CHARACTERIZATION AND MODULATION BY DRUGS AND OTHER EFFECTORS OF BOVINE LIVER MICROSOMAL FLAVIN MONOOXYGENASE (FMO)

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
THE DEPARTMENT OF BIOCHEMISTRY

JANUARY 2004

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

CHARACTERIZATION AND MODULATION BY DRUGS AND OTHER EFFECTORS OF BOVINE LIVER MICROSOMAL FLAVIN MONOOXYGENASE (FMO)

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January 2004, 107 pages

The flavin-containing monooxygenases (FMO; E.C.1.14.13.8) are microsomal NADPH and oxygen-dependent flavoprotein enzymes that catalyze the oxidation of a wide variety of xenobiotics, including drugs and environmental toxicants. Nucleophiles containing nitrogen, sulfur, phosphorus and selenium heteroatoms are the substrates of FMO.

Bovine liver microsomal FMO enzyme activity was characterized using methimazole as substrate, which is a highly specific substrate for FMO. From 12 different bovine liver samples, microsomes were prepared and the average specific activity of bovine liver microsomal FMO was found to be 2.37 ± 0.30 nmol/min/mg (Mean ± SE, n=12). The rate of reaction was linear up to 0.5 mg of bovine liver microsomal protein. The maximum FMO enzyme activity was detected at 37 °C and

at pH 8.0. Effects of detergents; Triton X-100 and Emulgen 913, on FMO activity were determined and found that enzyme activity increased by the addition of either detergent at all concentrations (0.1%-1.0%). The apparent V_{max} and K_m values of bovine liver microsomal FMO for methimazole substrate were found as 1.23 nmol/min/mg and 0.11 mM, respectively.

Thermostability of bovine liver microsomal FMO was studied at four different temperatures; 24 °C, 37 °C, 50 °C and 65 °C. The incubation time required for the complete loss of enzyme activity was 5 minutes at 65 °C, 10 minutes at 50 °C and 6.5 hours at 37 °C. 68 % of the activity was still detectable at the end of 53 hours at 24 °C. Bovine liver microsomal activity towards two drug substrates, imipramine and chlorpromazine, was also determined and found to be 3.73 and 3.75 nmol NADPH oxidized/min/mg, respectively. Effects of two drug substrates, imipramine and chlorpromazine, on bovine liver microsomal FMO-catalyzed methimazole oxidation activity was also studied and found that they inhibit FMO activity at all concentrations studied.

Modulation of bovine liver microsomal FMO activity was studied using three different heavy metal ions; Ni^{+2}, Cd^{+2} and Hg^{+2}. At all other concentrations studied for each heavy metal ion and at all substrate methimazole concentrations (0.1, 0.2, 0.5, 1.0 mM), FMO-catalyzed methimazole oxidation activity decreased compared to control activity. K_I values for Ni^{+2}, Cd^{+2} and Hg^{+2} were found to be 0.5 mM, 0.085 mM, 4.6 μM, respectively. From the Dixon plot, the pattern of inhibition for three heavy metal ions was observed to be noncompetitive.

Key words: FMO, flavin-containing monooxygenases, methimazole, imipramine, chlorpromazine, bovine liver microsome, characterization, modulation, effectors.

ÖZ

SIĞIR KARACİĞER MİKROZOMAL FLAVİN MONOOKSİJENAZ (FMO) 'IN KARAKTERİZASYONU VE İLAÇLAR İLE DİĞER EFEKTÖRLER TARAFINDAN MODÜLASYONU

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Flavin-içeren monooksijenazlar (FMO; E.C.1.14.13.8), ilaçlar ve çevresel toksinlerin de dahil olduğu birçok değişik yabancı maddelerin, NADPH ve oksijene bağımlı oksidasyonunu katalize eden mikrozomal flavoprotein enzimlerdir. Azot, sülfür, fosfor ve selenyum heteroatomu içeren nükleofiller FMO'nun substratlarıdır.

Sığır karaciğer mikrozomal FMO enzim aktivitesi, FMO enzimleri için çok özgün bir substrat olan metimazol kullanılarak karakterize edildi. 12 farklı sığır karaciğeri numunesinden mikrozomlar hazırlandı ve ortalama özgün FMO aktivitesi 2.37 ± 0.30 nmol/min/mg (Ortalama ± SH, n=12) olarak bulundu. Reaksiyon hızı 0.5 mg protein miktarına kadar doğru orantılı arttı. En yüksek FMO enzim aktivitesi 37 °C ve pH 8.0'de tespit edildi. FMO aktivitesi üzerine Triton X-100 ve Emulgen 913 deterjanlarının etkileri çalışıldı ve enzim aktivitesinin bu deterjanların herbirinin

reaksiyon ortamına eklenmesiyle tüm konsantrasyonlarda (0.1%-1.0%) arttığı gözlendi. FMO enziminin metimazol substratı için görünen V_{max} ve K_m değerleri sırasıyla 1.23 nmol/dak/mg and 0.11 mM olarak bulundu.

Sığır karaciğer mikrozomal FMO enziminin ısıya dayanıklılığı dört farklı sıcaklıkta çalışıldı; 24 °C, 37 °C, 50 °C ve 65 °C. Enzim aktivitesinin tamamen kaybolması için gereken inkübasyon süresi 65°C'de 5 dakika, 50 °C'de 10 dakika, 37 °C'de 6.5 saat olarak gözlendi. 24 °C'de 53 saatin sonunda halen %68 enzim aktivitesinin kaldığı görüldü. Sığır karaciğer mikrozomlarının imipramin ve klorpromazin gibi iki ilaç substratına karşı aktivitesi de ölçüldü ve sırasıyla 3.73 ve 3.75 nmol oksitlenmiş NADPH/dak/mg olarak tespit edildi. İmipramin ve klorpromazinin sığır karaciğer mikrozomal FMO enziminin metimazol oksidasyon aktivitesi üzerine etkileri belirlendi ve çalışılan tüm konsantrasyonların FMO enzim aktivitesini inhibe ettiği bulundu.

Sığır karaciğer mikrozomal FMO aktivitesinin modülasyonu üç farklı ağır metal iyonuyla, Ni^{+2}, Cd^{+2} ve Hg^{+2}, çalışıldı. Herbir ağır metal iyonu için çalışılan diğer tüm konsantrasyonlar ve tüm metimazol konsantrasyonlarında (0.1, 0.2, 0.5, 1.0 mM), FMO enziminin metimazol oksidasyon aktivitesinin kontrole göre azaldığı görüldü. Ni^{+2}, Cd^{+2} ve Hg^{+2} iyonları için K_I değerleri sırasıyla 0.5 mM, 0.085 mM, 4.6 μM olarak bulundu. Dixon eğrisinden üç ağır metal iyonu için de inhibisyon çeşidi nonkompetitif olarak gözlendi.

Anahtar kelimeler: FMO, flavin monooksijenaz, metimazol, imipramin, klorpromazin, sığır karaciğer mikrozomu, karakterizasyon, modülasyon, efektör.

TO MY FAMILY

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor Prof. Dr. Orhan ADALI for his invaluable guidance, continued advice and critical discussions throughout this study.

I would like to thank my co-supervisor Prof. Dr. Emel ARINÇ for her valuable comments and advice in this study.

I wish to thank my examining committee members Prof. Dr. Mesude İŞCAN, Assoc. Prof. Dr. Alaattin ŞEN and Assoc.Prof. Dr. Candan GÜRAKAN GÜLTEKİN for their suggestions and criticism.

I have special thanks to Birsen CAN DEMİRDÖĞEN and Şevki ARSLAN, from whom I've learned a lot and who were great and supportive friends.

I want to thank Azra BOZCAARMUTLU, Gülen ULUSOY, Tuğba BOYUNEĞMEZ and Haydar ÇELİK for their help and friendship.

Finally, I want to thank my grandparents, Ramazan & Şerife ACARTÜRK, and my uncle Atilla ACARTÜRK, for their support and encouragement throughout this study. I would like to thank my parents, Tülay & Kenan BÖLÜKBAŞI, my sister, Begüm BÖLÜKBAŞI, for their permanent support. I also would like to thank my cousin, Deniz BALCI for her help.

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∈-ACA	∈-Amino caproic acid
BSA	Bovine Serum Albumin
DF	Dilution Factor
DPT	Diphenyl sulfide
DTNB	5,5'-dithiobis (2-nitrobenzoate)
DTT	dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FMO	Flavin-containing monooxygenase
MI	Methimazole
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PMSF	Phenylmethylsulfonyl fluoride
TMA	Trimethylamine
TMAU	Trimethylaminuria
Tris	Tris (hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

Living organisms are continuously exposed to a great number of foreign compounds called xenobiotics through absorption, inhalation and ingestion. Following entry into the organism, those foreign compounds such as drugs, pesticides and many other chemicals are metabolized in the body. Usually, such metabolism converts the original compound into a more polar derivative to facilitate excretion. These foreign compounds are usually converted into less toxic ones; however, sometimes the product may be more toxic that may threaten the health. Many xenobiotics to which humans may be exposed in the environment are relatively chemically unreactive and non-polar. Therefore, enzyme systems act to introduce one or more "reactive centers" into the compound (Phase I metabolism) which promotes excretion of the compound directly then serve as a "handle" for the enzymatic attachment (conjugation or Phase II metabolism) of a more water-soluble group. The majority of Phase I metabolism is mediated by cytochrome P450s (CYPs). However, although CYPs catalyze the majority of Phase I metabolic reactions it has been recognized recently that another family of "drug-metabolizing enzymes", the flavin-containing monooxygenases (FMOs), also play significant roles.

The flavin-containing monooxygenases (FMO) and cytochrome P450 (CYP450)-dependent monooxygenases are the principle enzymes catalyzing the oxidation of lipophilic xenobiotics to electrophilic products capable of further metabolism or reaction with nucleophilic target molecules. Nnane and Damani, 2003, stated that both FMO and CYP450 play a significant role in sulphoxidation of sulfur

atoms that reside within or adjacent to aromatic ring systems. Similarities and differences between FMO and CYP450 enzymes are summarized in Table 1.1. Although FMO and CYP450 are involved in activation and detoxication reactions, CYP450 is the most important with regard to activation of toxicants (Arınç *et al*., 1991; Hodgson *et al*., 1999; Arınç *et al*., 2000). Both enzymes are microsomal and require NADPH and molecular oxygen for activity. FMO was first purified from pig liver microsomes in 1972 by Ziegler and Mitchell. The FMO is now known to exist in many species and in more than one organ. In recent years, it has been purified from liver microsomes of rat (Kimura *et al*., 1983), mouse (Sabourin *et al*., 1984; Sabourin and Hodgson, 1984), rabbit (Tynes *et al*., 1985), guinea pig (Yamada *et al*., 1990), macacque (Sadeque et al., 1993), from pulmonary (Tynes *et al*., 1985) and renal (Venkatesh *et al*., 1991) microsomes of mouse, and from brain microsomes of rat (Kawaji *et al*., 1995) and human (Bhagwat *et al*., 1996). FMOs are typically membrane-bound enzymes found in smooth endoplasmic reticulum and nuclear envelope of the cell, although cytosolic forms predominate in bacteria and unicellular organisms (Sum and Kasper, 1982; Schlenk, 1998). FMO activity, protein or mRNA have also been observed in several invertebrates, fish, reptile, avian and amphibian species (Schlenk, 1998). The physiological role of mammalian FMO is unknown although it has been observed that many heteroatom-containing chemicals in plants are efficiently oxygenated by FMO. A plant FMO-like enzyme has been characterized and it appears to possess many of the same functional properties of mammalian FMO (Cashman, 2002).

While the significance of human FMOs was reported in the early 1970s, it was not until the late 1980s and 1990s that FMOs were characterized by purification and cDNA cloning. FMOs (E.C.1.14.13.8) are microsomal NADPH-dependent flavoprotein enzymes that catalyze the oxidation of a wide variety of xenobiotics, including drugs, such as morphine, chlorpromazine, imipramine, tamoxifen and ranitidine, dietary constituents, environmental toxicants, and therapeutic agents (Hodgson *et al*., 1995; Bhamre *et al*., 1995; Kupfer and

Table 1.1 Similarities and differences between the flavin-containing monooxygenase (FMO) and cytochrome P450 (P450) (Hodgson *et al.,* 1999).

	FMO	**P450**
Co-substrates	NADPH, O_2	NADPH, O_2
Coupled enzymes	None	NADPH-P450 reductase Cytochrome b_5*
Cellular location	Microsomes	Microsomes, mitochondria
Xenobiotic inducers	None known**	Many, e.g. phenobarbital, TCDD
Inhibitors	Competitive substrates, only	Competitive substrates and mechanism based inhibitors, e.g. SKF525A
Isoforms	Few, only 6 known currently	Many, over 3000 known currently
Substrates	Some inorganics, organic compounds with N, S, Se, P heteroatoms	Organic compounds with and without N, S, Se, P heteroatoms

*Cytochrome b_5 is required only for some P450 isoforms and for some substrates

**Induction by sex steroids, glucocorticoids and dietary xenobiotics

Dehal, 1996; Stormer *et al*., 2000; Attar *et al*., 2003). Substrates are typically soft nucleophiles containing nitrogen, sulfur, phosphorus or selenium heteroatoms. In addition to nucleophilicity, size and charge are also important parameters (Bach, 2003). Evidence for six forms of the FMO gene exists, *FMO1- FMO6* (Cashman, 2002). However, *FMO6* does not appear to encode a functionally active enzyme (Krueger *et al*., 2001). Products of these genes exhibit overlapping, but distinct substrate specificity (Cashman, 1995). Considering the limited number of *FMO* gene products relative to other classes of drug metabolizing enzymes, e.g., the CYP450-dependent monooxygenases, the broad substrate specificity exhibited by FMOs is quiet remarkable. A major contributing factor to this property is the unique catalytic mechanism of these enzymes wherein a potentially reactive hydroperoxyflavin intermediate is formed in the presence of oxygen and NADPH, but in the absence of substrate (Poulsen and Ziegler, 1995). The broad substrate specificity of the FMOs, coupled with their documented expression in a highly species- and tissue-specific manner (Overby *et al*., 1992; Dolphin *et al*., 1996), suggests these enzymes make a significant contribution to the tissue-specific responses observed with some drugs and environmental toxicants. Interindividual variation has been observed for FMO expression. It has been mainly influenced by genetic factors. Environmental factors do not have a profound effect on FMO expression (Whetstine *et al*., 2000).

The human FMO is a prominent enzyme involved in the detoxication and elimination of drugs and chemicals. Therefore, an understanding of its substrate specificity, catalytic properties, molecular structure, expression patterns, gene regulation and polymorphisms would provide insight into features that would expedite drug discovery and development.

1.1 Physical Properties of FMO

1.1.1 Spectral Properties

Spectral studies demonstrate four spectrally different stable states of the flavin chromophore. The oxidized enzyme exhibits a typical flavoprotein spectrum with maxima at 385 and 450 nm (Ziegler *et al.,*1980). Increasing concentrations of $NADP^+$ shift both peaks to shorter wavelenght peak and 485 nm shoulder.

In the absence of oxygen, rapid complete reduction of the flavin occurs without detectable free radical or semiquinone formation. Reoxidation of the fully reduced flavoprotein yields a species with distinct spectral characteristics. It has a single absorbance band at 366 nm and is similar to that of the stable peroxy-FMN luciferin complex and of transient peroxy-FAD complexes found during oxidations catalyzed by hydroxybenzoate hydroxylase (Ghisla, 1978). This is indicative of a 4a flavin hydroperoxide complex. This species is stable for several hours at 2°C but decomposes rapidly above 25°C yielding oxidized flavoprotein and hydrogen peroxide.

1.1.2 Composition and Cofactor Requirement

Studies with hog liver FMO have shown that it is a polymeric protein with a monomer molecular weight of 65,000. The monomer migrates as a single band on SDS gel electrophoresis and amino terminal analysis indicates the presence of only one type of monomer. The FMO enzyme contains 1 mole FAD per monomer and is free of all metals except zinc (Poulsen, 1981).

The monoxygenase uses either NADH or NADPH as the cofactor; however, the K_m for NADH is at least ten times greater than that of NADPH. At saturating NADPH and oxygen, monooxygenase-catalyzed oxygenations of both sulfur and

nitrogen exhibit a distinct pH optimum at pH 8.3-8.4. The pH profile is essentially the same for all substrates and the velocity at pH 7.4 is 50 to 55 % of that at pH 8.4. However, with saturating NADH, the pH optimum shifts to 7.9-8.0. The Michaelis constants for NADPH and most substrates are only minimally affected by pH; however, the K_m for oxygen has marked pH dependence (Poulsen, 1981).

FMO exhibits a high degree of thermal lability and is easily inactivated at temperatures above 42°C. Kitchell *et al,* 1978, have shown that the flavoprotein undergoes several structural changes with increasing temperature, and that above 30°C these changes are irreversible. Thermal stability of FMO is increased in the presence of $NADP^+$ or NADPH and, in preparations completely free from endogenous inhibitors and proteases, the enzyme retains 95-98 % of its original activity after 20 minutes incubation at 37°C in the presence of a NADPH-generating system (Ziegler *et al.*, 1980). However, above 45°C irreversible denaturaion occurs in a few seconds.

1.2 Catalytic Properties of FMO

The mechanism of enzyme action involves an ordered addition of cofactors and substrates with an irreversible step between oxygen binding and binding of the oxygenatable substrate (Figure 1.1). Neither oxygen nor substrate are bound by the oxidized form of the enzyme and reduction of the flavin by NADPH must occur before oxygen can bind (Poulsen, 1981).

In the absence of substrate, FMO enzyme catalyzes an oxygen-dependent oxidation of NADPH to give $NADP^+$ and peroxide. The rate of H_2O_2 formation, resulting from turnover of the peroxyflavin, ranges between 3 and 10 % of the substrate oxygenation rate and is responsible for an apparent endogenous rate of oxygen reduction by NADPH. Addition of substrate to the peroxyflavin complex is the last step prior to oxygenation, and oxidation of the substrate by the flavin

Figure 1.1 Schematic representation of the kinetic mechanism for FMO. S and SO are oxygenatable substrates and oxygenated products, respectively (Taken from Poulsen, 1981).

hydroperoxide has been confirmed (Beaty and Ballou, 1981). All other mixed-function oxidases studied require binding of substrate before reduction by pyridine nucleotide, and the catalytic mechanism of the microsomal FMO is distinctly different. The proposed catalytic cycle of FMO is shown in Figure 1.2.

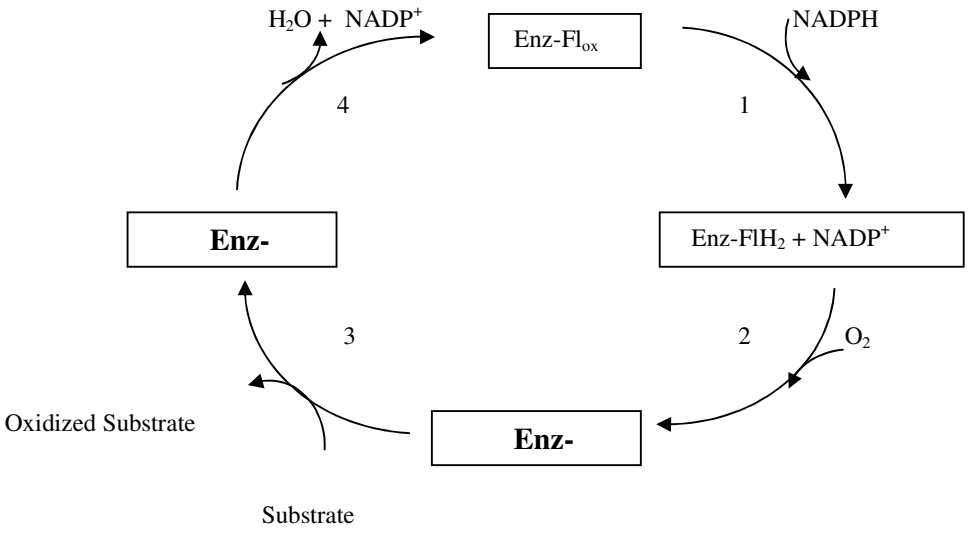


Figure 1.2 Proposed catalytic cycle of FMO (Taken from Cashman, 2002).

Steps 1 and 2 leading upto the hydroperoxy-flavoenzyme species (NADPH combination followed by molecular oxygen addition and hydroperoxy-flavoenzyme formation) are quiet rapid. Stabilization of this oxygenating species is a remarkable feature of this enzyme system and allows a wide variety of nucleophilic heteroatom-containing drugs and chemicals to attack the terminal hydroperoxy-flavoenzyme oxygen atom (step 3). The oxygenated product leaves the product-binding region in a fast step. Step 4 is the rate limiting step and since this step does not involve substrate, this accounts for the fact that good substrates for the FMO have similar V_{max} values. It is assumed that human FMOs follow the detailed steps of the catalytic cycle of animal models but this has not been rigorously examined (Cashman, 2002).

The mechanism for the catalysis of the FMO does not involve superoxide or free hydrogen peroxide; however, Rauckman *et al*., 1979, have shown that there is a small but detectable formation of superoxide during catalytic turnover. Neither superoxide nor H_2O_2 is involved in oxygenation since superoxide dismutase and catalase have no effect on any S- or N- oxygenations catalyzed by this enzyme.

1.3 Substrates of FMO

FMOs catalyze the oxidation of a great number of structurally diverse compounds that contain nitrogen, sulfur, phosphorus, selenium and other nucleophilic heteroatoms. Xenobiotic substrates include secondary and tertiary amines, secondary hydroxylamines, hydrazines, thioamides, thiocarbamides, sulfides, disulfides, thiols. There is a few known endogenous substrates, such as cysteamine and trimethylamine, of FMO. FMO generally converts lipophilic materials to more polar molecules that are more readily excreted. Often, this is a detoxication reaction whereby a chemical is transformed into an N-oxide or S-oxide that possesses sufficient polarity to terminate the pharmacological activity of the molecule. However, FMO can also bioactivate molecules to reactive metabolites that are electrophilic enough to covalently modify biologically relevant macromolecules.

1.3.1 Xenobiotic Substrates of FMO

It is straight forward to predict the product of FMO-mediated oxygenations because the products are predicted to be the same as those arising from treating the substrate with peroxide or peracid (Cashman, 2000). Thus, tertiary amines are converted into tertiary amine N-oxides and sulfides are converted into S-oxides. Xenobiotic substrates of FMO are listed in Table 1.2.

1.3.1.1 Sulfur Substrates

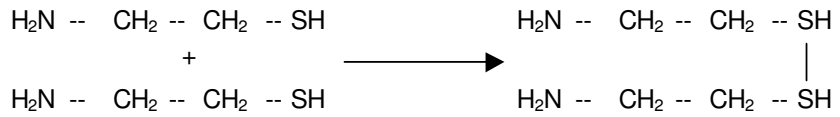
The flavin hydroperoxide moiety in the activated enzyme complex is a strong electrophile and should be capable of oxygenating any nucleophilic sulfur compound. Because of the enhanced nucleophilicity, the sulfur atom generally is the preferred site of FMO oxygenation in molecules that contain both sulfur and nitrogen atoms. However, the enzyme exhibits a high degree of specificity and readily discriminates between different types of sulfur nucleophiles. A common structural feature of most physiological sulfur compounds is the presence of one or more carboxyl groups and the monooxygenase is incapable of catalyzing oxygenation of any compound containing an ionized carboxyl group. This specificity is largely responsible for the physiological specificity of FMO (Poulsen, 1981). Examples of sulfur-containing organics oxygenation reactions by FMO are shown in Figure 1.3.

1.3.1.2 Nitrogen Substrates

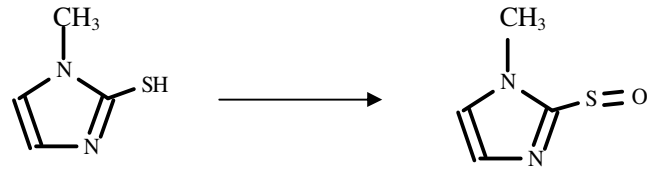
FMOs have been shown to metabolize nitrogen-containing heteroatoms. FMOs can oxidize primary, secondary, tertiary and heterocyclic amines and also hydrazines which are listed with examples in Table 1.2. In the presence of human liver microsomes it was observed that the primary amines were efficiently converted to their oximes through the intermediary of hydroxylamine (Cashman, 2000).

Table 1.2 Substrates of FMO (Taken from Ziegler, 1988; Hodgson *et al.*, 1995).

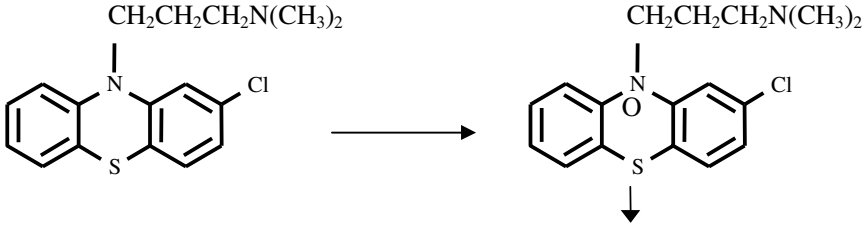
Substrate	Example
Organic Sulfur Substrates	
Thiols	Cysteine, Glutathione
Sulfides	Methionine, Dimethylsulfide
Disulfides	Cystamine, Butyl disulfide
Thioamides	Thioacetamide, Thionicotinamide
Thiocarbamides	Methimazole, Thiourea
Thiocyanates	Phenylisothiocyanate
Sulfoxides	Thiobenzamide sulfoxide
Sulfenic Acids	N-methylimidazole-2-sulfenic acid
Carbodithioic Acids	Phenylcarbodithionic acid
Organic Nitrogen Substrates	
Primary Amines	Tyramine, n-Octylamine
Secondary Amines	N-methylaniline, Perazine
Tertiary Amines	Nicotine, Imıpramine, Tamoxifen
Heterocyclic Amines	Xanomeline, Clozapine
Hydrazines	Phenylhydrazine
Organic Phosphorus Substrates	
Phosphines	Diethylphenylphosphine
Phosphonothioates	Fonofos
Organic Selenium Substrates	2-selenylbenzanilide
Boronic Acid	
Inorganics	HS^-, S_8, I^-, I_2



Cysteamine Cystamine



Methimazole Methimazole S-oxide



Chlorpromazine Chlorpromazine S-oxide

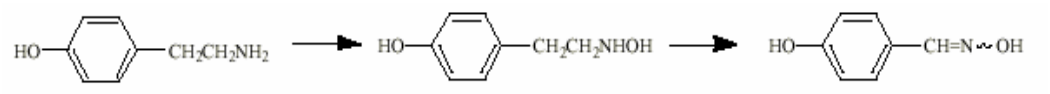
Figure 1.3 Examples of reactions catalyzed by FMO: Sulfur-containing xenobiotics (Adapted from Parkinson, 1996 and Cashman 2000).

N-oxygenation was largely dependent on human FMO3 although contribution from CYPs was also observed. Examples of nitrogen-containing organics oxygenation reactions by FMO are shown in Figure 1.4.

Biogenic amines such as tyramine are good substrates for the human FMO3. In the presence of human liver microsomes, tyramine is converted exclusively to the *trans* oxime in a process dependent on the human FMO3. Tyramine is sequentially N-oxygenated to the hydroxylamine, and then to the di-N-hydroxylamine that is spontaneously dehydrated to the *trans* oxime (Cashman, 2002).

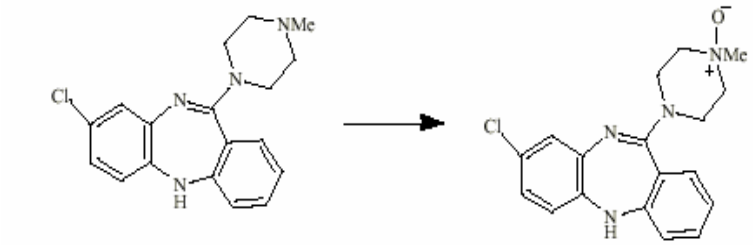
(S)-Nicotine is stereoselectively N-1'-oxygenated by human FMO3 to produce exclusively the *trans* nicotine N-1'-oxide (Figure 1.4). In contrast, animal FMO1 forms approximately a 50:50 mixture of *cis* and *trans* nicotine N-1'-oxide. That no *cis* nicotine N-1'-oxide is formed in the presence of adult human liver microsomes suggests that functional hepatic activity of human FMO1 is not present (Cashman, 2000). (S)-nicotine is quiet useful as a highly stereoselective *in vitro* probe of human FMO3 activity, but because of the relatively high K_m value, its usefulness as an *in vivo* probe is less than desired. For adults that smoke cigarettes, determination of the urinary (S)-nicotine N-1'-oxide levels and stereochemistry provides a stereoselective means of quantifying human FMO3.

Among the best substrates for FMO are nucleophilic cyclic tertiary amines. This may have something to do with the enhanced nucleophilicity of the nitrogen atom in the cyclic amine. As an example, clozapine is efficiently N-oxygenated by human FMO3. Caffeine has been also reported to be a substrate for human FMO (Cashman, 2000). However, on the basis of the lack of nucleophilicity of the nitrogen atoms in caffeine, it is unlikely that caffeine is a good substrate for human FMO.



Tyramine (hydroxylamine intermediate) Trans Oxime

(S)-Nicotine **Trans Nicotine N-1'-oxide**

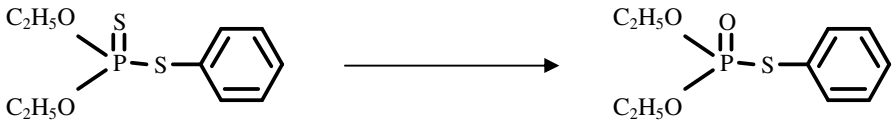


Clozapine Clozapine N-oxide

Figure 1.4 Examples of reactions catalyzed by FMO: Nitrogen-containing xenobiotics (Adapted from Cashman, 2000).

1.3.1.3 Phosphorus Substrates

Phosphines and phosphothioates are also substrates for FMO (Table 1.2). Fonofos, a highly toxic insecticide, is metabolized by both FMO and CYP450 to fonofos oxon (Figure 1.5). Fonofos was unusual in having a low K_m as well as low V_{max} for the purified mouse liver and kidney FMOs (Venkatesh *et al*., 1991).



Fonofos Fonofos oxon

Figure 1.5 Example of a reaction catalyzed by FMO: Phosphorus-containing xenobiotic (Adapted from Hodgson *et al*., 1999).

1.3.2 Endogenous Substrates of FMO

There is a few known endogeneous substrates, such as cimetidine and trimethylamine, for FMO. Cimetidine (Figure 1.6) has been shown to be stereoselectively S-oxygenated to cimetidine S-oxide by human FMO3 (Cashman *et al*., 1993). Cimetidine S-oxide is the major metabolite arising from FMO-dependent hepatic metabolism of cimetidine *in vivo*, presumably because of the nucleophilicity of the sulfur atom and because the imidazole moiety of cimetidine serves to inhibit CYP-dependent oxidation. Cimetidine S-oxygenation is a useful *in vivo* probe of human FMO3 activity and urinary cimetidine S-oxide is widely enough used in the clinical setting as to provide useful information about *in vivo* human FMO3 function (Cashman, 2000).

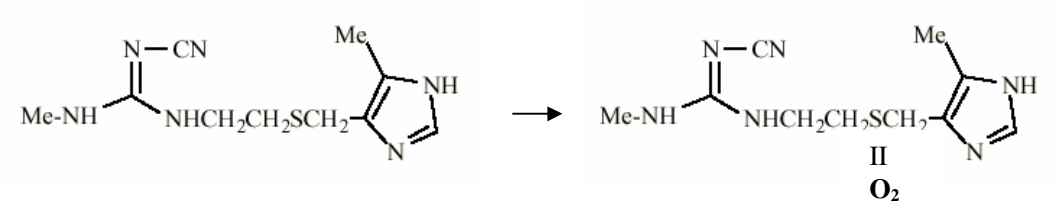


Figure 1.6 Cimetidine S-oxygenation by FMO (Adapted from Cashman, 2000).

With the recent reports showing a direct causative relationship between defective human *FMO3* genes and trimethylaminuria it has become clear that the human FMO3 is the exclusive catalyst for trimetylamine (TMA) N-oxygenations. TMA is present in high concentrations in the diet from the dietary precursor choline. To date, the most promising non-invasive probe of *in vivo* human FMO3 activity is the urinary TMA:TMA N-oxide ratio. Normal adults have TMA:TMA N-oxide ratios of 5:95. If the urinary TMA:TMA N-oxide ratio falls significantly below 10:90, then it is possible that a human FMO3 polymorphism is present (Cashman, 2000).

1.4 Trimethylaminuria

Trimethylaminuria, also known colloquially as the fish malodor syndrome, provides an excellent example of how genetically determined variability in the metabolism of a diet derived chemical, namely trimethylamine, can result in a distressing clinical condition. At a more general level it reflects how genetic constitution can adversely influence interactions with one's diet.

Biochemically, the disorder is characterized by the excretion of excessive amounts of a simple tertiary aliphatic amine, trimethylamine. Excess unmetabolized trimethylamine in the urine, sweat, breath and other body secretions confers a strong, very unpleasant body odor resembling that of rotting fish that can affect the

individual's ability to work or engage in social activities (Cashman *et al*., 2003). At its extreme, it can be accompanied by severe mental depression occasionally leading to suicidal tendencies These "greater-than-normal" amounts of trimethylamine are present due to a failure in removing it via the usual oxidation route to the non-odorous metabolite, trimethylamine *N*-oxide, owing to a mismatch in the enzymes capacity to undertake this reaction and the substrate load it has to process (Mitchell and Smith, 2001).

1.4.1 The First Clinical Reports

Historically, it is a fascinating fact that anecdotal descriptions of individuals with the fish malodor syndrome, or at least something very like it, have been recorded across various millennia and cultures.

The first clinical description of a case of fish malodor syndrome is attributed by Humbert and colleagues, 1970. The patient was a 6-year old girl with the clinical stigmata of Turner's syndrome, mild anemia, abnormal platelet function and decreased deformability of the red cells. And the child had a peculiar "fishy odor". This triggered off a search for trimethylamine, which was known to smell of fish. Biochemical studies following an oral challenging dose of trimethylamine showed that there was a marked increase in the excretion of the free amine in her urine as well as a pronounced exacerbation of her odor problem (Humbert *et al*., 1970). A subsequent study on a biopsy liver sample taken from this patient revealed a defective trimethylamine *N*-oxidizing system (Higgins *et al*., 1972). Over subsequent years, there appeared a number of sporadic reports of the fish malodor syndrome in both adults (Spellacy *et al*., 1979) and young children (Lee *et al*., 1976). These early studies showed that the condition could affect infants, children, and adults. This disease also causes other symptoms like depression, hypertension and disorders of amine metabolism.

1.4.2 Biochemical Features

Mitchell and Smith, 2001, stated that the outstanding biochemical feature in the fish malodor syndrome is the excessive excretion in the urine of un-oxidized trimethylamine. British individuals on the average Western diet excrete in the urine daily about 50 mg of trimethylamine *N*-oxide and about 1 to 2 mg of free trimethylamine; that is, the *N*-oxide accounts for greater than 90% of the total daily excretion of trimethylamine-related material. The total amounts vary, however, according to the composition of the diet and particularly if marine fish are eaten because they contain high levels of trimethylamine *N*-oxide.

Table 1.3 shows a summary of the pattern of urinary excretion of trimethylamine and its *N*-oxide for a group of unaffected controls, known heterozygotes (parents of children with fish malodor syndrome), and some patients with the fish malodor syndrome, either by following a normal diet or a challenge dose. The results are expressed as a percentage of the total trimethylamine related material excreted in the urine as the free amine on a normal diet as well as after an oral challenge "load" test (600 mg of trimethylamine). The finding for the two groups are also expressed in terms of the ratio: percentage trimethylamine/percentage trimethylamine *N*-oxide, which is diagnostic for a deficiency of *N*-oxidation. It can be seen that both the male and female patients excrete most (circa 70–80%) of the total trimethylamine derived from the diet as the free amine compared with only 4% found for healthy controls. The dysfunctional *N*-oxidation is clearly reflected in the high values seen for the ratio. After the challenge test, the fish malodor patients excreted even more free amine (90 %) whereas this remained unaffected for the control subjects. The heterozygotes , who coped with the normal dietary input and were indistinguishable from the unaffected controls, were shown to falter when challenged with the 600-mg oral load, and their *N*-oxidation capacities decreased; this is the basis of the challenge test for heterozygotes or carriers. There appears

Table 1.3 N-oxidation of TMA in fish malodor patients, heterozygotes (carriers), and affected individuals following a normal diet and a 600 mg TMA oral challenge dose (Taken from Mitchell and Smith, 2001).

Following a Normal Diet		
Group	Free TMA as a Percentage of Total Excretion	TMA / N-oxide Ratio
Fish Malodor Males = 4 Females = 9	 78.5 ± 13.3 71.8 ± 15.7	 3.65 2.55
Heterozygotes (n=13)	4.5 ± 1.8	0.047
Unaffected Controls (n=75)	4.3 ± 3.0	0.045
Following an 600 mg Challenge Dose		
Group	Free TMA as a Percentage of Total Excretion	TMA / N-oxide Ratio
Fish Malodor Males = 4 Females = 6	 91.5 ± 5.1 85.8 ± 7.1	 10.76 6.04
Heterozygotes (n=13)	24.3 ± 2.7	0.32
Unaffected Controls (n=75)	6.6 ± 3.0	0.071

to be a threshold for the appearance of fish malodor symptoms.The loss of "larger-than-normal" amounts of trimethylamine via the sweat and breath appears to occur in concordance with a urinary level of free trimethylamine of 10 mg/ml (18–20 mmol/mmol creatinine) or above. Depending upon urinary volumes, this correlates

with a urinary trimethylamine output of about 15 to 20 mg/day. In terms of frequency of occurrence, it should no longer be regarded as a "rare" disorder but more appropriately as an "uncommon" one. This metabolic syndrome has been found in several countries. The condition has been observed in both males and females.

1.4.3 Causative Mutations

TMAU is proposed to be an autosomal recessive inborn error of metabolism. The condition is thought to be uncommon, although it is distributed non-randomly in specific populations. Mutations of FMO3 gene causes the TMAU. The mutations described do not affect the FAD- or NADPH-binding domains.

Treacy *et al.*, 1998, stated that for individuals studied, compound heterozygous or homozygous individuals for the human FMO3 mutation P153L and E305X manifest the most severe phenotype. For P153L, C→T nucleotide transversion in codon 153 results in the conversion of proline to leucine. It is possible that this substitution in codon 153 causes protein tertiary structural changes that result in abnormal protein folding and an inactive human FMO3 *N*-oxygenase. For E305X, the substitution of a nonsense mutation (TAA) for the glycine (GAA) at position 305 is apparent. A genotype-phenotype correlation is emerging. Individuals homozygous or compound heterozygotes for the allele E305X appear to manifest a severe phenotype and disorders of biogenic amine metabolism. In normal humans, TMA is metabolized to the polar and nonodorous metabolite TMA N-oxide that is efficiently excreted in the urine. For normal individuals, the urinary TMA N-oxide to TMA ratio is 97:3. Homozygotes for E305X and P153L can have TMA N-oxide to TMA ratio as low as 10:90.

Akerman *et al.,*1999, stated that, in addition to P153 L and E305X mutations, two missense mutations are causatives of TMAU. The presence of a G→T nucleotide transversion in one of the FMO3 alleles at cDNA position 198 (c. 198 G→T), which

results in the conversion of codon 66 from methionine (ATG) to isoleucine (ATT; trivial name M66I). A C→T base change at position 1474 of the cDNA (c. 1474 C→T) has the effect of converting the amino acid 492 from arginine (CGG) to tryptophan (TGG; trivial name R492W). It is possible that the methionine of codon 66 may constitute an important secondary starting point for translation mechanisms, which would be perturbed by the isoleucine substitution. Equally possible, this substitution may result in abnormal protein folding, resulting in an inactive protein. The R492W mutation involves a hypermutable CpG site and is a nonconservative change in a highly conserved region of the FMO3 gene. This arginine at codon 492 is conserved in all FMO isoforms. Zhang *et al*., 2003, have shown that a mutation at codon 32 of FMO3 gene changed Glu for Lys. This mutation, E32K, abrogated the catalytic activity of the enzyme. There are also *FMO3* polymorphisms that cause TMAU. However, *FMO3* polymorphisms exhibit milder effects on N-oxidation activity than *FMO3* mutations.

1.4.4 Types of Trimethylaminuria

There are two major subtypes of fish malodor syndrome: first, those forms that are related to a dysfunction of the normal enzyme activity due to genetic, hormonal, or inhibitory-chemical influences; second, those forms arising from substrate overload of the enzyme activity (normal or depressed) such as an excess of dietary precursors of trimethylamine or variations in the gut microflora resulting in enhanced liberation of trimethylamine. Clearly, these are two intimately interrelated aspects. A substrate burden that is easily handled by one individual may become a substrate overload in another that has a decreased enzyme function for whatever reasons (Mitchell, 1999).

Primary Genetic Form The primary genetic form of the fish malodor syndrome is probably the best understood of the various forms of the disorder and accounts for a large proportion of the known cases. Human FMO3 is highly polymorphic and some

of the mutations, either alone, or in combination are associated with dysfunctional enzyme activity and the metabolic disorder (Dolphin *et al*., 1997; Treacy *et al*., 1998; Akerman *et al*., 1999). Primary trimethylaminuria is an inherited autosomic recessive disease due to mutations in the human FMO3 gene (Mazon *et al*., 2003).

Acquired Form There are at least three cases known of individuals with clinicaly and biochemically diagnosed fish malodor syndrome where the condition appeared to emerge in adult life. There was no previous history in childhood and there was no familial background to the disorder (Mitchell, 1999).

Transient Childhood Forms. A preterm infant (29-weeks old) who developed a fish odor while being fed a choline-containing food supplement has been described (Blumenthal *et al*., 1980). When the choline supplement was withdrawn the odor disappeared, and it failed to reappear at 8 months of age when the supplement was reinstituted. The authors attributed the fish odor associated with the choline-containing supplement to the immaturity of the *N*-oxidase enzyme (Mayatapek and Kohlmuller, 1998).

Transient Form Associated with Menstruation. Several of female patients told anecdotally of how their fishy odor seemed to intensify with the onset of menstruation (Ayesh *et al*., 1993). A systematic study has confirmed that in normal healthy women of menstruating age there occurs a short episode of trimethylaminuria just at the onset of and during menstruation that then disappears. This indicates possible hormone modulation of flavin monooxygenase activity (Mitchell and Smith, 2001).

Precursor Overload. A few cases of a transient form of the fish malodor syndrome have been attributed to precursor overload thereby saturating the existing levels of flavin monooxygenase. Trimethylamine is mostly derived from dietary precursors such as choline, carnitine, and trimethylamine *N*-oxide through enterobacterial

metabolism. Exposure to unusually high levels of such precursors may hasten a fish malodor syndrome (Mitchell and Smith, 2001).

1.4.5 Clinical Aspects of the Fish Malodor Syndrome

The psychosocial reactions appear to arise from the excretion of excessive amounts of malodorous trimethylamine in the sweat, breath, urine, and other bodily secretions thereby conferring a powerful and offensive body odor. The latter, as well may be imagined, can be highly destructive to personal, working, and career lives of the affected individuals. Some individuals become socially withdrawn and isolated and may go on to develop mental depression (Todd, 1979; Shelley and Shelley, 1984; Ayesh *et al.,* 1993). The condition can be particularly acute and severe for young children and adolescents who may be subject to ridicule, lose confidence, and even schooling. The severity of the syndrome is highly variable; in some individuals, it is severe, in others less so and sometimes it seems to be more episodic in nature. Another curious feature is that although some sufferers are cognizant of the smell of their condition others remain unaware (Ayesh *et al*., 1993).

1.4.6 Diagnosis

Mitchell and Smith, 2001, stated that until recent years, diagnosis was made on the basis of clinical symptoms and biochemical assays of urine samples for either trimethylamine alone or in combination with its *N*-oxide metabolite. An oral challenge test involving the administration of 600 mg of trimethylamine (as its hydrochloride salt) has also been found useful for investigating family pedigrees and identifying carriers of the metabolic disorder. Basically, one has to recognize that trimethylaminuria can arise from the interaction of two entities, namely a dysfunctional *N*-oxidation capacity (owing to genetic or nongenetic reasons) and the burden of trimethylamine available to be oxidized.

1.4.7 Treatment

There has been no systematic evaluation of the various treatments for the fish malodor syndrome. Many of the reports are anecdotal in nature or involve just small groups of patients. Attempts to reduce the intake of precursors of trimethylamine such as carnitine and choline, through dietary management, appear to have been successful in some patients but not in all. Choline restricted individuals should avoid whole eggs and egg yolk, organ meats, liver, most fish, most legumes and whole soybeans. However, choline is essential in humans and over-restriction can cause hepatic dysfunction. Avoiding indole-containing foods (brussels sprouts, broccoli, cabbage and cauliflower) could also help because these materials lead to the inhibition of human FMO3 (Cashman, 2002). It appears likely that dietary management might be most effective in mild to moderate forms of fish odor syndrome arising from particular mutations or haplotypes (Danks *et al*., 1976; Mitchell, 1996). Occasionally, a short course of neomycin and metronidazole to reduce the activity of the gut microflora and suppress the generation of trimethylamine have been said to be effective in some, but once again, not all cases (Treacy *et al*., 1995).

Apart from the obvious one of gene therapy with replacement of the human gene for FMO3 alternative approaches might embrace the following: use of gut absorbents, such as charcoal or ion-exchange resins; modify the gut flora to reduce the bacterial species responsible for the conversion of precursors to trimethylamine; incorporate micro-organisms “engineered” with human FMO3 into the gut flora, to oxidize any trimethylamine released to its non-odorous *N*-oxide; provide riboflavin supplements, a precursor of the FAD cofactor for flavin monooxygenase function, in an attempt to maximize any residual activity; and finally, from the cosmetic point of view, the development of “malodor suppressants” in hygiene products to disguise the offensive smell of trimethylamine.

1.5 FMO Gene Family

The human FMO gene family exists as a cluster. Human FMO1-FMO4 and FMO6 are encoded by 23-37 kb genes localized on chromosome 1q23- q25 (Dolphin *et al*., 1996). The human FMO5 gene is localized on chromosome 1q21.1 and is encoded by a 39 kb gene (Gelb *et al*., 1997). Human FMOs 1-4 have nine or ten exons, eight of which encode proteins.

The complete amino acid sequence of human FMO3 is given in Figure 1.7. Human FMOs are 532 to 558 amino acids in length, with specific amino acids highly conserved in all species, particularly residues 4 to 32 and 186 to 213, which contain the FAD- and NADPH- binding domains, respectively (Cashman, 1995). FMOs 1,2,3 and 5 have calculated molecular masses between 60,000 and 61,000. FMO 4 has molecular mass of more than 63,000. The estimated pI values of FMOs 2,3,4 and 5 are all in the basic range, between 8.3 and 9.1, but that of FMO1 (6.9) is neutral. All these data are listed in Table 1.4.

1	MTLGACGNFQ DHAEEGRASI YKSVFSNSSK EMMCFPDFPF PDDFPNFMHN SKIQEYIIAF
61	AKEKNLLKYI QFKTFVSSVN KHPDFATTGQ WDVTTERDGK KESAVFDAVM VCSGHHVYPN
121	LPKESFPGLN HFKGKCFHSR DYKEPGVFNG KRVLVVGLGN SGCDIATELS RTAEQVMISS
181	RSGSWVMSRV WDNGYPWDML LVTRFGTFLK NNLPTAISDW LYVKQMNARF KHENYGLMPL
241	NGVLRKEPVF NDELPASILC GIVSVKPNVK EFTETSAIFE DGTIFEGIDC VIFATGYSFA
301	YPFLDESIIK SRNNEIILFK GVFPPLLEKS TIAVIGFVQS LGAAIPTVDL QSRWAAQVIK
361	GTCTLPSMED MMNDINEKME KKRKWFGKSE TIQTDYIVYM DELSSFIGAK PNIPWLFLTD
421	PKLAMEVYFG PCSPYQFRLV GPGQWPGARN AILTQWDRSL KPMQTRVVGR LQKPCFFFH
481	LKLFAIPILL IAVFLVLT

Figure 1.7 The complete amino acid sequence of human FMO3 (ENTREZ accession XP_010618, submitted by the NCBI Annotation Project).

Table 1.4 Properties of members of human FMO family (Taken from Phillips et *al*., 1995).

	Human **FMO1**	Human **FMO2**	Human **FMO3**	Human **FMO4**	Human **FMO5**
Amino acid length	532	535	532	558	533
Molecular mass	60,306	60,903	60,047	63,338	60,225
pI	6.9	8.9	8.3	9.1	8.6

1.6 FMO Isoforms

While the significance of human FMOs was reported in the nearly 1970s (Ziegler and Gold, 1971), it was not until the late 1980s and 1990s that FMOs were characterized by purification and cDNA cloning. Now it has been known that at least five isoforms of human FMO exist, each encoded by its own gene. A sixth isoform (FMO6) has been putatively identified between FMO2 and FMO3 on human chromosome 1, from genomic DNA (ENTREZ accession AL021026, submitted by the Sanger Center); the gene is constitutively expressed but it is not known if functional protein is produced (Shephard *et al*., 1999).

"FMO" was used to designate the gene family and this root symbol is followed by an Arabic numeral to distinguish each member of the family (designated FMO1-6). With the exception of FMO6, that shares 71% amino acid sequence identity with FMO3, FMOs 1-5 exhibit between 50-58% amino acid identity across species lines (Cashman *et al*, 1999), listed in Table 1.5. Each of the six distinct human FMOs can be present in numerous tissues (Ziegler, 1993; Cashman, 2002). As human FMOs were categorized, the common names assigned to enzymes were

Table 1.5 Percent identities of the primary structures of members of human FMO family (Taken from Phillips et *al*., 1995).

	FMO1	**FMO2**	**FMO3**	**FMO4**	**FMO5**
FMO1	-	57	53	52	51
FMO2	57	-	56	54	56
FMO3	53	56	-	52	53
FMO4	52	54	52	-	52
FMO5	51	56	53	52	-

formalized and a nomenclature was adopted. The nomenclature was developed on the basis of sequence identity. If an FMO gene has a sequence identity with >82 % it is grouped within an orthologous form while nonorthologous forms exhibit between 50-80 % sequence identity. In keeping with International Union of Pure and Applied Chemistry (IUPAC) convention, the italicized root symbol is used to designate the gene and the nonitalicized root form is used to designate the mRNA, cDNA or protein (Cashman, 2002).

1.7 FMO Polymorphism

Unexpressed sequences, which are subject to little selective pressure, evolve so much faster than expressed sequences that they even accumulate significant numbers of sequence polymorphisms within a single species. Consequently, the evolutionary relationships among populations within a species can be established by determining how a series of polymorphic DNA sequences are distributed among them.

1.7.1 FMO3 Polymorphism

Evidence for six forms of the FMO gene exists but it is FMO3 that is the prominent form in adult human liver that is likely to be associated with the bulk of FMO-mediated metabolism. An understanding of the substrate specificity of human FMO3 is beginning to emerge and several examples of drugs and chemicals extensively metabolized by FMO3 have been reported. Expression of FMO3 is species- and tissue-specific, but unlike human CYP450, mammalian FMO3 does not appear to be inducible. Interindividual variation in FMO3-dependent metabolism of drugs, chemicals and endogenous materials is therefore more likely to be due to genetic and not environmental effects. A summary of genetic variation of human FMO genes is listed in Table 1.6 It is possible that allelic variation of human FMO3 causes abnormal metabolism of chemicals and has clinical implications for human drug metabolism. Human FMO3 allelic variation may eventually be shown to contribute to interindividual and interethnic variability in FMO3-mediated metabolism. Human FMO3 may be another example of an environmental gene that participates in a protective mechanism to help shield humans from potentially toxic exposure to chemicals. Heterogeneity in the relative frequencies of single and multiple site alleles, haplotypes and genotypes of the human FMO3 amongst various ethnic groups suggests population differences (Cashman, 2002).

Table 1.6 A summary of the genetic variation of human FMO genes.

FMO gene	Variation in genomic DNA	Variation in mRNA	Variation in Coding region
FMO 1	34	4	2
FMO 2	57	11	9
FMO 3	40	20	19
FMO 4	30	2	1
FMO 5	40	3	2

Genetic studies have shown that polymorphisms at the FMO3 gene locus play a role in the variation of the metabolism of certain amines including drugs, dietary agents and other xenobiotics. Further studies may eventually lead to insights into the role of FMO3 variation in therapeutic efficacy and possible drug interactions. On the basis what is known today, it is appearent that FMO3 has the most genetic variation that is relevant to function (Cashman, 2002).

Considerable interindividual variability in human FMO3 activity has been observed *in vitro*. Early studies with a highly characterized set of adult human liver microsomes showed about 6.6-fold variability in nicotine N-1'-oxygenation activity (Berkman *et al.*, 1995) and 2.4- to 2.7-fold variation in S-oxygenation of sulfides (Stevens *et al*., 1993). While some of the variability observed was undoubtedly due to thermal degradation of FMO from mishandling the tissue, or improper storage, some of the variability was due to genetic polymorphism.

Several variants in the coding region of human FMO3 have been observed and five of them (E158K, V257M, E308G, R492W and G180V) are associated with normal or slightly reduced TMA N-oxygenation activity. The first three variants are common polymorphisms and the next two are somewhat rarer. The possibility that two or more common polymorphisms when present in the FMO3 gene of the same individual markedly decreases human FMO3 functional activity is notable. It is possible that individuals with decreased human FMO3 activity have the potential to have altered toxicity of drugs or chemicals and induce adverse drug reactions (Cashman, 2002).

Three common variants are E158K (c. 488 G→A), V257M (c. 769 G→A) and E308G (c. 923 A→G). *In vitro* analysis of mutant FMO3 proteins expressed from the cDNAs of the two polymorphisms E158K and V257M demonstrated differences in substrate affinity for the variant alleles for the substrates 5-DPT, tyramine and TMA (Treacy *et al*., 1998). The polymorhism E308G also confers

decreased TMA oxidation in the compound heterozygous or homozygous state (Akerman *et al*., 1999). This substitution is a nonconservative change in a conserved amino acid, suggesting a functional significance. It was also identified that, using ranitidine as the test substrate, the genotype E308G *in cis* with E158K results in a decresaed ranitidine oxidation (Kang *et al*., 2000). The codon 257 polymorphism appears to show substantial differences in N-oxygenating kinetics for the biogenic amine substrate tyramine and TMA. This may have clinical consequences. For example, consumption of large quantities of cruciferous vegetables that contain substantial quantities of indolemethyl glucosinolates has been shown to inhibit human FMO3 *in vivo* and cause elevated levels of urinary TMA (Cashman et al., 1999). This effect may be more apparent in individuals that are homozygous or heterozygous for human FMO3 polymorhisms.

The maintanance of the prevalent codon 158 polymorphism in the white populations examined may not result solely from "genetic drift" but perhaps as a consequence of "molecular drive", whereby particular polymorphisms with selective advantages persist, for example, to combat exposure to plant toxins in particular geographic regions (Cashman *et al*., 1999). The distribution of the two codon 158 alleles is almost in equilibrium in these populations, suggesting that this may be an older polymorphism that is now balanced. Human V257M FMO3 is less prevalent and may represent a founder effect (Treacy *et al*., 1996).

Lambert *et al*., 2001, stated that the wild type haplotypes for those three polymorphisms occur most frequently. The E158K allele is the second most frequent polymorphism. E158K is usually linked to the wild type codon 158 and 308 alleles. These variant genotypes may predispose to mild TMAU under conditions of choline or TMA loading or with the use of FMO3 inhibitors and hormonal influences (Mitchell, 1999).

It was reported that the enzyme encoded by the human Glu-158 FMO3 polymorphic allele is more active for the tertiary amine substrates TMA and 5-DPT than the enzyme encoded by the less prevalent human Lys-158 FMO3 allele (Cashman, 1999; Treacy *et al*., 1998). The E to K amino acid substitution at position 158 significantly altered FMO3 activity *in vitro*. Subjects homozygous for K158 showed a significant decrease in V_{max} for benzydamine N-oxidation compared with the subjects carrying at least one wild type allele (EE158+EK158) according to the study of Stormer *et al*, 2000.

1.7.2 FMO2 Polymorphism

From its initial discovery as unique form of FMO in rabbit lung (Williams *et al*., 1984; Tynes *et al*., 1985), FMO2 has been identified as a major pulmonary isoform in a number of species including guinea pig, mouse and monkey (Lawton *et al*., 1990; Nikbakth *et al*., 1992; Cashman, 2000). Characterization of pulmonary FMO is of interest because of its potential role in the metabolism of environmental chemicals for which lung is a target organ or portal of entry.

FMO2 expression has been observed in pulmonary tissue from several species, but not human till 2000 (Whetstine *et al*., 2000). Two human *FMO2* point mutations have been reported: a cytosine to thymidine transition at position 1414 resulting in a premature stop codon and a thymidine insertion at position 1589 resulting in a frameshift. Whetstine *et al*., 2000, genotyped unrelated African-American, Caucasian and Korean individuals to define the frequency of these sequence variations and explore their significance. 26% African-Americans would exhibit pulmonary FMO2 enzyme activity; however, all of the tested Caucasians and Koreans were homozygous for the 1414T allele (Table 1.7).

Table 1.7 Frequency of the 1414>T Transition in Different Ethnic Groups (Taken from Whetstine *et al*., 2000).

Genotype frequency (%)					Allelic frequency (%)	
	Sample size	**CAG/ CAG**	**CAG/ TAG**	**TAG/ TAG**	**CAG**	**TAG**
Afr.-Ame.	180	2	44	134	13.3	86.7
Caucasians	52	0	0	52	0.0	100
Korean	100	0	0	100	0.0	100

The T1589 allele occured at frequencies of 6.9 and 13.0% in African-Americans and Caucasians, respectively, and appears to segregate with the 1414T allele (Table 1.8). Thus, it would have no further impact on FMO2 activity. Western blot analysis of pulmonary microsomes failed to detect immunoreactive protein in 1414T homozygotes. A heterozygotic individual did exhibit a single band of the expected size, but no detectable FMO activity in the corresponding lung microsomes. Sequence analysis, however, was consistent with the 1414C allele encoding an active FMO2 enzyme. FMO2 mRNA expression was observed in most individuals, but failed to correlate with genotype or protein expression. In summary, functional FMO2 is expressed in only a small percentage of the overall population. However, in certain ethnic groups, active pulmonary FMO2 enzyme will be present in a significant number of individuals.

In human, the major *FMO2* alleles encode a truncated, catalytically inactive protein, due to a cytosine to thymidine transition (1414C>T) resulting in a premature stop signal at codon 472 (Dolphin *et al*., 1998). Subsequently, another *FMO2* mutation, a single thymidine insertion (1588-1589insT), was identified which results in a frameshift that would alter the last six amino acids and cause an extra 23 amino

Table 1.8 Frequency of the T1589 Insertion in Different Ethnic Groups (Taken from Whetstine *et al*., 2000).

	Genotype frequency (%)				Allelic frequency (%)	
	Sample size	**TGC/ TGC**	**TGC/ TTG**	**TTG/ TTG**	**TGC**	**TTG**
Afr.-Ame.	175	155	16	4	93.1	6.9
Caucasians	23	19	2	2	86.9	13.1

acids to be added to the C-terminus of the protein. Given the different frequencies of the two mutant alleles, it was clear that these two mutations are not directly linked. Yet, all samples containing at least one T1589 insertion also contained at least one 1414T allele. In all instances, both mutations occured on the same allele, consistent with the frameshift occuring secondarily to the transition (Whetstine *et al*., 2000).The 1414C *FMO2* allele is designated *FMO2*1*, whereas the alleles containing the premature stop signal at codon 472 (1414T) is designated *FMO2*2A* and that containing both the stop codon and insertion mutation (1414T and T1589) *FMO2*2B*. Comparison of the FMO content with that from FAD determination suggests that truncated protein was not effectively binding FAD. Supplementation of FAD did not alter the situation. N-oxidation of DMA and S-oxidation of methimazole are reduced to undetectable levels by the truncation. FMO2.2A was membrane associated and lacked the ability to catalyze S-oxidation with methimazole as substrate. If residual capacity for S-oxidation is retained by truncated protein, it has been reduced to less than 0.1% of the activity observed with the full-length protein (Krueger *et al*., 2001).

FMO2 polymorphisms may enhance resistance or sensitivity to metabolic diseases, as well as alter drug efficacy or toxicity and the toxicity of numerous xenobiotics.

1.8 Factors Modulating FMO Expression and Activity

Adding to the complexity of species- and tissue-specific FMO expression, interindividual variation has been observed. Such interindividual variation in other drug metabolizing enzymes is known to be influenced by both environmental and genetic factors. However, the latter would appear to dominate in the case of the FMO. Unlike the cytochrome P450 and many of the conjugating enzymes, environmental factors do not have a profound effect on FMO expression (Cashman, 1995).

FMO expression may be influenced by hormonal and developmental factors, such as pregnancy, sex and age. However, in contrast to CYP450 monooxygenases FMO expression can not be induced by drugs or xenobiotics.

Gender has a role on FMO isoform expression in organisms. Hodgson *et al.*, 1999, stated that in mice liver FMO1 level was 2-3 times higher in the females than in that of males, while the FMO3 level is the same as FMO1 level in females but absent in males, probably due to testosteron repression. FMO5 level is the same in the liver of both gender. Therefore, in mice liver FMO3 is the gender-specific isoform, FMO1 is the gender-dependent isoform and FMO5 is the gender-independent isoform. Besides, when FMO3 levels were assessed in the liver and kidney of mice, rats, rabbits and dogs, only mouse and dog liver exhibited sex differences. This suggests that the testosteron repression of mouse liver FMO3 noted by Falls et *al*., 1997, is species- and tissue-specific.

In rat liver, FMO1 is expressed in males at higher levels than females. FMO3 and FMO5 expression has no difference between genders (Hodgson *et al*., 1999). In contrast to mouse liver FMO levels, rat liver FMO levels are positively regulated by testosterone and repressed by estradiol (Cashman, 1995). Species differences in rat

kidney microsomal FMO3 were evident in both sexes. The rat had 2- to 6-fold higher levels than dogs, mice and rabbits (Ripp *et al*., 1998).

Human liver microsomes exhibit some interindividual variability in FMO3 levels, but on average there are no differences between males and females (Ripp *et al*., 1998). In adult humans, FMO1 is expressed in kidney but not in liver, however in fetus its mRNA is abundant in both organs. FMO3 is expressed in the liver of adults, but absent or present in low amounts in foetal tissues (Phillips *et al*., 1995).

FMO activity is also modulated by some agents. Adalı *et al*., 1998 and Halpert *et al.,* 1998, stated that human FMO3 is subject to modulation by tricyclic antidepressants and other agents. Imipramine activates FMO3-catalyzed metabolism of methimazole at all substrate concentrations. This distinguishes FMO3 from rabbit FMO1 and FMO2, which are activated at high substrate concentration and inhibited at low substrate concentration, and pig FMO1, which is inhibited at all substrate concentrations. The response of FMO3 is also unique in that chlorpromazine is markedly more effective as a modulator than is imipramine. *N*-Octylamine, $MgCl_2$ and $HgCl_2$ all inhibit FMO3. Substitution of lysine for threonine at position 428 significantly alters the response of FMO3 to modulator without changing the kinetic parameters for the metabolism of the substrate. Activation by imipramine and chlorpromazine is reduced or abolished and inhibition, most obvious at low substrate concentrations, is observed. Inhibition might be caused by competition for the reactive oxygen rather than for some binding site (Wyatt *et al*., 1998). The activity of FMO3 can be modulated by large drug molecules as well as short-chain amines and metal ions. These data demonstrate that tricyclic antidepressants can activate, inhibit or have no effect on the FMO as a function of the FMO isoform, the specific tricyclic and its concentration and the concentration of the substrate.

1.9 Scope of This Study

All organisms are exposed constantly and unavoidably to man-made foreign chemicals such as drugs, industrial chemicals, pesticides and pollutants. Involvement of FMO enzymes in the metabolism of these chemicals has been reported in different tissues of several mammalian species to date. All these studies mainly focused on small laboratory animals such as rats, rabbits and mice. However, no data are available concerning the biochemical characterization of FMO enzymes in veterinary animals, such as bovine. Bovine is a common meat source in the world, especially in Turkey, and is therefore important for human diet and health.

This study was aimed to characterize the biochemical properties of bovine liver microsomal FMO and to investigate its role in the metabolism of drugs. Our laboratory has been involved in the purification and characterization of the components of microsomal CYP450-dependent monooxygenase system of sheep and bovine (Arınç and İşcan, 1983; Arınç, 1985; İşcan and Arınç, 1986, 1988; Adalı and Arınç, 1990; Adalı *et al*., 1996; Arınç and Çelik, 2002).

The aim of this research is to characterize bovine liver microsomal FMO activity in terms of time course, optimum temperature and pH, thermostability, effect of protein amount, effect of substrate concentration (determination of kinetic parameters, K_m and V_{max}), effects of various concentrations of different detergents, effects of modulators; imipramine and chlorpromazine as drug substrates and Ni^{+2}, Cd^{+2}, Hg^{+2} as heavy metal ions.

This study is the first concerning the extensive biochemical characterization of FMO from bovine liver microsomes and studying its modulation by drugs and other effectors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

ε-Amino caproic acid (ε-ACA), bovine serum albumin (BSA), chlorpromazine, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), imipramine, methimazole (N-methyl-2-mercaptoimidazole), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), phenylmethylsulfonyl fluoride (PMSF), tricine and tris[hydroxymethyl]aminomethane were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Acetic acid glacial, di-potassium hydrogen phosphate, ethylene diamine tetraacetic acid disodium salt (EDTA), glycerol, hydrochloric acid, magnesium chloride, mercury chloride, cadmium chloride, potassium dihydrogen phosphate, nickel chloride, sodium chloride and Triton X-100 were obtained from E. Merck, Darmstadt, Germany.

Emulgen 913 (polyoxyethyene nonyl phenyl ether) was a gift from Kao-Atlas Co., Ltd., Tokyo, Japan.

All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2 Methods

2.2.1 Preparation of Bovine Liver Microsomes

Livers from well-bled, healthy bovines were obtained from a local slaughterhouse (Mısırdalı Slaughterhouse, Sincan, Ankara) immediately after killing. The livers were placed in plastic bags packed in crushed ice. All subsequent steps were carried out at 0-4 °C or cold room. In the laboratory, the fatty and connective tissues were removed from livers. The livers were first rinsed with pre-cooled distilled water and then several times with pre-cooled 1.15 % KCl solution packed in crushed ice, to remove as much blood as possible. The tissues were drained on a filter paper, and then weighed to the nearest 0.1 gram, and cut into small pieces with scissors. The resulting tissue pieces were homogenized in 1.15 % KCl solution containing 2 mM EDTA (pH 7.2), 0.25 mM ε-ACA and 0.1 mM PMSF using a Potter-Elvehjem glass homogenizer packed in crushed ice, coupled motor (Black & Decker, V850, multispeed drill)-driven Teflon pestle at 2400 rpm. Five passes were made for three times with intervals of one minute for the homogenization of liver tissue. The volume of homogenization solution used was equal to three times the weight of liver tissue.

The liver homogenate was centrifuged at 9,500 rpm (10,800 × g) by using 12156 H type rotor in Sigma 3K30 Refrigerated Centrifuge (Saint Louis, Missouri, USA) for 25 minutes to remove cell debris, nuclei, and mitochondria in the pellet. After centrifugation, the supernatant fraction containing endoplasmic reticulum and other soluble fraction of the cell was filtered through double layers of cheesecloth in a Buchner funnel.

The microsomes were sedimented from the supernatant solution by centrifugation at 39,000 rpm (110,000 × g) for 60 minutes using type A-841 rotor in Sorval Combi Plus Ultracentrifuge (Du Pont Company, Wilmington, Delaware,

19898, USA). Then the supernatant fraction was discarded and the microsomal pellet was suspended in 1.15 % KCl solution containing 2 mM EDTA. The microsomes were re-sedimented by ultracentrifugation at 39,000 rpm (110,000 × g) for 50 minutes by using type A-841 rotor in Sorval Combi Plus Ultracentrifuge. The supernatant fraction was discarded again.

The washed microsomal pellet was resuspended in 25 % glycerol containing 1 mM EDTA. 0.5 ml of suspension solution was used for each gram of liver. Resuspended microsomes were homogenized manually using the Teflon-glass homogenizer to obtain a homogenous microsomal suspension. Then, these microsomal suspensions were gassed with nitrogen in plastic bottles and stored in liquid nitrogen.

2.2.2 Protein Determination

The protein concentrations in the microsomes were determined using the method described by Lowry *et al.*, 1951. Crystalline bovine serum albumin was used as a standard.

Bovine liver microsomes were diluted 200 times and aliquots of 0.1 ml, 0.25 ml, 0.5 ml were taken into test tubes. The final volume of test tubes was completed to 0.5 ml with distilled water. Standard tubes were also prepared using 0.5 ml BSA standards (as concentrations 0.02, 0.05, 0.10, 0.15 and 0.20 mg BSA/ml). Then, 2.5 ml of alkaline cupper reagent, consisting of 2 % cupper sulfate, 2 % sodium potassium tartarate and 2 % sodium carbonate in 0.1 N NaOH in a ratio of 1:1:100 in the given order, was added to each tube. All tubes were mixed with vortex and let to stand for 10 minutes at room temperature. Then, 2 N Folin-Phenol reagent was diluted 1:1 with distilled water and 0.25 ml of this reagent was added to each tube which was mixed within 8 seconds by vortex. The tubes were incubated for 30 minutes at room temperature. Finally, the intensity of color developed was measured

at 660 nm against reagent blank, using Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan).

A standard curve of absorbance versus BSA concentration (20 to 200 μg) was constructed in order to calculate the unknown protein concentration of the bovine liver microsomes.

2.2.3 Determination of Flavin-Containing Monooxygenase (FMO) Activity

FMO activity was determined using 3 different substrates; methimazole, chlorpromazine and imipramine. Methimazole/ DTNB assay was used for the characterization of bovine liver microsomal FMO activity.

2.2.3.1 Determination of FMO Activity Towards Methimazole

Methimazole is known to be a model artificial substrate for determination of FMO activity from different sources and it is S-oxygenated by FMO as shown in Figure 2.1. In this study methimazole/ DTNB assay described below was used to determine and characterize FMO activity of bovine liver microsomes.

Bovine liver microsomal FMO activity towards methimazole was determined using the method described by Dixit and Roche, 1984, with minor modifications. In this spectrophotometric assay, FMO activity can be accurately measured in whole cell homogenates without interference due to NADPH oxidase activities.

Methimazole has been shown to be a highly specific substrate for FMO and it is oxidized in the presence of oxygen and NADPH as shown in Figure 2.1.

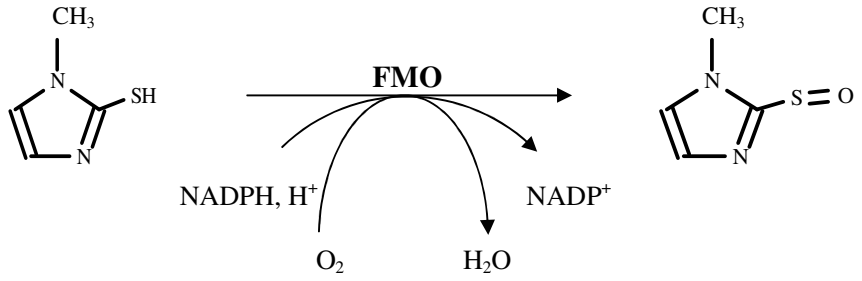
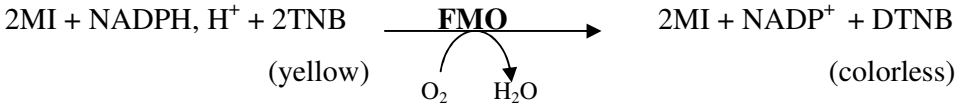


Figure 2.1 Methimazole S-oxygenation reaction by FMO.

This spectral assay was based on the observation that methimazole cannot reduce 5,5`-dithiobis (DTNB), but its conjugate disulfide is a strong oxidant that rapidly and completely oxidizes nitro-5-thiobenzoate (TNB) to DTNB. The measured reaction results in the oxidation of two molecules of TNB per catalytic turnover, as a result of the reactions shown in Figure 2.2

The reaction of the oxidized methimazole with TNB to generate DTNB can be summarized as follows:



TNB, which is formed in the assay medium from the reaction of DTNB with DTT, is yellow, having a maximum absorbance at 412 nm. Methimazole oxidation reaction results in the oxidation of TNB to colorless DTNB. Thus, FMO activity was measured by following the decrease in the amount of TNB, resulting from the oxidation of methimazole, at 412 nm using Shimadzu UV-160A UV-visible spectrophotometer (Shimadzu Corporation, Analytical Instruments Divison, Kyoto, Japan).

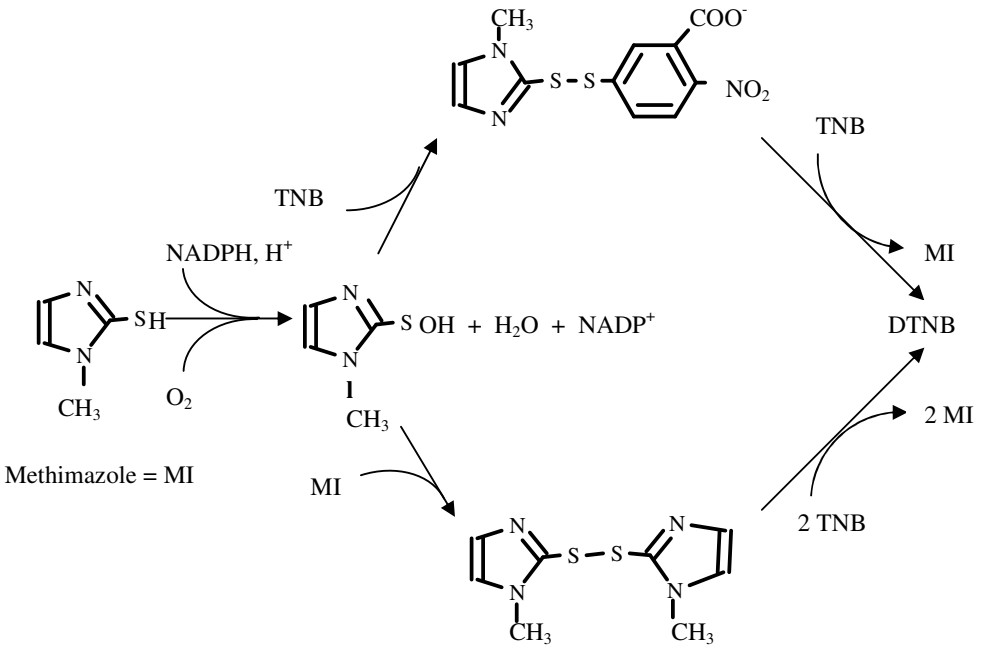


Figure 2.2 Oxidation of methimazole (S) by FMO, and the reaction of the oxidized product with TNB to generate DTNB.

Standard enzyme assay mixtures contained 100 µl of 0.5 M tris-HCl buffer, pH 8.0, 60 µl of 1 mM DTNB prepared in 0.1 M KPO_4, pH 8.0, 10 µl of 2 mM DTT, 10 µl of 10 mM NADPH, 250 µg bovine liver microsomal protein, 5 µl of 200 mM methimazole, and distilled water in a final volume of 1.0 ml. Final concentrations of reaction constituents are given in Table 2.1.

The reaction mixture, except substrate methimazole, was warmed to 37 °C in a spectrophotometric cuvette and the background rate was recorded. Reaction was

Table 2.1 Standard reaction medium for determining FMO activity using methimazole/ DTNB assay.

Stock Solutions	**Volume Added (µl)**	**Final Concentration in Reaction Medium**
0.5 M Tris-HCl, pH 8.0	100	0.05 M Tris-HCl pH 8.0
1 mM DTNB in 0.1 M KPO_4 pH 8.0	60	0.06 mM DTNB, 6 mM KPO_4
2 mM DTT	10	0.02 mM DTT
10 mM NADPH	10	0.1 mM NADPH
Microsomal Protein (25.8 mg/ml)	9.7	250 µg protein
10 % Triton X-100	10	0.1 % Triton X-100
200 mM Methimazole	5	1 mM Methimazole
Completed to 1 ml with distilled water		

initiated by the addition of methimazole to the cuvette and the decrease in absorbance at 412 nm was recorded for 3 minutes.

The rate of absorbance change (ΔA_{412}/min) was determined using the linear portion of the activity curve. The extinction coefficient (ε) of dissolved TNB was found to be 28.2 $mM^{-1} \times cm^{-1}$ at pH 8.4 (Dixit and Roche, 1984). Activity of enzyme was calculated as follows:

$$\text{Enzyme Activity (nmol/min/mg)} = \frac{\Delta A_{412}/\text{min}}{28.2} \times \frac{1000}{\text{mg protein}} \times \text{D.F.}$$

2.2.3.2 Determination of FMO Activity Towards Imipramine and Chlorpromazine

FMO-catalyzed imipramine and chlorpromazine oxidation was determined by following the substrate stimulated rates of NADPH oxidation, basically as described by Bhamre *et al.,* 1993.

Imipramine and chlorpromazine are psychoactive drugs that act on the central nervous system. The FMO-mediated metabolism of the antidepressant imipramine leads to the formation of its respective N-oxide, which is pharmacologically inactive. N-oxidation reaction of imipramine by FMO is shown in Figure 2.3.

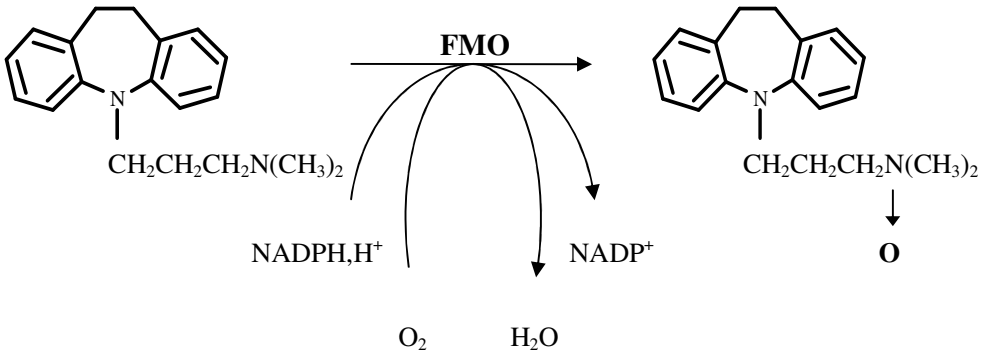


Figure 2.3 Imipramine N-oxidation reaction by FMO.

Chlorpromazine is metabolized to its respective S-oxide by FMO as shown in Figure 2.4. Typical reaction medium for determining bovine liver microsomal FMO activity towards imipramine or chlorpromazine consisted of 0.1 M Tricine buffer, pH 8.0 containing 1 mM EDTA, 0.1 mM NADPH, 0.1 % Triton X-100, 250 µg microsomal protein containing enzyme, and distilled water (Table 2.2).

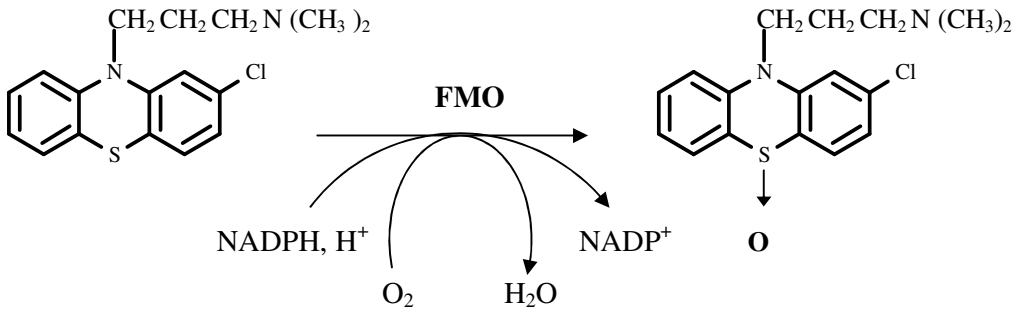


Figure 2.4 Chlorpromazine S-oxidation reaction by FMO.

Table 2.2 Typical reaction medium for determining FMO activity using imipramine or chlorpromazine as substrate.

Stock Solutions	**Volume Added (µl)**	**Concentration in Reaction Medium**
0.1 M Tricine pH 8.0 + 2 mM EDTA	500	0.1 M Tricine + 1 mM EDTA
10 mM NADPH	10	0.1 mM NADPH
Microsomal Protein (25.8 mg/ml)	9.7	250 µg protein
10 % Triton X-100	10	0.1 % Triton X-100
50 mM Imipramine or 100 mM Chlorpromazine	2	0.1 mM Imipramine or 0.2 mM Chlorpromazine
Completed to 1 ml with distilled water		

Reaction mixture was warmed to 37 °C in a spectrophotometric cuvette and the endogenous rate of NADPH oxidation was recorded. Then the reaction was initiated with the addition of substrate (either 0.1 mM imipramine or 0.2 mM chlorpromazine in reaction mixture) into the reaction medium and the decrease in absorbance at 340 nm was recorded for 3 minutes using Shimadzu UV-160A UV-visible spectrophotometer (Shimadzu Corporation, Analytical Instruments Divison, Kyoto, Japan).

Reaction rates were determined using extinction coefficient (ε) of 6.22 mM^{-1} x cm^{-1} for NADPH (Dixit and Roche, 1984). Activity of enzyme was calculated as follows:

$$\text{Enzyme Activity (nmol NADPH oxi./min/mg)} = \frac{\Delta A_{340}/\text{ min}}{6.22} \times \frac{1000}{\text{mg protein}} \times \text{D.F.}$$

2.2.4 Characterization and Effect of Some Modulators on Bovine Liver Microsomal FMO Activity

Characterization of bovine liver FMO activity was carried out using Methimazole/DTNB assay as described in section 2.2.3.1. Time course of bovine liver FMO, effect of protein amount, temperature, pH, and substrate concentration on FMO–catalyzed methimazole oxidation were studied. Also the effects of various concentrations of different detergents on enzyme activity, thermostability of the enzyme were determined. Besides, modulation of bovine liver microsomal FMO activity (using methimazole / DTNB assay) by drug substrates (imipramine and chlorpromazine) and other effectors (Hg^{+2}, Ni^{+2}, Cd^{+2} and Mg^{+2}) were studied in this concept. All experiments were carried out 2 or 3 times, each point in duplicates or triplicates and the error bars were drawn as standard error of means.

2.2.4.1 Effect of Protein Amount on Bovine Liver FMO Activity

The effect of protein amount on bovine liver FMO activity was determined by adding different amounts of microsomal protein to the reaction medium. The protein amounts used in reaction medium were changed from 136 µg to 680 µg. Five different concentrations of microsomal protein and duplicate of each protein concentration were used. Assays were conducted at pH 8.0 and the other conditions remained same as described in section 2.2.3.1.

2.2.4.2 Effect of Temperature on Bovine Liver FMO Activity

The effect of reaction temperature on bovine liver microsomal FMO activity was determined by changing the temperature of the reaction medium from 10 °C to 60 °C. The temperatures used were 10 °C, 20 °C, 25 °C, 30 °C, 37 °C, 45 °C, 50 °C, 55 °C, and 60 °C. Assays were conducted at pH 8.0 using 250 µg microsomal protein and the other conditions remained same as described in section 2.2.3.1.

2.2.4.3 Effect of Methimazole Substrate Concentration on Bovine Liver FMO Activity

Kinetic constants, K_m and V_{max} were determined by changing the methimazole concentrations from 0.1 mM to 2.0 mM. The other conditions were same as described under "Methods". The reaction rates obtained by increasing substrate (methimazole) concentration were used to draw Michaelis-Menten and Lineweaver-Burk plots for K_m and V_{max} determination.

2.2.4.4 Effect of pH on Bovine Liver FMO Activity

The effect of pH on bovine liver microsomal FMO activity was determined by following the enzyme activities at varying pH values in the range of 4 to 10. The assays were carried out using 0.5 M acetic acid-Na acetate buffer for pH 4.0, 5.0, and 6.0, 0.5 M Tris-HCl buffer for pH values 7.0, 8.0, 8.5, 9.0, and 0.5 M glycine-NaOH buffer for pH 10.0. The other conditions were the same as described under "Methods".

2.2.4.5 Effects of Various Concentrations of Different Detergents on Bovine Liver FMO Activity

The effects of 2 different detergents (Triton X-100 and Emulgen 913) at varying concentrations on bovine liver microsomal FMO activity were determined by methimazole oxidation method. Triton X-100 was added to the reaction medium at 0.1 %, 0.2 %, and 1.0 % final concentrations. Emulgen 913 was used at 0.1 % and 0.5 % final concentrations in reaction medium. Other conditions remained the same as explained under "Methods".

2.2.4.6 Thermostability of Bovine Liver FMO

The effect of temperature on bovine liver microsomal FMO enzyme stability was determined by incubating the bovine liver microsomes at 24 °C, 37 °C, 50 °C, and 65 °C, using a water bath. The duration of the incubation period varied from one temperature to the other. These microsomes were then used as enzyme source for methimazole oxidation assay, which was carried out at 37 °C as explained under "Methods". 500 µg microsomal protein per 1.0 ml reaction mixture was used in this experiment.

2.2.4.7 Effect of Magnesium Ion on Bovine Liver FMO Activity

The effects of magnesium on bovine liver microsomal FMO activity was studied by adding various concentrations of magnesium chloride ($MgCl_2$), 0.5 mM, 1 mM, 5 mM, 10 mM, 50 mM, 100 mM, 150 mM, 200 mM and 300 mM, into the reaction mixture. 500 µg microsomal protein per 1.0 ml reaction medium was used in this study. Reactions were initiated with addition of methimazole at a final concentration of 1.0 mM to the cuvette, and rates were recorded at 412 nm for 3 minutes.

2.2.4.8 Effects of Modulators on Methimazole Oxidation Activity of Bovine Liver Microsomal FMO

Drug substrates, imipramine and chlorpromazine, and heavy metal ions, Ni^{+2}, Cd^{+2} and Hg^{+2}, were used to study the modulation of bovine liver microsomal FMO activity using methimazole / DTNB assay.

2.2.4.8.1 Effects of Drug Substrates on Bovine Liver Microsomal FMO-catalyzed Methimazole Oxidation

The effects of imipramine and chlorpromazine on methimazole oxidation activity of bovine liver FMO were determined by adding varying concentrations of these substrates to the reaction medium. Imipramine was used at 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 0.75 mM, and 1.0 mM concentrations and chlorpromazine was added to the reaction medium at a final concentration of 1 µM, 10 µM, 50 µM, 100 µM, 200 µM, and 300 µM. Assays were conducted using 500 µg microsomal protein. Other conditions were same as described under “Methods”.

2.2.4.8.2 Effects of Heavy Metal Ions on Bovine Liver Microsomal FMO-catalyzed Methimazole Oxidation

In this study, the effects of heavy metal ions on methimazole oxidation activity of FMO were determined by using varying concentrations of 3 different metal ions. In the same experiment, substrate methimazole concentration was also changed from 0.1 mM to 1.0 mM during the methimazole / DTNB assay to study the modulation mechanism.

$NiCl_2$ was used at 5 μM, 10 μM, 100 μM and 200 μM concentrations. $CdCl_2$ was added to the reaction medium at final concentrations of 5 μM, 10 μM, 50 μM, 100 μM, 150 μM and 200 μM. $HgCl_2$ was used at 0.5 μM, 1.0 μM, 2.0 μM and 5.0 μM concentrations.

The reactions were carried out using a fixed methimazole concentration and changing metal ion concentration in the range given above. In this study, 3 or 4 different methimazole concentrations; 0.1 mM, 0.2 mM, 0.5 mM and 1.0 mM, were used. Reactions were initiated by the addition of methimazole and rates were recorded at 412 nm for 3 minutes.

CHAPTER 3

RESULTS

3.1 Bovine Liver Microsomal FMO Activity

FMO activity of bovine liver microsomes towards methimazole was quantitatively determined by continuously measuring the amount of TNB oxidized at 412 nm according to the method of Dixit and Roche, 1984. Liver FMO activity was calculated using a molar absorbance coefficient of 28,200 $M^{-1}cm^{-1}$. The activity of the FMO enzyme was determined using 12 different microsomal preparations of bovine liver. Protein concentrations (mg/ml), activities (nmol/min/ml) and specific activities (nmol/min/mg protein) of bovine liver microsomes were calculated, and listed in Table 3.1.

The specific activities of FMO from different microsomal preparations of bovine liver were found as 1.60, 1.38, 2.17, 2.18, 3.30, 2.71, 2.93, 4.95, 2.12, 1.21, 1.43 and 2.57. The average protein concentration, enzyme activity and specific activity were calculated as 25.8 ± 5.3 mg/ml, 61.4 ± 7.5 nmol/min/ml and 2.37 ± 0.30 nmol/min/mg protein (Mean ± SE, n=12), respectively.

3.2 Characterization of Bovine Liver Microsomal FMO Activity

Bovine liver microsomal FMO activity was characterized using methimazole/ DTNB assay. Characterization of the enzyme was carried out in terms of time course, optimum protein amount, optimum temperature, optimum pH, kinetic parameters, K_m and V_{max}. In addition to these, thermostability of the enzyme, effects of various

Table 3.1 FMO Activities From Different Bovine Liver Microsomes.

Bovine Liver Microsome	Protein Concentration (mg/ml)	Activity (nmol/min/ml)	Specific Activity (nmol/min/mg protein)
1	27.2	44.0	1.60
2	17.4	24.0	1.38
3	34.4	74.7	2.17
4	26.0	56.7	2.18
5	23.7	78.6	3.30
6	29.6	80.4	2.71
7	25.0	73.2	2.93
8	23.0	114.0	4.95
9	22.0	46.8	2.12
10	18.0	21.9	1.21
11	35.0	50.1	1.43
12	28.0	72.0	2.57
Mean ± SE	**25.8 ± 5.3**	**61.4 ± 7.5**	**2.37 ± 0.30**

concentrations of different detergents and metal ion, Mg^{+2}, on bovine liver microsomal FMO activity were determined. Besides, modulation of FMO activity by drug substrates, imipramine and chlorpromazine, and other effectors (Hg^{+2}, Ni^{+2}, Cd^{+2}) were studied. The activity of bovine liver microsomal enzymes towards drug substrates, imipramine and chlorpromazine, was also determined in this study. Most of the characterization studies were carried out in two or three experiments, each in duplicates and some in triplicates. The graphs were constructed from the means of these duplicate or triplicate determinations.

3.2.1 Time Course of Bovine Liver Microsomal FMO

The time course of a typical methimazole oxidation reaction catalyzed by bovine liver microsomal FMO is illustrated in Figure 3.1. The reaction was linear with time up to 17 minutes using the assay conditions given in Table 2.1. Bovine liver FMO catalyzed methimazole oxidation was followed for 3 minutes routinely throughout this study.

3.2.2 Effect of Microsomal Protein Amount on Bovine Liver FMO Activity

Increasing bovine liver microsomal protein amounts ranging from 136 μg to 680 μg were used in the reaction medium. Figure 3.2 shows the effect of microsomal protein amount on the rate of methimazole oxidation by bovine liver FMO. The reaction rate was linear with microsomal protein amount up to approximately 500 μg of protein per 1.0 ml of reaction mixture. Throughout this study depending on the type of experiment, standard microsomal protein concentrations of 250 μg/ml or 500 μg/ml of reaction mixture was used.

3.2.3 Effect of Reaction Temperature on Bovine Liver FMO Activity

The effect of incubation temperature on bovine liver FMO activity was studied by following the reaction rate at various temperatures in the range of 10 °C to 60 °C. The reaction mixture without microsomes was warmed to the desired temperature in a spectrophotometric cuvette and reaction was initiated by the addition of microsomes and then immediately substrate, methimazole. Reaction rate was recorded for 3 minutes at the test temperature as explained under “Methods”.

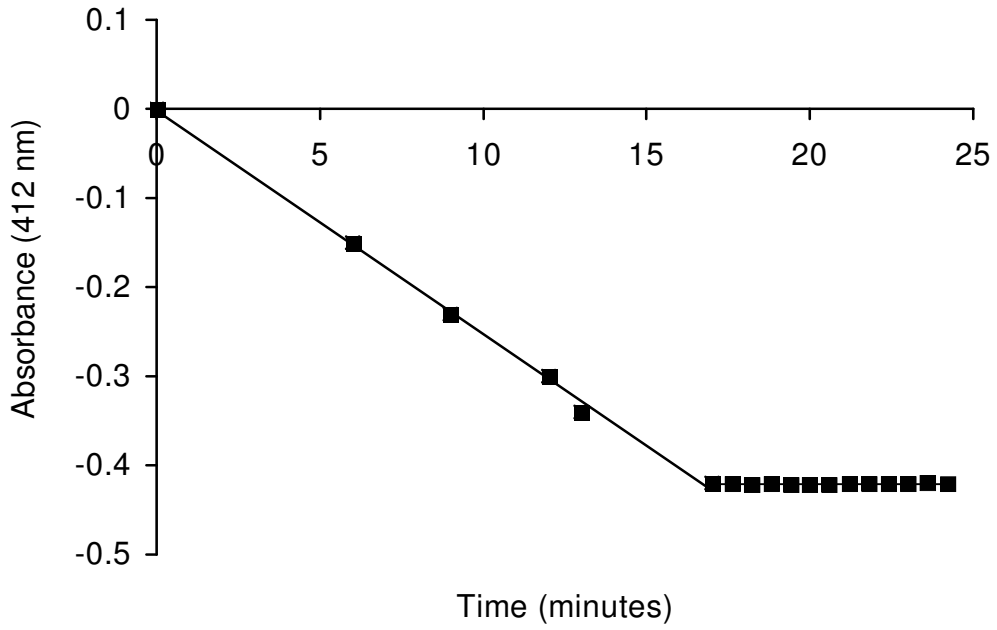


Figure 3.1 Time course of bovine liver FMO. The reaction mixture contained 0.05 M Tris-HCl buffer (pH 8.0), 0.06 mM DTNB, 0.02 mM DTT, 0.1 mM NADPH, 250 µg microsomal protein, 0.1 % Triton X-100 and 1.0 mM methimazole in a final volume of 1.0 ml. The assay mixture was warmed to 37 °C in a spectrophotometric cuvette and the endogenous rate was recorded. The reaction was initiated by addition of methimazole to the cuvette and the decrease in absorbance at 412 nm was recorded.

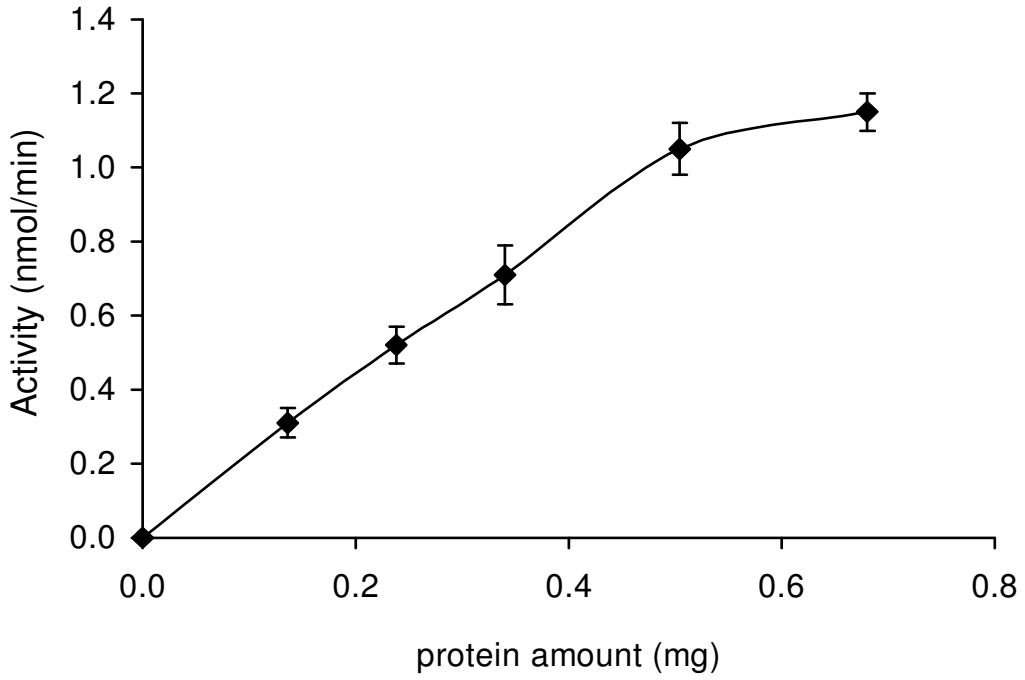


Figure 3.2 The effect of protein amount on bovine liver microsomal FMO activity. Reaction mixture contained 0.05 M Tris-HCl buffer, pH 8.0, 0.06 mM DTNB, 0.02 mM DTT, 0.1 mM NADPH, indicated amounts of microsomal protein, 0.1 % Triton X-100 and 1.0 mM methimazole in a final volume of 1.0 ml. The reaction was initiated by addition of methimazole and the rate was recorded at 412 nm for 3 minutes at 37°C.

Figure 3.3 illustrates the effect of temperature on bovine liver microsomal FMO activity. As shown in the figure, methimazole oxidation rate increased with increasing temperature up to 37 °C and the activity decreased above that point. Therefore, bovine liver microsomal FMO activity determinations were carried out at 37 °C throughout this study.

3.2.4 Effect of Methimazole Substrate Concentration on Bovine Liver FMO Activity

Effect of substrate concentration on bovine liver FMO activity was determined by increasing methimazole concentration from 0.1 mM to 2.0 mM. As shown in Figure 3.4, FMO activity was found to increase almost linearly with increasing substrate concentration until 0.2 mM methimazole. As the substrate concentration was further increased, the enzyme activity increased nonlinearly, and the enzyme seemed to be saturated with its substrate at or above 1.0 mM. As seen from the [S] versus Velocity graph, when the substrate concentration was increased beyond 1.0 mM, the rate of reaction and therefore FMO enzyme activity was found to be stable.

Figure 3.5 shows the Lineweaver-Burk plot derived from methimazole saturation curve. The plot was linear, suggesting simple Michaelis-Menten kinetics. The apparent V_{max} and K_m values were calculated from the double reciprocal plot as 1.23 nmol/min/mg and 0.11 mM respectively. We have used 1.0 mM methimazole, which is much higher than the K_m value of the enzyme, throughout the study.

3.2.5 Effect of pH on Bovine Liver FMO Activity

The effect of pH on bovine liver FMO activity was determined using buffers of different pH values changing from 4 to 10. Three different buffer systems were used: 0.5 M Acetic acid-Na acetate buffer for pH 4.0, pH 5.0 and pH 6.0; 0.5 M Tris-

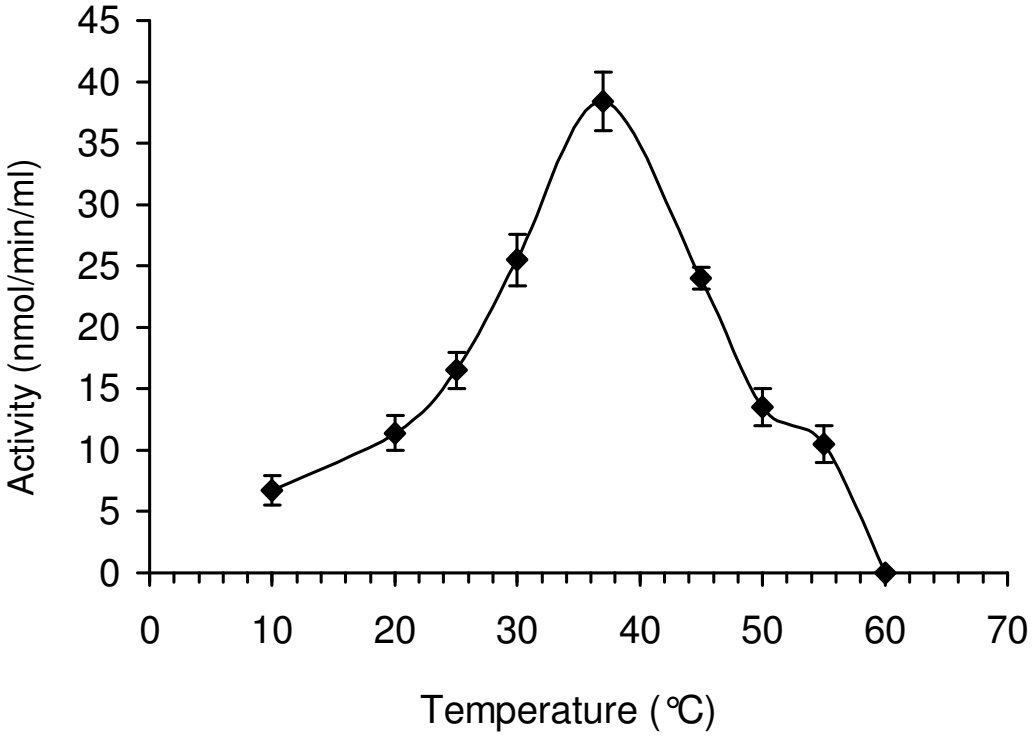


Figure 3.3 The effect of reaction temperature on bovine liver FMO activity. The constituents of the reaction mixture were the same as described in the legend of Figure 3.1. The assay mixture was warmed to the indicated temperature in a spectrophotometric cuvette and the endogenous rate was recorded. Reactions were initiated by the addition of methimazole to the cuvette and the decrease in absorbance at 412 nm was recorded for 3 minutes.

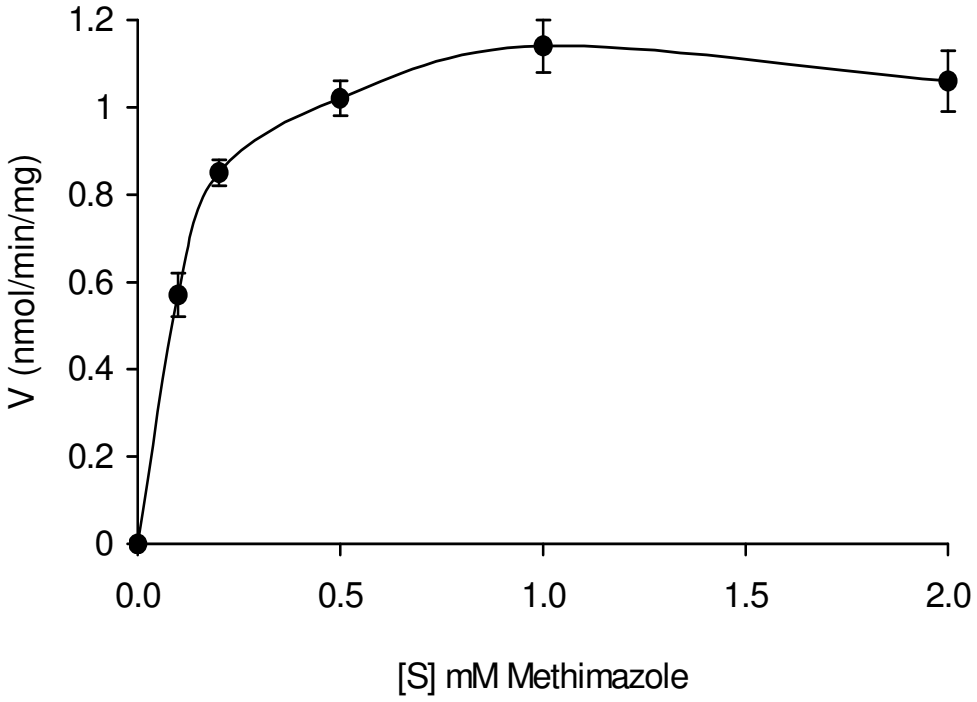


Figure 3.4 The effect of methimazole concentration on bovine liver microsomal FMO activity. Five different concentrations of methimazole, changing from 0.1 to 2.0 mM were used in the reaction medium. The reaction mixture contained 0.05 M Tris-HCl buffer, pH 8.0, 0.06 mM DTNB, 0.02 mM DTT, 0.1 mM NADPH, 250 µg microsomal protein, 0.1 % Triton X-100 and indicated amounts of methimazole in a final volume of 1.0 ml. The reactions were followed at 412 nm for 3 minutes at 37 °C.

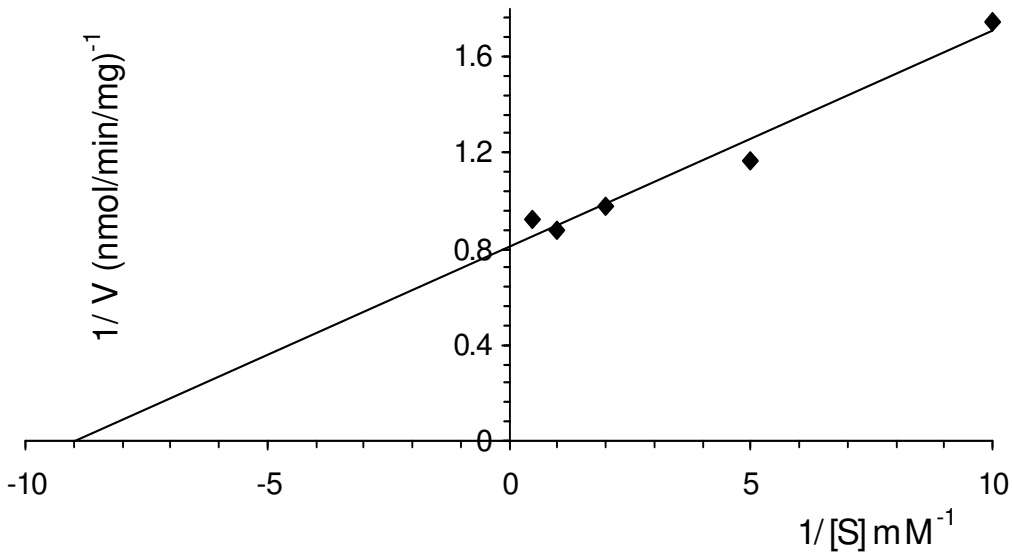


Figure 3.5 Lineweaver-Burk plot for FMO-mediated oxidation of methimazole by bovine liver microsomes. The conditions were identical to those given in the legend of Figure 3.4. The apparent K_m and V_{max} values of the reaction were 0.11 mM and 1.23 nmol/min/mg, respectively.

HCl buffer for pH 7.0, pH 8.0, pH 8.5 and pH 9.0; 0.5 M Glycine-NaOH buffer for pH 10.0.

Figure 3.6 illustrates the effect of pH on bovine liver microsomal FMO activity. The highest FMO activity was obtained at pH 8.0, using 0.5 M Tris-HCl buffer. Above and below pH 8.0 the rate of methimazole oxidation by bovine liver microsomal FMO decreased.

3.2.6 Effects of Various Concentrations of Different Detergents on Bovine Liver FMO Activity

Bovine liver microsomal FMO-catalyzed methimazole oxidation rate was recorded in the presence of two different detergents: Triton X-100 and Emulgen 913. Triton X-100 was added to the reaction medium at 0.1 %, 0.2 % and 1.0 % final concentrations. Effect of Emulgen 913 was determined by adding 0.1 % and 0.5 % final concentrations into the incubation mixture. Reactions were carried out using 250 μg microsomal protein per 1.0 ml assay mixture.

Figure 3.7 shows the effect of different detergent concentrations on bovine liver FMO activity. The FMO enzyme activity increased with the addition of either detergent at all concentrations. Among the detergents used, the most significant effect and highest FMO activity was observed with 0.5 % Emulgen-913 in the reaction medium. However, Emulgen-913 was not preferred because it is known to react with cytochrome P450s which are present at high amounts in the microsomal fractions. Therefore, 0.1 % Triton X-100 was chosen to be used in reaction medium in this study. Low concentration of the detergent was preferred in order to avoid inactivation of the proteins during long exposure to detergents.

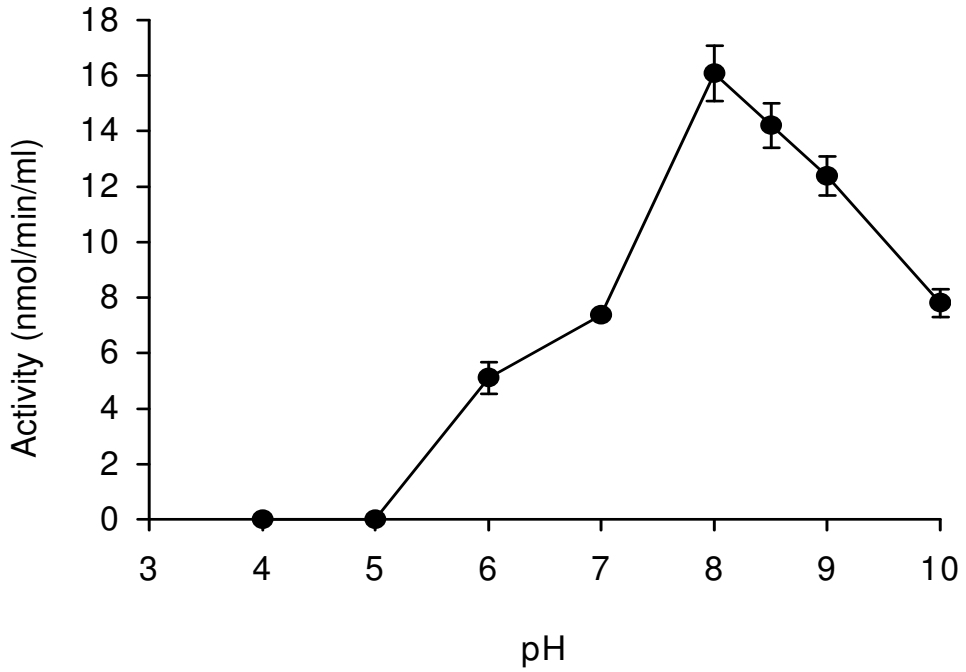


Figure 3.6 The effect of pH on bovine liver FMO activity. The reactions were carried out at the indicated pH values, using 0.5 M acetic acid-Na acetate buffer for pH 4.0, pH 5.0 and pH 6.0; 0.5 M Tris-HCl buffer for pH 7.0, pH 8.0, pH 8.5 and pH 9.0; 0.5 M glycine-NaOH buffer for pH 10.0. The buffers were prepared at room temperature. The other conditions were same as described under "Methods".

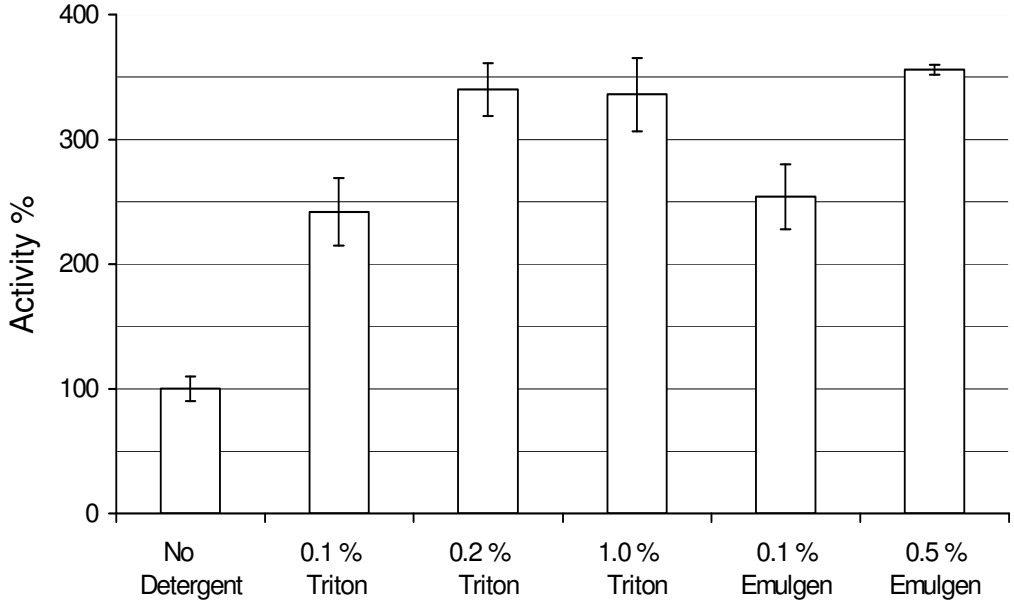


Figure 3.7 Effects of various concentrations of different detergents on bovine liver FMO activity. Triton X-100 in final concentrations of 0.1 %, 0.2 % and 1.0 % and Emulgen 913 in final concentrations of 0.1 % and 0.5 % were used in the reaction medium. The other conditions were same as described under "Methods".

3.2.7 Thermostability of Bovine Liver Microsomal FMO

Heat stability of bovine liver microsomal FMO enzyme stability was studied by incubating the bovine liver microsomes in a water bath at four different temperatures, 24 °C, 37 °C, 50 °C and 65 °C, for different periods of time. These microsomes were then used as enzyme source for methimazole oxidation assay, which was carried out at 37 °C as explained under "Methods".

Thermostability of bovine liver microsomal FMO at 65 °C is shown in Figure 3.8. 75 % of the initial activity was lost in 1 minute, and the activity was completely lost within 5 minutes at 65 °C.

Heat stability pattern of bovine liver microsomal FMO activity at 50 °C is shown in Figure 3.9. The activity of FMO decreased to one half of the initial enzyme activity in 1 minute. Within 5 minutes only 25 % of the initial activity was detectable. At the end of 10 minutes, FMO enzyme activity was completely lost at 50°C.

Figure 3.10 illustrates the thermostability pattern of bovine liver microsomal FMO at 37 °C. The enzyme activity decreased almost linearly in the first 3 hours. At the end of 6 hours only 10 % of the initial activity was detectable. FMO activity was completely lost at the end of 6.5 hours when incubated at 37 °C.

Heat stability of bovine liver microsomal FMO at 24 °C is shown in Figure 3.11. FMO activity decreased by 10 % at the end of two hours. 70 % of the initial enzyme activity remained after 1 day incubation. At the end of 53 hours, still 68 % of the initial enzyme activity was detectable at 24 °C.

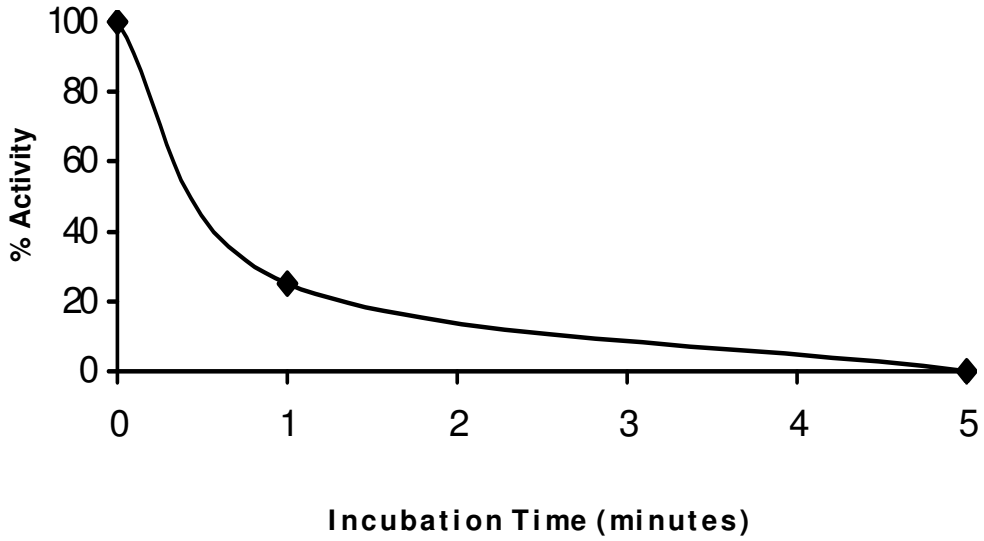


Figure 3.8 Heat stability of bovine liver microsomal FMO at 65 °C. The liver microsomes were incubated at this temperature in a water bath. Aliquots were taken after indicated periods of time and used as enzyme source for methimazole oxidation assay. The incubation mixture contained 0.05 M Tris-HCl buffer (pH 8.0), 0.06 mM DTNB, 0.02 mM DTT, 0.1 mM NADPH, 500 μg microsomal protein, 0.1 % Triton X-100 and 1.0 mM methimazole in a final volume of 1.0 ml. Reactions were followed for 3 minutes at 37 °C and rates were calculated as described under "Methods".

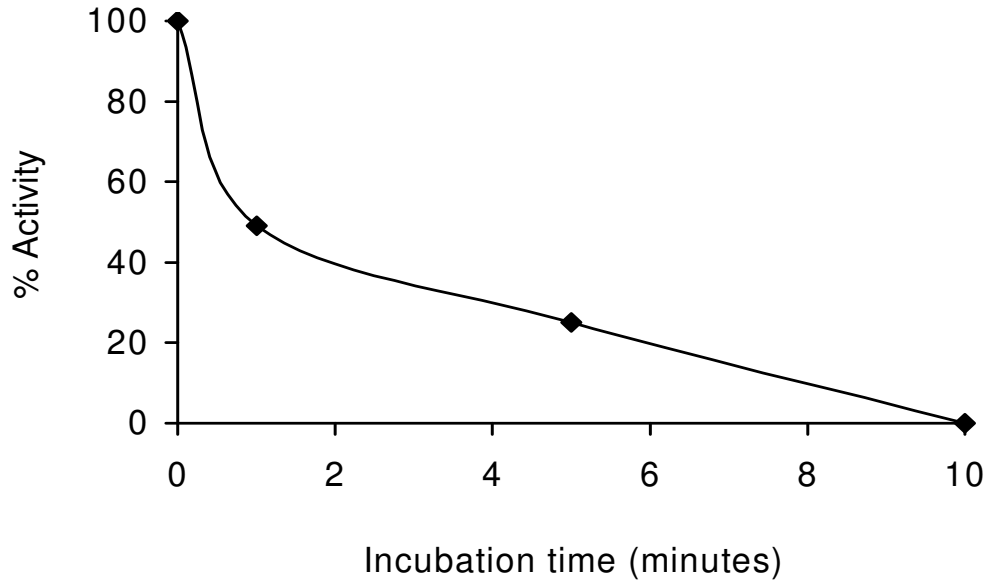


Figure 3.9 Heat stability of bovine liver microsomal FMO at 50 °C. The liver microsomes were incubated at this temperature in a water bath. Aliquots were taken after indicated periods of time and used as enzyme source for methimazole oxidation assay. The reaction mixtures were prepared as described in the legend of Figure 3.8. Reactions were followed for 3 minutes at 37 °C and rates were calculated as described under "Methods".

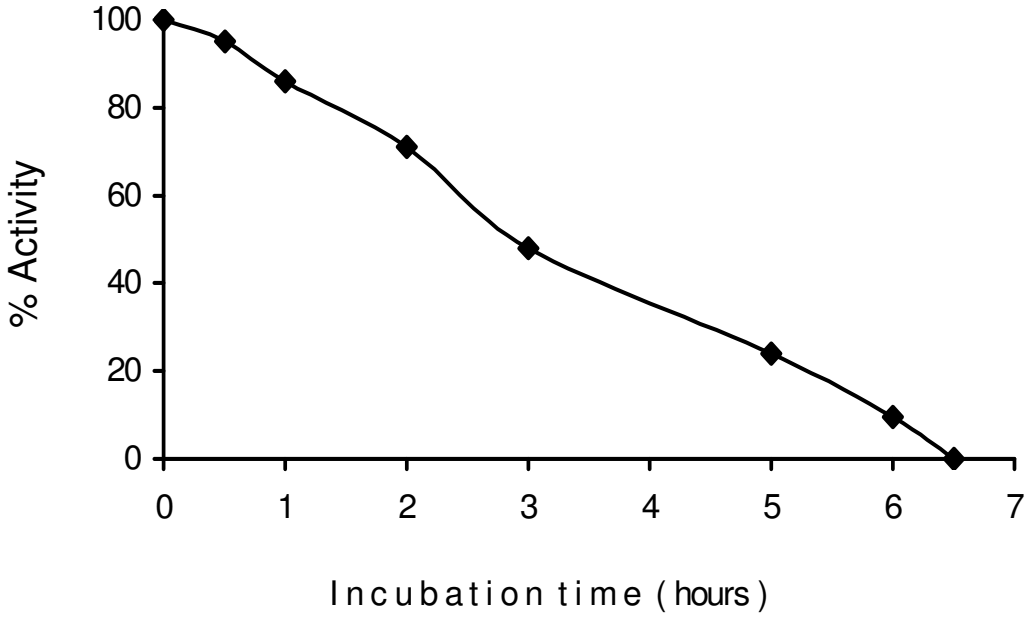


Figure 3.10 Heat stability of bovine liver microsomal FMO at 37 °C. The liver microsomes were incubated at this temperature in a water bath. Aliquots were taken after indicated periods of time and used as enzyme source for methimazole oxidation assay. The reaction mixtures were prepared as described in the legend of Figure 3.8. Reactions were followed for 3 minutes at 37 °C and rates were calculated as described under "Methods".

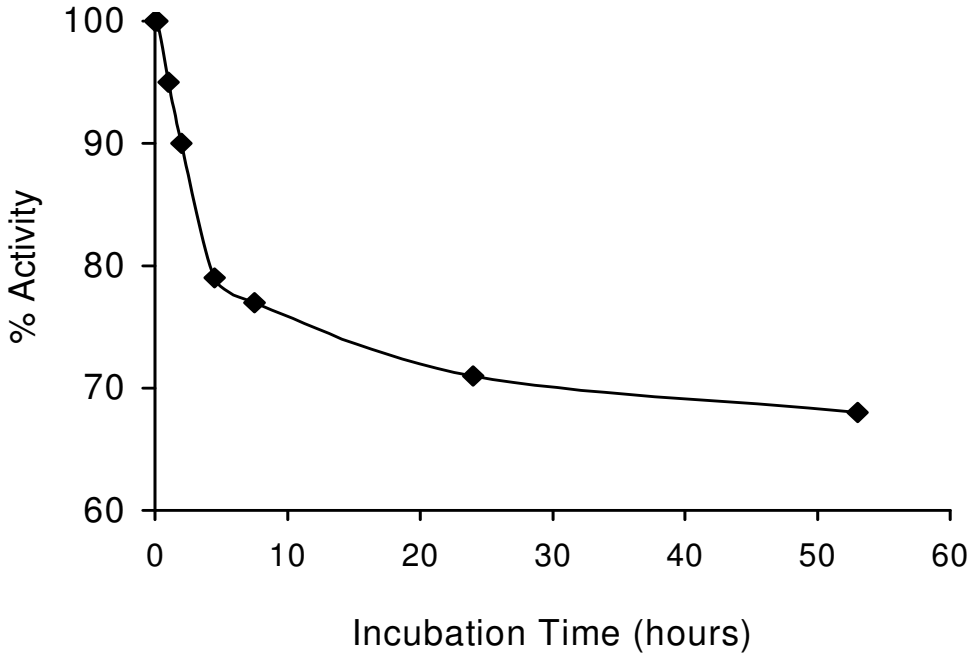


Figure 3.11 Heat stability of bovine liver microsomal FMO at 24 °C. The liver microsomes were incubated at this temperature in a water bath. Aliquots were taken after indicated periods of time and used as enzyme source for methimazole oxidation assay. The reaction mixtures were prepared as described in the legend of Figure 3.8. Reactions were followed for 3 minutes at 37 °C and rates were calculated as described under "Methods".

3.2.8 Effect of Magnesium Ion on Bovine Liver Microsomal FMO Activity

Effect of magnesium ion on bovine liver FMO activity was determined by adding $MgCl_2$ at concentrations ranging from 0.5 mM to 300 mM into the reaction medium.

As shown in Figure 3.12, bovine liver microsomal FMO activity was not changed by $MgCl_2$ up to a concentration of 1 mM. At 50 mM $MgCl_2$, 30 % of the initial activity was lost. At 200 mM $MgCl_2$, only 27 % of the initial activity remained. FMO was completely inhibited at 300 mM $MgCl_2$ concentration.

3.2.9 Metabolism of Drug Substrates by Bovine Liver Microsomal Enzymes

Bovine liver microsomal FMO activity was also determined using two drug substrates; imipramine and chlorpromazine using NADPH oxidation method, as described in section 2.2.3.2. The reaction was initiated by the addition of substrate (either 0.1 mM imipramine or 0.2 mM chlorpromazine in reaction mixture) into the reaction medium and the rate was recorded at 340 nm for 3 minutes at 37 °C.

Table 3.2 shows the activity of bovine liver microsomal enzymes towards drug substrates imipramine and chlorpromazine. Microsomal enzyme activities towards imipramine and chlorpromazine were found to be 85.8 and 86.4 nmol NADPH oxidized/min/ml, respectively. FMO activity towards methimazole, determined using methimazole/ DTNB assay as described in section 2.2.3.1, was also given in the same table for comparison. Enzyme activities were determined using the same microsomal preparation from bovine liver.

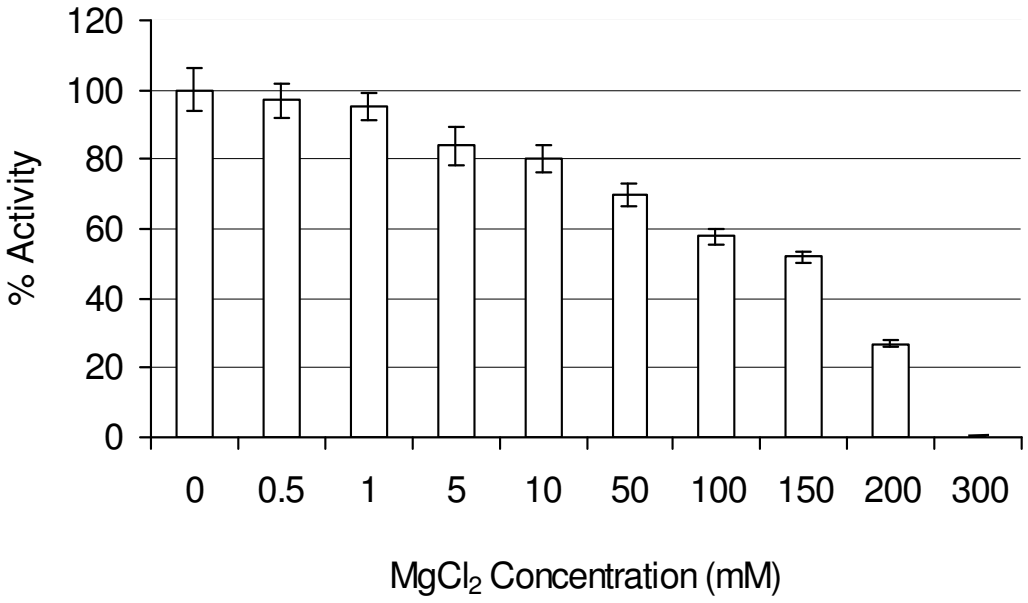


Figure 3.12 Effects of various concentrations of magnesium ion on FMO activity. The reaction medium contained indicated amounts of $MgCl_2$ in addition to the standard assay mixture constituents given under "Methods". The reactions were initiated with addition of 1.0 mM methimazole, and the rates were recorded for 3 minutes at 37 °C.

Table 3.2 FMO activity towards methimazole and two drug substrates, imipramine and chlorpromazine.

Bovine Liver Microsomes	Activity (nmol/min/mg)
Methimazole oxidation *	2.37
Imipramine oxidation **	3.73
Chlorpromazine oxidation ***	3.75

*** 1.0 mM methimazole in reaction medium was used. Activity was measured using methimazole/DTNB assay.**
**** 0.1 mM imipramine in reaction medium was used. Activity was measured using NADPH oxidation method.**
***** 0.2 mM chlorpromazine in reaction medium was used. Activity was measured using NADPH oxidation method.**

3.2.10 Modulation of Bovine Liver Microsomal FMO Activity by Drugs and Other Effectors

Modulation of bovine liver microsomal FMO activity by drugs and other effectors was studied by measuring the oxidation rate of methimazole using methimazole / DTNB assay. Bovine liver microsomal FMO-catalyzed methimazole oxidation was carried out in the presence of drug substrates (imipramine or chlorpromazine) and other effectors (Ni^{+2}, Cd^{+2}, Hg^{+2}). An appropriate amount of these modulators was added to the assay mixture and reaction was initiated with the addition of methimazole into the cuvette. Reaction rates were recorded at 412 nm for 3 minutes and calculated as described under "Methods".

3.2.10.1 Modulation of Methimazole Oxidation Activity of Bovine Liver Microsomal FMO by Imipramine

Imipramine effect on bovine liver microsomal FMO activity was determined using imipramine concentrations in reaction medium, ranging from 50 μM to 1 mM. A control without imipramine was also assayed. Figure 3.13 shows the

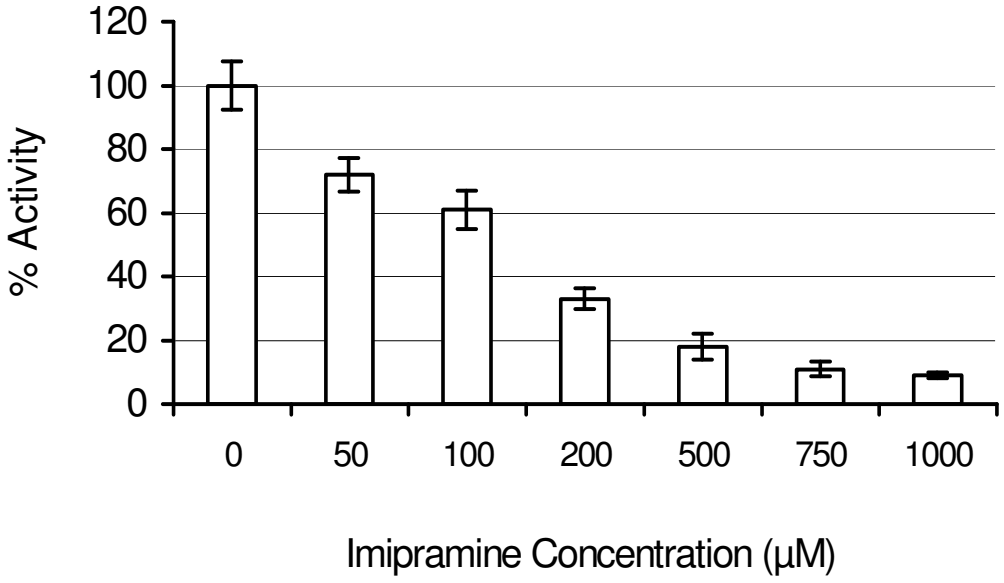


Figure 3.13 Effect of imipramine on FMO-catalyzed methimazole oxidation. The reaction medium contained indicated amounts of imipramine in addition to the standard assay mixture constituents given under "Methods". The reactions were initiated with addition of 1.0 mM methimazole and the rates were recorded for 3 minutes at 37 °C.

effect of imipramine on bovine liver microsomal FMO activity. FMO-catalyzed methimazole oxidation decreased to 72 % of initial enzyme activity at 50 μM. Inhibition by imipramine was more pronounced at concentrations higher than 100 μM. At 200 μM imipramine concentration, 67 % of the enzyme activity was inhibited. At 1 mM imipramine concentration, the enzyme was almost completely (91 %) inhibited.

3.2.10.2 Modulation of Methimazole Oxidation Activity of Bovine Liver Microsomal FMO by Chlorpromazine

A range of chlorpromazine concentrations, changing from 1 μM to 300 μM, was added to the reaction medium in order to determine the effect of chlorpromazine on bovine liver microsomal FMO-catalyzed methimazole oxidation. A control assay, without chlorpromazine, was also carried out.

As shown in Figure 3.14, chlorpromazine did not show a significant effect on bovine liver microsomal FMO activity at concentrations around 1 μM and 50 μM. At 100 μM chlorpromazine, FMO activity decreased sharply and only 40 % of the initial activity was detectable. At 200 μM and 300 μM chlorpromazine, less than 20 % of the initial activity remained.

3.2.10.3 Modulation of Bovine Liver Microsomal FMO Activity by Heavy Metal Ions

Nickel, cadmium and mercury ions were used to study whether these metal ions modulate the activity of FMO or not. Varying concentrations of each metal ion were added to the reaction medium containing a fixed methimazole concentration. The experiment was repeated using different concentrations of methimazole, changing from 1.0 mM to 0.1 mM (1.0 mM, 0.5 mM, 0.2 mM and 0.1 mM).

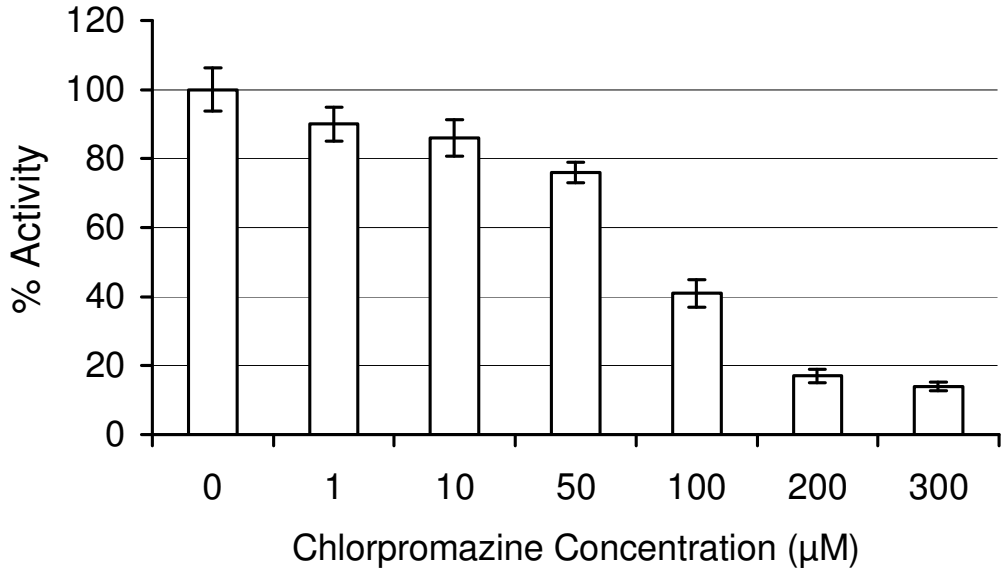


Figure 3.14 Effect of chlorpromazine on FMO-catalyzed methimazole oxidation. The reaction medium contained indicated amounts of chlorpromazine in addition to the standard assay mixture constituents given under "Methods". The reactions were initiated with addition of 1.0 mM methimazole and the rates were recorded for 3 minutes at 37 °C.

3.2.10.3.1 Modulation of Bovine Liver Microsomal FMO Activity by Nickel Ion

Modulation of methimazole oxidation activity of bovine liver microsomal FMO by nickel ion was determined by adding $NiCl_2$ at concentrations ranging from 0.005 mM to 0.2 mM into the reaction medium. This experiment was carried out using four different methimazole concentrations (1.0 mM, 0.5 mM, 0.2 mM and 0.1 mM). Figure 3.15 illustrates the change in FMO activity with increasing nickel concentration. An increase in activity at 0.005 mM $NiCl_2$ for each methimazole concentration used, indicated that the FMO enzyme was activated with this low nickel ion concentration. At all concentrations of methimazole, 60-70 % of the initial activity was still detectable with 0.2 mM $NiCl_2$ in reaction medium.

Figure 3.16 shows the Dixon plot, 1/V versus [I], in the presence of different fixed concentrations of substrate methimazole. The apparent K_I value was found to be 0.5 mM and the apparent V_{max} values for methimazole concentrations of 1.0 mM, 0.5 mM, 0.2 mM and 0.1 mM were calculated as 25.6, 23.0, 20.4 and 17.8 $(nmol/min/ml)^{-1}$, respectively. At all methimazole concentrations, 0.005 mM nickel ion activated the FMO enzyme. This figure informed the type of inhibition of FMO-catalyzed methimazole S-oxidation by nickel ion. K_I value remained same while V_{max} value differed for each concentration of methimazole. Therefore, the inhibition of bovine liver microsomal FMO activity by nickel ion was found to be noncompetitive type of inhibition.

3.2.10.3.2 Modulation of Bovine Liver Microsomal FMO Activity by Cadmium Ion

Modulation of methimazole oxidation activity of bovine liver microsomal FMO by cadmium ion was determined by increasing $CdCl_2$ concentration from 0.005 mM to 0.2 mM in the reaction medium. Three different methimazole concentrations

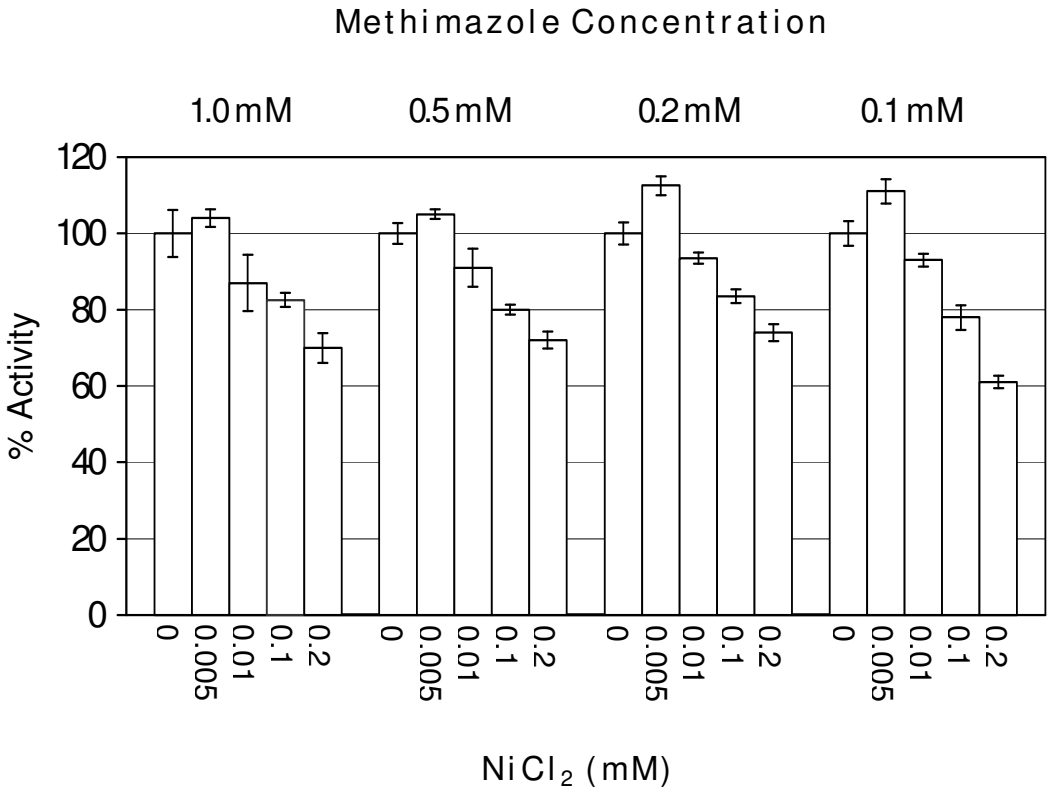


Figure 3.15 Modulation of bovine liver microsomal FMO activity by nickel ion. The reaction medium contained indicated amounts of $NiCl_2$ and methimazole. The other conditions remained same as described under "Methods". The reactions were initiated with addition of methimazole into the reaction medium and the rates were recorded for 3 minutes at 37 °C.

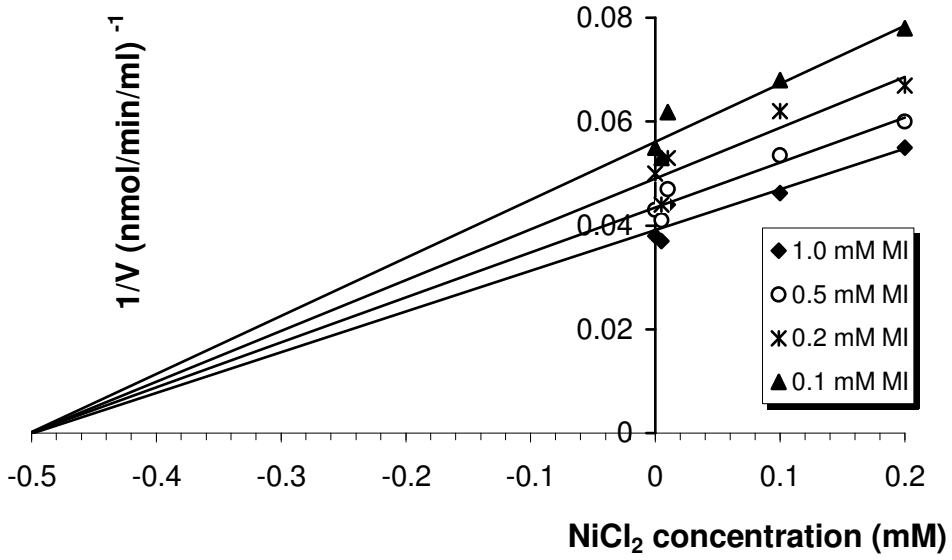


Figure 3.16 Dixon plot, 1/V versus [I], in the presence of different fixed concentrations of substrate methimazole. The conditions were identical to those given in the legend of Figure 3.15. The apparent K_I value for nickel ion was calculated to be 0.5 mM. Methimazole is abbreviated as "MI".

(1.0 mM, 0.5 mM, 0.2 mM) were used. Figure 3.17 shows the modulation of FMO activity by various concentrations of cadmium ion. When cadmium ion concentration was increased, the enzyme activity decreased gradually. For all methimazole concentrations used, the enzyme activity decreased to one half of the initial activity at 0.05 mM $CdCl_2$. The bovine liver microsomal FMO activity was completely inhibited by 0.2 mM $CdCl_2$.

Figure 3.18 shows the Dixon plot, 1/V versus [I], in the presence of different fixed concentrations of substrate methimazole. The apparent K_I value was found to be 0.085 mM and the apparent V_{max} values for methimazole concentrations of 1.0 mM, 0.5 mM and 0.2 mM were calculated as 19.0, 16.5 and 14.7 $(nmol/min/ml)^{-1}$, respectively. This figure also showed that inhibition of bovine liver microsomal FMO activity by cadmium ion was noncompetitive type of inhibition.

3.2.10.3.3 Modulation of Bovine Liver Microsomal FMO Activity by Mercury Ion

Modulation of methimazole oxidation activity of bovine liver microsomal FMO by mercury ion was determined by increasing $HgCl_2$ concentration from 0.5 μM to 5.0 μM in the reaction medium. In this experiment four different methimazole concentrations (1.0 mM, 0.5 mM, 0.2 mM, 0.1 mM) were used. As shown in Figure 3.19, FMO activity decreased with an increase of mercury ion concentration. At the highest $HgCl_2$ concentration studied, 5.0 μM, still 45 % of the initial enzyme activity was detectable at all methimazole concentrations.

Figure 3.20 shows the Dixon plot, 1/V versus [I], in the presence of different fixed concentrations of substrate methimazole. The apparent K_I value was found to be 4.6 μM and the apparent V_{max} values for methimazole concentrations of 1.0 mM, 0.5 mM, 0.2 mM and 0.1 mM were calculated as 22.6, 19.0, 16.4 and 13.9 $(nmol/min/ml)^{-1}$, respectively. This figure also showed that bovine liver microsomal FMO was inhibited by mercury ion noncompetitively.

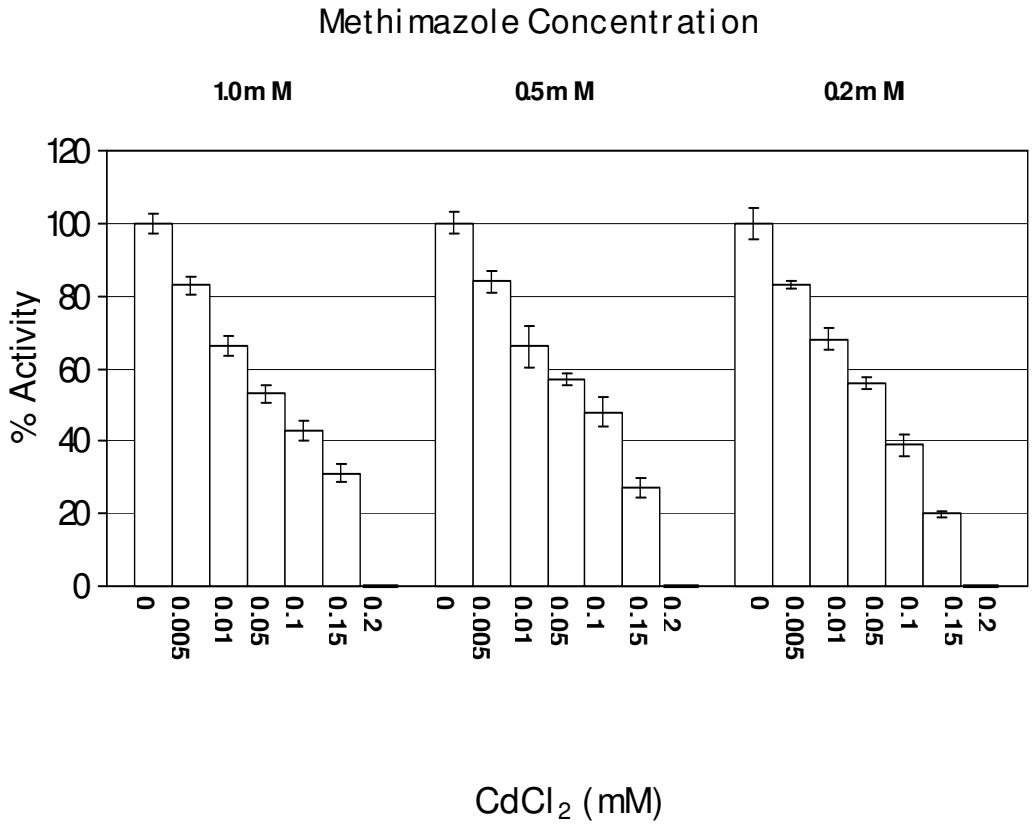


Figure 3.17 Modulation of bovine liver microsomal FMO activity by cadmium ion. The reaction medium contained indicated amounts of $CdCl_2$ and methimazole. The other conditions remained same as described under "Methods". The reactions were initiated with addition of methimazole into the reaction medium and the rates were recorded for 3 minutes at 37 °C.

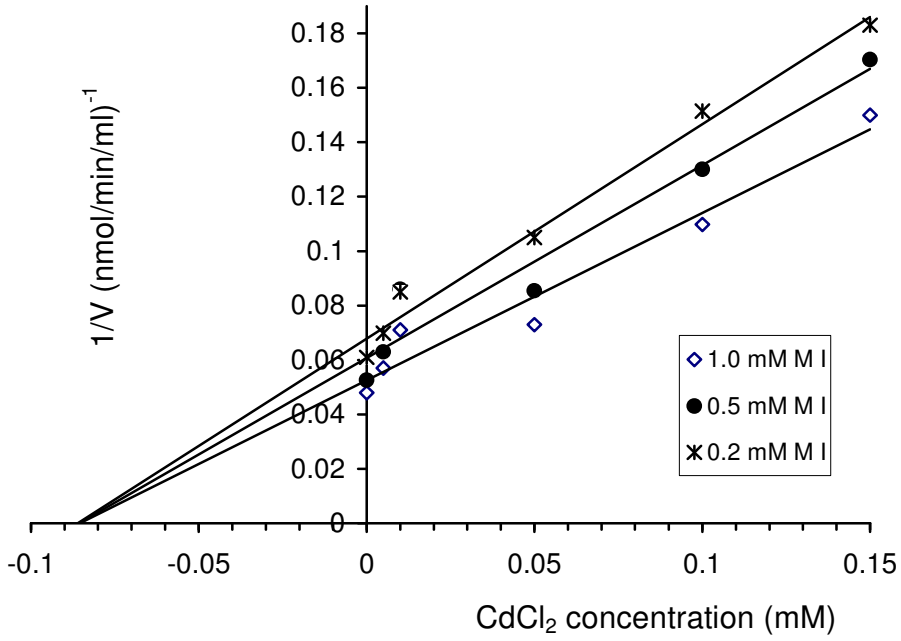


Figure 3.18 Dixon plot, 1/V versus [I], in the presence of different fixed concentrations of substrate methimazole. The conditions were identical to those given in the legend of Figure 3.15. The apparent K_I value for cadmium ion was calculated to be 0.085 mM. Methimazole is abbreviated as "MI".

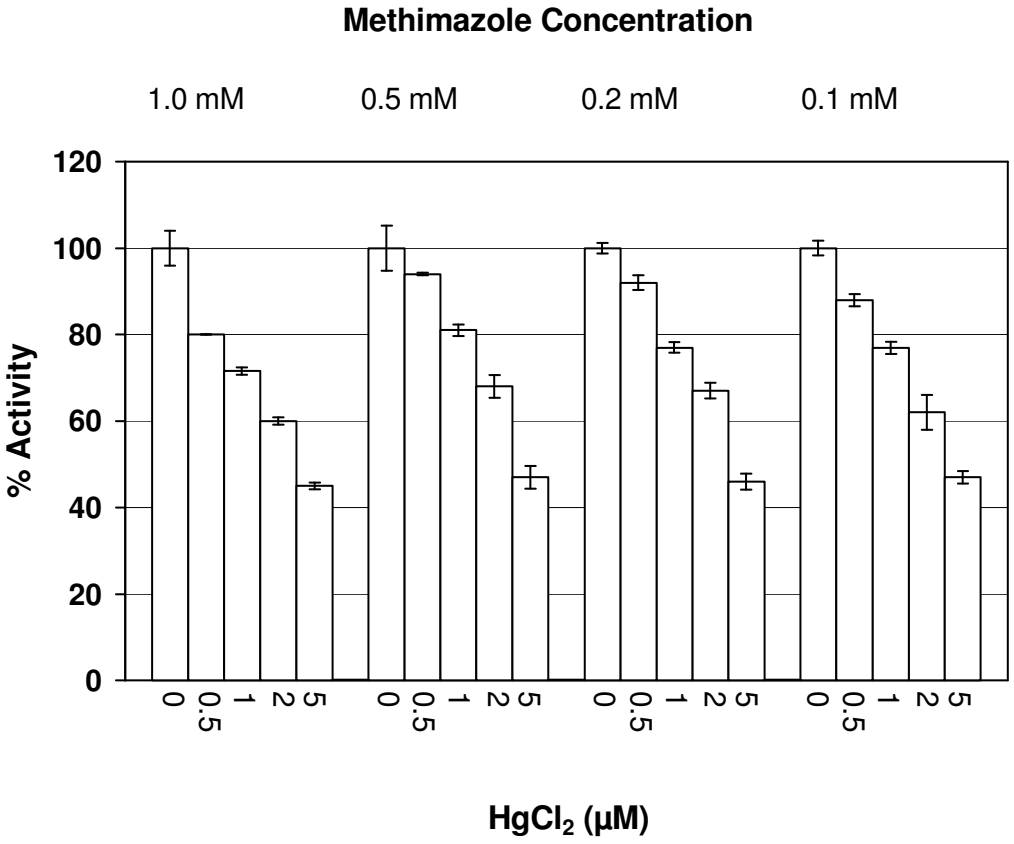


Figure 3.19 Modulation of bovine liver microsomal FMO activity by mercury ion. The reaction medium contained indicated amounts of $HgCl_2$ and methimazole. The other conditions remained same as described under "Methods". The reactions were initiated with addition of methimazole into the reaction medium and the rates were recorded for 3 minutes at 37 °C.

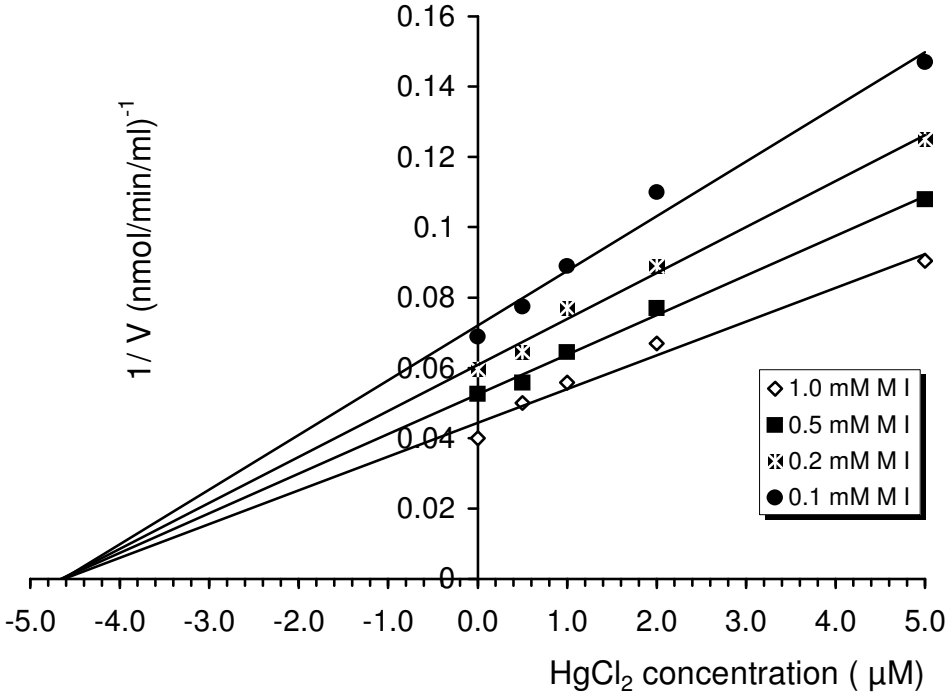


Figure 3.20 Dixon plot, 1/V versus [I], in the presence of different fixed concentrations of substrate methimazole. The conditions were identical to those given in the legend of Figure 3.15. The apparent K_I value for mercury ion was calculated to be 4.6 µM. Methimazole is abbreviated as "MI".

CHAPTER 4

DISCUSSION

The flavin monooxygenase of mammalian systems has been generally considered a detoxication enzyme converting nucleophilic heteroatom-containing chemicals into polar, more readily excreted metabolites. The beneficial effects of this process are thought to be participation in detoxication of foodstuffs and other xenobiotics that might otherwise be bioactivated by other enzyme systems.

FMOs require NADPH and oxygen for their activity. They are typically membrane-bound enzymes found in smooth endoplasmic reticulum and nuclear envelope of the cell, although cytosolic forms predominate in bacteria and unicellular organisms (Sum and Kasper, 1982; Schlenk, 1998). FMO was first purified from pig liver microsomes in 1972 by Ziegler and Mitchell. The FMO is now known to exist in many species and in more than one organ. In recent years, it has been purified from liver microsomes of rat (Kimura *et al*., 1983), mouse (Sabourin *et al*., 1984; Sabourin and Hodgson, 1984), rabbit (Tynes *et al*., 1985), guinea pig (Yamada *et al*., 1990), macacque (Sadeque *et al*., 1993), from pulmonary and renal microsomes of mouse (Tynes *et al*., 1985; Venkatesh *et al*., 1991), and from brain microsomes of rat (Kawaji *et al*., 1995) and human (Bhagwat *et al*., 1996). There are at least five isoforms of FMO (Philpot *et al*., 1999). A sixth form has been also identified, however, it is not known if functional protein is produced (Krueger *et al*., 2001). The expression of FMO is sex-, tissue- and species-dependent.

Most studies mainly focused on small laboratory animals such as rats, rabbits and mice. However, no data are available concerning the biochemical characterization of FMO enzymes in veterinary animals, such as bovine. Bovine is a common meat source in the world, especially in Turkey, and is therefore important for human diet and health. In addition, our laboratory has been involved in the purification and characterization of the components of microsomal CYP450-dependent monooxygenase system of sheep and bovine (Arınç and İşcan, 1983; Arınç, 1985; İşcan and Arınç, 1986, 1988; Adalı and Arınç, 1990; Adalı *et al*., 1996; Arınç and Çelik, 2002).

In this study, bovine liver microsomal FMO activity was characterized with respect to several properties, using methimazole as substrate. For this purpose, the method of Dixit and Roche, 1984, was used as described in "Methods". The DTNB/ Methimazole assay is a convenient, accurate and highly sensitive assay for measuring FMO activity. However, methimazole or closely related compounds are the only substrates that can be used for this assay. The activity of bovine liver microsomal FMO was determined using methimazole as substrate and the specific activities of FMO from 12 different microsomal preparation of bovine liver were found to be 1.60, 1.38, 2.17, 2.18, 3.30, 2.71, 2.93, 4.95, 2.12, 1.21, 1.43 and 2.57. The average specific activity was calculated as 2.37 ± 0.30 nmol/min/mg protein (Mean ± SE, n=12). A summary of the properties of bovine liver microsomal FMO is given in Table 4.1.

Bovine liver microsomal FMO activity was found to be time-dependent. The reaction rate was linear with time up to 17 minutes using the assay conditions given in Table 2.1. Dixit and Roche, 1984, have studied time course of FMO-catalyzed methimazole oxidation and reported that methimazole-dependent TNB oxidation catalyzed by samples of whole mouse liver homogenates was linear more than 10 minutes.

Table 4.1 A summary of the properties of bovine liver microsomal FMO.

PROPERTIES		BOVINE LIVER MICROSOMAL FMO
Activity Towards	Methimazole	2.37 nmol/min/mg
	Imipramine	3.73 nmol/min/mg
	Chlorpromazine	3.75 nmol/min/mg
Optimum Temperature		37 °C
Optimum pH		8.0
K_m and V_{max}		0.11 mM 1.23 nmol/min/mg
Effects of Detergents (% activity)	0.1 % Triton X-100	240 %
	0.5 % Emulgen 913	352 %
Thermostability (time required to decrease enzyme activity to zero)	65 °C	5 minutes
	50 °C	10 minutes
	37 °C	6.5 hours
	24 °C ∗	53 hours
K_I values for three heavy metal ions	Ni^{+2}	500 µM
	Cd^{+2}	85 µM
	Hg^{+2}	4.6 µM

∗ At the end of 53 hours, still 68 % of the activity was detectable.

The effect of microsomal protein amount on bovine liver FMO was determined in a range of 136 μg to 680 μg. The reaction rate was found to be linear with protein amount up to 500 μg protein per 1.0 ml of reaction mixture. Throughout this study, depending on the type of experiment, 250 or 500 μg/ml protein concentration was used. Dixit and Roche, 1984, showed that TNB-oxidation activity of female mouse liver FMO increased linearly with increasing protein concentration up to 1.0 mg protein. Another study carried out by Bhamre *et al.*, 1993, showed that rat kidney microsomal FMO activity increased linearly with increasing microsomal protein concentration, up to 400 μg protein.

The biochemical characterization of bovine liver microsomal FMO has also been carried out with respect to the response of this enzyme against different temperature and pH values. The effect of temperature on bovine liver microsomal FMO activity was determined with different temperatures ranging from 10°C to 60°C. The maximum activity was observed at 37°C. Bovine liver microsomal FMO enzyme activity decreased above this temperature due to enzyme denaturation. Demirdöğen and Adalı, 2004, have reported the optimum temperature of sheep liver microsomal activity as 37 ºC (Table 4.2).

The effect of pH on bovine liver microsomal FMO activity was determined using buffers of different pH values. The optimum pH for FMO-catalyzed methimazole oxidation was found to be 8.0, in this study. At pH 7.0, only one half of the FMO activity was observed compared to pH 8.0. However, at pH 9.0, more than 80 % of the activity was still detectable. Therefore, we can possibly state that acidic medium denatures FMO more effectively than the alkaline medium. In this study, the pH optimum, 8.0, of bovine liver microsomal FMO was found to be lower than the optimum pH obtained from other species. Hodgson *et al.*, 1984, reported pH optimum for purified mouse liver and lung FMO as 8.8 and 9.8, respectively; for purified rabbit liver and lung 8.6 and 10.0, respectively. Lattard *et al.*, 2001, has found the pH optimum for rat liver FMO as 8.9. Recently, Demirdöğen and Adalı,

Table 4.2 Comparison of some properties of bovine liver microsomal FMO with sheep liver microsomal FMO.

Properties	**Bovine Liver FMO**	**Sheep Liver FMO**
Optimum Temperature	37 °C	37 °C
Optimum pH	8.0	8.0
K_m	0.11 mM	0.118 mM

2004, have reported the pH optimum of sheep liver microsomal FMO as pH 8.0. The results obtained in this study with others have shown that FMO enzyme is more active near alkaline pH range of 8-9.

The kinetic studies were carried out using methimazole as substrate. The apparent K_m and V_{max} values calculated as 0.11 mM and 1.23 nmol/min/mg, respectively. Throughout this study, 1.0 mM methimazole which is much higher than the K_m value was used. Ziegler, 1980, has reported the K_m value for methimazole-oxidation activity of hog liver FMO as 5 μM. Bhamre, 1993, has found the K_m and V_{max} values for FMO-catalyzed methimazole oxidation in rat brain microsomes to be 0.8 mM and 31 nmol NADPH oxidized/min/mg protein, respectively. In another study by Bhamre, 1995, K_m and V_{max} values for methimazole oxidation activity of human brain FMO were found to be 17.1 μM and 29.8 nmol NADPH/min/mg protein, respectively. Adalı *et al*., 1998, have reported the K_m value for FMO-mediated oxidation of methimazole by human FMO3 as 30 μM. In 2004, Demirdöğen and Adalı have determined the K_m value of sheep liver FMO as 0.118

mM which is very close to the K_m value of bovine liver microsomal FMO. When K_m value of bovine liver microsomal FMO obtained in this study was compared with K_m values from other species, it was found that bovine liver microsomal FMO has a lower affinity towards methimazole than hog liver FMO, human FMO3 and human brain FMO, however, has a higher affinity towards methimazole than rat brain microsomal FMO.

Bovine liver microsomal FMO-catalyzed methimazole oxidation rate was recorded in the presence of two different detergents; Triton X-100 (0.1 %, 0.2 % and 1.0 % in reaction medium) and Emulgen 913 (0.1 % and 0.5 % in reaction medium). FMO enzyme activity increased with the addition of either detergent at all concentrations. The activation of microsomal FMO by detergents was apparently due to membrane perturbation, which resulted in increased cofactor or substrate accessibility to the enzyme. A summary of these results is listed in Table 4.1. The most significant increase in FMO activity was observed with 0.5 % Emulgen 913 in reaction medium. However, Emulgen 913 is known to react with CYP450s which are present in high amounts in microsomal preparations and therefore, Emulgen 913 was not preferred in this study. FMO activity increased with the increasing concentrations of Triton X-100 in the reaction mixture. With Triton X-100 at 0.1 % concentration in reaction medium, FMO activity was still much higher (2.5 fold) than the control. It has been known that high concentrations of detergents inactivate enzymes during long exposure. Therefore, the minimum concentration of Triton X-100 was routinely used for determination of FMO activity in the present study.

Thermostability of bovine liver microsomal FMO was studied at four different temperatures; 24°C, 37°C, 50°C and 65°C (Table 4.1). Preincubation of bovine liver microsomes at 65°C resulted in the complete loss of FMO activity within 5 minutes. At 50°C, although very drastic decrease of enzyme activity was observed in 1 minute of incubation, bovine liver microsomal FMO has shown activity for 10 minutes. At 37°C, the decrease in activity was much slower compared

to 65°C and 50°C. After 3 hours of incubation at 37°C 50 % of the initial FMO activity was still detectable. FMO activity was completely lost within 6.5 hours when incubated at 37°C. The decrease in enzyme activity became even slower at 24°C. 70 % of the initial enzyme activity remained after 1 day incubation. At the end of 53 hours, still 68 % of the initial FMO activity was detectable. Parkinson, 1996, stated that FMO is heat labile and can be inactivated in the absence of NADPH by warming microsomes to 50°C for 1 minute. In the present study, incubation time required to decrease bovine liver microsomal FMO activity by 50 % at 65°C, 50°C and 37°C were found to be 27 seconds, 1 minute and 3 hours, respectively. At 24°C, 68 % of the initial activity remained even at the end of 53 hours. The results obtained at 50°C and 65°C showed that bovine liver microsomal FMO is a heat labile enzyme.

The effect of magnesium ion on bovine liver microsomal FMO activity was determined by using $MgCl_2$ concentrations ranging from 0.5 mM to 300 mM in the reaction medium. The inhibitory effect of magnesium ion on bovine liver microsomal FMO activity was less pronounced up to a concentration of 1 mM. $MgCl_2$ in reaction medium. The concentration of $MgCl_2$ required for 50 % loss of activity was 150 mM. At 200 mM $MgCl_2$, only 27 % of the initial enzyme activity remained. 300 mM $MgCl_2$ in reaction medium completely inhibited FMO activity. Adalı *et al.*, 1998, have stated that $MgCl_2$ inhibited human FMO3 at all concentrations studied and the concentration of $MgCl_2$ required for 50 % loss of activity was between 10 and 20 mM.

In this study, in addition to methimazole, bovine liver microsomal enzyme activity towards drug substrates, imipramine and chlorpromazine were determined using NADPH oxidation method described under “Methods” and the activity towards imipramine and chlorpromazine were found to be 3.73 and 3.75 nmol NADPH oxi./min/mg, respectively. Besides, modulation of FMO-mediated methimazole oxidation was studied in the presence of drug substrates, imipramine and

chlorpromazine, and heavy metal ions, Ni^{+2}, Cd^{+2} and Hg^{+2}. A summary of these results was given in Table 4.1.

In the present study, all the studied imipramine concentrations (50 μM, 100 μM, 200 μM, 500 μM, 750 μM and 1000 μM) inhibited bovine liver microsomal FMO-catalyzed methimazole oxidation. Inhibition by imipramine was more pronounced at concentrations higher than 100 μM. At 200 μM imipramine only 33 % of the initial enzyme activity remained. 1.0 mM imipramine was almost completely (91 %) inhibited bovine liver microsomal FMO activity. Modulation of bovine liver microsomal FMO activity by chlorpromazine was also studied. Chlorpromazine did not show a significant effect on bovine liver microsomal FMO activity at concentrations around 1.0 μM and 50 μM. At 100 μM chlorpromazine, FMO activity decreased sharply and only 40 % of the initial enzyme activity was detectable. At 200 μM and 300 μM chlorpromazine, less than 20 % of the initial FMO activity remained.

Imipramine and chlorpromazine are known to be substrates for both FMO and CYP450s. However, methimazole is a specific substrate for FMO. Based on the observation of inhibition of methimazole oxidation catalyzed by only FMO in the presence of imipramine and chlorpromazine, we can possibly state that metabolism of these drug substrates are at least carried out by bovine liver microsomal FMO. However, the contribution of FMO to the metabolism of these drugs was not investigated. Ziegler *et al*., 1984, have demonstrated that the specific activity of pig liver FMO towards imipramine and chlorpromazine were 367 and 502 nmol NADPH/min/mg protein, respectively. Bhamre *et al*., 1995, have shown that psychoactive drugs, chlorpromazine and imipramine, exhibited high affinity (19.5 and 22.0 μM, respectively) for human brain FMO. In another study, Wyatt *et al*., 1998, have shown that imipramine markedly inhibits the activity of pig FMO1 at all concentrations of substrate studied, inhibits rabbit FMO1 and FMO2 at low substrate concentrations and activates the same isoforms at high substrate concentrations.

Adalı *et al.,* 1998, have stated that imipramine also activates human FMO3 but the effect requires relatively high concentrations and is not great. No inhibition is observed even at substrate concentrations as low as 10 μM. In contrast, chlorpromazine, which is only slightly more active than imipramine with FMO2, increases the activity of human FMO3 by nearly 2-fold at a concentration of 100 μM. These results demonstrate that tricyclic antidepressants can activate, inhibit or have no effect on the FMO as a function of the FMO isoform, the specific tricyclic and its concentration and the concentration of the substrate.

Nickel, cadmium and mercury ions were used to determine whether these heavy metal ions modulate the activity of bovine liver microsomal FMO or not. Varying concentrations of each metal ion were added to the reaction mixture containing a fixed methimazole concentration.

Modulation of FMO activity by nickel ion was determined by adding $NiCl_2$ at concentrations chancing between 0.005mM and 0.2 mM in the reaction medium. This assay was carried out using four different methimazole concentrations; 1.0 mM, 0.5 mM, 0.2 mM and 0.1 mM. For all methimazole concentrations studied, 0.005 mM $NiCl_2$ in reaction medium caused an increase in the activity of bovine liver microsomal FMO. However, FMO enzyme activity was inhibited by all other $NiCl_2$ concentrations studied. There was no significant difference in the amount of inhibition at different methimazole concentrations. The highest concentration of $NiCl_2$ studied, 0.2 mM, inhibited only 30-40 % of the initial FMO activity.

Modulatory effect of cadmium ion on bovine liver microsomal FMO activity was studied by adding different concentrations of $CdCl_2$, ranging from 0.005 mM to 0.2 mM in the reaction medium. This experiment was also carried out using three different methimazole concentrations (1.0 mM, 0.5 mM and 0.2 mM). All $CdCl_2$ concentrations studied, had inhibitory effect on methimazole-oxidation activity of bovine liver microsomal FMO. 0.2 mM nickel ion decreased FMO activity only by

30-40 %. However, the equal concentration of cadmium ion (0.2 mM) was enough to inhibit FMO activity completely. Therefore, we can possibly state that, compared to nickel ion, cadmium ion seems to be a more effective inhibitor due to a lower K_I value as explained later.

Modulation of bovine liver microsomal FMO activity by mercury ion was determined by increasing $HgCl_2$ concentration from 0.5 μM to 5.0 μM in the reaction medium. In this experiment, four different methimazole concentrations were used (1.0 mM, 0.5 mM, 0.2 mM and 0.1 mM). All tried concentrations of $HgCl_2$ inhibited FMO activity at the same extent for all methimazole concentrations. 5.0 μM of mercury ion decreased enzyme activity by about 50 %.

When the Dixon plots were drawn using 1/V versus [I] data, it was seen that the type of inhibition was noncompetitive for three heavy metal ions, Ni^{+2}, Cd^{+2} and Hg^{+2}. In this experiment, it has been observed that substrate methimazole concentration did not have an effect on inhibition of bovine liver microsomal FMO by these three metal ions because a classical noncompetitive inhibitor has no effect on substrate binding and vice versa. Substrate (S) and inhibitor (I) bind reversibly, randomly and independently at different sites. That is, I binds to enzyme (E) and to ES; S binds to E and to EI. However, the resulting ESI complex is catalytically inactive. Inhibitor might prevent the proper positioning of the catalytic center. The K_m value (measured as [S] required for 0.5 V_{max}) will be unchanged by a noncompetitive inhibitor because, at any inhibitor concentration, the enzyme forms that can combine with S (E and EI) have equal affinities for S. The net effect of a noncompetitive inhibitor is to make it appear as if less total enzyme is present. V_{max} in the presence of a noncompetitive inhibitor will be less than the V_{max} observed in the absence of inhibitor. This was also the case for these three metal ions since all V_{max} values were less than the controls (except the activation of FMO by 0.005 mM nickel ion). From the Dixon plots, $K_ı$ values were also calculated for nickel ion, cadmium ion and mercury ion as 500 μM, 85 μM and 4.6 μM, respectively.

Inhibition of bovine liver microsomal FMO by mercury ion was 18 times and 110 times more effective than cadmium and nickel ions, respectively. This means that mercury ion was a more potent inhibitor of bovine liver microsomal FMO than cadmium and nickel ions. Mercury ion may bind to regulatory site of FMO much more easily with a very low K_I value, compared to other two heavy metal ions.

CHAPTER 5

CONCLUSION

The present study was the first concerning the biochemical characterization of flavin monooxygenase (FMO) from bovine liver microsomes. Bovine liver microsomal FMO enzyme activity was characterized using methimazole as substrate, which is a highly specific substrate for FMO. From 12 different bovine liver samples, microsomes were prepared and the average specific activity of bovine liver microsomal FMO was found to be 2.37 ± 0.30 nmol/min/mg (Mean ± SE, n=12). The rate of reaction was linear up to 0.5 mg of bovine liver microsomal protein and for 17 minutes of incubation period. The maximum FMO enzyme activity was detected at 37 °C and at pH 8.0. Effects of detergents; Triton X-100 and Emulgen 913, on FMO activity were determined and found that enzyme activity increased by the addition of either detergent at all concentrations. The apparent V_{max} and K_m values of bovine liver microsomal FMO for methimazole substrate were found as 1.23 nmol/min/mg and 0.11 mM, respectively.

Thermostability of bovine liver microsomal FMO was studied at four different temperatures; 24 °C, 37 °C, 50 °C and 65 °C. The incubation time required for the complete loss of enzyme activity was 5 minutes at 65 °C, 10 minutes at 50 °C and 6.5 hours at 37 °C. 68 % of the activity was still detectable at the end of 53 hours at 24 °C. Bovine liver microsomal activity towards two drug substrates, imipramine and chlorpromazine, was also determined and found to be 3.73 and 3.75 nmol NADPH oxidized/min/mg, respectively. Effects of two drug substrates, imipramine and chlorpromazine, on bovine liver microsomal FMO-catalyzed methimazole oxidation activity was also studied and found that they inhibit FMO activity at all concentrations studied.

Modulation by effectors of bovine liver microsomal FMO activity was studied using three different heavy metal ions; Ni^{+2}, Cd^{+2} and Hg^{+2}. It was observed that nickel ion at very low concentration caused an increase in FMO enzyme activity. At all other concentrations studied for each heavy metal ion and at all substrate methimazole concentrations (0.1 mM, 0.2 mM, 0.5 mM, 1.0 mM), FMO-catalyzed methimazole oxidation activity decreased compared to control activity. K_I values for Ni^{+2}, Cd^{+2} and Hg^{+2} were found to be 0.5 mM, 0.085 mM, 4.6 μM, respectively. The pattern of inhibition for three heavy metal ions was observed to be noncompetitive.

This work revealed the presence of a form (or forms) of FMO in bovine liver microsomes. This study also showed the involvement of bovine liver microsomal enzyme FMO in the metabolism of drug substrates, imipramine and chlorpromazine. To our knowledge, FMO was not purified from bovine liver microsomes. Therefore, the results of this work, especially those concerning effects of detergents and modulators on enzyme activity, could be a good reference for future studies aiming to purify FMO isozymes from bovine liver.

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