Oxidative and electrophilic structural modification and catalytic regulation of human hydroxysteroid sulfotransferase 2a1 (hsult2a1)

Xiaoyan Qin

University of Iowa

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OXIDATIVE AND ELECTROPHILIC STRUCTURAL MODIFICATION AND CATALYTIC REGULATION OF HUMAN HYDROXYSTEROID SULFOTRANSFERASE 2A1 (hSULT2A1)

by

Xiaoyan Qin

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Human Toxicology in the Graduate College of The University of Iowa

December 2012

Thesis Supervisor: Professor Michael W. Duffel
ABSTRACT

Human hydroxysteroid sulfotransferase (hSULT2A1) catalyzes the sulfation of a broad range of endogenous (e.g., hormones, neurotransmitters, bile acids) as well as xenobiotic (e.g., drugs, environmental pollutants) compounds. Alteration in the catalytic activity of hSULT2A1 can lead to outcomes like endocrine disruptions or aberrant drug metabolism and xenobiotic toxicity. Oxidative and electrophilic stresses are known to cause physiological damage and be implicated as possible underlying pathologic mechanisms of a wide range of diseases. To examine the oxidative as well as electrophilic regulation of hSULT2A1, model oxidants (glutathione disulfide (GSSG), 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), diamide, tert-butyl hydroperoxide (TBHP)) and electrophiles such as quinone metabolites of polychlorinated biphenyls (PCB-quinones) and phenyl-p-benzoquinone (PBQ) were chosen for this study. Mechanistic studies correlating the enzyme structural modifications with alteration in the catalytic properties were performed to elucidate the catalytic regulative mechanism of an individual oxidant or electrophile.

Thiol oxidants including GSSG, DTNB, and diamide showed catalytic regulation of hSULT2A1. Changes in protein intrinsic fluorescence indicated conformational alterations in hSULT2A1 following the reaction with diamide. Binding properties of hSULT2A1 for its substrates were also altered after reaction with these thiol oxidants, which could be one major reason for the kinetic alteration due to oxidative modification. Formation of mixed disulfides with cysteines in hSULT2A1 was also identified as a result of reaction with GSSG and DTNB.

TBHP was chosen as a model for lipid peroxides, and reaction with this hydroperoxide decreased the catalytic function of hSULT2A1. The dissociation constant for binding of dehydroepiandrosterone (DHEA) was significantly altered with TBHP-pretreatment, but this did not affect the binding of 3’,5’-adenosine diphosphate (PAP) to the enzyme. Structural analysis identified cysteine sulfonic acids and methionine sulfoxide
formation after reaction of hSULT2A1 with TBHP, which could account for the alterations in the binding properties and the catalytic activity.

Both PCB-quinones and PBQ could regulate the catalytic activity of hSULT2A1. Although PCB-quinones only caused decreases in the catalytic activity at all concentrations tested, pretreatment with PBQ indicated that lower concentrations resulted in an increase in the catalytic activity of hSULT2A1 that was followed by a decrease in the catalytic activity of hSULT2A1 upon increasing the concentration of PBQ in the pretreatment. Differences in the dissociation constants of PAP after PBQ-pretreatment were also observed, indicating the key role played by these PCB-quinones in altering the binding of either PAP or the sulfuryl donors, PAPS. Adducts at cysteines in hSULT2A1 were formed following reactions with PCB-quinones and PBQ. Small amounts of cysteine sulfonic acids and methionine sulfoxides were also formed following reaction of the protein with PCB-quinones and PBQ.

Therefore, alterations in both the catalytic function as well as the structural properties of hSULT2A1 by interaction with oxidants and electrophiles may lead to changes in the metabolism of xenobiotics, as well as alterations in the endogenous balance of various steroid hormones. Such changes may be an important component in physiological damage that occurs under oxidative and electrophilic stress.

Abstract Approved: ________________________________
Thesis Supervisor

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December 2012

Thesis Supervisor: Professor Michael W. Duffel
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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph. D. thesis of

Xiaoyan Qin

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Human Toxicology at the December 2012 graduation.

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Thank you all.
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<tr>
<td>2-AAF</td>
<td>2-Acetylaminofluorene</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>3-MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>4-OH tamoxifen</td>
<td>4-Hydroxytamoxifen</td>
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<tr>
<td>α-OH tamoxifen</td>
<td>α-Hydroxytamoxifen</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilinonaphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>BSO</td>
<td>Bis(2-chlorethyl)-nitrosourea</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
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<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>Dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>Diamide</td>
<td>1,1’-Azobis(2,2-dimethylpropane)</td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycorticosterone</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-Dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EE</td>
<td>17α-ethynylestradiol</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric synthase</td>
</tr>
<tr>
<td>ERs</td>
<td>Estrogen receptors</td>
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<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
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<td>GSTA-6</td>
<td>Glutathione S-transferase A6</td>
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<td>H2O2</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>hSULT2A1</td>
<td>Human hydroxysteroid (alcohol) sulfotransferase 2A1</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibition concentration</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>k_cat</td>
<td>Catalytic constant (turnover number of an enzyme)</td>
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<tr>
<td>Kd</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>Ki</td>
<td>Inhibition constant</td>
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</table>
$K_m$ Michaelis constant
LC-MS Liquid chromatography mass spectrometry
MCP-1 Monocyte chemotactic protein
Met Methionine
NAC N-acetyl-cysteine
NAPQI N-acetyl-p-benzoquinone imine
NFAT Nuclear factor of activated T-cells
NF-κB Nuclear factor-κB
$O_2^-$ Superoxide anion radical
$OH^-$ Hydroxyl radical
ONOO$^-$ Peroxynitrite
PAP Adenosine 3’,5’-diphosphate
PAPS 3’-phosphoadenosine-5’-phosphosulfate
PBQ Phenyl-p-benzoquinone
PCB Polychlorinated biphenyl
2’-Cl-BQ 2’-cholorobiphenyl-2,5-benzoquinone
4’-Cl-BQ 4’-chlorobiphenyl-2,5-benzoquinone
3,6,4’-trCl-BQ 3,6,4’-chlorobiphenyl-2,5-benzoquinone
PCBs Polychlorinated biphenyls
PPARα Peroxisome proliferator-active receptor α
PSGL-1 P-selectin-glycoprotein ligand
PXR Pregnane X receptor
RNS Reactive nitrogen species
ROS Reactive oxygen species
RT-PCR Reverse transcription polymerase chain reaction
RXR Retinoid X receptor
S.D. Standard deviation
SF1 Steroidogenic factor 1
SNPs Single nucleotide polymorphisms
SULTs Cytosolic sulfotransferases
TBHP t-Butyl hydroperoxide
TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin
TNF-α Tumor necrosis factor-α
TPA Tissue plasminogen activator
Tris-HCl Tris (hydroxymethyl)aminomethane hydrochloride
$v$ Velocity
$V_{max}$ Maximum enzyme velocity
CHAPTER I
INTRODUCTION

Sulfotransferases

Sulfation of natural and synthetic chemicals including endogenous biomolecules, drugs, as well as environmental chemicals occurs widely. Biologically relevant sulfate conjugates were first discovered as a hallmark in the history of xenobiotic metabolism. Sulfated phenol was identified and characterized as the first conjugated chemical compound in human urine by Baumann in 1867 who isolated the compound from a patient who had been administered carbolic acid as an antiseptic treatment. At that time, however, the mechanism behind the sulfate conjugation was not clear. With the evolution of chemical as well as biochemical techniques, the enzyme responsible for catalyzing the sulfation of various biological compounds was identified and the cofactor 3’-phosphoadenosine-5’-phosphosulfate (PAPS) was isolated (Robbins and Lipmann, 1957). Sulfotransferases (SULTs) are the enzymes responsible for catalyzing the sulfation of various substrates. The sulfation is realized by the transfer of a sulfuryl group from the donor, PAPS or other less common ones, to a variety of amine and hydroxyl containing substrates (Figure 1).

Biological significance of sulfotransferases

Ever since the family of sulfotransferases was discovered, evidence of their physiological importance has emerged dramatically in the realms of detoxification, metabolism of endogenous compounds, as well as bioactivation. In most cases, sulfation works as a detoxification pathway, since the conjugated compounds are often more hydrophilic species that can be readily excreted via kidney or bile. However, the sulfate group could be a good leaving group, and the rest of the compound can be converted into an electrophilic cation which could react with nucleophilic sites on DNA and lead to mutations in DNA as well as carcinogenesis.
Endogenous compound metabolism and physiological impact

The sulfation reaction catalyzed by sulfotransferases is essential in organisms ranging from microbes to human. Two classes of sulfotransferases consist of cytosolic sulfotransferases as well as membrane associated sulfotransferase, and these participate in the biotransformation of endogenous compounds ranging from proteins, carbohydrates,
to small molecules including steroid hormones, neurotransmitters as well as bile acids. Sulfation of these substrates participates in numerous physiological functions.

Protein-tyrosine sulfotransferase is a membrane bound sulfotransferase that catalyzes the sulfation of a variety of chemokine receptors that are involved in leukocytes trafficking, angiogenesis, battling viral infection, as well as the host immune response in cancer. CCR5 and CXCR4 are two examples of chemokine receptors that are sulfated at tyrosine residues. Incubation of CCR5-expressing Cf2Th cells with a sulfation inhibitor can decrease the binding of chemokines to CCR5 (Farzan et al., 1999).

P-selectin-glycoprotein ligand (PSGL-1) is another example of a physiologically important protein that is sulfated in a reaction catalyzed by protein-tyrosine sulfotransferase. In the process of inflammation, leukocytes need to attach to the activated endothelial cells before transmigration into the blood vessel. Sulfation of PSGL-1 expressed on leukocytes is essential for the ligand to be recognized by P-selectin on endothelial cells (Kehoe and Bertozzi, 2000). The modulation of biological adhesion has long been a goal of pharmaceutical intervention in various pathological processes including inflammation, viral infections, arteriosclerosis and thrombosis, and tumor metastasis (Ward and Mulligan, 1994; Buckley and Simmons, 1997; Glinsky, 1998; Giddings, 1999). Being selectively involved in the biosynthesis of recognition epitopes crucial to the adhesive interactions of interest represents a biologically important role of sulfotransferases.

Besides tyrosine being a representative target of post-translational modification by sulfotransferase, carbohydrate moieties of glycoproteins in the extracellular matrix as well as on cell surface can be sulfated. This alters the physiochemical character of proteoglycans, particularly by rendering the compound a net negative charge at physiological pH as well as providing recognition determinants for many receptors (Hemmerich, 2001). Heparin sulfate is an example of a sulfated carbohydrate containing a defined hexasaccharide epitope that can bind to antithrombin and cause a
conformational change of antithrombin to elicit high affinity binding for its ligand thrombin, therefore stopping the blood clotting process (Rosenberg et al., 1997). Attracting proteins from the extracellular matrix, and bringing them to the close vicinity of their cell surface receptors to a form high local concentration is another important role heparin sulfate plays in the immune system-blood vessel interaction (Ihrcke et al., 1993).

Bile acids represent a large family of essential endogenous steroids with a broad range of physiological functions that include regulation of gene expression involved in the homeostasis of cholesterol, glucose and bile acids (Claudel et al., 2005; Kalaany and Mangelsdorf, 2006; Alnouti and Klaassen, 2008) as well as fat and fat-soluble vitamin absorption (Hofmann and Hagey, 2008). Bile acids, however, are also involved in a series of pathological events including carcinogenesis (Fukase et al., 2008) as well as liver toxicities such as cholestasis, bile duct infarction, and cirrhosis (Hofmann, 1999; Fickert et al., 2006). Sulfation of bile acids is an important metabolic pathway that alters the enterohepatic recirculation of bile acids and redirects them toward excretion in urine and feces (Alnouti, 2009).

Evidence from immunology and epidemiology, as well as clinical aspects, indicates that female sex hormones play an important role in the etiology and pathology of chronic immune/inflammatory diseases. As one current focus of study, estradiol (E2) can decrease the apoptosis of immune cells which can be considered as proinflammatory effect and lead to rheumatic diseases (Cutolo and Wilder, 2000). On one hand, E2 production can be regulated by estrogen sulfotransferase-catalyzed sulfation of estrone, which can lead to a decrease in the formation of E2. Sulfated E2 is also found in rheumatoid arthritis patients as well as osteoarthritis patients, which represents a proinflammatory signal which can be transported to inflamed synovial tissue (Schmidt et al., 2009). Another well known pathological role related to E2 is its reactivity toward estrogen receptors (ERs) in breast carcinoma. In hormone-dependent breast carcinoma, binding of E2 to ERs can activate not only multiple transcriptional events which can
finally lead to gene transactivation in the nucleus (Wang et al., 1999), and it can also cause extranuclear events like activation of many components in the signaling pathways such as insulin like growth factor 1 receptor, mitogen-activated protein kinase, and nitric oxide secretion (Cheskis, 2004; Levin, 2005). Sulfated estrone, being a compound that carries a negative charge at physiological pH, is eligible to be transported across the cell membranes of breast carcinoma cells (Miki et al., 2006), where sulfatase can catalyze removal of the sulfuryl group and transform it back to estrone. Besides breast carcinoma, other carcinomas like endometrial (Utsunomiya et al., 2004), prostate (Kapoor and Sheng, 2008), as well as colon carcinoma (Sato et al., 2009) are also closely related with estrogen levels in situ as well as estrogen sulfotransferase activities. This implies that sulfotransferases play a significant role in the regulation of endocrine balance and related carcinogenesis.

Dehydroepiandrosterone (DHEA) is another example of endogenous steroid substrate for sulfotransferases, and it is a common precursor of estrogens and androgens (Hanukoglu, 1992). DHEA and its sulfate are the most abundant circulating steroid hormones in humans, in which sulfated DHEA (DHEA-S) is the most common form in circulation and serves as a reservoir of DHEA (Baulieu et al., 1965). The relative level of DHEA to DHEA sulfate is primarily regulated by the equilibrium between sulfation catalyzed by sulfotransferases and desulfation catalyzed by sulfatases. Although there is no specific nuclear receptor for DHEA or DHEA-S characterized in humans, it is well recognized that multiple non-nuclear receptors are involved with eliciting physiological responses to DHEA. A membrane bound receptor was identified in endothelial cells, heart, liver and kidney which reacts with DHEA specifically with high affinity and can lead to stimulation of endothelial nitric synthase (eNOS) expression as well as activity (Liu and Dillon, 2002). This can further lead to downstream effects including stimulating endothelial proliferation, increasing flow-mediated dilation (Williams et al., 2004), reducing adhesion of monocytes to endothelial cells, amelioration inflammatory
responses, and the production of ROS (Kasperska-Zajac et al., 2009; Curatola et al., 2012). Clinical studies have revealed a protective effect of DHEA against cardiovascular diseases including ischemic heart disease and congestive heart failure (Beer et al., 1996; Feldman et al., 2001; Shufelt et al., 2010). In addition to the hormonal effect mentioned above, DHEA is also known as a neurosteroid that is synthesized in the brain independent of endocrine glands (Baulieu and Robel, 1998). Both DHEA and DHEA-S have broad and complex interactions with brain function including maintenance and division of human neuronal stem cells (Suzuki et al., 2004), promoting neurogenesis and neurite growth (Kimonides et al., 1998; Karishma and Herbert, 2002), and regulation of glutamate release in the prelimbic cortex related to anxiety and depression (Dong et al., 2009). DHEA-S in the brain is important in the memory process, and it has been shown to be a neuromodulator at the GABA receptor (Dubrovsky, 2005). In the human fetus, large amounts of DHEA are produced to support placental estrogen synthesis where human hydroxysteroid sulfotransferase (hSULT2A1) is abundantly expressed (Barker et al., 1994).

Dopamine is a major catecholamine neurotransmitter that can be metabolized by sulfation. By reacting with dopaminergic neurons in the brain, dopamine elicits its biological significance in multiple neurological functions including reward-related learning (Redgrave and Gurney, 2006), memory, cognition, and voluntary movements (Kori et al., 1995). Abnormal levels of dopamine have been associated with pathological disorders like schizophrenia and Parkinson’s disease (Stokes et al., 1999; Huttunen et al., 2008). Dopamine homeostasis is known to be regulated by conjugation reactions like sulfation and methylation (Mannisto and Kaakkola, 1999; Strott, 2002). By adding a sulfuryl group onto dopamine, it is commonly considered as an inactivation of the biological function of dopamine. Besides, the biotransformation by sulfation increases the overall hydrophilicity of dopamine thus facilitating excretion in the urine (Kienzl et al., 1990).
Xenobiotic metabolism and physiological significance

Cytosolic sulfotransferases are traditionally known as phase II drug metabolizing enzymes, or enzymes known for their ability to detoxify drugs and other xenobiotics. Besides detoxication and elimination of the parent compounds, activation of prodrugs as well as bioactivation of mutagenic xenobiotics comprise significant physiological effects mediated by sulfotransferases.

The scientific literature has indicated a large variety of drugs ranging from anticancer reagents (Gong et al., 2012), neurological treatments, and drugs of abuse (Tong et al., 2010; Schwaninger et al., 2011; Yoshinari et al., 2011), to the more commonly used antipyretic drug, acetaminophen (Mizuma et al., 1984) are metabolized and eliminated through conjugation reactions like glucuronidation and sulfation to make them more water soluble, thus facilitate their excretion through urine or feces.

Metabolic sulfation of drugs and other xenobiotics may require an antecedent oxidation step to form a hydroxyl group which can be further conjugated by sulfation. As one example of drug metabolism by sulfotransferases, acetaminophen is a well characterized drug known to be metabolized by this enzyme. As an easily available drug over-the-counter, accidental overdose of acetaminophen may occur. Hepatic failure can be caused by the active metabolite of acetaminophen formed via CYP450-dependent oxidation to the toxic quinone imine metabolite NAPQI (Nelson et al., 2007). By conjugation using sulfotransferase as well as glucuronosyltransferase, the plasma concentration of the parent compound could be decreased in a timely manner, and this can avoid significant formation of the toxic metabolite. As determined in human urine, 30 %-40 % of acetaminophen is excreted in the sulfated form (Patel et al., 1992), and sulfation is also important in the metabolism of acetaminophen in the human fetus (Adjei et al., 2008).

In addition to the participation of sulfotransferases in the metabolic pathways that help to eliminate xenobiotics from biological systems, this family of enzymes has other
important functions which should be reviewed with great attention as they not only affect
the physiological removal of xenobiotics, but also play a role in converting them into
more toxic metabolites. Sulfation has been known to be for many years associated with
the bioactivation of procarcinogens. Early studies on N-hydroxy-acetylamino fluorene, 1’-
hydroxysafrole and 1’-hydroxy-2’, 3’-dehydroestragole established the involvement of
sulfotransferase in the process of chemical induced carcinogenesis (Fuchs et al., 1981;
Fennell et al., 1985; Wiseman et al., 1985). A common reactive metabolite is formed by
the heterolytic cleavage of a sulfate group due to its electron-withdrawing property, and
this leaves an electrophilic cation behind. Nucleophiles like DNA can be the targets of
the resulting electrophilic cations, thus leading to mutagenesis. Major procarcinogens that
can be activated by sulfation usually carry the structure of benzylic or allylic alcohols,
aromatic hydroxylamines, or hydroxamic acids as these structures have the ability to
stabilize the resulting cation (Glatt, 1997). Due to the resonance stabilization of the
formed active metabolite, the cation does not necessarily bind to the nucleophiles at the
position where the sulfate group is lost. The site of modification can be a determinant of
the biological function of the covalent adduct. The well studied anti-breast cancer drug
tamoxifen, for example, is hydroxylated during cytochrome P450-catalyzed drug
metabolism in which two metabolites are generated: 4-hydroxytamoxifen (4-OH
tamoxifen) and α-OH tamoxifen (α-OH tamoxifen). Sulfation that occurs at the phenolic
site of 4-OH tamoxifen can lead to detoxification, whereas the sulfation at the allylic site
of α-OH tamoxifen will lead to bioactivation and the formation of reactive intermediates
(Figure 2) which can bind to DNA and lead to carcinogenesis (Shibutani et al., 2001;
Chen et al., 2002; Apak and Duffel, 2004).
Figure 2. Metabolism of tamoxifen by sulfation of the two oxidation products 4-OH tamoxifen and α-OH tamoxifen.
Classification and nomenclature of sulfotransferases

Two broad classes of sulfotransferases have been identified: one is the family of membrane-bound sulfotransferases that are located in the Golgi apparatus of the cell and are responsible for the sulfation of peptides, proteins, and glycosaminoglycans (on carbohydrates) to regulate their physiological function (Falany, 1997; Negishi et al., 2001); the other is the family of cytosolic sulfotransferases that are responsible for the metabolism of xenobiotics and small endogenous substrates like steroids, bile acids, and neurotransmitters (Coughtrie et al., 1998). The membrane-bound sulfotransferases have been implicated in several biological processes of great importance like leukocyte adhesion, anticoagulation as well as viral and bacterial entry into the cells (Chapman et al., 2004), and the catalytic mechanism is beginning to be explored as limited catalytic work had been done to this family of sulfotransferases (Danan et al., 2010). Major members in this family include tyrosylprotein sulfotransferase, as well as carbohydrate sulfotransferases like heparin sulfate O-sulfotransferase and heparin sulfate N-sulfotransferase (Jacobsson et al., 1979; Tornberg et al., 2011). Cytosolic sulfotransferases are involved in detoxication, hormone regulation, and drug metabolism (Hempel et al., 2005).

Considering the inconvenience that the inconsistency of nomenclature for sulfotransferases presented at the time, Blanchard and coauthors proposed a systematic nomenclature for the cytosolic sulfotransferases and denoted this superfamily of enzymes with the abbreviation “SULT” (Blanchard et al., 2004). The members of the SULT superfamily are further classified into families and subfamilies based on their amino acid sequence identity. SULTs that share at least 45 % amino acid sequence identity are assigned into same family, whereas the members that share at least 60 % sequence identity are assigned into same subfamily. Those with at least 97 % sequence identity are designated as the same isoform. The family members are designated with an Arabic number immediately after the name SULT (e.g. SULT1) and the subfamily names are
designated by alphabetical categories according to their time of discovery, for example, for SULT1 family, the first identified subfamily is named as SULT1A. Isoforms in one subfamily are denoted with an Arabic number following the subfamily name (e.g. SULT1A1) and the assignment of the first number in the subfamily was based on their first publication and the following ones are named sequentially according to their amino acid sequence similarity to the first member. However, in some minor cases like SULT2A1, due to the wide use of historical nomenclature, this rule is not strictly followed. The gene families of sulfotransferases basically follow the same rule as the protein, and are shown in italics for human genes (SULT1A1) and with the exceptions of mouse, and Drosophila which are in lower cases (e.g. Sult1a1). Different species are shown with a three-letter to five-letter code prefixed to the SULT name (e.g. (HUMAN)SULT1A1 ), or more recently, one single letter may be used to designate their species (e.g. hSULT1A1). Alleles of the same member of the subfamily are designated with *1, *2, etc., with the first identified sequence to be *1, whereas the synonymous single nucleotide polymorphisms (SNPs) which encode the same amino acid sequence are designated with *IA, *IB, etc. (Blanchard et al., 2004).

Up till now, 14 human cytosolic sulfotransferase genes within four gene families have been identified. Members from the families of SULT1, SULT2, SULT4 as well as SULT6 have been identified and characterized to reveal their biological significance (Blanchard et al., 2004; Freimuth et al., 2004). Although the family members share the similar structural characteristics, they appear to play distinctive roles in regulating metabolism. The SULT1 family comprises nine members divided into four subfamilies (1A1, 1A2, 1A3 and 1A4; 1C1, 1C2 and 1C3; 1B1 and 1E1). Members of SULT1 families are capable of catalyzing the sulfation of simple phenols, estradiols, thyroid hormones, neurotransmitters, as well as environmental contaminants and drugs (Gamage et al., 2003; Gamage et al., 2005; Lu et al., 2005). The SULT2 family has two identified protein-encoding genes which are translated into SULT2A1 and SULT2B1a, and
SULT2B1b that are capable of catalyzing the sulfation of steroids hormones, bile acids and other small molecules. SULT4B1 is the only member in this family identified in human which is highly conserved throughout species, and it is primarily expressed in brain. Although no specific substrate for SULT4B1 has been identified, several studies indicated its relationship with susceptibility to schizophrenia as well as being a regulative target by a peptidyl-prolyl cis-trans isomerase implicated in several neurodegenerative diseases (Falany et al., 2000; Meltzer et al., 2008; Mitchell and Minchin, 2009). SULT6B1 gene is primarily expressed in testis of primates, but physiological roles for this isoform are not yet clearly defined (Freimuth et al., 2004).

**Gene regulation and expression of sulfotransferases**

The expression of sulfotransferases is under precise control by specific modulators in different organs as well as tissues. As a prototype for research on the expression of drug metabolism enzymes, the regulation of cytochrome P450s (CYPs) family has been widely studied. An increasing body of literature indicates some similarities between the regulator pathways for SULTs and CYPs. However, considering the tissue specificity as well as species variability between the two, direct application of the knowledge about the transcriptional regulatory mechanisms from CYPs to SULTs is not possible. Transcription factors including steroid receptors (e.g. the glucocorticoid receptor, GR), the aryl hydrocarbon receptor (AhR), retinoid X receptor (RXR), constitutive androstane receptor (CAR), pregnane X receptor (PCR), farnesoid X receptor (FXR), and peroxisome proliferator-active receptor α (PPARα), as well as tissue-specific transcription factors like liver-enriched transcription factors and hepatic nuclear factors, are all capable of regulating the expression of sulfotransferases (Runge-Morris and Kocarek, 2005).

As one of the most studied transcription factors, AhR has been reported to be involved with the regulation of several different sulfotransferases. For example,
hSULT1E1 is expressed in human breast epithelial cells and is downregulated in many breast cancer cell lines. Its regulation by AhR has been examined by the treatment of cells with 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin (TCDD), and the mRNA levels for hSULT1E1 were decreased by 60\% (Puga \textit{et al}., 2000). Moreover, other major hepatic SULTs including hepatic hydroxysteroid sulfotransferase as well as aryl sulfotransferase were suppressed in livers of 3-methylcholanthrene- or \(\beta\)-naphthoflavone-treated rats and in isolated rat hepatocytes (Runge-Morris, 1998). However, species differences are present in the regulation of SULT genes. mSult2a1 as well as mSult2a2 in female mouse did not show a downregulation in the mRNA levels following TCDD pretreatment (Alnouti and Klaassen, 2008).

\textit{Polymorphism and pharmacogenetics of sulfotransferases}

Discoveries of polymorphisms in sulfotransferases date back to the 1980s when evidence in the thermal stability was observed in genetically different hSULT1A1 and hSULT1A3 in human blood platelets (Van Loon and Weinshilboum, 1984; Price \textit{et al}., 1988; Price \textit{et al}., 1989). From then on, a number of excellent reviews have focused on the phenotypic as well as the genotypic variants in sulfotransferases to see their implications in the overall homeostasis and pathological conditions in the biological system.

The identification of single nucleotide polymorphisms (SNPs) made it possible to investigate the genetic variations in relation to the activity of SULTs. Major families of SULTs including SULT1 and SULT2 are analyzed to examine their genetic variation and the physiological impact. hSULT1A1 expressed in blood platelets has demonstrated a variable enzymatic activity due to gender difference as well as a seasonal rhythm (Marazziti \textit{et al}., 1995; Nowell \textit{et al}., 2000). Ethnic variation in platelet sulfotransferase activity has also been reported, where there were significant differences between Spaniards and Finns (Brittelli \textit{et al}., 1999), and African-Americans had higher platelet
sulfotransferase activity compared to Caucasians (Anderson et al., 1988). Five common genetic polymorphisms of SULT1A1 at the 5'-flanking region were discovered to be present at different allele frequencies in Caucasian, African-Americans and Chinese groups. Platelet SULT1A1 enzymatic activity was significantly correlated with individual polymorphisms at the promoter region of different ethnic groups. Haplotypes were constructed, and it was demonstrated that the frequency of the haplotypes in different populations corresponded with the enzymatic activity difference (Ning et al., 2005). Polymorphisms in genes coding for hSULT1A1 as well as hSULT1E1 showed significant correlations with the concentrations of E₁, E₂ and DHEA in premenopausal women, and this might have profound influences in the etiology of certain cancers like breast, ovarian and endometrial cancers, as well as other conditions with high morbidity arising from hormone-sensitive tissues (Yong et al., 2010). Genetic variants can also affect the enzymatic activity of sulfotransferases like hSULT1A1 which plays an important role in anticancer drug metabolism that differences in anticancer effects were observed after tamoxifen treatment in patients with different alleles of hSULT1A1 (Falany et al., 1994; Nowell et al., 2002; Wegman et al., 2007).

Although polymorphisms in the SULT2 family have not been as widely studied as in the SULT1 family, two SNPs were identified with SULT2A1*1 (Lindsay et al., 2008). Five variant allozymes were detected for each SULT2B1, in which activities ranged from 64-88% of wild-type for SULT2B1a and from 76 to 98 % of SULT2B1b (Ji et al., 2007). The SULT2A1*2 and *3 allozymes showed decreased enzyme activity (Wood et al., 1996). In African-American populations, Ala261Thr is found to be of 13 % frequency and is believed to be significant in causing inter-ethnic differences in SULT2A1 activity (Thomae et al., 2002). Further analysis revealed that this variant is involved in the formation of the SULT2A1 dimer in vitro and is located in the conserved dimerization motif (Petrotchenko et al., 2001; Thomae et al., 2002).
Structural properties of sulfotransferases

The three dimensional structures of sulfotransferases are mostly conserved and are similar to those of nucleotide kinases (Kakuta et al., 1997). All the sulfotransferases are approximately spherical in shape with a single α/β fold which is formed with a central four or five-stranded β sheet and exterior surrounding α helices. Cytosolic sulfotransferases are mostly homodimers that consist of approximately 300 amino acid residues with the exception of mSult1e1 and rSULT1A1, where mSult1e1 is a monomer, and rSULT1A1 is known to form both homodimers and heterodimers (Kiehlbauch et al., 1995; Petrotchenko et al., 2001). The first solved crystal structure was the mSult1e1 with nucleotide reaction product 3’-phosphoadenosine-5’-phosphate (PAP) and the sulfuryl acceptor estradiol (E2) bound (Kakuta et al., 1998a). The active site and PAPS-binding residues are well conserved in all the sulfotransferases. While the PAPS-binding region is structurally conserved for all SULTs, the largest differences among the SULTs are in the substrate-binding region.

One of the major structural elements of the sulfotransferases contains a strand-loop-helix, where strand 8 and helix 6 define the PAPS/PAP binding site. The strand-loop-helix provides the major binding site for the 5’-phosphate group of PAPS and PAP, whereas residues from strand 8 and helix 6 bind the 3’-phosphate group. This specific binding structure is designated as the PSB loop, and it is well conserved through species. There are several key residues playing important roles in stabilizing the binding of PAPS and PAP. Using mSult1e1 as an example, Lys48, Ser49, Gly50 as well as Thr51 in the PSB loop and Thr52 from helix 3 are responsible for hydrogen-bonding with the oxygen atom in the 5’-phosphate group in PAPS and PAP. Among these residues, Lys48 has its distinctive and significant function to directly coordinate with the leaving oxygen, which indicates the role of this lysine in facilitating the dissociation of sulfuryl group by donating a proton to the leaving oxygen of PAP. This lysine residue is conserved in nearly all the sulfotransferases including the Golgi-membrane bound sulfotransferase. A
few exceptions have been identified with this lysine residue where it can be substituted by a functionally similar residue arginine (Kakuta et al., 1998b). The specific binding site for 3’-phosphate is constituted with strand 8 and helix 6, and this feature is conserved in all known structures of sulfotransferases (Bidwell et al., 1999; Dajani et al., 1999; Pedersen et al., 2000). The interactions of the sulfotransferase and the oxygen from the 3’-phosphate were established by the direct interactions with Arg130 and Ser138 of mSult1e1. Besides these key residues, Trp53 as well as Phe 229 stay parallel with the adenine part of PAP/PAPS to stabilize the adenine and help orient its direction (Kakuta et al., 1998b). Mutagenic studies on the key residues of flavonol 3-sulfotransferase indicated Arg140 as well as Arg276 are essential for the binding of the 3’-phosphate of PAPS to the enzyme. These residues correspond to of Arg130 and Arg257 in mSult1e1 and are involved in the catalytic regulation of flavonol 3-sulfotransferase (Marsolais and Varin, 1995).

Differences in the conformations as well as the key amino acid residues close to the substrate binding pocket are the important determinants for the substrate specificity for different families of sulfotransferases. By studying the crystal structure of the enzyme in the presence of its substrate, key binding components were identified. In the crystal structure developed for mSult1e1, three loops forming the hydrophobic substrate binding pocket were resolved, and the structural and chemical features of these substrate binding loops were superimposable in the structure of hSULT1A3 as well as hSULT2A1 (Bidwell et al., 1999; Pedersen et al., 2000). However, small changes in the key amino acid residues can regulate the substrate specificity of each type of sulfotransferase. For example, mSult1e1 has high specificity toward the sulfation of estradiol (E2), but it has only low activity for DHEA which is a good substrate for hSULT2A1. Superimposing a DHEA molecule model into the binding pocket of mSult1e1 indicated that its 19 methyl group, which is the major structural difference from E2, is too large to fit into the binding site due to a conflict with Tyr81 at the entrance of the binding pocket of E2. One single
mutation of the Tyr81 into a Leu, which has less steric hindrance at the gating position, exhibited a decreased $K_m$ for DHEA sulfation as well as an increased $k_{cat}$ of more than 10-fold compared to the wild type mSult1e1 (Petrotchenko et al., 1999).

Most cytosolic sulfotransferases are dimeric, and structural studies have provided some insights into the formation of the dimers. A highly conserved motif in SULTs near the C-terminus, KXXXTVXXXE, is essential for the dimerization of both homodimers as well as heterodimers in all the SULTs (Petrotchenko et al., 2001), and some evidence has been observed that the formation of the dimer complex is involved in the substrate inhibition characteristics of some cytosolic sulfotransferases (Cook et al., 2010a). mSult1e1 is known to be the only reported naturally occurring monomeric SULT due to mutations in the KTVE motif (Petrotchenko et al., 2001).

**Catalytic mechanism and substrate inhibition**

SULTs catalyze sulfation by transferring a sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate to the substrate. The catalytic mechanism of this reaction has been studied for several of the sulfotransferases, and, due to the conservation of key structural components as well as the strong degree of crystal structure homology, some basic concepts in this reaction can be widely applied to multiple members of sulfotransferases.

Since the first X-ray crystal structure of SULT indicated that the core structure of mSult1e1 resembled the nucleotide kinases, it was proposed that a similar in-line displacement catalytic mechanism might be in action (Kakuta et al., 1997). The active site and the transition state model mimicked by the mSult1e1-PAP-vanadate ion complex structure have provided structural support for this mechanism for the sulfuryl group transfer (Kakuta et al., 1998b).

Multiple studies have been focused on the kinetics of sulfation to determine whether the reaction proceeds through an associative bimolecular nucleophilic
substitution ($S_N2$)-like mechanism or by a dissociative unimolecular nucleophilic substitution ($S_N1$)-like mechanism. To date, it is clear that all the SULTs subjected to kinetic study were following a sequential mechanism where all the substrates bind to the enzyme prior to product release. Studies on the homogenous rSULT1A1 revealed rapid equilibrium random kinetics with a Random Bi Bi mechanism in which PAPS and $p$-nitrophenol bind to the enzyme independently (Duffel and Jakoby, 1981). Later studies on hSULT1E1 as well as hSULT2A1 confirmed this mechanism in the sulfation reaction using estradiol or DHEA as their substrates respectively (Zhang et al., 1998; Gulcan and Duffel, 2011). Meanwhile, kinetic experiments on sulfotransferases from monkey liver catalyzing the sulfation of monkey bile salts (Barnes et al., 1986), human brain aryl sulfotransferases (Whittemore et al., 1986), as well as flavonol sulfotransferase (Varin and Ibrahim, 1992), suggested an Ordered Bi Bi mechanism. Kinetic studies on hSULT1A1 utilizing isotopic exchange methods also supported an Ordered Bi Bi mechanism with PAPS binding first to the active site (Tyapochkin et al., 2008; Tyapochkin et al., 2009). A recent study on SULT1A1 discovered that the reverse reaction catalyzed by this enzyme was under a ping pong mechanism for PAP sulfation, however, any effects on the forward reaction are still unclear (Tyapochkin et al., 2011). The order of addition of substrates for hSULT2A1 appears to be dependent on the structure of the sulfuryl acceptor substrate (Cook et al., 2010b).

Indeed, crystal structures of hSULT2A1 with either the sulfuryl acceptor or the sulfuryl donor PAPS bound indicated the substrates can bind to the enzyme independently (Rehse et al., 2002; Chang et al., 2004; Lu et al., 2010). However, other work comparing the structures across a family of sulfotransferases has suggested that PAPS-binding can prime several flexible loops for binding of the substrate but not vice versa (Allali-Hassani et al., 2007).

By examining the crystal structure of mSult1e1, a transition state model was developed and the side-chain amino group of Lys48, which is conserved in all the
sulfotransferases is coordinated with both the oxygen from the phospho-sulfate as well as equatorial oxygen of a vanadate model (Kakuta et al., 1998b). The backbone nitrogen was interacting with the other equatorial oxygen. His108 in mSult1e1 also plays a key role in the sulfuryl transfer process, and this is conserved in all cytosolic sulfotransferases. Lys48 of mSult1e1 may protonate the bridge oxygen to assist in the dissociation of the sulfuryl group from the PAPS molecule, where His180 deprotonates the phenolic group to increase its nucleophilicity thus helping in its reaction with the sulfuryl group. Two other amino acid residues that have significant roles in the sulfuryl group transfer process are Lys106 and Ser138 of mSult1e1. The mechanism of hSULT1E1 has also been examined, as it is the human analog of mSult1e1. Lys105 in hSULT1A1 (homolog of Lys106 in the mouse enzyme) is found to stabilize the transition state, whereas Ser137 in hSULT1A1 (homolog of Ser138 in the mouse enzyme) helped stabilize the 3'-phosphate group of PAPS and hydrogen bond with Lys47 (homolog of Lys48 in mSult1e1) in the absence of an acceptor to prevent nonproductive PAPS hydrolysis (Pedersen et al., 2002) (Figure. 3).

Substrate inhibition is a common kinetic phenomenon seen with many drug metabolizing enzymes in which an increase in the substrate concentration will lead to a decrease in the rate of reaction (Korzekwa et al., 1998). Several possible substrate inhibition mechanisms are seen with sulfotransferases. One is a two-substrate binding site model in which a single subunit of enzyme can bind two substrate molecules simultaneously. In this model, it is presumable two binding sites for the substrates exist in one subunit of the enzyme with one being the catalytically active one while the binding of its substrate to the second binding site will decrease the sulfation rate (Zhang et al., 1998). Two p-nitrophenol molecules were identified in the active site of a crystallized SULT1A1 with PAP bound (Gamage et al., 2003). Based on a molecular modeling and site mutagenesis study, substrate inhibition by dopamine in SULT1A3 was identified to
be caused by the binding of two dopamine molecule in the active site (Barnett et al., 2004).

A second possible mechanism for substrate inhibition is due to the formation of a ternary dead-end complex \([E\cdot A\cdot B]\). The \([E\cdot A\cdot B]\) complex is nonproductive and can inhibit the catalytic reaction and the accumulation of the complex will slow the catalytic cycle significantly (Gamage et al., 2003; Gamage et al., 2005). Such a dead-end complex of \([E\cdot PAP\cdot Estradiol]\) was observed in a crystal structure of hSULT1A1 in which the substrate, estradiol, and the product, PAP, were bound to the enzyme in a nonproductive mode (Gamage et al., 2005). In the crystal structure of hSULT2A1, two different binding modes are seen for dehydroepiandrosterone (DHEA) in the active site. One study showed that one DHEA binding mode interacted with PAP binding and may contribute to the dead-end complex formation (Rehse et al., 2002), whereas another study found that both substrate-binding modes could form the dead-end complex and thus lead to substrate inhibition (Lu et al., 2008). The significance of the formation of a nonproductive ternary complex in the substrate inhibition implies that changes in the binding affinity of PAP may alter the substrate inhibition characteristics, since the PAP binding to the enzyme is directly related with the formation of this nonproductive complex. In one study on rSULT1A1, the reduced form of this enzyme showed substrate inhibition, but this was ameliorated when the enzyme was oxidized. The authors attributed this effect to the reduced affinity of the enzyme for PAP, and reduced overall lifetime of \([E\cdot PAP]\), due to the oxidation at specific cysteine residues (Marshall et al., 2000).

A third theory of the kinetic mechanism of substrate inhibition related with SULTs lies in the possibility that, at high concentrations, substrates may bind to the enzyme in a way (i.e. to an alternative site in relation to the reaction site) in which the resulting enzyme conformation is incompatible with PAPS binding. The resulting conformation preventing PAPS binding may yield a nonproductive conformer in which a
unique helix is formed upon the binding of substrate in an inhibition mode (Allali-Hassani et al., 2007).

Figure 3. Schematic representation of proposed catalytic mechanism for hSULT1E1
Human Hydroxysteroid Sulfotransferase hSULT2A1

The SULT2 family of cytosolic sulfotransferases was originally named as hydroxysteroid sulfotransferases or bile acid sulfotransferases, which also implies its substrate specificity. For human SULTs, the SULT2A subfamily has only one isoform, hSULT2A1, and this enzyme is responsible for catalyzing the sulfation of hydroxysteroids including DHEA, androgens, pregnenolone, as well as bile acids (Lyon et al., 1981; Comer et al., 1993; Falany et al., 1994; Forbes et al., 1995). Hydroxysteroid hormones are crucial in the development, maturation and physiological functioning of many vital organ processes. Meanwhile, hormones can be toxic under certain circumstances. Toxicity due to excessive biological action induced by hormones can lead to deleterious physiological responses manifested in cancer, mutagenicity or reproductive damage. Disturbance in the activity of hSULT2A1 may be directly related to the level of its substrates, like hydroxysteroid hormones and/or other xenobiotics that may affect the activity of those hormones. Regulation of the level of hydroxysteroids establishes a central role that hSULT2A1 plays in the homeostasis of hormones and maintenance of normal physiological functions.

Molecular characterization and tissue distribution

Human hydroxysteroid sulfotransferase (DHEA-ST; SULT2A1; EC 2.8.2.2) is the only member of SULT2A subfamily in humans. The gene for hSULT2A1 is located on the 19q13.3 region of chromosome 19, and it has 855 nucleotides that encode a 285 amino acid protein (Otterness et al., 1995a; Otterness et al., 1995b). A dimeric structure composed of two identical subunits was determined for the hSULT2A1 with each subunit having the mass of approximately 34 kDa (Falany et al., 1989; Rehse et al., 2002). Research on its stability and enzymatic activity has revealed that hSULT2A1 has highest catalytic activity for DHEA sulfation in the pH range from 7.0 to 9.0 and at approximately 40 °C (Kudlacek et al., 1997; Chang et al., 2001). Northern blot analysis
has shown that hSULT2A1 is highest in human liver, adrenal and small intestine (Otterness et al., 1992; Luu-The et al., 1995; Tashiro et al., 2000). Further analysis utilizing RT-PCR showed that the mRNA level of hSULT2A1 is high in steroidogenic organs like adrenal and ovary, androgen-dependant tissues like prostate tissue as well as in liver, stomach, small intestine and colon (Javitt et al., 2001). hSULT2A1 was also found to be expressed in human embryonic hepatocytes and continued to be expressed in these cells into adulthood. The expression of hepatic hSULT2A1 increases with advancing gestational age before reaching near-adult levels in the early postnatal period indicates an increased requirement for this enzyme during development (Barker et al., 1994).

Substrate specificity of hSULT2A1

hSULT2A1 has a broad substrate specificity ranging from endogenous hormones and bile acids to xenobiotics like drugs and carcinogens. Among a series of endogenous steroids, purified hSULT2A1 was reported to have the highest activity toward dehydroepiandrosterone (DHEA). The enzyme is also capable of catalyzing the sulfation other steroids including pregnenolone, androsterone, and estradiol. However, no activity was observed toward p-nitrophenol and dopamine, phenols that are known to be the substrates for SULT1 family members (Falany et al., 1989). hSULT2A1 is also known to be a major enzyme in bile acid detoxification. Recent studies have revealed structural relationships for the catalytic activities of hSULT2A1 towards individual bile acids. The hydroxyl group number in bile acids was reported to be inversely proportional to the catalytic efficiency of hSULT2A1, where the monohydroxyl, and the most toxic, bile acid (lithocholic acid) was sulfated with the highest efficiency in contrast to the trihydroxy, and the least toxic bile acid (cholic acid), which was sulfated with the least efficiency by hSULT2A1 (Huang et al., 2010). Procarcinogenic benzylic alcohols derived from polycyclic aromatic hydrocarbons like 6-hydroxymethylbenzo[a]-pyrene and
hycanthone are sulfated in reactions catalyzed by hSULT2A1, and this serves as a major metabolic bioactivation for environmental carcinogens (Glatt, 2000). hSULT2A1 also has a selective role in the N-sulfation of quinolone and other amine-containing drugs like ciprofloxacin, moxifloxacin, garenoxacin, and desipramine in humans (Senggunprai et al., 2009).

Genetic and catalytic regulation of hSULT2A1

Many xenobiotics as well as endogenous compounds are known to induce the transcription of hSULT2A1. Induction of hSULT2A1 by glucocorticoids through either the glucocorticoid receptor (GR) or the pregnane X receptor (PXR) was reported to be mediated by direct action on the promoter region of hSULT2A1 (Duanmu et al., 2002). Besides PXR, the constitutive androstane receptor (CAR) is also known to regulate the transcription and expression of hSULT2A1 through reaction with PXR and CAR-inducible composite element in the hSULT2A1 promoter region (Echchgadda et al., 2009). Retinoic acid receptor (RXR) potentially competes with GR for binding at the response element and has been shown to positively affect the expression of hSULT2A1 (Maiti et al., 2005). Species differences in genetic regulation were also seen with SULT2A1 when peroxisome proliferator-activated receptor (PPAR) could regulate the expression of the human enzyme hSULT2A1, but the ligand for this receptor failed to induce SULT2A1 in rat primary hepatocytes (Fang et al., 2005). Several xenobiotics that are known to have transcriptional regulatory effects like TCDD, 2-AAF, as well as 3-methylcholanthrene (3-MC) have been shown to reduce SULT2 activities and mRNA levels in rat liver (Runge-Morris, 1998) but failed to affect the expression of hSULT2A1 in a human colon carcinoma cell line (Lampen et al., 2004). Mechanistic studies have revealed that adrenal-specific expression of hSULT2A1 was linked to the ability of another orphan nuclear receptor steroidogenic factor 1 (SF1), to interact with the transcription factor GSTA-6 (Saner et al., 2005)
In addition to altering sulfation through changes in protein expression, the catalytic activity of hSULT2A1 can be regulated by interactions of the enzyme directly with small molecules like xenobiotics or endogenous compounds. Flavonoids and isoflavonoids are polyphenolic chemicals which are widely distributed in vegetables and fruits that are heavily consumed by human as well as other animals. Members of flavonoids are found to have regulative effects on sulfotransferases where hSULT2A1 is known to be inhibited by apigenin within the micromolar range. A 7-hydroxy-substituent is required for the inhibition effect due to myricetin, baicalein, galangin as well as 7-hydroxyflavone. Those flavonoids can all inhibit the sulfation reaction catalyzed by hSULT2A1 with fairly low values around 2-3 μM (Harris and Waring, 2008).

Another widely studied plant, liquorice, and its major active constituent, glycyrrhitin acid (GA), were found to elicit their overall biological effect through the pathway of inhibiting the catalytic activity of hSULT2A1. Such inhibition would lead to the generation of more active steroid hormones like DHEA, deoxycorticosterone (DOC), and other steroids expressed in human adrenocortical tissue. It was reported that the mRNA level of hSULT2A1 was not significantly altered after being treated with glycyrrhentin acid, however, a decrease in the catalytic activity of hSULT2A1 catalyzing the sulfation of DHEA as well as DOC was observed (Al-Dujaili et al., 2011).

In addition to natural small molecules that can regulate the catalytic activity of hSULT2A1, other chemicals like drugs and industrial pollutants can also have significant influence on this enzyme. The hydroxylated metabolites of polychlorinated biphenyls (PCBs), widely spread environmental chemicals, are reported to have inhibitory effects on hSULT2A1 as well. The different substitution patterns determined the nature of the effect on this enzyme, since 4’-OH PCB9 served as an inhibitor for hSULT2A1, while 4-OH PCB34 and 4’-OH PCB68 were substrates for this enzyme (Liu et al., 2006). More detailed structure-activity studies have elucidated structural features that are important in the interactions of OHPCB with hSULT2A1 (Ekuase et al., 2011).
An additional aspect of the regulation of hSULT2A1 has been observed with celecoxib. Following treatment of hSULT2A1 with celecoxib, the sulfation of 17α-ethynylestradiol (EE) was altered, and the sulfation position switched from the traditional 3-O-position to the 17β-O-position. Meanwhile, the overall catalytic activity was increased, and the sulfation catalyzed by treated SULT2A1 exceeded the native production by 3-4 fold (Cui et al., 2004).

Oxidative and Electrophilic Regulation of Physiological Macromolecules

The cellular environment is balanced between the oxidative state and the reductive state. Although the extracellular environment is predominantly reducing, an oxidized state within the cell could be extremely deleterious. Oxidative stress, defined as an excess production of reactive oxygen species (ROS) relative to antioxidant defense, has been shown to play an important role in the pathogenesis of vast number of diseases including atherosclerosis, heart failure, diabetes, hyperlipidemia, carcinogenesis, neurodegenerative diseases like Alzheimer’s disease, schizophrenia, obesity, and interference with male fertility (Jackson and Loeb, 2001; Park et al., 2007; Feletou et al., 2010; Arden and Sivaprasad, 2011; Ciobica et al., 2011; Tsutsui et al., 2011a; Tsutsui et al., 2011b; Tvrda et al., 2011). In addition to ROS, reactive nitrogen species (RNS) and electrophilic stress are also known to have great influence on the ability to regulate cellular processes as well as contribute to disease development.

Redox control of homeostasis as well as pathological conditions is known to be caused by ROS/RNS that are associated with lipid peroxidation, DNA damage, and formation of disulfide bonds in proteins. Superoxide anion radical (O$_2^-$) and its dismutation product, hydrogen peroxide (H$_2$O$_2$) are principal ROS that are derived primarily from the electron transport chain in mitochondria and other sources. Infectious disease states can be potent generators of ROS as well, in which mass production of oxidative bursts comprising cellular superoxide radicals and hydrogen peroxide by
neutrophils can lead to formation of hydroxyl radical (OH‘) and cause cellular component damage (Jackson and Loeb, 2001). Numerous reports have suggested that strand breaks and base-modification of DNA are caused by reaction of ROS directly on nuclear DNA (Lesko et al., 1980). Mechanistic studies indicated the formation of 8-oxo-dG after exposure of DNA to activated neutrophils or eosinophils, and the oxidative mechanisms caused by endogenous generated ROS and the oxidized nucleic acid are now being investigated to determine the etiology of various diseases (Shen et al., 2000; Lee and Pervaiz, 2011). Peroxynitrite (ONOO‘) is another potent oxidant that is known to cause DNA strand breaks, oxidation of DNA bases, deamination of G to A, and formation of 8-nitrodeoxyguanosine leading to damage to cellular functions (Burcham, 1999). Damage to DNA and other macromolecules can also occur from oxidation formation of other electrophilic mutagenic substances. For example, electrophilic lipid oxidation product propenals are capable of forming pyrimidopurinone adducts in DNA (Dedon et al., 1998).

In addition to modifications in DNA, oxidative stress can result in modifications within proteins that can lead to serious physiological outcomes. Major damage to proteins caused by ROS includes oxidation of the protein backbone, protein fragmentation, oxidation of amino acid side chains, generation of protein carbonyl derivatives as well as accumulations of oxidized proteins (Berlett and Stadtman, 1997). Structural modifications on proteins can lead to malfunction of the protein, altered enzymatic functions as well as aberrant binding properties of specific proteins followed by serious downstream effects like alteration of transcription events, impaired signaling pathways, or changes in cell adhesions.

Many reviews have focused on the mechanism of oxidative regulation of transcriptional events. Nuclear factor-κB is one essential and ubiquitous transcription factor which is activated in many cell types in response to growth stimuli and stress, leading to rapid induction of genes that encode a variety of growth and defense proteins. Redox manipulation due to ROS can regulate the activity of NF-κB as a result of
alterations in the binding properties of transcription factors to their target DNA sequences (Piao et al., 2005), and this may lead to altered cell growth or to tumorigenesis. Other transcription factors like AP-1 and NFAT are known to be regulated by ROS and have implications in several cellular effects that lead to neuronogenesis (Kennedy et al., 2012) and the expression of key proteins related with heart failure (Tsutsui et al., 2011b). A number of pro-inflammatory genes including cytokines MCP-1, IL-6, TNF-α, as well as IL-1α and IL-1β are also regulated by redox-controlled transcription factors like NF-κB (Gupta et al., 2011).

Besides directly targeting the transcriptional factors, ROS can affect physiological functions by attacking the proteins that regulate intracellular Ca^{2+} concentration, and this is known to be closely linked to multiple signaling cascades and cellular functions like fertilization, proliferation, development, learning and memory, contraction and secretion (Berridge et al., 2000). Important components regulating influx of extracellular calcium like voltage-gated Ca^{2+} channels, receptor operated channels, and Ca^{2+} release-activated Ca^{2+} channels have been determined to be affected by ROS. The altered calcium concentration can activate or inhibit the normal function of those channels, depending upon the cell type, and this may yield complex responses in inflammation and in the cardiovascular, nervous systems (Bogeski et al., 2011; Booth et al., 2011; Viola and Hool, 2011).

Apoptosis, which is an evolutionarily conserved programmed cell death process, is also known to be regulated by cellular redox state. Multiple reports have indicated that the weakened cellular antioxidant defense is related with an increased sensitivity of apoptosis with a depletion of cellular glutathione which serves as a major reducing component in the intracellular environment (Pierce et al., 1991; Staal et al., 1992). It has also been shown that acute apoptosis induced by cisplatin is partly due to the formation of cellular peroxide rather than the traditional DNA damage theory (Berndtsson et al., 2007).
Polyunsaturated fatty acids, being essential components of biological membranes, are also major targets of ROS induced peroxidative damage. The biological significance of this reaction includes the loss of unsaturated fatty acids of lipid membranes, the formation of a number of peroxidative lipid breakdown products (e.g. lipid hydroperoxides, fatty aldehydes and ketones) which are electrophilic and can react with other biomolecules (Plaa and Witschi, 1976; Slater, 1978; Slater, 1979; Tappel, 1980). The products formed during lipid peroxidation are known to be cytotoxic. Lipid peroxidation products like 4-hydroxy-nonenal, found in peroxidized liver microsomes, as well as malondialdehyde are known to cross link proteins and nucleic acids, and these reactions are related with carcinogenesis, neurodegenerative disease and other pathological conditions (Mukai and Goldstein, 1976; Cai et al., 2012; Perluigi et al., 2012).

Other reactive electrophilic species that contain α, β-unsaturated carbonyl groups or other reactive electrophilic functional groups range widely from environmental chemicals and drugs to nutritional factors. By reacting with cellular nucleophiles like DNA bases and protein sulphydryl groups, electrophiles can affect all levels of cellular events, with some processes in common with oxidative stress.

Environmental type-2 alkenes represent a large class of electrophiles that have extensive commercial and industrial applications. Acrylamide is one of the most widely studied type-2 alkenes, and it is responsible for neurotoxicity, pulmonary toxicity as well as hepatic and vascular toxicity (Beauchamp et al., 1985; Barber et al., 2001). Mechanistic studies have revealed that type-2 alkenes are soft electrophiles which can impair protein function by forming irreversible adducts with the soft nucleophilic thiolate sites of cysteines and lead to inhibition of NO signaling in the nervous system and cause neurotoxicity (LoPachin et al., 2008).

Major targets in proteins, like nucleophilic cysteines, histidines, lysines as well as methionines, are prone to oxidative damage as well as electrophilic modifications.
Specific interest in cysteine residues arises from the ability of cysteine to form oxidative reversible modifications as well as irreversible modifications. Reversible modifications include disulfide bond formation (-S-S-), sulfenic acid formation (-SOH), glutathioylation (-SSG), as well as nitrosylation (-SNO). Irreversible modification can be obtained through further oxidation from the sulfenic acid product to the sulfonic acid (-SO₃H) as well as through electrophilic modifications (Christians and Benjamin, 2012).

Different sulfur oxidations inside the cell are of great importance as they can affect both protein function through altering structural characteristics and binding properties. Glutathionylation has been identified to be an inactivation pathway in multiple proteins/enzymes including NFκB (Pineda-Molina et al., 2001), actin (Wang et al., 2001), tyrosine hydroxylase (Borges et al., 2002), protein kinase C-α (Ward et al., 2000), as well as protein phosphatase (Rao and Clayton, 2002). On the other hand, glutathionylation is associated with activation of proteins like glutathione S-transferase (Dafre et al., 1996), HIV-I protease (Davis et al., 1996a), and carbonic anhydrase II phosphatase (Cabisco and Levine, 1996). As an analogue of HIV-I protease, glutathionylation of human T-cell leukemia virus type 1 protease can inactivate this protein, however, this can be reversible with the reduction of the disulfide catalyzed by glutathione reductase. Additional studies have shown at least one cysteine or methionine is present near the regions of the dimer interface of all retroviral proteases, suggesting a conserved regulatory role for reversible Cys-S-glutathionylation (or Met sulfoxide formation) in viral proteases (Davis et al., 1996a; Davis et al., 1996b).

Intramolecular disulfide bond formation is known to be implicated in the thermodynamics of protein folding to stabilize the native conformation of a protein. However, as more evidence accumulates, disulfide bonds are also known to be a switch for regulation of protein functions. Transcription factor OxyR, which is involved in the oxidative adaption of bacteria, binds to DNA promoter regions to prevent transcription. The formation of a disulfide bond between two cysteines in the same OxyR protein
allows the recruitment of RNA polymerase and activation of transcription of target gene (Lee et al., 2004). Disulfide bond formation is also important in the immune response where a disulfide bond leads to conformational changes in CD4 that are required for fusion of viral and cell membrane (Matthias and Hogg, 2003).

*Oxidative regulation of sulfotransferases*

Previous studies have indicated that oxidative modification can alter the catalytic properties of a sulfotransferase, where sequential modification at cysteine residues can lead to different effects on the catalytic activity of rat sulfotransferase rSULT1A1 (Marshall et al., 1997; Marshall et al., 2000). It was first observed that the optimum pH value of rSULT1A1 differs for different substrates when subjected to oxidizing conditions. Even for the same substrate, prolonged storage could alter catalytic activity of the enzyme at same reaction pH when compared to the freshly isolated enzyme. Later observations revealed that partial oxidation of the enzyme can lead to a dramatic change from an acidic pH optimum to more physiological pH range for some substrates, and increases in the rate of sulfation can also occur. Changes in substrate specificities were also observed as a function of redox state of the enzyme’s environment (Marshall et al., 1997).

Glutathione disulfide (GSSG) was used as a model oxidant to study oxidative effects on the catalytic regulation of recombinant rSULT1A1. It was observed that the activity of rSULT1A1 was increased following the first hour of incubation of the enzyme with GSSG, but the activity was decreased with prolonged exposure to GSSG (Marshall et al., 1997). ESI-MS analysis revealed a sequential structural alteration of rSULT1A1 after GSSG pretreatment, and this was related to the regulation of the activity of this enzyme. Disulfide mapping of the tryptic peptides derived from GSSG-pretreated rSULT1A1 at the time of maximum activation identified a disulfide bond between Cys66 and Cys232 (Marshall et al., 1997). Prolonged coincubation of GSSG with rSULT1A1
can also lead to S-glutathiolation at Cys82, as well as the formation of another disulfide bond between Cys283 and Cys289, and these account for the catalytic inactivation of rSULT1A1 (Marshall et al., 1997). The rate enhancement seen with partial oxidation of the enzyme (i.e., oxidation of Cys66 and Cys232) has been attributed to alteration of the PAPS/PAP binding site (Marshall et al., 2000). Disulfide bond formation at Cys66 and/or Cys232 destabilized an inhibitory ternary complex of the enzyme, PAP, and the phenolic substrate (Marshall et al., 2000; Duffel et al., 2001), thus relieving substrate inhibition and enhancing the catalytic rate.

Oxidative regulation of the purified rSULT1A1 is directly correlated with regulation of the enzyme within intact cells. Direct comparisons between oxidative treatment of the enzyme in precision-cut tissue slices from rat liver and the purified enzyme have clearly shown that the pattern of changes in catalytic functions of the enzyme upon treatment with either diamide or t-butyl hydroperoxide are the same (Dammanahalli and Duffel, 2012). Moreover, studies utilizing rats exposed to high oxygen concentrations are also consistent with oxidative changes to the catalytic function of rSULT1A1 (Maiti et al., 2005).

Another member of sulfotransferase family 1, hSULT1E1, has also been found to be sensitive to oxidative modification using both recombinant protein as well as human cytosol or HepG2 cell cytosol. Significant alteration in the catalytic activity of hSULT1E1 was observed after preincubation of the enzyme with GSSG. Site-directed mutagenesis identified Cys83 in hSULT1E1 as the key amino acid responsible for GSSG inactivation of wild-type hSULT1E1 (Maiti et al., 2007).

Overall, oxidative modifications had been implicated in other families of sulfotransferases with an effect of regulating the enzyme activity observed in both recombinant enzymes, human cytosol or a whole cell system. In our research, recombinant hSULT2A1 was applied as a model system to study the oxidative as well as electrophilic modifications on the enzyme with different treatments. This was also used
as a model system to study the mechanism of modification as well as the structural regulative effect after conformational alterations occur to the protein.
CHAPTER II
STATEMENT OF THE PROBLEM

Cytosolic sulfotransferases are major drug metabolism enzymes that play important roles in the detoxication of xenobiotics and endogenous substances like bile acids and neurotransmitters, regulation of the homeostasis of endogenous hormones and bioactivation of some xenobiotics leading to enhanced therapeutic effects or adverse effects like carcinogenesis (Donovan et al., 1993; Falany, 1997; Glatt, 1997; Negishi et al., 2001; Nowell and Falany, 2006). Human cytosolic sulfotransferase hSULT2A1 (also known as hydroxysteroid sulfotransferase, alcohol sulfotransferase, DHEA-sulfotransferase, or bile-acid sulfotransferase) catalyzes the sulfation of endogenous steroids like dehydroepiandrosterone (DHEA), androsterone and bile acids is the sole member of the SULT2A subfamily in humans (Otterness et al., 1992; Weinshilboum et al., 1997; Duffel et al., 2001). Due to the biological significance of hSULT2A1 in the metabolism and regulation of endogenous as well as exogenous substances, the control of its catalytic activity is of great importance.

Oxidative stress and electrophilic stress, which can lead to the alteration of numerous biomolecules, is intimately related to the welfare of many essential functions and pathways in biological systems. Previous work done with one member of the SULT1 family has demonstrated that an alteration in the catalytic activity of rSULT1A1 (also known as rat aryl sulfotransferase IV) is regulated by the oxidative states of the cysteines in the protein. The amino acid sequence differences between rSULT1A1, with five cysteines, and hSULT2A1, which only possesses three free cysteines, suggested that the application of previous knowledge about rSULT1A1 to other families of SULT enzymes should be carried out with scrutiny.

The hypothesis for the current investigation is that the catalytic function of hSULT2A1 is regulated by agents that either alter the redox state of, or covalently modify, specific cysteines in the protein. To test the hypothesis, different thiol-oxidants
as well as model electrophiles were used to examine their effects on the structure and catalytic function of hSULT2A1. Electrophilic biphenyl quinones (both chlorinated and non-chlorinated) were also examined for their ability to alter the catalytic properties of hSULT2A1. Kinetic parameters were determined to elucidate the catalytic regulation of the enzyme by these oxidative and electrophilic treatments. Alterations in the binding properties of PAPS, PAP, and the substrate DHEA to the modified hSULT2A1 were determined and correlated with kinetic results. The structural modifications of hSULT2A1 were determined by liquid chromatography mass spectrometry (LC-MS) and these results were used to aid in the interpretation of kinetic and binding experiments. Finally, a computational modeling approach was applied, using the known crystal structure of hSULT2A1, to assess potential conformational alterations due to molecular modification by oxidants and electrophiles.
CHAPTER III
REGULATION OF THE CATALYTIC ACTIVITY OF hSULT2A1 BY FORMATION OF DISULFIDE BONDS

Introduction

Oxidative modifications that occur to proteins that are sensitive to the oxidative environment may lead to a change in the activity or function of the oxidized protein. Modifications induced by ROS/RNS can cause protein-protein cross-linking or protein carbonylation that is associated with permanent loss of function. These alterations at the structural level can lead to unfolding and degradation of the damaged proteins by the proteasomal system or cause progressive accumulation into cytoplasmic inclusions or extracellular aggregates as observed in most age-related neurodegenerative disorders (Dalle-Donne et al., 2008).

Many physiologically important proteins are regulated by oxidative states and the amino acid residue cysteine plays an important role in this regulation. NF-κB is one of the most widely studied proteins that have been implicated in the oxidative effects relevant to a series of pathological conditions like cancer and neurodegenerative diseases. One typical study has shown that DNA binding of NF-κB is inhibited by diamide treatment wherein oxidized cysteines form disulfide bonds in the protein. In this study, dithiothreitol (DTT) can reverse the effect of diamide, but it does not have any effect on the binding of DNA to the untreated NF-κB (Toledano and Leonard, 1991). Another study on the same protein also found that the DNA-binding affinity of NF-κB was decreased by diamide treatment, where either DTT-treatment or 2-mercaptoethanol-treatment, but not GSH, could increase the binding of DNA to NF-κB prepared under anaerobic conditions (Hayashi et al., 1993).

AP-1 is another transcription factor whose activation is a prerequisite to the growth factor and TPA-stimulated cell growth. DNA binding of AP-1 is known to be
increased by the reduction of a single conserved cysteine in the DNA binding domain of the proteins (Walker et al., 1993). Oxidative pretreatment with diamide and bis(2-chlorethyl)-nitrosourea (BSO) could stimulate the endogenous and inducible expression of c-fos and c-jun which are subunits of AP-1, thus increasing AP-1 binding (Bergelson and Daniel, 1994).

The reversible conjugation of glutathione to protein thiols, termed as protein S-glutathionylation, is involved in protein redox regulation which is known to have multiple implications in various biological functions and human diseases. Glutathione is a water-soluble tripeptide consisting of glycine, cysteine and glutamic acid (L-γ-glutamyl-L-cysteinylglycine). In mammalian cells, it is the predominant intracellular low molecular mass thiol, and it is also a strong electron donor. When under an oxidative environment, the molecule becomes oxidized to form glutathione disulfide. The GSH/GSSG ratio is normally closely regulated in cells. Disruption of this ratio can result in several cellular reactions that are involved in signal transduction and cell cycle regulation under conditions of oxidative stress (Schafer and Buettner, 2001). Protein sulfhydryl groups can be oxidized to form either intramolecular protein disulfides or intermolecular disulfides with low molecular weight thiols like homocysteine, cysteinylglycine, cysteine, and glutathione, and these are often defined as S-thiolated proteins (Eaton, 2006). Increased S-glutathionylated proteins like hemoglobin and actin are observed in different diseases like Diabetes mellitus and Freidreich’s ataxia (Niwa et al., 2000; Piemonte et al., 2001). Besides, research on oxidative regulation has also unveiled that glutathionylation on the specific cysteines in sulfotransferases can alter the catalytic activity of rSULT1A1 in a time dependent manner. Further analysis demonstrated the sequential formation of a glutathione adduct on a specific cysteine, as well as disulfide bond formation between two cysteines in this enzyme, can steadily increase the catalytic activity. Additional disulfide bond formation between two other
cysteines in rSULT1A1 led to a decrease in the catalytic activity of this enzyme (Marshall et al., 1997; Marshall et al., 2000).

Although catalytic regulation of family 1 SULTs such as rSULT1A1 and hSULT1E1 has been observed, the possibility of oxidative regulation of a family 2 SULT has not been examined previously. This study focuses on oxidative effects on the catalytic regulation of hSULT2A1 via disulfide bond formation. Alteration in the catalytic activity of hSULT2A1 may lead to numerous aberrant physiological effects including endocrine disruption and toxicological effects of changes in the metabolism of xenobiotics. Mechanistic details underlying the catalytic regulation of hSULT2A1 by oxidants may also help interpret the nature of the catalytic process of hSULT2A1. LC-MS was applied to identify the oxidative adducts formed on hSULT2A1, and kinetic and ligand-binding studies were used to explore the functional bases of these structural changes. Computational simulation of the structural analysis was also used to facilitate better understanding of the conformational and mechanistic implications in this regulation.

Materials and Methods

Chemicals: L-Glutathione (GSH), L-glutathione disulfide (disodium salt) (GSSG), 5,5’-Dithiobis(2-nitrobenzoic acid) (DTNB), dehydroepiandrosterone (DHEA), 1,1’-azobis(N,N-dimethylformamide) (Diamide), adenosine 3’,5’-diphosphate sodium salt (PAP), methylene blue, 2-mercaptoethanol and 4-vinylpyridine were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine 3’-phosphate, 5’-phosphosulfate (PAPS) was purchased from Sigma-Aldrich, and was further purified according to a previously described procedure (Sekura, 1981) to reach at least 98 % purity as determined by HPLC analysis. Sequencing grade modified trypsin and Glu-C (sequencing grade) were purchased from Promega (Madison, WI). 12% Tris-HCl precast SDS-PAGE gels (30 μL, 10 wells) were purchased from Bio-Rad Labs (Hercules, CA). Radiolabeled
[^3]H]dehydroepiandrosterone (94.5 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). 8-Anilinonaphthalene-1-sulfonic acid ammonium salt (ANS) was purchased from Fluka (Steinheim, Germany). All other chemicals used in this study were of the highest chemical purity commercially available.

Expression and purification of recombinant hSULT2A1

Human SULT2A1 was expressed in Escherichia coli BL21 (DE3) cells (Sheng and Duffel, 2003), and recombinant hSULT2A1 was obtained from the lysed E. coli cells through extraction and purification as previously described (Gulcan et al., 2008). After each chromatographic step in the purification procedure, fractions containing hSULT2A1 were identified with the previously described methylene blue assay using dehydroepiandrosterone (DHEA) as substrate (Nose and Lipmann, 1958; Sheng et al., 2001). Protein content was determined by the modified Lowry procedure with bovine serum albumin as standard (Bensadoun and Weinstein, 1976). Homogeneity of the purified protein was determined by SDS-PAGE with Coomassie brilliant blue staining. A single band with a 34 kDa relative molecular mass was observed, and this was consistent with the previously reported subunit mass of hSULT2A1 (Falany et al., 1989).

Pretreatment of hSULT2A1 with thiol-oxidants

Prior to incubation of hSULT2A1 with various oxidative reagents, the dithiothreitol (DTT) that remained from the purification of hSULT2A1 was removed by chromatography on a PD-10 column (1.45 x 5.0 cm; GE healthcare, Pittsburgh, PA). PD-10 columns were allowed to equilibrate to 4 °C for 1 hour prior to use. Four column volumes of 50 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 10 % (v/v) glycerol, and 0.05 % (v/v) Tween 20 were used to wash the column until the absorbance at 280 nm reached baseline. 1ml of purified hSULT2A1 (1 mg/ml) was added onto the column followed by elution with 5 ml of the Tris-HCl buffer mentioned above. hSULT2A1 eluent was collected when the UV absorbance at 280 nm first start to incline. The eluted
protein was concentrated to more than 1 mg/ml using a 10-ml Amicon stirred cell with PM-10 membrane (Millipore Corporation, Billerica, MA) (Liu et al., 2009). The concentration of the residual DTT was determined to be less than 0.01 mM by a standard assay for thiols (Jocelyn, 1987). Following the removal of DTT, hSULT2A1 was incubated with or without reduced glutathione (GSH), oxidized glutathione (GSSG), diamide, DTNB at appropriate concentrations in the 50 mM Tris-HCl buffer mentioned above at 25 °C for 1 hour. Solutions were saturated with argon before the incubation, and reactions were conducted in sealed tubes with an argon atmosphere above the solution.

**Kinetics of DHEA-sulfation catalyzed by hSULT2A1**

The kinetics of DHEA-sulfation were measured based on the quantification of DHEA sulfate (DHEA-S) formation catalyzed by hSULT2A1 as described previously (Gulcan and Duffel, 2011). Assays to determine the rate of sulfation of DHEA catalyzed by hSULT2A1 following treatment of the enzyme with oxidants (0.45 µM of hSULT2A1 in the preincubation with oxidants) were carried out in a total volume of 0.2 mL of 0.25M potassium phosphate at pH 7.4 containing 0.2 mM PAPS and the indicated concentration of a mixture of [3H]-DHEA and unlabeled DHEA. This reaction mixture was incubated in a 37 °C water bath for 2 minutes prior to addition of 30 ng of hSULT2A1 (in 2 µL volume) that had been subjected to the treatment with an oxidative reagent. The assay mixture was then incubated at 37 °C for either 3 or 4 minutes, with the reaction time adjusted to maintain initial velocity conditions. After incubation, 0.8 mL of 50 mM potassium hydroxide was added to end the reaction, and analysis of the DHEA-sulfate was carried out as previously described (Gulcan, 2011). For determination of IC$_{50}$ values, data obtained from activity assays were fit to a sigmoidal dose-response curve using SigmaPlot 11.0 (Systat Software, Chicago, IL) to calculate individual IC$_{50}$ values. The mean ± standard deviation of three replicates was determined for each assay.
**Ligand-binding studies**

The binding of ligands (e.g., DHEA and PAP) to the unmodified and oxidatively modified hSULT2A1 was determined using ANS as a fluorescent probe. The use of ANS to determine ligand-binding to hSULT2A1 was carried out as described previously for the study of rSULT1A1 with a modification using different concentrations of ANS by determining saturation under the conditions that were used in the assay (Figure 4) (Marshall et al., 1997; Liu et al., 2011). The binding studies were conducted in 0.25 M potassium phosphate buffer, pH 7.4, with a 200 μM final concentration of ANS and 2 μg of either oxidant-treated or untreated hSULT2A1 (an enzyme concentration of 12 nM based on the Mr of dimeric hSULT2A1) in a 1.0 ml total volume. The solution was placed in a quartz cuvette with 1.0 cm excitation path length and 0.4 cm emission path length. The mixture was then incubated at 37°C in a PerkinElmer LS55 luminescence spectrometer (PerkinElmer, Inc., Waltham, MA) for 2 min prior to titration. Various volumes of either a 1.0 mM or a 10 mM solution of DHEA in absolute ethanol were added to the enzyme-ANS mixture to make the final concentration of DHEA in the solution ranging from 0.2 μM to 60 μM. After each addition of DHEA, the solution was mixed well and 10s incubation time was allowed before taking the reading. Fluorescence emission was measured with an excitation wavelength at 410 nm and an emission wavelength at 480 nm. The entrance and the exit slits were both set at 5 nm. The fluorimeter shutters were closed when fluorescence measurements were not being made. A dilution factor for each addition of DHEA was applied in the final calculation of the change in fluorescence. All solutions were filtered with a Millex-GS 0.22 μm filter prior to use. Each concentration of DHEA in the titration was done in duplicate. The means and standard errors of $K_d$ values were calculated by fitting the data to a two-site binding equation corrected for non-specific binding (SigmaPlot 11.0, Systat Software, Chicago, IL).
Figure 4. Saturation determination of ANS in the presence of 2 µg/ml of hSULT2A1. Closed circles represent the titration of ANS into potassium phosphate buffer containing the Tris-HCl buffer for hSULT2A1 preservation (pH=7.4, 0.25 M sucrose, 10 % glycerol and 0.05 % Tween 20), where opened circles represent the titration of ANS into potassium phosphate buffer containing hSULT2A1 in the Tris-HCl buffer. Closed triangles represent the difference between the titration of ANS to the buffer only and the enzyme.
The intrinsic fluorescence of tryptophan residues in hSULT2A1 was also used to monitor the conformational change of the protein to reflect its interaction with ligands as described previously (Beechem and Brand, 1985; Gulcan and Duffel, 2011). Mechanistic as well as crystallographic studies have indicated that PAP and PAPS bind at the same binding site in each SULT (Yoshinari et al., 2001; Chapman et al., 2004; Wang and James, 2006). In our case, the binding at the site for PAPS and PAP was determined by monitoring the fluorescence change of hSULT2A1 by titration with increasing concentrations of PAP. The binding study was conducted in 0.25 M potassium phosphate buffer, pH 7.4, with an excitation wavelength of 290 nm and an emission wavelength of 347 nm. The entrance and the exit slits were set at 5 nm and 7 nm, respectively. Pretreated or untreated hSULT2A1 was added (15 nM final concentration based on the Mr of the dimeric protein) together with DHEA (0 μM, 0.5 μM, or 50 μM final concentration) into the potassium phosphate buffer in the quartz cuvette 20 min prior to the titration and placed in a 37 °C chamber attached to the spectrometer. Titration was carried out by addition of aliquots of a solution of PAP to the mixture to make final concentrations of PAP ranging from 0.5 μM to 300 μM. After each addition of PAP, the solution was mixed and incubated in the chamber for another 10 s before determining the fluorescence. A dilution factor for each addition of PAP solution was applied to calculate the change in fluorescence. All solutions used in this assay were filtered with a Millex-GS 0.22 μm filter prior to the experiment. Each concentration of the PAP titration was done in duplicate. The means and standard errors of $K_d$ values were calculated by fitting the data to a two-site binding equation (SigmaPlot 11.0, Systat Software, Chicago, IL).

**Determination of conformational changes upon oxidation of hSULT2A1**

Alteration in the overall structure of a protein may lead to small environmental changes around the amino acid residue tryptophan which are reflected in changes in fluorescence in response to the polarity of the surrounding area. When the conformational
change results in the tryptophan being exposed to a more polar environment, the intrinsic fluorescence decreases accordingly.

In this study, hSULT2A1 was first diluted in 0.25 M potassium phosphate buffer (pH 7.4) to reach a 0.25 μM final concentration based on the Mr of dimeric protein in 1.0 ml total volume in a quartz fluorimeter cell (Starna, 10 mm). Fluorescence changes upon addition of oxidants were monitored with a Spectra Max M5 spectrometer from Molecular Devices. The excitation wavelength was set at 290 nm, while the emission wavelength was at 347 nm. Temperature was set at 25 °C for all assays. Varying concentrations of diamide (i.e., 20 μM, 40 μM, 80 μM) as well as 1 mM GSH and 1 mM GSSG were added to the enzyme-containing buffer after the cell had been incubated in the chamber for 5 min separately. Fluorescence values were recorded every 5 min for the first 10 minutes and then every 10 min up until 90 minutes. 5mM DTT or 2-ME was added into the enzyme mixture immediately after recording the fluorescence data at 90 min. Following addition of DTT, measurements were made at 95 min, 100 min, 110 min and 120 min. Minor light absorption properties of diamide close to the excitation and emission wavelength of tryptophan can cause a filter effect in fluorescence measurements and, therefore, a correction equation was applied in this set of data analysis using the equation:

\[ F_{\text{obs}} = F_0 \times C \]

In this equation, \( F_{\text{obs}} \) is the observed fluorescence in the presence of filtration effect, \( F_0 \) is the original fluorescence and \( C \) represent the correction factor which is determined as below ((Birdsall et al., 1983)):

\[ C = 1 - 10^{-A/2.303A} \]

In which \( A \) is the absorption determined at 290 nm and 347 nm.
The structural modification of hSULT2A1 by thiol oxidants was detected and characterized by liquid chromatography-mass spectrometry. A PD-10 size-exclusion column was used to remove the DTT that remained in the purified preparation of hSULT2A1. The column was eluted with 50 mM Tris-HCl buffer, pH 8.0. The resulting enzyme was concentrated by ultrafiltration to approximately 2 µg/µL, and removal of DTT was verified as described above. Aliquots of hSULT2A1, each containing 15 µg of enzyme, were incubated with various thiol oxidants in a 25 µL volume at 25 °C for 1 hour followed by addition of 4-vinylpyridine into the solution (45 mM final concentration of 4-vinylpyridine). Following incubation of this mixture for another 1 hour, proteolytic digestion was carried out by adding 0.6 µg of sequencing grade trypsin in 50 mM Tris-HCl buffer (pH 8.0) and acetonitrile (final 10% v/v) in a total volume of 0.1 mL, and incubating at 37 °C for 16 h. In those cases where a secondary digestion was performed, 1µg of sequencing grade Glu-C was added immediately after the 16 h tryptic digestion, and the mixture was incubated at 25 °C for another 16 hours. All of the digestions were ended by adding 2 µL of concentrated acetic acid. All samples were subsequently analyzed by a Thermo LCQ Deca quadrupole ion trap mass spectrometer (ThermoElectron, San Jose, CA) interfaced with a Dionex LC (Dionex, Sunnyvale, CA). Data were collected using positive electrospray ionization over the mass range of 300-2000. A Supelco Discovery Bio Wide Pore C18 (2.1 x 150 mm) column (Sigma-Aldrich, St. Louis, MO) was utilized with a 10 µl injection volume. The LC mobile phase was 0.1% (v/v) formic acid in water (A) and acetonitrile with 0.1 % (v/v) formic acid (B). The initial gradient conditions were 5 % B, which was held for 2 minutes, and then the solvent composition was increased to 60 % B over 80 minutes at a flow rate of 20 µl/min.
Computational modeling studies on hSULT2A1

In order to understand the conformational impact of oxidative modifications on hSULT2A1, the Sybyl X program (Tripos; St. Louis, MO) was applied to predict the conformational changes in hSULT2A1 after oxidative modifications, and the predicted conformations were compared to the original structure using a protein alignment suite in the program.

The previously reported x-ray structure of hSULT2A1 with the cofactor PAP bound (Pedersen et al., 2000) was obtained from the Protein Data Bank (PDB file: 1EFH). The cofactor PAP was first extracted from the original structure followed by an optimization of the conformation using the Prepare Structure application. Since the structure was obtained as an x-ray crystal form, it is possible that the conformation may not be the most favored one outside of the crystal lattice. Thus the optimization included fixing the terminal ends by adding charges at the terminus, adding back missing hydrogens on the side chain of the amino acid residues, as well as fixing the side chain steric interactions in the structure followed by energy minimization. The Powell method was used for the initial optimization with a termination gradient of 0.5 kcal/(mol*A). The force field was set at the Tripos default value with no charges specified. The maximum number of iterations was set at 1500 for the calculation.

A glutathione (GSH) structure was built by the build protein apparatus in the biopolymer suite and was merged into the optimized hSULT2A1 structure with addition of a covalent bond to link the cysteine group in the GSH structure to the Cys55 or Cys199 of the protein. Bond angles were adjusted to avoid any side chain bumps and a regional minimization was first performed using a terminal gradient of 0.1 kcal/(mol*A) and the same force field as previously described. Then the overall stage energy minimization was conducted with same setting as the original structure with the exception to allow the C-alpha change.
The energy-minimized modified hSULT2A1 was aligned with the original structure hSULT2A1 (PDBFile: 1EFH) the protein backbones were compared to see the alterations in conformation, and the areas with the largest alterations were analyzed to reveal functional implications.

Experimental Results

*Regulation of catalytic activity in hSULT2A1 by disulfide bond formation*

To study the effect of catalytic function modification by disulfide bond formation, three model compounds that are known have the ability to form disulfide bond with cysteine thiols (GSSG, DTNB and diamide) were used. Previous reports had indicated GSSG could regulate the catalytic activity of rSULT1A1 by manipulating the oxidative states of cysteines in the protein. Either formation of a Cys66-glutathione disulfide or an intramolecular disulfide bond, Cys66-Cys232, could alter the protein conformation near the PAPS/PAP binding site near and lead to catalytic alterations (Marshall et al., 1997; Duffel et al., 2001).

DTNB is a versatile thiol-targeting reagent which has high specificity towards thiols and is widely used as detecting reagent for free thiol. In our study, we also exploited its thiol-modification function to form mixed disulfides with cysteines in hSULT2A1. As with GSSG, DTNB has been previously shown to alter the catalytic activity of rSULT1A1 (Marshall et al., 1998).

Diamide is a widely applied model small compound which is known to introduce oxidative stress by generating glutathione disulfides and other disulfide bonds (Wax et al., 1970). The usage of diamide is a well-established method to introduce disulfide bonds that lead to regulatory influences on cellular responses or to protein cross-linkage through modifications at free sulfhydryl groups at cysteines (Crawford et al., 1989). Diamide itself does not form stable adducts with sulfhydryls, but rather promotes their oxidation via an intermediate mixed sulfide (Lockwood, 2000). Thus, diamide was utilized in order
to examine both the ability of hSULT2A1 to form intramolecular disulfides and the effect of this alteration on catalytic function. Preliminary experiments showed a decrease in the catalytic activity of hSULT2A1 following incubation with either DTNB or diamide for periods up to 1 hour, but no significant change was observed after additional exposure to these oxidants (Figure 5). Later studies using intrinsic fluorescence to monitor the conformational change after oxidative treatment also indicated a continuous change in the structure of hSULT2A1 within one hour, but there were no significant additional changes after this. Thus, one hour pretreatment incubation was applied throughout this study as a standard.

Figure 5. Time-dependence of the pretreatment of hSULT2A1 with DTNB, and diamide. The enzyme was incubated for the indicated time at 25 °C, followed by 100- fold dilution into a standard assay for sulfation of 0.5 µM DHEA as described in Materials and Methods.
Cytosolic hSULT2A1 shares structural similarities with rSULT1A1 in their sulfuryl acceptor sites and PAP/PAPS binding sites. However, rSULT1A1 has 5 cysteines in each subunit, whereas hSULT2A1 only has 3 cysteines in each subunit. To compare the regulative effect of thiol oxidants on hSULT2A1 to rSULT1A1, 1mM glutathione disulfide (GSSG) was also used as a model compound to study the oxidative regulation of hSULT2A1. As shown in Figure 6, the effect of 1mM GSSG on catalysis was examined by coincubation with hSULT2A1 in the absence of any reducing reagent. Following dilution of the enzyme and oxidant into an assay for hSULT2A1, DHEA (concentrations ranging from 0.25 μM to 10 μM) was used as the substrate for the catalytic reaction. Significant differences between the catalytic activities of GSSG- and GSH-pretreated hSULT2A1 were found only when the DHEA concentration was below 1µM. Based on this finding, 0.5 μM DHEA was designated as the standard substrate concentration in all of the following experiments including IC$_{50}$ determination as well as the reversibility assays. It is also noteworthy that although pre-incubation with 1 mM GSSG resulted in a change in catalytic rate, incubation with 0.5 mM GSSG did not show alterations in the catalytic activity of hSULT2A1 after pre-incubation (data not shown).

The concentration-dependence of the effects of DTNB and diamide on the catalytic activity of hSULT2A1 was also examined. By preincubating hSULT2A1 with DTNB or diamide at concentrations ranging from 0 mM to 1 mM followed by dilution of the enzyme into a standard assay for sulfation of 0.5 μM DHEA, pseudo-IC$_{50}$ values for alterations in catalytic activity were determined to be 0.04 mM for diamide and 0.63 mM for DTNB in the preincubation mixture (Figure 7). DTNB downregulated the catalytic activity to 10 % of its original activity, while diamide could reduce the rate to 30 % of the original activity. Thus, DTNB and diamide were much more effective in reducing the catalytic activity than GSSG.
Figure 6. GSSG- and GSH-treated hSULT2A1 resulted in differences in its catalytic activity. The enzyme was incubated for 1 hour at 25 °C followed by 100 times dilution into a standard assay for sulfation of various concentrations of DHEA as described in Materials and Methods. Values are mean ± S.D. of 3 determinations. * indicates statistical significance by t-test ($p < 0.05$).

Figure 7. IC$_{50}$ determinations of diamide and DTNB pretreated hSULT2A1. The enzyme was incubated for 1 hour with various concentrations of oxidants at 25 °C followed by dilution in to a standard assay for DHEA sulfation. Values are mean ± S.D. of 3 determinations.
Reversibility of oxidative modification of hSULT2A1

Multiple reports in the literature have indicated the reversibility of oxidative modification of proteins including examples such as hSULT1E1 (Maiti et al., 2007), NFκB, IKK-β, and TNF receptor associated factor 6 (Pimentel et al., 2011). The effect of thiol oxidants on hSULT2A1 were examined to see if the oxidative effects could be reversed by restoring the reducing environment. After preincubation of hSULT2A1 with 0.5 mM diamide or 0.5 mM DTNB for 1 hour, 5mM DTT was added into the incubation immediately and the mixture was further incubated in 25 °C water bath for another one hour prior to activity assay for catalytic activity. Full recovery was observed for diamide-pretreated hSULT2A1 after restoring the reducing environment (Figure 8). After addition of DTT into the DTNB-pretreated hSULT2A1, the catalytic activity was brought back to approximately 80 % of the value seen with untreated hSULT2A1 (Figure 9). Glutathione disulfide (GSSG), another thiol-oxidant previously reported to have regulatory effects on rSULT1A1 (Marshall et al., 1997; Marshall et al., 2000), was also examined in our study; and very minor effects on catalytic function were observed when compared to the native enzyme. When using GSH-pretreated hSULT2A1 as control, GSSG showed a statistically significant decrease in the sulfation rate of hSULT2A1, and this effect can be reversed when 5 times concentrated DTT was added into the preincubation after 1 hour pretreatment with 1mM of GSSG (Figure 10). This finding was consistent with other previously reported cases where oxidative post-translational modifications could respond to redox signaling and was well controlled by the oxidative state of the compartment (Klatt et al., 1999; Pimentel et al., 2011).
Figure 8. Reversibility of the catalytic regulative effect of diamide pretreatment on hSULT2A1 by addition of DTT. The enzyme was incubated with 0.5 mM diamide at 25 °C for 1 hour followed by addition of 5 mM of DTT into the mixture and further incubated for another 1 hour. 100-fold dilution of pretreated enzyme was made into a standard assay using 0.5 μM DHEA. Values are mean ± S.D. of 3 determinations. Statistical significance by t-test (p value <0.001) is indicated as *. 
Figure 9. Reversibility of the catalytic regulative effect of DTNB pretreatment on hSULT2A1 by addition of DTT. The enzyme was incubated with 0.5 mM DTNB at 25 °C for 1 hour followed by addition of 5 mM of DTT into the mixture and further incubated for another 1 hour. 100-fold dilution of pretreated enzyme was made into a standard assay using 0.5 µM DHEA. Values are mean ± S.D. of 3 determinations. Statistical significance by t-test (p values) is shown.
Figure 10. Reversibility of the catalytic regulative effect of GSSG pretreatment on hSULT2A1 by addition of DTT. The enzymes were incubated with either 1 mM or GSH or 1 mM GSSG at 25 °C for 1 hour followed by addition of 5 mM of DTT into the mixture and further incubated for another 1 hour. 100-fold dilution of pretreated enzyme was made into a standard assay using 0.5 µM DHEA. Values are mean ± S.D. of 3 determinations. Statistical significance by t-test (p values) is shown.
Kinetic alteration of hSULT2A1 by oxidative treatment

The kinetic profile of treated as well as untreated hSULT2A1 catalyzing the formation of DHEA-S was examined using a broad range of substrate concentrations (0.5 μM-10 μM DHEA). For untreated hSULT2A1, an increase in the catalytic activity was observed in accordance with increasing concentration of DHEA up to approximately 2μM. This increase was followed by a decrease in the activity when further increases in the DHEA concentrations were made (Figure 11). Based on this characteristic kinetic profile, an equation for substrate inhibition kinetics (shown below) was applied to calculate the kinetic constants for untreated as well as oxidant-treated hSULT2A1.

\[
V = \frac{V_{\text{max}} \times [\text{DHEA}]}{K_m + ([\text{DHEA}] \times (1 + \frac{[\text{DHEA}]}{K_i}))}
\]

\(K_m\), \(V_{\text{max}}\), and \(K_i\) values of native hSULT2A1 were determined to be 178 ±35 nmol/min/mg, 1.3 ± 0.4 μM, 6.2 ± 2.4 μM, respectively, by fitting the sulfation rates to this equation. With GSSG-pretreatment, a slight increase in \(V_{\text{max}}\) value was observed compared to the GSH-pretreated enzyme, however, \(K_i\) and \(K_m\) values were not significantly changed (Table 1). A similar effect on the \(V_{\text{max}}\) value was observed for 0.5 mM DTNB-treated hSULT2A1 (268 ± 14 nmol/min/mg) (Table1 and Figure 12), whereas a decrease in \(V_{\text{max}}\) value was observed for 0.5 mM diamide-pretreated hSULT2A1 at 145 ± 5 nmol/min/mg (Table 1 and Figure 13). As shown in Table 1, there were no significant differences in the \(K_m\) values observed in all the oxidative pretreatment cases when compared to the untreated enzyme. Although no significant change in the calculated \(K_i\) value was observed for the GSSG-pretreated enzyme compared to the untreated hSULT2A1, DTNB- as well as diamide- pretreated hSULT2A1 both exhibited increases in \(K_i\) values compared to the untreated hSULT2A1. This indicated that a loss of substrate inhibition was occurring during the oxidative modification.
Figure 11. Kinetics characterization of hSULT2A1 catalyzing the sulfation of DHEA with 200 µM PAPS. Values are mean ± S.D. of 3 determinations.

Table 1. Summary of kinetic alteration of different thiol oxidant pretreated hSULT2A1 catalyzing the sulfation of DHEA with the presence of 200 µM PAPS

<table>
<thead>
<tr>
<th></th>
<th>hSULT2A1 only</th>
<th>GSH treatment</th>
<th>GSSG treatment</th>
<th>Diamide treatment</th>
<th>DTNB treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vmax(nmol/min/mg)</td>
<td>178±35</td>
<td>191±15</td>
<td>278±33</td>
<td>145±5</td>
<td>268±14</td>
</tr>
<tr>
<td>Km(µM)</td>
<td>1.3±0.4</td>
<td>0.3±0.07</td>
<td>1.4±0.3</td>
<td>1.5±0.2</td>
<td>2.7±0.4</td>
</tr>
<tr>
<td>Ki(µM)</td>
<td>6.2±2.4</td>
<td>10±2.2</td>
<td>5.5±1.2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Note: hSULT2A1 was pretreated with or without oxidants at 25 °C for 1 hour followed by 100 times dilution into a standard assay to determine the sulfation rate using various concentrations of DHEA ranging from 0.25 µM to 10 µM. N/A in the table indicated a $K_i$ value that is greater than $10^6$ µM, and thus no significant substrate inhibition.
Figure 12. Kinetic characterization of 0.5 mM diamide-pretreated hSULT2A1 catalyzing the sulfation of DHEA in the presence of 200 µM PAPS. The enzyme was incubated with either 0.5 mM diamide at 25 °C for 1 hour followed by 100-fold dilution into a standard assay using various concentrations of DHEA ranging from 0.25 µM to 10 µM described in Material and Methods. Values are means ± S.D. of 3 determinations.

Figure 13. Kinetic characterization of 0.5 mM DTNB-pretreated hSULT2A1 catalyzing the sulfation of DHEA with 200 µM PAPS. The enzyme was incubated with either 0.5 mM diamide at 25 °C for 1 hour followed by 100-fold dilution into a standard assay using various concentrations of DHEA ranging from 0.25 µM to 10 µM described in Material and Methods. Values are means ± S.D. of 3 determinations.
Alterations in binding properties of substrate and products upon oxidative pretreatment of hSULT2A1

Alterations observed in the kinetic character of hSULT2A1 in terms of the loss of substrate inhibition indicated changes in the binding properties of DHEA, PAPS, and/or PAP to the enzyme, since the binding of substrates and products was previously determined to be essential in the formation of a dead end complex in hSULT2A1 (Zhang et al., 1998; Gulcan and Duffel, 2011). Binding properties of the substrate, DHEA, as well as the product PAP were determined for both the native as well as the oxidant-treated forms of hSULT2A1.

Binding of DHEA to untreated as well as the oxidant-pretreated hSULT2A1 was examined by displacement of the fluorescent probe ANS. DHEA (final concentrations ranging from 0.1 μM to 15 μM) dissolved in absolute ethanol was used in titrations to displace ANS that was previously bound to hSULT2A1. The percentage change of fluorescence was calculated and applied to a two-site binding model with non-specific binding to obtain the binding constants of DHEA binding to the oxidant-treated or native hSULT2A1. Preliminary control experiments showed that ANS bound well to native hSULT2A1 and was displaced by DHEA. The $K_d$ values for DHEA bound to the untreated hSULT2A1 that were determined by the ANS assay (Figure 14) were consistent with previously described $K_d$ values for DHEA binding to hSULT2A1 that were determined using an intrinsic fluorescence assay (Gulcan and Duffel, 2011). Pretreatment of the enzyme with 1 mM GSSG changed the binding characteristics of DHEA as manifested in the loss of a second binding constant describing the binding of DHEA to the pretreated enzyme. The $K_{d1}$ was 1.0 ± 0.1 μM and this was very close to $K_{d1}$ seen for the untreated enzyme. Pretreatment of hSULT2A1 with 0.5 mM diamide resulted in the loss of the second binding constant as well. Again, the $K_{d1}$ was similar to both the GSSG-treated as well as the untreated enzyme at 0.9 ± 0.1 μM. When the enzyme was pretreated with 0.5 mM DTNB, no significant change was observed in terms of DHEA binding,
Figure 14. Binding curve of DHEA bound to untreated hSULT2A1. $K_d$ values were calculated by fitting the data to a two-site binding model. Two determinations were made at each DHEA concentration.
where the same $K_d$ values were observed for the pretreated enzyme compared with the native hSULT2A1 (Table 2).

Since PAPS is an important co-substrate in SULT-catalyzed reactions, changes in the binding of the corresponding product, PAP, were examined following pretreatment of the enzyme with oxidants. PAP was used in this group of studies, as it shares the same binding characteristics as PAPS, and it is also a key component of substrate inhibition (Gulcan and Duffel, 2011). By fitting the fluorescence change percentage to a two-site binding model, a $K_{d1}$ of $1.9 \pm 0.2 \mu M$ and a $K_{d2}$ of $540 \pm 100 \mu M$ were observed with the untreated enzyme. Pretreatment of the enzyme with $1 \text{mM GSH}$ showed no significant change in either $K_{d1}$ or $K_{d2}$ values (Table 3). Pretreatment of the enzyme with $1 \text{mM GSSG}$ had a slight effect on the first binding site ($K_{d1}=2.4 \pm 0.3 \mu M$) when compared to the GSH-pretreatment, but this was not significantly different from the untreated enzyme. However, the $K_{d2}$ value of PAP-binding to the GSSG-pretreated hSULT2A1 was $250 \pm 38 \mu M$, and this was significantly decreased compared to the untreated enzyme. Both $200 \mu M$ diamide-pretreatment as well as $500 \mu M$ DTNB-pretreatment showed a similar alteration in PAP-binding to the GSSG-pretreated enzyme (Table 3).

PAP-binding to the oxidant-treated hSULT2A1 was also examined in the presence of either $0.5 \mu M$ or $50 \mu M$ DHEA. In the case of untreated enzyme, with both $0.5 \mu M$ and $50 \mu M$ DHEA present, $K_{d1}$ values were not significantly different from those obtained without DHEA present (Table 3-5). However, $K_{d2}$ values were all similarly lowered when compared to those without DHEA present. $1 \text{mM GSH}$ pretreatment with DHEA present showed an increased $K_{d2}$ when either $0.5 \mu M$ or $50 \mu M$ DHEA was present whereas no significant change was observed in $K_{d1}$ values (Table 3-5). The increase in the $K_{d2}$ values for GSH pretreatment may indicate a slight amount of oxidative modification can happen to the enzyme during the one hour preincubation period and this was consistent with our previous observation in the difference between the catalytic activity of GSH-pretreated enzyme and the untreated enzyme. Pretreatment with $1 \text{mM}$
GSSG increased both $K_{d1}$ (44 ± 27 μM) and $K_{d2}$ (> 1000 μM) for PAP-binding in the presence of 50 μM DHEA (Table 4), however, in the presence of 0.5 μM DHEA, only a minor effect on $K_{d2}$ was observed (Table 5). 500 μM DTNB treatment had no effect on PAP binding in the presence of 50 μM DHEA (Table 4) but showed a significant increase in both $K_{d1}$ and $K_{d2}$ in the presence of 0.5 μM DHEA (Table 5). 200 μM Diamide-pretreatment had no significant effect on PAP binding in the presence 0.5 μM of DHEA, however a slight increase in both $K_{d1}$ and $K_{d2}$ was observed in the presence of 50 μM DHEA (Tables 4 and 5).

Table 2. $K_d$ values of DHEA binding to oxidant-pretreated or untreated hSULT2A1. $K_d$ values were obtained by fitting the binding data to a two site binding model.

<table>
<thead>
<tr>
<th>DHEA binding</th>
<th>untreated hSULT2A1</th>
<th>1 mM GSSG</th>
<th>0.5 mM diamide</th>
<th>0.5 mM DTNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(\mu M)$</td>
<td>0.7±0.1</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>$K_{d2}(\mu M)$</td>
<td>12±5.0</td>
<td>N/A a</td>
<td>N/A a</td>
<td>11±8.0</td>
</tr>
</tbody>
</table>

a No $K_{d2}$ was determined for DHEA binding

Table 3. $K_d$ values of PAP binding to oxidant-pretreated or untreated hSULT2A1 determined by fitting the data to a two site binding model.

<table>
<thead>
<tr>
<th>PAP binding</th>
<th>untreated</th>
<th>1 mM GSH</th>
<th>1 mM GSSG</th>
<th>0.2 mM diamide</th>
<th>0.5 mM DTNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(\mu M)$</td>
<td>1.9±0.2</td>
<td>0.9±0.3</td>
<td>2.4±0.3</td>
<td>2.0±0.3</td>
<td>3.1±1.1</td>
</tr>
<tr>
<td>$K_{d2}(\mu M)$</td>
<td>540±100</td>
<td>661±194</td>
<td>250±38</td>
<td>340±54</td>
<td>325±52</td>
</tr>
</tbody>
</table>
Table 4. $K_d$ values of PAP binding to oxidant-pretreated or untreated hSULT2A1 in the presence of 50 µM DHEA determined by fitting the data to a two site binding model.

<table>
<thead>
<tr>
<th>PAP binding</th>
<th>untreated</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mM GSH</td>
</tr>
<tr>
<td>$K_{d1}$(µM)</td>
<td>1.1±0.3</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>$K_{d2}$(µM)</td>
<td>264±36</td>
<td>453±63</td>
</tr>
</tbody>
</table>

Table 5. $K_d$ values of PAP binding to oxidant-pretreated or untreated hSULT2A1 in the presence of 0.5 µM DHEA determined by fitting the data to a two site binding model.

<table>
<thead>
<tr>
<th>PAP binding</th>
<th>untreated</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mM GSH</td>
</tr>
<tr>
<td>$K_{d1}$(µM)</td>
<td>0.9±0.2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>$K_{d2}$(µM)</td>
<td>248±92</td>
<td>328±94</td>
</tr>
</tbody>
</table>
Conformational alteration upon oxidative pretreatment

Disulfide formation in hSULT2A1 resulting from pretreatment with thiol-oxidants may lead to conformational changes in the protein structure. These effects would then cause changes in the binding properties of hSULT2A1 for the substrate, the co-substrate PAPS, or the product PAP. With the help of intrinsic fluorescence to determine protein conformational changes, we were able to evaluate the impact of disulfide bond formation on the protein structure of hSULT2A1. These experiments contained the same enzyme concentration that was applied in the pretreatment step of the catalytic activity determinations.

The concentrations of diamide used in this study were determined by the previous IC$_{50}$ assays. The concentration of diamide at IC$_{50}$ (40 µM), two times of the IC$_{50}$ (80 µM), and half of the IC$_{50}$ (20 µM) were applied in this conformational study. By monitoring the fluorescence change with time, 20 µM of diamide did not significantly change the conformation of hSULT2A1 within a 90 min time period, where 40 µM and 80 µM diamide manifested significant changes in the observed intrinsic fluorescence of the modified hSULT2A1 (Figure 15). This indicated alterations of the tryptophan environment in the protein, and, therefore, conformational changes upon diamide treatment at those concentrations. For the treatment with diamide, it is important to notice that the conformational change occurred in a time-dependent and concentration-dependent manner. With the addition of the reducing reagent DTT, untreated hSULT2A1 did not show any response to the reducing environment in terms of the intrinsic fluorescence change. In contrast, the diamide-treated hSULT2A1 showed an immediate recovery of the intrinsic fluorescence back to the original level seen for the enzyme (Figure 15).

With the addition of 1mM of GSH, no significant change in the intrinsic fluorescence of hSULT2A1 was observed (Figure 16).
Surprisingly, with 1 mM GSSG coincubation, where an intrinsic fluorescence decrease was expected, there was only a small change in the intrinsic fluorescence of hSULT2A1 (Figure 17). It was not possible to utilize this method of analysis for treatment of the enzyme with DTNB due to its high absorption at a wavelength overlapping with the excitation wavelength of tryptophan. Moreover, since the concentration of DTNB keeps changing in the time-dependent reaction with the enzyme, it was not possible to apply a correction factor.
Figure 16. Time dependent intrinsic fluorescence change of hSULT2A1 caused by 1 mM GSH addition. 1 mM GSH diluted into hSULT2A1-containing buffer at time zero in a quartz cuvette followed by addition of 5 mM 2-ME at 90 minutes. The change in fluorescence was monitored every 5 to 10 minutes. Values are the means ± S.D. of 3 determinations.

Figure 17. Time dependent intrinsic fluorescence change of hSULT2A1 caused by 1 mM GSSG addition. 1 mM GSSG was diluted into hSULT2A1-containing buffer at time zero in a quartz cuvette followed by addition of 5 mM 2-ME at 90 minutes. The change in fluorescence was monitored every 5 to 10 minutes. Values are the means ± S.D. of 3 determinations.
Structural modification of hSULT2A1 identified by LC-MS

Structural modifications of hSULT2A1 following pretreatment with oxidants were analyzed by LC ESI quadrupole ion trap mass spectrometry. 4-Vinylpyridine (45 mM) was added after each set of oxidative treatments to alkylate remaining free cysteine thiols and, thereby, preclude any possible thiol modification shuffling during the later digestion steps. hSULT2A1 without oxidative pretreatment was also incubated with 45 mM vinylpyridine to serve as the control for all the later oxidative treatments. Three 4-vinylpyridine adducted cysteines including Cys55, Cys199 and Cys154 were found in tryptic peptides peaks at 65.56 min, 20.03 min, and 75.72 min respectively in the LC-MS chromatography. Pretreatment with GSSG resulted in extra peaks at retention times of 22.51 min and 71.03 min corresponding to glutathione mixed disulfides at Cys199 and Cys55, respectively. Double digestion with trypsin followed by Glu-C confirmed the findings from the single tryptic digestion with an additional Cys154-glutathione mixed disulfide adduct identified in the chromatograph. After diamide treatment, single tryptic digestion showed a tryptic fragmentation resulting from the loss of all three 4-vinylpyridine adducted cysteines and a disulfide bond linked Cys55 with Cys199 was found as the (M+4H)$^{4+}$ ion with low abundance. Double digestion using both trypsin and Glu-C produced smaller peptides, and this confirmed the findings that diamide-pretreatment resulted in the formation of disulfide bond-linked two peptides which contained Cys55 and Cys199 (Table 6). With DTNB, our LC-MS results showed that all the three cysteines in hSULT2A1 were modified by TNB, and this was observed both from single-digestion as well as the double-digestion experiments. All the data on modification of the structure of hSULT2A1 by thiol-oxidants are summarized in Table 6 showing the modified peptides, the digested peptide sequence, the predicted mass, and the mass found in our LC-MS analysis.
Table 6. LC-MS detection of peptides that contain oxidative modified cysteines in hSULT2A1 after oxidative pretreatment

<table>
<thead>
<tr>
<th>Modified Cysteine</th>
<th>Peptide Structure</th>
<th>Predicted Mass (M+H)^+</th>
<th>Mass found</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSSG treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys199-GS</td>
<td>GS-(ICQFLGK)</td>
<td>1113.94</td>
<td>1113.41 (M+H)^+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>557.47 (M+2H)^2+</td>
</tr>
<tr>
<td>Cys55-GS</td>
<td>GS-(SGTNWLAEILCLMHSK)</td>
<td>2108.39</td>
<td>1054.73 (M+2H)^2+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>703.6 (M+3H)^3+</td>
</tr>
<tr>
<td>Cys199-GS *</td>
<td>GS-(ICQFLGK)</td>
<td>1113.94</td>
<td>1113.43 (M+H)^+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>557.43 (M+2H)^2+</td>
</tr>
<tr>
<td>Cys55-GS *</td>
<td>GS-(ILCLMHSK)</td>
<td>1249.57</td>
<td>1249.41 (M+H)^+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>625.49 (M+2H)^2+</td>
</tr>
<tr>
<td><strong>Diamide treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys199-Cys55</td>
<td>(TIEKICQFLGK)(SGTNWLAWILCLMHSKGD AKWIQSVPIW)</td>
<td>4746.47</td>
<td>1188.28 (M+4H)^4+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>792.64 (M+6H)^6+</td>
</tr>
<tr>
<td>Cys199-Cys55</td>
<td>(TIEKICQFLGKTLEPEELNLITGK)(SGTNWLAEILCLMHSK)</td>
<td>4474.38</td>
<td>1119.82 (M+4H)^4+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>747.04 (M+6H)^6+</td>
</tr>
<tr>
<td>Cys199-Cys55 *</td>
<td>(ICQFLGK)-(ILCLMHSK)</td>
<td>1749.94</td>
<td>1749.2 (M+H)^+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>875.65 (M+2H)^2+</td>
</tr>
<tr>
<td><strong>DTNB treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys199-TNB</td>
<td>TNB-(ICQFLGK)</td>
<td>1005.62</td>
<td>1005.33 (M+H)^+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>503.35 (M+2H)^2+</td>
</tr>
<tr>
<td>Cys55-TNB</td>
<td>TNB-(DEDVIILTPKSGTNWLAEILCLMHSK)</td>
<td>3286.75</td>
<td>1096.51 (M+3H)^3+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys199-TNB</td>
<td>TNB-(SGTNWLAEILCLMHSK)</td>
<td>2000.07</td>
<td>1999.51 (M+H)^+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000.6 (M+2H)^2+</td>
</tr>
<tr>
<td>Cys55-TNB</td>
<td>TNB-(ICQFLGKTLEPEELNLIKNSFQSMK)</td>
<td>3307.81</td>
<td>1103.27 (M+3H)^3+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys199-TNB</td>
<td>TNB-(SWEEYFEWFCQGTLYGSW FDHIHGWM)</td>
<td>4021.84</td>
<td>1341.52 (M+3H)^3+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1006.49 (M+4H)^4+</td>
</tr>
</tbody>
</table>
Sybyl-X was applied in our computational study to estimate the conformational changes due to disulfide bond formation in hSULT2A1. Sybyl-X is a versatile molecular modeling program that enables researchers to do small molecule modeling and simulation, macromolecular modeling and simulation, cheminformatics, lead identification as well as lead optimization. In this study, macromolecular modeling and simulation were extensively used to address the critical structural aspects of conformational alterations occurring after formation of either protein-protein or protein-small molecule disulfide bonds.

By comparing the energetically optimized structures before and after modification, GSSG-adducted hSULT2A1 showed a slight switch in the protein C-backbone around the position of modification (Figure 18). This conformational change also spread into the binding pocket for PAPS and PAP. Additional structural changes were seen near amino acid Lys44 and His99, which are known to play a key role in the transfer of sulfuryl
group from the donor PAPS to the substrate. However, the backbone structures close to the sulfuryl acceptor binding site (i.e., DHEA binding sites), as well as the entrance for binding of DHEA did not have any significant alteration according to this computational simulation (Figure 18). It was also worth noticing that the glutathione tri-peptide side chain that conjugated to Cys55 is pointing out toward the exterior of the globular structure of hSULT2A1, which coincidently lies in the pathway from which PAPS/PAP comes into its binding site.

By connecting two key targets of our interest, cysteine 55 and cysteine 199, to build an intramolecular protein disulfide, the energy optimization revealed an overall alteration in the C-backbone structure of hSULT2A1. Key components including the PAPS/PAP binding site, catalytic functional residues like Lys44, His99, Ser129, as well the DHEA binding site were all altered upon formation of disulfide bond between Cys55 and Cys199 (Figure 19). This indicated a possible relationship between the conformation change in hSULT2A1 related to oxidative modification and the catalytic function as well as substrate binding characteristics of the enzyme.
Figure 18. Sybyl-X simulation of glutathione-protein disulfides at Cys199 and Cys55 hSULT2A with the colored structure indicated PAP binding site. Green structures indicated the adducted glutathione side chain. Differences in the backbone structure of hSULT2A1 before and after the modification were illustrated in blue and orange color.
Figure 19. Sybyl-X simulation of disulfide formed between Cys55 and Cys199 with two small yellow dots indicating the Cys positions, the colored structure indicating the PAP and large colored dots indicate key catalytic components: Ser129 and Lys 44 in green and His99 in orange. Differences in the backbone structure of hSULT2A1 before and after the modification were illustrated in blue and orange color.
Discussion

Oxidative regulation of sulfotransferases has been an interesting component of understanding the characteristics of this family of enzymes and assessing their function in pathological states and under conditions of oxidative stress. Cysteine, being one of the major targets of oxidative attack, is prone to oxidative modification which can further lead to alterations in protein function, especially during oxidative stress (Ghezzi et al., 2005; Vinther et al., 2011). Studies targeting oxidative regulation of the catalytic activity had been done in family 1 sulfotransferases including SULT1E as well as SULT1A. Members of these families have been shown to be catalytically regulated by thiol-glutathionylation (Marshall et al., 1997; Maiti et al., 2005; Maiti et al., 2007). In the present study, we demonstrated that the catalytic activity of hSULT2A1, as one important member of the SULT2 family, could also be modulated through oxidations at cysteine residues. In comparison to previous studies in which Cys66 and Cys232 of rSULT1A1 were key cysteines whose oxidative state could regulate the catalytic activity of rSULT1A1, Cys55 and Cys199 represent the analogous cysteines in hSULT2A1. By preincubation with various thiol oxidants like GSSG, DTNB, and diamide, the catalytic activity of hSULT2A1 was decreased. In addition, this decrease in the catalytic activity could be reversed when the reducing environment of the enzyme was restored, with approximately 100 % restoration of activity achieved after DTT addition to oxidatively modified hSULT2A1. In previous work done with hSULT1E1, the authors stated that hSULT2A1 did not respond to oxidative regulation because it lacked a key cysteine in the catalytic active site (Maiti et al., 2007). However, in our experiments, GSSG did cause catalytic modification of hSULT2A1. Among several differences, one of the major reasons causing this discrepancy was that they were using a high concentration (2.0 μM) of DHEA as substrate (Chen et al., 2006). According to our results, GSSG only affects the catalytic activity of hSULT2A1 when the substrate concentration is below 1.0 μM. This could be due to the substrate inhibition of hSULT2A1 by DHEA, in which
inhibitory effects are seen when DHEA concentration is higher than 1.0 to 1.5 μM. In addition, the exact enzyme concentration was not determined in their work since they were using whole liver cytosol to determine the catalytic activity change of individual sulfotransferase.

Previous literature had indicated that GSSG could regulate the catalytic activity of rSULT1A1 by manipulating the oxidative states of cysteines. Either forming a Cys66-glutathione disulfide or an intramolecular disulfide bond between Cys66 and Cys232 could alter the protein conformation near the PAPS/PAP binding site, and this could further lead alterations in reaction velocity (Marshall et al., 1997; Duffel et al., 2001). In our experimental results, a mixed disulfide with glutathione was found at both Cys55 and Cys199 of hSULT2A1, however, residual vinylpyridine-adducted Cys55 and Cys199 as well as Cys154 were still detectable from the LCMS chromatograph for GSSG-pretreated hSULT2A1. This indicated that GSSG caused a partial oxidation of hSULT2A1 under the conditions used. These structural modifications altered the binding characteristics of both substrate DHEA and PAP. A decrease in the $K_{d2}$ value for DHEA after GSSG-treatment, and an increase in $K_d$ values for PAP-binding was observed. As mentioned before, the effect of DHEA on the binding of PAPS/PAP was examined under oxidative conditions since DHEA was known to have effects on PAPS/PAP binding under reducing conditions. Our results showed that without DHEA, PAPS/PAP only showed a minor difference in the $K_{d2}$ value before and after GSSG-pretreatment. However, when 0.5 μM DHEA (i.e., the same concentration as in our kinetic assays) was added into the PAPS/PAP-binding assay, an increase in the $K_{d1}$ value was observed. This phenomenon also was true for 50 μM DHEA, which is known to saturate hSULT2A1, and significant increases in both $K_{d1}$ and $K_{d2}$ values were observed. We hypothesized that the increase in $K_{d1}$ value and decrease in $K_{d2}$ value of PAP/PAPS binding, as well as the alteration in substrate binding, indicated a loosened initial binding of these compounds to the enzyme and led to destabilization of the PAP-enzyme-substrate ternary complex, a key
component of substrate inhibition. On the other hand, at a lower concentration of DHEA under the conditions of initial velocity, no substrate inhibition occurs. This effect of lowered binding affinity is manifested in decreased binding of both substrate and PAPS, ultimately leading to a decrease in the Vmax of the catalytic reaction. By looking at the crystal structure information (Pedersen et al., 2000; Rehse et al., 2002), Cys55 and Cy199 are in close vicinity to the PAPS/PAP binding pocket, however, a glutathione mixed disulfide at Cys199 may be too large to fit into the cavity. Thus it may only cause the glutathione moiety to point out toward the surface of the protein molecule.

DTNB is a versatile reagent that has high specificity towards thiols and is widely used as a detecting reagent for free thiol. In our study, we also exploited this thiol-modification function to disulfides with cysteines in hSULT2A1. All three cysteines in hSULT2A1 formed mixed disulfides with TNB. By a computational simulation, the TNB-Cys55 disulfide had the greatest impact on the PAPS/PAP binding site by inducing conformational alteration in the backbone of the protein close to this site. This conformational change turned out to have no effect on DHEA binding, but it caused increases in the values of both $K_{d1}$ as well as $K_{d2}$ of PAPS/PAP binding in the presence of 0.5 μM DHEA. These changes in the binding of the PAP can lead to an elimination of the substrate inhibition complex as evident from the increased $K_i$ in the kinetic assays. A decrease in the binding affinity at the PAP/PAPS binding site also means that, under the conditions of low DHEA concentration, the overall catalytic activity of hSULT2A1 could be compromised due to lower affinity for PAP. It is also important to note that the formation of TNB-protein mixed disulfides may also serve as a model for other small molecules reacting with cysteine in hSULT2A1, and thus aid in predicting their potential effects.

LC-MS structural analysis revealed that a disulfide bond was formed between Cys55 and Cys199 in hSULT2A1 after diamide treatment. Our preliminary data also indicated a conformational variation upon oxidative treatment, and this change was
dependent upon the concentration of diamide as well as incubation time. Among the three oxidative agents applied in this study, diamide introduced the largest conformational change as measured by intrinsic fluorescence assay and modulated by computational analysis. GSSG hardly had any effect on fluorescence change, and modeling of the effects of DTNB indicated that it could make a change to the conformation of the enzyme, but that change was not as significant as with the diamide-pretreated hSULT2A1. When the LC-MS results were combined with computational simulation, it was clear that the disulfide bond between Cys55 and Cys199 in hSULT2A1 resulting from diamide-treatment caused a significant alteration in the carbon backbone of the protein close to the PAP/PAPS binding site. The kinetic effect of this modification was that substrate inhibition was abolished by the formation of the intramolecular disulfide bond. Substrate binding studies revealed that the binding of DHEA was altered by a decrease in $K_{d2}$ after diamide pretreatment. The binding of PAP was examined as well, which turned out to depend on the presence of DHEA where, with 0.5 μM DHEA present, only a minor alteration in binding was observed. However, a slight increase in $K_{d2}$ for PAP-binding was observed both in the absence of DHEA and with 50 μM DHEA present. Interestingly, an increase in the $K_i$ value determined by kinetic analysis (i.e., less substrate inhibition) was observed due to this altered binding, however, the overall $V_{max}$ was only slightly decreased when compared to the untreated enzyme.

Existing literature had shown that the binding of a second substrate to the enzyme hSULT1A1 could be the reason for substrate inhibition as well for the SULT family (Gamage et al., 2006). Our results here demonstrated increases in $K_i$ values following GSSG- and diamide-treatment could be related to the lower $K_{d2}$ for DHEA binding. This also suggested the possibility of two substrate binding sites in hSULT2A1 in a similar manner to those in rSULT1A1 (Gamage et al., 2006). However, for DTNB treatment, $K_{d2}$ of DHEA binding showed no significant change compared to the untreated enzyme, and this indicated a different mechanism in the regulation of hSULT2A1. The regulatory
effect seen from DTNB-pretreatment might be due to the increase in the $K_d$ values for PAPS/PAP binding.

Another possible mechanism for this catalytic regulation could come from conformational alterations that interfere with the key amino acids involved in the catalytic step. Modifications of the cysteines in hSULT2A1 were determined to have conformational effects on the binding cavity of PAPS/PAP as simulated by computational analysis in which the loops surrounding PAPS/PAP were shifted after modification. These studies also indicated that the positions of key residues in the catalytic step of hSULT2A1, including His99, Ser129, and Lys44, might be altered such that the efficiency of sulfuryl group transfer might be disturbed and lead to a compromised overall catalytic activity.

Earlier studies with rSULT1A1 had indicated that the oxidative effects on the purified enzyme could be extrapolated to the enzyme within intact whole cells or tissue of living animals in which the oxidant had the same effect on the catalytic ability of rSULT1A1 in tissue as their purified form (Dammanahalli and Duffel, 2012). This serves as a model for us to seek further information on the physiological impact of this kind of oxidative effect on human health through the effects of oxidative stress on hSULT2A1 or other SULT family members. Such future investigation will be essential to determining the potential roles for these oxidative effects on metabolism of drugs or other xenobiotics and on regulation of the activity of endogenous hormones that are substrates for these enzymes.
CHAPTER IV
REGULATION OF THE CATALYTIC ACTIVITY OF hSULT2A1 BY REACTION WITH TERT-BUTYLHYDROPEROXIDE

Introduction

Oxidation of cellular lipids has been an intensive research area for many years, and advances in understanding the complex nature of lipid peroxidation and its potential biological significance have been the result of increased interest and efforts drawn to this field. Research has revealed that lipid peroxidation is implicated in the etiology of various human diseases including cardiovascular disease (Berliner and Heinecke, 1996), diabetes (Silverstein and Febbraio, 2009), chronic alcohol exposure (Yang et al., 2010), lung diseases (Imai et al., 2008), neurodegenerative disorders (Simonian and Coyle, 1996), and cancer (Klaunig et al., 2010). As observed in the different diseases mentioned above, lipid peroxides and their downstream products have also been widely used as biomarkers in the early diagnosis of pulmonary diseases, neurodegenerative disease and other diseases related to oxidative stress (Janssen, 2008; Galasko and Montine, 2010; Milatovic et al., 2011; Reed, 2011).

The reaction of reactive oxygen species and reactive nitrogen species (ROS/RNS) with cellular membranes leads to the production of lipid peroxidation, and this usually involves hydrogen-atom abstraction by peroxyl or alkoxyl radicals, oxygen addition to carbon radicals, peroxyl radical fragmentation or rearrangement, peroxyl radical addition to carbon-carbon double bonds or cyclization, and peroxy-peroxy termination (Niki, 2009; Gruber et al., 2011). Besides autoxidation of unsaturated fatty acids and radiation-induced lipid peroxidation, a number of enzymes (e.g., cyclooxygenase (COX), cytochrome P450 (CYP450), lipoxygenase (LOX), and others) are also known to play important roles in endogenous lipid peroxidation reactions. Such reactions are also
related to important biological functions like pro-inflammatory and anti-inflammatory responses (Massey and Nicolaou, 2011).

The biological significance of lipid peroxidation lies not only in the loss of unsaturated fatty acids of lipid membranes, but also in the formation of varieties of lipid peroxidation breakdown products including lipid hydroperoxides, fatty aldehydes like hexanal, ketones, and malondialdehyde (Plaa and Witschi, 1976; Kappus and Sies, 1981). These reactive lipid peroxidation breakdown products are implicated in the modification of cellular components such as DNA and proteins, as well as in the initiation of further lipid peroxidation.

Lipid peroxide products ranging from reactive epoxides to aldehydes are known to react with cellular DNA. The major aldehyde products of lipid peroxidation include acrolein, 4-hydroxynonenal (4-HNE), and malondialdehyde (MDA). These reactive substances react with DNA to form exocyclic adducts and cause unrecognizable bases that lead to errors during replication. Crotonaldehyde and acrolein can also react with DNA to form N^2-(3-hydroxybut-1-ylidene)dG and acrolein-dG as propane adducts (Pan and Chung, 2002). When acrolein, crotonaldehyde, and HNE are converted to epoxyaldehyde, they can also react with DNA to form etheno-adducts with both pyrimidines as well as purines. The altered bases in DNA that have reacted with reactive aldehyde products of lipid peroxides mainly produce base pair substitution mutations. Etheno-dA is highly mutagenic and can lead to AT->GC transitions, AT-> TA or AT->CG transversions (Pandya and Moriya, 1996). In addition, modified dC and dG with etheno adducts are known to lead to both transversions as well as transitions (Cheng et al., 1991; Moriya et al., 1994).

In addition to the modification of nuclear DNA, lipid peroxide products are also capable of reacting with cellular proteins, and this can lead to damage or alteration in their functions. The reactive aldehyde MDA is known to form Ne-(2-propenol) on lysines (Kato et al., 1999), while HNE modifies protein histidine, lysine, or cysteine through an
\(\alpha,\beta\)-unsaturated double bond and forms a hemiacetal structure by Michael addition (Kawai et al., 2004). Functional groups of proteins can also react with oxidation products of polyunsaturated fatty acids, and this occurs especially with carbonyl derivatives of these lipid peroxide products.

Peroxides are oxidants that are capable of reacting with thiols to form sulfenic acids (RSOH). Sulfenic acids have both nucleophilic and electrophilic reactivity and are generally unstable and highly reactive (Poole et al., 2004). In a cellular environment, especially in the vicinity of a protein free thiol, a sulfenic acid is prone to react with the thiol to form a disulfide bond, thus leading to alteration in the protein structure and function. The stabilization of a sulfenic acid in a protein requires the absence of a thiol in the same area. Furthermore, limited solvent accessibility and presence in a non-polar environment in the protein can also contribute to the stabilization of sulfenic acids (Roos and Messens, 2011). Cysteine sulfenic acids can be further oxidized to sulfinic acids or sulfonic acids in the presence of an excess amount of oxidants like lipid peroxides. These higher oxidative states of cysteines may also be involved in the alteration of protein activity, and they may increase the susceptibility of protein to aggregation and degradation (Reddie and Carroll, 2008).

As previously mentioned, hSULT2A1 has three free cysteines in each subunit of the dimeric molecule, and these are reactive toward thiol oxidants that are capable of forming disulfide bounds. In this section, \(t\)-butylhydroperoxide (TBHP) was used as a model for lipid peroxides in order to look at the potential oxidative as well as the electrophilic effects that lipid peroxides may have on the catalytic regulation of hSULT2A1. In addition, kinetic analyses and substrate-binding determinations were performed to examine the mechanism of this catalytic regulation by TBHP. LC-MS analysis was also utilized to search for the exact molecular modifications that occur at specific amino acids with an emphasis on sulfur-containing amino acids like cysteines and methionines in hSULT2A1. With computational simulation, the conformational
effects of TBHP-modification were studied, and these effects were applied to aid in a mechanistic explanation of the catalytic regulative effect of TBHP on hSULT2A1.

Materials and Methods

Chemicals: tert-butyl hydroperoxide (TBHP) (70 % w/w), adenosine 3′,5′-diphosphate sodium salt (PAP), dehydroepiandrosterone (DHEA), methylene blue, 2-mercaptoethanol, 4-vinylpyridine, and ammonium iron(II) sulfate hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine 3′-phosphate, 5′-phosphosulfate (PAPS) was purchased from Sigma-Aldrich and further purified according to a previously described procedure (Sekura, 1981) to reach at least 98 % purity as determined by HPLC analysis. Sequencing grade modified trypsin and Glu-C (sequencing grade) were purchased from Promega (Madison, WI). 12 % BIO-RAD Tris-HCl (30 μL, 10 wells) precast gels were purchased from Bio-Rad Labs (Hercules, CA). [3H]Dehydroepiandrosterone (94.5 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). 8-Anilinonaphthalene-1-sulfonic acid ammonium salt (ANS) was purchased from Fluka (Steinheim, Germany). All other chemicals used in this study were of the highest chemical purity commercially available.

Pretreatment of hSULT2A1 with TBHP

Prior to incubation of hSULT2A1 with various oxidative reagents, the dithiothreitol (DTT) that remained from the purification of hSULT2A1 was removed by chromatography on a PD-10 column (1.45 x 5.0 cm; GE healthcare, Pittsburgh, PA). The PD-10 column was allowed to sit in the cold room prior to use for 1 hour in order to let the particles equilibrate to the 4 °C ambient temperature. 50 mM Tris-HCl, pH 7.4, containing 0.25 M Sucrose, 10 % (v/v) Glycerol, and 0.05 % (v/v) Tween 20 was used to wash the column for (usually 4 column volumes) until the absorbance at 280 nm reached baseline. A 1 mL aliquot of hSULT2A1 (1 mg/ml protein concentration) was added onto the column followed by elution with 5 ml of the Tris-HCl buffer mentioned above. The
eluate containing hSULT2A1 was collected when the UV absorbance at 280 nm first start to incline. The eluted protein was concentrated to more than 1mg/ml using a 10-ml Amicon stirred cell with PM-10 membrane (Millipore Corporation, Billerica, MA) (Liu et al., 2009). The concentration of the residual DTT was determined to be less than 0.01 mM by a standard assay for thiols (Jocelyn, 1987). Following the removal of DTT, hSULT2A1 was coincubated with different concentrations of TBHP in the 50 mM Tris-HCl buffer mentioned above at 25 °C for 1 hour. Solutions were saturated with argon before the incubation, and assays were conducted in sealed tubes with an argon atmosphere above the solution.

Concentration determination of TBHP

TBHP is not stable in aqueous solutions, and it is known to decompose slowly. Therefore, it is recommended to assay the concentration of the stock solution of TBHP before utilizing it in experiments and at least once every two months. To assay the concentration of TBHP, 50 µL of 100-times diluted TBHP was first coincubated with 50 mL of 1mM (NH₄)₂Fe(SO₄)₂ for 5 minutes. An aliquot of 1 ml of 2 % KSCN in 0.1 N HCl was subsequently added to the mixture and further incubated for another 15 minutes. UV absorption at 480 nm was monitored for the TBHP solution incubated as described above. The equation A=εlc was used to calculate the TBHP concentration, where A is the absorption intensity monitored at 480 nm, ε is the molar absorptivity (57300 M⁻¹cm⁻¹ in this case), l is the path length and is (1cm in this case), and c is the concentration to be determined (Cohen, 1967; Mihaljevic et al., 1996).

Computational modeling study

To better understand the structural as well as the catalytic implications of TBHP -modification, computational simulation of the oxidative modification of hSULT2A1 was carried out using the Tripos Sybyl-X program. The previously reported x-ray structure with the cofactor PAP bound (Pedersen et al., 2000) was obtained from the Protein Data
Bank (PDB file 1EFH). Primary optimization of the crystal structure was previously described in Chapter III.

Cysteine sulfonic acids were built using the bond and atom module in Sybyl-X on Cys55, Cys199 as well as Cys154. Methionine sulfoxide was also constructed at Met16, Met137, Met223 and Met57. Bond angles were adjusted to avoid any side chain obstruction, and a regional minimization was first performed using a terminal gradient of 0.1 kcal/(mol*A) and the same force field as described in Chapter III. Then the overall stage energy minimization was conducted with same setting as the original structure with the exception to allow changes in the C-alpha backbone.

Energy-minimized modified hSULT2A1 was aligned with the original structure of 1EFH and the protein backbones were compared to see the alterations in conformation and those areas with the largest alterations were analyzed to reveal their functional implications.

Other experimental procedures

Enzyme expression and purification, determination of rates of sulfation of DHEA, ligand-binding assays, conformational monitoring assays, as well as LC-MS determination of structural modifications after TBHP-pretreatment of the protein were the same as described in Chapter III, with the exception of the use of TBHP in place of GSSG, DTNB, or diamide.

Experimental Results

Regulation of the catalytic activity of hSULT2A1 by TBHP

As a model compound for lipid peroxides, TBHP was examined to determine its ability to regulate the catalytic activity of hSULT2A1. Concentrations of TBHP ranging from 0.01 mM to 10 mM were incubated with hSULT2A1 at 25°C water bath for 1 hour in the absence of any reducing reagents. Catalytic activity of TBHP-modified hSULT2A1
was determined by 100-fold dilution of the TBHP-pretreated enzyme into a previously described assay using \(^3\)H-DHEA as the substrate (Chapter III). The results showed that at low concentrations of TBHP-pretreatment (e.g., 0.01 mM and 0.1 mM), there was no significant change in the catalytic activity of hSULT2A1 compared to the untreated group. However, when increasing the pretreatment concentration to 1 mM, there was a significant decrease in the catalytic activity of the TBHP-pretreated hSULT2A1 (Figure 20).

As previously observed in the studies on disulfide bond formation in Chapter III, the reversibility of modification of hSULT2A1 with TBHP was examined. A ten-fold molar excess of DTT was used in attempt to reverse the oxidation of hSULT2A1 by TBHP. The hSULT2A1 was first incubated with 0.5 mM TBHP in 25 \(^\circ\)C water bath for 1

![Figure 20. Catalytic activity of hSULT2A1 following pretreatment with different concentrations of TBHP. TBHP-pretreated hSULT2A1 was diluted into a standard sulfation assay using 0.5 \(\mu\)M DHEA as substrate. * indicates the statistical significance \((p < 0.05)\) in the catalytic activity of the TBHP-pretreated group compared to the untreated enzyme. Values are the means ± S.D. of 3 determinations.](image-url)
hour, and, when this enzyme was diluted into a standard assay for sulfation of DHEA, it showed a statistically significant decrease in the catalytic activity compared to the untreated control group (Figure 21). However, when DTT was added to the TBHP-pretreated hSULT2A1, and the mixture was incubated for another 1 hour at 25 °C and there was an increase over the catalytic activity of the TBHP-pretreated hSULT2A1 (Figure 21, p < 0.001). DTT reversed the effect of TBHP-pretreatment to a level similar to that seen with the untreated enzyme. It is also worth noticing that there was still a small difference between the TBHP-treated and untreated enzyme when both were incubated with DTT (Figure 21).

With these experiments, it is clear that the catalytic ability of hSULT2A1 can be modified in the manner of a decrease in the specific activity following pretreatment of the enzyme with TBHP. Furthermore, this alteration is partially reversible with the restoration of the reducing environment.

*Alteration of the kinetics of hSULT2A1-catalyzed reaction following treatment with TBHP*

The kinetic profiles of treated- as well as untreated-hSULT2A1 catalyzing the formation of DHEA-S were examined using a broad range of concentrations of the substrate DHEA (0.5 μM-10 μM). For untreated hSULT2A1, an increase in the catalytic activity was observed in accordance with the increasing concentration of DHEA up to approximately 2 μM, and this was followed by a decrease in the activity with further increases in the DHEA concentration. Based on this characteristic kinetic profile, the substrate inhibition kinetic equation that was described in the previous chapter was applied. After pretreatment with 0.5 mM TBHP, the modified hSULT2A1 was subjected to the same kinetic assay using the same substrate concentration range as in the control group above. In contrast to the enzyme that had not been exposed to TBHP, no substrate inhibition was observed for the TBHP-pretreated hSULT2A1. The kinetics of DHEA-
sulfation catalyzed by the TBHP-pretreated hSULT2A1 was better described using the original Michaelis-Menten equation shown below.

\[
\nu = \frac{V_{\text{max}}[\text{DHEA}]}{K_m + [\text{DHEA}]}
\]

Figure 21. 5 mM DTT addition reverses the catalytic alteration effect induced by TBHP. Black bars represent hSULT2A1 without any treatment; gray bars represent TBHP pretreated hSULT2A. * indicates a statistically significant difference in the catalytic activity of the TBHP-pretreated group compared to the untreated enzyme (\(p < 0.001\)). Values are the means ± S.D. of 3 determinations.
With TBHP pretreatment, the $K_m$ for the catalytic reaction was determined to be $0.78 \pm 0.09 \, \mu M$, while the $V_{\text{max}}$ is $121 \pm 3.8 \, \text{nmol/min/mg}$ where constants were similar to those observed with the untreated hSULT2A1 ($V_{\text{max}} \, 178 \pm 35 \, \text{nmol/min/mg}$, $K_m \, 1.3 \pm 0.4 \, \mu M$ and $K_i \, 6.2 \pm 2.4 \, \mu M$) (Figure 22).

![Figure 22](image_url)

Figure 22. Kinetic profile of DHEA sulfation catalyzed by TBHP pretreated hSULT2A1 fitting to a michaelis-menten equation. The top panel (A) is the kinetic profile of untreated hSULT2A1 as a comparison to TBHP pretreated hSULT2A1 (B). Values are mean ± S.D. of 3 determinations.
Effects of TBHP-pretreatment on binding of DHEA and PAP to hSULT2A1

As stated in the previous chapter, binding properties of substrates to hSULT2A1 relate intimately with the kinetic characteristics of sulfation. Thus, binding of both a substrate DHEA, as well as a product PAP, to hSULT2A1 following reaction of the enzyme with TBHP were examined to see the ability of oxidative modification induced by lipid peroxides to alter the binding affinities of the enzyme.

The binding of DHEA to 0.8 mM TBHP-pretreated hSULT2A1 was determined by titrating the fluorescent probe ANS bound to the enzyme with increasing concentrations of DHEA. The fluorescence change was then fit to either a single-site or a two-site binding model using SigmaPlot. The equilibrium dissociation constant $K_d$ for DHEA binding to hSULT2A1 that had been pretreated with TBHP was determined to be $1.62 \pm 0.12 \, \mu\text{M}$, in contrast to $0.7 \pm 0.1 \, \mu\text{M}$ for untreated hSULT2A1. There was no detectable binding of DHEA to a second site in the TBHP-pretreated hSULT2A1.

PAP-binding to TBHP-pretreated hSULT2A1 was determined by directly titrating the TBHP-pretreated hSULT2A1 with increasing concentrations of PAP and monitoring the intrinsic fluorescence of the enzyme to indicate the change of the protein structure in response to the binding of PAP to the enzyme (Table 7). Based on previous observations that the presence of DHEA can affect the binding of PAP to hSULT2A1 (Gulcan, 2010), PAP binding to TBHP pretreated hSULT2A1 was also monitored with the presence of either 0.5 μM or 50 μM of DHEA (Tables 8 and 9).

Without the presence of DHEA, both $K_{d1}$ and $K_{d2}$ of PAP-binding were decreased after TBHP-pretreatment when compared to the binding of PAP to untreated hSULT2A1. With the presence of 0.5 μM of DHEA, the $K_{d1}$ for PAP-binding was not significantly changed, but a slight increase in $K_{d2}$ was seen when compared to the binding of PAP to the untreated hSULT2A1. In the presence of 50 μM of DHEA, the $K_{d1}$ for PAP-binding was slightly increased, but no significant change in the $K_{d2}$ of PAP binding was observed after TBHP-pretreatment.
Table 7. Equilibrium dissociation constants for PAP binding to hSULT2A1 with and without TBHP pretreatment.

<table>
<thead>
<tr>
<th>PAP binding</th>
<th>Untreated hSULT2A1</th>
<th>TBHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(\mu M)$</td>
<td>1.9±0.2</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>$K_{d2}(\mu M)$</td>
<td>540±100</td>
<td>308±50</td>
</tr>
</tbody>
</table>

Table 8. Equilibrium dissociation constants for PAP binding to hSULT2A1 with and without TBHP pretreatment with the presence of 50 μM DHEA.

<table>
<thead>
<tr>
<th>PAP binding with 50 μM DHEA</th>
<th>Untreated hSULT2A1</th>
<th>TBHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(\mu M)$</td>
<td>1.1±0.3</td>
<td>2.5±0.9</td>
</tr>
<tr>
<td>$K_{d2}(\mu M)$</td>
<td>264±36</td>
<td>309±86</td>
</tr>
</tbody>
</table>

Table 9. Equilibrium dissociation constants for PAP binding to hSULT2A1 with and without TBHP pretreatment with the presence of 0.5 μM DHEA.

<table>
<thead>
<tr>
<th>PAP binding with 0.5 μM DHEA</th>
<th>Untreated hSULT2A1</th>
<th>TBHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(\mu M)$</td>
<td>0.9±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>$K_{d2}(\mu M)$</td>
<td>248±92</td>
<td>330±76</td>
</tr>
</tbody>
</table>
Conformational alterations upon TBHP-pretreatment of hSULT2A1

Conformational changes of hSULT2A1 play important roles in the binding of substrates to the enzyme as well as in the catalytic process which involves the stabilization of substrates and the transfer of sulfuryl group from the donor PAPS. Thus, the conformational changes following TBHP-pretreatment of the enzyme were examined. By monitoring the intrinsic fluorescence change of hSULT2A1 from the time point of TBHP addition up to 90 minutes, a steady decrease in the fluorescence of hSULT2A1 in the presence of TBHP was observed. However, only at 90 minutes there was a significant difference of intrinsic fluorescence of hSULT2A1 with or without TBHP present. There was an increase in the intrinsic fluorescence after the addition of the reducing reagent 2-ME, resulting in no statistically significance between the untreated enzyme group and the treated enzyme group at 10 minutes following reduction (Figure 23). With the slight change in the intrinsic fluorescence, several possibilities may be considered as the reason for this small alteration in the conformation of hSULT2A1 by TBHP. The alteration in the intrinsic fluorescence is due to the change of the polarity of protein tryptophan environment. The change in polarity of the ambient environment of tryptophan can be due to the change in the overall protein conformation that will expose selected tryptophans to a more hydrophilic environment and lead to a decrease in the intrinsic fluorescence of those tryptophans. It is also possible that the modification by TBHP may cause modifications on cysteines or other residues that are close to the tryptophan sites and create a local change of polarity that affects the intrinsic fluorescence of tryptophan.
Figure 23. Conformational alteration of hSULT2A1 after TBHP pretreatment. The enzyme was diluted into 0.25 M potassium phosphate buffer (pH 7.4) and monitored by fluorescence at the wavelength indicated in the methods section. The fluorescence change was monitored every 5 to 10 minutes. 2-mercaptoethanol (2ME) was added at 90 minutes (final concentration of 2ME was 5mM) and the fluorescence change was further monitored for 30 minutes. Statistical significance in the difference between the catalytic activity of the TBHP-pretreated group and the untreated enzyme was indicated by a * . Values are the means ± S.D. of 3 determinations.
Structural modifications of hSULT2A1 identified by LC-MS

Besides the overall conformational change of hSULT2A1 determined by protein intrinsic fluorescence change, specific modifications at the amino acid level were also monitored by LC-MS assays after reaction of the enzyme with TBHP. 15μM of reduced hSULT2A1 was coincubated with 32 mM TBHP in a Tris-HCl buffer (pH = 8.5) at 25°C for one hour prior to subsequent digestion and LC-MS analysis.

Multiple peptides that contained cysteine and methionine were found to be modified compared to the untreated control (Table 10). Peptides that contained Cys55 or Cys199 were found to be modified into cysteine sulfonic acids. Besides, Met57, Met 223, Met16 as well as Met137 were also found to be modified and converted to the corresponding methionine sulfoxides. However, one methionine, Met120, was found to be intact after TBHP pretreatment. Double-digestion using a secondary cleavage enzyme, Glu-C, produced smaller peptides that yielded better resolution with lower ionization states. Double digestion confirmed the findings for both of the sulfonic acids at Cys55 and Cys199 as well as the formation of the Met sulfoxides.

Computational simulation of the structural effects of TBHP- treatment on hSULT2A1

As seen in the previous section, molecular modifications like the formation of sulfonic acids at cysteines, as well as methionine sulfoxides, were identified by LC-MS methods. A molecular model was constructed for the oxidative modifications on selected cysteines and methionines in hSULT2A1 in which sulfonic acid groups were added onto Cys55 and Cys199, while Met57, Met16, Met137, Met223 were converted to sulfoxides, respectively. Energy optimization was performed after building functional groups onto the original hSULT2A1 structure (Figure 24). Following optimization, an alignment of the two structures was carried out to compare the backbones of hSULT2A1 with and without oxidative modification by TBHP. By simply viewing the backbone alteration of hSULT2A1 with the oxidized cysteines and methionines, there was no significant change
Table 10. Cysteine and methionine modification after TBHP pretreatment for hSULT2A1

<table>
<thead>
<tr>
<th>Modification identified</th>
<th>Peptide Structure</th>
<th>Predicted Mass (M+H)⁺</th>
<th>Mass found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonic acid Cys55 + Met57 Sulfoxide</td>
<td>(SGTNWLAELC LMHSKGDAKW1 QSVPIWER)-O4</td>
<td>1866.89 (M+H)⁺</td>
<td>1866.66 (M+H)⁺, 934.284 (M+2H)²⁺</td>
</tr>
<tr>
<td>Sulfonic acid Cys55+ Met 57 Sulfoxide(double digestion)</td>
<td>(SGTNWLAELC LMHSK)-O4</td>
<td>1866.89 (M+H)⁺</td>
<td>1866.8 (M+H)⁺, 933.94 (M+2H)²⁺</td>
</tr>
<tr>
<td>Sulfonic acid Cys55(double digestion) + Met 57 Sulfoxide</td>
<td>(ILCLMHSK)-O4</td>
<td>1008.50 (M+H)⁺</td>
<td>1008.43 (M+H)⁺, 504.96 (M+2H)²⁺</td>
</tr>
<tr>
<td>Sulfonic acid Cys199</td>
<td>(TIEKICQFLGK)-O3</td>
<td>1327.69 (M+H)⁺</td>
<td>1327.70 (M+H)⁺, 664.35 (M+2H)²⁺</td>
</tr>
<tr>
<td>Sulfonic acid Cys199</td>
<td>ICQFLGK-O3</td>
<td>856.44 (M+H)⁺</td>
<td>856.44 (M+H)⁺, 428.72 (M+2H)²⁺</td>
</tr>
<tr>
<td>Sulfonic acid Cys199(double digestion)</td>
<td>(ICQFLGK)-O3</td>
<td>856.44 (M+H)⁺</td>
<td>856.36 (M+H)⁺, 472.71 (M+2H)²⁺</td>
</tr>
<tr>
<td>Met120 unmodified</td>
<td>AKVIYLMR</td>
<td>993.59 (M+H)⁺</td>
<td>993.57 (M+H)⁺, 497.29 (M+2H)²⁺</td>
</tr>
<tr>
<td>Methionine223 sulfoxide</td>
<td>(NSSFQSMK)+O</td>
<td>944.42 (M+H)⁺</td>
<td>944.41 (M+H)⁺, 472.71 (M+2H)²⁺</td>
</tr>
<tr>
<td>Methionine16 sulfoxide</td>
<td>MSDDFLWFEGI AFPTMGRSETLR + O</td>
<td>2869.34 (M+H)⁺</td>
<td>2869.30 (M+H)⁺, 718.37 (M+4H)⁴⁺</td>
</tr>
<tr>
<td>Methionine137 sulfoxide</td>
<td>DVLVSGYFFWK NMK + O</td>
<td>1749.87 (M+H)⁺</td>
<td>1749.83 (M+H)⁺, 583.95 (M+3H)³⁺</td>
</tr>
<tr>
<td>Met120 unmodified</td>
<td>VIYLMR</td>
<td>1161.66 (M+H)⁺</td>
<td>1161.65 (M+H)⁺, 581.32 (M+2H)²⁺</td>
</tr>
</tbody>
</table>
Figure 24. Sybyl-X simulation of hSULT2A1 with oxidized cysteines and methionines. The C-alpha backbone of the oxidized protein overlaps with that of the unmodified hSULT2A1, blue lines represent the backbone of unmodified hSULT2A1; orange lines represent the hSULT2A1 with modification. Yellow balls indicate the position of cysteines while orange balls indicate the positions of methionines. Met120 is highlighted in purple. Backbones of hSULT2A1 before and after modification are illustrated in orange and blue which were overlapping on each other.
in the backbone of the modified enzyme, since it overlapped with the original structure perfectly. Side chain modification should be taken into consideration, however, as some modified cysteines and methionines are close to the substrate binding sites. As shown in Figure 24, Met120 (highlighted in purple) was the sole methionine that was not modified after reaction with TBHP. By examining its position in the 3D-structure displayed, Met120 resided in the center of the protein with a fairly hydrophobic environment. This may be a reason for the inaccessibility of Met120 to oxidative modifications by TBHP-treatment. Two of the methionines that are close to the PAP binding site, Met57 and Met223, were oxidized to methionine sulfoxides after TBHP-pretreatment, while two other methionines Met16 and Met137 that were also converted to methionine sulfoxides sit at the entrance for the DHEA-binding site in hSULT2A1.

**Discussion**

*tert*-Butyl hydroperoxide (TBHP) has been widely applied in the study of oxidative stress at both cellular and subcellular levels and serves as a model for lipid hydroperoxides. Exposure of cells to TBHP can result in a significant increase in ROS generation, the formation of other lipid peroxidation products, and cytotoxicity. DNA strand breakage resulting from TBHP-treatment was also reported to stem from the oxidation of cysteines in Ca^{2+}-dependent endonuclease that can lead to the activation of this protein (Aherne and O'Brien, 2000). Moreover, methionine oxidation following treatment with TBHP has been implicated in multiple studies on the functional regulation of several monoclonal antibodies (Chumsae *et al.*, 2007; Wei *et al.*, 2007; Ji *et al.*, 2009).

The catalytic activity of hSULT2A1 was previously identified to be regulated by thiol-oxidants that are capable of forming disulfide bonds. To further investigate the catalytic regulation of hSULT2A1 through potentially different mechanisms, TBHP was applied in this study to see if a model hydroperoxide could alter the catalytic activity of the enzyme. It was also anticipated that additional information could be obtained in
regard to the structural activity relationships for the specific amino acid residues in the enzyme whose oxidative states may play important roles in the regulation of the overall activity of the enzyme.

The ability of TBHP to alter the catalytic function of the enzyme was first confirmed by incubating TBHP with hSULT2A1. The specific activity of hSULT2A1 catalyzing the sulfation of DHEA was significantly decreased following incubation of hSULT2A1 (0.45 µM, calculated based on monomeric MW) with 1 mM TBHP for 1 hour at 25 °C. Increasing the pretreatment concentration of TBHP led to further decreases in the specific activity of hSULT2A1. Although this provided evidence of a catalytic alteration due to TBHP, it did not address the basis for this regulation. Further kinetic assays monitoring the specific activity of hSULT2A1 in response to increasing concentrations of the substrate DHEA indicated a loss of substrate inhibition in the sulfation reaction catalyzed by TBHP-pretreated hSULT2A1. Several theories for substrate inhibition have been previously proposed for hSULT2A1. One of those centers on the formation of a ternary complex consisting of the sulfation product PAP, the substrate DHEA and hSULT2A1 (Gulcan and Duffel, 2011). Another proposed basis for substrate inhibition of the enzyme is related to the binding modes of substrate DHEA where two modes exist in hSULT2A1 named “open” and “closed” conformations (Lu et al., 2008; Cook et al., 2010b). The open conformation is capable of binding two DHEA molecules to hSULT2A1 while the closed conformation can only bind one DHEA molecule binding. Another theory has been proposed where the formation of the homodimer of hSULT2A1 is associated with the allosteric binding site of DHEA in the open conformation (Cook et al., 2010a).

In order to understand the reason for the loss of substrate inhibition observed with TBHP-pretreated hSULT2A1, binding assays were performed to see if the binding of DHEA and PAPS/PAP was involved. It was found that DHEA only bound to a single site in hSULT2A1 after TBHP-pretreatment. This was consistent with previous proposed
hypotheses that the loss of DHEA allosteric binding is related with the loss of substrate inhibition. By further examining the amino acid modifications after TBHP-pretreatment, two of the modified methionines, Met16 and Met137, are at the entrance sites for DHEA. Previous mutagenesis studies on hSULT2A1 had shown that mutation of Met137 to a larger amino acid like tryptophan can eliminate the substrate inhibition (Lu et al., 2008). In this case, we hypothesize that the formation of methionine sulfoxide on the side chain of both Met16 and Met137 may alter DHEA-binding to hSULT2A1 after TBHP pretreatment.

PAP was applied as a model in determining the nucleotide substrate (PAPS)-binding to hSULT2A1 before and after TBHP-pretreatment. PAP and PAPS bind to the same binding site in hSULT2A1, and previous work has indicated similarities in the binding properties of PAP and PAPS (Gulcan and Duffel, 2011). Although the low-affinity binding of PAP was slightly decreased (i.e., small increase in $K_{d2}$) by TBHP treatment, addition of either a saturating concentration of DHEA or a lower concentration (where substrate inhibition does not occur) exhibited no alteration in PAP binding. As determined by LC-MS studies, the oxidative modification at cysteine sites may play a role in the alteration in the binding of PAPS/PAP as they are close to the PAP binding site, and other modifications at Cys55 and Cys199 through disulfide bond formation have effects on the binding of PAPS/PAP. However, with a random mechanism, the binding of DHEA can occur in the absence of the binding of PAPS/PAP. This binding of DHEA may stabilize the conformation of hSULT2A1, so that PAPS and PAP bind with higher affinity to hSULT2A1 (i.e., a significant decrease in $K_{d2}$ is seen). In addition, minor alterations close to the PAPS/PAP binding site (e.g., those seen with oxidative modification at Cys55 and Cys199) can no longer regulate the binding of PAPS/PAP.

Conformational alterations of hSULT2A1 with TBHP-treatment were determined by monitoring the intrinsic fluorescence change in hSULT2A1. Although the results have shown a slight decrease in the monitored intrinsic fluorescence over time with TBHP
present, this could be a site-specific effect of methionine oxidation, as many tryptophan residues are close to methionines. Further computational simulation confirmed this possibility by substituting native cysteines as well as methionines in the protein with cysteine sulfonyl acids and methionine sulfoxides, and no significant change in the backbone structure of the modified hSULT2A1 was observed.

Oxidation of cysteines is predominantly known as a sequential process with the formation of lower oxidation product sulfenic acids first, and subsequent oxidation to convert the sulfenic acids to sulfinic acids and sulfonic acids. Although LC-MS data indicated sulfonic acid formation at cysteines after TBHP pretreatment, considering the overnight digestion step after the preincubation of hSULT2A1 with TBHP, it is possible that the sulfonic acid is the final product of cysteine oxidation which is related with the incubation time for the proteolytic digestion. Although an alkylation reagent 4-vinylpyridine was added following the incubation step of hSULT2A1 with TBHP, if the sulfenic acid was formed in 1 hour, 4-vinylpyridine will have no effect of blocking the cysteines for further modifications. Thus, on the basis of the LC-MS analysis, it is hard to predict what is the actual modification that occurs to the enzyme and has the catalytic regulatory effect within 1 hour preincubation of hSULT2A1 with TBHP. Nevertheless, by looking at the reversibility assays in which hSULT2A1 was coincubated with TBHP for 1 hour prior to catalytic assays, there was a significant decrease in the catalytic activity catalyzing the sulfonation of DHEA. With the addition of the reducing reagent 2-ME, the decrease in catalytic activity was reversed. As sulfonic acids and sulfinic acids are modifications related with protein oxidation that are not able to be reversed by treatment with 2ME, this evidence points to the formation of sulfenic acids within the one hour. It is also possible that the sulfenic acid can react with a nearby cysteine to form an intramolecular disulfide between Cys55 and Cys199 as observed in the diamide pretreated hSULT2A1. While this is also known to be reversible by restoring the
reducing environment of the modified enzyme, such a peptide disulfide was not detected in the LC-MS studies.
CHAPTER V
REGULATION OF THE CATALYTIC ACTIVITY OF hSULT2A1 BY ELECTROPHILIC PCB-QUINONES

Introduction

Polychlorinated biphenyls (PCBs) are a large family of industrial chemicals that were widely used since the early 1930s in applications such as transformers, hydraulic fluids, lubricating oils, coolants, plasticizers, building materials and many others. Although the production and most uses of PCBs were banned by the United States and many other countries in the 1970s, and by the Stockholm Convention on Persistent Organic Pollutants in 2001 (Porta and Zumeta, 2002), they are still present in the environment and are relatively resistant to biodegradation.

Various effects on human health have been observed following exposure to PCBs. Reports have indicated a relationship between high-level exposure to PCBs and child developmental abnormalities including improvement in cognitive and sensory functions, motor and auditory functions and hypomineralized enamel defects of permanent teeth (Wigle et al., 2008). Other studies have also suggested an association of prenatal or lactational exposure to PCBs with respiratory symptoms (Roosli, 2011). PCBs are also known to be linked with the development of autoimmunity, where persons who live close to relevant industry tend to have elevated antithyroid antibodies. In addition, individuals that were exposed to PCBs through poisoned cooking oils, or fish eaters in contaminated areas, are more prone to other diseases including liver and lung cancers, endocrine disruptions (e.g., abnormalities in thyroid hormones), higher incidence of stillbirth, and increased incidence of diabetes (Crinnion, 2011).

Numerous studies have been conducted to explore the key toxicological relationships between PCB exposure and disease development. It is found that PCBs influence biological systems in a complicated manner that ranges from gene expression
and functional protein manipulation to impairment of the cell cycle. For example, PCBs are suspected to have implications in the development of cardiovascular diseases like atherosclerosis by stimulating pro-inflammatory pathways in the vascular endothelium caused by the down-regulating of peroxisome proliferator-activated receptor (PPAR) signaling (Hennig et al., 2005), and up-regulating of proinflammatory genes including E-selectin, intercellular adhesion molecule-1 (ICAM-1), and chemoattractant protein-1 (MCP-1) (Choi et al., 2003). Subchronic dietary exposure of rats to Aroclor 1254, an industrial mixture of different PCBs, can lead to induction of various hepatic drug metabolizing enzymes CYP1A1 as well as CYP1A2 (Dragnev et al., 1994). Gene mutations have been observed when PCB 3 was applied to Chinese hamster v97 cells. Two major metabolites, including PCB hydroquinone and the further oxidation product PCB-quinone were further examined and the PCB-quinone was found to be the ultimate mutagen to induce chromosome and genome mutations (Zettner et al., 2007).

Quinones represent an important family of biologically active electrophiles that can either react with cellular nucleophiles or initiate cellular redox cycling, thus eliciting various physiological outcomes. Cellular targets including proteins and DNA are known to have their function altered by reaction with quinones. Polyaromatic quinones like the environmental pollutant phenanthrenediones, can inhibit a number of functional proteins like protein tyrosine phosphatase CD45 and protein tyrosine phosphatase 1B (Wang et al., 2001). Nucleophilic amino acids like lysine, cysteine as well as histidine are all known to be modified by quinones (Guengerich et al., 2001; Casini et al., 2002; Labenski et al., 2009). The formation of quinone-amino acid adducts may influence the color, taste, and aroma of foods, altering their solubility and digestibility (Bittner, 2006). Electrophiles other than quinones may interact with cellular components through mechanisms related to those of quinones. Products of lipid peroxidation like acrylamide, acrolein, and 4-hydroxy-2-nonenal (4-HNE) are all in the class of electrophilic type-2 alkenes which are highly reactive toward cellular nucleophiles, and such reactions are hypothesized to be
the reasons for a number of pathological conditions like neurotoxicity and hepatic
diseases (LoPachin et al., 2008; Galligan et al., 2012).

When PCBs enter into biological systems, the CYP450 family is often responsible
for their oxidation to hydroxylated PCBs. When two such oxidations occur, it can lead to
the formation of a hydroquinone. Either autoxidation or through enzymes like
peroxidases or prostaglandin synthase can convert PCB hydroquinones to PCB quinones
(McLean et al., 1996; Oakley et al., 1996; Srinivasan et al., 2001) and elicit a broad
range of physiological impacts at different levels from DNA mutation and protein
expression to regulation of functional proteins.

DNA adducts formed with PCB-quinones have been detected wherein the
conjugation is formed through a Michael addition preferentially to guanosine followed by
stabilization through enolization (Zhao et al., 2004). DNA strand-breaks have also been
characterized in cellular systems after treatment with PCB-quinones (Srinivasan et al.,
2001).

The reactivity of PCB-quinones toward sulfhydryl functional groups plays an
important role in cellular toxicity. It was well established that the binding of GSH to
PCB-quinones could be one of the major reasons for depletion of cellular antioxidative
response which could finally lead to cell death (Srinivasan et al., 2002). It is also
hypothesized that the binding of PCB quinones to the enzyme topoisomerase impairs this
enzyme and leads to a modulation in catalytic function (Bender et al., 2006).

Since we have previously observed effects on hSULT2A1 by modification of
cysteines with thiol oxidants, three PCB-quinones and a non-chlorinated biphenyl
quinone were examined as model electrophiles to evaluate the potential for reaction of
quinones with electrophilic sites that regulate the catalytic activity of hSULT2A1.
Materials and Methods

Chemicals: adenosine 3’,5’-diphosphate sodium salt (PAP), methylene blue, dehydroepiandrosterone (DHEA), 2-mercaptoethanol, 4-vinylpyridine, and ammonium iron(II) sulfate hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine 3’-phosphate, 5’-phosphosulfate (PAPS) was purchased from Sigma-Aldrich and further purified according to a previously described procedure (Sekura, 1981) to reach at least 98% purity as determined by HPLC analysis. Sequencing grade modified trypsin and Glu-C (sequencing grade) were purchased from Promega (Madison, WI). 12% BIO-RAD Tris-HCl (30 μL, 10 wells) precast gels were purchased from Bio-Rad Labs (Hercules, CA). [3H]Dehydroepiandrosterone (94.5 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). 8-Anilinonaphthalene-1-sulfonic acid ammonium salt (ANS) was purchased from Fluka (Steinheim, Germany). N-acetyl-L cysteine was purchased from Acros Organics (Pittsburgh, PA). 2’-chlorobiphenyl-2,5-benzoquinone (2’-Cl-BQ), 4’-chlorobiphenyl-2,5-benzoquinone (4’-Cl-BQ), and 3,6,4’-chlorobiphenyl-2,5-benzoquinone (3,6,4’-triCl-BQ) were graciously provided by Dr. Hans Lehmler and his lab. Phenyl-p-benzoquinone (PBQ) was purchased from MP biomedicals (Solon, OH) (Figure 25) and recrystallized from ethanol prior to use. All other chemicals used in this study were of the highest purity commercially available.

Pretreatment of hSULT2A1 with PCB-quinones

Prior to incubation of hSULT2A1 with various quinones, dithiothreitol (DTT) that remained from the purification of hSULT2A1 was removed by chromatography using a PD-10 column (1.45 x 5.0 cm; GE healthcare, Pittsburgh, PA) as described in Chapters III. Following the removal of DTT, hSULT2A1 was coincubated with different concentrations of PCB-quinones or PBQ in the 50 mM Tris-HCl buffer described in Chapter III for preincubations at 25 °C for 1 hour. Solutions were saturated with argon
before the incubation, and assays were conducted in sealed tubes with an argon atmosphere above the solution.

*Inhibition of hSULT2A1 activity by PCB-quinones*

The kinetics of DHEA sulfation was measured based on the quantification of DHEA sulfate (DHEA-S) formation catalyzed by hSULT2A1 as described previously (Gulcan and Duffel, 2011). Assays to determine the rate of sulfation of DHEA catalyzed by hSULT2A1 were carried out using the dilution method described in Chapter III. To test the direct inhibition effect of PCB-quinones, 50 nM of individual PCB-quinones was added to the mixture in different reactions to determine any inhibitory effect of the sulfation catalyzed by hSULT2A1. The mean ± standard deviation of three replicates was determined for each assay.

*Computational modeling study*

To better understand the structural as well as the catalytic implications of reaction with quinones, computational simulation of this modification on hSULT2A1 was carried out using the Tripos Sybyl X program. The x-ray structure previously reported by Pedersen et al with cofactor PAP bound (1EFH) (Pedersen *et al.*, 2000) was obtained from the Protein Data Bank. Primary optimization of the crystal structure was previously described in Chapter III.

Structures of PCB-quinones were built using the drawing module in Tripos Sybyl X, followed by connecting them through a thioether linkage to either Cys55 or Cys199 at the 4-position of the quinone ring. In addition, methionine sulfoxides were also constructed on Met137, Met223, and Met228, as observed in LC-MS studies. Bond angles were adjusted to avoid any side chain bumps and a regional minimization was first performed using a terminal gradient of 0.1 kcal/(mol*A) and the same force applied field as previously described in Chapter III. Then, the overall stage energy minimization was conducted with same setting as the original structure with the exception to allow C-alpha
changes. Energy-minimized modified hSULT2A1 was aligned with the original structure of 1EFH and the protein C-alpha backbones were compared to see the alterations in conformation and the area with the largest alteration was analyzed to reveal potential functional implications.

_N-acetyl-cysteine model study_

In order to reveal the potential structures resulting from reaction of PCB-quinones with cysteines in hSULT2A1, reactive products were examined using a model compound, N-acetyl-cysteine (NAC), which possesses the thiol group that was observed to react with PCB-quinones in previous sections of this study.

An equal volume of 4 mM 3,6,4’-triCl-BQ (dissolved in DMSO) was added dropwise into a 4 mM NAC solution in 100 mM potassium phosphate buffer (pH =7.4) to reach a final concentration of 2 mM 3,6,4’-triCl-BQ. The mixture of 3,6,4’-triCl-BQ and NAC was incubated in a 37 °C water bath for 15 min. Analysis of the adducts was performed on a Waters Q-Tof Premier mass spectrometer interfaced with Waters Acquity UPLC system using a 4.6 mm i.d. × 25 cm 5 µm Grace Alltima HP C-18 column. The mobile phase used was 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in acetonitrile (B) delivered at a flow rate of 300 μl/min. The gradient profile was set as 65 % A (35 % B) isocratic held from 0 to 2 min, increased B to 80 % from 2 to 30 min, and then the isocratic elution with 20 % A (80 % B) for another 50 min. A sample volume of 20 µL sample solution was injected using an autosampler which is part of the UPLC system. The UV profile was monitored at 275 nm for this gradient elution. MS data were collected over the full scan positive mode at a M/Z range of 300-700.

_Other experimental procedures_

Enzyme expression and purification, ligand binding assays, catalytic regulation of hSULT2A1 by quinones, conformational monitoring assays, as well as LC-MS determination of structural modifications after PCB-quinones-pretreatment were the same
as described in Chapter III, with the exception of the use of individual PCB-quinones and PBQ in places of GSSG, DTNB, or diamide.

4'-chlorobiphenyl-2,5-benzoquinone (4'-Cl-BQ)

2'-chlorobiphenyl-2,5-benzoquinone (2'-Cl-BQ)

3,6,4'-chlorobiphenyl-2,5-benzoquinone (3,6,4'-triCl-BQ)

Phenyl-p-benzoquinone (PBQ)

Figure 25. Structures of PCB-quinones and PBQ used in this study
Experimental Results

**Catalytic regulation of hSULT2A1 by PCB-quinones and PBQ**

As model compounds for electrophiles, PCB-quinones and PBQ were examined to see their ability to regulate the catalytic activity of hSULT2A1. Increasing concentrations of PCB-quinones (from 0.25 µM to 5 µM) were used in preincubations with hSULT2A1 at 25 °C for 1 hour. Following 100-fold dilution, into a standard assay, catalytic activity of the PCB-quinone-modified hSULT2A1 was determined by the assay using 0.5 µM \(^3\)H-DHEA as the substrate. Decreases in the catalytic activity of hSULT2A1 were observed after PCB-quinone-pretreatment (Figure 26). 3,6,4’-triCl-BQ regulated the activity of hSULT2A1 at relatively low concentrations compared to the other PCB-quinones tested in this study. While the other two PCB-quinones have different effective concentrations for regulating the catalytic activity of hSULT2A1, all three PCB-quinones exhibited statistically significant decreases in the enzymatic activity starting at 1 µM concentration in the pretreatment incubation.

Previous studies had shown that hSULT2A1 is reversibly inhibited by many hydroxylated PCBs (Ekuase et al., 2011). Our catalytic assays were carried out by 100-fold dilution of the pretreated enzyme into the reaction mixture. This means that for hSULT2A1 pretreated with 5 µM PCB-quinones, there could be as much as 50 nM PCB-quinone present in the reaction examining sulfation of DHEA. Thus, there exists the possibility that the decrease in the catalytic activity of hSULT2A1 was a result of reversible inhibition by PCB-quinones that was not dependent upon the covalent binding of the PCB-quinone to the enzyme. To preclude the possibility that the decrease in the catalytic activity of hSULT2A1 after PCB-quinone pretreatment purely came from the reversible inhibition by competing with substrate binding, an inhibition experiment was performed without the preincubation step. As seen in Figure 26, there was no significant decrease in the catalytic activity in the present of either 50 nM 2’-Cl-BQ or 50 nM 4’-Cl-
BQ. For the assay with 50 nM 3,6,4’-triCl-BQ present, there was a statistically significant decrease in the catalytic activity of hSULT2A1 catalyzing the sulfation of 0.5 μM DHEA. Nevertheless, the extent of this decrease can only account for a small portion of the total decrease in the activity of the enzyme that was pretreated with 3,6,4’-triCl-BQ (Figure 27).

The effect of PBQ on the catalytic function of hSULT2A1 was also examined (Figure 28). The results showed an increase in the catalytic activity of hSULT2A1 when incubated with relatively low concentrations of PBQ (e.g., 0.5 μM and 1.0 μM). However, when the concentration of PBQ in the preincubation was increased, the catalytic activity of hSULT2A1 decreased.

The kinetic characteristics of DHEA-sulfation catalyzed by the hSULT2A1 pretreated with PCB-quinones and PBQ were also analyzed. The kinetic profile of untreated hSULT2A1 was determined as previously described and was characterized using a substrate-inhibition kinetic equation. After pretreatment with either PCB-quinones or PBQ, the modified hSULT2A1 was subjected to kinetic assay using the same substrate concentration range as for the untreated enzyme, and attempts were initially made to determine the kinetic constants using the same substrate inhibition equation as that was used for the untreated enzyme. However, no substrate inhibition was observed for the 2’-Cl-BQ-, 3,6,4’-triCl-BQ-, or PBQ-pretreated hSULT2A1. Thus, the kinetics of DHEA-sulfation catalyzed by hSULT2A1 pretreated with those three compounds were better described using the Michaelis-Menten equation, as described in the previous chapters.

The kinetic constants for the quinone-pretreated hSULT2A1 are compared to those of the untreated enzyme in Table 11. Compared to untreated hSULT2A1, 2’-Cl-BQ-, 4’-Cl-BQ- and PBQ-pretreated enzyme all had higher $V_{\text{max}}$ values and slightly higher $K_m$ values. The 3,6,4’-triCl-BQ-pretreated hSULT2A1 had lower $V_{\text{max}}$ and $K_m$ values compared to the untreated enzyme. Although there was a $K_i$ determined by the
fitting for 4'-Cl-BQ pretreated hSULT2A1, it is higher compared to the $K_i$ observed with the untreated enzyme.

Figure 26. Catalytic activity of hSULT2A1 follows pretreatment of the enzyme with different concentrations of PCB-quinones and 100-fold dilution into an assay for sulfation of 0.5 µM DHEA. The statistical significance ($p < 0.05$) of changes in the catalytic activity of the PCB-quinones pretreated group compared to the untreated enzyme is indicated by an *. Values are the means ± S.D. of 3 determinations.
Figure 27. Test of the inhibitory effect of PCB-quinones on hSULT2A1 without pre-incubation but at a relevant 100-fold dilution of the highest concentration used for time-dependent pre-incubation experiments. The statistical significance (p < 0.05) in the catalytic activity of the PCB-quinone-pretreated group compared to the untreated enzyme is indicated by an *. Values are the means ± S.D. of 3 determinations.
Table 11. Summary of kinetic constants for hSULT2A1-catalyzed sulfation of DHEA following pretreatment of enzyme with PCB-quinones and PBQ.

<table>
<thead>
<tr>
<th></th>
<th>Untreated hSULT2A1</th>
<th>Pretreatment</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2'-Cl-BQ</td>
<td>4'-Cl-BQ</td>
<td>3,6,4'-triCl-BQ</td>
<td>PBQ</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg)</td>
<td>178±35</td>
<td>349±15</td>
<td>372±46</td>
<td>115±2</td>
<td>239±9.5</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>1.3±0.4</td>
<td>3.3±0.3</td>
<td>4.1±0.7</td>
<td>0.9±0.06</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>$K_i$ (µM)</td>
<td>6.2±2.4</td>
<td>N/A</td>
<td>17.2±5.6</td>
<td>N/A*</td>
<td>N/A*</td>
</tr>
</tbody>
</table>

*No substrate inhibition detected
The binding affinities of substrates affect the catalytic properties of hSULT2A1 and play an important role in the substrate inhibition seen in the hSULT2A1-catalyzed sulfation of DHEA. As noted above, substrate inhibition characteristics of hSULT2A1 were determined to be altered following pretreatment of the enzyme with quinones. The binding of substrate DHEA as well as the product PAP to the modified enzyme were monitored to elucidate the mechanism of this catalytic regulation by PCB-quinones and PBQ.

The binding of DHEA to hSULT2A1 following reaction of the enzyme with PCB quinones or with PBQ was determined by displacement of the fluorescence probe ANS with increasing concentrations of DHEA. The change in fluorescence intensity was then fit to a two-site binding model. Equilibrium dissociation constants $K_{d1}$ and $K_{d2}$, were determined by the fitting, and the results are summarized in Table 12. The results showed little change in the binding of DHEA to PCB-quinone-pretreated as well as PBQ-pretreated hSULT2A1.

PAP-binding to the modified hSULT2A1 was determined by direct titration with increasing concentrations of PAP to PCB-quinone- and PBQ-pretreated, and untreated hSULT2A1. The intrinsic fluorescence of hSULT2A1 was monitored to observe changes in the protein conformation in response to the binding of PAP to the enzyme (Table 13). Significant changes in the binding of PAP were observed after reaction with the PCB-quinones or PBQ.

### Table 12. Equilibrium dissociation constants for DHEA-binding to hSULT2A1 with and without pretreatment of enzyme with 10 µM PCB-quinones or PBQ.

<table>
<thead>
<tr>
<th>DHEA binding</th>
<th>Untreated hSULT2A1</th>
<th>Pretreatment</th>
<th>2'-Cl-BQ</th>
<th>4'-Cl-BQ</th>
<th>3,6,4'-triCl-BQ</th>
<th>PBQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}$ (µM)</td>
<td>0.7±0.1</td>
<td>0.6±0.1</td>
<td>0.6±0.1</td>
<td>0.3±0.03</td>
<td>0.5±0.08</td>
<td></td>
</tr>
<tr>
<td>$K_{d2}$ (µM)</td>
<td>12±5µM</td>
<td>23±50</td>
<td>20±10</td>
<td>15±0.6</td>
<td>23±14</td>
<td></td>
</tr>
</tbody>
</table>
quinone. The binding of PAP to both 2’-Cl-BQ-pretreated enzyme as well as 4’-Cl-BQ-pretreated enzyme was totally abolished, and no binding constants could be determined. 3,6,4’triCl-BQ-pretreatment caused significant decrease in both $K_{d1}$ and $K_{d2}$ for PAP-binding, and this indicated a tighter binding of PAP to the modified enzyme. For the PBQ-pretreated hSULT2A1 there was no significant change in the PAP-binding after reaction at 10 µM PBQ, but when the pretreatment concentration of PBQ was 1µM, dissociation constants for PAP binding, including both $K_{d1}$ and $K_{d2}$, increased when compared to both the untreated hSULT2A1 and the 10 µM PBQ pretreated hSULT2A1 (Figure 29). Based on previous observations that the presence of DHEA can affect the binding of PAP to either untreated hSULT2A1 or to the hSULT2A1 altered by disulfide bond formation, PAP-binding to PCB-quinone- and PBQ-pretreated hSULT2A1 was also monitored in the presence of both 0.5 µM and 50 µM of DHEA (Table 14, Table 15). Although the binding of PAP to hSULT2A1 following reaction of the enzyme with either 2’-Cl-BQ or 4’-Cl-BQ occurred in the presence of DHEA, there was still a significant change in the binding characteristics of PAP compared to the modified enzymes. For 2’-Cl-BQ-pretreated hSULT2A1, the $K_{d2}$ for PAP was still greater than what could be reliably determined by protein intrinsic fluorescence.

Increases in both $K_{d1}$ and $K_{d2}$ in the presence of 0.5 µM of DHEA were observed for 2’-Cl-BQ-pretreated hSULT2A1. 4’-Cl-BQ-pretreated hSULT2A1 also demonstrated a significant decrease in the binding of PAP, as seen by the increase in $K_{d1}$. For 3,6,4’triCl-BQ-pretreated hSULT2A1, little change was noted in either $K_{d1}$ or $K_{d2}$ in the presence of 0.5 µM or 50 µM DHEA. For PBQ-pretreated hSULT2A1, there was no change in the binding constants observed in the presence of 0.5 µM of DHEA. However, when the concentration of DHEA was increased 50 µM, the binding of PAP to PBQ-modified enzyme was significantly weakened, as manifested by elevated $K_{d1}$ and $K_{d2}$ values.
Table 13. Equilibrium dissociation constants for PAP-binding to hSULT2A1 with and without pretreatment of the enzyme with 10 µM PCB-quinones or PBQ.

<table>
<thead>
<tr>
<th>PAP binding</th>
<th>Untreated hSULT2A1</th>
<th>Pretreatment</th>
<th>2’-Cl-BQ</th>
<th>4’-Cl-BQ</th>
<th>3,6,4’-triCl-BQ</th>
<th>PBQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(µM)$</td>
<td>1.9±0.2</td>
<td>N/A$^a$</td>
<td>N/A$^a$</td>
<td>0.9±0.3</td>
<td>0.7±0.9</td>
<td></td>
</tr>
<tr>
<td>$K_{d2}(µM)$</td>
<td>540±100</td>
<td>N/A$^a$</td>
<td>N/A$^a$</td>
<td>105±12</td>
<td>452±206</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ No binding of PAP detected

Table 14. Equilibrium dissociation constants for PAP-binding to hSULT2A1 in the presence of 50 µM DHEA with and without pretreatment of the enzyme with 10 µM PCB-quinones or PBQ pretreatment.

<table>
<thead>
<tr>
<th>PAP binding with 50 µM DHEA</th>
<th>Untreated hSULT2A1</th>
<th>Pretreatment</th>
<th>2’-Cl-BQ</th>
<th>4’-Cl-BQ</th>
<th>3,6,4’-triCl-BQ</th>
<th>PBQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(µM)$</td>
<td>1.1±0.3</td>
<td>1.2±0.5</td>
<td>2.1±0.7</td>
<td>1.3±0.3</td>
<td>5.8±2.0</td>
<td></td>
</tr>
<tr>
<td>$K_{d2}(µM)$</td>
<td>264±36</td>
<td>N/A$^a$</td>
<td>136±29</td>
<td>218±52</td>
<td>553±237</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $K_{d2}$ greater than 1M

Table 15. Equilibrium dissociation constants for PAP-binding to hSULT2A1 in the presence of 0.5 µM DHEA with and without pretreatment of the enzyme with 10µM PCB-quinones or PBQ pretreatment.

<table>
<thead>
<tr>
<th>PAP binding with 0.5 µM DHEA</th>
<th>Untreated hSULT2A1</th>
<th>Pretreatment</th>
<th>2’-Cl-BQ</th>
<th>4’-Cl-BQ</th>
<th>3,6,4’-triCl-BQ</th>
<th>PBQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{d1}(µM)$</td>
<td>0.9±0.2</td>
<td>3.3±0.6</td>
<td>4.8±2.3</td>
<td>1.8±0.4</td>
<td>1.3±0.4</td>
<td></td>
</tr>
<tr>
<td>$K_{d2}(µM)$</td>
<td>248±92</td>
<td>407±81</td>
<td>270±45</td>
<td>180±39</td>
<td>268±72</td>
<td></td>
</tr>
</tbody>
</table>
Figure 29. Binding constants for PAP binding to untreated hSULT2A1, 1 µM PBQ pretreated hSULT2A1 and 10 µM PBQ pretreated hSULT2A1.
Structural modifications of hSULT2A1 identified by LC-MS

Regulatory effects of PCB-quinones as well as PBQ on hSULT2A1 were observed as both alterations in the catalytic activity and changes in the binding properties of those modified enzymes. Specific modifications at the amino acid level were determined by LC-MS after reaction of the enzyme with PCB-quinones and PBQ. 15 μM of reduced hSULT2A1 was coincubated with 250 μM of PCB-quinones or PBQ in Tris-HCl buffer (pH = 8.5) at 25 °C for one hour prior to subsequent digestion and LC-MS analysis.

Adducts at Cys55 as well as Cys199 indicating a Michael-type addition to the quinone were observed with 4’-Cl-BQ as well as 2’-Cl-BQ (Table 16). For 3,6,4’-triCl-BQ-pretreated hSULT2A1, a dichlorinated adduct was found in the digested peptides containing Cys55 and Cys199, thus indicating a chlorine-displacement reaction. In LC-MS analysis of the PBQ-pretreated hSULT2A1, an adduct was found on the peptide containing Cys55, but no adduct was observed on Cys199. However, of all of the peptides containing 4-VP-adducted cysteines were diminished after PCB treatment.

In addition to the cysteine adducts seen following reaction of the hSULT2A1 with PCB-quinones and PBQ, small amounts of peptides containing Cys55 sulfonic acid were observed throughout the samples of hSULT2A1 that had undergone reaction with PCB-quinones and PBQ. In addition, selected methionines like Met223 and Met228 and Met137 were found to be oxidized to form methionine sulfoxides (Table 16).

Computational simulation of structural modifications caused by PCB-quinone treatment

LC-MS studies have revealed the location of adduct formation by reaction of hSULT2A1 with PCB-quinones as well as PBQ. In order to explore potential consequences to the structure of the protein, a molecular model was constructed with the modifications on selected cysteines and methionines in hSULT2A1. The 4’-Cl-BQs were linked at the 4 position of the quinone ring to both Cys55 and Cys199, and both Met223
Table 16. Cysteine and methionine modification after PCB-quinone and PBQ pretreatment for hSULT2A1

<table>
<thead>
<tr>
<th>Modified Cystine</th>
<th>Peptide Structure</th>
<th>Predicted Mass (M+H)^+</th>
<th>Mass found</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3,6,4’-triCl-BQ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys55-Di-Cl-BQ</td>
<td>Di-Cl-BQ-</td>
<td>3711.83</td>
<td>1237.66 (M+3H)^3+</td>
</tr>
<tr>
<td></td>
<td>(DEDVIIITYPKSNTNWLAIELCLMHSK)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys199-Di-Cl-BQ</td>
<td>Di-Cl-BQ-</td>
<td>2923.56</td>
<td>1461.97 (M+2H)^2+</td>
</tr>
<tr>
<td></td>
<td>(RTIEKICQFLGKTELPEELNLILK)</td>
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<td></td>
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<tr>
<td>Cys199-Di-Cl-BQ</td>
<td>Di-Cl-BQ-</td>
<td>2451.18</td>
<td>1225.95 (M+2H)^2+</td>
</tr>
<tr>
<td></td>
<td>(ICQFLGKTELPEELNLILK)</td>
<td></td>
<td>817.21 (M+3H)^3+</td>
</tr>
<tr>
<td>Cys55-O3</td>
<td>Di-Cl-BQ-</td>
<td>2222.05</td>
<td>1112.51 (M+2H)^2+</td>
</tr>
<tr>
<td></td>
<td>(SGTNWLAIELCLMHSKGD)</td>
<td></td>
<td>556.99 (M+4H)^4+</td>
</tr>
<tr>
<td></td>
<td>O3-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(SGTNWLAIELCLMHSKGD)</td>
<td></td>
<td></td>
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<tr>
<td><strong>2’-Cl-BQ</strong></td>
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<td>Cys55-2’-Cl-BQ</td>
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<td>2020.90</td>
<td>1010.32 (M+2H)^2+</td>
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<td>(SGTNWLAIELCLMHSK)</td>
<td></td>
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<tr>
<td>Cys199-2’-Cl-BQ</td>
<td>2’-Cl-BQ-</td>
<td>1024.43</td>
<td>1024.31 (M+H)^+</td>
</tr>
<tr>
<td></td>
<td>(ICQFLGK)</td>
<td></td>
<td>512.62 (M+2H)^2+</td>
</tr>
<tr>
<td>Cys55-O3</td>
<td>O3-</td>
<td>2222.05</td>
<td>1112.51 (M+2H)^2+</td>
</tr>
<tr>
<td></td>
<td>(SGTNWLAIELCLMHSKGD)</td>
<td></td>
<td>556.99 (M+4H)^4+</td>
</tr>
<tr>
<td></td>
<td>O3-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(SGTNWLAIELCLMHSKGD)</td>
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<td><strong>4’-Cl-BQ</strong></td>
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<td>(ICQFLGK)</td>
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<tr>
<td>Cys199-4’-Cl-HQ</td>
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<td>1210.42 (M+2H)^2+</td>
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<tr>
<td>Cys55-O3</td>
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</tr>
<tr>
<td></td>
<td>(SGTNWLAIELCLMHSKGD)</td>
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</tr>
<tr>
<td></td>
<td>O3-</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>(SGTNWLAIELCLMHSKGD)</td>
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<td>BQ-</td>
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<td></td>
<td>662.98 (M+3H)^3+</td>
</tr>
<tr>
<td>Cys55-O3(Double Digestion)</td>
<td>O3-</td>
<td>2222.05</td>
<td>1112.51 (M+2H)^2+</td>
</tr>
<tr>
<td></td>
<td>(SGTNWLAIELCLMHSKGD)</td>
<td></td>
<td>556.99 (M+4H)^4+</td>
</tr>
<tr>
<td><strong>All treatments</strong></td>
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<td></td>
</tr>
<tr>
<td>Met137 sulfoxide</td>
<td>O-(DVLVGSYFFWKNMK)</td>
<td>1749.87</td>
<td>875.24 (M+2H)^2+</td>
</tr>
<tr>
<td>Met223 sulfoxide</td>
<td>O-(NSSFQSMK)</td>
<td>944.42</td>
<td>944.38 (M+H)^6</td>
</tr>
<tr>
<td>Met228 sulfoxide</td>
<td>O-(MSNYSLLSVDYVVDK)</td>
<td>1748.85</td>
<td>1748.57 (M+H)^7</td>
</tr>
</tbody>
</table>
and Met228 were converted to sulfoxides. Energy optimization was performed after building these functional groups into the original hSULT2A1 structure (Figure 30).

The optimized hSULT2A1 structure, without any modification, was aligned with modified hSULT2A1. The C-alpha backbones of hSULT2A1, with and without modification, were compared. By simply viewing the backbone alteration of hSULT2A1 with the adducted cysteines and oxidized methionines, a change in the backbone close to Cys55 and Cys199 was observed with the modified enzyme. The alteration in the backbone close to the modification sites spanned the region where PAPS/PAP binds. It should also be noted that side chain modifications should be taken into consideration, as some modified residues are close to the substrate-binding sites. For example, Met137 is at the entrance site of hSULT2A1 for the substrate DHEA. On the other hand, aromatic rings of PCB-quinones may have electronic or steric properties that, after adduction with Cys55 and Cys199, result in the ring system interacting with the protein to alter either the binding of substrates and products the enzyme or the overall catalytic process.

**N-acetyl-cysteine model study**

The reaction of 3,6,4'-triCl-BQ with N-acetylcysteine (NAC) was studied as a model system to obtain more information on the type of protein adducts that might occur at cysteine residues in proteins. As described above in the Materials and Methods section of this chapter, an equal volume of 4 mM 3,6,4'-triCl-BQ in DMSO was added dropwise to 4 mM NAC solution in 0.1 M potassium phosphate buffer (pH=7.4).

After reaction of the 3,6,4'-triCl-BQ with NAC, LC/MS analysis was performed to separate reaction products that were formed (Figure 31, Table 17). A profile (UV detection) of the reaction products was first determined by an HPLC method. This profile indicated that the peaks in Figure 30 and Figure 33 at retention times up to and including (M+H)^+ of 413.9990 was detected by LC-MS. This molecular ion also showed a typical
Figure 30. Sybyl simulation of hSULT2A1 with 4-Cl-BQ adducts on cysteines and with oxidized methionines. Blue lines represent the backbone of unmodified hSULT2A1; orange lines represent the hSULT2A1 with modification. Yellow balls indicate the position of modified cysteines (i.e., Cys55 and Cys199) while orange balls indicate the positions of oxidized methionines (e.g., Met 223, Met 137 and Met 228).
two-chlorine isotope pattern confirming the loss of one chlorine atom from 3,6,4′-triCl-BQ (Figure 32). Furthermore, another product with a mass ion corresponding to di-NAC adducts being formed with 3,6,4′-triCl-BQ was also detected at a later retention time; it had an (M+H)$^+$ of 543.066. The product was a hydroquinone and the isotopic pattern shown for this structure was changed to that of a typical one-chlorine pattern, confirming the loss of two chlorine atoms with adduct formation (Figure 33). In addition, di-NAC adducts were found at retention times of 49 min and 53 min with a di-chlorinated PCB-quinone structure (theory (M+H)$^+$ of 575.0116). This indicated that both Michael–type addition and displacement reactions can happen at the same time when an excess amount of thiols are present with respect to 3,6,4′-triCl-BQ (Figure 32). Other major mass peaks in Figure 31 and 34 are either degradation products of NAC-PCB-quinone or aggregation products of NAC-quinone adduct. A m/z peak at (M+H)$^+$ of 672.5257 did not show any chlorine isotopic pattern, and the mass was even higher than a tri-NAC-benzoquinone adduct which made it hard to assign the exact molecular identity in this case. At longer incubation times, even at 4 °C for another 1 hour, a similar LC chromatogram system was observed (Figure 34). However, at a retention time of 49 min, the major product shifted from a single NAC-adducted PCB-quinone to a double NAC-adducted PCB-quinone (theory (M+H)$^+$ of 541.0498) (Figure 35).

In summary, these results indicates that reaction of 3,6,4′-triCl-BQ with thiols can occur at more than one position, by more than one mechanism, and form multiple adducts. Both single and double NAC adduct can be formed on the same PCB-quinone molecule, either displacing the chlorine atom on the phenyl ring, or by Michael type addition. This may have implications for forming protein-protein crosslinks through the PCB quinones or for creating mixed adducts containing a protein cysteine and a small molecular weight thiol (e.g., glutathione) bound to the same PCB-quinone. Further structural studies will be needed to unambiguously determine the chemical structures of these protein adducts in terms of the exact position on the PCB-quinone where the NAC adduct is formed.
Figure 31. LCMS determination of NAC adducts formation with 3,6,4’-triCl-BQ.
Figure 32. MS spectrum at 49 min corresponding to the mass of NAC adducts formed with PCB-quinone.
Figure 3. MS spectrum at 70 min corresponding to the mass of double-NAC-adduct formed with PCB-hydroquinone.
Figure 34. LCMS determination of NAC adducts formation with 3,6,4'-triCl-BQ after 2 hours incubation.
Figure 35. MS spectrum at 49 min corresponding to the mass of a double-NAC-adduct formed with PCB-quinone after 2 hours incubation.
Table 17. Structures identified by LCMS following the reaction of NAC with 3,6,4'-triCl-BQ

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Mass identified (M+H)^+</th>
<th>Isotopic pattern</th>
<th>Structure</th>
<th>Calculated mass (M+H)^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>49.24</td>
<td>413.9990</td>
<td>2Cl</td>
<td>[Structure Image]</td>
<td>413.9969</td>
</tr>
<tr>
<td><strong>49.20</strong> (2 hours)</td>
<td>541.0498</td>
<td>1Cl</td>
<td>[Structure Image]</td>
<td>541.0506</td>
</tr>
<tr>
<td>49.24</td>
<td>575.0121</td>
<td>2Cl</td>
<td>[Structure Image]</td>
<td>575.0116</td>
</tr>
</tbody>
</table>
Discussion

Electrophiles represent a large group of biologically active compounds that are capable of reacting with nucleophilic sites on cellular molecules like DNA, proteins, and lipids. Electrophilic modifications to proteins are known to cause toxicities by forming adducts on amino acids that alter key functional roles that the modified proteins play in signal transduction (Dickinson et al., 2004), energy production (Echtay et al., 2003), mitochondrial respiration (Humphries et al., 1998), or cell death signaling (Kondo et al., 2002). In this study, PCB-quinones and PBQ were used as model electrophiles to study the impact on hSULT2A1.

The catalytic assays using different PCB-quinone pretreatments revealed different regulative effects depending on the different structural properties of the compounds applied in this study. As seen in the catalytic analysis of hSULT2A1 that had been pre-treated with PCB-quinones, the effects of the individual PCB-quinones are different. Although all the PCB-quinones demonstrated an inhibitory effect in regulating the catalytic activity of hSULT2A1, 2'-Cl-BQ had the highest IC$_{50}$, while 3,6,4'-triCl-BQ...
had the lowest IC$_{50}$. This indicated that different structural and physical properties of individual PCB-quinones may affect the extent to which the enzyme is modified. A slight decrease in the sulfation rate after directly adding 3,6,4’-triCl-BQ into DHEA sulfation assay using native hSULT2A1 implied either a reversible inhibition or a reaction with the 3,6,4’-triCl-BQ that was too rapid for the analytical method used. A small decrease in the first binding constant determined by assay of DHEA-binding to 3,6,4’-triCl-BQ-pretreated hSULT2A1 might be related to this inhibition.

In the kinetic studies, 4’-Cl-BQ and 2’-Cl-BQ demonstrated similar effects in terms of changing the kinetic constants for the substrate DHEA. $V_{\text{max}}$ was elevated for both the 4’-Cl-BQ- and the 2’-Cl-BQ-pretreated enzyme. This increase in $V_{\text{max}}$ might be partly due to the loss, or attenuation in, the substrate inhibition that is caused by the pretreatment with either 4’-Cl-BQ or 2’-Cl-BQ. For 3,6,4’-triCl-BQ-pretreated hSULT2A1, a different alteration in the $V_{\text{max}}$ of sulfation was observed, wherein a significant decrease in $V_{\text{max}}$ was seen after 3,6,4’-triCl-BQ pretreatment. Although no $K_i$ was detectable for the 3,6,4’-triCl-BQ-pretreated hSULT2A1, this did not result in elevating the $V_{\text{max}}$ of the sulfation reaction. Instead, $V_{\text{max}}$ of the reaction catalyzed by 3,6,4’-triCl-BQ pretreated enzyme was slightly decreased compared to the untreated enzyme. This could indicate a different mechanism of modification caused by 3,6,4’-triCl-BQ.

As identified in the reactions catalyzed by PCB-quinone-pretreated hSULT2A1, the regulation of the catalytic activity by PCB-quinones was closely related with the loss of substrate inhibition. Further analyses were carried out to look into the mechanism of this regulation. Several theories related to substrate inhibition in sulfotransferases have been previously proposed. The formation of ternary complexes in SULT1A1 and SULT1E1 that contain product PAP and the sulfuryl acceptor substrate has been regarded as one of the major reasons for substrate inhibition in these SULTs (Duffel and Jakoby, 1981; Zhang et al., 1998). Moreover, the importance of these ternary complexes in the
substrate inhibition of hSULT2A1 has also been established (Gulcan and Duffel, 2011). It has also been proposed that there are two binding modes for the substrate DHEA in hSULT2A1, named “open” and “closed” conformations of the enzyme; the open conformation of hSULT2A1 is capable of binding two DHEA molecules, while the closed conformation only accommodates binding of one DHEA molecule (Lu et al., 2008; Cook et al., 2010b). An additional component of this proposal is that the formation of the homodimer of hSULT2A1 is associated with the allosteric binding site of DHEA in the open conformation, since the substrate inhibition was not seen in an engineered monomer of hSULT2A1 (Cook et al., 2010a). In our study, only 3,6,4′-triCl-BQ-pretreatment altered the first binding constant of DHEA binding to the enzyme. However, when we explored the binding of PAP to the enzyme, it was significantly altered by pretreatment of hSULT2A1 with PCB-quinones. After PCB-quinone pretreatment, 2′-Cl-BQ and 4′-Cl-BQ abolished PAP-binding to the enzyme while the 3,6,4′-triCl-BQ-pretreated enzyme still had the ability to bind PAP after modification, but the affinity of PAP for the enzyme was increased (i.e., lower values for the dissociation constants) when adding the substrate DHEA into PAP-binding assays (to better imitate usual reaction conditions), the binding of PAP to the hSULT2A1 modified by either 2′-Cl-BQ or 4′-Cl-BQ was restored but with a slightly elevated $K_d$, indicating less binding affinity of the cofactor compared to that seen with the untreated enzyme. As determined by LC-MS, 4′-Cl-BQ and 2′-Cl-BQ modified the enzyme similarly by forming adducts at both Cys55 and Cys199 in hSULT2A1. In the presence of substrate DHEA, $K_d$ values for PAP binding to the 3,6,4′-triCl-BQ pretreated hSULT2A1 did not show a significant change in the presence of DHEA. In the LC-MS analysis, a dichlorinated adduct was detected in the 3,6,4′-triCl-BQ pretreated hSULT2A1 which can be formed through a substitution type of reaction.

Phenyl-benzoquinone was used as a model for other biphenyl-quinones, as it shares the same core structure of the PCB-quinones, but without chlorine atoms. It
showed a marked difference from the PCB-quinones in that low concentrations of PBQ in the pretreatment resulted in an increase in the catalytic activity, but increased concentrations of PBQ yielded a decrease in the catalytic activity. Binding of DHEA to PBQ-pretreated hSULT2A1 was not significantly altered compared to that of the untreated enzyme. However, PAP-binding was significantly altered when a lower PBQ concentration was used in the pretreatment. A significant increase in the binding of PAP both in $K_{d1}$ and $K_{d2}$ was observed after PBQ pretreatment. As previously stated, PAP-binding assays was used to determine PAPS-binding to the enzyme as well. Thus, low affinity binding of PAP may also reflect a lower affinity binding of PAPS to the enzyme after reaction with PBQ, and this may play an important role in the catalytic regulation in the PBQ-pretreated hSULT2A1 as well as in the PCB-quinone-pretreated hSULT2A1.

In order to study the reaction of protein cysteines with PCB-quinones, N-acetyl-cysteine (NAC) was applied as a model. Previous studies on the reactivity of 4’-Cl-BQ with NAC showed that single- or double-NAC adducts were formed by Michael-type additions, and both quinone and hydroquinone final products were identified (Wangpradit, 2009). In our experiments, we examined the ability of 3,6,4’-triCl-BQ to form adducts with NAC. Both single- and double- NAC adducts were detected following reaction of NAC with 3,6,4’- triCl-BQ. In addition to Michael-type adduct formation, our LCMS study also revealed the loss of a chlorine atom in conjunction with the formation of two NAC adducts from which at least one should be formed through a chlorine displacement reaction. These suggested that the reaction of 3,6,4’-triCl-BQ with cysteines proceeded through either chlorine atom displacement or through a Michael-type addition. This model study provided a mechanistic basis for the hypothesis that multiple adducts containing sulfhydryl functional groups, either in a protein or in small molecules, could be formed on the same PCB-quinone. This also implied a possible mechanism of PCB-quinone modification on both the protein structures and the catalytic activity of hSULT2A1.
As seen with the LCMS analysis of tryptic digested hSULT2A1, several methionines (e.g., Met223, Met228, Met137) and Cys55 were also oxidized following the reaction of hSULT2A1 with either PCB-quinones or PBQ. This indicates the possibility that the protein-tethered PCB-quinones or PBQ underwent redox cycling and generated reactive oxygen species that could lead to further oxidation of cysteines or methionines in the protein. Previous study had indicated the formation of semi-quinone radicals when glutathione was reacted with PCB-quinone (Song, 2009). Subsequent reactions of oxygen molecules with the radicals could yield reactive oxygen species like superoxide radicals ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH$^-$). Meanwhile, tethered hydroquinones on peptide thiols (e.g., GSH) could also be oxidized to corresponding quinones and lead to the next round of redox cycling.
CHAPTER VI
CONCLUSIONS

Human hydroxysteroid sulfotransferase (hSULT2A1) has been examined in this dissertation to demonstrate the catalytic regulative effects of oxidative as well as electrophilic modifications on this enzyme at the molecular level. This will help with the prediction of the metabolism of xenobiotics (e.g., drugs or toxicants) or endogenous substances (e.g., hormones, bile acids) catalyzed by this group of enzymes in response to oxidative or electrophilic stress. By using this approach, it also leads to better understanding of the catalytic process of hSULT2A1 in a mechanistic perspective as key amino acid residues are modified in this study which could cause alterations in the binding modes of substrates to the enzyme and change in rates of catalysis.

Thiol oxidants including GSSG, DTNB as well as diamide, which were known to form mixed or intramolecular disulfide bonds, were utilized to examine their regulatory effects on hSULT2A1. Following the reaction of hSULT2A1 with GSSG, DTNB or diamide, the catalytic activity of hSULT2A1 was consistently decreased when using 0.5 \( \mu \text{M} \) DHEA as the substrate. Further analysis has shown a conformational alteration in diamide-pretreated enzyme which could be caused by a intramolecular disulfide bond linking Cys55 and Cys199 close to the PAPS binding site. This was confirmed by an LCMS analysis of tryptic digested hSULT2A1 following reaction with diamide. GSSG- and DTNB-pretreatment also caused structural modifications at Cys55, Cys199 and Cys154 in hSULT2A1 as determined by LCMS study of digested enzyme following the reaction of the enzyme with those two thiol oxidants. These alterations of the protein lead to changes in the binding of substrates to the enzyme. Diamide-pretreatment primarily caused changes in DHEA-binding to the modified enzyme, GSSG-pretreatment caused alterations in the binding affinity of both DHEA and PAPS/PAP, while DTNB-pretreatment only caused changes in PAP binding. These alterations in the substrate binding to the modified enzyme could further lead to changes in the catalytic
characteristics (e.g., substrate binding property alteration, kinetic alteration). A computational simulation also confirmed the findings that protein conformation changes could be induced by mixed disulfide formation or intramolecular disulfide formation that could affect the backbone structure of the protein or the entry route through which substrates came into their binding sites.

Reactivity of lipid peroxides towards hSULT2A1 was studied by utilizing TBHP as a model compound. Catalytic alteration was achieved by reaction of hSULT2A1 with TBHP and showed a significant decrease in the catalytic activity when 0.5 µM DHEA was used as the substrate. This effect was reversed upon restoring the reducing buffer conditions to the TBHP-modified hSULT2A1. LCMS analysis revealed cysteine oxidations as well as methionine oxidations following the reaction of hSULT2A1 with TBHP. The oxidations of both cysteines and methionines affected the binding of the substrates, especially DHEA, to the enzyme. This could further lead to kinetic alteration in catalyzing the sulfation of DHEA by oxidized hSULT2A1 which was determined to be a result of changes in substrate inhibition properties of the reaction. Computational simulation of oxidized hSULT2A1 (sulfonic acid cysteines and oxidized methionines) indicated potential roles for the methionine sulfoxides in altering the entrance for DHEA in hSULT2A1, thus changing the binding properties of DHEA and the overall catalysis.

Beside oxidative modification of hSULT2A1 that was studied in this dissertation, electrophilic modification of the enzyme was also studied utilizing PCB-quinones and PBQ as model compounds. PCBs represent a large group of persistent organic pollutants in our living environment. The toxicological mechanisms of PCBs are still in the process of elucidation, but it is increasingly clear that the toxicity of some lower chlorinated PCBs is associated with their metabolites. Here we have used one group of these metabolites, PCB-quinones, to better understand its potential effects on hSULT2A1. Three PCB-quinones and one phenyl-\(p\)-benzoquinone were chosen as model compounds to examine the influence of electrophilic modifications on hSULT2A1.
After reaction of hSULT2A1 with PCB-quinones and PBQ, the catalytic activity of the enzyme was consistently decreased in a concentration-dependent manner. LCMS determination identified PCB-quinone- as well as PBQ- adducts formed on both Cys55 and Cys199 of hSULT2A1. Sulfonic acid cysteines and methionine sulfoxides were also observed after reaction of the enzyme with PCB-quinones and PBQ. Binding properties of PAPS/PAP were significantly altered after PCB-quinone or PBQ pretreatments. This leads to the loss of substrate inhibition characteristics in the catalytic step of DHEA sulfation and also to a decrease in the catalytic activity of this enzyme. Computational simulation using 4’-Cl-BQ as model treatment provided a good estimation of the conformational alterations as well as the relative locations of the adducted 4’-Cl-BQ. It was clear that conformational changes in the PAPS/PAP binding site, as well as interruption in the substrate binding pocket, were contributing to the catalytic alteration after 4’-Cl-BQ adduction. Further analysis using N-acetyl cysteine as a model compound to study the reactivity of 3,6,4’-triCl-BQ towards cysteine thiols revealed some aspects of the chemical mechanism of this modification. The results showed that either one or two NAC adducts could be formed on the same 3,6,4’-triCl-BQ molecule through either Michael addition or displacement reactions.

From the experimental observations, both (e.g., GSSG, DTNB, diamide, TBHP) and electrophiles (PCB-quinones and PBQ) can regulate the catalytic activity of hSULT2A1. Key amino acid residues like cysteines and methionines that are prone to oxidative modification play an important role in this regulation. Both oxidative and electrophilic modifications on Cys55 and Cys199 can have conformational influences that alter the c-alpha backbone positions as well as the key catalytic residues like Ser129 and Lys44. Alterations in these structural characteristics of hSULT2A1 will lead to changes in substrate binding as well as the catalytic sulfuryl transfer step. Modifications that have larger side chains can reach into the gateway of the PAPS/PAP binding site and show changes in the association and dissociation of PAPS/PAP in the enzyme. Further
analysis also has identified that methionine sulfoxide formation at the entrance of substrate DHEA can cause changes in the binding properties of DHEA to hSULT2A1. These structural and binding alterations can further lead to enzyme kinetic changes such as alteration in the $k_{\text{cat}}$ or substrate inhibition properties, thus result in overall changes in the catalytic activity of hSULT2A1.

In this dissertation, three key cysteines were identified to have catalytic regulative effects in hSULT2A1, however, with the treatment procedure used in this work, specific function and the reactivity of individual cysteines were not fully uncovered. Future studies employing site-directed mutation at selected residues like cysteines or methionines, followed by oxidative or electrophilic treatment, may reveal the functional implications for the modifications on each different residue. At this stage, with the mutated enzymes, additional studies on the kinetics and residue selectivity for the reaction of various electrophiles with the protein will give a more complete picture of the structural features guiding reactions of individual cysteine residues with different treatments. The mutation study could also identify the extent to which these residues are important in the catalytic steps of hSULT2A1.

In order to extrapolate the catalytic regulative effect of oxidative and electrophilic stress observed with recombinant protein to a larger biological system, especially to examine the toxicological effect caused by different PCB-quinones, a whole cell system with known expression of hSULT2A1 should be applied in future work. This would better mimic the real situation of a complicated metabolizing system in the cell, and it would also help to observe possible downstream biomarkers for PCB-quinone toxicities. Further extraction of the modified enzyme from the cell system could also help confirm the findings to see if the quinone adducts and the oxidation products observed with the recombinant protein are also the major products of protein modifications in the cells.


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