Cloning, Sequencing, Distribution, and Expression in *Escherichia coli* of Flavin-containing Monoxygenase 1C1

EVIDENCE FOR A THIRD GENE SUBFAMILY IN RABBITS*

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Two full-length cDNA clones (2.2 kilobases) encoding a newly recognized form of mammalian flavin-containing monooxygenase (FMO) have been isolated from independent libraries constructed with mRNA from different rabbits. The cDNAs encode a polyepitope of 533 amino acids which contains two putative pyrophosphate binding domains and a hydrophobic carboxyl terminus characteristic of FMOs. This sequence is 52 and 57% identical to sequences of the rabbit "hepatic" and "pulmonary" FMOs, respectively, and 55% identical to the sequence of "liver form 2" published recently by Ozols (Ozols, J. (1991) Arch. Biochem. Biophys. 290, 103–115). cDNA for the new FMO (FMO 1C1) hybridizes with two species of mRNA, one of 2.6 kilobases and one of about 5.4 kilobases, from liver or kidney, but not lung. Guinea pig, hamster, rat, and mouse all express this form of FMO in liver, kidney, and lung. FMO 1C1 has been tentatively characterized following expression in *Escherichia coli*. It is inactive with methimazole as substrate but highly active with n-octylamine. The temperature lability, responses to ions and detergent, and pH optimum of FMO 1C1 are similar to values reported for hepatic FMO. Sequence comparisons and analysis of rabbit and human genomic DNA indicate that FMO 1C1, as well as the pulmonary and hepatic FMOs, comprise a single gene family made up of distinct gene subfamilies (A, B, C, D, . . . N), each appearing to contain a single gene. A nomenclature, based on these interrelationships and following the same designations used for classifying cytochromes P-450, is proposed.

Mammalian microsomal monoxygenases have the capacity to metabolize a remarkable variety of exogenous chemicals including many drugs, pesticides, and industrial by-products. This oxidative process results primarily in product elimination, but it can also yield reactive intermediates that bind covalently to DNA, RNA, and protein with toxic consequences. Although the best known of these monooxygenases are those comprising the cytochrome P-450 system (1), a second family of enzymes, the flavin-containing monooxygenases (FMOs: EC 1.14.13.8), is beginning to emerge.

Localized to many mammalian tissues, the FMOs catalyze the NADPH- and oxygen-dependent metabolism of numerous xenobiotics that have nitrogen-, sulfur-, and phosphorus-containing functional groups with polarizable, electron-rich centers (2, 3). The first FMO to be characterized in any detail was a form present in pig liver (4). Subsequently, similar "hepatic" FMOs were isolated from rat (5), mouse (6), rabbit (7, 8) and guinea pig (9) liver. A second type of FMO, the "pulmonary" form, has been isolated from rabbit (7, 10) and mouse (7) lung. The hepatic FMO is expressed in liver, kidney, and lung of several species (11, 12), whereas the pulmonary form is expressed in lung and kidney but not in liver (12). It is now known that the pulmonary and hepatic forms of the FMO expressed in rabbit are products of related but distinct genes and that their primary sequences are 56% identical (13). Based on sequence comparisons, orthologs of the hepatic FMO are expressed in pig (14) and human (15).

In addition to the pulmonary and hepatic forms of the FMO, evidence for additional members of this gene family exists. Two forms of the FMO have been purified from rabbit liver and sequenced. The sequence of one (16) is highly similar (>99% identity) to that derived for the hepatic FMO, whereas that of the second (17) is related equally (about 55% identity) to the sequences of both the hepatic and pulmonary forms. Two forms of the FMO have also been isolated from guinea pig liver (9) but have not been shown to be products of different genes.

Recently, we have compared the kinetics of the pulmonary and hepatic FMOs expressed in COS-1 cells with those of the enzymes present in pulmonary and hepatic microsomal preparations, and our results indicate that multiple forms of the FMO are present in both tissues (18). To explore further the possibility that additional forms of the FMO exist, we have carried out low stringency screening of a rabbit liver cDNA library with probes isolated from the 5'-portions of the cDNAs encoding the pulmonary and hepatic enzymes. We report the isolation and sequencing of a cDNA encoding a third FMO, the tissue and species distribution of its mRNA, and the characteristics of the enzyme expressed in *Escherichia coli*. The sequence derived from the third cDNA species does not match that of the hepatic or pulmonary forms (13) or that reported by Ozols for a possible second gene product in liver (17). A comparison of the mRNA levels for three forms of the FMO indicates that expression of these enzymes varies significantly with tissue and species. Each enzyme appears to

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L08449.

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‡ The abbreviations used are: FMO(s), flavin-containing monooxygenase(s); Tricine, N-tris(hydroxymethyl)methylglycine; kb, kilobases.
Flavin-containing Monooxygenase 1C1

represent a distinct gene subfamily that contains a single gene.

**MATERIALS AND METHODS**

**Isolation of mRNA and Genomic DNA**—Total RNA was obtained from liver, kidney, and lung of rabbits (adult male New Zealand White; Dutch Farms, Denver, PA) by a modification (19) of the methods of Chirgwin et al. (20) and Glisin et al. (21). Polyadenylated mRNA was isolated by chromatography on oligo(dT)-cellulose (22). Standard procedures (23, 24) were used for the isolation of genomic DNA from rabbit liver. Human genomic DNA from placenta was purchased (Clontech; San Diego, CA).

**Construction and Screening of a cDNA Library**—Double-stranded cDNA was synthesized from rabbit liver mRNA using a kit available commercially (Pharmacia LKB Biotechnology Inc.). A phage library was constructed in Xzap II (Stratagene; La Jolla, CA) after selection of eDNA species between 1.5 and 4.0 kilobases. The library was screened (10^7 recombinant plaques) in duplicate by plaque hybridization with rabbit hepatic 5'-cDNA (a 735-base pair EcoRI-KpnI fragment) and rabbit pulmonary 5'-cDNA (a 521-base pair EcoRI-FstI fragment) labeled with [3P]dCTP (Amersham Corp.) by the random priming method (25). Prehybridization was carried out for 3–4 h in 6X SSC, denatured salmon sperm DNA (100 μg/ml), 4X Denhardt’s, 0.5% SDS, and 50% formamide. Hybridization (10^6 cpm/ml) was carried out overnight at 37°C in the same solution. Hybridized filters were washed twice in 1X SSC, 0.1% SDS for 1 h at 37°C and then subjected to autoradiography for 12–24 h at -80°C. Isolated clones were then screened under conditions of high stringency and signals compared with those obtained with known pulmonary and hepatic clones. Clones undergoing significant loss of signal at high stringency were investigated further.

**DNA Sequencing**—EcoRI-restricted fragments of the isolated cDNA clones were subcloned into pBluescript (Stratagene) and sequenced (Sequenase Version 2.0; U.S. Biochemical Corp.) by the dideoxy chain termination method (26). Reactions were primed with oligonucleotides complementary to specific regions within the cDNA insert of clone and ligated into pKKHC that had been digested with NcoI and PstI and subjected to autoradiography. The truncated cDNA was then digested with Ncol and PstI and ligated into pKKHC that had been digested with the same enzymes. The resulting plasmid (pKK1C1) was used to transform competent *E. coli* strain JM109 or XL1. Colonies transformed with pKK1C1 or vector alone were grown at 37°C in LB medium plus ampicillin (50 μg/ml) to an optical density of 0.4–0.5. Isopropyl 1-thio-β-D-galactopyranoside was then added (final concentration, 1 mM), and the cells were grown overnight at 30°C. Cells were harvested by centrifugation at 2,000 X g for 5 min, resuspended in 10 ml TE-glucose (50 mM glucose, 25 mM Tris, pH 8.0, and 0.5 mM EDTA) containing lysozyme (1 mg/ml), and incubated for 20 min at 4°C. Cells were harvested again by centrifugation at 2,000 X g for 5 min, resuspended in 3 ml of lysis buffer (100 mM KCl, 50 mM KF, pH 8.0, and 1 mM EDTA), and sonicated (four 30-s pulses separated by 1-min periods of cooling). Cell debris were removed by centrifugation for 5 min at 2,000 X g, and the supernatant fraction produced was centrifuged at 100,000 X g for 25 min to obtain the 100,000 X g particulate fraction. The 100,000 X g particulate fraction was resuspended at in KF (50 mM, pH 7.4) containing glycerol (20%), and EDTA (1 mM), and stored at -70°C.

**Characterization of Expressed FMO 1C1**—Expressed FMO 1C1 was detected by electrophoresis of the *E. coli* 100,000 X g particulate on polyacrylamide gels in the presence of SDS (SDS-polyacrylamide gel electrophoresis) and staining with Coomassie Blue (31). Flavin content (FAD and FMN) was determined fluorometrically by the method of Faeder and Siegel (32), hydrogen peroxide production by the method of Hildebrandt et al. (33), and hydroxyamine-reducing equivalents by the method of Belanger et al. (34). Standard curves for this assay were determined with known amounts of N-benzylhydroxylamine. Assays for H2O2 and hydroxyamine equivalents were carried out in the absence of EDTA.

FMO activity was determined spectrophotometrically (Amino DW2A UV/VIS spectrophotometer) with methimazole as the substrate (35). The assay contained Tricine/KOH (0.1 M, pH 8.4) or potassium phosphate (0.1 M, pH 7.4), EDTA (1 mM), 5,5'-dithiobis(nitrobenzoic acid) (0.06 mM), diethiothreitol (0.2 mM), NADPH (0.1 mM), and microsomal protein (0.15–0.20 mg/ml). Reactions were initiated by the addition of substrate after obtaining a stable base line of absorbance at 412 nm. For substrates other than methimazole, the rates of reaction were monitored by following NADPH oxidation at 340 nm. In all cases protein concentrations were determined by the method of Lowry et al. (36).

**RESULTS**

**Isolation and Sequencing of cDNA Clones Encoding FMO Enzymes Expressed in Liver**—A rabbit liver cDNA library (I) in Xzap II was screened in duplicate with 5'- pulmonary and hepatic FMO cDNA probes under conditions of low stringency. Positive clones were then screened at high stringency, and a single clone (la) that did not hybridize strongly with either probe was isolated. Hybridization of clone la was nearly undetectable with the hepatic probe and was weak with the pulmonary probe under conditions of high stringency. Results of control experiments showed little difference between low and high stringency for hybridization of known pulmonary and hepatic clones with the 5'-probes. These results indicated that clone la was neither a hepatic nor a pulmonary FMO clone but was likely related to both. Digestion of clone la with EcoRI restriction endonuclease yielded three fragments, a result not obtained with clones for the hepatic or pulmonary FMOs. The EcoRI fragments were isolated and subcloned into pBluescript vector for nucleotide sequencing. The strategy used for the complete sequencing of Ia is shown in Fig. 1. The cDNA insert of clone la contains 2,196 bases with a 5'-flanking region of 98 bases, an open reading frame of 1,599 bases terminating in a TAG stop codon, and a 3'-flanking region of 496 bases (Table 1). A consensus polyadenylation signal (AAATAAA) was found 28 bases 5' of a polyadenylated tail. The open reading frame encodes a polypeptide of 533 amino acids (Table 1), with a molecular mass of 59,885 Da and a pl of 8.82. Sequences thought to be associated with pyrophosphate binding (GGXGXXG/A) are located at residues 9-14 and 192-197. The sequence derived from clone Ia is 52% identical with the hepatic FMO sequence, 57% identical to...
Positions in a number of flavoproteins other than the FMOs and form 2 as FMO 1D1. The human "FMO1" (15) and pig hepatic FMO (14) are IA1 orthologs as determined by their apparent associations with rabbit FMO 1Al) and their apparent relationships with the interrelationships among the primary sequences. The same sequences are also located in similar structures are very similar, with percent identities ranging from 52 to 57% (Table 1).

Alignment of the four primary sequences also reveals that FMOs 1B1 and 1C1 share a 3-residue gap corresponding to positions 318–320 of the consensus sequences (317–319 of 1Al and 316–318 of 1D1), that FMO 1C1 is missing consensus residue 346, and that FMO 1D1 is missing consensus residues 427 and 429. Some improvement can be made in the alignment of the final 35 carboxyl-terminal residues, but only with an excessive number of insertion/deletions.

Although absolute structural identity for the four FMOs is only about 30%, high degree of overall structural relatedness is shown by a comparison of their hydrophathy profiles (Fig. 2). The profiles are very similar and have five putative membrane-associated regions in common. A sixth hydrophobic region is found in all except 1B1. The greatest hydrophobic character, possibly associated with a membrane anchor, is found at the carboxyl terminus of each sequence.

Comparison of FMO Sequence Identities and Proposed Nomenclature—The amino acid sequence derived from the nucleotide sequence of clone Ia, and those of the hepatic FMO (13), pulmonary FMO (13), and form 2 FMOs (17) are compared in Table II. The interrelationships among the primary structures are very similar, with percent identities ranging from 52 to 57% (Table II). According to the parameters used for development of a nomenclature for cytochrome P-450 (1), these relationships describe a single gene family (designated 1) and four subfamilies (designated A, B, C, and D), each containing a single gene (designated 1). Although the identification of form 2 at the nucleotide level has been made in the rabbit but not yet reported, comparisons of amino acid sequences alone indicate that form 2 is likely the product of a distinct gene. Because of these considerations we will refer to the hepatic enzyme as FMO 1A1, the pulmonary enzyme as FMO 1B1, the enzyme encoded by clone Ia as FMO 1C1, and form 2 as FMO 1D1. The human "FMO1" (15) and pig hepatic FMO (14) are IA1 orthologs as determined by their sequences (which are 86 and 87% identical, respectively, to that of rabbit FMO 1A1) and their apparent associations with single genes.

Structural Comparisons of FMOs 1A1, 1B1, 1C1 and 1D1—The amino acid sequence identities among the FMOs are all less than 60%, but their primary structures share several specific and a number of general properties. Most notably, in all four sequences the GXGXGG and GXGXXG/A sequences, which have been associated with the binding of FAD and NADP, respectively (38), are located at identical positions (Table III). The same sequences are also located in similar positions in a number of flavoproteins other than the FMOs (Table III). Alignment of the primary sequences of the four FMOs (Table IV) shows that the frequency of completely conserved residues is highest in the amino-terminal region (50% for the first 67 positions) and lowest in the carboxyl-terminal region (20% for the final 60 positions). With the exception of a 5-residue peptide (FATGY) starting at consensus position 331, no common peptides of more than 3 residues are found beyond position 51. The FATGY peptide is also present in pig FMO 1A1 (14), human FMO 1A1 (15), and guinea pig FMO 1B1 (39).

Analysis of mRNA from livers, lungs, and kidneys of rabbit, rat, mouse, hamster, and guinea pig by hybridization with full-length cDNA probes for FMO 1A1, 1B1, and 1C1 is shown in Fig. 4. Hepatic and renal samples from each species contained mRNA that hybridized with the probes for FMO 1A1 and FMO 1C1. However, large differences among the liver to kidney ratios for the two mRNAs were noted. In rabbit, guinea pig, and hamster, hepatic expression of mRNA for FMO 1A1 was greater than renal expression, but the opposite was true for rat and mouse. Hepatic expression of FMO 1C1 was similar to renal expression in the hamster but greatly exceeded renal expression in all other species. The results also show highly variable expression of mRNA for FMO 1A1 and FMO 1C1 in lung. FMO 1A1 mRNA was detected in pulmonary samples from guinea pig, hamster, mouse, and rat. With both mouse and rat, pulmonary expression of mRNA for FMO 1A1 exceeded hepatic expression. Trace amounts of FMO 1A1 mRNA may also have been detected in the samples (10 μg) from rabbit lung. Similar species differences were noted for the pulmonary expression of mRNA for FMO 1C1, although in guinea pig, hamster, and mouse the relative amounts of 1C1 mRNA were much less than the amounts of 1A1 mRNA. With the exception of the mouse, multiple bands of mRNA from lung were detected with the probe for FMO 1B1. The most complex pattern, that for rabbit, has been described previously (13). Trace amounts of 1B1 mRNA were also detected in renal samples from hamster and mouse. No 1B1 mRNA was detected in the sample (1 μg) of RNA from rabbit kidney, although it has been seen clearly in larger (5 μg) samples (13). There was no detectable hybridization of the probe for FMO 1B1 with hepatic mRNA from any species.

Fig. 1. Strategy used for sequencing cDNAs encoding rabbit liver FMO 1C1. The clones depicted (Ia, 2,196 bases; IIa, 2,183 bases) were isolated from independent libraries. Sequences were obtained from EcoRI fragments in pBluescript through the use of T3 and T7 universal primers (arrows with closed squares) and directly through the use of specific oligonucleotide primers (arrows with vertical lines).

\[ \text{Position in a number of flavoproteins other than the FMOs and form 2 as FMO 1D1. The human "FMO1" (15) and pig hepatic FMO (14) are IA1 orthologs as determined by their apparent associations with rabbit FMO 1Al) and their apparent relationships with the interrelationships among the primary sequences. The same sequences are also located in similar structures are very similar, with percent identities ranging from 52 to 57% (Table II). According to the parameters used for development of a nomenclature for cytochrome P-450 (1), these relationships describe a single gene family (designated 1) and four subfamilies (designated A, B, C, and D), each containing a single gene (designated 1). Although the identification of form 2 at the nucleotide level has been made in the rabbit but not yet reported, comparisons of amino acid sequences alone indicate that form 2 is likely the product of a distinct gene. Because of these considerations we will refer to the hepatic enzyme as FMO 1A1, the pulmonary enzyme as FMO 1B1, the enzyme encoded by clone Ia as FMO 1C1, and form 2 as FMO 1D1. The human "FMO1" (15) and pig hepatic FMO (14) are IA1 orthologs as determined by their sequences (which are 86 and 87% identical, respectively, to that of rabbit FMO 1A1) and their apparent associations with single genes.}

\[ \text{Structural Comparisons of FMOs 1A1, 1B1, 1C1 and 1D1—The amino acid sequence identities among the FMOs are all less than 60%, but their primary structures share several specific and a number of general properties. Most notably, in all four sequences the GXGXGG and GXGXXG/A sequences, which have been associated with the binding of FAD and NADP, respectively (38), are located at identical positions (Table III). The same sequences are also located in similar positions in a number of flavoproteins other than the FMOs (Table III). Alignment of the primary sequences of the four FMOs (Table IV) shows that the frequency of completely conserved residues is highest in the amino-terminal region (50% for the first 67 positions) and lowest in the carboxyl-terminal region (20% for the final 60 positions). With the exception of a 5-residue peptide (FATGY) starting at consensus position 331, no common peptides of more than 3 residues are found beyond position 51. The FATGY peptide is also present in pig FMO 1A1 (14), human FMO 1A1 (15), and guinea pig FMO 1B1 (39).} \]
TABLE I

Nucleotide and derived amino acid sequences from cDNA encoding a third form of flavin-containing monooxygenases expressed in rabbit liver

<table>
<thead>
<tr>
<th>Species</th>
<th>% Identity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>98%</td>
<td>FMO 1A</td>
</tr>
<tr>
<td>Rabbit</td>
<td>97%</td>
<td>FMO 1B</td>
</tr>
<tr>
<td>Rabbit</td>
<td>95%</td>
<td>FMO 1C</td>
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</table>

**Analysis of DNA for Genes Encoding FMOs 1A, 1B, and 1C**—Southern blot analysis of rabbit genomic DNA by hybridization with selective 3' probes for FMO 1A (StuI-EcoRI fragment, bases 1346–2062), FMO 1B (PstI-EcoRI fragment, bases 1368–2611), and FMO 1C (StyI-EcoRI fragment, bases 1403–2196) is shown in Fig. 5. DNA restricted with HindIII, EcoRI, or PstI hybridized as single bands with one exception; analysis of HindIII-digested DNA for FMO 1C gave two bands. These bands, at ~3.8 and ~2.3 kb, are consistent with HindIII restriction sites at bases 1735 and 1858, close enough together to give the appearance of a single cut.

**Table II**

Comparison of the primary structures of the mammalian microsomal flavin-containing monooxygenases and a proposed nomenclature

<table>
<thead>
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<th>Species</th>
<th>Trivial name</th>
<th>No.</th>
<th>% Identity 1</th>
<th>% Identity 2</th>
<th>% Identity 3</th>
<th>% Identity 4</th>
<th>Proposed name</th>
<th>Reference</th>
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<tr>
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<td>Hepatic FMO</td>
<td>1</td>
<td>56</td>
<td>52</td>
<td>54</td>
<td>87</td>
<td>86</td>
<td>55</td>
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<tr>
<td>Rabbit</td>
<td>Pulmonary FMO</td>
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<td>57</td>
<td>56</td>
<td>57</td>
<td>58</td>
<td>86</td>
<td>55</td>
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<tr>
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<td>Hepatic FMO</td>
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<td>55</td>
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<td>55</td>
<td>55</td>
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<tr>
<td>Pig</td>
<td>Hepatic FMO</td>
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*This sequence is reported in the present work.*

Analysis of DNA for Genes Encoding FMOs 1A, 1B, and 1C—Southern blot analysis of rabbit genomic DNA by hybridization with selective 3' probes for FMO 1A (StuI-EcoRI fragment, bases 1346–2062), FMO 1B (PstI-EcoRI fragment, bases 1368–2611), and FMO 1C (StyI-EcoRI fragment, 1403–2196) is shown in Fig. 5. DNA restricted with HindIII, EcoRI, or PstI hybridized as single bands with one exception; analysis of HindIII-digested DNA for FMO 1C gave two bands. These bands, at ~3.8 and ~2.3 kb, are consistent with HindIII restriction sites at bases 1735 and 1858, close enough together to give the appearance of a single cut.

The 3' probes for rabbit FMOs 1A, 1B, and 1C also produced single bands when hybridized to genomic DNA from other species. The results obtained with human DNA restricted with HindIII, EcoRI, and PstI are shown in Fig. 6. As seen with rabbit, the bands of DNA detected with the three
### TABLE 1

**FAD and NADP binding domains of the mammalian flavin-containing monoxygenases and several related flavoproteins**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>AA</th>
<th>FAD-binding domain</th>
<th>AA</th>
<th>NADP-binding domain</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMO 1A</td>
<td>Pig</td>
<td>4</td>
<td>RVAIGVAGSGLAS1KCCLEEGLEPTCFER</td>
<td>186</td>
<td>SVIVTGNSGSDTDFAVEAHLKVKVFLSTT</td>
<td>14</td>
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<tr>
<td>FMO 1A</td>
<td>Rabbit</td>
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<td>RVAIGVAGSGLAS1KCCLEEGLEPTCFER</td>
<td>186</td>
<td>RVLVTGNSGSDTDFAVEAHLKVKVFLSTT</td>
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<tr>
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<td>RVAIGVAGSGLAS1KCCLEEGLEPTCFER</td>
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<td>RVLVTGNSGSDTDFAVEAHLKVKVFLSTT</td>
<td>15</td>
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<tr>
<td>FMO 1B</td>
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<td>RVLVTGNSGSDTDFAVEAHLKVKVFLSTT</td>
<td>18</td>
</tr>
<tr>
<td>FMO 1B</td>
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<td>4</td>
<td>RVAIGVAGSGLAS1KCCLEEGLEPTCFER</td>
<td>186</td>
<td>RVLVTGNSGSDTDFAVEAHLKVKVFLSTT</td>
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<td>FMO 1C</td>
<td>Rabbit</td>
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<td>RVLVTGNSGSDTDFAVEAHLKVKVFLSTT</td>
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<tr>
<td>FMO 1D</td>
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<td>JUCD</td>
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<td>RTVVGGQGADGFLNLARWGEAFAINW</td>
<td>40</td>
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</table>

**Consensus**

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### TABLE IV

**Alignment of primary structures of FMO 1A1, FMO 1B1, FMO 1C1, and FMO 1D1**

A consensus sequence shows amino acids in all positions where at least three of the FMO sequences are in agreement; all other positions are designated by asterisks. Positions of complete agreement are shown by the dark boxes, and pyrophosphate binding sites are shown by dark bars. The data for 1A1 and 1B1 are from Lawton et al. (13) and for 1D1 are from Ozols (17).

**Probes are distinctly different with no cross-hybridization observed.**

**Expression of FMO 1C1 in E. coli**—Expression of FMO 1C1 in *E. coli* (strains JM109 and XL1 Blue) was monitored by SDS-polyacrylamide gel electrophoretic analysis of the 100,000 × g particulate fraction. The Coomassie Blue staining pattern of the fraction from transformed cells showed a significant band corresponding to the molecular mass of FMO 1C1 (~62,000 Da) which was not seen with the control cells (Fig. 7). In addition, the intensity of a band at ~20,000 Da was increased significantly following transformation with FMO 1C1. In contrast, with *E. coli* strain XL1 the intensity of this band was equally high with both the vector alone and FMO 1C1-transformed cells (Fig. 7). The FMO 1C1 expressed
in JM109 cells was examined in detail, and the results are reported below. No differences have been noted between these results and those of preliminary experiments carried out with FMO 1C1 expressed in XL1 cells.

The 100,000 × g particulate fraction from JM109 cells expressing the 62-kDa protein and from control cells was analyzed for bound flavin in the form of FMN and FAD. The FAD content of the particulate fraction from the transformed cells was much greater (675 ± 62 versus 161 ± 19 pmol/mg of protein) and the FMN content slightly lower (43 ± 5 versus 70 ± 9) in the three independent recombinant and control preparations examined. Assuming that the difference in FAD content (514 pmol/mg of protein) was equivalent to the enzyme concentration (1 mol of FAD/mol of enzyme), FMO 1C1 accounted for an average of 3.2% of the membrane protein in three different transformations.

The catalytic activity of expressed FMO 1C1 was examined with methimazole, chlorpromazine, prochlorperazine, imipramine, N,N-dimethylaniline, cysteamine, trimethylamine, triethylamine, n-decylamine, n- nonylamine, and n-octylamine as potential substrates. Only with the last two compounds was any activity observed. Increases in NADPH oxidation with n-octylamine and n-decylamine (3 mM each) were 6.5 and 3.8 nmol·min⁻¹·mg of protein⁻¹, respectively. The capacity of the E. coli system to support other FMO-catalyzed reactions was demonstrated by the activity of expressed FMO 1B1 with methimazole as substrate (not shown). The turnover number, approximately 20 nmol of product·min⁻¹·nmol of FMO 1B1⁻¹, was similar to that observed for purified FMO 1B1 with thiourea as substrate (21.6) as calculated from the data of Tynes et al. (39).

The FMO 1C1-mediated oxidation of NADPH observed with n-octylamine could have been a result of substrate metabolism or an uncoupling reaction involving breakdown of the hydroperoxyflavin-enzyme intermediate or both (39).
Therefore, we investigated the formation of \( \text{H}_2\text{O}_2 \) (the product of uncoupling) and hydroxylamine equivalents (one product of substrate metabolism) catalyzed by FMO 1C1 in the presence of \( n\)-octylamine. Similar experiments were carried out with expressed FMO 1B1, which is known to metabolize \( n\)-octylamine (39), and the results are shown in Fig. 8. With FMO 1B1, formation of \( \text{H}_2\text{O}_2 \) and hydroxylamine equivalents was associated with \( n\)-octylamine-induced rates of NADPH oxidation (12.8 nmol of NADPH oxidized·min\(^{-1}\)·nmol of FMO 1B1\(^{-1}\)). Each accounted for approximately 40% of the overall activity, the remainder likely a result of further metabolism or breakdown of the \( N\)-hydroxy metabolite. These results are very similar to those obtained with purified 1B1 (39). In contrast, increased rates of formation of \( \text{H}_2\text{O}_2 \) and hydroxylamine equivalents were not observed in association with \( n\)-octylamine-induced oxidation of NADPH catalyzed by FMO 1C1. The lack of \( \text{H}_2\text{O}_2 \) formation ruled out an uncoupling reaction (added \( \text{H}_2\text{O}_2 \) could be recovered quantitatively from standard incubations containing expressed FMO 1C1) and indicated that metabolism of \( n\)-octylamine must have occurred. The failure to detect hydroxylamine equivalents will be discussed below.

The metabolism of \( n\)-octylamine catalyzed by expressed FMO 1C1 in the particulate fraction from \( E. coli \) was further characterized with respect to a number of parameters (Fig. 9). The \( \text{pH} \) optimum for the reaction was \( \approx 9.0 \) (Fig. 9A), activity was inhibited in a time-dependent manner by 100 mM \( \text{MgCl}_2 \) and 1% sodium cholate (Fig. 9C), and the enzyme was heat-labile; complete loss of activity was observed with treatment at 45 °C for 5 min. (Fig. 9D). The \( K_m \) and \( V_{\text{max}} \) for the reaction, as determined from results obtained with concentrations of \( n\)-octylamine between 0.2 and 5 mM, were \( \sim 1.5 \) mM and \( \sim 11 \) nmol of NADPH oxidized·min\(^{-1}\)·mg of protein\(^{-1}\) (Fig. 9B). Based on a FMO 1C1 concentration of 0.53 nmol/mg in the preparation examined, as determined from the FAD content, the catalytic constant was 20.8 nmol of NADPH oxidized·min\(^{-1}\)·nmol of FMO 1C1\(^{-1}\). At concentrations of \( n\)-octylamine greater than 10 mM, the activity of FMO 1C1 was less than predicted, and at concentrations greater than 20 mM inhibition was observed.

**DISCUSSION**

Recent findings (17, 18) indicate that more than one gene product is involved in FMO activity expressed in rabbit liver. We have investigated this by screening rabbit liver cDNA libraries with probes constructed from the regions of highest identity (5'·coding) between the cDNAs encoding the rabbit liver (1A1) and lung (1B1) FMOs. Two identical cDNA clones that encode a third form of rabbit FMO, FMO 1C1, were isolated from independent libraries. Like the previously identified forms of FMO, 1C1 contains the characteristic FAD and NADP pyrophosphate binding domains and a markedly hydrophobic carboxyl terminus. The amino acid sequence derived for FMO 1C1 is 57 and 52% identical to the sequences of FMO 1A1 and FMO 1B1, respectively. The derived sequence of FMO 1C1 is also 55% identical to the sequence of the form 2 rabbit liver FMO purified by Ozols (17). These relationships indicate that form 2 is a product of a distinct gene and can be designated FMO 1C1.

**Fig. 6. Analysis of human genomic DNA by hybridization with cDNAs encoding FMO 1A1, 1B1, and 1C1.** The details of this analysis are the same as those listed in the legend for Fig. 5 with the exception that human hepatic genomic DNA (20-μg samples) was used.

**Fig. 7. Expression of FMO 1C1 in E. coli.** Samples (120 μg of protein) from the 100,000 × g particulate fractions from isopropyl-β-D-galactopyranoside-induced cultures of \( E. coli \) were electrophoresed on polyacrylamide gels in the presence of SDS and stained with Coomassie Blue. \( E. coli \) (strain JM109, lanes 2 and 4; strain XL1 Blue, lanes 3 and 5) was transformed with recombinant FMO 1C1 cDNA (lanes 4 and 5) or with nonrecombinant pKKHC vector (lanes 2 and 3). The standards run in lane 1 were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine plasma albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). The arrow points to the expressed FMO 1C1.

**Fig. 8. Metabolism of \( n\)-octylamine catalyzed by FMO 1B1 and 1C1 expressed in \( E. coli \).** Rates of NADPH oxidation (NADPH, solid bars), \( \text{H}_2\text{O}_2 \) formation (\( \text{H}_2\text{O}_2 \), stippled bars), and formation of hydroxylamine equivalents (RNOH, cross-hatched bars) were determined in the absence (basal rates) and presence of \( n\)-octylamine. All samples were from 100,000 × g particulate fractions of \( E. coli \) (strain JM109) transformed with recombinant FMO 1B1 or 1C1. Activities are reported as a percent of the rate of oxidation of NADPH obtained in the presence of \( n\)-octylamine.
detected in two different cDNA libraries, the level of FMO that observed for FMO 1Bl which is also associated with 

of glutathione reductase and the FMOs reveals absolute con-

... Dolphin and kidney. For example, renal expression of FMO 1Al is 

of mRNA levels. Analysis of mRNA also showed large species 

5.4 kb) are associated with FMO 1C1. Based on numbers of clones 

striking similarity among the NADP binding sequences (see 

or as percent control (panels C and D). Samples used for the temperature stability experiment (panel D) were heated at 

45 °C for the times indicated and then stored on ice until assayed at 37 °C. The samples examined were from the 100,000 

× g particulate fraction of E. coli (JM109) transformed with recombinant FMO 1C1.

1D1 and that the FMO gene family consists of at least four 

subfamilies.

Analysis of mRNA indicates that FMO 1C1, like FMO 1A1, 

is expressed in liver and kidney but not in lung of rabbits. Unlike FMO 1A1, however, two species of mRNA (2.6 and 

5.4 kb) are associated with FMO 1C1. These transcripts appear to be encoded by a single gene, a finding similar to 

that observed for FMO 1B1 which is also associated with multiple species of mRNA. Based on numbers of clones 
detected in two different cDNA libraries, the level of FMO 1C1 expression in rabbit liver is only about 25% that of FMO 1A1. Similar results were obtained with qualitative analysis 
of mRNA levels. Analysis of mRNA also showed large species differences in the expression of FMO 1A1 and 1C1 in liver and kidney. For example, renal expression of FMO 1A1 is 

clearly greater than hepatic expression in the rat and mouse. In these species, therefore, the so-called liver FMO appears to 

be a kidney FMO. Dolphin et al. (15) have shown that expression of FMO 1A1 in human liver is also relatively low. Analysis of genomic DNA from rabbits and humans indicates that interpretation of the expression results is not complicated by the presence of more than one gene in each subfamily, at least in the case of FMOs 1A1, 1B1, and 1C1.

FMO 1C1, like FMO 1A1 and 1B1, contains putative FAD and NADP binding domains that compare favorably with 
those present in glutathione reductase and a number of other flavoproteins (see Table III). Characteristic of the FAD and 

NADP binding domains of glutathione reductase is a pyrophosphate-binding ββββ fold centered around a highly conserved sequence, GXGXXG/A (37). Comparison of the 30 residues that form the entire pyrophosphate binding domains of glutathione reductase and the FMOs reveals absolute conservation of key residues in the FAD binding region and a striking similarity among the NADP binding sequences (see 

Table III). All of these sequences contain a hydrophilic residue located exactly 4 residues from the 1st glycine of the 
GXXGXXG sequence, a core of six predominantly hydrophobic residues, and a negatively charged residue (Asp or Glu) located exactly 18 residues from the 3rd glycine. This residue forms a hydrogen bond with the 2' OH group of the FAD adenine ribose (37). In the NADP binding domain the corresponding residue at the carboxyl terminus of the β-strand is neutral, presumably to accommodate the negatively charged 2 phosphophate group (37). All of the FMOs contain a neutral threonine at the position (214 for 1A1 and 1B1, 215 for 1C1, and 213 for 1D1) equivalent to Ile-217 of glutathione reductase. The two binding domains of the FMOs and flavoprotein disulfide oxidoreductases are the only segments of these proteins which display any significant overall sequence identity.

The absolute conservation of two pentapeptides (-EGLEP- beginning at residue 25 and -FATGY- beginning at residue 
328 of FMO 1C1) in the primary structures of the mammalian FMOs suggests that these sequences may have some vital 
structural or functional role. Computer searches of the SwissPro data base revealed that one of these peptides (FATGY) is present in the bacterial FMO, lysine N'-hydroxylase (40). It is of interest that lysine N'-hydroxylase is also the only nonmammalian FMO thought to form a stable, substrate-independent, C(4α)-hydroperoxylavin intermediate (41) similar to that formed by the mammalian FMOs (42). In contrast, other bacterial flavin-containing enzymes, like p-hydroxybenzoate hydroxylase (43), melilotate hydroxylase (44), salicylate hydroxylase (45), and luciferases (46), form short lived or metastable intermediates only in the presence of substrate. A possible role for the FATGY peptide in the formation of a stable intermediate is under investigation.

Despite the fact that FMO 1C1 exhibits all of the structural properties characteristic of FMOs 1A1 and 1B1, its substrate
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specification appears to be highly restricted. Compounds that are metabolized by FMO 1A1 and FMO 1B1, like methimazole (13), and compounds metabolized only by FMO 1A1, like imipramine and chlorpromazine (10), are not substrates for FMO 1C1. In fact, we were able to elicit activity (measured as NADPH oxidation) only with n-octylamine, which is a substrate for FMO 1B1 but not 1A1. Lack of production of either hydrogen peroxide or hydroxylamine equivalents indicated that the increased NADPH oxidation was not caused by uncoupling and that metabolism of the n-octylamine was likely proceeding to the oxime metabolite (47). Notwithstanding its limited substrate specificity, the activity of FMO 1C1 in the particulate fraction from E. coli responds to changes in pH, temperature, detergent levels, and ion concentrations in the same manner as FMO 1A1, not FMO 1B1.

With the cloning and expression of FMO 1C1, three forms of the enzyme, each with a distinct substrate specificity, can now be expressed in E. coli and examined in detail. Work in progress employing site-directed mutagenesis and chimeric constructions should allow for the identification of specific structural features that determine the functional identities of the FMOs. In particular, we are examining the structural elements that appear to restrict substrate access to FMO 1C1.

REFERENCES