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RECENT STUDIES ON THE STRUCTURE AND FUNCTION OF MULTISUBSTRATE FLAVIN-CONTAINING MONOOXYGENASES

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INTRODUCTION

Interest in the oxidation of xenobiotics catalyzed by flavin-containing monooxygenases has expanded dramatically over the past decade. Studies on their exceptional substrate range, wide tissue distribution, and the existence of multiple forms are perhaps the major developments that have stimulated recent work on these enzymes. In addition, the reports by Smith and his associates on the polymorphism of the N-oxygenation of trimethylamine (1) and nicotine (2) in humans apparently due to a genetic defect in one or more of the flavin-containing monooxygenases has fueled further interest in the role of these enzymes in the metabolism of xenobiotics.

This review focuses primarily on recent work on the number of isoforms (based largely on differences in primary structures deduced from cDNAs) present in major organs of entry, possible methods for estimating substrate specificity and activity of different isoforms in crude tissue preparations, and methods for manipulating hepatic concentration (activity) of flavin-containing monooxygenases with dietary xenobiotics. The latter work, while still in very early stages, will undoubtedly have a significant impact on this area of drug metabolism by providing a convenient model for assessing the effect of changes in the concentration of these enzymes on the metabolism of a specific

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drug in live animals. It also demonstrates for the first time that flavin-containing monooxygenases, like virtually all other enzymes of detoxication, are induced by xenobiotics.

However, before discussing recent progress on these aspects, a brief review is given of the catalytic mechanism responsible for the exceptionally broad range of substrates accepted by these enzymes. A clear understanding of the chemical steps in the catalytic cycle is essential for insights into structural features that may determine, at least in part, the substrate specificity of different forms of the flavin-containing monooxygenases. The unusual catalytic cycle also distinguishes these enzymes from other monooxygenases bearing flavin prosthetic groups and is the only basis for their classification as a distinct subgroup of flavin-dependent monooxygenases. The criteria for classifying a flavoprotein with these multisubstrate flavin-containing monooxygenases along with problems of nomenclature are discussed. Although the nomenclature committee of the International Union of Biochemistry discourages the use of acronyms for enzymes, the abbreviation FMO is so widely used, and apparently accepted, that this usage is continued in this review.

PROPERTIES DISTINCTIVE TO FMOs

Flavin nucleotides are exceptionally versatile molecules that in different protein environments, either alone or in conjunction with other functional groups, have a role in many different redox reactions involving the metabolism of xenobiotics. Most of these are well known and include reductases (3, 4), oxidases (5, 6), as well as monooxygenases (7, 8). Here we are concerned only with a subgroup of the flavin-dependent monooxygenases that share a mechanism distinctly different from all other known monooxygenases (9, 10). Because the mechanism of these enzymes is the defining characteristic responsible for virtually all of their properties of primary interest to pharmacologists and toxicologists, the catalytic cycle of these flavoenzymes is summarized in the following section.

Mechanism

The catalytic cycle shown in Figure 1, based on detailed kinetic and spectral studies (11–13) with FMO purified from pig liver microsomes and to a more limited extent on FMO from rabbit lung microsomes (14), illustrates the major steps characteristic of these enzymes. Unlike all other monooxygenases bearing flavin or other prosthetic groups, the oxygenatable substrate is not required for dioxygen reduction by NADPH (15). FMO is apparently present within cells in the very reactive 4a-hydroperoxyflavin form and any soft nucleophile that can make contact with this potent monooxygenating agent (16) will be oxidized. The product (SO), formed by oxygen transfer from the

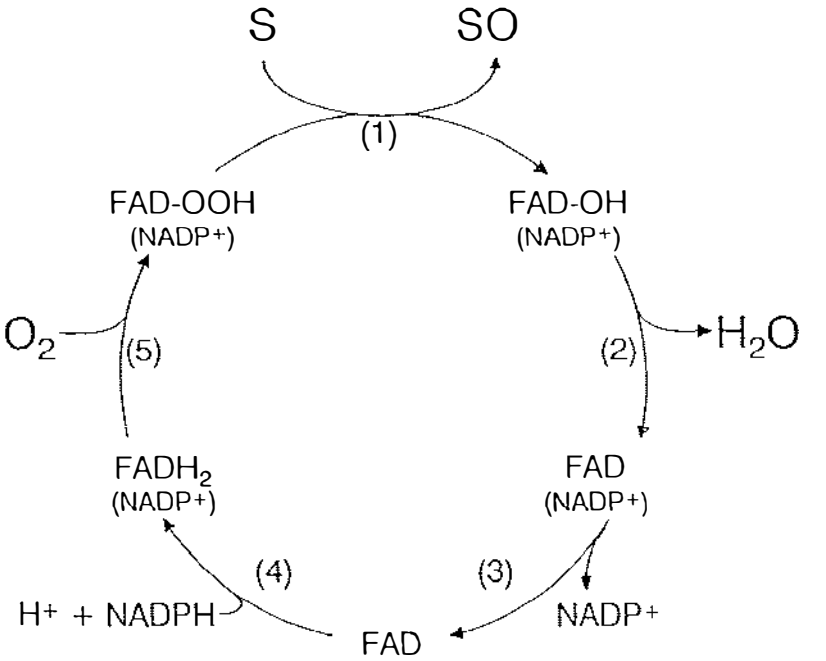

Figure 1 Major steps in the catalytic cycle of FMO. S and SO are the xenobiotic substrate and its oxygenated product, respectively. (Figure from Ref. 15, by permission.)

hydroperoxyflavin to the nucleophile, is released immediately. Steps 2–5 simply regenerate the enzyme-bound oxygenating agent from NADPH and oxygen and, as stated above, the xenobiotic substrate is not required for any of these steps.

A single point of contact between the xenobiotic and the terminal oxygen of the hydroperoxyflavin is all that is required for product formation. It is this property, unique to FMOs, that is responsible for the extraordinary broad substrate specificity of these flavoenzymes. Because the energy required to drive the reaction is present in the enzyme before it encounters the xenobiotic, precise fit usually required to lower the energy of activation of an enzyme-catalyzed reaction is not necessary and FMOs catalyze, often with equal facility, the oxidation of compounds as dissimilar as iodide (17), boronic acids (17), phosphines (18), most functional groups bearing sulfur or selenium (10, 19), as well as a host of synthetic and naturally occurring amines and hydrazines (8, 20).

The catalytic cycle (Figure 1) consistent with such broad specificity limits possible models describing differences in the substrate specificities of FMO

Table 1 Classification of mammalian flavin-containing monooxygenase based on primary structure

Class	Species	Level	Genbank #	Ref.
1A1	Pig	cDNA	M32031	26
	Rabbit	cDNA	M32030	27
	Rabbit	protein		28
	Human	cDNA	M64082	29
	Rat	cDNA	M84719[a]	
1B1	Rabbit	cDNA	M32029	27
	Rabbit	protein	—	30
	Guinea pig	cDNA	—	31
1C1	Rabbit	cDNA	—	32
	Rabbit	protein		33
1D1	Rabbit	cDNA		32
	Rabbit	protein		35
	Human	cDNA	M83772	34
1E1	Rabbit	cDNA	—	32
	Human	cDNA	—	36

[a] Sequence published only in GENBANK by K. Itoh.

isoforms and militates against imprecise use of terms to describe enzyme-catalyzed reactions. For example, the use of high or low "affinity" to describe differences based on K_m is never correct and these expressions are especially inappropriate with enzymes such as FMOs, which require only a single point of contact between enzyme and the xenobiotic substrate. Despite the lack of tight binding, FMO-catalyzed reactions are fully enzymic and follow saturation (Michealis-Menton) kinetics (11). While a single point of contact between the xenobiotic substrate and the enzyme-bound oxidant will often suffice, this does not preclude more complex interactions with some substrates of a specific FMO. For example, it is very probable that substrate binding lowers the energy of activation for the oxidation of cyclohexanone by the bacterial cyclohexanone monooxygenase (21), but the oxidation of organic sulfur compounds and boronic acids catalyzed by this flavoprotein, like the mammalian FMOs, probably requires only a single point of contact between the enzyme and substrate for catalysis. In addition, the observation that K_m for a homologous series of substrates bearing the same functional group often decreases with increasing lipophilicity (22) does not invalidate the preceding analysis. This may simply be due to nonspecific absorption of the more lipophilic analogs on the enzyme.

ISOFORMS

Definitive evidence for the existence of more than one form of FMO in a single species was reported at about the same time by Williams et al (23) and Tynes et al (24). Both groups described the isolation of a flavin-dependent monooxygenase from rabbit lung microsomes that was distinctly different in substrate specificity and immunochemical properties from liver FMO. Subsequent studies (25) quickly demonstrated that tissues from other species also contained two or more immunochemically different forms and in the past two years a number of isoforms clearly different in primary structure have been described.

The primary structures of five FMO isoforms from the rabbit (Table 1) are from 52–57% identical. The direct amino acid sequence determined on four forms of FMO isolated from rabbit tissues by Ozols (Table 1) agree with the sequences deduced from their cDNA and the few differences noted may be due to allelic variants or to experimental error. Orthologs of the rabbit FMO isoforms, present in other mammals, including humans, are from 86–88% identical in primary structure to the rabbit forms. Preliminary work by Philpot and his associates (32) obtained by blot analysis of genomic DNA suggests that all five genes present in the rabbit are also present in humans, guinea pigs, hamsters, rats, and mice.

The sequence identities based on the mean score of all published mammalian FMO calculated using the MACAW sequence-analysis program (37) provided by The National Center for Biological Information (NCBI-NIH) is illustrated graphically in Figure 2. The sequence around the flavin (residues 9 to 14) and the NADPH (residues 191 to 196) binding sites are virtually identical in all forms of mammalian FMO. The region from 325 to 400 is also highly conserved but there is almost no sequence identity near the C-terminus. The relationship between structure and function at regions other than the putative FAD and NADPH binding sites is not known.

It should be emphasized that at present the definition of FMO is based only on function and not on structure. While the more than 50% identities of the mammalian flavoproteins listed in Table 1 is strong evidence for similarity in mechanism, this may not be true in other vertebrates or organisms even further removed from mammals. For example, a search of the NCBI-NIH data bank for proteins bearing sequences similar to one or more of the mammalian FMOs identifies a bacterial cyclohexanone monooxygenase in addition to a number of functionally different flavoproteins. While there is no question that the cyclohexanone monooxygenase from *Acinetobacter* is mechanistically similar to and can be properly classified as an FMO, its primary structure (38) is only about 30% identical to its nearest mammalian relative. On the other hand, sequence similarities of pig liver FMO to glutathione reductase and some

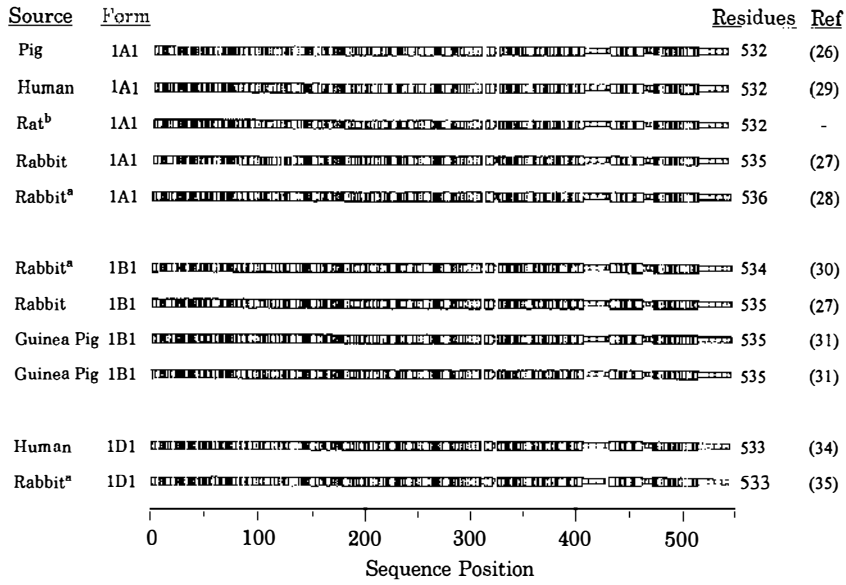

Figure 2 Mean score identities of the amino acid sequences of all published mammalian FMOs. Dark bands represent regions that are more than 90% identical in primary structure. (*a*) Sequence determined from isolated protein. All others deduced from cDNA. (*b*) sequence published only in GENBANK by K. Itoh.

other flavoproteins are also close to 30% (due primarily to the FAD and NADPH binding regions) but they are functionally very different from FMO. Attempts to identify an FMO solely from sequence similarities deduced from cDNA without any information as to the catalytic properties of the expressed protein should at present be restricted to those that are at least 80–85% identical with an FMO of known function.

Nomenclature Based on Structure

The existence of multiple forms of FMO in a single species creates problems in nomenclature that were discussed at length at an informal meeting of participants at the symposium on Multisubstrate Flavin-containing Monooxygenases held at the 1992 FASEB meeting in Anaheim, California. Although one method of naming FMO isoforms is not completely satisfactory at this time, the participants were strongly encouraged to adopt the method introduced by Philpot and his associates patterned after the nomenclature used for P450-dependent monooxygenases (39). In this system the 52–57% identity in the primary structures of the five forms of FMO in the rabbit (Table 1) describes a gene family designated "1. This gene family contains five

subfamilies labeled A through E and different gene products in the same subfamily are also designated by Arabic numerals. Thus, the known isoforms in the rabbit are named rabbit FMO-1A1, -1B1, -1C1, -1D1, and -1E1. Orthologs from other species that are at least 85% identical in primary structure to one of these rabbit forms are given the designation for that class as illustrated in Table 1.

Because of clear evidence that more than one form is often present in a single tissue and tissue-selective expression varies with species, the use of "liver" or "lung" FMO should be dropped, along with other trivial designations such as form 1, form 2, etc. Although this system of nomenclature based on structure is straightforward and should be adopted whenever possible, amino acid sequence cannot give any definitive information on similarities or differences in substrate specificities in different tissues or species. At present a single system of nomenclature does not meet the requirements of all investigators and the following section describes another method for the classification of these enzymes based on differences in substrate specificities that can be used to classify FMO activities (isoforms) in crude tissue preparations.

Isoform-selective Substrates

The substrate specificities of only a few FMO isoforms have been examined in detail and direct comparisons have been limited largely to rabbit 1B1 (14, 40) and pig 1A1 (9, 40). Although from different species, the comparisons of these two forms identified qualitative differences in substrate specificities that could be exploited to devise enzymic methods for measuring the distribution of FMO isoforms in crude tissue preparations by activity measurements. For example, structure-activity studies carried out with both organic nitrogen and sulfur nucleophiles demonstrated (40) that access to the 4a-hydroperoxyflavin is quite different in these two FMO isoforms. Compounds bearing substituents within 3Å of the nucleophilic heteroatom that were more than 8Å in their longest axis were totally excluded by the rabbit FMO-1B1 but not by pig FMO-1A1. Thus, the overall size of the nucleophile appears to be a major factor limiting access to the 4a-hydroperoxyflavin in different FMO isoforms, and by simply measuring the oxidation of a functional group bearing substituents of increasing size, such differences should be readily identifed. To test this possibility, Athena Guo in my laboratory examined the oxidation of five thiocarbamides that varied in surface areas from 91 to 326 square Å in reactions catalyzed by microsomes from different tissues and species and, as anticipated, the rates decreased with increasing substrate size with all preparations tested (41). The pattern was also species- and tissue-specific and, without exception, microsomal preparations that did not catalyze the oxidation of a thiocarbamide intermediate in size did not

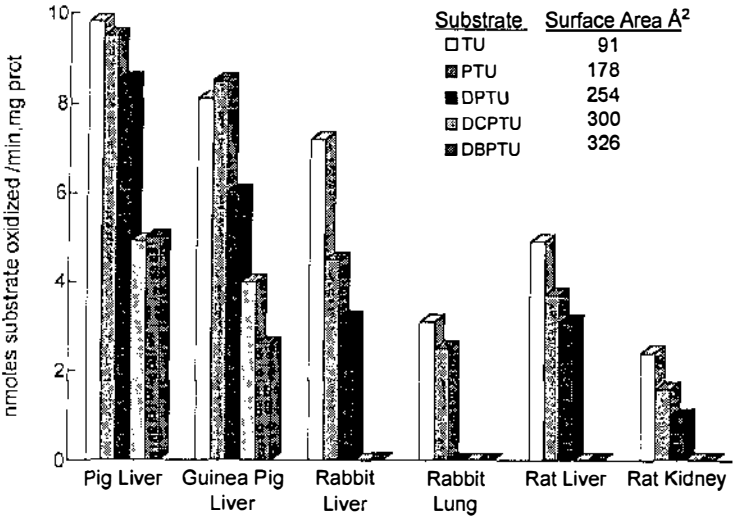

Figure 3 Species and tissue differences in FMO catalyzed oxidation of thiocarbamides that differ in size. (*a*) Abbreviations used are: thiourea TU, phenylthiourea PTU, 1,3-diphenylthiourea DPTU, 1,3-bis(3,4-dichlorophenyl)-2-thiourea DCPTU, 1,1-dibenzyl-3-phenyl-2-thiourea, DBPTU.

accept larger thiocarbamides, whereas all smaller analogs were readily oxidized. The species and tissue differences in thiocarbamide substrate specificities, illustrated graphically (Figure 3), show some striking differences. Only microsomes from pig and guinea pig liver catalyze the oxidation of thiocarbamides larger than 1,3-diphenylthiourea and only the latter can distinguish between 1,3-bis-(3,4-dichlorophenyl)-2-thiourea and the somewhat larger 1,1-dibenzyl-3-phenyl-2-thiourea. While all preparations except rabbit lung microsomes catalyzed the oxidation of 1,3-diphenylthiourea, the rates were always less than those with either phenylthiourea or thiourea. Surprisingly, preparations from rabbits and rats were able to distinguish between phenylthiourea and thiourea. Tissues from these species apparently contain a FMO isoform that accepts small substrates such as N,N-dimethylaniline or methimazole (41, 42) but ones as large or larger than phenylthiourea are excluded.

Nomenclature Based on Activity

These differences in rates as a function of substrate size suggest that there are at least five distinct forms of FMO—arbitrarily labeled A through E—that can be identified by such activity measurements. Form A catalyzes the oxidation of all five thiocarbamides whereas form E only accepts substrates

smaller than phenythiourea. The size cutoffs for isoforms between these extremes labeled B, C, and D are illustrated in Figure 4.

Other than the major forms purified from pig liver and rabbit lung, the relation between those identified only by activity or by structure is not known at present. While pig liver form A (Figure 4) is identical to 1A1 (Table 1) and rabbit D with 1B1 (Table 1), the structural isoforms of 1A1 in rat and rabbit tissues were either not present in amounts detectable by activity measurements or they differ in substrate specificity from the pig liver 1A1. The latter possibility is the more likely and until the substrate specificity of a specific isoform has been determined with the purified protein, conclusions as to substrate specificities across species must be interpreted with caution.

On the other hand, size restrictions for substrates accepted by different isoforms determined with thiocarbamides can be extrapolated to substrates bearing other functional groups. For instance, isoforms D and E that exclude 1,3-diphenylthiourea also will not catalyze N-oxygenation of impramine, chlorpromazine, or other tricyclics where the side chain nitrogen is within 3Å from the tricyclic ring system whose dimension through its longest axis exceeds that of 1,3-diphenylthiourea (40, 41). In addition, the species distribution of isoforms determined by activity with thiocarbamides also offers an explanation for the differences in the role of FMO in the N-oxidation of pyrolizine alkaloids by microsomes from different species. The overall size of an alkaloid as large as senecione would be readily accepted only by isoforms A and B. These forms are present in detectable amounts only in liver microsomes from pig and guinea pig and it is, therefore, not surprising that the contribution of FMO to the N-oxidation of this alkaloid is high in guinea pig liver (43) but negligible in rat liver (44).

The preceding examples illustrate the type of information on substrate specificities that can be deduced from activity measurements with thiocarba-

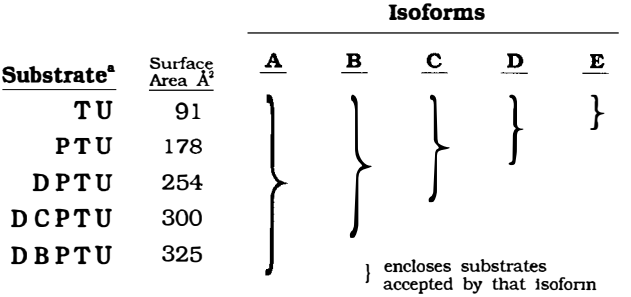

Figure 4 Specificities of FMO isoforms as a function of substrate size.(*a*) See legend to Figure 3 for definition of abbreviations.

mides that differ in size. The procedure developed for measuring the oxidation of thiocarbamides to their sulfenic acids (42) is also relatively simple and quite sensitive. Furthermore, with liver tissue from mammals activity measurements can be carried out with whole homogenates. In other tissues with activities much lower than liver, reliable measurements can be obtained with particulate fractions freed from most of the cytosol by one centrifugation. This simplifies tissue preparation and avoids most of the problems involved in quantitative recovery of the endoplasmic reticulum of a tissue homogenate in the microsomal fraction isolated by differential centrifugation.

The proposed nomenclature based on differences in the oxidation of thiocarbamides that differ in size has obvious limitations in that activity is not necessarily associated with a single entity. For instance, it is conceivable that different structural isoforms of FMO could have the same substrate size limits and activity measurements alone could easily miss or underestimate the total number of FMO gene products in a species. These measurements do, however, provide a convenient and rapid method for measuring the size limits of substrates accessible to these enzymes in crude tissue preparations and this information can be used to predict access of other types of xenobiotic soft nucleophiles to the active sites of FMO isoforms present in that tissue preparation. In addition, because these flavoproteins discriminate among xenobiotic soft nucleophiles by excluding nonsubstrates rather than selectively binding their substrates, it is very likely that the substrate specificity of isoforms A through E will be the same across species and tissues. Until methods for measuring the expression of FMO isoforms at the protein level along with measurements of their substrate specificities are developed, a dual system of nomenclature emphasizing different properties of these enzymes appears necessary. A nomenclature based on structure alone is especially useful for defining genetic and evolutionary relationships, whereas the nomenclature based on differences in substrate specificity stresses function and will be of most interest to investigators studying the metabolism of xenobiotics.

Species and Tissue Distribution

While most of the studies on FMO have been carried out with mammalian tissues, it has been known for some time that flavoproteins similar in mechanism to mammalian FMOs are present in *Acinetobacter* (21) and *Trypanosomi* (45). The presence of enzymes similar to FMO in these organisms suggests that multisubstrate flavoproteins are probably widely distributed in living organisms but literature on FMOs in species other than mammals is quite sparse. However, a number of reports, most very recent, demonstrate that dimethylaniline N-oxidase and methimazole S-oxidase

(activities characteristic of FMO) are present in tissues from marine invertebrates (46–48), marine elasmobranchs (49), and freshwater fish (50). Tissue distribution studies by Schlenk & Buhler also show that FMO is readily detectable in the visceral mass and gill tissues of oysters (48) and in liver, kidney, and gill microsomes of the rainbow trout (51). These authors also conclude that the bioactivation of the pesticide, aldicarb, is catalyzed primarily by FMO in the trout. These studies indicate that FMO in marine animals, as in mammals, is concentrated in the major organs of entry and undoubtedly plays a significant role in the oxidative metabolism of xenobiotic soft nucleophiles in these species.

Recent studies on the tissue distribution of FMOs in mammals have focused primarily on structural isoforms identified as described in a preceding section, but how this is related to function is largely unknown. However, there is some rather compelling evidence that mammalian FMO isozymes that differ in substrate specificities are expressed in different tissues as a function of development or hormonal manipulations. For instance, in rats the isoforms that exclude impramine are apparently under somewhat different hormonal control in liver, lung, and kidney from those that accept this tricyclic antidepressant (52). A report by Lemoine et al (53) also suggests that isoforms catalyzing N-oxygenation of imipramine are absent in adult human liver, whereas forms that accept N,N-dimethylaniline are readily detected. On the other hand, kidney microsomes from adult humans catalyze rapid N-oxidation of imipramine, and in humans this organ may be the major site for the N-oxidation of imipramine and chlorpromazine, which are major metabolites in the serum of humans treated orally with these tricyclic antidepressants (54). Human kidney microsomes also catalyze rapid oxidation of sulindac sulfide (55) and it is possible that, unlike most other mammals, the substrate specificities of human kidney FMOs is broader than that of these flavoenzymes in the liver. The observations (29) that an FMO 88% identical to the pig liver enzyme in amino acid sequence is expressed in adult human kidney and fetal liver but not in liver from adults, is also quite suggestive. However, until the substrate specificities of the human FMO isoforms are more clearly defined, the similarity in structure of the human adult kidney and fetal liver FMO to the pig liver FMO-1A1(A) does not necessarily mean that their substrate specificities will be the same.

In addition, the contribution of FMO isoforms to the metabolism of xenobiotic soft nucleophiles present in other human tissues is essentially unknown. Until more is known about the expression of FMO isoforms in different tissues, speculation as to the loss of which isoform is responsible for the genetic polymorphism in the metabolism of trimethylamine also appears premature.

INDUCTION OF FMO

Studies on the modulation of FMO in vivo have been largely restricted to developmental (56, 57) and hormonally controlled changes (14, 53, 58) or to decreased activity upon food restrictions (59–61). An early attempt (62) to induce FMO by treating rats with phenobarbital either produced no change or decreased activities characteristic of FMO and it was assumed that these flavoenzymes are not regulated by exposure to xenobiotics. However, at the time these experiments were carried out it was not known that FMO only catalyzes the oxidation of soft nucleophiles and the possible induction of these enzymes by dietary xenobiotics was therefore overlooked.

The first indication that FMO may be regulated by dietary xenobiotics was obtained by Kaderlik et al (63) who found that there was a selective, rapid drop in FMO protein and activity in the liver of rats maintained on total parental nutrition for as little as two days and by seven days FMO was only 20–25% of controls. A similar but a somewhat smaller decrease in FMO activity was observed in rats maintained on a semisynthetic oral diet up to nine days and activity was restored (induced) by adding an extract of rat chow to the semisynthetic diet. These results suggest that FMO is probably induced by one or more organic nitrogen or sulfur xenobiotic soft nucleophiles so abundant in food derived from plants (64, 65). It is likely that FMO and other enzymes regulated by xenobiotic soft nucleophiles are already maximally induced in animals maintained on commercial rat chow and induction can be observed only after the animals are maintained on a diet relatively free from such xenobiotics. While the compounds were not identified, Kaderlik (unpublished observations) found that commercial rat chow contained no less than 290 nmoles/gm of FMO substrates with K_ms of 40μM or less based on total extract-dependent oxygen consumption catalyzed by purified pig FMO-1A1(A). This method cannot accurately measure the concentrations of substrates with much higher K_ms but they were probably present and the total concentration of xenobiotic soft nucleophiles in rat chow appears substantial.

Nnane & Damani have recently measured the effects of diets relatively free from xenobiotics on the pharmacokinetics of ethylmethylsulfide (66) and trimethylamine (67) in rats. The in vivo dispositions of both xenobiotics were significantly altered in animals on a semisynthetic diet and the decreased clearance of both correlated quite well with the changes in the concentration (activity) of liver FMO. Whether the semisynthetic diets affect some FMO isoforms more than others is not known, but this can be readily tested in future studies.

In addition, this work offers a convenient live animal model for studying the effects of changes in FMO concentrations on the metabolism of drugs and other xenobiotic substrates for these enzymes. However, it should be

emphasized that the semisynthetic diets will very likely affect glucuronyl transferases (68) and perhaps S-methyl transferases (69) that also catalyze detoxication of xenobiotic soft nucleophiles. Correlations between changes in clearance of a drug and FMO concentrations do not necessarily prove that oxidation is necessary for clearance.

ROLE IN METABOLISM

The functions of FMO in the metabolism of xenobiotics has been discussed in considerable detail in several reviews (8–10, 70–72) and only some of the more recent work on the role of these enzymes in metabolism of organic nitrogen or sulfur xenobiotics that significantly extend or highlight earlier studies is taken up here. In general, the problems of defining the contributions of these enzymes to the metabolism of a xenobiotic, outlined previously (8), still remain. While the manipulation of liver FMO by diet and potential induction of specific isoforms by different xenobiotics as described in the preceding section may help, at present the possible contribution of FMO to the oxidative metabolism of xenobiotics is often based on eliminating other monooxygenases, principally the P450 family of monooxygenases. Such an approach, however, assumes that FMO and P450-dependent monooxygenases are the only enzymes present in microsomes that can catalyze NADPH and oxygen-dependent oxidation of xenobiotics. While this may be true for some (perhaps most) xenobiotics subject to metabolic oxygenation, the studies of Sausen & Elfarra (73) suggest that the microsomal monooxygenase catalyzing the S-oxygenation of cysteine S-conjugates is quite different in properties from P450-dependent monoxygenases or any of the known FMOs. The assumption that this enzyme is a variant of FMO with very different substrate specificities may be correct but until it is purified and characterized, the possibility that it is a new type of xenobiotic multisubstrate monooxygenase cannot be ruled out.

The N-oxygenation of trialkylamines is a significant route for the oxidative metabolism of a large number of medicinal amines and naturally occurring alkaloids with basic side chains (8, 71) and formation of an aliphatic N-oxide is usually good presumptive evidence for a role of FMO in the metabolism of a specific xenobiotic amine. Because the N-oxidation of most acyclic aliphatic tertiary amines is catalyzed largely, if not exclusively, by FMOs, only recent studies on amines that are metabolized primarily by N-oxygenation are taken up here.

Amines

The role of FMO in the metabolism of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydrapyridine (MPTP) has been determined in mice (74, 75), rat

hepatocytes (76), and cultured mouse astrocytes (77). These studies indicate that MPTP, a known substrate for purified pig FMO-1A1 (78), is also N-oxygenated both in vivo and in vitro by enzymes with the properties of FMO. For example, the formation of the N-oxide by mouse astrocytes was stimulated by N-octylamine and inhibited by alternate substrates for FMO, and in live mice preadministration of an alternate substrate for FMO such as methimazole blocks N-oxygenation and increases toxicity by shunting more MPTP to neural monoamine oxidase, where it is oxidized to the ultimate toxin, MPP+ (N-methyl-4-phenylpyridinium). These studies clearly demonstrate that N-oxygenation can be a major route for the detoxication of aliphatic tertiary amines. In addition, the rather high rate of MPTP N-oxide formation by cultured mouse astrocytes indicates that an enzyme similar in catalytic properties to liver FMO in this species is present in neural tissue in significant quantities. The presence of FMO in microsomal fractions from brain tissue had been reported earlier by Bhamre & Ravindranath (79).

Although trialkylamine oxides are usually sufficiently stable to isolate (54) from biological materials, a report by Cashman (80) demonstrates that 3,4-dimethyoxystyrene, the major metabolite produced by the microsomal catalyzed oxidation of verapamil, is formed by decomposition of the enzymically generated N-oxide. Unlike most other trialkylamine oxides, verapamil N-oxide is surprisingly susceptible to thermal decomposition by Cope elimination.

Cashman and his associates have also measured the enantioselective N-oxidation of chlorpheniramine (81) and zimeldine (82) by purified pig liver FMO-1A1 and by liver microsomes from rats and pigs. The data suggest that the N-oxygenation of both drugs is catalyzed exclusively by FMO. Pig liver microsomes, like the purified FMO, had a marked preference for the Z enantiomer of zimeldine and for the D form of chlorpheniramine; whereas rat liver microsomes preferentially catalyzed N-oxygenation of the E enantiomer of zimeldine. Because the enantiomers of these drugs differ in some pharmacological properties, the marked species differences in enantioselective N-oxygenation may contribute to species differences in some of the effects of these drugs.

Marked species differences have been reported in the N-oxidation of methapyriline (83), the cardioprotective drug, stobadine (84), and of the pyrrolizidine alkaloid, senecionine (43). With the last two drugs activity of guinea pig liver is five to ten times that of rat or rabbit microsomes, which, as mentioned earlier, is consistent with differences in the specificities of the major FMO isoforms present in the liver of these species. The biological basis for such differences is not known, but the exceptionally high activity in the guinea pig indicates that across species factors other than diet may be involved in the expression of FMO isoforms.

Xenobiotics Bearing Sulfur

Most functional groups bearing sulfur show substrate activity with FMOs purified to homogeneity from liver microsomes of several species (8, 70, 72). However, unlike tertiary alkylamines, the oxidation of organic sulfur compounds does not produce metabolites that are unique to FMO. The sulfenium cation radical is more stable than most alkylamine cation radicals and oxidation of organic sulfur compounds by sequential one-electron or by a direct two-electron oxidation usually produces the same products (85). However, by the use of inhibitors and/or alternate substrates it is usually possible to determine the contribution of various peroxidases, oxidases, or monooxygenases to the oxidation of organic sulfur xenobiotics. Most of the information available on the functions of FMOs on the metabolism of xenobiotic sulfur compounds comes from the studies of Hodgson and his associates and this work has been recently reviewed (8, 10, 70, 72). Hodgson et al (86) have also recently described problems of measuring FMO catalyzed S-oxygenations in preparations containing detergents.

Because of facile nonenzymic oxidation of functional groups bearing sulfur, biochemical mechanisms are often difficult to define unambiguously. However, most investigators agree that thiourea and other aliphatic thiocarbamides such as ethylene thiourea and the closely related 2-mercaptoimidazole (methimazole), are oxidized exclusively via FMOs. Recent studies on the covalent binding of ethylene thiourea and of methimazole to microsomal proteins described by Hui et al (87) and Decker & Doerge (88, 89) are consistent with the known properties of their respective oxidation products produced via FMO. Both are oxidized to their sulfenates, which in the absence of GSH react with protein thiols yielding mixed disulfides. While the sulfenates of thiocarbamides and 2-mercaptoimidazoles are similar in properties, their further oxidation (also catalyzed by FMO) yields sulfinates that are very different in some chemical properties. Ethylene formamidine sulfinate is not only far more stable than the aromatic N-methylimidazole-2-sulfinate but also produces different products upon hydrolysis. The latter sulfinate hydrolyzes to sulfite and N-methylimidazole in less than 3-4 minutes at pH 7.4, 37°C (L. L. Poulsen, unpublished studies). On the other hand, the more stable sulfinate of ethylene thiourea, like other formamidine sulfinic acids, reacts with water or protein amino groups forming the respective urea or protein bound guanidino adducts and sulfoxalate (90). The differences in the reactivity of the metabolically formed sulfinic acids and not differences in routes of formation may account for the differences in toxicity of thiocarbamides and 2-mercaptoimidazoles.

The stereoselective sulfoxidation of asymmetric sulfides catalyzed by purified FMOs and liver microsomes has been determined with a series of

alkyl p-tolyl sulfides (91) and dithiolanes (92, 93). Studies on the mechanisms for the dithiolane S-oxygenation by liver, kidney, and lung microsomes suggest that the diastereoselective and enantioselective S-oxygenation of dithiolanes is catalyzed primarily by FMOs. The studies of Rettie et al (91) indicate that the extent of enantioselective sulfoxidation depends on the isozyme employed and the steric bulk of the alkyl substituent. These investigators also found that, for the most part, the active site chirality of the microsomal FMOs is retained in the purified enzymes.

FUTURE DIRECTIONS

Predicting the future course of research in a rapidly developing area can be treacherous but there are several obvious directions that should give new insights into the role of these flavoenzymes in the metabolism of xenobiotics. For example, the preparation of monospecific antibodies should stimulate work on the expression of FMO isoforms in different tissues of humans and/or animal models frequently used to study drug metabolism. Such preparations should also facilitate studies on modulation of FMO isoforms as a function of development or exposure to various environmental toxins.

More detailed information on the substrate specificities of FMO isoforms in a single species or across species should be another active area of research over the next five years. While the use of isoform selective substrate probes can provide some useful information, ultimately the specificities of each FMO isoform and its species homologues must be determined with purified preparations. However, the interpretation of data collected with isolated enzymes may not always be straightforward. For example, pig FMO-1A1 bears a carbohydrate adduct on asparagine 120 (K. K. Korsmeyer, J. R. Cashman, private communication) and there is some evidence that substrate specificity of this flavoenzyme is determined, at least in part, by the presence and/or structure of the carbohydrate adduct. It is thus likely that the specificities of proteins isolated from clones of genes expressed in prokaryotes may be different from the gene product expressed in mammals. Even data on substrate specificities collected with preparations isolated from mammalian tissue are not completely free from problems. Because of the unusual catalytic mechanism of these flavoenzymes, even small perturbations of the protein region(s) controlling access to the 4a-hydroperoxyflavin can lead to qualitative changes in substrate specificity. For example, Poulsen (unpublished observations) has observed that detergents used to extract these enzymes from microsomes sometimes open up the active site to slightly larger substrates than accepted by the membrane-bound enzyme. While only compounds at or near the size cutoff for a specific isoform are affected, the "all or none" difference in activity with such compounds can be dramatic. Because of these

and other problems inherent to enzymes that do not selectively bind substrates, conclusions as to similarities or differences in substrate specificities of purified FMO isoforms must be carefully evaluated.

The contribution of these flavoenzymes to the metabolism of drugs and other xenobiotics in live animals will undoubtedly receive increasing attention. While the potential use of diet (66, 67) and/or pretreatment with xenobiotic soft nucleophiles to alter FMO activities in tissues of live animals may prove useful, metabolic cycling common to many of the xenobiotic oxidation products produced via FMO (8) may complicate interpretation of data collected with live animals. However, the lack of an inhibitor selective for FMOs remains the major impediment to definitive studies on the function of these enzymes in the metabolism of xenobiotics. While this is generally true of enzymes that have apparently evolved to catalyze the detoxication of a range of different xenobiotics, the resistance of FMO to inactivation by xenobiotics is remarkable. Unlike the P450-dependent monooxygenases that are readily inactivated by a variety of amines, hydrazines, and sulfur compounds common in many plants, all plant extracts examined in this laboratory over the past ten years appeared completely free from inhibitors of FMO. This resistance to inhibition by naturally occurring xenobiotics suggests that these flavoenzymes have evolved to oxidize and deactivate xenobiotic soft nucleophiles that so readily inactivate not only P450-dependent but other heme enzymes bearing similar porphyrin prosthetic groups. This may be the reason that the tissue distribution of the P450- and flavin-dependent monooxygenases are similar. An integrated approach to determine how these and other enzymes of detoxication cooperate in the metabolic detoxication of xenobiotics could be a productive area of future research.

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