Expression and Characterization of a Modified Flavin-containing Monooxygenase 4 from Humans*

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The inability to obtain flavin-containing monooxygenase 4 (FMO4) in heterologous systems has hampered efforts to characterize this isoform of the FMO gene family. Neither the human nor the rabbit ortholog of FMO4, each of which has been cloned and sequenced, has been expressed. Attempts to achieve expression of FMO4 have been made with Escherichia coli, baculovirus, yeast, and COS systems. The cDNAs encoding FMO4 have extended coding regions compared with those encoding other FMO isoforms. The derived amino acid sequences of FMO1, -2, -3, and -5 from all species examined contain about the same number of residues (531–535 residues), whereas the derived sequences of human and rabbit FMO4 contain 558 and 555 residues, respectively.

We have investigated whether the elongation of the FMO4 coding region is related to the inability to achieve expression. The cDNA encoding human FMO4 has been modified by a single base change that introduces a stop codon at the consensus position. This modification allows for expression in E. coli. Lack of expression of intact FMO4 is caused by a problem that occurs following transcription, a problem that is overcome completely by relocation of the stop codon 81 bases to 5′ of its normal position. Truncated FMO4 is expressed as an active enzyme with characteristics typical of an FMO isoform.

Possible functional changes resulting from altering the 3′-end of an FMO were investigated with human FMO3. Elongation of the coding region of the FMO3 cDNA to the next available stop codon (FMO3*) resulted in the expression of an enzyme with properties very similar to those of unmodified FMO3. Elongation of FMO3 lowered the level of expression in E. coli but did not eliminate it. As with FMO4, the difference in expression levels between FMO3 and elongated FMO3 (FMO3*) appears to be related to translation rather than transcription. The functional characteristics of FMO3 and FMO3* are not significantly different.

The flavin-containing monooxygenase (FMO, EC 1.14.13.8) gene family encodes a minimum of five isoforms that catalyze

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‡ The abbreviations used are: FMO, flavin-containing monooxygenase; FMO3*, elongated FMO3; FMO4*, truncated FMO4; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; Tridine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

The present work demonstrates that an active form of human FMO4 can be expressed in E. coli transformed with a
truncated cDNA. The recombinant enzyme has been characterized and found to exhibit the functional properties associated with other members of the FMO gene family. In addition, evidence is presented in contradiction to our previous results that suggested alternative splicing in the 5'-region of the FMO transcript.

**MATERIALS AND METHODS**

Cloning of Human FMO3 and FMO4—A human cDNA library constructed from hepatic mRNA isolated from the liver of an adult male has been described previously (16). The cDNA library was 1 × 10⁶ plaques/µL. The library was screened with a 620-bp pair 5'-fragment (EcoRI) of the cDNA for rabbit FMO3 (12) random labeled (25) with [α-32P]dCTP (Boehringer Mannheim). Positive plaques were hybridized and hybridized in 6 × SSC, 100 µg/ml salmon sperm, 4 × Denhardt's solution, 0.2% SDS, and 50% formamide. Subsequent to overnight hybridization at 37 °C, the lifts were washed twice under conditions of low stringency (1 × SSC and 0.1% SDS) for 30 min at 37 °C. Fifteen positive plaques were isolated to purity and characterized by restriction mapping based on published sequences (14–16) and end sequencing with T3 and T7 primers. Seven clones containing complete coding regions were identified, four for FMO3, one for FMO4, and two for FMO5.

Sequencing of Human FMO3 and FMO4—Oligonucleotide primers were used to obtain sequences from both strands of three full-length FMO3 clones and one FMO4 clone (26). Primers (19 for FMO3 and 10 for FMO4) were based on published sequences (14, 15). Primers for these experiments and others described below were synthesized in our laboratory (381A PCR MATE; Applied Biosystems, Foster City, CA). Sequencing data were analyzed and aligned using the software package from Genetics Computer Group, Inc. (27) and by comparison with published data (14, 15).

Preparation of E. coli Expression Vector (pJL2)-FMO cDNA Constructs—A 1715-base fragment of the cDNA encoding FMO3 (coding region plus 115 bases of the 5'-flank and a single base (C) 5' of the start codon) was excised from pBluescript (Stratagene, La Jolla, CA) with NcoI and Scal and inserted into pJL2-FMO3-pJL restricted with Ncol and EcoRV. The vector, pJL-2, is a derivative of pKK223-2 (Pharmacia Biotech Inc.) in which the origin of replication is changed, and a translation enhancer sequence is inserted between the ribosome binding site and the start codon (28). The coding regions of elongated FMO3 (FMO3*), normal FMO4, and truncated FMO4 (FMO4*) were isolated and found to exhibit the functional properties associated with other members of the FMO gene family. In addition, evidence is presented in contradiction to our previous results that suggested alternative splicing in the 5'-region of the FMO transcript.

Expression of Human FMO4 and FMO3—The sequence we obtained for human FMO4 differs with that published by Dolphin et al. (15) at only two positions: C for T in the second base of codon 323 (changes valine to alanine) and C for T in the second base of codon 502 (changes valine to alanine). These differences could easily be related to several factors, including allelic variation, polymerase infidelity, and sequencing errors. In contrast, our sequence for human FMO3 differs markedly from the sequence of human FMO3 obtained from cDNA (14)2 and a PCR product used for expression by Lomri et al. (36). Alignment of these sequences shows 14 base substitutions and 17 positions where alignment requires introduction of a gap into one of the sequences (Table I). These changes alter the derived amino acid sequence at 20 positions and shorten the protein from 533 to 532 residues. Comparison of our sequence with a second sequence of human FMO3 found in GenBank (sequence Z47552, submitted by C. T. Dolphin, T. E. Cullingford, E. A. Shepherd, R. L. Smith, and I. R. Phillips) shows none of these differences, although four other base substitutions are noted: T for C in the third base of codon 43 (retains phenylalanine), G for A in the first base of codon 158 (changes glutamine to lysine), T for C in the third base of codon 239 (retains phenylalanine), and A for G in the third base of codon 486 (changes valine to isoleucine). The alignment of our sequence with that of rabbit FMO3 (12) is also free of gaps (Table I).

Expression of Human FMO4 and FMO3—Expression of recombinant full-length FMO4 (FMO4), truncated FMO4 (FMO4*), or nonrecombinant pJL2 containing pJL2 vector control, was induced at 37 °C in LB medium plus ampicillin (50 µg/ml) to an absorbance of 0.4–0.5 at 600 nm. Isopropyl O-thio-galactoside (IPTG) was then added to a final concentration of 1 mM, and the cells were grown overnight at 30 °C. Cells were harvested by centrifugation at 20,000 × g for 5 min (all centrifugation steps were done at 4 °C unless otherwise specified) and resuspended in 10 ml of ice-cold lysis buffer (0.8 M KCl, 50 mM KPi, pH 7.4, and 1 mM EDTA) containing lysozyme (1 mg/ml). After incubation for 30 min on ice with occasional gentle inver-...
(FMO4*), full-length FMO3 (FMO3), or elongated FMO3 (FMO3*). The 100,000 × g particulate fractions were prepared from IPTG-induced cultures, examined for FMO expression, and characterized. Results were compared with those obtained with the 100,000 × g particulate fraction from E. coli transformed with plg-L-2 vector alone.

Expression of FMO4 and FMO4*—Fractions from E. coli transformed with FMO4, FMO4*, or plg-L-2 vector alone (plg) were examined for evidence of FMO expression. Samples of protein electrophoresed on polyacrylamide gels were stained with Coomassie Blue. The pattern and intensity of the protein bands in the relevant region (50–60 kDa) obtained with the plg L or FMO4 samples were virtually identical (Fig. 1A, lanes 1 and 4). In contrast, the sample from the FMO4* preparation contained a band of protein corresponding to ~60 kDa (Fig. 1A, lane 5) that was not evident in the samples of plg L or FMO4 (Fig. 1A, lanes 1 and 4). Antibodies to FMO3 that cross-react with FMO1, -2, and -5 were used for immunoblot analysis of the FMO4 and FMO4* samples. These antibodies detected clearly the protein of ~60-kDa mobility observed with the FMO4* sample (Fig. 1B, lane 5). In contrast, no immuno-reactive protein was evident with the plg L or FMO4 samples (Fig. 1B, lanes 1 and 4).

Expression of FMO3 and FMO3*—Bands of protein not present in the plg L sample were detected by staining the FMO3 and FMO3* samples with Coomassie Blue (Fig. 1A, lanes 2 and 3). However, expression of the ~57-kDa protein in the FMO3 (lane 2) sample was clearly greater than expression of the ~59-kDa protein in the FMO3* sample (lane 3). The intensities of staining obtained with antibodies to FMO3 on immunoblots pointed to a similar difference between the amounts of FMO3 and FMO3* expressed (Fig. 1B, lanes 2 and 3).

Metabolism of Methimazole by FMO4 and FMO4*—The 100,000 × g particulate fractions were used to examine the activities of FMO4 and FMO4* with methimazole as the substrate (Table II). With FMO4 or plg L preparations, no activity was detected. The activity of recombinant FMO4* preparations at a methimazole concentration of 1 mM was 1.15 ± 0.08 nmol of product min⁻¹ mg of protein⁻¹ (n = 4), and recombinant FMO4* had a specific activity of 2.3 nmol of product min⁻¹ mg of protein⁻¹. Metabolism of methimazole catalyzed by FMO4* conformed to Michaelis-Menten kinetics. The apparent Kₘ for the reaction was 3.3 mM and the Vₘₐₓ was about 4 nmol of product min⁻¹ mg of protein⁻¹. The specific activity and Vₘₐₓ calculations were based on a flavinadeninedinucleotide content of 746 pmol/mg protein in the FMO4* preparation compared with 241 pmol/mg in the plg L preparation.
metabolism of methimazole catalyzed by FMO3 and FMO3* (Table II). Rates of metabolism at 1 mM methimazole were 38.3 ± 1.9 (n = 5) nmol of product min⁻¹ mg of protein⁻¹ for FMO3 and 10.4 ± 0.6 (n = 5) nmol of product for FMO3*. Specific activities (nmol of product min⁻¹ nmol of FMO⁻¹) were 60.1 and 44.6 for FMO3 and FMO3*, respectively. Metabolism by either form of the enzyme conformed to Michaelis-Menten kinetics, with apparent Kₘ values of between 25 and 35 μM and V_max values of about 58 (FMO3) and 45 (FMO3*) nmol of product min⁻¹ nmol of FMO⁻¹. Flavin adeninedinucleotide contents were 878 pmol/mg for FMO3, 475 pmol/mg for FMO3*, and 241 pmol/mg for the vector alone.

Characterization of FMO4*, FMO3, and FMO3*—The effects of a number of factors on the metabolism of methimazole catalyzed by FMO4*, FMO3, and FMO3* were compared. The optimum pH for the reaction catalyzed by FMO4* was found to be near 10.2. The responses of FMO3 and FMO3* to pH were nearly identical, with optimum pH near 9.5 (Fig. 2). The activities of all three enzymes were found to be moderately temperature-sensitive (more stable than has been noted for FMO1 but less stable than FMO2). FMO3 and FMO3* responded to heat in a similar fashion and reached 50% loss of activity with an exposure to 45°C of between 2 and 3 min (Fig. 3). FMO4* was somewhat less labile and reached 50% inhibition after ~4 min of heat exposure (Fig. 3).

The effects of n-octylamine or sodium cholate on the activities of FMO3 and FMO3* were very similar; FMO3 and FMO3* were inhibited 39 and 31%, respectively, by n-octylamine and 60 and 55%, respectively, by sodium cholate (Fig. 4). However, FMO3* was inhibited somewhat less than FMO3 by magnesium chloride (33% versus 18%) and was not activated to the same extent by imipramine (4 versus 16%). The effects of all four agents on the activity of FMO4* differed from those observed with either FMO3 or FMO3*: less inhibition (25%) by n-octylamine, greater inhibition by magnesium chloride (54%), nearly complete inhibition by sodium cholate (91%), and inhibition (14%) rather than activation by imipramine (Fig. 4).

Transcription of FMO3 and FMO4 in E. coli—Lack of expression of FMO4 in E. coli—Lack of expression of FMO4 in E. coli and reduced expression of FMO3 following elongation to form FMO3* were examined to determine whether the fault could be localized to problems associated with transcription or translation. RNA was isolated from E. coli transformed with FMO3, FMO3*, FMO4, FMO4*, or pJL vector alone and examined with [32P]-labeled cDNA probes for FMO3 and FMO4 (Figs. 5 and 6). Patterns and amounts of RNA detected were very similar when results obtained for FMO3 and FMO3* or FMO4 and FMO4* were compared. No evidence of abnormal or absent transcript was seen in the case of FMO4, and no decrease in transcript amount was apparent with FMO3*.

Alternative 5'-Splicing of FMO4—Previous results from our laboratory indicated that FMO4 undergoes alternative splicing in the 5'-region of the transcript (12). We have repeated the

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**TABLE II**

| Isoform | FADa | Activityb | Kinetic constants |
|---------|------|-----------|------------------|
|         | mg protein⁻¹ | nmol FMO⁻¹ | Kₘapp (μM) | V_max (nmol)]
| FMO3   | 878  | 38.3 ± 1.9 (n = 5) | 60.0 | 25  | 58  |
| FMO3*  | 475  | 10.4 ± 0.6 (n = 5) | 44.6 | 35  | 45  |
| FMO4   | 746  | 1.2 ± 0.1 (n = 4)  | 2.3  | 3300 | 4   |
| Vector | 241  | Not determined     |      |      |     |

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a FAD content is reported as pmol/mg protein.
b The activity is nmol methimazole metabolized × min⁻¹ × nmol FMO⁻¹.

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**Fig. 2. Effect of pH on the metabolism of methimazole catalyzed by FMO3, FMO3*, and FMO4*.** The activities of expressed FMO3 ( ), FMO3* ( ), and FMO4* ( ) are presented as a function of pH. Buffer conditions are described under “Materials and Methods.”

**Fig. 3. Effects of treatment with heat on the activities of FMO3, FMO3*, and FMO4*.** Samples of expressed FMO3 ( ), FMO3* ( ), and FMO4* ( ) were treated at 45°C for various times. Subsequently, they were analyzed for their ability to catalyze the metabolism of methimazole. The relationship between activity and time of treatment is shown.

PCR analysis of cDNA synthesized from rabbit liver mRNA with the same primers altered to introduce restriction sites for subcloning into pBluescript and subsequent sequencing. The primers correspond to the extreme 5'-end and bases 273–291 of the FMO4 cDNA. Results of PCR amplification again produced two bands, the expected one of ~300 base pairs and a predicted one of ~230 base pairs (12). The larger band was identified by sequence analysis as corresponding exactly to the first 291 bases of the FMO4 cDNA. In contrast, the sequence of the smaller band was found to be unrelated to FMO4. A second antisense primer (bases 292–309), used with the same sense primer, also formed a band of ~300 base pairs but did not produce a smaller band. These findings indicate that FMO4 does not undergo alternative splicing at the 5'-end and that our previous conclusion was based on an artifactual result (12).

**DISCUSSION**

An inability to achieve heterologous expression of FMO4 has been experienced with both the human and rabbit orthologs of this enzyme (12, 24). This has frustrated efforts to characterize
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Fig. 4. Effects of several agents on activities catalyzed by FMO3, FMO3*, and FMO4*. The effects of n-octylamine, MgCl2, sodium cholate, and imipramine on the metabolism of methimazole catalyzed by FMO3 ( ), FMO3* ( ), and FMO4* ( ) are shown. Results are presented as percentage of control for each FMO isofom.

Fig. 5. Hybridization of probe for FMO3 with RNA isolated from E. coli. RNA was isolated from E. coli transformed with FMO3*-pJL (lane 2, 0.5 μg; lane 3, 1.0 μg), FMO3-pJL (lane 3, 0.5 μg; lane 6, 1.0 μg), or pJL-L2 (lane 1, 0.5 μg; lane 4, 1.0 μg) electrophoresed on agarose, transferred to a nylon membrane, and reacted with 32P-labeled cDNA for FMO3. Autoradiography was done as described under “Materials and Methods.”

Attempts to define the reasons for the lack of expression of FMO4* in E. coli are now underway. The present findings indicate quite clearly that the problem is not associated with transcription and is likely a function of translation. Data based on RNA modeling suggest that high potential for hybridization involving the 3'-end of the FMO4 coding region could theoretically interfere with translation. Whether this impediment to translation is encountered in vivo and is overcome by some regulatory process remains to be determined.

Results with FMO3 were not so profound. Elongation of FMO3 to the next available stop codon (72 bases to the 3'-end) did decrease expression by 3-4-fold, but FMO3* was still produced in amounts that were at least 250 times the level of immunochemical detection. Differences of this magnitude are often seen when different vectors (pJL-L2 versus pKK) or E. coli cell types (XL-1 versus JM-109) are used (11). Also, the expression of FMO3* reported here is actually about five times greater than that of FMO3 reported by Lomri et al. (37), who used a pTrc99A vector and E. coli strain NM522. Examination of transcript levels, however, does indicate that decreased expression of FMO3 following elongation is associated with translation and is not due to transcriptional differences related to vector and cDNA or construct and cell strain compatibility.

FMO4* expressed in E. coli is detectable as an active enzyme as well as a protein. Characterization of recombinant FMO4* shows a number of general properties exhibited by other FMO isoforms, with the exception of FMO2: sensitivity to heat, MgCl2, sodium cholate, and n-octylamine. The enzyme catalyzes the oxidation of methimazole, but with very little enthusiasm (Km = 3.3 mM). It is possible that unmodified FMO4 would be significantly more active than FMO4*, but our experience with COOH-terminal modifications of other FMO isoforms argues against this. As seen in the present work, elongation of FMO3 has little if any effect on activity. Results with FMO4 also indicate that the COOH-terminal region is not important to the catalytic activity of FMO isoforms (21).

Although methimazole is among the best substrates for every known FMO isoform, it is metabolized efficiently only by FMO1 (Km = 3 μM) and FMO3 (Km = 30 μM). The marginal activities of FMO4* (Km > 5 μM) and FMO5 (Km > 5 μM) or, for that matter FMO2 (Km = 300 μM) brings into question their classification as “drug-metabolizing enzymes.” On the other hand, the widespread distribution and high degree of structural conservation exhibited by these enzymes does suggest that they are of some functional importance. Further studies on FMO4, particularly with respect to its expression in the brain (24), will be greatly aided by the development of monospecific antibodies made possible by the successful expression of FMO4* in E. coli.

3 M. K. Wyatt and R. M. Philpot, unpublished observations.
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