ELUCIDATING THE ROLE OF MEMBRANES IN MODULATING THE THERMODYNAMICS AND KINETICS OF SUBSTRATE BINDING TO THROMBOXANE SYNTHASE

BY

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THESIS

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ABSTRACT

Thromboxane synthase (CYP5A1) is a membrane-bound cytochrome P450. It synthesizes thromboxane A$_2$ (TXA$_2$) from PGH$_2$, which is a potent mediator of platelet aggregation and vasoconstriction. TXA$_2$ has been implicated in a wide range of pathophysiological disease conditions. Though it is a membrane protein, most of its biochemical characterization has been performed in soluble reconstituted systems. Membranes have been shown to play an important role in the structure and function of membrane proteins. This work presents the first report on membrane incorporated CYP5A1 and its biochemical characterization. To achieve this, native-like lipid bilayers called nanodiscs were used to obtain monomeric and stabilized protein. The protein was successfully expressed in a bacterial expression system and the purified protein was incorporated into nanodiscs. This system was functionally characterized using CO binding, substrate binding and activity assays. The specific activity of the protein was evaluated both in nanodiscs and detergent solubilized system and was shown to increase by 100% in Nanodiscs. In order to understand the role of the membrane in modulating the electrostatic environment of the protein, redox potentials of substrate free and substrate bound CYP5A1 in membranes was measured. This is also the first account of an endoperoxide substrate like PGH$_2$ bound to a protein. We noticed that the redox potential of the protein in Nanodiscs were not very different from those in detergents showing lack of modulation of the active site electrostatics by the membrane environment. The kinetics of substrate analog (U44069 and U46619) and cyanide binding to CYP5A1-nanodiscs was measured using stopped flow to evaluate the conformational change in the protein in lipid bilayers of Nanodiscs. It was determined that the kinetic parameters of binding of the substrate analogs to CYP5A1-nanodisc system were different from detergent solubilized state. Moreover, the accessibility of cyanide to the active site of the protein also changed in CYP5A1-nanodiscs as compared to detergent solubilized systems. These results show that the conformation of CYP5A1 is different in Nanodiscs which may account for the increased activity of the protein in the lipid bilayers.
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CHAPTER 1: INTRODUCTION TO THROMBOXANE SYNTHASE

Thromboxane synthase (CYP5A1) is an endoplasmic reticulum membrane protein that belongs to the cytochrome P450 superfamily of enzymes. In general, cytochrome P450 enzymes are mono-oxygenases that catalyze reactions involved in drug metabolism, synthesis of cholesterol, steroids and many other lipids. CYP5A1 is highly expressed in the lung, platelets, kidney, stomach, duodenum, colon and spleen. CYP5A1 is mainly known for its function in platelets and is involved in the production of Thromboxane A\(_2\) (TXA\(_2\)). TXA\(_2\) is a biologically active metabolite of arachidonic acid, which is a ω-6 fatty acid found in diet (Figure 1.1). It is formed from the prostaglandin H\(_2\) (PGH\(_2\)) by the action of CYP5A1. CYP5A1 expression has been found to be closely associated with inflammatory diseases. It is widely implicated in a range of disease conditions like cardiovascular disease, arthritis, cancer and many others due to its effects in promoting platelet aggregation and vasoconstriction. A wide variety of human cancers have found increased expression of cyclooxygenase (COX) in corresponding tissues thereby increasing interest in its downstream metabolites like TXA\(_2\).

1.1 Pharmaceutical potential of CYP5A1

Thromboxane synthase catalyzes the isomerization of PGH\(_2\) to the chemically unstable metabolite, TXA\(_2\). TXA\(_2\) is a potent inducer of vasoconstriction and platelet aggregation. There has been development of thromboxane synthase inhibitors and TXA\(_2\) receptor antagonists as TXA\(_2\) has been associated with various cardiovascular and pulmonary disease conditions. Its key role as a platelet activator and aggregator has been implicated in cardiovascular conditions like unstable angina, stroke and progression of ischemic injury after coronary artery occlusion. In other intestinal, renal and pulmonary tissues, it has been shown to affect blood flow distribution and airway caliber. Release of TXA\(_2\) in excess in these tissues has been shown to initiate ulcer, pulmonary hypertension, renal and bronchoconstriction. It has also been linked to pregnancy complications causing conditions like preeclampsia resulting in reduced uteroplacental blood and premature delivery (1, 2).

The evidence for the existence of an endoperoxide structure for the formation of prostaglandins was discovered as early as 1965, though it was not until 1973 that PGH\(_2\) was isolated. These endoperoxides were then seen to have distinctive effects on platelets and
smooth muscle contractions and were later classified as a new group of compounds called thromboxanes. TXA<sub>2</sub> was then detected and implicated in the contraction of porcine coronary arteries due to thrombin-stimulated platelets. TXA<sub>2</sub> was found to be an immediate response of activated platelets and that the release was complete in 15 seconds. Intensive research has been ongoing in this area to elucidate the biochemistry, physiology and pharmacology of these compounds (3).

**Role in Hypertension:** Studies with inhibitors of thromboxane synthase have suggested a possible role of thromboxane synthase in increasing blood pressure in spontaneously hypertensive rats. Thromboxane B<sub>2</sub>, which is a stable hydrolysis product of TXA<sub>2</sub> was measured in both the serum of whole blood and platelet enriched blood in experimental and control rats. Severe hypertension was seen to develop in rats with prior vascular damage leading to speculations that TXA<sub>2</sub> played an important role (4). Severely hypertensive rats were found to contain platelets with low survival, which when injected into stroke resistant spontaneously hypertensive (SRSH) rats showed normal survival patterns. This study suggested that the low platelet survival was due to vascular abnormalities. Injection of a thromboxane synthase inhibitor UK 38485 resulted in delaying severe hypertension in the experimental rats but did not prevent stroke. Therefore TXA<sub>2</sub> was shown to have a role in elevating the blood pressure but its sole inhibition did not prevent a stroke from occurring.

**Role in Coronary artery disease:** Formation of TXA<sub>2</sub> in unstimulated monocytes from patients with unstable angina, stable effort angina and control was studied to examine the role of TXA<sub>2</sub> in the occurrence of myocardial ischemia (5, 6). It was found that samples from patients with unstable angina have a higher concentration of TXA<sub>2</sub> than those with effort angina or controls. CYP5A1 inhibitor and TXA<sub>2</sub> receptor antagonist picotimide was found to be more effective in reducing the number of angina attacks, silent ischemic episodes and duration of ischemia even though it had a lower effect on the inhibition of TXA<sub>2</sub> formation in circulating monocytes than aspirin. Therefore TXA<sub>2</sub> formed by monocytes has been shown to have a significant role in the development of myocardial ischemia in patients with unstable angina.
**Role in Thrombolysis:** During coronary thrombolysis, a marked increase in platelet activation was seen which decreased the response of patients to thrombolytic therapy (7). Coronary thrombolysis is mainly used for the treatment of patients with acute Q wave myocardial infarcts. The main problems facing this therapy include the time required for the thrombolysis and re-occlusion of the artery after therapy. Inhibition of TXA₂ by aspirin or other combinations of drugs led to accelerated reperfusion and less cyclic flow variations. To test whether thrombin induced acute re-occlusion of infarct related arteries, argatroban, which is a thrombin inhibitor, was used on canine thrombolysis models. Increase in thromboxane B₂ were observed after reperfusion by the thrombin inhibitor. Therefore response to thrombin inhibitors was weakened by TXA₂ formation. This problem was again countered with the use of a TXA₂ inhibitor in combination with the thrombin inhibitors. Tissue type plasminogen activator is usually used for patients with acute myocardial infarction (8). Using this technique, reperfusion was seen to be followed by gradual cycles of occlusions followed by blood flow until a persistent occlusion occurred. TXA₂ and serotonin receptor antagonists in combination were found to prevent re-occlusions throughout experimental times.

**Role in Preeclampsia:** Preeclampsia is caused due to an imbalance between prostacyclin synthase and TXA₂. Plasma levels of prostacyclin and thromboxane were determined in normal, mild preeclampsic and severe preeclampsic pregnancies (9, 10). Normal pregnancies were seen to favor a higher prostacyclin to thromboxane ratio. The ratio was decreased slightly in mild preeclampsia and to a greater extent in severe preeclampsia. In both mild and severe preeclampsia, it was found that the prostacyclin levels were decreased to the same extent, but only in the severe cases was thromboxane shown to increase significantly.

**1.2 Review of Mechanism of action**

CYP5A1 along with Prostacyclin synthase (PGIS) belongs to a class of compounds called prostanoids, which are a family of key signaling molecules. They play a role in inflammation, autoimmune disorders, shock syndromes, physiologic regulation like growth and
differentiation. As these enzymes are synthesized and released on demand, they are subject to rapid formation and degradation making them difficult to study. CYP5A1 is considered a non-classical cytochrome P450. The classical cytochrome P450 enzymes are monooxygenases that are involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. CYP5A1 is considered a non-classical cytochrome P450 in the sense that it does not need an electron donor to catalyze its reaction. It catalyzes its reaction by isomerization of its substrate.

**Structural Studies:** Nucleotide sequence of human platelet cDNA was used to deduce the primary structure of CYP5A1. It is a 533 amino acid long chain with a molecular weight of 60487 Da. It only exhibits a maximum of 40% homologous identity with the rest of the P450s. It still has the highly conserved cysteine residue near the heme binding site of P450s was found at residues 472-492 (11). The mutant form of the protein where the cysteine was replaced by a serine was found to be inactive, though it was expressed at the same level (12). The gene expresses two transcript variants named TXS I and TXS II. TXS II was found to contain a 163 bp deletion at the cysteine containing peptide sequence during alternate splicing thus rendering it non-functional (13). Both forms of this enzyme were found to be present in different organs of the body. Thus this was concluded to be part of a regulatory mechanism to limit production of CYP5A1. Mutations in the residues, Asn-110, Trp-133, Arg-137, Arg-413, and Arg-478 were found to affect the heme binding of the protein and also reduced the enzymatic activity down to 5% of the wild type (14). The mutated proteins also lost their ability to bind substrate analogs and were more prone to digestion by proteases. Based on a few similarities to P450 terp, it was predicted that the A ring propionate in CYP5A1 was hydrogen bonded to Asn-110, Arg-413, and Arg-478 and the D ring propionate to Trp-133 and Arg-137.

**Polymorphisms:** The human CYP5A1 was found to contain 13 exons over a 150 kb range. The haploid human genome contained only one transcript of the CYP5A1 gene. Transcription of this gene was found to be mainly TATA dependent though it has multiple start points for transcription. The ninth exon of the gene contained a repetitive dinucleotide CA sequence that exhibited allelic polymorphisms (15).
Expression of soluble form: Though CYP5A1’s pharmaceutical potential has been identified; its reaction mechanism has not yet been extensively studied. This is primarily due to its tendency to aggregate and oligomerize in solution.

In order to better understand the mechanism of this protein, it has been purified in bacterial expressions systems. From the hydropathy plot of the protein sequence, the hydrophilic and hydrophobic residue profile was obtained. Using this plot the major transmembrane regions in the N-terminal region of the gene could be determined and modified to include more hydrophilic peptide sequences. A hydrophilic sequence, MAKKTSS was added to replace the major hydrophobic transmembrane region (16).

Characterization of recombinant CYP5A1: The modified CYP5A1 showed a typical Soret peak at 416 nm and purified modCYP5A1/2C5-his exhibited an absorption spectrum consistent with a low-spin P450, with a Soret peak at 416 nm and α and β bands at 567 and 534 nm, respectively. The dithionite-reduced protein with carbon monoxide bound showed a major peak at 450 nm and a shoulder at 420 nm which was eliminated in the difference spectra. This indicated that the heme was incorporated in the modified protein without any structural perturbation (17).

A coupled assay was also performed to determine whether PGH₂ would be converted to MDA and HHT. Formation of HHT and MDA, as indicated by the absorption maxima at 234 and 268 nm, respectively, was observed in the presence, but not in the absence, of the chimeric protein. The formation of reaction products TXB₂, MDA and HHT were monitored to assess their stoichiometry. The products were found in a molar ratio of 1.0:1.0:1.0 similar to the wild type CYP5A1. \( K_m \) and \( V_{max} \) were found to be 32.9 ± 5.5 μM and 2.33 ± 0.37 μmol MDA/min/mg of protein compared to the wild type CYP5A1 which had a \( K_m \) of 22.5 ± 4.5 μM and a \( V_{max} \) of 35.1 ± 3.2 μmol MDA/min/mg of protein. To assess the binding characteristics of the modified protein, absorption spectra of different substrate analogs and inhibitors were monitored. As expected, binding of substrate analogs showed a blue shift in the Soret peak and that of inhibitors showed a red shift. The dissociation constant of U44069 was 16 μM, close to that of wild type, 21 μM.
**Mechanism hypothesis:** CYP5A1 catalyzes approximately equal amounts of two distinct reactions when reacted with its substrate PGH₂.

\[ \text{PGH}_2 \rightarrow \text{TXA}_2 \]

\[ \text{PGH}_2 \rightarrow \text{HHT} + \text{MDA} \]

**Mechanism 1:** TXA₂ is the biologically active compound that is rapidly converted to its biologically inactive form, TXB₂. It is a potent vasoconstrictor and inducer of platelet aggregation. The first reaction occurs through the isomerization of PGH₂. The second reaction is a fragmentation reaction which results in the formation of 12-1-hydroxy-5, 8, 10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA). The biological functions of MDA and HHT are still unclear, though MDA has been found to form adducts with amino groups of proteins and DNA which have been detected in atherosclerotic lesions of human aorta. These products are formed in a 1:1:1 (TXA₂: MDA: HHT) ratio. Different mechanisms have been tried to justify the formation of these products. The initial reaction starts from arachidonic acid and from it the formation of PGH₂. PGH₂ contains a relatively weak epidioxy bond which is the reaction center for the initiation of all subsequent steps. It can be cleaved in different ways to yield a total of five different products (18). Two of the products, PGD₂ and PGE₂ are formed by the heterolytic cleavage of the epidioxy bond. Formation of TXA₂ is more complex. Heme thiolate ligands were suggested to be involved because of their ability to transfer electrons from peroxides to organic compounds (19). To elucidate the characteristics of substrate binding and catalytic mechanisms of the heme moiety, different substrates and inhibitors were bound to the protein and their absorption spectra studied. The addition of substrate did not convert the Fe (III) into a high spin complex as would be expected with other P450s. It was postulated that the epidioxy ligand replaced the water ligand at the sixth coordination center and hence only subtle spectral changes could be observed through substrate binding. From inhibitor binding studies, it was concluded that the substrate bound to the Fe center through its epidioxy bridge and the side chain was above the heme plane at an angle.

Ortho substituted inhibitors did form a high coordination, high spin shift to 390 nm which could have been due to steric hindrance and displacing of the endogenous water
ligand at the sixth coordination site. The mechanism proposed by Hecker and Ullrich uses radical intermediates to explain the reaction. From EPR and other optical studies, it was concluded that CYP5A1 reacts with the C-9 oxygen of the endoperoxide bond. Oxy radicals are formed first, which are then rearranged to carbon radicals. These could then rapidly be converted into carbocations by the ferrylthiolate or iron (III) thyl structures formed as intermediates.

CYP5A1 also converts PGH$_3$ into TXA$_3$ but interestingly cannot convert PGH$_1$ into TXA$_1$. It rather converts them into HHT and MDA. Other compounds like PGG$_2$, 8-iso-PGH$_2$, 13(S)-hydroxy-PGH$_2$, and 15-keto-PGH$_2$ were also applied to test its substrate specificity. CYP5A1 converted PGH$_1$, 8-iso-PGH$_2$, 13(S)-hydroxy-PGH$_2$ and 15-keto-PGH$_2$ into the corresponding heptadecanoic acid (C$_{17}$) and MDA, whereas the corresponding thromboxane derivative was formed only from PGG$_2$, PGH$_2$, and PGH$_3$ together with the corresponding C$_{17}$ metabolite and malondialdehyde in a 1:1:1 ratio. This led to the suggestion that HHT was formed through a common intermediate for all these reactions. The endoperoxide bond is cleaved first followed by the rapid formation of [S- . . . Fe$^{''''}$ . . . O-R] species which gives rise to an alkoxy radical. The next step was found to be a β-scission pathway through isotope studies, which showed that the C-H bond at the C-12 position was not cleaved during this synthesis. This radical intermediate could then follow one of two steps leading to the formation of TXA$_2$ or MDA and HHT. The intermediate when decomposed, formed MDA and HHT or undergoes a Fe (IV) oxidation reaction to form a carbocation and finally rearrange to form TXA$_2$. Use of other substrates further validated their model. The 15-keto group in conjugation with the double bond at C-13 position destabilized the C-12 radical intermediate leading to the production of only MDA and HHT, the fragmentation products. The shifting of the double bond to 14-15 position and hydroxyl group to C-13 position remarkably decreased all reactions. The alkoxy radical undergoes a β-scission and subsequent ionic rearrangement to form TXA$_2$. Therefore homolytic cleavage of the endoperoxide bond forms Cpd II with the alkoxy radical whereas heterolytic cleavage forms Cpd I which is either a porphyrin radical or an amino acid radical and an alcohol.
ROOH + Fe (III) → RO₂⁺ + Fe (IV) → O
ROOH + Fe(III) → ROH + [Por]^{+} \cdots Fe(IV) → O
or [X]^{+} \cdots Fe(IV) → O

**Mechanism 2:** In this mechanism proposed by Yanai and Mori (19) the pathway proceeds through the formation of Fe (III)-π cation radical intermediates. Density functional studies were performed to reveal that the Fe (III) porphyrin system reduced the activation energy of the endoperoxide bond cleavage by 34 kJ/mol. The endoperoxide C (9) oxygen atom attaches to the Fe(III) active site of the porphyrin and an alkoxy radical intermediate is formed by homolytic cleavage. An allyl radical is then formed by the cleavage of C (11)-C (12) bond and a one electron transfer of the 6 membered ring leads to the formation of TXA₂. A Fe (III) or any metal containing catalysts with one electron acceptor ability can lead to the formation of TXA₂.

**Reactive intermediates:** The binding of CYP5A1 with its substrate analog U44069 is a two-step binding process which gives a K_d value of 28 μM (20). Similarly binding of nitrogen based ligands like Imidazole and Clotrimazole also follow this two-step binding process where the K_obs of the fast phase is linearly dependent on the concentration of substrate or inhibitor and that of the slow phase is independent of the concentration. Rapid scan stopped flow analysis or freeze quench EPR spectroscopic studies failed to reveal the radical intermediates formed. The reaction of CYP5A1 metabolism was then concluded to be very rapid with no or little accumulation of products. CYP5A1 transforms 15-HPETE to 13-OH-14, 15 -EET and also to 15-ketoeicosatetraenoic acid (15-KETE) and 15-hydroxyeicosatetraenoic acid (15-HETE) (11). Homolytic scission leads to the formation of 13-OH-14, 15 -EET and 15-KETE, whereas heterolytic scission is needed for the formation of 15-HETE. 60% of the 15-HPETE added was homolytically cleaved by CYP5A1. Homolytic cleavage was 1.4 times faster than heterolytic cleavage by CYP5A1.

The intermediates formed from this reaction were investigated using rapid scan stopped flow. The spectral changes observed show that there are two reactive intermediates, one with a blue shifted soret peak (species B) and is a CYP5A1-15-HPETE complex and the other one species C, which was postulated to be a mixture of Cpd I and Cpd II.
**Active site characteristics:** To determine the conformational changes and 3D structure of PGH$_2$ in the active site of CYP5A1, a high resolution NMR spectroscopy of its substrate analog U44069 was performed. From these experiments, it was found that the triangle shape of the free U44069 was changed to a more compact structure when it was in the bound state (21). The active site of CYP5A1 could fit the compact structure of the substrate analog but not its free form. This shows that PGH$_2$ also undergoes some structural change when it binds to CYP5A1.

CYP5A1 coordination with PGH$_2$ substrate analogs U44069 and U46619 showed a few interesting results (22). Coordination with U44069 was found to be in a 6-coordinate low spin state in accordance with previous studies, while those with U46619 showed results contrary to previous studies, indicating that it too was in a 6-coordinate low spin complex rather than a 5 coordinate high spin state (23). Main vibrational peaks at 1374, 1503, 1564, 1587 and 1638 cm$^{-1}$ were seen for CYP5A1 using high-frequency Raman resonance spectroscopy. Addition of both U44069 and U46619 did not alter these vibrational modes to a significant extent. Hence CYP5A1 when bound to these substrate analogues was found to be in a 6c/ls state as opposed to the 5c/hs state proposed earlier for U46619 based on absorption spectra. Earlier mechanisms favoring binding of the O (9) oxygen of U44069 over that of O (11) of U46619 to CYP5A1 were questioned in light of this new evidence. Although U46619 was shown to be more flexible and prone to alter the protein environment compared to U44069.

Based on the structural similarity to P450cam, the heme pocket in the active site was seen to have two propionate groups that interact with the protein matrix. The resting enzyme shows one main propionate bending mode, while the addition of substrate analogs increases a second propionate bending mode and decreases the intensity of the first one. Based on different vibrations seen for the substrate analogs, U44069 with the O (9) binding was postulated to disrupt the 6-propionate group due to its carboxylate side chain. U46619 on the other hand, due to a different orientation did not interact with the propionate groups with its carboxylate side chain but did disrupt the hydrogen bonding network of the propionate groups due to its bulky side chain.
1.3 References


2.1 Effect of membranes on proteins in general

While CYP5A1 is an endoplasmic reticulum membrane protein (24), all of its analyses to date have been performed in soluble reconstituted systems. The enzyme being catalytically competent in these systems, they have provided great insight into the mechanism and function of this protein. Though the complex conditions of the cell membrane environment cannot be mimicked, recent advances in technology have made possible the study of membrane proteins in lipid bilayers that come closer to the natural environment of the proteins in question. These studies have proven necessary due to effects the lipid composition and state of the bilayer can have in modulating enzyme activity (25-27). The presence of specific types of phospholipids has also been hypothesized to stabilize kinetically distinguishable conformations of cytochrome P450s, and modulate their affinities for substrates.

In addition to the differences in the composition, lipid bilayers are also known to be complex systems in a dynamic state with lateral, rotational, and transverse movements (28). The bilayer exposes the proteins to different regions of hydrophobicity while travelling transversely through it. The composition of lipids in a membrane has been shown to play an important role in fluidity of the membrane (29). This membrane fluidity in turn affects the activation energy of enzyme function (30). For example, the activity of CYP3A4 can be modulated by the presence of anionic lipids and the biophysical characteristics of CYP3A4 change in lipid bilayers (31). The protein displays monophasic reduction kinetics as opposed to multiphasic kinetics in detergents. And many membrane proteins also need specific anionic or cationic lipids for their function. Like the integral membrane protein tissue factor which requires acidic phospholipids to recruit a soluble factor VIIa necessary for the initiation of a blood coagulation cascade(32). Therefore to account for the effect of
membranes on CYP5A1 in its native-like environment and to understand the role of this microenvironment on CYP5A1 function, we have incorporated CYP5A1 into lipid bilayers of Nanodiscs. As a first step we use CYP5A1-Nanodiscs to measure the activity of CYP5A1 in neutral lipids. This initial study will elucidate the importance of lipids in modulating the activity of CYP5A1.

2.2 Model lipid bilayers of Nanodisc for functional interrogation of CYP5A1

In this study we used Nanodiscs to biochemically elucidate the function of CYP5A1 in lipid bilayers. Nanodiscs are essentially lipid bilayers held together with the help of membrane scaffold proteins (33). Incorporating a CYP protein into Nanodiscs mimics the endogenous membrane environment of the protein (34). Nanodiscs have been proven to be excellent for the solubilization and biochemical investigation of many diverse membrane protein systems including G-protein coupled receptors (GPCRs). Moreover, this technology has enabled the stabilization of membrane proteins both in solution (35-39) and on surfaces (38, 40-43).

2.3 Composition of platelet membranes

A selective increase in thromboxane synthase activity has been found in the intracellular membrane elements of platelets, known as dense tubular membranes (24). These changes in activity have been suggested to be due to differences in lipid composition of the inner and surface membranes of platelets (44). An analysis of four glycerophospholipids: phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC), found in membranes, shows that the inner platelet membranes are enriched in PC and PI, PS remained constant, while PE is decreased when compared to the surface membrane. Inner membranes of platelets also appear to be depleted of sphingomyelin in comparison to the outer surface membrane. This enrichment of thromboxane synthase activity associated with blood platelet inner membranes may be attributed to the differences in lipid composition.
Even though the pharmaceutical potential of this protein has been well established, its mechanism yet remains to be characterized. This is mainly due to it being a transmembrane protein causing it to aggregate in solution. Recently nano-scale lipid like bilayers called Nanodiscs has been developed by Sligar lab, which will be used here to incorporate and solubilize the protein. The functional properties of the protein can then be investigated in vitro through biophysical assays. The chemical mechanisms of this enzyme can play a significant role in developing therapeutics and unraveling the nature of inflammatory disease conditions in the human body.

2.4 Materials and methods

**Materials:** Human CYP5A1 gene was obtained from Origene. The bacterial strain DH5α was obtained from Invitrogen. Ampicillin, arabinose, chloramphenicol, IPTG and Ni-NTA resin were bought from Gold Biotechnology. δ-Aminolevulinic acid and hematin were obtained from Frontier Scientific. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. Amberlite XAD 2 was acquired from Supelco. Arachidonic acid, PGB<sub>2</sub>, TXB<sub>2</sub>, U44069 and U46619 were obtained from Cayman Chemicals. COX-2 was kindly provided by Ben Orlando and Dr. Michael Malkowski. All other chemicals were purchased from Sigma-Aldrich or Fisher Chemicals.

**Gene modification:** Two modifications were made to the CYP5A1 human gene, which was obtained from Origene. For the first construct, the gene was N-terminus modified and a truncation of 1-29 amino acids was made. There is an N-terminus insert MALLAVF in its place which favors expression in *E.coli* and also a His-tag at the C-terminus. An Ndel and XbaI site have been engineered at the N-terminus and C-terminus respectively. The modified gene is in pAr5 (modified pCWori) which has an N-terminus Ndel site. pTGro7 + pGroE are co-transformed with the modified gene into DH5alphaF’1Q Max Efficiency cell line. The co-transformed cell has both ampicillin and chloroamphenicol resistance. For the second construct, the first 1-29 amino acids of the human CYP5A1 gene were truncated and replaced with the hydrophilic MAKKTSS sequence along with an addition of a histidine tag at the C terminus to aid in purification (45).
Expression and purification of CYP5A1: The first 1-29 amino acids of the human CYP5A1 gene were truncated and replaced with the hydrophilic MAKKTSS sequence along with an addition of a histidine tag at the C terminus to aid in purification (45). The construct was inserted into a pCWori vector and co-transformed with pTGro7 (chaperonin system) into DH5α cells. The recombinant CYP5A1 construct was expressed and purified according to published protocols with certain modifications (46). Briefly, the cells were grown in terrific Broth (TB) media until they reached an O.D. of 0.8-1.0 and were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.5 mM delta-aminolevulinic acid (δ-ALA) and 4 mg/L arabinose. They were grown for 44 hours at 26°C and 160 rpm. The harvested cells were resuspended in Buffer A (0.1 M potassium phosphate pH 7.4, 10% glycerol, 0.1 M sodium chloride) containing 2 mM magnesium chloride, 1 mM phenylmethanesulfonylfluoride (PMSF), approximately 1 mg each of D’Nase and R’Nase for 1 hour and lysed 5 times using a 1/8’ tip Heat Systems Ultrasonics (Qsonica, Newtown, CT) sonicator at 80% duty cycle with 1 minute rest between cycles. The solution was then centrifuged using a Beckman ultracentrifuge (Beckman Coulter, Brea, CA) at 35K rpm for 1 hour and the pellet was resuspended in Buffer A containing 2% Lubrol overnight to solubilize CYP5A1. The sample was again centrifuged at 35K rpm for 1 hour before loading the supernatant onto a Ni-NTA column pre-equilibrated with Buffer A. The column was first washed with 3X column volumes of Buffer A containing 2% Lubrol and then incubated with 5X column volume of buffer B (0.1 M potassium phosphate, 10% Glycerol, 0.2% Lubrol) containing 10 mM Histidine, 5 mM ATP, 10 mM MgCl₂ and 150 mM KCl to separate any co-purifying GroEL for 2 hours. CYP5A1 was then eluted using 0.1 M potassium phosphate, 10% Glycerol, 0.1 M NaCl, 0.2% Lubrol containing 100 mM Histidine.

Assembly of CYP5A1-Nanodiscs: CYP5A1-Nanodiscs were assembled from a mixture of CYP5A1, membrane scaffold protein (MSP) and POPC lipids by removing the detergent using Amberlite (47). Membrane scaffold protein (MSP1D1) has a Histidine tag preceded by a highly specific Tobacco Etch Virus (TEV) protease recognition site. The Histidine-tag was cleaved from the protein using TEV protease and is referred to as MSP1D1 (-). POPC lipids stock concentration was determined by phosphate analysis. Small aliquots of lipids were
dried using nitrogen gas to remove residual chloroform and were solubilized using 100 mM sodium cholate. MSP1D1 (-) was added in a ratio of 65:1 (lipids: MSP (-)) and the solution incubated at 4°C for 1 hour on a shaker. Detergent solubilized CYP5A1 was then added in a ratio of 15:1 (CYP5A1: MSP (-)) and incubated for a further of 1 hour. Biobeads were added to the final mixture and left overnight to remove detergent and initiate the formation of the Nanodiscs. After addition of the biobeads and overnight incubation, the lipids: MSP (-): protein mixture was added to a Ni-NTA column pre-equilibrated with 0.1 M potassium phosphate buffer (pH 7.4). The column was washed with 5X column volumes of buffer B (0.1 M potassium phosphate pH 7.4, 0.1 M NaCl) containing 20 mM Histidine. The monomeric protein: MSP (-) fractions were then eluted using buffer B containing 100 mM Histidine, thus effectively separating empty Nanodiscs from the CYP5A1-Nanodiscs. This sample was then subjected to size exclusion chromatography using a Superdex 200 10/300 column (GE Life Sciences, Piscataway NJ) coupled to a high performance liquid chromatography (HPLC) system with an Alliance 2695 analytical separation module (Waters, Milford MA) and a Waters 996 photodiode diode array detector (Waters, Milford MA). The Nanodiscs were run on the SEC column with an isocratic mobile phase of 0.1 M potassium phosphate buffer at a 0.5 ml/min flow rate. The Nanodisc peak eluted at approximately 26 minutes. The concentrated CYP5A1-ND was stored at -80°C in the presence of 10% glycerol.

**Characterization of CYP5A1 in Nanodiscs:** CYP5A1-Nanodiscs (CYP5A1-ND) were characterized using different techniques. SDS-PAGE was used to determine the incorporation of CYP5A1 into the Nanodiscs and its purity. The Nanodiscs separated out on the PAGE gel as two distinct bands at 56 kD and 23 kD for CYP5A1 and MSP1D1 (-) respectively. The purified CYP5A1-Nanodisc constructs were analyzed using UV-visible spectra. The construct had a typical peak at 417 nm and the α and β bands (Q-bands) at 536 nm and 570 nm respectively. The protein was reduced using dithionite in the presence of methyl violagen (used at 1/40th of the protein concentration) and the Fe(II) CO spectra was measured for all the different constructs as done by Omura and Sato (48) with minor modifications. The sample was diluted to a final concentration of 5 μM with buffer A or buffer B. Methyl violagen was added at one fortieth the concentration of the protein. This
mixture was purged with nitrogen gas for 20 minutes to remove traces of oxygen and sodium dithionite was then added anaerobically to reduce the protein. CO-purged 0.1 M potassium phosphate buffer was added and the spectrum recorded using a Cary Bio 300 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara CA). The CYP5A1-ND were further tested by studying the binding to PGH\textsubscript{2} substrate analogs 9,11-dideoxy-9α,11α-epoxymethano Prostaglandin F2α (U44069) and 9,11-dideoxy-9α,11α-methaneoepoxy Prostaglandin F2α (U46619).

**Activity assay of CYP5A1:** Thromboxane Synthase converts PGH\textsubscript{2} into TXA\textsubscript{2} and also forms HHT and MDA as fragmentation products in a separate reaction (49). To test for the activity of the recombinant protein, the amount of TXB\textsubscript{2} formed was determined using a LC-MS method (50) along with the formation of HHT and MDA monitored spectroscopically at 234 nm and 268 nm respectively (51). As PGH\textsubscript{2} is very unstable at room temperature and degrades fast in aqueous solution, a coupled assay was used to produce PGH\textsubscript{2} from COX-2 (provided by Dr. Michael Malkowski) and arachidonic acid (AA) in the reaction mixture. 50 nM COX-2 was first reconstituted with 1 µM hematin to produce active COX-2. 50 nM CYP5A1 was then added to the reaction mixture and incubated for 2 minutes at 23°C. 5 µM arachidonic acid (AA) was added to initiate the reaction and the kinetics of the reaction was monitored immediately for 300 seconds at 234 nm corresponding to HHT formation. A spectrum was also recorded at the end of the kinetics measurement to determine the amount of HHT and MDA formed using their respective extinction coefficients of 33 mM\textsuperscript{-1} cm\textsuperscript{-1} and 31.5 mM\textsuperscript{-1} cm\textsuperscript{-1}. The reaction was terminated by the addition of 300 µL ice cold ethyl acetate. The internal standard PGB\textsubscript{2} was added at this point and the reaction mixture was shaken for a few minutes. It was then centrifuged at 3000 rpm for 5 minutes to separate the organic and aqueous phases. The organic solvent was then extracted and evaporated to dissolve the resultant residue in 100 µL of ice cold 40% acetonitrile: 60% water. This sample was analyzed for quantitation of TXB\textsubscript{2} using liquid chromatography-tandem mass spectrometry (LC/MS/MS) method with prostaglandin B\textsubscript{2} (PGB\textsubscript{2}) as an internal standard. Saturating concentrations of the substrate (arachidonic acid) was used to
calculate the reaction $V_{\text{max}}$ (nmol/min/nmol of CYP) for CYP5A1 activity. Rates are reported as the mean ± SE.

**Liquid chromatography- tandem mass spectrometry for quantitation of TXB$_2$:** Liquid chromatography-tandem mass spectrometry (LC/MS/MS) was employed for quantitation of TXB$_2$, and PGB$_2$ parent ions [M + H]$^+$ and their corresponding fragment ions. The LC/MS/MS system consisted of a Waters Alliance 2795 analytical high performance liquid chromatography separation module (Waters, Milford, MA) coupled to an electrospray ionization mass spectrometer (Waters Quattro Ultima, Waters, Milford, MA) operated in positive mode. The sample was analyzed using a reversed-phase C18, 1.3 Å, 2.1 mm x 20 mm and 2.5 µm pore size column (Waters, Milford, MA). The MRM data acquisition consisted of monitoring the following analytes in positive mode using these transitions (parent ion → fragment ion, cone voltage, collision voltage): PGB$_2$ (333.3 → 175, 30 eV, 20 eV and 333.3 → 235, 35 eV, 20 eV) and TXB$_2$ (369.3 → 169, 25 eV, 18 eV and 369.3 → 195, 25 eV, 14 eV) all with 100 ms dwell time. Quantitation of TXB$_2$ was determined using the peak areas normalized to the internal standard (PGB$_2$ 100 ng/ml) from a previously calculated standard curve. Analytical data was processed using Waters Mass Lynx software (version 4.1).

**Redox potential measurements:** The oxidation reduction potentials of the protein were determined photochemically by reductive titrations (52-55). The protein was used in a 3 µM concentration with 10 mM EDTA, 0.5 µM Safranin T and 20 nM methyl viologen. A 300 Watts halogen lamp was used to photoreduce the protein and dye system. The absorbance was recorded after irradiating the cuvette for varying lengths of time. Safranin T with an $E_m$ = -289 mV was used as the indicator dye so that the estimated redox potential difference between the protein and dye would be less than 60 mV. The sample was purged with ultrapure nitrogen for 20 minutes prior to irradiation. The final reduced state was reached by adding sodium dithionite and the data was analyzed according to the Nernst equation.

The potential of the dye at each point in the titration is given by the Nernst equation:

$$E_D = E_{OD} + \frac{RT}{nF} \log_{10}(f_{\text{ox}-\text{Dye}}/f_{\text{red}-\text{dye}})$$

for Safranin T (Equation 1).
Assuming that the reaction reaches equilibrium after each titration, the $E_D$ of the dye equals that of the protein:

$$E_D = E_{OP} + \frac{RT}{nF} \log_{10}\left(\frac{f_{ox-P}}{f_{red-P}}\right)$$ (Equation 2)

Where $R$ stands for the Universal gas constant, $T$ for temperature in Kelvin, $n$ for the number of electrons transferred, $F$ for Faraday’s constant, $f_{ox-P}/f_{red-P}$ fraction of oxidized protein to reduced protein and $f_{ox-Dye}/f_{red-dye}$ fraction of oxidized dye to reduced dye.

The data was fit to multiple wavelengths for the protein and dye using a MATLAB subroutine given in the supplementary material. The log plot of the fraction of oxidized to reduced protein versus the $E_D$ of safranin T gives a straight line. The percentage reduced protein to the $E_D$ of dye was fit to the Nernst curve using the equation

$$% R = \frac{100}{1+10^{\left(\frac{(E_D-E_{op})}{0.59}\right)}}$$

where $% R$ is the percentage of reduced protein, $E_D$ is the equilibrium potential of the solution determined by monitoring the dye redox state, $E_{OP}$ is the midpoint reduction potential of the protein. The redox potentials were also verified by an oxidative titration where dithionite was used to reduce the protein completely in the first step and potassium ferricyanide was then used to oxidize the sample back to its oxidized state in the presence of the dye. This experiment was repeated for substrate free and both the substrate analogs bound to CYP5A1.

**Equilibrium constant determination for cyanide binding to the protein active site:** The dissociation constants of cyanide binding to substrate free and substrate analog bound CYP5A1 were determined by steady state binding measurements. Determination of the equilibrium constants was done by titrating ~2 μM protein with increasing concentrations of cyanide until saturation was reached. The spectrum of the solution was recorded after each cyanide addition. The steady state binding experiment was performed separately with substrate free and substrate analogs U44069 and U46619 bound protein. The difference spectra obtained was used to calculate the dissociation constant $K_D$ of cyanide binding to the protein in the substrate free and substrate bound state. The binding data was fitted to one site binding hyperbolic equation.

$$\Delta A_{446-415nm} = \Delta A_{max}[S] / (K_D + [S])$$ (Equation 3)
Where $\Delta A_{446-415 \text{nm}}$ is the difference in the maximum and minimum values of the absolute difference spectra, $[S]$ is the cyanide concentration and $K_D$ is the dissociation constant of cyanide binding to CYP5A1.

**Transient state kinetics of cyanide binding to protein active site measured using stopped-flow**: The rate of cyanide binding to CYP5A1 active site in the presence and absence of substrate analogs were determined using the Applied Photophysics SX.18MV stopped flow spectrophotometer where CYP5A1 substrate bound and free was loaded into syringe 1 and syringe 2 contained cyanide with the same concentration of substrate as syringe 1. Protein concentrations used were typically ~2 μM. Observed rate constants were determined at a minimum of three different substrate concentrations with increasing concentrations of substrate analogs U44069 and U46619 (0, 15 µM, 35 µM) added to the protein. In the syringe 2, a high concentration of cyanide (40 mM) was used in order to ensure pseudo first order binding kinetics. The solutions in both the syringes were allowed to mix with an instrument dead time of 750 ms and the data were obtained on a logarithmic scale for 5s. The $\Delta A_{447-415 \text{nm}}$ was calculated from the difference spectra for all the time points for each sample and the data was fit to a double exponential equation using Origin Pro 8.6.

$\Delta A_{447-415 \text{nm}} = A_0 + A_1 e^{k_1} + A_2 e^{k_2}$ (Equation 4)

Where $A_1$ and $A_2$ are the amplitudes of the fast and slow phases of the reaction respectively and $k_1$ and $k_2$ are the rates of fast and slow phases of binding to the protein which are determined from the fitting the data obtained to equation 4.

### 2.5 Results

**Expression and purification**: The first construct made with the MALLAVF sequence in place of the first 1-29 truncated amino acids produced a very low yield of 0.1mg/L of protein, which is considered very low for protein analysis. A second construct was made in an attempt to increase the yield of the protein expressed. As described in the materials and methods section, a hydrophilic sequence was used, which dramatically increased the yield of the protein to ~1mg/L.
The purification protocol was also modified at each step to optimize the yield. The protein has a characteristic reddish brown color due to the heme moiety. The soret of the protein is at 417 nm, as with most of the proteins in the Cytochrome P450 superfamily. These have been used in the optimization steps described below as a test for characterizing the protein.

Elution: Imidazole was initially used to elute the bound protein from the Ni-NTA column. The reddish brown color of the solubilized protein was dramatically reduced after purification through the column. Upon troubleshooting, it was found that the imidazole used to elute the protein in fact bound to it. Hence making it unfit for any further assay reactions. Thus, 50mM Histidine was used for the consequent procedures.

Solubilizing detergent: The solubilizing agent used initially was Triton. The protein was then substrate bound and analyzed using a UV-Vis spectrophotometer. The spectra showed the protein to be in a bound state and it was suspected that triton was the one bound to it. Therefore, another detergent Lubrol PX was used for solubilizing.

Column chromatography buffers: The column buffers were also then changed accordingly to reduce the amount of detergent used during column chromatography [1]. All the optimization steps performed are listed in Table 2.1. The yields obtained with different purification protocols are shown in a graph in Figure 2.1.

**Nanodisc incorporation:**

**Phospholipid analysis:** POPC lipid stocks were made to incorporate the CYP5A1 protein into Nanodiscs. Phosphate analysis using a phosphate standard was performed to obtain the Nanodisc concentration from the lipid stocks. The average concentration of the lipid stocks was calculated as 101.58 nmoles/µL. Empty Nanodiscs were then prepared as a result of the positive and consistent concentrations. The symmetrical peak was obtained at the expected time of 26.75 minutes.

**Membrane Scaffold Protein:** In all of the experiments with Nanodiscs, the membrane scaffold protein, MSP1D1 was used. These proteins have been derived from the Human Apolipoprotein A1 sequence. These proteins contain a TEV cleavage site and a His tag, and these proteins hold the lipid bilayer together.
**Protein incorporation into Nanodiscs:** The first construct showed incorporation into Nanodisc structures but had low efficiency due to aggregated state of the protein. To disaggregate the protein, different detergents like CHAPS, Tween 20 were used and it was found that Lubrol had a better efficiency in terms of disaggregating the protein. Another method of decreasing the hydrophobic character of the protein was used, which replaced the N terminus first 1-29 amino acids with the construct MAKKTSS rather than the MALLAVF previously used, as described in the sections above. Along with a dramatic increase in yield of the protein expression, this modification also resulted in better Nanodisc incorporation. The protein aggregate peak seen at 15 minutes, is much less pronounced than with the first construct. The N-terminal truncation of the protein might be postulated to decrease the stability of the Nanodisc due to the protein not being able to stay in the disc for long periods of time. Therefore collected Nanodisc peaks were re-injected after a period of 1 week to monitor the stability of the Nanodiscs, and the Nanodiscs were seen to be stable over this period of time.

A corresponding protein peak at 417 nm was seen for the Nanodisc peak at 25.40 minutes. Figures 2.2A and B show the SDS-PAGE and HPLC chromatogram which demonstrate the successful incorporation of purified homogenous and monomeric CYP5A1 into Nanodiscs. The soret of the Nanodisc incorporated CYP5A1 was also seen at 417 nm. These CYP5A1-Nanodiscs were also stable as seen by HPLC chromatograms (data not shown) and were tested for the characteristic feature of cytochrome P450s to bind to CO in the ferrous state. Figure 2.3 shows the Fe (II)-CO spectra of CYP5A1-Nanodiscs which has a prominent peak at 450 nm.

**Activity assay:**

*CYP5A1- COX-2 coupled activity assay in membrane bilayers:* A coupled activity assay was used to measure the activity of CYP5A1-Nanodiscs (56). Briefly, COX-2 with hemin was mixed with CYP5A1-Nanodiscs. Arachidonic acid was added to initiate the reaction. PGH₂ was therefore generated *in situ* due to the reaction between heme reconstituted COX-2 and arachidonic acid. The PGH₂ formed in this reaction was utilized as a substrate by CYP5A1, which in turn converted it into TXA₂, MDA and HHT (Figure 2.4) (57). We monitored the
three products formed using different methods. HHT and MDA formation were monitored using UV-Vis spectroscopy at wavelengths 234 nm and 268 nm respectively. The amount of TXB₂ formed was determined by a modified LC/MS/MS method as described in the materials and methods section. The rate and amount of formation of HHT, MDA and TXB₂ was monitored over a period of 10 minutes by different methods.

_CYP5A1 enhanced activity in membranes:_ The rate of product formation (product/min/mg) of HHT, MDA and TXB₂ in detergent solubilized CYP5A1 and in Nanodisc are given in Figure 2.5. The values for HHT and MDA fall within the range of previously obtained rates (46, 51, 58). In coupled assay, HHT is increased in Nanodiscs ratio by 20% (ratio 1.2 ± 0.3), MDA by 60% (ratio is 1.6 ± 0.3) and TXB₂ by 130% (ratio 2.3 ± 0.3). The increased activity of CYP5A1 in Nanodisc may be the result of several factors described below.

_Firstly_, the native lipid bilayer environment of the Nanodisc for the proteins CYP5A1 and COX-2 might lead to an increase in conformational stability which in turn leads to increases in the expected product formation. In fact COX-2 oxygenase activity goes up by 20% when it is incorporated into Nanodiscs (unpublished data). As this is a coupled activity assay, the increase in activity from both COX-2 and CYP5A1 can synergistically contribute to the increase in product formation. Moreover, PGH₂ is an unstable hydrophobic molecule. Hence, it is possible that the presence of the lipid bilayers of Nanodiscs facilitates the effective shuttling of PGH₂ from COX-2 to CYP5A1 before PGH₂ denatures in aqueous solution.

_Secondly_, it is probable that the electrostatic environment of the membrane modulates the CYP5A1 active site to thermodynamically stabilize the intermediate formed during PGH₂ isomerization and therefore favors the formation of the expected products. Previously, we have shown that the electrostatics of the membrane environment can modulate the redox potential of cytochrome P450 reductase (CPR) in such a way that it promotes the effective electron transfer from CPR to CYP3A4 in membranes (39). Hence we measured the redox potential of CYP5A1 in membranes in the presence of different substrates to understand the electrostatic modulation of the active site of CYP5A1 by the membrane environment.
Thirdly, we speculate that CYP5A1 adopts a more native-like conformation in membranes as compared to detergent solubilized state therefore we also measure the direct activity of CYP5A1 using synthesized PGH₂ in detergents and lipid bilayers. In this direct assay of addition of PGH₂, HHT is decreased in Nanodiscs by 30% (ratio 0.7), MDA by 30% (ratio is 0.71) and TXB₂ increases by 30% (ratio 1.3 ± 0.3).

*CYP5A1, direct PGH₂ assay*: To observe the extent of membrane effects purely due to the membrane, on CYP5A1, PGH₂ was directly added to CYP5A1 in another type of activity assay.

The results from these experiments were similar to those from the coupled activity assay. The physiological product of the reaction, TXB₂ was again seen to be 100% greater in Nanodiscs than in the detergent solubilized system. The amount of HHT and MDA formed was interestingly lower in Nanodiscs than in the soluble system, though the HHT and MDA ratios remained to be 1:1 in both the cases (Figure 2.6). From these experiments it can be seen that CYP5A1 in Nanodiscs produces a greater amount of its physiologically relevant product TXB₂, and less of its side or fragmentation products. The enzyme is more efficient and has a greater turnover number when it is incorporated into a native like membrane environment.

**Substrate Binding**: CYP5A1-Nanodiscs were also tested for their substrate binding properties. CYP5A1 is expressed in platelets and binds specifically to PGH₂ (59). As PGH₂ is labile in aqueous solution, most studies on substrate coordination to the active site are studied using two substrate analogs, U44069 (where the C-11 oxygen atom of the PGH₂ analog is replaced by a methyl group) and U46619 (where C-9 oxygen of the PGH₂ analog is replaced by a methyl group). Previous studies have shown the coordination of CYP5A1 to U44069 is in a six coordinate low spin state as seen by UV-Vis spectroscopy while the coordination with U46619 is in a six-coordinate low spin state contrary to the five coordinate high spin state seen spectrally (23). These analogs were used to discern substrate binding characteristics of the active site. The binding of U44069 to CYP5A1 showed its characteristic trough and peak at 426 nm and 409 nm respectively (Figure 2.7A) (17). The spectral shift produced by U44069 shows that the -O- atom (at C9 position) of the
molecule binds to the heme active site. The binding of U46619 showed a trough and peak at 418 nm and 395 nm respectively (Figure 2.7B) (17). U46619 produces a typical spectra characteristic of a high spin state where the -CH₂- group (at C9 position) faces the heme active site of CYP5A1. PGH₂ (the native substrate) shows spectral characteristics similar to U44069, which is indicative of binding of an endoperoxide to the active site (18). The binding of these two substrates fit to a one site binding hyperbolic equation and the spectral dissociation constant of U44069 and U46619 binding to CYP5A1-Nanodiscs were determined to be 6 µM and 5 µM respectively. The binding of U44069 is found to be tighter in Nanodiscs compared to 14 µM in detergents.

**Redox potentials:** To probe the electrostatics around the heme active site in CYP5A1, we measured the redox potentials of the substrate free and substrate analogs bound CYP5A1. As shown in figure 2.8, the substrate free redox potential of CYP5A1 is -282 ± 2 mV. This value is slightly more positive than the usual values obtained for soluble P450s or detergent solubilized P450s (54, 60). The addition of its native substrate analog U44069 resulted in an Eₘ of -284.5 ± 0.5 mV (Figure 2.9) while the binding of the other substrate analog U46619 produced a small positive shift to -270 ± 5 mV (Figure 2.10). Considering that the error range of these measurements was ±5 mV, there was not a significant change in the redox potentials on binding either substrate. The substitution of the water at the active site by the endoperoxide oxygen of U44069, does not change the redox potential while the addition of U46619 where the methyl group binds close to the heme center is accompanied by a redox potential change of ~ +10 mV. The spin state change on binding U46619 was ~ 14% and hence we do not expect a dramatic redox potential change as these two parameters have been shown to be linked for both soluble and membrane bound proteins (60, 61).

Different mechanisms have been proposed to explain the formation of these three products. Ullrich and Hecker (Ref) postulated the formation of an Fe(IV)-porphyrin intermediate and density functional theory (DFT) studies by Yanai and Mori (62) proposed that CYP5A1 may also isomerize PGH₂ via the Fe(III)-porphyrin π-cation radical intermediate. In both pathways the reaction proceeds through homolytic cleavage of the endoperoxide O-
O bond of PGH₂ to give an alkoxy radical. This first step of binding of the endoperoxide to CYP5A1 has been shown to have the highest activation energy (63). Additionally, unlike most classical cytochrome P450s, the addition of native substrate PGH₂ to CYP5A1 does not convert the Fe(III) of CYP5A1 into a high spin complex (58). This is due to the epidioxy ligand (PGH₂), being a weak field ligand that replaces water as a ligand at the sixth coordination center. In either of the two cases mentioned above, stabilization of the higher redox state Fe (IV) for the substrate bound protein might be correlated with the higher activity of CYP5A1 with respect to its ability to isomerize PGH₂. In other words, CYP5A1 bound to substrate is less likely to be reduced and therefore its redox potential (tendency to get reduced) should be lower. This was shown to be true as binding of both U44069 and U46619 to CYP5A1 did not change the redox potential considerably when compared to substrate binding in other systems.

In order to understand the role of membranes in modulating the redox potential of CYP5A1, we also measured the redox potential of substrate free, U44069 bound and U46619 bound CYP5A1 in a detergent solubilized state. CYP5A1 when substrate free gave a redox potential of -300 ± 10 mV. The redox potentials of U44069 bound CYP5A1 was measured to be -318 ± 10 mV and that of U46619 bound was -307 ± 10 mV in detergent (Figure 2.11). This is opposite to the trend seen in Nanodiscs towards less negative midpoint potentials upon addition of substrate. Overall all the redox potentials are close to each other. So it is possible that the membranes are not modulating the redox potential of CYP5A1 to facilitate the stabilization of its PGH₂ bound intermediate. Therefore we conclude that this modest modulation of the redox potential of CYP5A1 cannot possibly account for the 100% increase in CYP5A1 catalyzed activity. The structure of CYP5A1 was modeled in Phyre2 (64) and this was used to obtain the orientation of CYP5A1 in membrane. The resultant model structure of CYP5A1 in membranes shows that the protein in not deeply embedded in the membrane (Figure 11). Therefore, the active site would be further away from the lipid bilayer and would not be influenced substantially by the electrostatics of the membrane.
**Cyanide binding:** The protein may adopt a different conformation in Nanodisc as compared to its state in solution, leading to the observed increase in product formation. In order to explore the active site accessibility, we measured the access of cyanide to the ferric state of CYP5A1 is its substrate and bound form. In this case the rate of cyanide binding was measured as a function of substrate analog concentration to probe how substrate binding blocks the cyanide access to the active site (65).

**Equilibrium cyanide binding studies to CYP5A1:** We measured the equilibrium $K_D$ of cyanide binding to the protein in the presence and absence of a substrate. U44069 is a substrate analog of PGH₂ and is not metabolized by CYP5A1 but is an excellent model compound to study how PGH₂ binds to the heme center. CYP5A1 in Nanodiscs bound to U44069 has a dissociation constant of 6 µM, in contrast to 15 µM in detergent solubilized systems. This shows that the binding of the substrate analog is more than two fold tighter in Nanodiscs than previously reported in other systems.

Equilibrium cyanide binding studies to CYP5A1 were performed at 6°C to probe the active site accessibility. The dissociation constant $K_D$ determined for cyanide binding to substrate free CYP5A1 in Nanodiscs is 12 mM and that of U44069 bound CYP5A1 in Nanodiscs is 37 mM and U46619 bound is 17 mM. For detergent solubilized CYP5A1, the $K_D$ of cyanide binding values changed from 18 mM for substrate free to 28 mM for U44069 bound CYP5A1 and 22 mM for U46619 bound CYP5A1. The substrates were added at saturating levels to both the systems (~60 µM). As shown in Table 2.2, this is a dramatic increase in the $K_D$ of binding for CYP5A1-Nanodisc when compared to similar systems studied earlier. The effect is more pronounced in Nanodiscs indicating that the conformation of the substrate binding pocket in CYP5A1-Nanodiscs changes dramatically upon substrate binding, which might hinder the active site access to cyanide binding. Therefore, the $K_D$ of cyanide binding to U44069 bound CYP5A1 in Nanodiscs is higher comparatively. An alternative hypothesis is that cyanide binding has easy access in substrate free CYP5A1 in both detergent and Nanodiscs, but the substrate binding pocket is narrower in Nanodiscs when blocked by substrate, leading to a lower $K_D$ of cyanide binding as seen in some other systems (65-67).
To investigate the dynamics of the active site conformation upon substrate binding, the cyanide binding experiments were conducted with another substrate analog, U46619. As previously mentioned, U46619 has a C (11) substitution instead of an oxygen atom. There have been discrepancies in literature as to how PGH₂ binds to the heme iron. Many theories suggest that PGH₂ binds using both the O (9) and the O (11) atoms though it preferentially uses an O (9) binding to the Fe (68). The \( K_D \) of cyanide binding to CYP5A1 with U46619 bound in Nanodiscs is 17 mM and in detergent is 22 mM which is similar to the 12 mM and 18 mM values respectively in substrate free CYP5A1. As it can be clearly seen from this data, U46619 hinders the binding of cyanide in Nanodiscs to a lower extent than does U44069.

*Kinetics of cyanide binding to CYP5A1 - amplitude of fast phase:* We further measured the rate of cyanide binding to CYP5A1 in the presence and absence of substrates similar to the equilibrium state measurements. The data for the kinetics of cyanide binding fit to a double exponential equation. The first phase of the reaction is defined as the fast phase that is related to the association of cyanide to the active site. It is speculated that the second or slow phase is correlated to the slow conformational change that exists between different states of the protein (69). Figure 2.1D and E shows the percentage of amplitude in the fast phase of the cyanide binding to CYP5A1 with both the substrate analogs. In CYP5A1-Nanodiscs, when bound to U44069, we observed a dramatic decrease in the percentage fast phase from 72% at substrate free to 33% when saturating amounts of substrate is present. Interestingly, in detergent solubilized system, there is not much variation in this fast phase amplitude as it stays constant around ~50%. This is an indication that the conformation adopted in membrane bilayer allows U44069 to block the active site, which prevents rapid cyanide binding. The kinetics data agrees with our equilibrium binding data, that the \( K_D \) of binding increases when U44069 binds to CYP5A1 in Nanodiscs.

The trend for the other substrate U46619 is not significantly different in solution when compared to membranes. Interestingly, the detergent system shows a much greater variation from 78% at substrate free to 18% at saturating concentration of U46619. The fast phase amplitude of the Nanodisc system changes from 66% at substrate free to 40% at saturating concentration of substrate. However, the binding of U46619 is not very different
in the soluble and membrane systems. The rates of cyanide association calculated for both the fast and slow phases of the reaction at increasing concentrations of U44069 and U46619 are shown in Figures 2.12F and G respectively.

**Kinetics of cyanide binding to CYP5A1- rates of association:** The slow phase rate of all the systems with any concentration of substrate bound, is very similar. There are however multiple differences in the association rate during fast phase (Figure 2.12F and G). In CYP5A1-Nanodiscs, the rate of association in the fast phase for U44069 decreases as the concentration of substrate is increased, while in detergent solution, it remains constant. This is very similar to the trend observed for the percentage of amplitude in the fast phase as discussed in the above section. For the binding of U46619, the trend in closing off the active site to cyanide binding appears to be absent in both detergents and Nanodisc systems, as there is no significant difference in their fast phase rates of association.

It is clear from the cyanide accessibility studies that the protein adopts a different conformation state in lipid bilayer membranes. The binding of U44069 (the native-like substrate) reduces the accessibility of cyanide to the active site. Therefore it is highly probable that the increase in the isomerization of PGH₂ into TXB₂, MDA and HHT by CYP5A1-Nanodiscs can be attributed to the change in conformation of the CYP5A1 in Nanodiscs and not from electrostatic modulation by the membrane.

### 2.6 Conclusion

The thermodynamics and kinetics of substrate analog binding to CYP5A1 in model lipid bilayer Nanodiscs was elucidated in this work. The coupled activity of CYP5A1 with COX-2 in membranes was measured, which was shown to be enhanced in lipid bilayers by 130% for TXB₂ formation. The increase in activity is attributed to several possible factors and different techniques were further employed to study some of the factors.

In the Nanodisc lipid membrane environment, both CYP5A1 and COX-2 presumably adopt more native-like conformations. We believe that this stabilizing effect of the membrane environment leads to the increase in product formation. In a separate work, it was established that COX-2 oxygenase activity increases by ~20-30% in Nanodiscs.
(unpublished data). As this is a coupled activity assay, the increase in activity can be ascribed to the synergistic action of both COX-2 and CYP5A1 in membranes, leading to the increase in the amount of products formed. To gauge the effect of membranes on CYP5A1 alone, a direct activity assay was used with CYP5A1. It was seen that the TXB$_2$ formation increased by a mere 30% compared to the 130% in the coupled assay. The incorporation of COX-2 and CYP5A1 in membranes, accounts for only a part of the increased TXB$_2$ formation, seen in the coupled assay. Another possible mechanism which accounts for the dramatic increase seen in the coupled assay is an effective shuttling of the substrate PGH$_2$ through the membrane. Eicosanoids generated in the prostanoid synthesis pathway are partially hydrophobic and very labile in aqueous solution. Therefore the presence of the membrane environment possibly aids in shuttling the COX-2 product PGH$_2$, through the membrane, to CYP5A1 in Nanodiscs.

It was shown in a previous study, that the electrostatic modulation of the active site in Cytochrome P450 Reductase (CPR), can lead to higher stability of active intermediates formed in the reaction, which increases the product generation. Hence the redox potential of CYP5A1 in the presence of different substrates in reconstituted system and in membranes was measured, to understand the electrostatic modulation of the active site of CYP5A1 by the membrane environment. The redox potential of substrate free and substrate bound CYP5A1-Nanodisc were ~20mV more positive than the detergent solubilized state. This change in redox potential is very low and does not account for the dramatic increase seen in product formation.

CYP5A1 interacts with the membrane both through the amino-terminal membrane-anchoring domain and the helix F and F–G loop region. When the N-terminus is truncated and residues in the F and G helices are substituted, a soluble CYP5A1 protein can be engineered. As CYP5A1 is embedded in the membrane in its native state, it is important to understand if the membrane induces CYP5A1 to a different conformation, which might play a role in enhancing the activity of CYP5A1. The equilibrium steady state cyanide binding to CYP5A1 bound to native-like substrate U44069 and to U46619 was measured. The binding of U44069 is tighter and binding of cyanide is inhibited to a greater extent, especially when
in Nanodiscs. This is also evident from the kinetics of cyanide binding as shown in sections above. Therefore, from these experiments we propose that there is a substantial conformational change in the CYP5A1 when incorporated into Nanodiscs that may explain its enhanced activity towards the isomerization of its native substrate PGH$_2$. 


2.7 References


Figure 1.1 Prostaglandin pathway from Arachidonic acid
Table 2.1. Optimization conditions for CYP5A1 purification protocol

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<tr>
<th>Step</th>
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<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
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<td>From plate</td>
<td>From stock</td>
<td>From stock</td>
</tr>
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<td>Media for primary culture</td>
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<td>LB</td>
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<td>Incubator shaker conditions for media</td>
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<td>37°C, 220 RPM</td>
<td>37°C, 220 RPM</td>
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<td>Terrific Broth</td>
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<td>Incubator shaker conditions for media</td>
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<td>1.0mM IPTG, 0.5mM δ-ALA, 4g Arabinose</td>
<td>1.0mM IPTG, 0.5mM δ-ALA, 4g Arabinose</td>
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<td>160 RPM</td>
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<td>Resuspension buffer (Buffer A)</td>
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<td>Dnase, Rnase</td>
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<td>5X, 80% Duty cycle, Output 4, 1/8&quot;, In Falcons</td>
<td>5X, 80% Duty cycle, Output 4, 1/8&quot;, In Falcons</td>
<td>5X, 80% Duty cycle, Output 4, 1/8&quot;, In Falcons</td>
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Table 2.1 (cont.)

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<td>Ni-NTA</td>
<td>Ni-NTA, DEAE</td>
<td>Ni-NTA, DEAE</td>
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<td>0.1M Potassium phosphate buffer, 10% Glycerol, 0.01% Lubrol, 5mM Histidine</td>
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<td><strong>Elution</strong></td>
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<td>0.1M Potassium phosphate buffer, 10% Glycerol, 0.01% Lubrol, 50mM Histidine</td>
<td>0.1M Potassium phosphate buffer, 10% Glycerol, 0.01% Lubrol, 50mM Histidine</td>
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Figure 2.1. Yields of CYP5A1 using different expression and purification protocols.
Figure 2.2. Characterization of CYP5A1-Nanodiscs. (A) CYP5A1 was incorporated into Nanodiscs and purified using size exclusion chromatography-HPLC. SDS-PAGE was used to verify the isolation of CYP5A1-Nanodiscs. Column 2 shows the protein molecular weight standards and column 1 shows two bands that corresponds to ~56 kDa for CYP5A1 and ~24 kDa corresponds to the molecular weight of MSP1D1(-). (B) Size exclusion HPLC chromatogram of CYP5A1 in MSP1D1(-) Nanodiscs recorded at 417 nm.
Figure 2.3. CO-Binding difference spectrum of CYP5A1 in Nanodiscs. The protein in Nanodiscs exhibited ~100% P450 content which is indicative of a well-folded and functional cytochrome P450.
Figure 2.4. Formation of products from PGH$_2$ by Thromboxane synthase
Figure 2.5. (Left) Schematic of COX-2 docking on the membrane bilayers of CYP5A1-Nanodiscs and PGH$_2$ shuttling through the membrane. (Right) Coupled Activity measurement of CYP5A1 in aqueous solution and in Nanodiscs. CYP5A1 converts PGH$_2$ into MDA, HHT and TXB$_2$ in a coupled assay where PGH$_2$ is formed in situ from arachidonic acid (AA) via COX-2. The amount of HHT and MDA formed were measured using UV-Vis spectroscopy while TXB$_2$ formation was measured using LC/MS/MS. The relative amount of HHT, MDA and TXB$_2$ formed in CYP5A1-Nanodisc is higher than the amount formed by CYP5A1 in aqueous solution. Moreover, the rate of formation of both HHT and MDA is higher for CYP5A1 Nanodiscs compared to CYP5A1 in aqueous solution. The HHT formed is 20% higher, MDA is 60% higher and the TXB$_2$ formed is 130% higher in Nanodiscs as compared to detergent solubilized.
Figure 2.6. **Activity measurement by direct PGH₂ addition of CYP5A1 in aqueous solution and in Nanodiscs.** The amount of HHT and MDA formed were measured as described previously. The relative amount of HHT and MDA formed in CYP5A1-Nanodisc is surprisingly 33% lower than the amount formed by CYP5A1 in aqueous solution, but the TXB₂ formed remains 100% higher in Nanodiscs.
Figure 2.7: (A) Steady state binding of U44069 to CYP5A1-Nanodiscs. The $K_D$ is determined to be 6 µM in Nanodiscs and 15 µM in detergent solubilized state (Inset: Difference spectrum of U44069 bound CYP5A1-Nanodiscs) (B) Steady state binding of U46619 to CYP5A1-Nanodiscs. The $K_D$ is determined to be 5µM (Inset: Difference spectrum of U46619 bound CYP5A1: ND)
Figure 2.8: The redox titration of CYP5A1 in Nanodiscs is conducted in 100 mM phosphate buffer pH 7.4 at 25°C for substrate free. The change in the absorption maxima on light reduction were used to calculate the percentage of reduced protein. The potential is measured using safranin T dye based method using light based reduction as well as dithionite based reduction. The Nernst plot (25° C) for single electron redox function is reported for the substrate free CYP5A1 and substrate bound forms. The zero intercept gives $E^{''}$, the redox potential of the protein and the slope divided by 59 mV yields n, the number of electrons involved in the reaction which is 0.8 – 1 for the measurements indicated. This figure corresponds to the redox titration of CYP5A1 in Nanodiscs in substrate free and substrate bound state. The redox potential of the substrate free CYP5A1-Nanodisc is -282 ± 2 mV.
Figure 2.9: The redox titration of CYP5A1 in Nanodiscs is conducted in 100 mM phosphate buffer pH 7.4 at 25°C for 60 µM substrate U44069 bound protein. The redox potential of the substrate U44069 bound is -284.5 ± 0.5 mV.
Figure 2.10: The redox titration of CYP5A1 in Nanodiscs is conducted in 100 mM phosphate buffer pH 7.4 at 25°C for 60 μM substrate U46619) bound protein. The redox potential of the substrate U46619 bound is -270 ± 5 mV.
Figure 2.11: The redox titration of CYP5A1 in solubilized system at pH 7.4 at 25°C for substrate free and 60 µM substrate (U44069 or U46619) bound protein. The same method as used for CYP5A1 ND was used. (A) (B) (C) corresponds to the redox titration of CYP5A1 in Nanodiscs in substrate free and substrate bound state. The redox potential of the substrate free CYP5A1 is \(-300 \pm 10\) mV, U44069 bound is \(-318 \pm 10\) mV and U46619 bound is \(-307 \pm 10\) mV.
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<td>(70)</td>
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Table 2.2: Equilibrium state cyanide binding to various CYP enzymes.
Figure 2.12. Cyanide binding to substrate free and substrate bound CYP5A1 in detergents and in Nanodiscs. (A) UV-Vis spectra of steady state binding titration of cyanide to CYP5A1 in Nanodiscs. (B) Difference spectra of data shown in figure 3A. (C) The change of absorbance (ΔA) from 447 nm to 415 nm was calculated for each titration and plotted against the corresponding cyanide concentration. Data was fitted with Origin Lab to the single binding isotherm. (D) The percentage of amplitude in the fast phase of the rate of the cyanide binding to CYP5A1 versus increasing concentration of substrate U44069. (E) The percentage of amplitude in the fast phase of the rate of the cyanide binding to CYP5A1 versus increasing concentration of substrate U46619. Rates of fast and slow phase of cyanide binding to CYP5A1, $k_{fast}$ and $k_{slow}$ versus increasing concentrations of substrate are reported for (F) U44069 and (G) U46619.