ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 317, No. 1, February 20, pp. 275–284, 1995

Characterization of Flavin-Containing Monooxygenase 5 (FMO5) Cloned from Human and Guinea Pig: Evidence That the Unique Catalytic Properties of FMO5 Are Not Confined to the Rabbit Ortholog[1]

Lila H. Overby,* Alan R. Buckpitt,*† Michael P. Lawton,* Emmanuel Atta-Asafo-Adjei,* Johannes Schulze,*‡ and Richard M. Philpot*,[2]

*Molecular Pharmacology Section, Laboratory of Cellular and Molecular Pharmacology, National Institutes of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; †Department of Pharmacology and Toxicology, University of California, Davis, California 95616-5224; and ‡Walter Straub Institute for Pharmacology and Toxicology, Ludwig Maximilians-University of Munich, D-80336 Munich, Germany

Received October 17, 1994, and in revised form November 28, 1994

Several full-length clones encoding the human and guinea pig orthologs of flavin-containing monooxygenase 5 (FMO5) have been isolated from libraries constructed with hepatic mRNA. The clones were detected by hybridization with the cDNA encoding FMO5 expressed in rabbit. The human and guinea pig cDNAs encode for proteins of 533 amino acids that contain putative pyrophosphate binding domains characteristic of mammalian FMOs. The sequences derived for the human and guinea pig FMO5 proteins are 87% identical and are 85 and 82% identical, respectively, to the sequence of rabbit FMO5. As is the case with other FMOs, FMO5 in human and guinea pig is encoded by multiple transcripts. Rabbit FMO5 expressed in *Escherichia coli* was purified and used to elicit antibodies in goat. These antibodies detected FMO5 in samples from livers of adult humans, rabbits, and guinea pigs and fetal livers of humans. The human and guinea pig forms of FMO5 were expressed in *E. coli* and characterized. Neither enzyme effectively catalyzed the metabolism of methimazole, a general FMO substrate; however, both were active with *n*-octylamine. The responses of the human FMO5 and guinea pig FMO5 to detergent, ions and elevated temperature are all similar to the responses described for rabbit FMO5. These results indicate that the unique properties of FMO5 from rabbit are species-independent and that this form of the flavin-containing monooxygenase is not readily classified as a drug-metabolizing enzyme.

The flavin-containing monooxygenases (FMO, EC 1.14.13.8)[3] catalyze the oxidation of nucleophilic nitrogen, sulfur, and phosphorus atoms in a wide variety of compounds (1, 2). Members of this gene family, five of which have been identified (3), are found primarily associated with endoplasmic reticulum and are present in most organs of all mammalian species examined. Like other so-called "phase I" enzymes, most notably members of the cytochrome P450 supergene family (4), the FMOs can serve to increase the polarity of a host of xenobiotics, including many pesticides and drugs, as a prerequisite for excretion. However, FMO-catalyzed reactions may form highly reactive, toxic products with some substrates (5). Although no clear roles for the FMO in the metabolism of endogenous compounds have been established, several physiologically relevant substrates have been identified (6, 7). FMOs have also been implicated in the detoxification of some dietary chemicals and a deficiency in the enzyme is likely the cause of trimethylaminuria, a genetic disease of humans (8).

The first purification of a FMO was reported by Ziegler and co-workers in 1972 (9). Although this form of the enzyme, which was isolated from pig liver, served as the "model" FMO for many years, FMOs purified

[1] Sequence data from this article have been deposited with the GenBank Libraries under Accession Nos. FMO5-L37080 (human) and FMO5-L37081 (guinea pig).

[2] To whom reprint requests should be addressed at NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709. Fax: (919) 541-1898.

[3] Abbreviations used: SDS, sodium docecyl sulfate; SSC, standard sodium citrate; FMO, flavin-containing mnooxygenase; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactoside.

0003-9861/95 $6.00

from liver, kidney, and lung of mice (10, 11), rats (12), guinea pigs (13), and rabbits (14) were eventually studied. Characterization of these preparations indicated the existence of multiple forms of the enzyme, a possibility suggested originally on the basis of the differential effects of heavy metals on FMO-catalyzed metabolism in rabbit hepatic and pulmonary microsomal preparations (15). Immunochemical differences between forms of the enzyme expressed in liver and lung provided additional evidence of multiplicity (14, 16).

Cloning, sequencing, and characterization of cDNAs encoding the FMOs from pig liver (FMO1)[4] and rabbit liver and lung (FMO1 and FMO2) demonstrated conclusively the existence of related but distinctly different FMO genes (17, 18), and sequences of purified FMOs (FMO1 and FMO3) showed that at least two distinct forms of the enzyme are present in rabbit liver (19, 20). Two additional forms of the FMO (FMO4 and FMO5) have now been identified (21, 22).

FMOs 1, 2, 3, and 5 have been characterized following expression in *Escherichia coli* (21–23). As shown previously with the purified enzymes (24–26), FMO1 and FMO2 differ catalytically in that tricyclic antidepressants, like imipramine and chlorpromazine, are substrates only for FMO1, and short-chain, primary amines, like *n*-octylamine, are substrates only for FMO2. In general, however, FMO1 and FMO2 have very broad and highly similar substrate specificities. Both the catalytic and physical characteristics of FMO3 are similar to those of FMO1 (21). The properties of FMO5, however, are clearly unique (22). Unlike the other forms of the enzyme, FMO5 does not metabolize substrates like methimazole and thiobenzamide. In fact, FMO5 from rabbit appears to be inactive with substrates other than a limited number of short-chain amines (22).

The properties of FMO5 suggest either that it has not evolved as a "drug-metabolizing" enzyme in the same manner as FMOs1, 2, and 3, or that the rabbit enzyme is unique and somehow compromised. As a first step in pursuing this question, we have now cloned and characterized FMO5 orthologs from human and guinea pig.

METHODS AND MATERIALS

Isolation of mRNA and construction of cDNA libraries. Liver tissue was removed from an adult male (8–10 weeks) Hartley guinea pig (Charles River Breeding Laboratories, Wilmington, MA) and frozen immediately as described (18). A sample of adult, male human liver was obtained from a tissue bank maintained by Glaxo, Inc. (Research Triangle Park, NC). Total RNA was isolated by a modification (27) of the methods of Chirgwin *et al.* (28) and Glisin *et al.* (29), and mRNA was purified according to Aviv and Leder (30). cDNA libraries were constructed in Uni-Zap XR (31) using *Xho*I–*Eco*RI linkers (Stratagene, La Jolla, CA) after selection of cDNA ranging from 1.5 to 6.5 kb. The human liver cDNA library was amplified from $\sim 10^5$ plaques; the guinea pig library was used unamplified.

Screening of cDNA libraries. The guinea pig and human liver cDNA libraries were screened with a rabbit FMO5 cDNA (*Not*I/*Sac*I, bases 1–1249) (22). The probe (FMO5r) was labeled by the random primer method of Feinberg and Vogelstein (32) using the random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Plaque lifts were prehybridized and hybridized in a solution of 6× SSC, 100 μg salmon sperm DNA/ml, 4× Denhardt's, 0.5% SDS, and 50% formamide. Hybridization (10^6 cpm/ml; $\sim 2 \times 10^8$ cpm/μg probe) was done under moderate-stringency conditions (37°C, overnight), and the filters washed at 37°C in 1× SSC/0.1% SDS solution. Positive clones were isolated, screened to purity, and excised with the ExAssist/SOLR system into pBluescript phagemid (Stratagene).

Nucleotide sequencing of clones. Double-stranded plasmid DNA was isolated and the cDNA inserts were end sequenced (Sequenase Version 2.0, United States Biochemical, Cleveland, OH) by the dideoxy chain termination method (33) using T_3 and T_7 primers. Full-length sequences were obtained by subcloning *Eco*RI, *Sac*I, and *Hin*dIII fragments and sequencing with T_3 and T_7 primers, or with internal oligonucleotide primers. Full-length FMO5 cDNAs from two clones were sequenced and equivocal areas resequenced using 7-deaza-dGTP or dITP (United States Biochemical) to resolve band compressions associated with certain regions of the DNA templates. Sequence data were analyzed and aligned with the software package from Genetics Computer Group, Inc. (34).

Analysis of mRNA. Hepatic mRNA from livers of humans and guinea pigs was analyzed by the Northern blot procedure. Messenger RNA was electrophoresed in 1% agarose gels containing 0.5% methyl mercury (35), transferred to nylon membranes, and baked. The membranes were prehybridized for a minimum of 4 h at 42°C and then hybridized with a human FMO5 5′ probe (*Eco*RI/*Xho*I, bases 1–709) or a rabbit coding region probe (*Ava*I/*Spe*I, bases 56–1882) labeled by the random primer method (5×10^6 cpm/ml) at 37°C overnight. Hybridized membranes were washed at 42°C for 2 × 30 min in 1× SSC/0.1% SDS solution, and exposed to autoradiography film at −70°C for ~1 day.

Analysis of message transcripts by the polymerase chain reaction. Reaction mixes were made using the GENEAMP kit (Perkin–Elmer Cetus, Norwalk, CN) following the manufacturer's specifications. Approximately 1 ng of cDNA, synthesized from guinea pig liver polyadenylated mRNA, was used for each reaction mix. Primers were synthesized on a 381A PCR MATE (Applied Biosystems, Foster City, CA). Amplification was carried out in a Perkin–Elmer Cetus thermocycler. An initial denaturation step was done for 5 min at 94°C before the addition of *Taq* polymerase enzyme (hot start procedure). Thirty cycles, consisting of denaturation (1 min, 94°C), annealing (1 min 15 s, 45°C), and elongation (1 min, 72°C plus an additional 1 s added each subsequent cycle) were carried out. Amplified fragments were separated on a 2% agarose gel.

Construction of E. coli expression vectors. The vector pJL-2, which is a derivative of the commercially available pKK233-2 (Pharmacia), was used. In pJL-2 the pBR322 origin of replication is replaced with one from pUC, and a translation enhancer sequence is inserted between the ribosome binding site and the start codon (36). In addition, the *Pst*I cloning site has been replaced by an *Eco*RV site (21). These modifications limit the vector to *Xba*I, *Nco*I, *Eco*RV, and *Hin*dIII sites for insertion of cDNAs. Digestion of pJL-2 with *Xba*I and *Eco*RV results in a 5′ *Xba*I site and a 3′ blunt-ended site that facilitates insertion of cDNAs with internal *Nco*I, or *Hin*dIII sites as found in the cDNAs encoding human and guinea pig FMO5. To clone the cDNA encoding guinea pig FMO5 (FMO5g) into pJL-2, an *Xba*I restriction site was added 5′ of the start codon (sense primer: 5′-GCTCTAGACCAT GA CCA AGAAAAGGATTGCGG-3′) and a *Sma*I blunt site was added 3′ of the stop codon (antisense primer: 5′-TCCCCCGGGATATCTAAG-CATAAGCCA-3′). For the cDNA encoding human FMO5 (FMO5h) an *Xba*I restriction site was added 5′ of the start codon (sense primer:

[4] The nomenclature used in this manuscript follows that devised by Lawton *et al.* (3).

5'-GGTCTAGACCATGACTAAGAAAAGAATTG-3') and a *Sma*I site added 3' of the stop codon (antisense primer: 5'-TCCCCCGGG-ACAACTAGAAGTAAGCTATAAT-3'). The *Xba*I/*Sma*I-digested PCR fragment of FMO5g or FMO5h was ligated into pJL-2 obtained following digestion with *Xba*I and *Eco*RV and the resulting plasmids were called pJL-FMO5g and pJL-FMO5h. All PCR products were sequenced completely.

Expression in E. coli. *E. coli* strain XL-1 cells transformed with pJL-2, pJL-FMO5h, pJL-FMO5g, or pKK-FMO5r (22) were grown to midlog phase at 37°C and then overnight at 30°C (150 rpm) in the presence of 1 mM IPTG to induce expression of recombinant proteins. *E. coli* subcellular fractions were isolated as described (23).

Analysis of cDNA-expressed proteins. The particulate fractions (50 μg) of pJL-2, pJL-FMO5h, pJL-FMO5g, and pKK-FMO5r were electrophoresed on an 8% SDS/polyacrylamide gel and stained with Coomassie blue. Activities of pJL-FMO5h, pJL-FMO5g, and pKK-FMO5r (~100 μg protein) were determined in Tricine/KOH (0.1 M, pH 8.4), EDTA (1 mM), and NADPH (100 μM) with *n*-octylamine (22) or methimazole as substrate (37). Kinetics of methimazole metabolism were determined from results obtained by the addition of increasing amounts of methimazole (2–15 mM final concentrations) to the sample cuvette. To assess temperature stability, washed microsomes were first heated for 5 min at 45°C and then transfered to an ice-cold Eppendorf tube. Sodium cholate (1%) or $MgCl_2$ (100 mM) was added to the sample and reference cuvettes prior to determination of reaction rates. Reactions were initiated by the addition of *n*-octylamine after obtaining stable rates of baseline NADPH oxidation.

Purification of FMO5r, antibody production, and immunoblotting. *E. coli* transformed with pKK-FMOr were recovered from 1–2 liters of medium, and the membrane fraction was isolated as described above. The membrane fraction was resuspended (20 mg protein/ml) in phosphate buffer (10 mM, pH 7.4) containing EDTA (0.1 mM) and 20% glycerol (buffer A) and solubilized by the addition of Emulgen 911 (0.5%, final concentration). Undissolved material was removed by centrifugation at 100,000*g* for 1 h. FMO5 was isolated from the supernatant in the precipitate formed between 5.5 and 12.5% polyethylene glycol. The precipitate was diluted to 10 mg protein/ml with buffer A and applied to a column of DEAE cellulose equilibrated with buffer A containing dithiothreitol (1 mM). FMO5 was recovered in the flow through by elution with buffer A or in the first fractions eluted with a KCl gradient (0 to 1 M) in buffer A and was concentrated by precipitation with 12.5% polyethylene glycol.

Antibodies to FMOr were raised in goats. Acrylamide gel electrophoresis and immunoblotting of hepatic microsomal samples were done by the methods of Laemmli and Favre (38) and Towbin (39), respectively. The antisera was used at a 1:1000 dilution.

Analytical methods. Flavin content (FAD and FMN) was determined fluorometrically by the method of Faeder and Siegel (40). Protein was determined by the method of Lowry *et al.* (41).

RESULTS

Cloning of FMO5 from guinea pig and human liver cDNA libraries. The guinea pig library (2.5×10^5 plaques) was screened at low stringency with the cDNA encoding the rabbit FMO5 and twenty plaques were selected for further analysis. Rescreening at higher stringency yielded 10 positive clones. These clones were purified and 9 were identified by end-sequencing as homologous to rabbit FMO5. Four clones (4, 8, 10, and 11) were judged to contain intact coding regions and two (4 and 8) were sequenced completely (Fig. 1). The sequence of clone four contains 2691 bases, 1599 in the coding region, 231 in the 5'-flanking region, and 861 in the 3'-flanking region. The coding region and 3'-flanking sequences of clone 8 are identical to those of clone 4 with the exception that the 3'-flanking region is shorter by 377 bases. The sequences of the 42 bases immediately 5' of the start codon are also identical; however, the sequence of the 21 bases 5' of that point deviates from that of clone 4. The sequence of the 5'-flanking region of clone 10 matches that of clone 8 and extends 11 bases further (Fig. 1, Table I). The 5' ends of clones 1, 2, 3, 5, 11, and 16, all of which were end-sequenced, do not extend to the point were clones 8 and 10 deviate from clone 4 (Fig. 1). Clones 4 and 8 encode for a protein of 533 amino acids that contains two putative pyrophosphate binding domains in the positions characteristic for the mammalian FMOs (Table II). The amino acid sequence derived for the protein encoded by clones 4 and 8 is 82% identical to the sequence of rabbit FMO5.

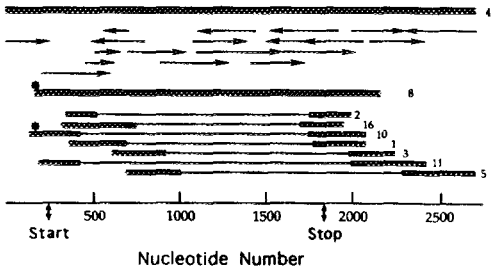

FIG. 1. Sequencing strategy for cDNA clones encoding the guinea pig ortholog of FMO5. The complete sequencing strategy for clone 4 is shown. Clone 8 was sequenced in a manner similar to that used for clone 4. All other clones were end-sequenced as shown. The positions where the sequences of clones 8 and 10 deviate from that of clone 4 (32 bases 5' of the start codon) are marked by asterisks (*).

Approximately 1.5×10^5 plaques from the amplified human liver library were screened with the cDNA encoding rabbit FMO5. Eight clones were isolated and purified and six of these were determined by end-sequencing to be homologous to rabbit FMO5. Of the six, five were distinct clones and two were duplicates. Two clones (1 and 8) were sequenced (Fig. 2, Table I) and found to be identical with the exception of five bases inserted into clone 8 at a position 31 bases 5' of the start codon. Clones 10 and 19 also contained these five bases. Clone 8 contained a coding region of 1599 bases, a 5'-flanking region of 75 bases and a 3'-flanking region of 642 bases. The 3' ends of all four clones terminate within 22 bases of each other. The amino acid sequence derived for the protein encoded by clones 8 and 1 contains 533 residues and is 85% identical to the sequence of rabbit FMO5 and 87% identical to the sequence of guinea pig FMO5 described above. The human and guinea pig sequences contain two GxGxxG putative pyrophosphate binding domains in exactly the same positions as those found in the sequence of rabbit FMO5 and other FMO isoforms.

The nucleotide sequences of rabbit, human, and guinea pig FMO5 are compared in Table I and the amino

TABLE I

Complete Nucleotide Sequences of FM05 from Guinea Pig, Human, and Rabbit[a]

GPG	1	GAAAAACTTAGACGCTGCTGCTGCTGATGAT	31
GPG	32	GATGGTAGTGGTTGCGGTGATTTTCTGTGCCAAGCAGATCTAAACCACGTTTTCTCTCGGCTAAGGACAGAAAATTTATGCGGAGCCGTGAGTGGGCGGG	131
RAB	1	TCACAGCATCCGGCGGCTCGCAGCGGCGGGACATCGCGGACGTTCGCGCAGGCGGACTAGTGACTTCCACGCAAGACAGGCGACGCTGCCGGGAGACC	98
GPG	132	TGTTGGCTGTCAGC GC GGACGTC CAA CCTTCC CATTGGC ATTGCTGTTGTTA AGTGA C AGA T AC A T A A CT T	231
GPG[b]		*TTG GGC CTTCG AGA AGTG CGCAG*	
HUM	1	C CACT GA GGATCTGTTGAGCGCTTCAG A AGGCG ACAGG GACACTA T A A CT	75
RAB	99	ATGGCCGGGAAAAGAGTGGCGGTGATTGGGGCGGGAGCGAGCGGGCTGGCTTGCATCAAGTGCTGCCTGGAAGAGGGCTTGGAGCCCGTCTGCTTCGAAA	198
GPG	232	___A AA GA T A GA T T CT C CT A G A C T T	331
HUM	76	___A TAA A T T GA T CT C G A A A T T	175
RAB	199	GGACCGACGACATCGGAGGTCTGTGGAGGTTCCAGGAAAGTCCCGACGAAGGAAGGGCCAGTATCTACAAATCCGTGATCATCAACACCTCCAAGGAGAT	298
GPG	332	GT CT T G C A A T A C A A T T T	431
HUM	176	T T G C A T A T A T T T A	275
RAB	299	GATGTGCTTCAGTGACTACCCCATCCCGGATCATTTTCCTAACTTCATGCACAACTCCCAGGTCCTGGAGTACTTCAGGATGTACGCCAAAGAATTTGGT	398
GPG	432	C A A A T A T C A T T C	531
HUM	276	T A A A C T TG T T AC	375
RAB	399	CTTCTGAAGTATATTCAGTTTAAGACCACTGTGTGCAGTGTGAAGAAGCGGCCTGATTTCTCCACGTCGGGCCAATGGGAGGTGCTGACTGAGTGCGAAG	498
GPG	532	A C C A C A A T A G AG C ACAT	631
HUM	376	A GA A TG T A A G C A CT	475
RAB	499	GGAAAAAGGAGAGTGCTGTCTTCGATGGGGTCCTGGTTTGCACCGGCCATCACACCAGTGCTCACCTGCCACTGGAAAGCTTCCCTGGGATTGAGAAGTT	598
GPG	632	C A GTG A T T CA A C A A C T A A A	731
HUM	476	TGAA T A A T A T A T A	575
RAB	599	CAAAGGGCAGTACTTGCACAGTCGAGACTATAAGAACCCAGAGAAATTCACTGGAAAGAGAGTCATTGTCATTGGCATTGGGAATTCTGGAGGGGACCTG	698
GPG	732	A C T G AGC TG A A	831
HUM	576	C GG A A T	675
RAB	699	GCTGTGGAGATCAGCCACACAGCCAAGCAGGTCTTCCTCAGCACCAGGAGAGGGGCTTGGATCATGAATCGTGTCGGCGACCATGGATATCCTATTGATA	798
GPG	832	A A A T G T AT TC A AA A C C C G	931
HUM	676	A T A T C A G T C GC G	775
RAB	799	TACTGCTGTCTTCTCGATTTAGTCAATTTTTGAAGAAGATTACTGGTGAAACAATAGCAAATTCATTTTTGGAAAGAAAGATGAACCAAAGGTTTGACCA	898
GPG	932	GT G CAT T TCA A ACT CC T T T G A G A C A TG A	1031
HUM	776	GT T C C CA T A ATG CTG CC T T CAA A A A	875
RAB	899	TGCAATGTTTGGTCTGAAGCCTAAACACAGAGCTTTGAGTCAACACCCAACAGTAAATGATGACCTGCCAAATCGTATCATTTCTGGCTCTGTCAAGATC	998
GPG	1032	AG C AA C G A A AG ATG G G G	1131
HUM	876	A C C G T CT TG G AG G	975
RAB	999	AAAGGAAATGTGAAGGAATTCACAGAAACAGCTGCCATATTTGAAGATGGCTCCAGGGAGGATGACATTGATGCTGTTATTTTTGCCACAGGCTATAGCT	1098
GPG	1132	C A G G G A C G T	1231
HUM	976	G G G C	1075
RAB	1099	TTTCCTTTCCTTTTCTTGAAGACTCTGTCAAAGTGGTGAAAAACAAGGTATCTCTGTATAAAAAGGTCTTCCCCCCTAACCTGGAAAAGCCAACTCTTGC	1198
GPG	1232	GA G T T A A GA	1331
HUM	1076	GA A G T C C A C T G	1175
RAB	1199	AATCATAGGCTTGATCCAGCCCCTGGGAGCCATTATGCCCATTTCAGAGCTCCAAGCACGATGGGCCACCCTAGTGTTTAAAGGGCTAAAGACTTTACCC	1298
GPG	1332	A T T G C TGTT A A A A G	1431
HUM	1176	T T A G C T AG A T A G	1275
RAB	1299	TCACAAAGTGAAATGATGACAGAAATATCTCAGGTTCAAGAGAAAATGGCAAAAAGGTATGTGGAGAGCCAACGCCATACCATTCAGGGAGACTACATAG	1398
GPG	1432	G A A C G G A C T A C	1531
HUM	1276	G G A A C G T AC	1375
RAB	1399	AGACCATGGAAGAAATTGCTGATTTGGTGGGGGTCAGGCCAAATTTGCTGTCTCTGGCTTTCACCGACCCCAGGCTGGCATTACAATTACTTTTGGGACC	1498
GPG	1532	A G G T T A C C A C T T A C A G T C T	1631
HUM	1376	T GC C C C T A C T AC	1475
RAB	1499	CTGTACTCCAGTCCATTATCGTCTCCAGGGCCGTGGAAAGTGGGATGGGGCTCGGAAAACCATCCTTACCGTAGAAGATCGGATCAGGAAGCCTCTGATG	1598
GPG	1632	A C C A C C A GG T T A AC T T T AT	1731
HUM	1476	C A C G A C A G T C AC T C	1575
RAB	1599	ACAAGAGTCACGGAAAGCAGTAACTCTGTGACCTCGATGATGACAATGGGCAAGTTTATGCTAGCTATTGCTTTCTTAGCCATAGCTGTGGTTTATTTTT	1698
GPG	1732	AA T AG C T CA GTT AGCAG A CT TGT C G T T AT A C GC _	1831
HUM	1576	AGTT G GT A T A CA A C C T T AT A A C C C_	1675

TABLE I—*Continued*

```
RAB 1699  AGCTGTCCCTTTGTCATTGCCTCTGCTTTCATTGGGAAGCTTAACTTAGAGAGAGATACCTTCAGAATTTTACAAGATCAAATGACCCTCCTCTTTCAAA  1798
GPG 1832  __A A   A     A    CTGAA    G         G TC      TGCC TTAGAACTTGAAAA ATTC GT  C CT AC GTCA A TGCC  1831
HUM 1676  __T    TA     C    CTGTT              TC ACAGAT CCT CAGAATCTG CGAG TTGAC  TCAGTTT A AT GCCCAG    1775

RAB 1799  TTGCCCCATTTCTCTTTCAAAAGCATTAATTCTCTCTTCATTTTCCTACAGTGAGATCCAAGCTTTTCATTTGCACTAAGCATCTCCTCACCTCTCATGA  1898
GPG 1932  CCAAATTC   TATC  TC   AGC  TT  T  CTACAA GAAAT     GTTT  TTG ATGAACTT CCATT CTCT T GAT A TTT TCTTCTT  2031
HUM 1776  A CTA TT AATGTC CTTTCGAA GC T AA TCAC TTCC  TT CCTACA TGAAACCTG     CA  TGTA T A TCA CTC CTTC A TCAT  1875

RAB 1899  GCCTTCACTTTCTCTCTCCAGAGCAGCTCGGGTACTCTTAGTCATCTTTGTATGTCCCTAGCAGAGTAGTTGACATTTGGCTGGTGTTTAACCAATGTTT  1998
GPG 2032  ATTA_T_AA AT ATGAT   CATCT  AA_AA_T A  GTGT AT C TAT C GATAG TTTG   T A GAGC CAAT TTGT  G TT T ATAC   2131
HUM 1876   ATCCGT AC   TC  TGT GTA T C TA ACTGGGAGC   GG ACTCT T AGTC T TTT   TG CTTTAGCA AGTTCT GACATGTGGTAGG  1975

RAB 1999  GGTGTTGTGGCTCAAAGTCTGTTTTTGTATGGGAAATGACTGACTGTATAACTCTGCTTGGGATGGAATTTGGTTTTCCATTATTTTTGTCTTTAACATT  2098
GPG 2132  ATAAA_AAATG AT TT TCTGAGA A___AA_  G A AGT T  GTCTTACAATGG CAT     TAAGACA A AG  ACACAAGACA  CT TT C  2231
HUM 1976  T CT AA AAA GTTT  TGT  A CAA  TTT TGGT GG  GA   AGT AGCATCG TATAAA TCGCTTAC C ACG  AC C TCT C G T GG  2075

RAB 2099  ATAACAAATGTATGTTTCCTGAGAAATAAGATTAATAATGACCTTCGTAATTGTAGACAAATAAATACTTAAGTTACTTTGTTCTACATGCCAAAAAA   2196
GPG 2232  C  TTC T AATGTACCTGGT ATTGGC AG  TTATTCCTTT C T  AGGAAT TGTCTCTTT CT A CAAATAA ATACT T CACTTCTGGCTGG  2331
HUM 2076  T TGATTT C   TAGAAGCTCA TT T  T  TT TT TCATA TA   C AA T TGTT CCTG GAG TAAG GAAATAATGTTCCTA   T GTTG  2175

GPG 2332  ATAGATCAGTTGAATAATTTAGCATTATCAATAAATTCTACTTGGCTGAGATATATGAAAATTTTAAATTGGCTGTCCTATCTTTTTTTAAAATCTTAGT  2431
HUM 2176  TATGTATCTAAGATAAGACATATAGATGCTTAAGACATTTTGTTTCACTTGCTATTCACTAGTGTACTTGAAACATGGTCATTTTTAGCCCTTTTCCTTA  2275

GPG 2432  TCCCCCATTAGGATCCAGCAAGACATTAGTTTACAAAGTAAGTGGCTGCTAACCCTTGGTGGTGTGAGCTATTAATGTGACTTCTCTCCCCCAACTCTTT  2531
HUM 2276  GGAACCATGTCTTTATTTTCTCAATAAAGAAATTACTTTCA                                                            2316

GPG 2532  CTCTTTTTCCTATAAATAGAGCCTAAAAAAAATTCAAAGGGGTCATTTACATCTTAAATTAGGATTTAACTAAGTCAACATAACTAACTCAATATAAGAT  2631

GPG 2632  ACTAACTCAATATAAGAGTATTCTGCTTACACAGTGTAATGAAATAAAAAACATCCCAGT                                          2691
```

[a] The sequences are numbered with the most 5′ base designated as 1. Only bases that differ from those of the rabbit sequence (RAB) are given for the human (HUM) and guinea pig (GPG) sequences. The human sequence is for clone 8 and it contains five underlined bases (40–44, ACAGG) that are not present in the sequence of clone 1. The start codons (ATG) and stop codons (TAG) are underlined. Also underlined are the 11 putative 3′ cleavage signals present in the 3′-flanking region of the guinea pig sequence starting at bases 2035, 2061, 2136, 2160, 2306, 2345, 2362, 2623, 2625, 2641, and 2675. The sequence of rabbit FM05 is from Atta-Asafo-Adjei *et al.* (*J. Biol. Chem.* **268,** 9681–9689, 1993).

[b] This line shows the variation in the guinea pig sequence that exists to the 5′ of base 191 in clone 10.

acid sequences are shown in Table II along with the sequence recently obtained from purified FMO5 by Ozols (42). The sequence from the protein, which is missing a C-terminal peptide of 20 residues, differs from the derived sequence at five other positions (Table II).

Analysis of mRNA from humans, guinea pigs, and rabbits for expression of FMO5. Samples (5 μg) of hepatic mRNA from rabbit, guinea pig, and human were examined with a probe prepared from a *Spe*I–*Ava*I restriction fragment (bases 56–1924) of the cDNA for rabbit FMO5 (Fig. 3A). Two bands of mRNA (~2.6 and 3.8 kb) were detected in the human sample and a very broad band of mRNA (~1.8–3.0 kb) was detected in the guinea pig sample. As reported previously (22), two FMO5 transcripts are present in samples of rabbit hepatic mRNA.

An *Eco*RI–*Xho*I fragment (bases 1–642) from the cDNA for human FMO5 was used as a probe to examine further the human and guinea pig transcripts. The identity between the human and guinea pig FMO5 cDNAs in this region is exceptionally high (~90%), and cross-hybridization with transcripts for other FMOs can be avoided by using this probe at relatively high stringency. Again, two distinct transcripts were detected in samples from human (Fig. 3B). With samples from guinea pig, a broad band was still detected although it was now limited to ~2.2–2.8 kb (Fig. 3B). The appearance of multiple bands with the guinea mRNA sample agrees with the finding of cDNAs having of 3′-flanking regions of various lengths (Fig. 1). In support of the latter, the 3′-flanking region of guinea pig FMO5 cDNA (clone 4) contains eleven putative 3′ cleavage signals, three of which are in the predominate AATAAA form (Table I). Additional evidence of several guinea pig FMO5 transcripts was multiple bands obtained upon PCR amplification of the FMO5 3′-flanking region in cDNA synthesized from guinea pig hepatic mRNA. In this experiment a single 3′-primer (poly-A) and a single 5′ primer, derived from sequence near the stop codon, produced six to eight amplified bands ranging from 200 to 800 bases in length (data not shown).

Results of PCR amplification also indicate that the two different 5′-flanking regions sequences of the guinea pig cDNAs (4 vs 8 and 10) are products of distinct transcripts. Primers specific for the variant 5′ sequences of clones 4 and 10 were used with a common 20 base coding region primer, located 569 bases 3′ of the start codon, to amplify single bands of appropriate lengths. These two amplified bands were identified by restriction analysis as derived from guinea pig FMO5 transcripts.

Expression of human, guinea pig, and rabbit FMO5 in E. coli. Expression of FMO5 in *E. coli* (XL1 Blue) was detected by analysis of the 100,000*g* particulate fraction (10 μg) by SDS–PAGE. The patterns of staining by Coomassie blue showed a major band from transformed cells

TABLE II

Amino Acid Sequences of FM05 from Guinea Pig, Human, and Rabbit[a]

```
RAB-cDNA   1  MAGKRVAVIG AGASGLACIK CCLEEGLEPV CFERTDDIGG LWRFQESPDE   50
RAB-Prot   1  --  K    _ _____                                        48
GPG-cDNA   1   TK  I   _ G V__ SS                  SA           N E    50
HUM-cDNA   1   TK  I   _ G V__ SS     V                         N E    50

RAB-cDNA  51  GRASIYKSVI INTSKEMMCF SDYPIPDHFP NFMHNSQVLE YFRMYAKEFG  100
RAB-Prot  49                                                          98
GPG-cDNA  51                              Y        H                  100
HUM-cDNA  51                              Y        A             D    100

RAB-cDNA 101  LLKYIQFKTT VCSVKKRPDF STSGQWEVLT ECEGKKESAV FDGVLVCTGH  150
RAB-Prot  99                                                         148
GPG-cDNA 101             N                V   H   TKVD    A M       150
HUM-cDNA 101       R           Q           V   S    GDE      M      105

RAB-cDNA 151  HTSAHLPLES FPGIEKFKGQ YLHSRDYKNP EKFTGKRVIV IGIGNSGGDL  200
RAB-Prot 149           K                                  _______      198
GPG-cDNA 151   N                      F          A       VI _______    200
HUM-cDNA 151   N                      F          G        I _______    200

RAB-cDNA 201  AVEISHTAKQ VFLSTRRGAW IMNRVGDHGY PIDILLSSRF SQFLKKITGE  250
RAB-Prot 199                                                         248
GPG-cDNA 201                    S   L    K     T V       TY  S  L Q   250
HUM-cDNA 201      Q                 L     y    A   F   L TH IW  C Q   250

RAB-cDNA 251  TIANSFLERK MNQRFDHAMF GLKPKHRALS QHPTVNDDLP NRIISGSVKI  300
RAB-Prot 249                                                         298
GPG-cDNA 251  SLS AYV KQ   E    E           M                A M  V  300
HUM-cDNA 251  SL  KY  K  I      E                   L           L  V  300

RAB-cDNA 301  KPNVKEFTET AAIFEDGSRE DDIDAVIFAT GYSFSFPFLE DSVKVVKNKV  350
RAB-Prot 299   G ▲(KEL)                                               351
GPG-cDNA 301   G                                  D                  350
HUM-cDNA 301   G                                  D              I   350

RAB-cDNA 351  SLYKKVFPPN LEKPTLAIIG LIQPLGAIMP ISELQARWAT LVFKGLKTLP  400
RAB-Prot 352                                                         401
GPG-cDNA 351              R                       G   V Q            400
HUM-cDNA 351              R                       G     Q            400

RAB-cDNA 401  SQSEMMTEIS QVQEKMAKRY VESQRHTIQG DYIETMEEIA DLVGVRPNLL  450
R B-Prot 402                                                         451
GPG-cDNA 401       A  T KA  EI      D           Q       EF   K       450
HUM-cDNA 401       A    KA  EID                 D    LA               450

RAB-cDNA 451  SLAFTDPRLA LQLLLGPCTP VHYRLQGRGK WDGARKTILT VEDRIRKPLM  500
RAB-Prot 452                                                         501
GPG-cDNA 451        K    K FF      I      P    H    A    TY        N  500
HUM-cDNA 451        K    H         I   V  P         A    TD           500

RAB-cDNA 501  TRVTESSNSV TSMMTMGKFM LAIAFLAIAV VYF                    533
RAB-Prot 502              M ------- ---------- ---                   514
GPG-cDNA 501   E  K   M V AV T C     VV F  IM A A                    533
HUM-cDNA 501    V R S M   T  I       L  F  II A                     533
```

[a] The complete derived sequence for rabbit FM05 is shown (RAB-cDNA). Residues that differ from those of the rabbit cDNA-derived sequence are shown for the sequences from rabbit protein (RAB-Prot) and guinea pig (GPG-cDNA) and human (HUM-cDNA) cDNA-derived sequences. Deletions relative to the rabbit-derived sequence are shown as dashes (—). Insertions relative to the rabbit derived sequence are shown in parenthesis and are indicated by an arrowhead (▲). The putative pyrophosphate-binding sequences (GxGxxG) are underlined. The rabbit sequence derived from cDNA (R-cDNA) is from Atta-Asafo-Adjei *et al.* (*J. Biol Chem.* **268,** 9681–9689, 1993) and the rabbit sequence from purified protein (R-Prot) is from Ozols (*Biochemistry* **33,** 3751–3757, 1994).

that was absent in samples from control cells (Fig. 4A). With pKK-FMO5r the mobility of the band corresponded to a molecular weight of ~62,000 Da, and with pJL-FMO5h and pJL-FMO5g the bands corresponded to 60,000 and 58,000 Da, respectively (Fig. 4A). High levels of expression, particularly where FMO5 is clearly the major protein in the membrane fraction (FMO5h and FMO5g), are evident. The mobilities of the expressed proteins were the same as the mobilities of the corresponding hepatic microsomal proteins detected by im-

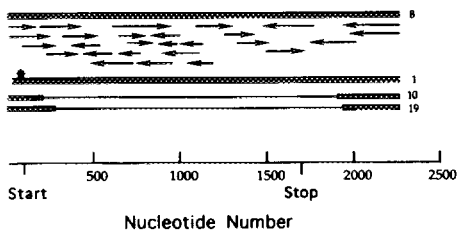

FIG. 2. Sequencing strategy for cDNA clones encoding the human ortholog of FMO5. The complete sequencing strategy for clone 8 is shown. Clone 1 was sequenced in a manner similar to that use for clone 8. All other clones were end-sequenced as shown.

munoblotting of human, guinea pig, and rabbit samples with antibody to rabbit FMO5 (Fig. 4B). Antigen for this antibody was purified from *E. coli* transformed with pKK-FMOr as described under Methods and Materials. The antibody does not cross-react with FMO1, FMO2, or FMO3 (not shown). The results show that FMO5 is expressed in both adult and neonatal human liver as well as in livers of rabbits and guinea pigs. With guinea pig two bands of very similar mobility appear to be detected in the liver microsomal preparation. This may indicate the presence of allelic variants with behaviors similar to those we have described for FMO2 in both guinea pig and rabbit (43).

Increases in flavin content related to transformation of FMO5 were 0.59, 1.04, and 1.06 nmol/mg protein for

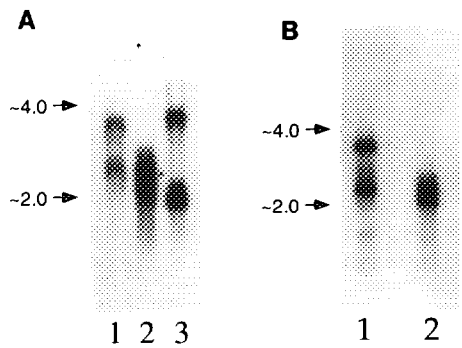

FIG. 3. (A) Analysis of mRNA isolated from liver of human, guinea pig, and rabbit for expression of FMO5 transcripts. Samples of mRNA (5 μg) purified from adult human (lane 1), guinea pig (lane 2), and rabbit (lane 3) liver were electrophoresed in agarose (1%) containing methyl mercury, transfered to nylon membrane, and hybridized with a ^{32}P-labeled *SpeI–AvaI* coding region fragment (bases 56–1924) of the cDNA for rabbit FMO5. Autoradiography was done by standard procedures. (B) Analysis of mRNA isolated from liver of human and guinea pig by hybridization with a high-identity probe obtained from the cDNA for human FMO5. Samples of mRNA (3 μg) purified from human (lane 1) and guinea pig (lane 2) liver were electrophoresed in agarose (1%) containing methyl mercury, transfered to nylon membrane, and hybridized with a ^{32}P-labeled *EcoRI–XhoI* 5′ fragment (bases 1–642) of the cDNA for human FMO5. Autoradiography was done by standard procedures.

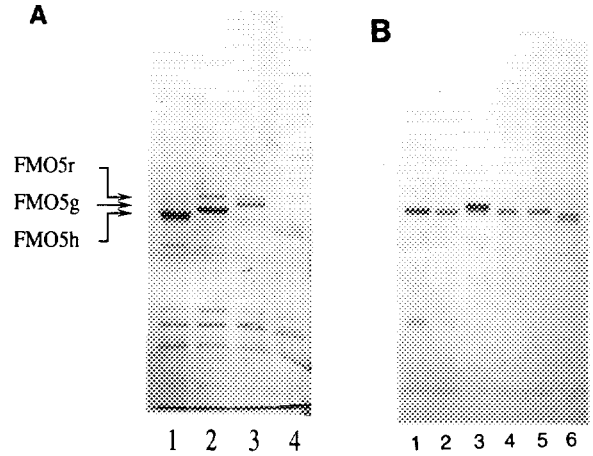

FIG. 4. (A) Expression human, guinea pig, and rabbit FMO5 in *E. coli*. Samples (10 μg) of the membrane fraction prepared from *E. coli* induced by treatment with isopropyl 1-thio-β-D-galactopyranoside were electrophoresed on polyacrylamide gel in the presence of SDS and stained with Coomassie blue. *E. coli* (strain XL1 Blue) was transformed with pJL-FMO5h (lane 1), pJL-FMO5g (lane 2), pKK-FMOr (lane 3), or with nonrecombinant pJL vector (lane 4). (B) Expression of FMO5 in liver of human, guinea pig, and rabbit. Samples (20 μg) of hepatic microsomal protein were electrophoresed on polyacrylamide gel in the presence of SDS, transfered to nitrocellulose, and stained using the antibody to rabbit FMO5. Adult liver samples from human (lanes 1 and 2), rabbit (lane 3), and guinea pig (lane 6) are shown along with samples from human fetal liver (lanes 4 and 5).

pKK-FMO5r, pJL-FMO5h, and pJL-FMO5g, respectively. Based on a 1:1 molar ratio of FAD to FMO and the molecular weights listed above, we calculate that 3.7% (rabbit), 6.2% (human), and 6.1% (guinea pig) of the protein in the *E. coli* particulate fractions was FMO5.

Characterization of human, guinea pig, and rabbit FMO5. Human, guinea pig, and rabbit FMO5 expressed in *E. coli* were examined for their ability to metabolize methimazole, a known substrate for FMO1, FMO2, and FMO3. Although some activity was noted with all three orthologs of FMO5 (Fig. 5), maximum activity was much less than that observed with other forms of the enzyme (21, 44). All three FMO5 isoforms exhibited exceptionally high apparent K_ms (3 to 10 mM) for this substrate (Fig. 5).

Activities with *n*-octylamine were much higher than those with methimazole; 22.8 nmol NADPH oxidized $\times$ min^{-1} $\times$ nmol FMO^{-1} for rabbit FMO5, 26.1 for human FMO5, and 38 for guinea pig FMO5. These rates are within the range expected for maximum velocities associated with mammalian FMOs. Characterization of the FMO5 enzymes with *n*-octylamine as substrate showed that the activities of all three exhibit a time-dependent sensitivity to sodium cholate (1%). However, the extent of the maximum inhibition by cholate does differ; 50% for rabbit FMO5, 65% for human FMO5, and 100% for guinea pig FMO5 (Fig. 6). Human FMO5 and guinea pig

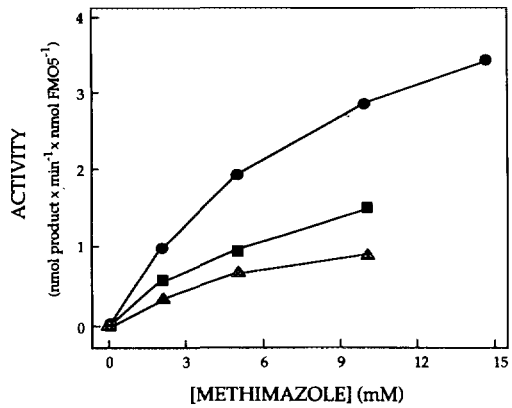

FIG. 5. Metabolism of methimazole catalyzed by FMO5 expressed in *E. coli.* Rates of methimazole metabolism (nmol product × min^{-1} × nmol FMO5^{-1}) catalyzed by human FMO5 (●), guinea pig FMO5 (▲), and rabbit FMO5 (■) are shown for different concentrations of substrate.

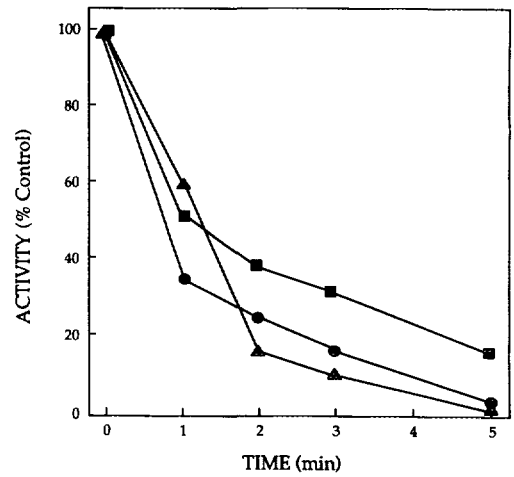

FIG. 7. Effects of elevated temperature on the activities of FMO5 expressed in *E. coli.* The effects of elevated temperature on the metabolism of *n*-octylamine catalyzed by human (●), guinea pig (▲), and rabbit (■) FMO5 are shown as a function of time. Samples were heated at 45°C for the times indicated and then stored on ice until assayed at 37°C.

FMO5 were somewhat more sensitive than rabbit FMO5 to $MgCl_2$, although 100% inhibition is obtained for all three enzymes within 6 min of treatment (Fig. 6). The time-dependent sensitivity of the three FMO5 enzymes to elevated temperature (45°C) is shown in Fig. 7. In general, the behavior of all three was similar with complete inhibition approached by treatment for five min.

DISCUSSION

Five isoforms of the FMO, each the product of a distinct gene, have been identified in rabbit. Reasons for this multiplicity are not clear, although some tissue selective expression is apparent (14, 16, 18, 21, 22) and differences among catalytic specificities with xenobiotics as substrates have been noted. The latter is particularly true in the case of FMO5. Although the kinetics (apparent K_ms) associated with FMOs 1–3 can vary significantly with some substrates (21, 44), their specificities differ only somewhat—FMO1 and FMO3 metabolize tricyclic antidepressants (22, 26) and FMO2 metabolizes short chain amines (24, 25); otherwise, all three isoforms share the same list of substrates. In contrast, activity with FMO5 has been observed only with a few low-molecular-weight primary amines (22).

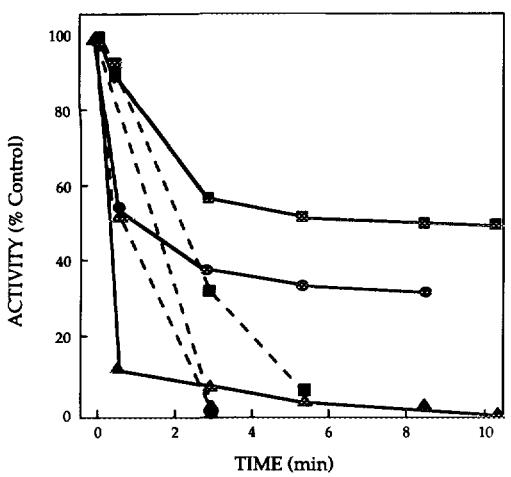

FIG. 6. Effects of cholate and $MgCl_2$ on the activities of FMO5 expressed in *E. coli.* The effects of 1% sodium cholate (—) and 100 mM $MgCl_2$ (---) on the metabolism of n-octylamine catalyzed by human (●), guinea pig (▲), and rabbit (■) FMO5 are shown as a function of time.

The characteristics of FMO5 from rabbit suggested two possibilities: first, the rabbit ortholog of FMO5 is not representative of FMO5 in general; second, FMO5 is not readily classified as a "drug metabolizing" enzyme, although is it clearly a member of the FMO gene family. Given the results we have obtained with FMO5 from human and guinea pig, the first of these possibilities can be excluded.

Clones encoding FMO5 were isolated from cDNA libraries constructed with hepatic mRNA from liver of human and guinea pig. Like FMO5 from the rabbit, the sequences derived for the human and guinea pig enzymes contain 533 amino acids, characteristic FAD and NADP pyrophosphate binding domains, and hydrophobic carboxy-termini. The human sequence and guinea pig sequence are 87% identical to each other and 85% (human) and 82% (guinea pig) identical to the rabbit sequence. Like all other forms of FMO, the identities with all known homologous forms are between 52 and 57%. These sequences and structural relationships clearly identify the human and guinea pig FMOs as orthologs of rabbit FMO5.

The cDNA-derived sequences for the human, guinea pig, and rabbit FMO5 orthologs differ in length from the sequence reported for FMO5 purified from rabbit liver by 19 residues (42). This difference is accounted for by the derived sequences being longer by 20 residues at their C-termini and by 2 residues at their N-termini and by an insertion at amino acid 304 (derived sequence) of three residues (KEL) in the sequence of the protein relative to the derived sequences. A similar insertion also appears at the same position in the protein sequence of FMO3 (20, 21). Likely, the three added residues are an artifact of the protein sequencing procedure (21). The virtually identical mobilities of the enzymes expressed in *E. coli* and the hepatic microsomal enzymes indicates that C-terminal processing does not account for the 20 residues missing from the sequence of the FMO protein. Likely the C-terminal peptide was not recovered from the reverse-phase HPLC column used for separation of digests, a possibility discussed by Ozols (20).

Similar to the case for other FMO isoforms, there are multiple transcripts for human and guinea pig FMO5. This is particularly complicated in the case of the guinea pig, which exhibits both 5′ and 3′ alternative processing. The variability at the 3′ end appears to be associated with replicate 3′ cleavage signals. However, the origin of the two 5′ variants remains unexplained; we have not detected an insertion sequence as we did with the variant 5′ transcripts of FMO4 (21). In any case, these multiple transcripts all appear to be derived from single genes (18).

The catalytic activities of all three orthologs of FMO5 were found to be similar. Methimazole, a good to excellent substrate for other FMO isoforms, is a poor substrate at best for the FMO5 isoforms. In contrast, *n*-octylamine, a substrate previously thought to be specific for FMO2, was readily metabolized by all three. In addition, all three orthologs of FMO5 are characterized by their similar responses to $MgCl_2$, sodium cholate, and elevated temperature. These results establish that the unique properties of FMO5 are not species-specific and that rabbit FMO5 is not an aberrant form of the enzyme. The species-independent nature of the FMO5 characteristics and its apparent lack of efficiency as a drug-metabolizing enzyme suggest that this FMO isoform may have some physiological function. Clearly, FMO5 is expressed in liver and can be detected easily at the protein level in all three species examined. It is interesting that FMO5 appears to be the only FMO isoform expressed in both adult and neonatal human liver. In contrast, FMO1 appears to be expressed only in neonatal liver (45) and FMO3 only in adult liver (46, and unpublished). The involvement of the FMO in the regulation of prenylated proteins has recently been suggested (47), and a possible role for FMO5 in this process is being investigated.

REFERENCES

1. Ziegler, D. M. (1980) *in* Enzymatic Basis of Detoxication (Jakoby, W. B., Ed.), pp. 201–227, Academic Press, New York.
2. Ziegler, D. M. (1988) *Drug Metab. Rev.* **19,** 1–32.
3. Lawton, M. P., Cashman, J. R., Cresteil, T., Dolphin, C. T., Elfarra, A. A., Hines, R. N., Hodgson, E., Kimura, T., Ozols, J., Phillips, I. R., Philpot, R. M., Poulsen, L. L., Rettie, A. E., Shephard, E. A., Williams, D. E., and Ziegler, D. M. (1994) *Arch. Biochem. Biophys.* **308,** 254–257.
4. Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., and Nebert, D. W. (1993) *DNA Cell Biol.* **12,** 1–51.
5. Hajjar, N. P., Hodgson, E. (1982) *Biochem. Pharmacol.* **31,** 745–752.
6. Sausen, P. J., and Elfarra, A. A. (1990) *J. Biol. Chem.* **265,** 6139–6145.
7. Ziegler, D. M., and Poulsen, L. L. (1977) *Trends Biochem. Sci.* **2,** 79–81.
8. Ayesh, R., Smith, R. L. (1990) *Pharmacol. Ther.* **45,** 387–401.
9. Ziegler, D. M., and Mitchell, C. H. (1972) *Arch. Biochem. Biophys.* **150,** 116–125.
10. Sabourin, P. J., Smyser, B. P., and Hodgson, E. (1984) *Int. J. Biochem.* **16,** 713–720.
11. Venkatesh, K., Levi, P. E., and Hodgson, E. (1991) *Biochem. Pharmacol.* **42,** 1411–1420.
12. Kimura, T., Kodama, M., and Nagata, C. (1983) *Biochem. Biophys. Res. Commun.* **110,** 640–645.
13. Yamada, H., Yuno, K., Oguri, K., and Yoshimura, H. (1990) *Arch. Biochem. Biophys.* **280,** 305–312.
14. Tynes, R. E., Sabourin, P. J., and Hodgson, E. (1985) *Biochem. Biophys. Res. Commun.* **126,** 1069–1075.
15. Devereux, T., Philpot, R. M., and Fouts, J. R. (1977) *Chem.-Biol. Interact.* **18,** 277–287.
16. Williams, D. E., Ziegler, D. M., Nordin, D. J., Hale, S. E., and Masters, B. S. S. (1984) *Biochem. Biophys. Res. Commun.* **125,** 116–122.
17. Gasser, R., Tynes, R. E., Lawton, M. P., Korsmeyer, K. K., Ziegler, D. M., and Philpot, R. M. (1990) *Biochemistry* **29,** 119–124.
18. Lawton, M. P., Gasser, R., Tynes, R. E., Hodgson, E., and Philpot, R. M. (1990) *J. Biol. Chem.* **265,** 5855–5861.
19. Ozols, J. (1990) *J. Biol. Chem.* **265,** 10289–10299.
20. Ozols, J. (1991) *Arch. Biochem. Biophys.* **290,** 103–115.
21. Burnett, V. B., Lawton, M. P., and Philpot, R. M. (1994) *J. Biol. Chem.* **269,** 14314–14322.
22. Atta-Asafo-Adjei, E., Lawton, M. P., and Philpot, R. M. (1993) *J. Biol. Chem.* **268,** 9681–9689.
23. Lawton, M. L., and Philpot, R. M. (1993) *J. Biol. Chem.* **268,** 5728–5734.
24. R. E. Tynes, Sabourin, P. J., Hodgson, E., and Philpot, R. M. (1986) *Arch. Biochem. Biophys.* **251,** 654–664.
25. L. L. Poulsen, Taylor, K., Williams, D. E., Masters, B. S. S., and Ziegler, D. M. (1986) *Mol. Pharmacol.* **30,** 680–685.
26. D. E. Williams, Hale, S. E., Muerhoff, A. S., and Masters, B. S. S. (1985) *Mol. Pharmacol.* **28,** 381–390.
27. Gasser, R., Negishi, M., and Philpot, R. M. (1988) *Mol. Pharmacol.* **32,** 22–30.
28. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
29. Glisin, V., Crkvenjakov, R., and Byus, C. (1974) *Biochemistry* **13,** 2633–2637.
30. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.

31. Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. (1900) *Nucleic Acids Res.* **16,** 7583–7600.
32. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
33. Sanger, F., Nicklen, S., and Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
34. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395.
35. Bailey, J. M., and Davidson, N. (1976) *Anal. Biochem.* **70,** 75–85.
36. Pernecky, S. J., Larson, J. R., Philpot, R. M., and Coon, M. J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2651–2655.
37. Dixit, A., and Roche, T. E. (1984) *Arch. Biochem. Biophys.* **233,** 50–63.
38. Laemmli, U. K., and Favre, M. (1973). *J. Mol. Biol.* **80,** 575–599.
39. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci USA* **76,** 4350–4354.
40. Faeder, E. J., and Siegel, L. M. (1973) *Anal. Biochem.* **53,** 332–336.
41. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
42. Ozols, J. (1994) *Biochemistry* **3,** 3751–3757.
43. Nikbakht, K. N., Lawton, M. P., and Philpot, R. M. (1992) *Pharmacogenetics* **2,** 207–216.
44. Lawton, M. P., Kronbach, T., Johnson, E. F., and Philpot, R. M. (1991) *Mol. Pharmacol.* **40,** 692–698.
45. Dolphin, C., Shephard, E. A., Povey, S., Palmer, C. N. A., Ziegler, D. M., Ayesh, R., Smith, R. L., and Phillips, I. R. (1991) *J. Biol. Chem.* **266,** 12379–12385.
46. Lomri, N., Gu, Q., and Cashman, J. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1685–1689.
47. Park, S. B., Howald, W. N., and Cashman, J. R. (1994) *Chem. Res. Toxicol.* **7,** 191–198.