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Review

Some distinctions between flavin-containing and cytochrome P450 monooxygenases

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Abstract

This minireview summarizes information concerning the differences and similarities of the human flavin-containing- (FMO, E.C. 1.14.13.8) and the cytochrome P450-monooxygenases (CYP, E.C. 1.14.14.1). Human FMO oxygenates soft nucleophiles. CYP mainly catalyzes C–H abstraction but also oxidizes nitrogen- and sulfur-containing compounds. Both FMO and CYP generally convert lipophilic compounds into more hydrophilic metabolites. The mechanism by which each monooxygenase operates is quite distinct. Sometimes, CYP or FMO bioactivate chemicals to reactive metabolites but to date, drug toxicity thus far observed in the clinic is mainly the result of CYP-dependent oxidation. Both FMO and CYP possess genetic variability that may contribute to inter-individual variability observed for drug metabolism. In contrast to CYP, FMO is not induced or readily inhibited and potential adverse drug–drug interactions are minimized for drugs prominently metabolized by FMO. These properties may provide advantages in drug design, and by incorporating FMO detoxication pathways into drug candidates, more drug-like materials may emerge.

Keywords: Amine-, sulfide-oxygenation; Enzyme mechanism; Genetic variation; Drug development

The flavin-containing monooxygenase (FMO) (E.C. 1.14.13.8) and cytochrome P450 (CYP) (E.C. 1.14.14.1) both catalyze the NADPH-dependent N- or S-oxygenation of heteroatom-containing compounds but each microsomal monooxygenase system is distinct. It was assumed that these reactions were only catalyzed by CYP [1] but like CYP, after FMO was purified to homogeneity, it was shown that the action of FMO was distinct from CYP [2]. Similar to CYP, the FMO is considered to have evolved as a xenobiotic detoxication catalyst to protect mammals from lipophilic nucleophilic plant chemicals in the early environment [3,4]. While FMO requires soft nucleophiles as substrates, CYP can oxidize non-nucleophilic substrates. Similar to CYP, FMO is involved in the oxygenation of a wide range of heteroatom-containing compounds. Generally, FMO converts lipophilic nucleophiles to more polar, readily excreted metabolites and generally decreases the pharmacological activity of the compound. Although CYPs generally convert lipophilic compounds to more hydrophilic materials, they can also oxidize compounds to electrophilic reactive metabolites that can have significant consequences for toxicity. In contrast to CYP where a significant number of compounds inhibit the enzyme, FMO is rarely inhibited. In fact, examples of reactive metabolites that are formed by FMO but do not inhibit FMO have been shown to traverse to CYP and inactivate that enzyme system [5]. Probably because of the difference in enzyme mechanism and regulation of CYP compared with FMO, CYP appears to participate in more metabolic reactions that result in toxicity.

Nomenclature

The number of mammalian FMOs thus far characterized is relatively small compared with the number of

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CYPs. The total number of CYP DNA sequences found in nature is greater than 500 with about 57 for human CYPs. The large number of CYPs may indicate the importance of the significant number of physiological substrates for CYP [4]. In contrast, the number of functional human FMOs is five with an additional six pseudogenes (i.e., *FMO* 7P, 8P, 9P, 10P, and 11P) at a different chromosomal location, about 4 Mb telomeric of the original *FMO* gene cluster [6]. To date, few physiological substrates have been identified for FMO. Similar to CYPs, FMOs with sequence identity >40% have been grouped in the same family (i.e., 1, 2, etc.) [7,8]. For the five forms of functional human FMO (i.e., FMO1 to FMO5), sequence identity ranges between 52% and 60%, with the exception of FMO3 and FMO6 that share 71% identity. CYPs with greater than 59% identity are grouped into subfamilies. Generally, CYPs are defined by spectral properties or sequence similarity and FMOs are mainly grouped by sequence similarity. Like CYP, within a gene family, similar patterns of *FMO* intron/exon organization exist, supporting the notion that the *FMO* gene family probably arose as duplication of an ancestral gene or via a series of independent gene duplication events [6].

Substrate metabolism differences

Thus far, the literature suggests that CYP is responsible for the lion's share of drug and chemical metabolism [4]. For example, in humans, CYP3A4 appears to be the dominant CYP and contributes to over 60% of the metabolism of drugs reported to date. On the other hand, FMO-mediated metabolism has not been associated with a large number of human drugs. The reason for this may be because of the thermal instability of the FMO and the manner in which metabolic incubations have been carried out. For example, initiating metabolic incubations to a pre-warmed hepatic preparation by addition of NADPH generally causes a loss of FMO activity and an inadvertent metabolic shift to CYP [9]. As discussed below, significant inter-individual variation of FMO functional activity has been observed in hepatic microsomes and use of well-characterized hepatic (or other tissue) preparations is essential [10]. Verification of FMO activity with recombinant enzyme is a second useful step [9]. Selective functional substrates such as TMA [11], benzydamine [12,13], (*S*)-nicotine [14], cimetidine [15], itopride [16], clozapine [17], and ranitidine [18] have been used to characterize FMO in in vitro studies. While some of these substrates are also oxidized by CYP, there are various approaches used to distinguish CYP and FMO activities. For example, running microsomal incubations at pH 8.4–9.4 generally abrogates CYP activity (pH optimum of 7.4) and affords conditions for relatively selective FMO activity measurements (pH optimum 9–10). FMO substrates such as nicotine and cimetidine, for example, have shown a 6.6-fold variation in (*S*)-nicotine N-1′-oxygenation activity [14] or a 2.4-fold variation in cimetidine *S*-oxygenation activity [15]. These studies were conducted with microsomes where immunoreactivity and selective functional FMO substrate oxygenation activity were highly correlated [17], and this is a prerequisite for these types of studies.

Another way to distinguish CYP and FMO functional activity is to use thermally inactivated microsomes. In the absence of NADPH, FMO is quite unstable at 50 °C, for example, while under similar conditions, microsomes retain about 85% of the CYP functional activity [9]. Of course, it is important to distinguish microsomes that are highly competent in functional FMO activity from those that are not (i.e., low FMO activity from mishandling of the tissue or improper preparation of the microsomes or storage of the microsomes). Postmortem inactivation of human FMO3 probably accounts for the majority of the loss of FMO functional activity and of the lack of congruence between selective functional FMO3 activity and FMO3 immunoreactivity. Another way to distinguish CYP- from FMO-mediated metabolism is to examine metabolism in the presence of high concentrations of a detergent such as Emulgen that abrogates CYP activity but does not significantly alter FMO activity [19]. As described below, certain chemicals have overlapping substrate avidity for FMO and CYP (i.e., (*S*)-nicotine, dimethylaniline) while other compounds are exclusive substrates for FMO (i.e., TMA) or inhibitors of CYP and substrates for FMO (i.e., cimetidine).

Catalytic mechanism and protein structure

The mechanisms of CYP and FMO catalysis have been summarized elsewhere [20,21]. Even though there are overlapping substrates accepted and similar products formed, the mechanism that each enzyme system uses is quite different. For example, the first step of CYP is thought to be addition of substrate to the enzyme followed by electron transfer from the flavoprotein NADPH-CYP reductase to the substrate-bound CYP. Then electrons flow to the FMN prosthetic group and then sequentially to the CYP to ultimately afford a reactive iron-oxo species, although other peroxy forms of the hemoprotein have been proposed also to be oxidants involved in CYP-dependent metabolism [4]. In contrast, the first step for FMO is reduction of the FAD by NADPH. The next step is formation of a C4a-hydroperoxyflavin by addition of molecular oxygen to the reduced FAD. Therefore, when the substrate is accepted by FMO the enzyme is already in an active form (i.e., a cocked gun) ready to effect oxygenations. FMO is

distinct from CYP in that FMO forms a relatively stable hydroperoxy flavin intermediate [22,23]. CYP forms a relatively unstable ferrous-O_2 complex that can decompose and generate superoxide anion or hydrogen peroxide. The protein environment of FMO apparently protects the hydroperoxy flavin from decomposing, conserving NADPH, and affording an efficient two-electron oxygenating agent for nucleophiles with the appropriate size and shape. Although CYP mainly engages in C-H bond abstraction, it can also effect N- or S-oxidations. For CYP-mediated heteroatom oxidation, oxygen rebound occurs to afford oxides of nitrogen or sulfur compounds. CYP-mediated heteroatom oxidation may be more complex than oxygen rebound to a radical cation. Nevertheless, the mechanism is more complicated than FMO-mediated oxygenation and CYP likely provides opportunity for relatively electrophilic intermediates (i.e., radicals) to form. In addition to the property that FMO forms a relatively stable hydroperoxy flavin, another property is that relatively reactive metabolites formed by FMO do not inactivate the enzyme [1,23] but sometimes leave the FMO product binding domain and inhibit or covalently modify proximal proteins including CYP [24,25]. Only a few true competitive inhibitors of FMO have been reported [26,27], and most of the apparent FMO inhibition is due to alternate substrate competitive inhibition. Another important property of FMO is that the enzyme also retards the acceptance of biological nucleophiles such as glutathione or cysteine [28,29] and does not participate in futile cycles of NADPH consumption and non-productive disulfide formation. The recalcitrance of FMO to biological nucleophile oxygenation has supported the idea that FMO conserves nonproductive NADPH utilization. However, futile cycles for FMO and CYP do exist (i.e., amine oxidation and retro-reduction back to amine) and may be particularly relevant to toxicological aspects of drug metabolism. Another distinction between FMO and CYP is that, barring steric constraints, nucleophilic sites in molecules that are readily oxygenated by peroxides or peracids are also selectively oxygenated by FMO [5] but this is not always the case for CYP. Hence, it is often straightforward to predict initial FMO oxygenation products. The lack of inhibition of FMO coupled with the fact FMO is not normally induced suggests FMO may be a useful metabolic enzyme in drug metabolism to decrease the dependence on CYP and possibly decrease adverse drug–drug interactions that are the result of CYP-mediated processes [10].

Regulation and species differences

Various physiological factors including cofactor supply and diet can regulate FMO expression. Unlike certain CYPs that perform important physiological function and that can be closely regulated, FMO deficiencies have not been associated with profound toxicities or fatalities. However, loss-of-function mutations for *FMO3* cause a condition called trimethylaminuria, the inability to detoxicate and deoderate trimethylamine (an exclusive substrate for FMO3) [30]. Additional CYPs that are non-selective and oxidize a broad spectrum of substrates may have evolved to metabolize substances encountered in the diet or from the environment. In contrast to FMO, these broad spectrum CYPs are often inducible [31]. Very few reports of FMO induction have appeared although the effect of hormones on modulating FMO activity has been described [32]. Functionally active FMO protein expression has been shown to be both tissue- and species-dependent [23,33]. Thus, in human liver, FMO3 and 5 are the prominent species but in rat liver, considerable FMO1 is present [5]. Mouse liver FMO shows enormous variation as a consequence of gender [34]. Consequently, a small animal experimental model of human FMO has not been reported thus far. Transcriptional regulation of FMO involving receptors that bind ligands and interact with DNA has not been as widely studied as other forms of regulation. In contrast, transcriptional regulation of CYP appears to share a common type of regulation. For example, the Ah receptor is important in CYP1A1 regulation [35]. A number of mechanisms to regulate CYP including enhancement of mRNA stability, modulation of heme degradation, enzyme phosphorylation, and protein-protein interactions (i.e., cyt b_5) [4] either do not occur for FMO or have not been reported. Not much has been reported about enhancement of FMO mRNA stability or selective stimulation of translation of FMO mRNA transcripts.

The FMO cofactor, FAD, is tightly bound and is not apparently rapidly turned over in vivo. Like CYP, there is relatively little evidence that post-transcriptional regulation of FMO is important. In contrast to CYPs where mechanisms of ubiquitin-dependent degradation (CYP3A) or vacuolar lysosomal degradation (CYP2B1) have been observed [36], little has been reported about the endoplasmic reticulum-associated degradation of FMOs. Although FMOs have a consensus sequence for N-glycosylation and mass spectrometry studies have shown that FMO1 is selectively N-glycosylated at Asn 120 [37], like CYP19 (i.e., aromatase) the functional significance of this is not certain. It is possible that N-glycosylation is associated with membrane localization but the successful expression of FMO cDNA in bacteria suggests that N-glycosylation is not essential for functional activity. Considerably more information about CYP structure is known compared with FMO. The highly hydrophobic nature of FMOs with apparent multiple internal sites of membrane association has confounded attempts to attain structural information using traditional approaches. Some modeling has been

done for FMO but much more structural information is needed. Thus, mammalian CYP apparently has a membrane association motif while FMO utilizes multiple internal membrane association regions.

Genetic variation

CYPs sometimes show substantial effects of single nucleotide polymorphisms (SNPs) on functional activity and dramatic effects on humans and experimental animals. For example, CYP2D6 and CYP2C19 SNPs show large effects on metabolism of debrisoquine and (*S*)-mephenytoin, respectively [38,39]. FMO SNPs have also been associated with dramatic functional differences in selective functional enzyme activity [40]. However, confounding the influence of genetic variability on CYP-dependent metabolism is the possibility for enzyme induction or inhibition. Because FMO is not readily induced or inhibited, changes in FMO functional activity as a consequence of environmental influences are not likely to be dramatic. As a result, significant variation of metabolism of drugs or chemicals by FMO is probably due to genetic differences. While the number of SNPs reported for FMOs is less than that for CYPs, nevertheless, significant numbers of SNPs are known. For example, based on the *Homo sapiens* chromosome 1 working draft sequence from locus link of GenBank, *FMO1*, *FMO2*, *FMO3*, *FMO4*, and *FMO5* have 34, 57, 40, 30, and 40 genomic DNA variants [41]. This translates into 2, 9, 19, 1, and 2 coding region variants for FMO1, FMO2, FMO3, FMO4, and FMO5, respectively. Several alleles are prominent SNPs in all populations examined to date (i.e., alleles 158, 257, and 308). Decreases in selective functional oxygenation in vitro for FMO3 cDNA-expressed activities have been observed for 158, 308, and 158/308 double mutants [40]. As described below, some of these FMO variants modulate drug or chemical metabolism in vivo. A rare SNP only observed in individuals of African origin (i.e., FMO3 360P) has been associated with an "extensive metabolizer" phenotype [40]. The FMO3 360P SNP could contribute to greater metabolism of FMO-mediated metabolism and possibly greater drug clearance in individuals possessing this SNP. To date, and in contrast to CYP, few examples of observations have been made related to FMO SNPs and alterations in in vivo drug metabolism. Determination of the importance of *FMO3* SNPs was increased after detection of abnormal TMA metabolism in humans. TMA N-oxygenation provides a selective means to phenotype human FMO3 [30] in vivo and study trimethylaminuria, because it is readily available from a large number of dietary precursors and is exclusively N-oxygenated by FMO3.

Another polymorphism affording very great FMO3 functional activity could arise from gene duplication. This has not been observed for FMO3 but SNPs in the promoter region have been associated with an 8-fold increase in promoter activity [42].

Yet another way that inter-individual *FMO* functional diversity is determined is by expression of splice variants. Extensive splice variants have been observed in prominent adult human drug metabolizing tissues (i.e., adult and fetal liver, kidney, and fetal and adult brain) [43]. To date, most FMO splice variants either caused a frame-shift or lacked essential functional sites and were not capable of encoding a functional enzyme. A common in-frame deletion variant encoding deletion of 63 amino acids was identified for *FMO1*, *FMO3*, *FMO4*, and *FMO5* but cDNA-expression of this 63 amino acid variant for a number of FMOs did not afford an active enzyme [43].

Inter-individual differences of FMO

In adult liver tissue, FMO3 attains expression levels approaching 60% of the major CYP3A in adult human liver [44]. FMO1 is not present in adult liver in appreciable quantities but in fetal liver has expression levels of approximately 32% of CYP3A7 based on relative activity measurements [45]. Although a large number of examples of human drugs have not been reported to be metabolized by FMO, nevertheless, considerable FMO enzyme is present in the adult liver. As discussed above, because FMO is not induced, inter-individual differences are presumably due to genetics, and because FMO3 and FMO5 are the prominent forms in adult human liver, these FMOs likely determine the contribution of FMO to drug and chemical metabolism. However, FMO5 appears to possess narrow substrate specificity and a relatively low activity and thus, genetic variation of FMO3 may largely determine inter-individual variability due to FMO. Microsome correlation analysis of the FMO-mediated oxygenation of a substrate with FMO immunoreactivity with well-characterized microsomes is generally a good starting point to examine a role of FMO in compound metabolism [9].

Thus far, a few cases (i.e., benzydamine, (*S*)-nicotine, ranitidine, and cimetidine), have shown that FMO3 possesses significant inter-individual variability in drug or chemical metabolism in vivo [15,18,46,47]. Thus, smokers infused with (*S*)-nicotine-d_2 or smokers administered (*S*)-nicotine via the dermal route varied 3.3-, 11.3-, and 7.1-fold, respectively [47]. Inter-individual variability for cimetidine oxygenation is less than that for nicotine based on pharmacokinetic parameters (i.e., AUC, $t_{1/2}$ and CL in the range of 1.7- to 2.6-fold) and this could also be due to *FMO* genetic variability. Both in vivo (*S*)-nicotine and cimetidine oxygenations can be used for determination of the stereoselectivity of FMO3 in vivo [48]. In studies of ranitidine, humans showed a 2-fold

variation in urinary *N*-oxide as a function of FMO3 genotype [18].

Role in drug development

It may be advantageous to have a significant contribution of FMO to the metabolism of a drug [10,49]. This may be because by decreasing the dominance of CYPs in the metabolism of a drug by increasing the involvement of FMO, this may lead to fewer adverse drug–drug interactions that are the result of CYP action. Because inter-individual variability of FMO is likely due to genetic variation and not inhibition or induction, genetic screening of *FMO* may allow an easier prediction of abnormal drug metabolism. The lack of substrate specificity for FMO by commonly used drugs may also contribute to a decrease in adverse drug–drug interactions. Because the majority of drugs in use today do not serve as FMO substrates, they will not undergo alternate substrate competitive inhibition of new drug candidates metabolized by FMO and this should ultimately lead to new classes of drugs with decreased adverse drug–drug interactions. Decreasing potential drug–drug interactions by developing drug candidates that are metabolized by multiple enzyme systems (including FMO) that decrease the reliance on one enzyme system may be advantageous.

In summary, CYP and FMO are both monooxygenases and quite distinct from a mechanistic standpoint but share several common features: both enzymes are broad spectrum monooxygenases that appear to sacrifice considerable enzyme velocity to bind a wide range of substrates. CYP and FMO both differ in the types of substrates accepted, although they share a similar general function. While the catalytic rate of FMO-mediated oxygenation is nominally about twice that of the catalytic rate for a typical CYP substrate [20], FMO-mediated metabolism appears to be centered on highly nucleophilic substrates and CYP accepts considerably less nucleophilic compounds. CYP oxidizes compounds via sequential one-electron processes while FMO oxygenates compounds with a two-electron mechanism and this may contribute to the greater prevalence of toxicity observed for CYP-dependent metabolism compared with FMO. While both CYP and FMO have shown considerable genetic variation, it has been the CYP genetic variants that have been associated with the majority of toxic consequences observed to date.

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