IDENTIFICATION OF ACTIVE FLAVIN-CONTAINING MONOOXYGENASE ISOFORM 2 IN HUMAN LUNG AND CHARACTERIZATION OF EXPRESSED PROTEIN

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ABSTRACT:

Full-length human (hFMO2.1) and monkey (mFMO2) flavin-containing monooxygenase proteins, which share 97% sequence identity, were produced by baculovirus-mediated expression in insect cells and assayed for S-oxygenation under conditions known to affect FMO activity. Both enzymes demonstrated maximal activity at pH 9.5; but hFMO2.1 retained significantly more activity than mFMO2 did at pH 9.0 and higher. hFMO2.1 also retained significantly more activity than mFMO2 did in the presence of magnesium and all detergents tested. Although hFMO2.1 had more residual activity after heating at 45°C than mFMO2, under some conditions, both had less than 10% of control activity, whereas expressed rabbit FMO2 retained over 50% activity. Screening for NADPH-oxygenation by hFMO2.1, indicated that substituted thioureas with a small cross-sectional area (2.4–4.3 Å) are good substrates, whereas 1,3-diphenylthiourea (11.2 Å) was not oxygenated. We confirmed the presence of hFMO2.1 in lung tissue from a heterozygous individual (hFMO2*1/hFMO2*2A) by Western analysis and confirmed activity by S-oxygenation. These microsomes also demonstrated a heat-associated loss of activity similar to expressed hFMO2.1. The heat sensitivity of hFMO2.1 may partially explain why activity in post mortem human lung samples has previously been unreported. Individuals that have the FMO2*1 allele-encoding full-length hFMO2.1 may exhibit altered drug metabolism in the lung.

The mammalian flavin-containing monooxygenases (FMOs; EC 1.14.13.8) are a family (each family having a single member) of xenobiotic-metabolizing enzymes that bind FAD as a prosthetic group and NADPH as a cofactor. Substrates are structurally diverse compounds containing a soft nucleophile; although this is commonly nitrogen or sulfur (Ziegler, 1993; Cashman, 1995; Cashman et al., 2000), other nucleophiles, such as some selenium-containing compounds, are also substrates (Chen and Ziegler, 1994). Metabolism by FMO generally yields metabolic products that are more polar and less toxic or less biologically active than the parent xenobiotic, as is the case for tertiary amines (Ziegler, 1984; Damani, 1988). However, bioactivation, often involving sulfur oxygenation, sometimes results (Ziegler, 1991; Cashman, 1995; Genter et al., 1995).

Proteins from four forms of FMO have been confirmed (FMOs 1–3 and 5) by immunodetection in human tissue samples (Haining et al., 1997; Myers et al., 1997; Overby et al., 1997; Whetstine et al., 1999; Yeung et al., 2000). Expression of protein from FMO4 and a recently identified sixth isoform on human chromosome 1 (ENTREZ accession AL021026) has not been demonstrated yet. FMO isoforms can be distinguished on the basis of patterns of tissue and developmental expression as exemplified by human isoforms 1 and 3 (Dolphin et al., 1996). In addition, there are isoform differences in substrate specificity determined, in part, by the size and shape of the nucleophilic xenobiotic and isomorph-dependent stereoselectivity (Poulsen and Ziegler, 1995; Cashman, 1998).

In humans, the predominant mammalian lung FMO isoform, FMO2, contains a premature stop codon encoding production of an inactive protein lacking 64 AA (Dolphin et al., 1998). Genotyping studies (Whetstine et al., 2000) performed on lymphocytes and lung tissue from human donors indicate that, although Caucasian (n = 52) and Asian (n = 100) populations are homozygous for the allele-encoding truncated protein (hFMO2*2A), 26% of the African American population (n = 180) have at least one copy of the allele-encoding full-length protein (hFMO2*1; hFMO2*2A, hFMO2*2A, and hFMO2*2A). hFMO2.2A refer to the human alleles and proteins for full-length and truncated FMO isoform 2, respectively). Additional work by our laboratory (unpublished data) indicates that this allele also exists in the Hispanic population, although additional samples from this ethnic group and others are necessary to determine the true allelic prevalence.

Due to the broad range of FMO substrates, individuals from polymorphic subpopulations that express the hFMO2*1 allele may have altered drug and xenobiotic metabolism. Studies demonstrating that full-length human FMO2 (hFMO2.1) protein is catalytically active in
humans are lacking. However, baculovirus expressed hFMO2.1 is active toward methimazole and has enhanced activity in the presence of magnesium (Dolphin et al., 1998), in accord with what has been observed for other FMO2 orthologs (Lawton et al., 1991; Lawton and Philpot, 1993; Krueger et al., 2001).

Failure to detect activity (N-oxygenation of N,N-dimethylaniline) in human lung microsomes by our laboratory (Whetstine et al., 2000) probably stems largely from a lack of samples from individuals expressing the hFMO2*1 allele and perhaps from use of poor substrates. We have hypothesized that hFMO2.1 may lose activity as a consequence of elevated temperature, which may occur during post mortem recovery of tissue. This would be contrary to the relative thermal stability displayed by the rabbit FMO2 ortholog, rFMO2 (Williams et al., 1985), widely considered to be a model for the FMO2 isoform. Studies conducted by our laboratory (Krueger et al., 2001) with the monkey ortholog (mFMO2) demonstrate that it can lose most of its activity after 5 min at 45°C. Since the AA sequences of mFMO2 (Yueh et al., 1997) and hFMO2*1 (Dolphin et al., 1998) share 97% identity, we predicted that the human ortholog would also be sensitive to thermal inactivation.

In this article, we describe heterologous baculovirus expression and enzymatic characterization of hFMO2.1. Although hFMO2.1 was similar to mFMO2, these two orthologs were distinguishable from each other and rFMO2, on the basis of thermal sensitivity, in assays of S-oxygenation. In addition, expressed hFMO2.1 and mFMO2 differed significantly in their ability to perform S-oxygenation at elevated pH (at or above pH 9.0) and in their response to magnesium and detergent supplementation during assay. Furthermore, we make the first report of active FMO protein in lung microsomes from a human donor and demonstrate a heat-associated loss of activity, similar to expressed hFMO2.1.

Materials and Methods

Detergents [CHAPS (catalog no. C3023), cholic acid SDS, and tergitol NP9], assay components [all substrates, NADPH, EDTA, 5,5-dithiobis-(2-nitrobenzoate) (DTNB), dithiothreitol, tricine (catalog no. T0377), and potassium phosphate], FAD, phenylmethylsulfonyl fluoride (PMSF), and trypan blue were from Sigma (St. Louis, MO). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, MA). DH5α blue were from Sigma (St. Louis, MO). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, MA). DH10Bac cells, Spodoptera frugiperda (Sf9) insect cells, SI-900 II SFM, cellfetin, and antibiotics) were from Invitrogen (Carlsbad, CA). Acrylamide and nitrocellulose membranes were from Bio-Rad (Richmond, CA).

Cloning of hFMO2.1. A cDNA clone of the hFMO2*1 allele (Whetstine et al., 2000) was obtained as a gift from R. N. Hines (Medical College of Wisconsin, Milwaukee, WI). Full-length cDNA was gel-purified following restriction enzyme digestion of DNA with PstI/HindIII. A recombinant cDNA clone was created by ligation to PstI/HindIII-cut pFastBac1 vector DNA. Recombinant plasmid DNA was used to transform DH10Bac competent cells to produce recombinant bacmid.

Other Clones. Full-length mFMO2 and rFMO2 cDNA clones were ligated into pFastBac1 and pFB1-BNE (modified pFastBac1), respectively, as described elsewhere (Krueger et al., 2001). Transforming DH10Bac with pFastBac1 DNA devoid of foreign DNA inserts produced control bacmid.

Production of Viral Stocks, Protein Expression, and Purification. SI-900 II SFM insect cells were maintained in SI-900 II SFM in shaker flasks, according to the manufacturer’s recommendations, SI-900 II SFM cells were transfected with recombinant bacmid DNA (hFMO2*1, mFMO2, rFMO2, and control bacmid) and cellfetin. Recombinant baculovirus was harvested 72 h post-transfection and was amplified to prepare high-titer secondary and tertiary viral stocks (1 ml of primary or secondary per 50 ml of cells at 2 × 10^6 cells - ml ^{-1}). Recombinant protein was produced by infecting SI-900 II SFM cells (2 × 10^6 cells - ml ^{-1}) with a volume of amplified virus (5–10 ml/100 ml of cells) that resulted in nearly complete cell death, but little cell debris, 96 h post-infection. Cell death was assessed with trypan blue (DeLuca, 1965). FAD was supplemented (10 μM - ml ^{-1}) during expression.

Insect cells were harvested, and microsomes were isolated (Krueger et al., 2001) and resuspended in storage buffer (10 mM potassium phosphate, pH 7.6, 20% glycerol, 1 mM EDTA, 0.4 mM PMSF). Protein concentration (Lowry et al., 1951) and flavin content were assayed (Fader and Siegel, 1973) and used to estimate FMO-specific content.

Protein Isolation from Human Samples. Frozen human lung samples were obtained from organ donors through the International Institute for the Advancement of Medicine (Exton, PA). DNA was isolated and genotyped (Whetstine et al., 2000) with respect to hFMO2*1 and hFMO2*2A alleles. Microsomes were prepared (Guenengerich, 1989) from two individuals homozygous for hFMO2*2A (H9 and HL0292) and a heterozygous hFMO2*2A/hFMO2*1 (H6) individual and were resuspended in storage buffer before determination of protein concentration.

Additional Samples. Lung microsomes were prepared from three female New Zealand white rabbits (Rabbit Research Institute, Oregon State University, Corvallis, OR) and three female Rhesus macaque (Oregon Regional Primate Research Center, Beaverton, OR), and protein content was determined.

Antibodies and Western Detection. Primary polyclonal antibodies to FMO isoforms were used to characterize recombinant hFMO2.1 and lung microsomes from human tissue donors. They included commercially available (GENTEST, Woburn, MA) antibodies to human FMO1, FMO3, and FMO5 (hFMO1, hFMO3, and hFMO5) and custom antibody to expressed mFMO2 (Whetstine et al., 2000; Krueger et al., 2001) [hFMO1, hFMO3, hFMO5, mFMO2, and rFMO2 are human, monkey, and rabbit FMO with species (lowercase letter) and isoform (number) indicated]. The antibodies toward human FMOs were diluted according to the manufacturer’s recommendations. The anti-mFMO2 antibody was used at a dilution of 1:50,000 unless otherwise specified. Secondary antibody was horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad). Efforts aimed at reducing cross-reactivity of the anti-mFMO2 antibody to non-FMO2 proteins included optimization of antibody dilution (from 1:10,000 to 1:100,000) and absorption of cross-reacting proteins from the primary antibody. Microsomal proteins (675 μg each) from baculovirus expressed rabbit FMO1 (rFMO1) and female Sprague-Dawley rat liver (as a source of FMO1 and FMO3) were denatured and separated on SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose. Membranes containing 0, 75, or 600 μg of expressed rFMO1 and rat liver microsomes each were blocked, then incubated with 50 ml of primary antibody buffer containing 1.0 μl of mFMO2 antisera for 1 h. We calculated that 75 μg of expressed rFMO1 microsomes contained 4 μg of rFMO1 protein (determined by FAD analysis) and that 75 μg of rat liver microsomes contained at least 0.15 μg of FMO1 and 0.25 μg of FMO3 (estimates from Western blots are the minimal content because we did not have authentic rat FMO1 and FMO3). The desorbed antibodies were recovered and were used to detect expressed mFMO2, hFMO2.1, rFMO1, and hFMO3 from replicate Western blots. The relative detection of FMO2 versus non-FMO2 proteins was compared to determine whether cross-reactivity to FMO1 and FMO3 proteins was selectively reduced.

Western blots were used to assess quality and quantity of expressed proteins, to examine antibody sensitivity and selectivity, and to determine the profile of FMO proteins in human lung samples. Proteins were separated, blotted, detected, and quantitated as previously described (Krueger et al., 2001).

FMO Catalytic Activity. Methimazole-dependent S-oxygenation (Dixit and Roche, 1984) was used to monitor FMO-specific enzyme activity, using a Cary 300 Bio UV-visible double-beam spectrophotometer (Varian, Palo Alto, CA). Reaction mixture (100 mM tricine/0.1 mM EDTA; 0.06 mM DTNB in 100 mM potassium phosphate, pH 8.0; 2.5 μM dithiothreitol; 0.1 mM NADPH) and protein (20–100 μg of expressed FMO, 500 μg of expressed control, or up to 1000 μg of lung microsomal protein in 1.0 ml of reaction mixture) were added to sample and reference cuvettes. The mixture was equilibrated at 37°C for 3 min before addition of methimazole to the sample cuvette. Absorbance was monitored at 412 nm. Standard conditions were pH 8.5 (at 37°C) and 2.0 mM methimazole. Methimazole concentrations from 0.2 to 5.0 mM were used to estimate apparent V_max and K_m from Lineweaver-Burke plots. Two batches
of each protein were assayed for methimazole-dependent S-oxidation; assays were performed in duplicate. The threshold of detection for this assay with our equipment is approximately 0.035 nmol of substrate \( \cdot \) min\(^{-1}\).

Nonstandard conditions were used to further characterize hFMO2.1. The effect of pH was examined by substituting tricine/EDTA stocks prepared at pH 8.0 to 10.5 for the standard buffer. The effect of detergents (0.5–1%) and magnesium (at 100 mM) was tested by addition to standard buffer just before the 3-min equilibration. Thermal lability was assessed following preincubation for 5 min at 45°C. Proteins were prepared at 0.2 \( \mu \)g \( \cdot \) ml\(^{-1}\) (expressed protein) or 3.0 \( \mu \)g \( \cdot \) ml\(^{-1}\) (expressed protein and lung microsomes) in tricine buffer (100 mM tricine, pH 8.5, 1 mM EDTA) or storage buffer. Following incubation at 45°C, samples were immediately transferred to ice until assayed for residual activity. Activity observed under nonstandard conditions was normalized to standard conditions (100%) for each batch or individual. Velocities less than or equal to zero were taken to be zero. When the deviation of an assay (parameter reported by the Cary Software) was greater than or equal to the observed change in absorbance, the activity was also assumed to be zero.

Further preliminary characterization of expressed hFMO2.1 was made following the rate of substrate-dependent NADPH oxidation at 340 nm (Ziegler and Poulsen, 1978) to screen for activity toward potential S- and N-containing substrates. Compounds that were screened include substituted thioureas and thio carbamides, several amines, and recreational and prescription drugs. Assays were performed using a Cary 300 Bio UV-visible double-beam spectrophotometer. Each assay was prepared three times with a single batch of microsomes (0.07 mg of protein, 0.1 nmol of expressed hFMO2.1) at pH 9.5 for all substrates (in 1.0 ml of reaction mixture). The threshold of detection with our equipment is approximately 0.161 nmol of substrate \( \cdot \) min\(^{-1}\) for this assay, a 4.6-fold higher threshold than the methimazole assay.

Statistical analyses were performed on the methimazole S-oxidation data using the mixed procedure in SAS version 8.1 (SAS Institute, Inc., Cary, NC). Each experiment had a two-way factorial treatment design, with batch (or individual) of each species split into subportions to create the experimental units for studying a second factor (pH level, additive type, or conditions during heat treatment). Therefore, the analysis of variance models were those for a split-plot design with species as the whole plot factor and the second factor as the subplot factor. All analyses were performed on the percentage of standard scale in which residuals were acceptable relative to linear model assumptions. Standard data were removed for the analysis. To get a standard design-based estimate for variance components and Satterthwaite approximations for denominator degrees of freedom were both used.

Results

Analysis of Baculovirus Expressed Constructs. Active hFMO2.1 protein was produced from cDNA constructs in a baculovirus expression system for use in enzyme characterization studies. Expressed mFMO2, rFMO2, and control proteins were also made to facilitate interpretation of results. Microsomal proteins isolated from infected Sf9 insect cells were used as the basis for our studies.

We assayed microsomes for FAD content and, after subtracting FAD content measured from control infections, estimated the FMO content of recombinant proteins (Table 1). Batch-dependent variation in the FMO content of hFMO2.1 was related to the amount of virus used for the infection (viral amplification, tertiary versus quaternary; and viral load during infection, 7 versus 10 ml of virus \( \cdot \) 100 ml\(^{-1}\) of cells). The end result was an FMO content that ranged from 5.2 to 13.8% of the microsomal protein. By contrast, mFMO2 from secondary and tertiary viruses (both from 10 ml of virus \( \cdot \) 100 ml\(^{-1}\) of cells) yielded microsomes with an FMO content of 6.0 to 7.9%.

We used the methimazole assay (Dixit and Roche, 1984) to characterize enzyme activity since the assay is simple and widely used and metabolism is regarded as FMO-specific (Poulsen et al., 1979; Ziegler, 1993). No activity was detected from microsomes from control infections of Sf9 cells (data not shown).

Expressed rFMO2 has a reported \( K_m \) for methimazole from 264 to 315 \( \mu \)M (Lawton et al., 1991; Lawton and Philpot, 1993), whereas expressed hFMO2.1 has an apparent \( K_m \) of approximately 411 \( \mu \)M (Dolphin et al., 1998). These estimates compare favorably with the published \( K_m \) of 411 \( \mu \)M for native rFMO2 (Lawton et al., 1991). Our own estimates of \( K_m \) values for mFMO2 and hFMO2.1 are substantially higher (Table 1), ranging from 1000 to 1500 \( \mu \)M. Although the \( K_m \) estimates for mFMO2 and hFMO2.1 were not significantly different from each other, they are approximately twice the level we recently reported for rFMO2 and mFMO2 (Krueger et al., 2001).

We have determined that older batches of expressed rFMO2 and mFMO2 used in our earlier publication and newly expressed batches of both of these proteins are now yielding \( K_m \) estimates similar to the values in Table 1. We traced the entire recent rise in \( K_m \) to a new lot of tricine (the earlier lot was purchased in the late 1980s, whereas the new lot was purchased in 2000), after performing identical assays with old and new protein batches in each of the two tricine lots (data not shown). Limited assays with borrowed tricine (same catalog number, purchased in 1993) and assays performed with Tris buffer both yielded \( K_m \) values similar to those observed with our newest lot of tricine (not shown). Our data do not rule out the possibility of a substrate-specific interaction with tricine as the cause of the original elevation in estimated \( K_m \). We tested two lots of methimazole; although no differences were observed in our assay results (data not shown), both batches of methimazole were of recent origin (1999 and 2000). In addition to the rise in \( K_m \), we have also observed an ortholog-specific change in observed activity that only occurred at pH 10.0 (unpublished results); but, other assays we performed were unaffected. We have not yet determined why our estimates do not agree with those of other investigators; however these differences have no effect on the normalized results reported in the rest of this paper. From comparison of calculated estimates made with older and newer protein batches, using the new lot of tricine, we have concluded that \( K_m \) values for mFMO2, hFMO2.1, and rFMO2 are not substan-

### Table 1

FMO content and kinetics of methimazole-dependent S-oxidation for expressed hFMO2.1 and mFMO2

<table>
<thead>
<tr>
<th>MFO Content</th>
<th>Percent FMO2</th>
<th>( V_{\text{max}} )</th>
<th>( K_m )</th>
<th>( V_{\text{max}}/K_m )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>hFMO2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.85</td>
<td>5.2</td>
<td>23.2</td>
<td>1.46</td>
<td>15.9</td>
</tr>
<tr>
<td>2</td>
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<td>13.8</td>
<td>14.3</td>
<td>1.01</td>
<td>14.2</td>
</tr>
<tr>
<td>mFMO2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.30</td>
<td>7.9</td>
<td>33.7</td>
<td>1.53</td>
<td>22.1</td>
</tr>
<tr>
<td>2</td>
<td>0.99</td>
<td>6.0</td>
<td>40.4</td>
<td>1.48</td>
<td>27.2</td>
</tr>
</tbody>
</table>

* \( V_{\text{max}} = \) nanomoles \( \cdot \) minute\(^{-1}\) \( \cdot \) nanomole\(^{-1}\) of MFO; \( K_m = \) millimolar determined at pH 8.5 using methimazole at 0.2, 0.5, 1.0, 2.0, and 5.0 mM; \( R^2 = \) Lineweaver-Burke correlation coefficients.
Assays were performed with microsomes from two batches of each clone using tricine buffer at the indicated pH values. Each batch was assayed twice at each pH. Mean velocity calculated under standard conditions was designated as 100% for each batch, and the remaining results were normalized accordingly. Mean-normalized clone activity and S.E. are shown. Velocities for 100% were 21.6 and 23.0 nmol · min⁻¹ · mmol⁻¹ for mFMO2 and 10.7 and 11.9 nmol · min⁻¹ · mmol⁻¹ for hFMO2.1.

Initially different from each other (not shown). Velocities observed with hFMO2.1 were consistently lower than what we observed for mFMO2 (Table 1; Fig. 1-3), so hFMO2.1 was also somewhat less efficient than mFMO2.

Recombinant hFMO2.1 and mFMO2 demonstrated the same overall response to changes in the pH of the buffer (Fig. 1) in the range from pH 8.0 to 9.5. Both had maximal activity at pH 9.5 (140% for mFMO2 and 188% for hFMO2.1) and 45 to 60% activity at pH 8.0. At pH 9.0 and above, however, the relative activity of hFMO2.1 was significantly higher than that observed for mFMO2 (p < 0.05). This difference in response reached a maximum at pH 10.5 (p < 0.0001); on average hFMO2.1 retained full activity, whereas activity was barely detectable from mFMO2. We did not initially assess activity at pH 7.5 since we expected that activity would be low and would not discriminate between these orthologs. Subsequent assays at pH 7.5 have confirmed our expectations; hFMO2.1 and mFMO2 have 18 and 23% activity, respectively (results not shown).

Modulators of FMO Activity. Expressed hFMO2.1 responded to magnesium and detergents with significantly (p < 0.0001) enhanced enzyme activity (Fig. 2), a response previously observed in the presence of magnesium (Dolphin et al., 1998). Recombinant hFMO2.1 had the greatest enhancement of activity in response to magnesium (nearly 3-fold) and an approximate 2-fold increase in response to detergents (170–205%). Modulators also significantly (p ≤ 0.004) altered the activity of expressed mFMO2, as reported earlier (Krueger et al., 2001). In the case of cholic acid, mFMO2 activity is decreased to 75% of standard conditions. Ortholog differences were highly significant (p ≤ 0.0001) for all treatments. A profile similar to hFMO2.1 has been documented for expressed and purified rFMO2 in the presence of magnesium (Lawton and Philpot, 1993; Krueger et al., 2001) and cholic acid (Williams et al., 1985; Lawton and Philpot, 1993; Krueger et al., 2001), although the response to magnesium is of a lesser magnitude. Response to detergents is dependent on detergent, detergent concentration, substrate assayed, and the specific FMO isoform or ortholog (Venkatesh et al., 1991); thus, in assays performed with other detergents or other substrates, hFMO2.1 might respond like mFMO2 rather than rFMO2.

Assessment of Potential hFMO2.1 Substrates. Several in vitro substrates of S- and N-oxidation by expressed hFMO2.1 were identified by monitoring substrate-dependent NADPH oxidation (Table 2). We performed the assays at pH 9.5 to improve the sensitivity of this assay. Since expressed hFMO2.1 was 1.88-fold more active at pH 9.5 than it was at pH 8.5 (Fig. 1), raising the pH of the assay effectively lowered the threshold of detection from 0.161 to 0.086 nmol of NADPH · min⁻¹. The smaller thioureas and thio-carbamides were good substrates for hFMO2.1. The calculated Kₘ decreased from 25...
The blot demonstrates that antibody to mFMO2 is both selective toward FMO and sensitive toward FMO2 orthologs.

Efforts to reduce or eliminate FMO1 and FMO3 cross-reactivity from the anti-mFMO2 antibody were unsuccessful (not shown). The optimal dilution of the antibody that did not limit FMO2 detection was 1:20,000 to 1:50,000. Our attempt to strip out cross-reacting antibodies from 1.0 µl of antiserum, used reasonable levels of adsorbing proteins (combined FMO1 and FMO3 was 4.4–35.2 µg) but yielded no improvement in ortholog selectivity. Further attempts to make this antibody ortholog-specific are likely to be unsuccessful.

Since we could not reduce antibody cross-reactivity to multiple FMO isoforms, we performed Western blots of expressed proteins and human lung microsomes with our antibody to mFMO2 and with commercially available anti-human FMO antibodies (1, 3, and 5) to determine the spectrum of FMOs in lung. Anti-mFMO2 antibody did not cross-react with any non-FMO proteins present in lung microsomes or Sf9 microsomes (Fig. 5A), and no FMO was detected from either H9 or control microsomes. Based on densitometry obtained from the Western blots, we estimate anti-mFMO2 detects 90 to 100% of hFMO2.1.

Our data with antibodies to hFMO1 and hFMO3 demonstrated that these proteins were absent from H6 and H9. Anti-hFMO1 antibody did not cross-react with expressed mFMO2 or hFMO2.1; no FMO1 was detected from the human lung samples, although the antibody did cross-react with non-FMO proteins in lung and Sf9 microsomes (Fig. 5B). Anti-hFMO3 detected non-FMO proteins from lung but not from Sf9 cells (Fig. 5C). In addition, the antibody was strongly cross-reactive with expressed mFMO2 and hFMO2.1 and detected FMO in H6 but not H9 lung microsomes. The pattern of detection and relative intensity of the detected bands suggests that there is no FMO3 present in the lung microsomes; it is likely that all FMO detected by this antibody to hFMO3 can be attributed to cross-reactivity of the antibody with hFMO2.1.

Antibody to hFMO5 cross-reacted with a limited number of non-FMO proteins but not with either of the expressed FMO2 proteins (Fig. 5D). This antibody weakly cross-reacted with proteins in both H6 and H9 with the approximate molecular weight of FMO, indicating another FMO isoform may be present in lung, perhaps FMO5. This is consistent with work indicating that hFMO5 mRNA is present at low levels in lung (Shephard et al., 1999). However, the low level of hFMO5 that we detected, combined with the reported unfavorable kinetics for methimazole metabolism by this isoform (Fig. 3B) and monkey (p < 0.0001) lung microsomes in both buffers; but the magnitude of difference between monkey and H6 microsomes was not significant (p = 0.08). Although residual activity of monkey and H6 microsomes was somewhat lower than it was from recombinant protein, residual rabbit activity was similar regardless of the treatment.

Confirmation of hFMO2.1 as the Source of Methimazole Metabolism. Genotyping studies have demonstrated that our antibody to expressed mFMO2 detects hFMO2.1 protein from Western blots of human lung preparations from individuals with at least one hFMO2*1 allele (Whetstone et al., 2000). We prepared a multi-isoform FMO Western blot to characterize the anti-mFMO2 antibody and found that it cross-reacted with every isoform tested (Fig. 4). However, not all isoforms or all variants of a particular ortholog were detected with equal efficiency. For isoforms that we could determine FMO content, mFMO2 ~ hFMO2.1 > rFMO2 > hFMO1 > hFMO3 > rFMO1.

The blot demonstrates that antibody to mFMO2 is both selective toward FMO and sensitive toward FMO2 orthologs.
Ntempt to detect hFMO2*2A and mFMO2. The response of hFMO2.1 to pH and detergents was more similar to hFMO2.1 to elevated temperatures was between mFMO2 and rFMO2, distinguishable under a range of conditions. Although the response of hFMO2.1 was similar to effectors of FMO activity. However, since AA sequences of hFMO2.1 and mFMO2 are 97% identical, we distinguished orthologs, which share 85% AA identity, are readily distinguished under a variety of test conditions (Krueger et al., 2001). Although we detected activity toward methimazole in lung microsomes from hFMO2*2A/hFMO2*2A (monkey), there was no thermal inactivation of H6. There was no thermal inactivation of H6. The only published report of active FMO protein chimeras involving more than a single AA interchange used pig and rabbit FMO1 successfully (Whetstine et al., 2000). Although we also performed that assay with 1.0 mg of protein · ml⁻¹, this may have been below the threshold of detection with this substrate. We demonstrated that both expressed hFMO2.1 and lung microsomes from H6, under some conditions, lose the majority of their capacity to metabolize methimazole due to a heat-associated decline of activity. We have speculated that failure to detect FMO activity from human lung microsomes could be partially due to post mortem heat inactivation. We do not know what protection may be afforded by excised lung tissue or how long donor samples were held at any given temperature. However, our results with expressed hFMO2.1 lend credence to the hypothesis that this protein may be partially heat inactivated in lung tissue from some donors; although based on our estimate of hFMO2.1 content in lung microsomes (8.8 pmol/mg), there was no thermal inactivation of H6.

Preliminary screening of S- and N-containing compounds for their substrate specificity of expressed hFMO2.1 determined by NADPH oxidation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (μM)</th>
<th>V (nmol · min⁻¹ · mg⁻¹)</th>
<th>Kₘ (μM)</th>
<th>Vₘₐₓ (nmol · min⁻¹ · mg⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Oxygination</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiourea</td>
<td>5–500</td>
<td>71a</td>
<td>25</td>
<td>51</td>
<td>0.98a</td>
</tr>
<tr>
<td>Ethylthiourea</td>
<td>10–1000</td>
<td>70</td>
<td>14</td>
<td>50</td>
<td>0.95</td>
</tr>
<tr>
<td>1-Phenylthiourea</td>
<td>20–500</td>
<td>35</td>
<td>4</td>
<td>25</td>
<td>0.99</td>
</tr>
<tr>
<td>1,3-Diphenylthiourea</td>
<td>250</td>
<td>N.A.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioacetanilide</td>
<td>200</td>
<td>110.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiobenzamide</td>
<td>200</td>
<td>69.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Oxygination</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Trimethylamine</td>
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<td>47.0</td>
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<tr>
<td>N-Dodecylhydroxylamine</td>
<td>200</td>
<td>35.0</td>
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</tbody>
</table>

N.A., no activity detected.

* R² = Lineweaver-Burke correlation coefficients.

Discussion

Comparisons made with mFMO2 and rFMO2 have demonstrated that these orthologs, which share 85% AA identity, are readily distinguishable under a variety of test conditions (Krueger et al., 2001). Since AA sequences of hFMO2.1 and mFMO2 are 97% identical, we expected similar responses to effectors of FMO activity. However, characterization demonstrated that hFMO2.1 and mFMO2 are clearly distinguishable under a range of conditions. Although the response of hFMO2.1 to elevated temperatures was between mFMO2 and rFMO2, the response of hFMO2.1 to pH and detergents was more similar to that observed for rFMO2 (Krueger et al., 2001) than it was to mFMO2.

Although we detected activity toward methimazole in lung microsomes from a hFMO2*2A/hFMO2*2A individual (H6), an earlier attempt to detect N-oxidation activity using dimethylaniline was not successful (Whetstine et al., 2000). Although we also performed that assay with 1.0 mg of protein · ml⁻¹, this may have been below the threshold of detection with this substrate. We demonstrated that both expressed hFMO2.1 and lung microsomes from H6, under some conditions, lose the majority of their capacity to metabolize methimazole due to a heat-associated decline of activity. We have speculated that failure to detect FMO activity from human lung microsomes could be partially due to post mortem heat inactivation. We do not know what protection may be afforded by excised lung tissue or how long donor samples were held at any given temperature. However, our results with expressed hFMO2.1 lend credence to the hypothesis that this protein may be partially heat inactivated in lung tissue from some donors; although based on our estimate of hFMO2.1 content in lung microsomes (8.8 pmol/mg), there was no thermal inactivation of H6.

Preliminary screening of S- and N-containing compounds for their ability to be oxidized by hFMO2.1 was accomplished by following NADPH oxidation. We followed the reaction at pH 9.5 to compensate for the lower sensitivity provided by this general assay. Like previous studies performed with rFMO2 (Nagata et al., 1990; Guo et al., 1992), hFMO2.1 is active toward thiourea compounds with a small cross-sectional area but is inactive toward 1,3-diphenylthiourea, which has a larger cross-section (11.2 Å). However, unlike rFMO2, which has a 10-fold increase in Kₘ for 3, the Kₘ of hFMO2.1 decreased 6-fold when 1-phenylthiourea was the substrate rather than thiourea (2.4 Å). A similar ortholog distinction between human and pig FMO1 was recently reported (Kim and Ziegler, 2000). The only published report of active FMO protein chimeras involving more than a single AA interchange used pig and rabbit FMO1.
identify drugs that may require specific dosing regimens in affected ethnic groups and individuals.

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References

Whetstone JR, Yauh M-F, McCarter DG, Williams DE, Park C-S, Kang JH, Cha Y-N, Dolphin (Wyatt et al., 1998). In the absence of a crystal structure, the documentation of ortholog differences in which underpinnings are likely to trace to the substrate binding site is significant. Our preliminary work indicates that chimeras generated using mFMO2, hFMO2.1, and rFMO2 will be active (data not shown). Although differences in response to pH, detergents, and heat can be screened for, there is no reason to assume a priori that any of these differences will provide structural insight. However, substrate-specific differences might yield structural clues, relevant to isoform and ortholog differences in substrate metabolism.

The finding that thioureas, thioacetanilide, and thiobenzamide are substrates for hFMO2.1 is a critical finding. These compounds undergo FMO-dependent S-oxidation to reactive sulfenic and sulfonic acid derivatives, resulting in covalent binding, GSH oxidation, and toxicity (Ziegler, 1991; Cashman, 1995). Not only is the lung an important route of entry for some of these compounds (e.g., ethylene thiourea), but also a number of thioureas and related compounds are known lung toxins (Cashman et al., 1982; Houeto et al., 1995). Since the hFMO2*1 allele occurs in approximately 26% of the African-American population (Whetstone et al., 2000), there is a possibility that this polymorphism could result in ethnic differences in metabolism of these potential toxins, in addition to small drugs. If so, knowing the allelic composition of this gene may eventually prove useful in identifying those individuals at increased risk from certain environmental toxicants.

This study has not only identified viable hFMO2.1 protein from a known heterozygote but also demonstrates the inherent difficulty of performing enzyme studies from donor tissue. In vitro production of hFMO2.1 circumvents these problems by providing a high-yield, enzyme-specific stock of reproducible quality amenable to study. An effort to screen potential drugs for metabolism by hFMO2.1 should...


