Non- P450 Oxygenases

Flavin-Containing Monooxygenases
(microsomal FMOs)

Molybdenum Hydroxylases
(cytosolic AO and XO)

Monoamine Oxidases
(mitochondrial MAO-A, MAO-B)

Aldehyde Dehydrogenases  (cytosolic and mitochondrial ALDHs)
Useful literature


• Hutzler et al., Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. *Expert Opin. Drug Metab. Toxicol.* 2013 Early on-line.


Why should we care about non-P450 pathways?

- P450-mediated metabolism dominates oxidative metabolic clearance of drugs, and so, multiple co-administered drugs can be competing for the same clearance pathways, leading potentially to serious drug-drug interactions.
- Genetic polymorphisms that reduce P450 function are well documented and can also cause serious drug-gene interactions, at least for low therapeutic index drugs.
- For both reasons, designing away from P450-dependent clearance mechanisms could be considered a useful strategy during early drug discovery.
- Moreover, as pharmaceutical companies have tried to design ligands for increasingly more complicated biological targets, there has been an inevitable increase in the lipophilicity and molecular volumes of new chemical entities, which have become that appear to be associated with safety failures in the clinic.
- Therefore, replacement of easily incorporated carbocyclic rings (benzene, naphthyl) rings with heteroaromatic rings (pyrimidine, pthalazine, etc) has become an increasingly employed strategy to reduce lipophilicity.
- This has had the effect of switching away from P450 metabolism and towards, especially, aldehyde oxidase as the main metabolic enzyme.
Outline

• History/General Enzyme Characteristics
• Structure/Catalytic Mechanism
• Multiplicity/Regulation
• Substrates and Reaction Pathways
• In Vitro Methodologies for;
  - differentiating FMO-mediated from P450-mediated Catalysis
  - identifying AO- *versus* XO-dependent catalysis
  - discriminating between MAO-A and MAO-B catalysis
FMO History

**1960s**  A liver microsomal enzyme system (E.C. 1.14.13.8) that utilizes NADPH and molecular oxygen to convert N,N’-dimethylaniline to N,N-dimethylaniline N-oxide is described by Dr. Daniel Ziegler and colleagues.

![Reaction diagram](image)

**1970s**  Ziegler’s enzyme’, purified from hog liver, shown to contain flavin, but no heme, thereby distinguishing this flavin-containing monooxygenase (FMO) from the microsomal hemoprotein-containing cytochrome P450s. [1980s – ‘lung’ FMO]

**1990s**  FMO Nomenclature Committee names hog liver FMO as FMO1 and the ‘lung’ form as FMO2. Other forms with <60% sequence identity are named with ascending arabic numerals, FMO3, 4, 5 in all mammals.
## Comparison of General Properties: FMO vs P450

<table>
<thead>
<tr>
<th></th>
<th>FMO</th>
<th>P450</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Function</strong></td>
<td>Monooxygenase</td>
<td>Monooxygenase</td>
</tr>
<tr>
<td></td>
<td>[ S + O_2 + NADPH + H^+ \rightarrow SO + H_2O + NADP^+ ]</td>
<td></td>
</tr>
<tr>
<td><strong>Reducing cofactor</strong></td>
<td>NADPH</td>
<td>NADPH</td>
</tr>
<tr>
<td><strong>Cellular Location</strong></td>
<td>Microsomal</td>
<td>Microsomal</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td>60-65 kDa</td>
<td>50-60 kDa</td>
</tr>
<tr>
<td><strong>Substrates</strong></td>
<td>Oxidizes at N,S,P</td>
<td>N,S,P and C</td>
</tr>
<tr>
<td><strong>Prosthetic group</strong></td>
<td>FAD</td>
<td>Heme</td>
</tr>
</tbody>
</table>
FMO Catalytic Cycle

1. NADP$^+$ oxidized to NADPH, $H^+$
2. FAD-OH oxidized to FAD
3. FADH$_2$ reduced to FAD
4. $O_2$ oxygenase activity
Aspects of FMO Catalysis

- The reaction mechanism is ordered, i.e. NADPH, oxygen and oxidizable substrate add to the enzyme before any of the products leave.
- The enzyme-bound hydroperoxyflavin [FAD-OOH] is a very stable, albeit relatively weak oxidant.
- Release of water and/or NADP$^+$ is believed to be the rate-limiting step.
- FMO can oxidize practically any soft nucleophile that the enzyme’s FADOOH active center encounters.
- In general, uncharged and single positively charged substrates can gain access, in preference to negatively charged and multiple positively charged compounds.
• Crystal structures available only for soluble yeast and bacterial forms of the enzyme that have 20-30% sequence similarity to microsomal FMO.

• All, however, use NADPH as cofactor, and possess conserved cofactor binding sequence and an FMO identifying motif.

• FMO structure has two domains with FAD (green) and NADPH (red) bound near the interface.
• Asn 91 (conserved in all FMOs) may stabilize the C4a-hydroperoxide through polar interactions

• NADP+ may also stabilize by shielding the hydroperoxide from solvent
FMO Multiplicity

- Five active genes, *FMO1-FMO5*, are present in most species.
- *FMO6* is inactive due to alternative splicing.

- *FMO1*, *FMO2* and *FMO3* are the best characterized enzymes.
FMO1: General Characteristics

• The majority of our knowledge of the FMO enzymes is derived from detailed biochemical studies conducted in the 1970s and ‘80s on hog liver FMO1 - the first FMO to be purified.

• FMO1 is characterized by its thermolability and promiscuous substrate specificity, which is the widest of all of the FMO isoforms.

• FMO1 is the dominant form of the enzyme in the liver of most experimental animal species, but is not expressed in adult human liver.

• In humans, liver FMO1 is a fetal enzyme, but is also expressed at relatively high levels in adult kidney.
FMO2: General Characteristics

• Generally found at highest levels in lung tissue of experimental species.

• FMO2 is also characterized by its unusual thermostability.

• Structure-function data obtained with phenothiazines and rabbit FMO2 suggest the C4a-hydroperoxyflavin lies about 6-8 angstroms below the enzyme surface in a channel no more than 8 angstroms in diameter.

• Enzyme is currently the focus of crystallization efforts.

• Genetic polymorphisms in humans, commonly Q472X, renders human FMO2 inactive in all but a small proportion of the population.
FMO3: General Characteristics

• The major FMO enzyme present in adult human liver.

• Possesses reasonably broad substrate specificity, but is more selective than FMO1; i.e. FMO1 selectivity < FMO3 < FMO2.

• Trimethylamine (TMA) is the classical endogenous substrate.

• In 1° trimethylaminuria (TMAU), affected individuals cannot metabolize TMA and so excrete the odoriferous compound in their urine, sweat and breath due to TMAU mutations in the FMO3 gene.
FMO4 and FMO5: General Characteristics

• FMO4 appears to be ubiquitously expressed at low levels in a variety of tissues, but little is known of its substrate specificity, due largely to difficulties in expression of the recombinant enzyme.

• FMO5 is a major FMO form present in human liver, but exhibits little or no catalytic activity towards common FMO substrates.
Regulation of FMO Expression/Activity

- FMO protein expression is **not significantly induced** by pretreatment of animals with common P450 inducers such as polycyclic hydrocarbons or anticonvulsant drugs like phenobarbital.

- High levels of Ah receptor-dependent induction of FMO2 and FMO3 mRNA has been observed in mice, but FMO **activity increased modestly**.

- FMO activity **can be modulated** by sex steroids and glucocorticoids.
  - estradiol/progesterone up regulate FMO2 in rabbit lung,
  - glucocorticoids induces FMO2 in rabbit kidney,
  - testosterone suppresses FMO3 in male mice.
FMO Reactions

- Microsomal FMOs oxidize substrates containing nucleophilic nitrogen, sulfur, phosphorous and selenium atoms.
- The prototypical FMO reaction is N-oxygenation of a tertiary amine, e.g. trimethylamine, N,N-dimethylaniline, to the respective N-oxide metabolite, as shown below.

FAD-OOH: FMO active oxygenating species

![Diagram of FMO reactions](image)
Examples of N-Containing Drugs Metabolized Predominantly by FMO

**BENZYPAMINE** - ~20% of dose excreted in urine as N-oxide

**MOCLOBEMIDE** - ~30% of dose excreted in urine as N-oxide

**ITOPRIDE** - ~75% of dose excreted in urine as N-oxide
Other N-containing Drug Substrates

• While FMO has the capacity to metabolize a vast range of amine-containing compounds, a significant contribution to the metabolic clearance of drugs approved in the US is much more limited.

• Examples include; ranitidine, olanzapine, pargyline, xanomeline and chlorpheniramine.
FMO-catalyzed N-oxygenation

• FMOs can also convert 1° and 2° amines to hydroxylamine, nitrone and oxime metabolites.

\[
R-\text{H}_2\text{C}-\text{NH}_2 \rightarrow R-\text{H}_2\text{C}-\text{NH} \rightarrow R-\text{HC}==\text{N} \]

\[
R-\text{H}_2\text{C}-\text{NH} \downarrow R_1 \rightarrow R-\text{H}_2\text{C}-\text{N} \downarrow R_1 \rightarrow R-\text{HC}==\text{N} \downarrow R_1
\]

• In general, these FMO reactions are considered detoxification pathways.
• However, some secondary hydroxylamines, e.g. those derived from 3,3-iminodipropionitrile and N-deacetyl ketoconazole, have been associated with neurotoxicity and hepatotoxicity, respectively.
FMO-Catalyzed S-Oxygenation

- FMOs catalyze sulfoxide formation from sulfides and (less efficiently) sulfone formation from sulfoxides,

- Other S-containing drug substrates include; cimetidine, methimazole, ethionamide and SM-12502.
FMO-catalyzed Bioactivation

- Most commonly associated with metabolism of S-containing substrates, e.g. thioureas, that can be converted sequentially to reactive sulfenic and sulfinic acids.

- Classical substrates activated in this fashion are the hepatotoxins, thioacetamide and thiobenzamidine.

- A drug example is ethionamide, which requires bioactivation by a *M. tuberculosis* FMO for its antitubercular activity, whereas organ toxicities may be due to human FMO-dependent (off-target) bioactivation.
Tools for Identifying FMO Catalysis in Microsomes

- P450s can catalyze essentially all the same reactions as FMO.
- Main goal is to differentiate FMO activity from that of P450 in microsomes.
- Approach is to
  (A) selectively inhibit P450 or,
  (B) selectively activate or inactivate FMO activity.
Diagnostic inhibitors for FMO?

- No inhibitory antibodies have generated against any FMO isoform.
- No mechanism-based inhibitors of FMO have been described.
- Reversible inhibitors include alternate substrates, such as methimazole.

- However, methimazole is not a specific inhibitor for FMO.
- Other potentially isoform-selective alternate substrates are imipramine (FMO1) and trimethylamine (FMO3).
(A) Selective inhibition of P450

1. Use a P450 reductase antibody.
   - All P450s use the same reductase to transfer electrons from NADPH.

2. Use a mechanism-based inhibitor of the P450s, e.g. 1-aminobenzotriazole (ABT) is a pan inhibitor of microsomal P450, but not FMO.
   - Relies on P450-mediated conversion of ABT to benzyne, which then reacts with P450 heme.
(B) Selective inhibition of FMO

1. Exploit thermolability of FMOs (except FMO2)
   - Heat microsomes at 45oC in the absence of NADPH for ~2 min selectively inactivates FMO1 and FMO3.

2. Exploit FMOs relative insensitivity to non-ionic detergents.
   - Treatment of microsomes with 0.2% Lubrol or Emulgen will inactivate P450s, but not FMOs.
Molybdenum Hydroxylases

Xanthine oxidase (XOR; XO) and aldehyde oxidase (AOX1, AO) are separate gene products exhibiting ~50% amino acid homology. There is only one gene for each in humans.

The enzymes have a complex tripartite structure, comprising two identical subunits of ~145 KDa each.

The enzyme complex typically shuttles electrons from substrates (i.e., the substrate gets oxidized in the process) to an electron acceptor, usually oxygen, (although XD can use NAD⁺). N-oxides, sulfoxides, aromatic nitro compounds and some others can also be reduced.

\[
\text{RH} + \text{H}_2\text{O} \rightarrow \text{ROH} + 2e^- + 2\text{H}^+ 
\]
Substrate Specificity – Guanine derivatives

In the main, XO and AO target sp²-hybridized carbon atoms rendered electron-deficient by a nitrogen atom to which they are linked by a double bond, i.e. -CH=N.

The major substrates for XO and AO are nitrogen heterocycles. Xanthine is the prototypic substrate for XO and allopurinol is a selective XO inhibitor.

XO and AO bioactivate 6-deoxy guanine prodrugs for the anti-viral, penciclovir. This strategy was helpful because the active agents were poorly bioavailable.

XO also metabolizes the anticancer drug, 6-MP (minor pathway).
Additional heterocyclic substrates for AO

- **34, Caffeine**
  - In vivo probe for XO?

- **CYP1A2**
  - AO

- **Zaleplon**
  - Short-acting hypnotic

- **AO**
  - **5-Oxo-Zaleplon**

- **Carbazeran**
  - <5% oral bioavailability, rapid metabolism

- **SGX 523**
  - Renal toxicity due to insoluble lactam metabolite

- **Zaleplon**
  - Renal toxicity due to insoluble lactam metabolite
Metabolism of Iminium ions by AO

AO also plays an important role in the detoxification of potentially reactive iminium ions that are often generated by P450 or MAO from cyclic tertiary amines.

Lactam metabolites are the result, e.g. AO-catalyzed formation of the nicotine metabolite, cotinine, that is formed in by sequential P450/AO metabolism.
Metabolism of Aldehydes by AO

AO can catalyze the oxidation of aldehydes to carboxylic acids, and both endogenous (e.g. retinaldehyde) and xenobiotic aldehydes (below) may be substrates. As with iminium ions, these processes can be sequential.

Note: AO-catalyzed oxidation of aldehydes may not be a highly significant process in vivo. Other enzymes like ALDHs and P450s likely play a role.
Reductive Metabolism by AO (may not happen in vivo!)

AO and XO can catalyze reductive metabolism (in vitro) in the presence of a good electron donor such as xanthine (XO) and 2-hydroxypyrimididine or N-methylnicotinamide (both AO), and in the absence of air.

Ring scissions

Prodrug activation

Two and 6-electron reductions!
Substrate probes for AO

Substrate probe - **Pthalazine**

![Phthalazine](image)

**Internal standard**

2-methyl-4(3H)-quinazolinone (IS)

**Substrate probe – DACA**

![DACA](image)

**Human liver cytosol**

Km is 4-8 µM
Vm is 2-4 nmol/mg/min

Barr and Jones, *DMD* (2011, 2013)
Diagnose chemical inhibitors for AO

Menadione – classic AO inhibitor, but P450 inhibitor too, and cytotoxic to hepatocytes.

Raloxifene – very potent (low nM), uncompetitive AO inhibitor, MBI for CYP3A4, so avoid in hepatocytes.

Hydralazine – selective time-dependent inhibitor of AO. Low potency (Ki ~80 µM), but can use at 25 µM in hepatocytes to estimate fraction metabolized by AO with no effect on P450s (Strelevitz et al., DMD 2012).

Vanillin – alternative (aldehyde) substrate, also a ‘classical AO inhibitor.'
Inter-species, inter-organ and inter-individual variability

**General dogma:**
AO activity is high in humans and monkeys, low in rodents, and absent in dogs.

**Caveats:**
- Large strain difference in rats and mice.
- Gender differences in mice, with males exhibiting 3-4x activity of females.

**Human liver cytosolic activities:**
- Efforts to scale in vitro activity to in vivo typically under-predict AO-mediated clearance.
- Is this due to enzyme lability after processing or to significant extra-hepatic AO catalysis? Note: High AO activities in the human intestine.

**Polymorphisms in human AO:**
- Common coding SNPs at N1135S (13-26%) and H1297R (5-6%).
- These are possible rapid metabolizers (Hartmann et al., *DMD*, 2012).
Catalytic Mechanism

Active site contains the exotic cofactor - molybdopterin. Substrates (RH) react at the Mo (VI) centre.

Catalysis is initiated by base-assisted, nucleophilic attack of the Mo^{VI}–OH group on the electron deficient carbon of the N-C bond, with concerted hydride transfer to the Mo=S group. Hydrolysis of the Mo-O-C bond by water releases oxidized substrate (ROH).
# MAO: General Characteristics

<table>
<thead>
<tr>
<th></th>
<th>FMO</th>
<th>AO</th>
<th>MAO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td>Microsomal</td>
<td>Cytosolic</td>
<td>Mitochondrial (outer membrane) 50 kDa</td>
</tr>
<tr>
<td><strong>Monomer Size</strong></td>
<td>55-65 kDa</td>
<td>145 kDa</td>
<td></td>
</tr>
<tr>
<td><strong>Catalytic center</strong></td>
<td>FAD N-terminal, GxGxxG motif</td>
<td>Mo-pterin (plus an FAD-subunit)</td>
<td>FAD C-terminal covalent cysteiny</td>
</tr>
<tr>
<td><strong>Reducing ‘Cofactor’</strong></td>
<td>NADPH</td>
<td>Substrate</td>
<td>Substrate</td>
</tr>
<tr>
<td><strong>Typical Amine Substrates</strong></td>
<td>Tertiary amines</td>
<td>N-Heterocycles</td>
<td>Primary amines</td>
</tr>
<tr>
<td><strong>Xenobiotic induction</strong></td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Tissue distribution</strong></td>
<td>Liver, Intestine</td>
<td>Liver, Intestine</td>
<td>Nerve terminals (presynaptic sympathetic) Liver, intestine</td>
</tr>
<tr>
<td><strong>Multiplicity (humans)</strong></td>
<td>FMO 1-5</td>
<td>AO (and XO)</td>
<td>MAO-A, MAO-B</td>
</tr>
</tbody>
</table>
Examples of common MAO substrates

Primary amine neurotransmitters are typically MAO-A substrates

Xenobiotics, including environmental toxins, e.g. MPTP, are often MAO-B substrates

However, molecular size is a determinant, with MAO-A typically preferring larger molecules

MAO catalyzes oxidative deamination (cleavage of C-N bond) to form aldehydes
Oxidation of Amines to Imines by MAO-bound FAD
Concerted (polar) mechanism
This mechanism parallels the SET mechanism of P450-mediated N-dealkylation.
Supported by MAO inactivation by cyclopropyl and cyclobutylamines.
Hydrogen atom abstraction (HAT) mechanism(s) also proposed for MAO catalysis.
Metabolism of drugs by MAO

- Not very many examples,
  - drugs with moieties resembling the **indoleamine moiety of 5HT**
  - see citalopram (previous section), an SSRI.

---

- **Sumatriptan** is a 5HT<sub>1</sub>-receptor agonist used therapeutically for the treatment of migraine. In humans, it is metabolized to a single phase 1 metabolite - the indoleacetic acid. This oxidative deamination is catalyzed by liver **MAO-A**. In vitro studies with selective mechanism-based inhibitors (clorgyline for MAO-A) ruled out catalysis by P450 or MAO-B (Dixon et al. *Biochem. Pharmacol.* 47:1253 (1994)).
Diagnostic MAO Inhibitors

MAO-A  Clorgyline

MAO-B  Deprenyl

- Structural basis for inhibitor (and substrate) selectivities is the Y→I change shown above (Geha, RM et al., JBC, 2001).
Crystal structures reveal that the MAO-A has one large substrate cavity (550 Å³), whereas MAO-B’s active site is divided into two, with (400 Å³ substrate cavity and a 290 Å³ entrance cavity ‘gated’ by Tyr326 and Ile199.

Milczek et al., *FEBS J* (2011)
Mechanism-based inactivation of MAO by acetylenes

Covalently bound to the N-5 position on FAD
Bindi et al., *J. Med Chem*, 2004
ALDEHYDE DEHYDROGENASE

- ALDHs catalyze the NAD\(^+\)-dependent irreversible oxidation of aldehydes.
- R-CHO ----> R-CO\(_2\)H
- Catalytic cysteine residue, oxygen comes from activated water molecule
- Homotetramer, 4 subunits of 55kDa
- >20 ALDH genes coding for enzymes with with broad substrate specificities exist in the human genome.
- ALDH1-3 are most closely associated with xenobiotic metabolism.

**ALDH2** - Mitochondrial form, mainly responsible for the metabolism of ethanol-derived acetaldehyde.
ALDH3A1 is expressed at high levels in tumors and, form that of the enzyme that, along with ALDH1A1/5A1, may determine cellular response to the anticancer drug, cyclophosphamide (CP)