CHARACTERIZATION OF EXPRESSED FULL-LENGTH AND TRUNCATED FMO2 FROM RHESUS MONKEY

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ABSTRACT:

Flavin-containing monoxygenase (FMO, EC 1.14.13.8) is a family of xenobiotic-metabolizing enzymes that catalyze the oxygenation of a wide variety of xenobiotics that contain a soft nucleophile, most commonly nitrogen and sulfur (Ziegler, 1993; Cashman, 1995). Mammals express five FMOs that are developmentally regulated in a sex-, tissue-, and species-specific manner (Hines et al., 1994; Lawton et al., 1994; Falls et al., 1995, 1997; Lee et al., 1995; Shehin-Johnson et al., 1996). Elucidation of the regulatory region of rabbit FMO2 suggests that tissue-specific transcription factors may regulate lung-specific expression (Hines et al., 1994; Lawton et al., 1994; Dann et al., 1996; Larsen-Su et al., 1999), indicating possible hormonal regulation.

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Abbreviations used are: FMO, flavin-containing monoxygenase; FMO2*1, FMO2, human alleles and proteins for full-length and truncated human FMO2, respectively; hFMO2, mFMO2, and rFMO2, human, monkey, and rabbit FMO isoform 2, respectively (where -471 and -535 indicate the number of AA in the truncated and full-length proteins, respectively); TNB, nitro-5-thiobenzoate; DTNB, 5,5-dithiobis-(2-nitrobenzoate); CHAPS, 3-[3-cholamidopropyl(dimethylammonio)]-1-propanesulfonate; DMA, [UL-14C]dimethylammonio]-1-propanesulfonate; FAD, flavin adenine dinucleotide; PMSF, phenylmethylsulfonyl fluoride; dNTP, 2′-deoxyxynucleoside-5′-triphosphate; PCR, polymerase chain reaction; Sf9, Spodoptera frugiperda; HPLC, high-performance liquid chromatography.

Flavin-containing monoxygenase (FMO, EC 1.14.13.8) is a family of xenobiotic-metabolizing enzymes that catalyze the oxygenation of a wide variety of xenobiotics. FMO2 is highly expressed in the lung of most mammals examined, but the protein has only recently been detected in humans, presumably due to a premature stop codon at AA472 in most individuals. In this study, full-length (mFMO2-535) and 3′-truncated (mFMO2-471) monkey FMO2 protein, produced by cDNA-mediated baculovirus expression, were characterized and compared with baculovirus-expressed rabbit FMO2 (rFMO2-535). Although baculovirus-expressed mFMO2-535 had properties similar to FMO2 in monkey lung microsomes and had catalytic properties similar to rFMO2-535, the expressed proteins differed in a number of properties in S-oxygenation assays. Both enzymes had the same pH optima (pH 9.5); however, mFMO2-535 quickly lost activity at higher pH values whereas rFMO2-535 retained the majority of its activity. Also, mFMO2-535 was significantly less stable at elevated temperatures and in the presence of cholic acid but had greater activity in the presence of magnesium. mFMO2-535 had higher apparent Km and Vmax/Km values than rFMO2-535 did in N-oxygenation assays. mFMO2-471 was correctly targeted to the membrane fraction, but N- and S-oxygenation was not detected. Since the AA sequence identity of mFMO2 and human FMO2 is 97%, our results with mFMO2-535 suggest that individuals carrying the allele encoding full-length FMO2 are likely to have in vivo FMO2 activity. Such activity could result in marked differences in the metabolism, efficacy, and/or toxicity of drugs and xenobiotics for which lung is a portal of entry or target organ.

From its initial discovery as a 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; Nikbakht et al., 1992; Yueh et al., 1997). Although the FMO2 isoform can metabolize many general substrates of FMO, it is inactive toward certain tertiary amines such as imipramine and chlorpromazine that are metabolized by FMO1 (Williams et al., 1984). In addition, FMO2 is capable of N-oxygenation of some primary alkylamines (Poulsen et al., 1986; Tynes et al., 1986) and exhibits isoform-specific stereoselectivity in S-oxygenation of alkyl-substituted p-tolyl sulfides (Rettie et al., 1995). We previously reported that rabbit FMO2 is developmentally regulated by age and pregnancy and that FMO2 levels were correlated with plasma concentration of progesterone and corticosterone at late gestation and early postpartum, respectively (Lee et al., 1995; Larsen-Su et al., 1999), indicating possible hormonal regulation. Elucidation of the 5′ regulatory region of rabbit FMO2 suggests that tissue-specific transcription factors may regulate lung-specific expression of FMO2 (Shehin-Johnson et al., 1996).

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. Early attempts by our laboratory to generate human FMO2 knocked-out mice and to subclone the DNA encoding human FMO2 have been unsuccessful. Two human FMO2 cDNA sequences have been reported in GenBank, one with GenBank 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).

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laboratory to detect FMO2 protein (by Western analysis and enzyme assay) from human lung microsomes were not successful (unpublished). Therefore, we focused instead on a close human relative, the rhesus monkey lung FMO2 (mFMO2), for the study of pulmonary FMO2. We previously cloned mFMO2 from a rhesus monkey lung cDNA library. Positive identification as an ortholog of rabbit FMO2 (rFMO2) was made by cDNA comparison, immunohistochemical analysis of protein, and FMO substrate specificity (Yueh et al., 1997). Recently, human FMO2 (hFMO2) cDNA was isolated and characterized (Dolphin et al., 1998). Their sequence analysis indicated that the major allele encodes a polypeptide that lacks 64 amino acid residues, resulting from a C to T amber mutation at codon 472 (FMO2*2A), compared with the FMO2 ortholog in other mammals. The C allele (FMO2*1), which encodes full-length protein (FMO2*1), occurs in 26% of African-Americans, but it is absent in Caucasians and Asian-Americans (Whetstine et al., 2000).

In this study, we established a heterologous expression system for production of functional mFMO2-535 protein, and then went on to create a truncated mFMO2 (mFMO2-471) analogous to FMO2*2A, by site-directed mutagenesis. Expressed mFMO2-535 resembled the rabbit ortholog immunohistochemically; however, in-depth characterization of mFMO2-535 using an assay for methimazole-dependent nitro-5-thiobenzoxate (TNB) oxidation identified a number of differences compared with rFMO2-535. While mFMO2-471 was immunohistochemically detectable, our results confirm that the C terminus is essential for enzyme stability and detectable catalytic activity.

Materials and Methods

Triton N101, tergitol NP9, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) (catalog no. C3023, CS849), blue dextran, hydroxyapatite, flavin adenine dinucleotide (FAD), methimazole, NADPH, isocitrate, isocitrate dehydrogenase, EDDA, [UL-3C]/[N-3dimethylamylamine (specific activity, 6.2 μCi/μmol) (DNA), 5,5-dithiobis(2-nitrobenzoate) (DTNB), diithiothreitol, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). (S)-5'-H1Nicotine (32 Ci/mmol) was a gift from Dr. Mark Shigenaga (University of California, San Francisco, CA). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, MA). Taq polymerase, Expand High Fidelity enzyme, buffers, and 2'-deoxyuridinose-5'-triphosphate (dNTP) mixture for PCR were from Roche Molecular Biochemicals (Indianapolis, IN). DH5α, and the components of the Bac-to-Bac baculovirus expression system, including Max Efficiency DH10bac competent cells, Spodoptera frugiperda (Sf) insect cells, SF-900 II serum-free medium, cellficient reagent, blue-gal, gentamicin, and Puc/M13 primers, were obtained from Life Technologies (Gaithersburg, MD). Acrylamide, nitrocellulose, and horseradish peroxidase-conjugated secondary antibodies for Western blotting were purchased from Bio-Rad (Richmond, CA). GeneClean II was from BIO 101 Inc. (La Jolla, CA). Hyperfilm MP and enhanced chemiluminescence kit were from Amersham Pharmacia Biotech (Arlington Heights, IL). Oligonucleotides for PCR were synthesized by the Center for Gene Research and Biotechnology (Oregon State University). PCR was performed in a 96-well Robocycler (Strategene, La Jolla, CA). FAD determinations were made on a PerSeptive Biosystems (Framingham, MA) CytoFluor Series 4000 fluorescent plate reader.

Cloning of mFMO2. The Bac-to-Bac baculovirus expression system was used to express mFMO2-535 cDNA obtained from plasmid pBK-CMV-FMO2, a positive clone from cDNA library screening (Yueh et al., 1997). Plasmid DNA was digested with EcoRI/XbaI to yield a 1.6-kbp full-length cDNA, which was subsequently ligated to EcoRI/XbaI cut pFastBac1, placing the mFMO2-535 cDNA downstream of a baculovirus promoter derived from the polyhedrin gene. Recombinant plasmid was isolated and used to transform DH10Bac Escherichia coli cells, which contain the baculovirus shuttle vector (bacmid) with a mini-att T7N target site, as well as the helper plasmid that transposes the gene insert from pFastBac1 together with flanking baculovirus sequences into the bacmid. White recombinant bacmid colonies were visualized on LB agar plates containing the chromogenic substrate blue-gal. High molecular weight DNA was prepared from recombinant bacmid clones. PCR was performed with PUC/M13 primers using DNA from recombinant colonies as template, and PCR products were visualized by UV light subsequent to electrophoresis through a 1% agarose gel (0.5 μg/ml of ethidium bromide, 40 mM Tris acetate, 2 mM EDTA), to confirm transposition of the clone into the bacmid. A negative control vector-bacmid clone was produced by transposing pFastBac1 into the bacmid without any foreign DNA inserted, and it was subsequently used to produce virus for control infections.

Since the identity between the mFMO2 and hFMO2 coding regions is more than 97% (Dolphin et al., 1998), the truncated mFMO2 was produced as a model of the human allele, using mFMO2-535 cDNA as a template for PCR-directed mutagenesis. A pair of primers (forward primer: 5′-AAG TTT GAT GCA TGG CAA AGA AGG TAG; reverse primer: 5′-CTA GTC TAG ACT AAT AGG AGT GTG AGG G) was used to introduce two restriction enzyme sites (BamHI at the 5′; Xbal and a stop codon at amino acid residue 472) in mFMO2-535 cDNA. PCR was performed with an enzyme mixture of Taq + pfu polymerase (Expand High Fidelity) to provide proof reading activity. After an initial denaturation step at 94°C for 2 min, 30 cycles including denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and polymerization at 72°C for 1 min for each cycle, and an additional 7 min at 72°C in the final cycle were performed. The PCR-generated fragment, containing truncated mFMO2-471 cDNA, was digested with BamHI/XbaI. The resultant 1422-bp fragment was isolated from an agarose gel with a GeneClean kit following electrophoresis, then ligated into a BamHI/XbaI-cleaved pFastBac vector. The sequence of the truncated clone was confirmed (Sanger et al., 1977).

Cloning of rFMO2. We worked with a rabbit cDNA clone (provided by R. N. Hines, The Medical College of Wisconsin, Milwaukee, WI) initially cloned in pUC19. We originally re-engineered this clone so that the ATG start site was embedded in an Ncol restriction site, for use in an E. coli expression vector, pET-30a. This was accomplished by PCR-directed mutagenesis with a forward primer (5′-GGT ACC CCC ATG GCA AAG AGG GTG GCA GT) that created the Ncol site and a reverse primer (5′-CGG ATC CCT AGA ACC ATT GC) that created a BamHI site downstream of the stop codon. PCR conditions were as follows: 99°C denaturation for 13 s, 54°C annealing for 45 s, and 68°C extension for 3, 5, and 7 min for cycles 1 to 10, 11 to 20, and 21 to 30, respectively. Reactions contained Expand High Fidelity polymerase (0.26 units/10 μl), magnesium (1.5 mM), dNTPs (10 mM, each), primers (0.3 μM), and template DNA (2.5 ng/10 μl) in 1× High Fidelity buffer. The PCR fragment was digested with Ncol/BamHI and was cloned into pCR2.1 (Invitrogen, San Diego, CA) in DH5α, and was subsequently sequenced. For baculovirus expression, the clone was digested with Ncol/HindIII and was gel purified. pFastBac1 was engineered to be compatible with the rabbit clone by creating an NcoI site in the multiple cloning region. Two complimentary primers were designed to produce a 19-bp overlap with BamHI and EcoRI sticky-ends and an internal Ncol site (forward primer: 5′-GAT CCA TGC ATG GCA GCA GC; reverse primer: 5′-AAT TCG TGC GAC CAT GGA TCA TG), pFastBac1 was digested with BamHI and EcoRI to drop out an 18-bp fragment between these two sites. The cut vector was gel purified. The two primers were heated in ligation buffer at 95°C for 2 min followed by 2 min at each 70, 66, 62, 58, and 54°C, and 4 min at 50°C before chilling on ice. The adaptor was ligated with BamHI/EcoRI cut pFastBac1 in a standard ligation reaction to create a new plasmid (pFB1-BNE), which was transformed into DH5α. The new vector DNA was digested with Ncol/HindIII, gel purified, and ligated with the Ncol/HindIII fragment containing the full rFMO2-535 open reading frame. Bacmid was produced and confirmed as already described.

Viral Preparation and Protein Expression. Sf9 insect cells were routinely maintained in SF-900 II serum-free medium containing 10 mg/liter gentamicin in a 500-ml sterile shaker flask at 27°C on an orbital shaker (125 rpm). SF9 cells (1 × 10⁶) were seeded into 35-mm wells of a 6-well plate with 2 ml of medium/well. Then they were transfected with recombinant bacmid (mFMO2-535, mFMO2-471, and rFMO2-535) via carrier cellfectin reagent according to the manufacturer’s protocol (Life Technologies). Recombinant baculovirus was harvested at 72 h post-transfection and was amplified (1–2 ml of primary virus/100 ml) in Sf9 cells in log phase (2 × 10⁶ cells/ml) to prepare high titer virus stocks. After 3 to 7 days, cells were pelleted to remove debris, and the
viral supernatant was collected. Sixty milliliters of secondary virus was used to infect 600 ml of SD cells in suspension cultures (2 × 10^6 cells/ml). FAD was supplemented (10 μg/ml) for expression. Insect cells were harvested at 96 h postinfection, washed with buffer containing 20 mM potassium phosphate (pH 7.4) and 137 mM sodium chloride, repelleted, and stored at −80°C until further use.

Protein Isolation and Western Analysis of Expressed Proteins. Microsomes were prepared from the insect cell pellets (Guenigerich, 1989). Microsomes were resuspended in 10 mM potassium phosphate (pH 7.6), 20% glycerol, 1 mM EDTA, and 0.4 mM PMSF (buffer A) and homogenized with a motor-driven Teflon-tipped pestle on ice. The suspension was centrifuged at 10000 g for 10 min at 4°C to remove large cellular debris. The balance of the supernatant was centrifuged at 100,000g for 80 min at 4°C to pellet the microsomes. The cytosol was removed and an aliquot saved, while the pellets were thoroughly resuspended in approximately 3 pellet-volumes of buffer A.

Expressed mFMO2-535 protein was further purified with some modifications to procedures reported for FMO3 (Haining et al., 1997). All procedures were performed at 4°C, with as little exposure to light as possible. Insect cell membranes (about 40 mg from 600 ml of expression) were solubilized in a final volume of 15 ml of buffer A with 1% Triton-N101 nonionic detergent. This mixture was homogenized with a motor-driven Teflon-tipped pestle, then stirred for 60 min on ice, and centrifuged at 100,000g for 80 min to remove insoluble material. The solubilized microsomes were diluted with additional buffer A to a final concentration of 0.5% detergent, then applied directly to a column of 10 ml of blue dextran pre-equilibrated with buffer B (buffer A containing 0.5% Triton-N101). The column was washed with buffer B, followed by buffer B with 100 mM NaCl overnight. FMO was eluted with buffer B containing 500 mM NaCl, and fractions were collected. Fractions containing flavoprotein, identified by FAD spectral profile with a scanning (350–500 nm) spectrophotometer were pooled and dialyzed with buffer A. The dialyzed fraction was applied to a 1.5-ml hydroxyapatite column that was previously equilibrated with buffer A. The column was washed overnight with buffer A and eluted with 400 mM potassium phosphate (pH 7.4), 20% glycerol.

The FMO-containing fractions were identified as described above, pooled and dialyzed in 50 mM Tris buffer (pH 7.5), 0.1 mM EDTA, 0.1 mM PMSF, 20% glycerol, and stored at −80°C.

The quantity of FMO recovered was calculated from the absorbance measured at 440 nm using an extinction coefficient for flavin of 11.5 cm M−1 M−1, and a calculated molecular mass (from actual amino acid sequence) of 60,922 Da for mFMO2-535.

The protein concentration of insect cell lysates, cytosolic and microsomal fractions, as well as solubilized purified protein was assayed (Lowry et al., 1951) with bovine serum albumin as standard. The flavin content of mFMO2-535, mFMO2-471, and rFMO2-535 was determined (Fader and Siegel, 1973) for the microsomal fractions, whereas a flavin scan was used for purified mFMO2-535 and rFMO2-535 (from rabbit lung).

Proteins, including purified rabbit FMO2 (from rabbit lung) or purified recombinant mFMO2-535 as standards, were separated on 8% acrylamide gels by SDS polyacrylamide electrophoresis (Laemmli, 1970) and transferred to nitrocellulose. Membranes were blocked and then incubated with guinea pig anti-rabbit FMO2 IgG or rabbit anti-mouse FMO2 antisera (see below). After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-guinea pig IgG or goat anti-rabbit IgG, respectively, as the secondary antibody. FMO2 was visualized by enhanced chemiluminescence on Hyperfilm. Quantitation was performed by densitometry of Hyperfilm using an HP ScanJet IIcx/T flatbed scanner (Hewlett Packard, Palo Alto, CA) with NIH film. Quantitation was performed by densitometry of Hyperfilm using an HP ScanJet IIcx/T flatbed scanner (Hewlett Packard, Palo Alto, CA) with NIH film. Quantitation was performed by densitometry of Hyperfilm using an HP ScanJet IIcx/T flatbed scanner (Hewlett Packard, Palo Alto, CA) with NIH film.
indicating that the truncated protein does not effectively bind FAD.

mF2-471 and the control infections were not substantially different, (Table 1) for the full-length proteins but was lower for truncated FAD determination was in general agreement with the Western data weights based on actual sequence data. FMO content calculated from FAD per mol of FMO (Ziegler, 1980) and calculated molecular

(Fader and Siegel, 1973), using the known relationship of 1 mol of FMO from two separate batches of expressed mFMO2-535; lanes 7 and 8 contained 2.0 μg of microsomes from two separate batches of expressed mFMO2-471. B, lanes 1 to 4 were loaded with 5.0, 3.0, 1.5, and 0.75 pmol of purified rFMO2-535 from rabbit lung, respectively; lanes 5 and 6 contained 1.0 μg of microsomes from two separate batches of expressed rFMO2-535.

(not shown).

bacmid viral infection did not produce any cross-reacting protein (data

Enzyme Activity.

Results

Identification of Expressed FMO2. On average we recovered approximately 7.5 mg of microsomal protein from 100 ml of infected insect cells (range was from 3–14 mg per 100 ml). Western analysis of cytosolic and microsomal proteins demonstrated that expressed mFMO2-535, mFMO2-471, and rFMO2-535 were correctly targeted to the membrane fraction; mFMO2-535 and rFMO2-535 migrate with the same apparent molecular weight under denaturing polyacrylamide electrophoresis conditions (data not shown). Each of our FMO2 antibodies (anti-monkey and anti-rabbit) was able to detect FMO2 proteins from the other species. Western blots of the microsomal proteins demonstrated efficient expression of FMO2 for all clones (Fig. 1; Table 1) with FMO content of some preparations as high as 2 nmol of FMO/mg of microsomal protein (12%). A control vector-bacmid viral infection did not produce any cross-reacting protein (data not shown).

Expression was also assessed by means of FAD determination (Fader and Siegel, 1973), using the known relationship of 1 mol of FAD per mol of FMO (Ziegler, 1980) and calculated molecular weights based on actual sequence data. FMO content calculated from FAD determination was in general agreement with the Western data (Table 1) for the full-length proteins but was lower for truncated mF2-471. In fact, the flavin content and the FAD:FMN ratios of mFMO2-535 and rFMO2-535 were very similar (Table 3). There was some batch-to-batch variation in the calculated specific activity for each clone, but no substantial differences between the rabbit and monkey clones. Specific activities from 40.9 to 51.2 and 36.1 to 50.0 nmol · min⁻¹ · mg⁻¹ of microsomal protein were measured for rFMO2-535 and mFMO2-535, respectively. These rates are substantially higher than those observed for microsomes prepared from animals. The specific activity in nonpregnant female rabbit lung microsomes is 5.8 ± 1.7 nmol · min⁻¹ · mg⁻¹ (Williams et al., 1985), and in nonpregnant female rhesus lung microsomes it is 1.2 ± 0.4 nmol · min⁻¹ · mg⁻¹ (Yueh et al., 1997); therefore, the high rates we observe from expressed protein verify a high level of FMO overproduction in the baculovirus expression system.

We also made estimates of specific activity on the basis of FMO content determined by either Western blotting or flavin content (Fader and Siegel, 1973) and calculated the apparent V_max and K_m for each method (Table 3). The calculated specific activity varied somewhat depending on which method was used to estimate the FMO content; however, there was broad general agreement in the estimates. The V_max estimated for expressed rFMO2-535 (mean of 58.3 and 37.2 min⁻¹ calculated using estimates of FMO content from Westerns and FAD determination, respectively) somewhat exceed rates previously reported (ca. 30.5 min⁻¹) for FMO2 purified from rabbit lung assayed under standard conditions (Williams et al., 1985). The calculated K_m values for mFMO2-535 and rFMO2-535 for metabolism of methimazole were very similar by all methods of calculation, ranging from 733 to 818 μM, which is nearly double the K_m of 411 μM reported for both native rabbit FMO2 (Lawton et al., 1991) and expressed human FMO2.1 (Dolphin et al., 1998).

We were unable to detect activity in protein from either mFMO2-471 or the vector-bacmid control infection with the methimazole-dependent S-oxygenation assay of Dixit and Roche (1984); this result is in agreement with results from expressed truncated human protein (FMO2.2A) (Dolphin et al., 1998). We have calculated that this assay

| TABLE 1 | FMO content of microsomes determined from Western blots and flavin assay |
|-----------------------------------------------|
| Western Blottinga | Flavin Assayb |
| nmol FMO · mg⁻¹ | Percent Protein | nmol FAD · mg⁻¹ | Percent Protein | Ratio FAD:FMN |
| Protein | | |
| rFMO2-535 | 1.01 (0.86–1.16) | 62. (5.2–7.1) | 1.58 (1.25–1.91) | 9.7 (7.6–11.7) | 81.8 (65.3–98.2) |
| mFMO2-535 | 1.85 (1.00–2.07) | 9.4 (6.1–12.7) | 1.70 (1.46–1.93) | 10.4 (8.9–11.8) | 30.4 (25.6–35.1) |
| mFMO2-471 | 0.44 (0.29–0.58) | 2.2 (1.6–2.8) | 0.16 (0.16–0.16) | 0.9 (0.9–0.9) | 9.4 (7.0–11.8) |
| Control | N.D. (N.D.) | N.A. (N.A.) | 0.16 (0.09–0.22) | N.A. (N.A.) | 7.5 (1.3–13.6) |

N.D., none detected; N.A., not applicable.
a The appropriate form of purified FMO2 was included at known concentrations to enable calculations. The mean and (range) of two batches of expressed protein is reported for all determinations.
b The FMO content was determined by flavin assay (Fader and Siegel, 1973) assuming all FAD is associated with the FMO protein, and the following relationships derived from calculated molecular weights of the proteins: 16.37 nmol of FAD · mg⁻¹ of rFMO2-535, 16.42 nmol of FAD · mg⁻¹ of mFMO2-535 and 18.62 nmol of FAD · mg⁻¹ mF2-471.

substrate for mFMO2-535, with an apparent K_m of 1700 μM (ca. 3-fold higher than the K_m for rFMO2-535); however, the apparent V_max with mFMO2-535 was an order of magnitude greater than the rabbit ortholog (Table 2). The net result is that V_max/K_m with nicotine as the substrate is 3.4 times higher for the monkey compared with the rabbit FMO2.

There was also an ortholog distinction with respect to the kinetics of DMA N-oxygenation. DMA was also a better substrate of rFMO2-535 with respect to apparent K_m (310 and 670 μM for rabbit and monkey, respectively); however, again a higher apparent V_max was observed with mFMO2-535. N-Oxygenation by mFMO2-471 and the vector-bacmid control was not detected for either DMA or nicotine (detection limits were 0.002 and 0.004 nmol · min⁻¹, respectively).

The kinetics of methimazole-dependent S-oxygenation by microsomal mFMO2-535 and rFMO2-535 were very similar (Table 3). There was some batch-to-batch variation in the calculated specific activity for each clone, but no substantial differences between the rabbit and monkey clones. Specific activities from 40.9 to 51.2 and 36.1 to 50.0 nmol · min⁻¹ · mg⁻¹ of microsomal protein were measured for rFMO2-535 and mFMO2-535, respectively. These rates are substantially higher than those observed for microsomes prepared from animals. The specific activity in nonpregnant female rabbit lung microsomes is 5.8 ± 1.7 nmol · min⁻¹ · mg⁻¹ (Williams et al., 1985), and in nonpregnant female rhesus lung microsomes it is 1.2 ± 0.4 nmol · min⁻¹ · mg⁻¹ (Yueh et al., 1997); therefore, the high rates we observe from expressed protein verify a high level of FMO overproduction in the baculovirus expression system.

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We were unable to detect activity in protein from either mFMO2-471 or the vector-bacmid control infection with the methimazole-dependent S-oxygenation assay of Dixit and Roche (1984); this result is in agreement with results from expressed truncated human protein (FMO2.2A) (Dolphin et al., 1998). We have calculated that this assay
TABLE 2
Kinetics of N-oxygenation for purified lung rFMO2-535 and expressed microsomal mFMO2-535

<table>
<thead>
<tr>
<th></th>
<th>DMA N-Oxygenation</th>
<th>(S)-Nicotine N'-Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$ (μmol/10 min)</td>
</tr>
<tr>
<td>rFMO2-535</td>
<td>310</td>
<td>10.1</td>
</tr>
<tr>
<td>mFMO2-535</td>
<td>670</td>
<td>38.2</td>
</tr>
</tbody>
</table>

*Turnover for mFMO2-535 expressed in insect microsomes assumes a specific content of 1.7 nmol FMO/mg protein (Table 1).

The correlation coefficients for the lines used to estimate $K_m$ and $V_{max}$ were as follows: DMA N-oxygenation, $r = 0.994$ and 0.936 for rFMO2-535 and mFMO2-535, respectively; (S)-nicotine N'-oxygenation, $r = 0.999$ for rFMO2-535 and mFMO2-535.

TABLE 3
Kinetics of methimazole-dependent S-oxidation for expressed rFMO2-535 and mFMO2-535

<table>
<thead>
<tr>
<th></th>
<th>Western Estimate</th>
<th>Flavin Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}$ (μM)</td>
<td>$K_m$ (μM)</td>
</tr>
<tr>
<td>rFMO2-535</td>
<td>58.3 (55.5–61.1)</td>
<td>809 (800–818)</td>
</tr>
<tr>
<td>mFMO2-535</td>
<td>38.7 (30.5–46.8)</td>
<td>760 (750–769)</td>
</tr>
</tbody>
</table>

* $V_{max}$ used either the Western or the flavin estimate of FMO content = nmol min$^{-1}$ mg$^{-1}$ of microsomal protein, estimated from Western blotting (Table 1); so if there is residual activity it is less than 0.1% that of mFMO2-535.

The assay was performed with microsomes from two separate batches from each clone as described under Materials and Methods using Tricine buffer at the indicated pH values. Velocities calculated from assays performed under standard conditions were designated as 100% for each batch of each clone, and the results from the test conditions were normalized accordingly. Results shown are the mean normalized results from the separate batches. The error bars show the maximal observed activity. Velocities for 100% were 27.5 and 29.2 min$^{-1}$ for mFMO2-535 batches and 29.5 and 37.1 min$^{-1}$ for rFMO2-535 batches using estimates of FMO content derived from the flavin assay (Table 1).

The activity was better if the samples were maintained at a higher protein concentration. Storage buffer had a more protective effect than did protein concentration (e.g., on average, mFMO2-535 retained 43% activity at low concentration in storage buffer but had only 29% activity heated at high concentration in Tricine buffer), and the effects were additive. rFMO2-535 retained more activity than did mFMO2-535 under all conditions examined. Under optimal conditions (high concentration/storage buffer), rFMO2-535 retained 95% activity while mFMO2-535 retained 65% activity. Under the least protective conditions, rFMO2-535 still maintained 58% activity while activity was barely detectable with mFMO2-535 microsomes. The clone exhibited significantly different responses to heat when the protein concentration/buffer composition was varied (interaction contrast: $p = 0.025$, 4 df); however, they differed significantly only when...
Assays were performed as described (see Materials and Methods) with standard assay conditions. Protein to be assayed was diluted to a concentration of 0.2 μg/μl (dilute) or 3.0 μg/μl (concentrated) in either Tricine buffer (100 mM Tricine, pH 8.5, 1 mM EDTA) or storage buffer (10 mM potassium phosphate pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.4 mM PMSF) before heating (5 min at 45°C then iced). Velocities were normalized as described in Fig. 2, with normalization to the unheated treatment. The error bars show the maximal observed activity. Velocities for 100% with unheated, undiluted microsomal samples were 26.7 and 24.2 min⁻¹ for mFM02-535 batches and 28.0 and 34.5 min⁻¹ for rFM02-535 batches.

When we supplemented 100 mM MgCl₂ or any of three different detergents (Fig. 4). However, the two clones responded significantly differently to additives (clone by additive interaction: \( p = 0.0015, 4 \text{ df} \)). Relative to the no additive response, the biggest difference between rFM02-535 and mFM02-535 was due to cholic acid (\( p = 0.0032, 1 \text{ df} \)), which actually depressed the activity observed with mFM02-535. mFM02-535 had enhanced activity in the presence of CHAPS and tergitol NP9 that was not significantly different from that of rFM02-535. There was also a significant difference in the response of rFM02-535 and mFM02-535 to MgCl₂ supplementation (\( p = 0.023, 1 \text{ df} \)). While MgCl₂ increased S-oxidation of mFM02-535 and rFM02-535, mFM02-535 batches experienced a higher mean level of stimulation (237 versus 188%).

With regard to the enhancement of activity we observed with CHAPS, this response is apparently linked to the manufacturing stimulation (237 versus 188%).

While MgCl₂ increased S-oxidation of mFM02-535 and rFM02-535, mFM02-535 batches experienced a higher mean level of stimulation (237 versus 188%).

Discussion

We report here the efficient expression of FMO2 in a baculovirus-mediated system that makes it possible to produce quantities of protein for our future studies including preliminary assessment of the properties of human FMO2.1, determination of gene structure-function relationships, and the study of specific mutations. Expressed mFM02-535 possessed similar catalytic activity to that of expressed and native rFM02-535 purified from lung microsomes with respect to S-oxidation of methimazole, and it was actually somewhat more efficient at N-oxidation of the two substrates (DMA and nicotine) examined, as measured by \( V_{\text{max}}/K_{\text{m}} \).

The amino acid sequence predicted from the cloned FMO2 cDNA (Yueh et al., 1997) has 97% identity with the reported human FMO2*1 allele (Dolphin et al., 1998). mFM02-471, which lacks the 64-amino acid residues of the C terminus, was produced in baculovirus to mimic human FMO2.2A, and it was compared with expressed mFM02-535 protein. No difference in subcellular distribution was found, suggesting that this C-terminal peptide is not required for membrane association. This further confirms the findings from an expressed rabbit FMO2 mutant missing the final 26 residues of the C terminus (Lawton and Philpot, 1993). However, while this mutant maintained its membrane association like mFM02-471, it also retained enzyme activity, whereas mFM02-471 did not.

Comparison of the FMO content predicted from Western blotting with that from FAD determination led us to believe that the truncated protein was not effectively binding FAD. Supplementation of FAD to the assay for S-oxidation did not alter the activity of either the active mFM02-535 or the mFM02-471 truncated proteins; however, we lacked a positive control for this assay variation (e.g., an expressed FMO known to lack FAD and activity, whose activity could be restored with supplementation). Nonetheless, our finding that N-oxidation of DMA and S-oxidation of methimazole is reduced to undetectable levels by the truncation is in complete agreement with published work on expressed human FMO2.2A (Dolphin et al., 1998). They also demonstrated that FMO2.2A was membrane associated and lacked the ability to catalyze S-oxidation with methimazole as substrate. Our studies indicate that if residual capacity for S-oxidation is retained by mFM02-471, it has been reduced to less than 0.1% of the activity observed with mFM02-535.

The FMO2 isoform has often been characterized as stable at high pH, thermal stable, and stimulated by detergents and magnesium. Our results with the monkey FMO2 ortholog demonstrate that these gen-
eralizations have their limitations. We measured a sharp drop in activity by mFMO2-535 at pH values that do not appreciably decrease the activity of rFMO2-535, a nearly complete loss of activity by mFMO2-535 in response to thermal conditions that eliminate only about 40% of rFMO2-535 activity, and some effector-specific ortholog differences.

We have demonstrated that there are significant ortholog differences in activity in response to 1% cholate when methimazole is used as the test substrate. Venkatès and coworkers (1991) demonstrated that the increase or decrease in relative activity caused by the presence of detergent was dependent on the detergent used, the mouse FMO isoform under study, and the specific substrate under study. Thus, ortholog differences between rFMO2-535 and mFMO2-535 may well show a different pattern if other substrates are paired with the detergents we studied.

That more ortholog differences have not previously been identified may be due to the generally small spectrum of conditions investigated, the choice of parameters tested (most investigators test for response to magnesium), the orthologs under study, or a failure to carefully control test parameters when comparing orthologs (i.e., concentration and buffer composition during heat treatment). In addition, as our experiments with CHAPS demonstrate, slightly different chemical preparations can produce very different results.

Active protein produced from chimeric constructs could be useful in assigning protein properties to specific regions of the amino acid sequence. Our efforts to produce active rabbit FMO1 × FMO2 protein (data not shown) and work by others (D. M. Ziegler and L. L. Poulsen, personal communication) indicate a general inability to produce active protein from interisoform chimeras involving large sequence stretches. By contrast, ortholog chimeras can be produced that are active (Wyatt et al., 1998). Thus, it should be possible to produce active protein from mFMO2-535 × rFMO2-535 chimeras; we have demonstrated here that such chimeras could be assayed for numerous differences.

Genotyping experiments have demonstrated that 26% (Whetstine et al., 2000) of people of African American descent carry the FMO2*1 allele, and these individuals are in theory capable of expressing catalytically active FMO2.1 enzyme. Identification of full-length protein in some human samples by Western blotting indicates that there are at least two phenotypes of hFMO2 (full-length and undetectable). The genotyping results, produced in cooperation with Dr. Hines’ laboratory, showed that only individuals who expressed FMO2.1 protein had the FMO2*1 allele encoding full-length protein. The DMA assay revealed no catalytically active (at least at the present detection limit) FMO2 in lung microsomes from any phenotype. There are at least two explanations for this observation. We have demonstrated here that mFMO2-535 is more thermolabile than the rabbit ortholog. If human FMO2.1 is also thermolabile, post-mortem degradation before freezing of lung tissue may significantly reduce or eliminate catalytic activity but leave the primary structure intact. We also find in the present study that, based on K_{in}, DMA is not a particularly good substrate for mFMO2. Again, if the primate orthologs are similar in this respect, the lack of DMA-N-oxygenation previously reported in human microsomes known to contain FMO2.1 (Whetstine et al., 2000) may not be definitive. Therefore, potential substrates for N- or S-oxygenation other than those examined here should be tested for human FMO2 metabolism to determine whether this polymorphism affects drug metabolism in humans.

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