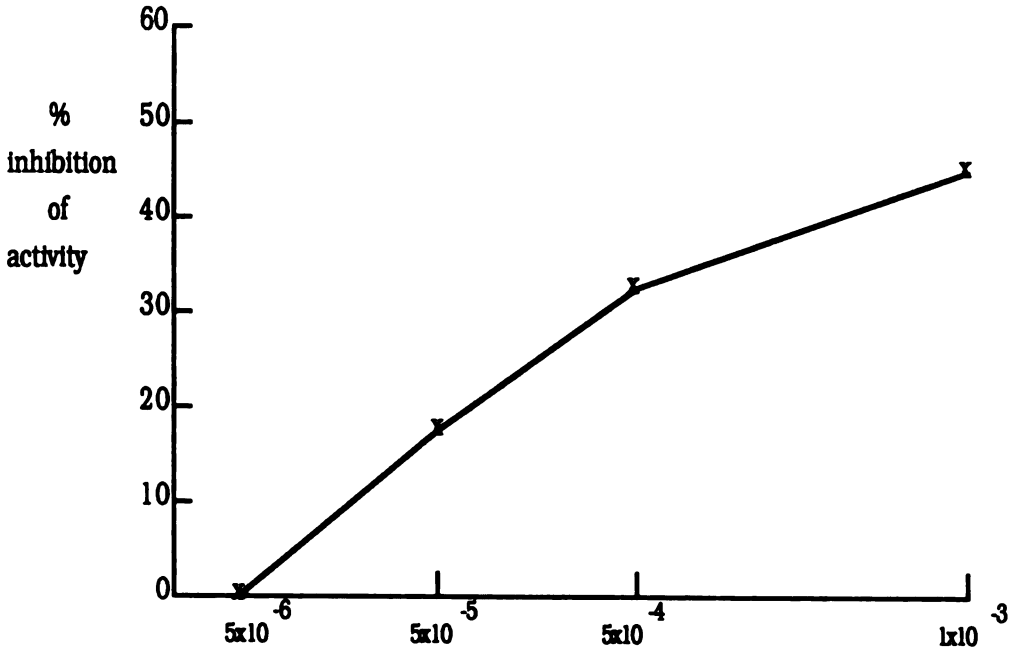
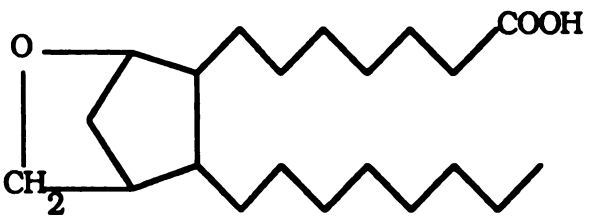
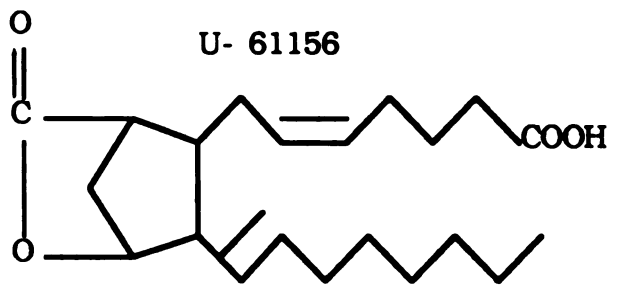
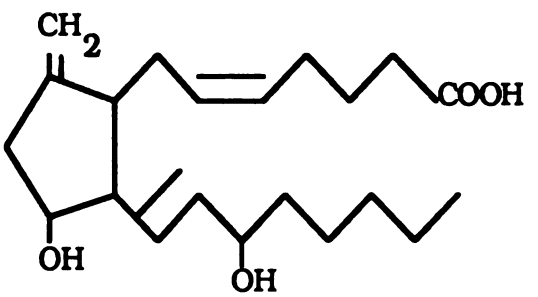

60
50
40
30
20
10
0
% inhibition of activity
5x10 -6
5x10 -5
5x10 -4
1x10 -3
- log [FBP]

Figure 15

solutions were prepared at 50 mM in absolute ethanol, and 1 ul was added to the 100 ul reaction mixtures (for a final concentration of 0.5 mM). Control reactions also contained 1 ul ethanol. PGH_2 (48 uM) was then added to initiate the reactions. Nonenzymatic reactions containing the inhibitors were also performed, and the amounts of product formed were subtracted from the values obtained in the enzymatic reactions. All of the compounds tested inhibited PGH-PGE isomerase by at least 20 or 30%, except for the commonly used thromboxane receptor antagonist U-44069, which had no effect on PGH-PGE isomerase activity. The structures of the three analogs having the greatest inhibitory effect and the percent of inhibition by them is listed in Table 7. U-48271 was the most effective inhibitor, but only inhibited 58% of the activity of the immobilized PGH-PGE isomerase. U-61156 and U-51570 also inhibited about one-half of the enzyme activity. These three analogs do not appear to have any structural features in common.

Table 7. Substrate analogs that inhibit immobilized PGH-PGE isomerase.

Analog Name and Structure	% inhibition
U-48271 	58
U- 61156 	53
U-51570 	45

DISCUSSION

Only one microsomal protein (M_r = 17,500) appeared to be bound to IgG_1(*hei*-7)-Protein A-agarose as determined by elution and separation of the proteins by SDS-PAGE (Figure 11), and only IgG_1(*hei*-7)-Protein A-agarose samples containing this bound protein catalyzed the formation of PGE_2 from PGH_2. However, the possibility exists that other proteins that do not stain with silver reagent, or that are hidden under one of the other protein bands could also be present. An additional control, in which microsomes are passed over a column containing a nonspecific IgG_1, should also have been included in this experiment.

This immobilized PGH-PGE isomerase, required GSH for optimal activity, and showed linear product formation both over time and with the amount of enzyme present in the reaction (Figure 12). The immobilized enzyme was stable for 4 hrs on ice when incubated in the presence of GSH, but lost about one-half of its activity when GSH was absent from the incubation buffer (Figure 14), indicating that, as in the case of solubilized microsomes, GSH stabilizes the enzyme.

The activity of the immobilized PGH-PGE isomerase was optimal between pH 7.0 and 8.1 (Figure 13). In contrast, PGH-PGE isomerase activity in solubilized microsomes was greater at pH 8.0 than at pH 7.0 (observation only). The pH-activity profiles of immobilized enzymes often show displacements relative to the pH-activity profiles of the soluble enzymes (111,112). These effects can usually be explained by factors that act to limit proton diffusion or cause proton partitioning in the system. Substrate diffusion limitation can also effect the pH profile, causing a broadening of the activity peak. Unfortunately, the activities of the immobilized and soluble forms of the

IgG_1(*hei*-7)-reactive PGH-PGE isomerases cannot be directly compared. The enzyme has not been purified in soluble form in the absence of IgG_1(*hei*-7), and solubilized microsomes cannot be used for comparison because more than one protein in the microsomes has PGH-PGE isomerase activity (Chapter II).

The term Km apparent (Kmapp) should be used here to describe the immobilized enzyme activity in place of the Michaelis-Menton constant, Km. The Kmapp for the substrate, PGH_2, was 387 uM at pH 8.0 and 0.5 mM GSH. The Kmapp for the cofactor, GSH, was 40 uM at pH 8.0 and 48 uM PGH2, and was 170 uM at 640 uM PGH_2. As with the pH-activity profile, the Michaelis constant for an immobilized enzyme is usually different from that of the free enzyme in solution (111,112). The Km of an immobilized enzyme is usually greater than that of the soluble enzyme due to diffusion phenomena. In the case of the IgG_1(*hei*-7)-reactive PGH-PGE isomerase, the Kmapp of 387 uM is about 9-fold greater than was reported for the PGH-PGE isomerase activity immunoprecipitated from solubilized microsomal proteins (5), and is much greater than the Km for PGH_2 of 10 uM reported in solubilized bovine microsomes (3).

Two non-steroidal anti-inflammatory drugs partially inhibited PGH-PGE isomerase activity in SVG microsomal proteins, and flurbiprofen also partially inhibited immobilized PGH-PGE isomerase (Figure 15). Their structures are very different than the substrate for PGH-PGE isomerase, so the mechanism of their effect is unknown. FBP and flufenamic acid are competitive inhibitors of cyclooxygenase, so the physiological significance of their effect on PGH-PGE isomerase activity is also unknown. Inhibition of PGH-PGE isomerase in addition to cyclooxygenase might be important in tissues like the renal cortex where the level of PGH-PGE isomerase activity to cyclooxygenase activity is very high (79).

The three S-blocked GSH analogs had no effect on the activity of immobilized PGH-PGE isomerase. Glyoxalase I, another enzyme that requires GSH as a cofactor, is inhibited by S-blocked GSH compounds (97). Glyoxalase I binds GSH at the active site

through three interactions (97). The tripeptide part of GSH binds at the active site, and is responsible for the substrate specificity of the enzyme. The second interaction is hydrophobic, and has been demonstrated by the increased inhibition of the enzyme as the alkyl chain length of S-substituted GSH analogs is increased. The third interaction of GSH with Glyoxalase I involves hydrogen bonding to metal-bound water molecules. Therefore, the SH function of GSH is not directly involved in the binding of GSH to Glyoxalase I, but is involved in the enzyme reaction. Thus, S-blocked GSH analogs inhibit the enzyme by binding at the active site. If GSH binds to PGH-PGE isomerase through the SH group, S-blocked GSH analogs would not inhibit the reaction because they would not be able to bind to the enzyme. Formaldehyde dehydrogenase, another enzyme that requires GSH as a cofactor, is not inhibited by the S-alkylglutathiones, but is inhibited by ophthalmic acid, a GSH molecule without the SH group (98). In this case, the S-alkylglutathiones are probably not bound to the enzyme due to steric hindrance by the alkyl group. The effect of ophthalmic acid on PGH-PGE isomerase was not determined.

Three substrate analogs were identified that inhibited about one-half of the PGH-PGE isomerase activity (Table 7). The degree of inhibition might be increased by increasing the ratio of inhibitor to PGH_2 in the reactions, or by preincubating the inhibitor with the enzyme. No common structural element among these three compounds could be identified. If GSH interacts with PGH_2, perhaps the element that they have in common is their ability to form a complex with GSH that binds to the enzyme.

CHAPTER V

THE ROLE OF GSH IN THE PGH-PGE ISOMERASE REACTION

GSH has previously been identified as a cofactor in the isomerization of PGH by microsomal PGH-PGE isomerase (3). However, the role of GSH in the PGH-PGE isomerase reaction has never been examined using purified enzyme. In the experiments described in this chapter, I have used PGH-PGE isomerase immobilized on IgG_1(*hei*-7)-Protein A-agarose to examine the effects of GSH on the stability and catalytic activity of the enzyme. The effect of sulfhydryl modifying reagents on the immobilized PGH-PGE isomerase was also examined.

MATERIALS AND METHODS

Materials. Mercuric chloride, *p*-hydroxy mercuribenzoate, methyl methanethiosulfonate, and 4,4'-dithiodipyridine were purchased from Sigma Chemical Co. All other materials were as described in Chapters II, III, or IV.

Isolation of PGH-PGE Isomerase. The procedure used for the immobilization of PGH-PGE isomerase by attachment to IgG_1(*hei*-7)-Protein A-agarose was as described in Chapter IV. In some experiments GSH was removed from the immobilized enzyme. To do this, agarose gel to which the enzyme was bound was washed with 10-12 volumes of MSB+NaCl, and then washed with 8-12 volumes of MSB without GSH. It would typically take 10-15 min from the beginning of the GSH-free wash until the samples were assayed for PGH-PGE isomerase activity. Other reagents were then added back to aliquots of the immobilized enzyme, or the columns were washed with 5-6 volumes of buffer containing these reagents.

PGH-PGE Isomerase Activity Assays. This procedure was as described in Chapter IV.

Quantitation of GSH. Immobilized enzyme (and gel without enzyme) was prepared as usual in buffer containing either 50 uM GSH or no GSH. PGH-PGE isomerase activity assays containing 17 ul aliquots of gel, with and without enzyme, were performed for 0, 30, and 60 seconds at room temperature. The concentration of PGH_2 in each reaction was 50 uM. Reactions were stopped by acidification to pH 3.0 with 18 ul of

100 mM citric acid, and the supernatants were separated from the gels by centrifugation for 30 sec. Then 60 ul of the 118 ul reaction volume was added to 190 ul of a solution containing 125 uM 4,4'-dithiodipyridine (4,4'-DTDP) in 25 mM KH_2PO_4. The absorbance was measured at 324 nm exactly 2 min after the samples were added to the 4,4'-DTDP solution, and the amount of GSH in the samples was calculated using an extinction coefficient of 19,600 M^{-1}. Reaction blanks (i.e., gels with and without enzyme reacted for 0, 30, or 60 sec, but without GSH) were used to zero the spectrophotometer before each identical reaction containing GSH.

RESULTS

The Effect of Increasing GSH Concentrations on PGH-PGE Isomerase Activity. PGH-PGE isomerase activity was measured as a function of the concentration of GSH at different concentrations of PGH_2 (Figure 16). The Km for GSH of immobilized PGH-PGE isomerase appeared to increase as the concentration of PGH_2 increased. The Km for GSH at pH 8.0 was 40 uM at 48 uM PGH_2, and was 170 uM at 640 uM PGH_2. The Km for PGH_2 was 387 uM at 0.5 mM GSH (Figure 17). The few other data points obtained indicate that the Km for PGH_2 may remain at about the same value for concentrations of GSH greater than or equal to 50 uM. The small amount of PGE_2 formation observed in the reactions containing less than 5.0 uM GSH was probably due to the intrinsic activity of the enzyme, and, as shown in the next paragraph, does not represent time-dependent product formation by the enzyme. Intrinsic enzyme activity is the activity remaining when exogenous GSH is not present in the enzymatic reaction, and may represent isomerase activity due to some GSH that is tightly bound to the enzyme.

The time course of the PGH-PGE isomerase reaction in the presence and absence of exogenous GSH is shown in Figure 18. In this experiment, 17 ul of gel (either with or without bound enzyme) was assayed using 0 or 0.5 mM GSH at 0, 30, and 60 sec. PGE_2 formed in the nonenzymatic reactions was subtracted from the enzymatic reactions before the data were plotted. PGH-PGE isomerase in the presence of 0.5 mM GSH showed increasing product formation over time. However, PGH-PGE isomerase without exogenous GSH showed the same level of activity at 0, 30, and 60 seconds. When looking at only 30 sec reactions, as in the above kinetic data, there appears to be PGE_2 formation by PGH-PGE isomerase in the absence of GSH, when, in fact, there is actually

Figure 16. PGH-PGE isomerase activity as a function of the concentration of GSH at different concentrations of PGH2. Immobilized PGH-PGE isomerase was washed and resuspended in GSH-free buffer, and 17 ul aliquots of gel were removed for enzyme assays. The different concentrations of GSH were then added back to the samples, and the samples without GSH were assayed first. PGE2 formation in nonenzymatic reactions was subtracted from the enzymatic results.

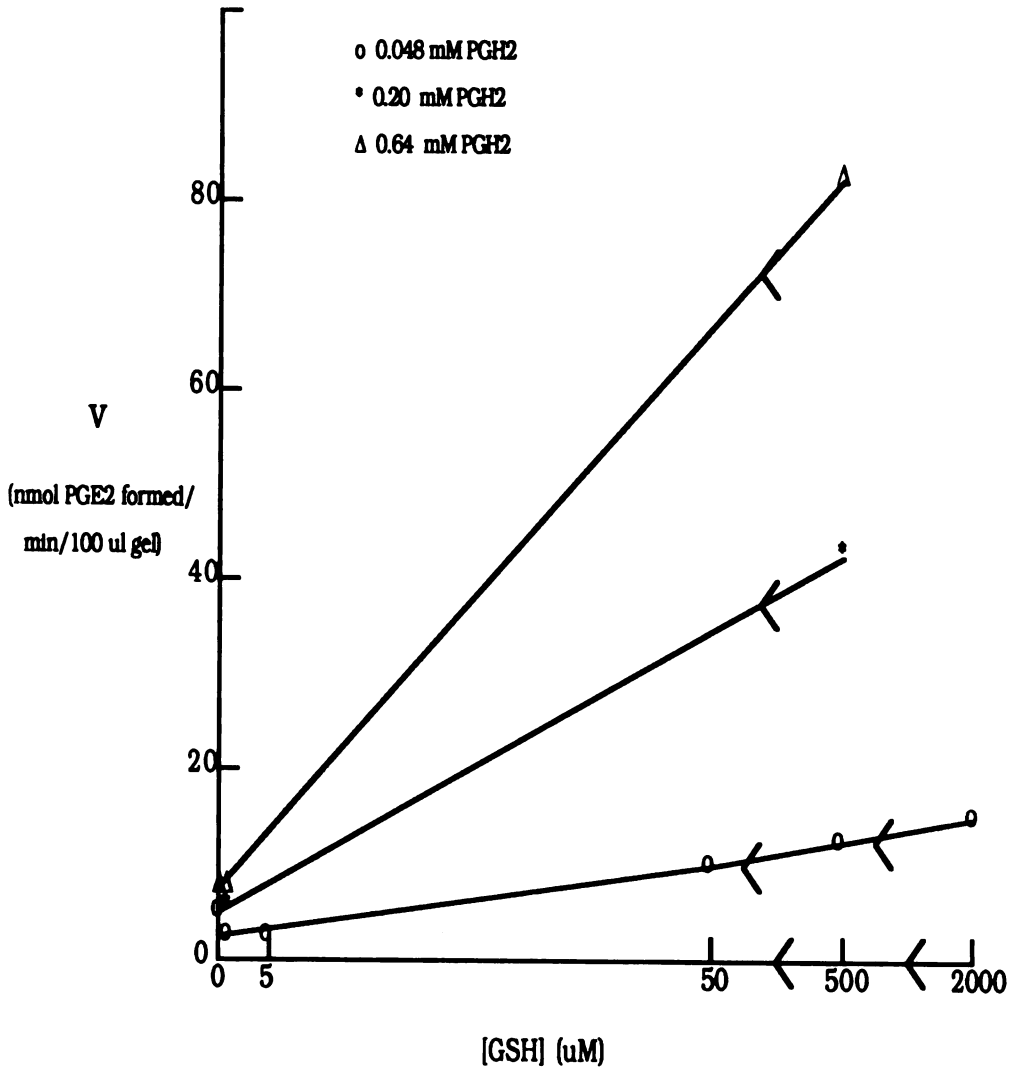

o 0.048 mM PGH2
* 0.20 mM PGH2
Δ 0.64 mM PGH2
V
(nmol PGE2 formed/
min/100 ul gel)
80
60
40
20
0
0
5
50
500
2000
[GSH] (uM)

Figure 16

Figure 17. PGH-PGE isomerase activity as a function of PGH2 concentration at different concentrations of GSH. Experimental details are as described in Figure 16.

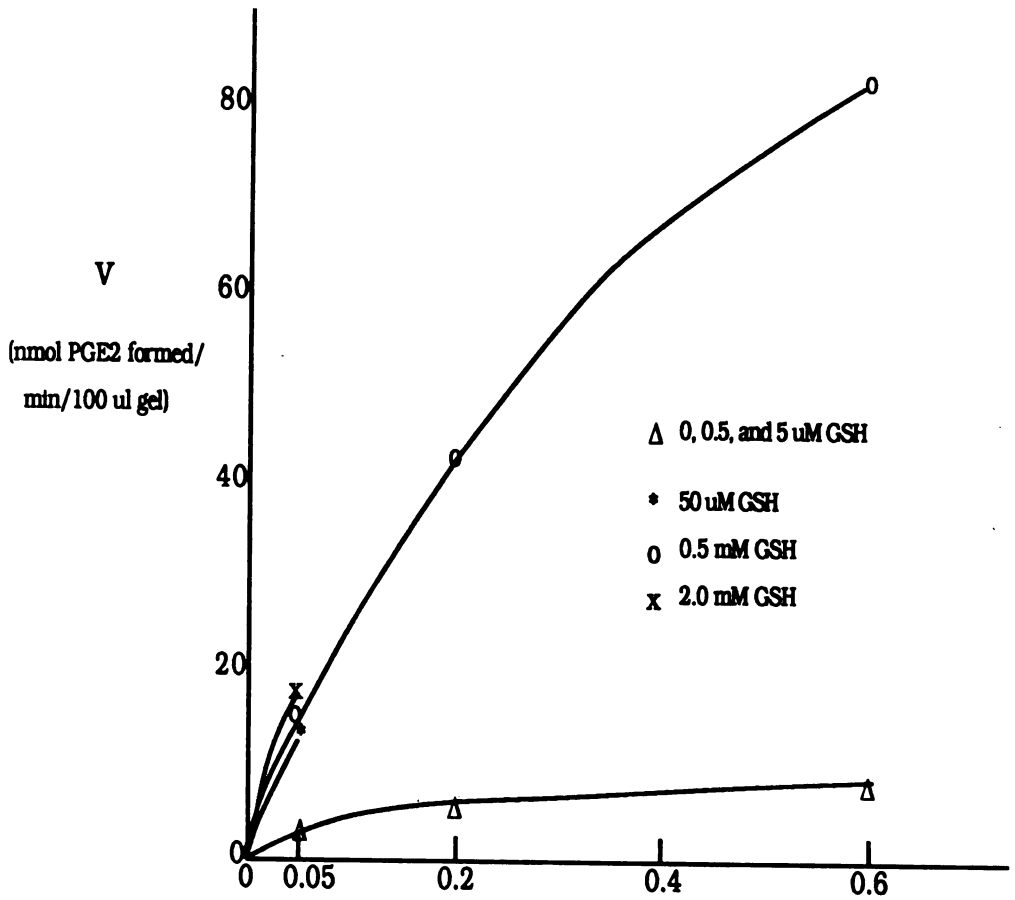

V
(nmol PGE2 formed/
min/100 ul gel)
80
60
40
20
0
0
0.05
0.2
0.4
0.6
Δ 0, 0.5, and 5 uM GSH
* 50 uM GSH
o 0.5 mM GSH
x 2.0 mM GSH
[PGH2] (mM)

Figure 17

Figure 18. PGH-PGE isomerase shows no time-dependent formation of PGE2 in the absence of GSH. PGH-PGE isomerase bound to 17 ul of gel, in the presence or absence of 50 uM GSH, was assayed for isomerase activity in reactions of 0, 30, and 60 sec. Reactions were initiated by the addition of 4.8 nmol ^{3}H-PGH2. PGE2 formation in identical nonenzymatic reactions were subtracted from the enzymatic results.

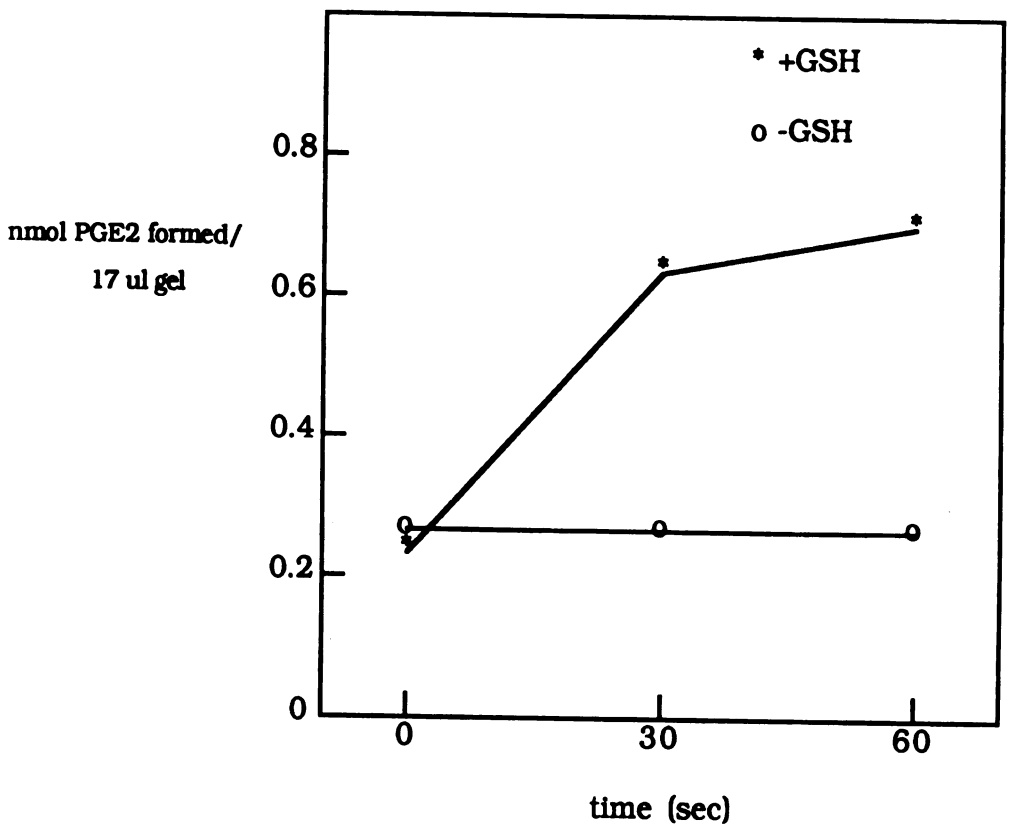


Figure 18

no time-dependent formation of PGE_2 without GSH.

GSH is Specifically Required by PGH-PGE Isomerase for Both Activity and Stability. GSH was removed from the immobilized enzyme, and aliquots of the gel placed into test tubes containing GSH, DTT, cysteine (final concentrations were 1 mM), or no thiol compound. Samples without enzyme were prepared identically. Aliquots of 17 ul of gel incubated with the different thiol compounds were then assayed for PGH-PGE isomerase activity both in the presence and absence of 1 mM GSH (Figure 19). In the presence of DTT or without any thiol, PGH-PGE isomerase activities were 22% and 16%, respectively, of the isomerase activity in the presence of 1 mM GSH. When cysteine was substituted for GSH in the reaction no product formation could be measured.

In the same experiment, the stability of the PGH-PGE isomerase activity was measured after a two hour incubation of the enzyme in the presence of 1 mM GSH, DTT, cysteine, or in the absence of thiol (Figure 20). DTT and cysteine stabilized the isomerase activity over the two hour period only slightly more than buffer containing no thiol compound, in which only 48% of the initial isomerase activity was retained. PGH-PGE isomerase incubated in the presence of 1 mM GSH, however, maintained 93% of its initial activity after a 2 hour incubation on ice. Although only two thiol compounds were tested, this data implies that the PGH-PGE isomerase specifically requires GSH for both maximal activity and for stability.

S-Methyl GSH Cannot Replace GSH in the PGH-PGE Isomerase Reaction. Three columns containing immobilized PGH-PGE isomerase were washed with 8 volumes of MSB+NaCl and 6 volumes of GSH-free buffer, and then with 6 volumes of GSH-free buffer or buffer containing either 2 mM GSH or 2 mM S-methyl GSH. Aliquots of gel with the bound enzyme were removed and assayed for PGH-PGE isomerase activity in buffer containing either 2 mM or 4 mM GSH or 2 mM S-methyl GSH (Table 8). Control

Figure 19. GSH is specifically required for PGH-PGE isomerase activity. GSH was removed from the immobilized enzyme and replaced with no thiol, GSH, DTT, or cysteine. PGH-PGE isomerase activity of the enzyme in the different buffers was then measured in the presence and absence of GSH.

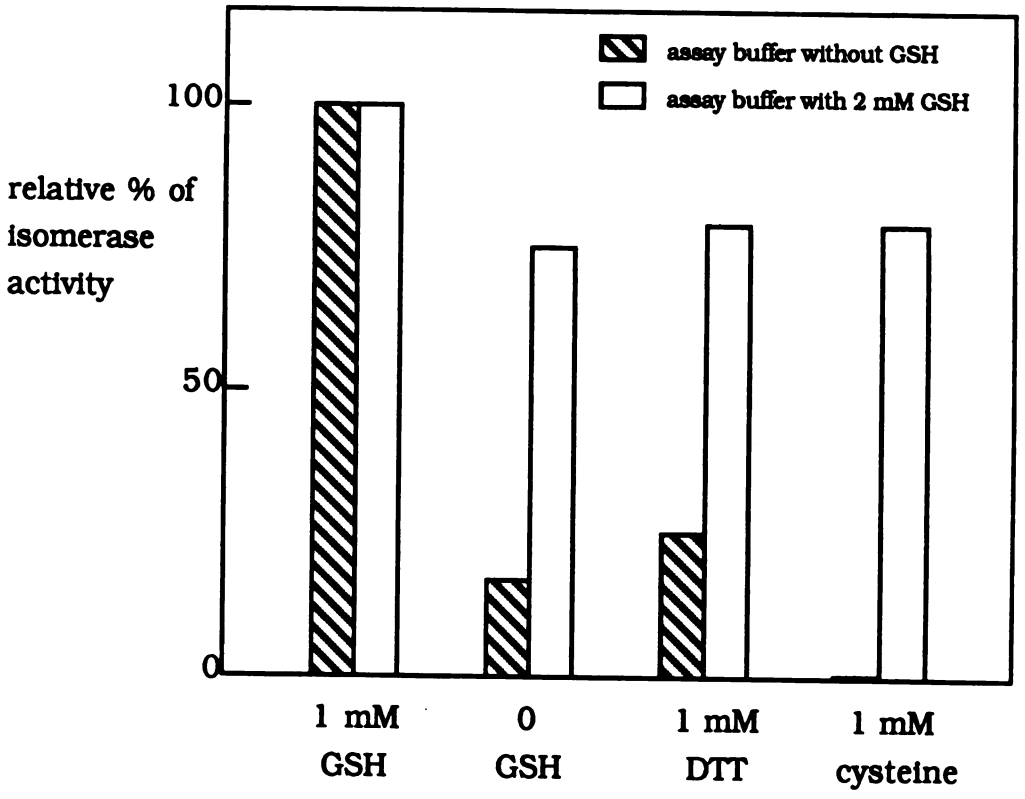

assay buffer without GSH
assay buffer with 2 mM GSH
100
50
0
relative % of isomerase activity
1 mM GSH
0 GSH
1 mM DTT
1 mM cysteine

Figure 19

Figure 20. GSH is specifically required for the stability of PGH-PGE isomerase. Immobilized PGH-PGE isomerase was incubated in buffers containing 1 mM GSH, DTT or cysteine, or in buffer without thiol. PGH-PGE isomerase activity bound to 17 ul of gel was measured at the indicated times using assay buffer containing 2 mM GSH.

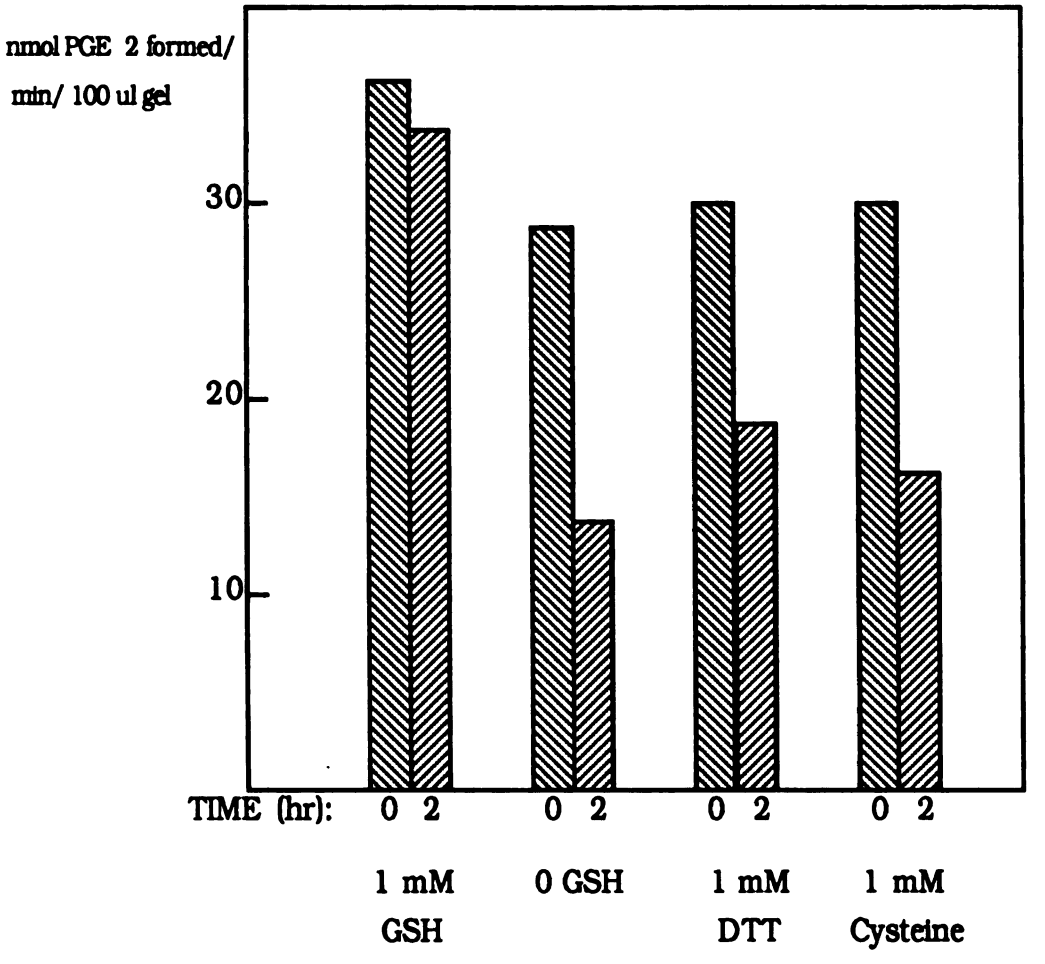

nmol PGE 2 formed/
min/ 100 ul gel
30
20
10
TIME (hr):
0 2
0 2
0 2
0 2
1 mM
GSH
0 GSH
1 mM
DTT
1 mM
Cysteine

Figure 20

reactions without enzyme were subtracted from each result. All reactions containing 1 or 2 mM GSH showed about the same amount of PGH-PGE isomerase activity (tubes no. 1,2,4,5, and 6), regardless of the presence or absence of S-methyl GSH. However, when only S-methyl GSH was present in the reaction (tubes no. 3 and 7) only 15-25% of the isomerase activity relative to the activity in the presence of GSH was observed. This is about the same amount of activity that was measured in the absence of GSH in the previous experiment, and also on other occasions. Again, S-methyl-GSH showed no inhibitory effect on PGE_2 formation when present in the assay in equimolar amounts with GSH. These results indicate that S-methyl GSH, like cysteine and DTT, cannot substitute for GSH in the PGH-PGE isomerase reaction.

GSH is not Consumed During the PGH-PGE Isomerase Reaction. No oxidation of GSH occurred during the course of the PGH-PGE isomerase reaction when thiol concentrations were monitored using the aromatic disulfide 4,4'-DTDP (4,4'-dithiodipyridine). Reactions were performed in the presence of 50 uM GSH and 50 uM PGH_2. Details of the procedure are given in the Methods section of this chapter. No change in absorbance was detected over the time course of the reaction in samples with or without PGH-PGE isomerase, indicating that GSH is not oxidized during the reaction (Figure 21). PGH-PGE isomerase activity by the immobilized enzyme used in this experiment was also determined.

Absorbance values at 234 nm for these samples ranged from 0.250 to 0.255, a variation of only 2%. At 60 sec about 10% of the PGH_2 in the enzymatic reaction had been converted into PGE_2. Therefore, this technique is sensitive enough to detect whether GSH is consumed stoichiometrically as PGE_2 is formed, but may not be sensitive enough to detect the oxidation of very small amounts of GSH during the reaction.

Sulfhydryl Modifying Reagents Inhibit PGH-PGE Isomerase. The effect on

Table 8. Substitution of S-methyl GSH for GSH in the PGH-PGE isomerase activity assay.

	Column Wash Buffer	Assay Buffer	nmol PGE2 formed/ min/17 ul gel	% of the 2mM GSH Reaction
1)	2 mM GSH	2 mM GSH	2.52	100
2)	2 mM GSH	2 mM S-Methyl	2.89	115
3)	2 mM S-Methyl	2 mM S-Methyl	0.62	25
4)	2 mM S-Methyl	2 mM GSH	2.50	99
5)	2 mM S-Methyl	4 mM GSH	2.47	98
6)	0 GSH	2 mM GSH	3.14	125
7)	0 GSH	2 mM S-Methyl	0.47	19 (15)

Three columns of PGH-PGE isomerase immobilized on IgG1(*hei*-7)-Protein A-agarose were washed with 6 volumes of GSH-free buffer, and then with 6 volumes of one of the three column wash buffers. 17 ul aliquots of gel (51 ul) were placed in test tubes, 50 ul of the specified assay buffer added, and each reaction started by the addition of PGH2. Control reactions using IgG1(*hei*-7)-Protein A-agarose without enzyme were subtracted from each enzymatic reaction.

Figure 21. GSH is not oxidized during the course of the PGH-PGE isomerase reaction. The concentration of GSH in the PGH-PGE isomerase reactions was determined using 4,4'-DTDP. The concentration of GSH in both the enzymatic and nonenzymatic reactions remained about 50 uM, the initial concentration, whether the reactions were stopped at 0, 30, or 60 sec (bars). The initial concentration of PGH2 in the reactions was also 50 uM. About 14% of the PGH2 was converted to PGE2 during the course of the reaction (——).

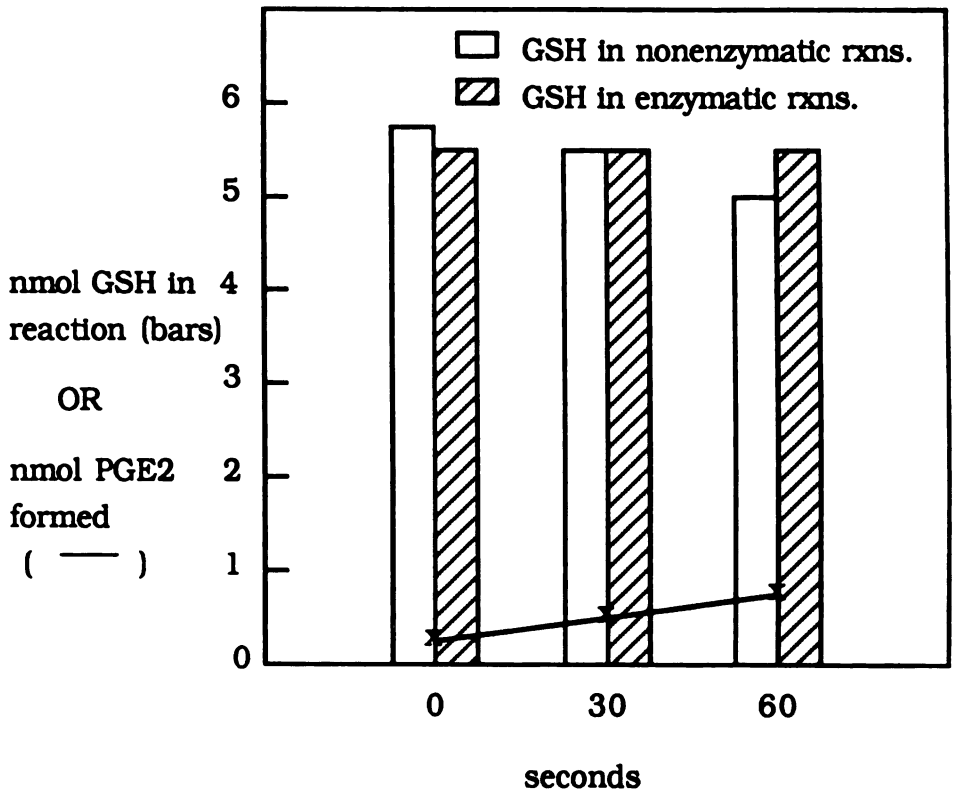

GSH in nonenzymatic rxns.
GSH in enzymatic rxns.
nmol GSH in reaction (bars)
OR
nmol PGE2 formed
(——)
6
5
4
3
2
1
0
0
30
60
seconds

Figure 21

PGH-PGE isomerase activity of three compounds that bind to protein sulfhydryl groups was determined (Figure 22). In this experiment, IgG_1(*hei*-7)-Protein A-agarose both with and without bound enzyme was washed with 8 volumes of GSH-free buffer. Aliquots of the gel were then incubated with the sulfhydryl reagents on ice for 8-10 min in the absence of GSH (unless otherwise indicated), and then assayed for isomerase activity in the presence of GSH. Mercuric chloride ($HgCl_2$), the only compound tested at 10^{-3} M, completely abolished PGH-PGE isomerase activity. At 10^{-4} M both $HgCl_2$ and methyl methanethiosulfonate (MMTS) inhibited PGH-PGE isomerase by about 50%. *p*-Hydroxy mercuribenzoate (*p*-HMB) was slightly less effective as an inhibitor, but all three compounds produced approximately the same degree of inhibition when tested at 10^{-4} and 10^{-6} M.

Figure 22. Sulfhydryl reagents inhibit PGH-PGE isomerase. GSH was removed from the immobilized PGH-PGE isomerase, and the enzyme was incubated with each of the three reagents for 8-10 min on ice. PGH-PGE isomerase activity was then measured in the presence of 1 mM GSH. The empty bars on the graph represent samples where 1 mM GSH was included in the incubation of the enzyme with the sulfhydryl reagent.

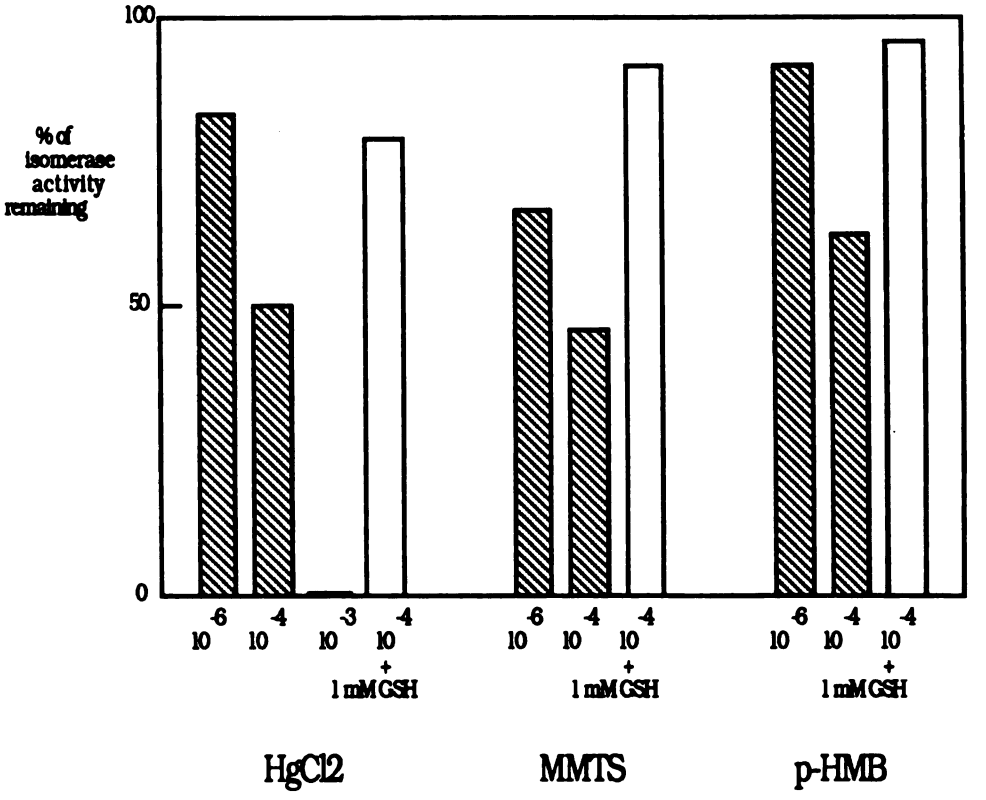

100
50
0
% of isomerase activity remaining
10 -6
10 -4
10 -3
10 -4
+ 1 mM GSH
10 -6
10 -4
10 -4
+ 1 mM GSH
10 -6
10 -4
10 -4
+ 1 mM GSH
HgCl2
MMTS
p-HMB

Figure 22

DISCUSSION

The Km for GSH increased as the concentration of PGH_2 increased in the PGH-PGE isomerase reaction, while the Km for PGH_2 remained constant for any concentration of GSH greater than or equal to 50 uM. While no firm conclusions can be made from the meager kinetic data presented in Figures 16 and 17, it appears that PGH-PGE isomerase does not exhibit Michaelis-Menton kinetic behavior with regard to the substrate, PGH_2, or with regard to the cofactor, GSH. Under physiological conditions the concentration of GSH is usually >1 mM (95). Since the Km for GSH is only 50 uM, the data indicates that the availability of the substrate, PGH_2, is the factor regulating the rate of the PGH-PGE isomerase reaction. Phenomena influencing these measurements due to the fact that an immobilized enzyme was used in the activity assays were discussed in Chapter IV.

PGH-PGE isomerase activity was assayed at three different reaction times in the presence and absence of exogenous GSH (Figure 18). PGH-PGE isomerase in the presence of 0.5 mM GSH showed a time-dependent increase in the formation of PGE_2. When GSH was removed from the reaction, however, the small amount of PGE_2 formed at time zero did not increase. Intrinsic activity of the enzyme due to the binding of some GSH at or near the active site of the PGH-PGE isomerase could explain these results. A small amount of intrinsic activity is obtained with enzyme that has bound GSH, but once the enzyme turns over the GSH diffuses away from the enzyme and cannot be replaced when there is no GSH (or very small concentrations of GSH) in the assay buffer. In the time zero reactions, the GSH already bound to the enzyme could react with enough substrate to produce the 15-20% product formation measured in the absence of exogenous

GSH. Such intrinsic activity by PGH-PGE isomerase could explain the constant, small amount of PGE_2 formed in all the reactions containing 0 to 5 μM GSH (Figure 17).

Cysteine and DTT could not replace GSH as a cofactor in the PGH-PGE isomerase reaction (Figure 19). When DTT was substituted for GSH, PGH-PGE isomerase activity was only slightly greater than when no GSH was present in the reaction. However, when cysteine was substituted for GSH in the reaction, the the isomerase activity measured in the 0 GSH reaction was abolished. It is possible that the large excess of cysteine, in the absence of GSH, could displace enzyme-bound GSH. Cysteine does not appear to have any other effect on PGH-PGE isomerase activity, because about the same amount of enzyme activity was measured in the cysteine and DTT buffers when GSH was present in the activity assay.

Whether enzyme-bound GSH is responsible for the PGH-PGE isomerase activity observed in GSH-free buffer could be tested. The immobilized enzyme could be incubated with ^{35}S-GSH, and then washed with buffer with or without cysteine. If the loss of ^{35}S from the column coincided with the loss of PGH-PGE isomerase activity upon addition of cysteine to the wash buffer, it would support the hypothesis that GSH is binding to the PGH-PGE isomerase.

Analysis of the reaction products of PGH-PGE isomerase activity assays that contained DTT, in both the enzymatic and nonenzymatic reactions, showed that about 50% of the total radioactivity migrated below PGE_2 on the TLC plate. This section of the TLC lanes usually contains less than 8% of the total radioactivity. The identity of the compound migrating in this part of the TLC plate was not identified, but was probably $PGF_{2\text{-alpha}}$. The nonenzymatic formation of $PGF_{2\text{-alpha}}$ from PGH_2 in the presence of dithiols has been observed before (100). If DTT interacts in some way with PGH_2 to form $PGF_{2\text{-alpha}}$, that might explain why it cannot substitute for GSH in the isomerase reaction. Large amounts of $PGF_{2\text{-alpha}}$ were not formed in the reactions containing cysteine, however, and cysteine also could not substitute for GSH in the PGH-PGE isomerase

reaction. This observation though does raise the question of whether the different thiol compounds might interact with PGH_2 in different ways, and of whether a GSH-PGH_2 intermediate is involved in the PGH-PGE isomerase reaction. GSH does form adducts with PGA_2 (114), and with the substrates of the other reactions in which it is involved as a cofactor (95-99). A possible intermediate would be a hemimercaptal formed between GSH and PGH_2, the formation of which could be monitored by measuring the absorbance 240 nm (115). It is not known whether the enzyme would have to be present for the formation of such a complex. One experiment was performed where PGH_2 and GSH were mixed without enzyme and the absorbance at 240 nm monitored. However, the PGH_2 degraded so rapidly in the aqueous solution that no stable absorbance values could be measured. Also, the concentration of PGH_2 that would probably be required in the reaction to monitor hemimercaptal formation would be greater than the concentration that is soluble in aqueous solution. If such an intermediate exists, it could possibly be isolated from the acidified aqueous phase of the enzyme reaction. Many ^3H-compounds would probably be present in the aqueous phase, so the use of ^{35}S-GSH would greatly enhance the isolation of a GSH-PGH_2 adduct. Also, adduct formation could possibly be predicted from kinetic analyses as it was for Glyoxalase I (113).

GSH was also specifically required for the stability of immobilized PGH-PGE isomerase activity (Figure 20). No precedent could be found where a specific thiol compound was required by an enzyme only for the maintenance of the thiol status of the enzyme. It seems more likely that GSH is specifically required to stabilize PGH-PGE isomerase activity because it is a cofactor binding at or near the active site of the enzyme, rather than the possibility that it is just nonspecifically maintaining the thiol status of the enzyme. For example, the presence of the cofactor, NADH, when covalently attached at the active site of alcohol dehydrogenase has been shown to have the effect of stabilizing enzyme activity (116,117). S-methyl GSH could not substitute for GSH in the PGH-PGE isomerase reaction (Table 8), which indicates either that the SH group of GSH is involved

in the catalysis, or that the methyl group sterically hinders the binding of S-methyl GSH to the enzyme (See the discussion regarding formaldehyde dehydrogenase in Chapter IV.)

Results obtained by previous investigators (3) were confirmed when measurements showed that GSH was not oxidized during the course of the PGH-PGE isomerase reaction. The previous determination, however, had been made using only a partially purified enzyme.

Three compounds that bind to sulfhydryl groups in proteins were found to inhibit PGH-PGE isomerase in a dose-dependent manner (Figure 22). The two mercury compounds, $HgCl_2$ and *p*-HMB, bind irreversibly to protein sulfhydryl groups (118). $HgCl_2$ can also interfere with reactions in which a thiolester is formed (95). MMTS, on the other hand, is a very small molecule that binds reversibly to free sulfhydryl groups (119). It is probably the least likely sulfhydryl-modifying reagent to cause inhibition of enzyme activity for reasons other than the blocking of SH groups. Other larger groups can cause inhibition of activity due to steric hindrance, or due to perturbation of the three dimensional structure of the protein. The fact that all three reagents produced about the same degree of inhibition of isomerase activity indicates that they are probably causing their effect by blocking some protein sulfhydryl group(s) required for PGH-PGE isomerase activity. The problem here is that the effect of the three reagents cannot be directly compared unless the kinetics of inhibition by each reagent are known. The degree of inhibition of PGH-PGE isomerase by the sulfhydryl reagents would probably be even greater if their time of incubation with the enzyme were increased. PGH-PGE isomerase was incubated with the sulfhydryl reagents for only 8-10 min. A more commonly used incubation time is 60 min. But even with the short incubation times used in this experiment, significant inhibition was obtained suggesting a role of enzyme sulfhydryl groups in the PGH-PGE isomerase reaction.

Many of the results in this chapter confirm data obtained by other investigators using solubilized microsomes, or partially purified enzyme. But there are several pieces of

data that differ from previous reports. The kinetic data show a lower Km for GSH and a higher Km for PGH_2 than was reported previously. Ogino *et al.*(3) reported that thiol compounds other than GSH, at a concentration of 1 mM, stabilized PGH-PGE isomerase activity during a two hour incubation on ice. Such an effect was not observed here. Ogino *et al.* had used only a partially purified bovine enzyme in their experiments, which could account for these differences.

SUMMARY

Glutathione had previously been proposed to be a cofactor in the enzymatic formation of PGE from PGH by microsomal PGH-PGE isomerase. The enzyme, however, had never been purified to verify the role of GSH in the reaction. Three monoclonal antibodies had been prepared by Dr. Tanaka that precipitated PGH-PGE isomerase activity from solubilized SVG microsomes. I have identified the antigens reactive with two of the antibodies. The immunochemical and kinetic studies of these two proteins indicate that they are different enzymes.

The two most important factors for the stabilization of PGH-PGE isomerase activity in solubilized microsomes were determined to be: 1) solubilization of the microsomal proteins with 10 mM CHAPS rather than with Triton X-100; and 2) maintenance of fresh GSH in the solubilized proteins. Attempts to purify PGH-PGE isomerase by immunoaffinity chromatography have been unsuccesful. However, the protein reactive with IgG_1(*hei*-7) has been isolated from the other microsomal proteins by immobilizing the antibody and enzyme on Protein A-agarose. This immobilized enzyme requires GSH for activity and stability, and shows linear product formation both over time and with increasing amount of enzyme. By using such an immobilized enzyme, GSH can be removed from the enzyme, to examine its role in the PGH-PGE isomerase reaction. Some non-steroidal antiinflammatory drugs and PGH_2 analogs were shown to partially inhibit PGH-PGE isomerase.

The data obtained on the role of GSH in the PGH-PGE isomerase reaction indicates that GSH is a cofactor in the reaction. When GSH is removed from the enzyme all time-dependent formation of PGE_2 is abolished. DTT or cysteine cannot substitute for

GSH in the reaction. PGH-PGE isomerase loses about one-half of its activity over a two hour period when incubated without exogenous GSH. DTT and cysteine do not stabilize the enzyme to the extent that GSH does.

Unfortunately, sufficient evidence was not obtained to unequivocally define the mechanism by which GSH acts in the PGH-PGE isomerase reaction. It was confirmed that GSH is not oxidized during the course of the PGH-PGE isomerase reaction. The lack of inhibition by S-blocked GSH's suggests that the SH group of GSH may be involved in the binding of GSH to the enzyme. The inability of S-methyl GSH to substitute for GSH in the reaction suggests that the SH group of GSH may also be involved in the catalysis. However, another explanation is that the alkyl group may make the S-methyl GSH too bulky to fit into the enzyme binding site. Other GSH analogs need to be tested for these effects. Two good candidates to be tested are opthalmic acid and homoGSH. Opthalmic acid, a GSH without the thiol group, inhibits glyoxalase I and formaldehyde dehydrogenase, two other enzymes that require GSH as a cofactor. HomoGSH, a compound in which the glycine residue of GSH is substituted by ß-alanine, is the only thiol other than GSH that reacts with formaldehyde dehydrogenase.

Inactivation of PGH-PGE isomerase activity by sulfhydryl-modifying reagents has been observed by many investigators. I have verified that the PGH-PGE isomerase reactive with IgG_1(*hei*-7) is also sensitive to inactivation by sulfhydryl reagents. Especially significant is its inhibition by MMTS, a small compound that reversibly binds to protein SH groups. The effect of compounds that interact with dithiols, such as cadmium chloride and arsenite, should also be tested.

A lot of questions remain to be answered regarding the mechanism of action of GSH in the PGH-PGE isomerase reaction. The data do suggest that GSH is bound to the enzyme possibly through the SH group. Mixed disulfides of proteins with GSH are quite stable, and could be responsible for the specific stabilizing effect of GSH on PGH-PGE isomerase. No evidence for the formation of a GSH-PGH_2 adduct has been found. Due

to the swiftness of the reaction, any interaction between GSH and PGH_2 probably occurs at the active site of the enzyme.

One mechanism that fits with the current data, and is also compatible with the 1,2-hydride shift mechanism proposed by Lands *et al.* is shown in Figure 23. In this model, a dithiol is present at the active site of the enzyme. A sulfhydryl group of the enzyme exerts a nucleophilic attack on the substrate molecule to form a hemimercaptal intermediate. The reaction could then proceed by a 1,2-hydride shift in the substrate, followed by an intramolecular thiol/disulfide exchange at the active site.

Alternatively, the enzyme may bind GSH so that the SH group of GSH attacks the substrate. Further studies of the effects of GSH analogs in the PGH-PGE isomerase reaction may determine which mechanism is correct.

Figure 23. **Proposed mechanism for the role of GSH in the PGH-PGE isomerase reaction.**

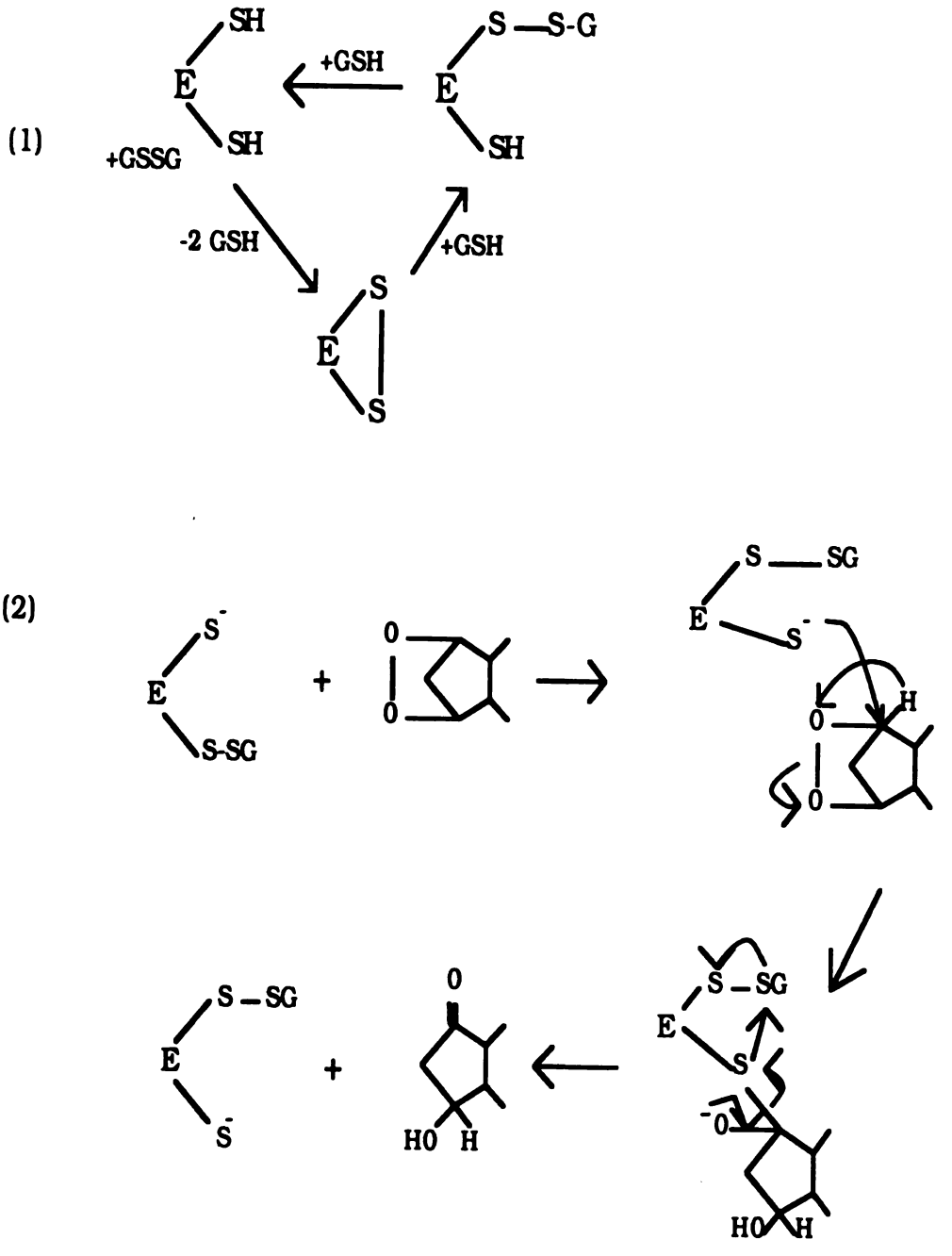

(1)
SH
E
SH
+GSH
S—S-G
E
SH
+GSSG
-2 GSH
+GSH
S
E
S
(2)
S⁻
E
S-SG
+
0
0
S—SG
E
S⁻
H
0
0
S—SG
E
S⁻
+
0
HO H
S—SG
E
S
-0
HO H

Figure 23

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