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Isoform Specificity of Trimethylamine *N*-Oxygenation by Human Flavin-Containing Monooxygenase (FMO) and P450 Enzymes

SELECTIVE CATALYSIS BY FMO3

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ABSTRACT. In the present study, we expressed human flavin-containing monooxygenase 1 (FMO1), FMO3, FMO4t (truncated), and FMO5 in the baculovirus expression vector system at levels of 0.6 to 2.4 nmol FMO/mg of membrane protein. These four isoforms, as well as purified rabbit FMO2, and eleven heterologously expressed human P450 isoforms were examined for their capacity to metabolize trimethylamine (TMA) to its *N*-oxide (TMAO), using a new, specific HPLC method with radiochemical detection. Human FMO3 was by far the most active isoform, exhibiting a turnover number of 30 nmol TMAO/nmol FMO3/min at pH 7.4 and 0.5 mM TMA. None of the other monooxygenases formed TMAO at rates greater than 1 nmol/nmol FMO/min under these conditions. Human fetal liver, adult liver, kidney and intestine microsomes were screened for TMA oxidation, and only human adult liver microsomes provided substantial TMAO-formation (range 2.9 to 9.1 nmol TMAO/mg protein/min, N = 5). Kinetic studies of TMAO formation by recombinant human FMO3, employing three different analytical methods, resulted in a K_m of 28 ± 1 μM and a V_{max} of 36.3 ± 5.7 nmol TMAO/nmol FMO3/min. The K_m determined in human liver microsomes ranged from 13.0 to 54.8 μM. Therefore, at physiological pH, human FMO3 is a very specific and efficient TMA *N*-oxygenase, and is likely responsible for the metabolic clearance of TMA *in vivo* in humans. In addition, this specificity provides a good *in vitro* probe for the determination of FMO3-mediated activity in human tissues, by analyzing TMAO formation at pH 7.4 with TMA concentrations not higher than 0.5 mM. BIOCHEM PHARMACOL **56**;8:1005–1012, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. flavin-containing monooxygenase; trimethylamine; benzydamine; *N*-oxidation; trimethylaminuria; cytochrome P450

Trimethylaminuria or "fish-odor syndrome" is a human disorder characterized by an impaired ability to convert the malodorous TMA§ to its odorless *N*-oxide (TMAO) [1]. TMA is derived *in vivo* from dietary sources such as choline and carnitine, or by reduction of TMAO in the gut [2, 3]. In humans, TMA is metabolized exclusively to TMAO with up to 60 mg/day excreted in the urine of healthy people and less than 5% excreted as the parent compound [4, 5]. The reaction is widely considered to be catalyzed by the FMO enzyme system (EC 1.14.13.8) [6, 7], which is known to support the oxygenation of a wide variety of heteroatom-containing compounds. To date, five distinct forms of mammalian FMO have been identified, and according to the present nomenclature each member of this protein family is designated by an Arabic numeral (FMO1–FMO5) [8]. The human FMO isoforms exhibit distinct developmental and tissue-specific expression patterns. FMO3 is recognized as the major hepatic form in adults, whereas FMO1 is expressed in kidney and fetal liver, but not in adult liver [9, 10].

Because TMA is cleared substantially by hepatic first-pass metabolism [11] and liver disease influences TMA metabolism [12], FMO3 appeared likely to catalyze the majority of TMAO formation *in vivo*. Indeed, a mutation in the *FMO3* gene, which decreases TMA metabolism, has been described recently [13]. However, no data are available on the contribution of individual human FMO and P450 isoforms to TMA *N*-oxygenation. Therefore, the primary objective of this study was to express human FMO1, FMO3, FMO4, and FMO5 and determine the

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§ *Abbreviations:* BA, benzydamine; BAO, benzydamine *N*-oxide; FMN, flavin adenine mononucleotide; FMO, flavin-containing monooxygenase; P450, cytochrome P450; TMA, trimethylamine; and TMAO, trimethylamine *N*-oxide.

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isoform selectivity and kinetics of TMAO formation. In addition, the capacity for TMAO formation in microsomes of human adult liver, kidney and intestine, and human fetal liver was determined, using radiolabeled TMA and a new, specific HPLC method with radiochemical detection.

MATERIALS AND METHODS

Chemicals

TMA · HCl, TMAO and lumazine were obtained from Aldrich. [^{14}C]TMA · HCl (sp. act. 1.7 mCi/mmol), BA · HCl, FAD, and FMN were purchased from Sigma. [^{14}C]TMAO was synthesized as previously described [5, 14], but without purification. Briefly, [^{14}C]TMA · HCl, dissolved in diluted sodium hydroxide, was oxidized with hydrogen peroxide in a closed vial. [^{14}C]TMA was oxidized completely to [^{14}C]TMAO within 24 hr at room temperature as determined by HPLC analysis with radiochemical detection. BAO and dazidamine were provided by the F. Angelini Research Institute.

Biological Materials

Microsomal preparations of five human liver samples, prepared and characterized as previously described [15], were used. Samples of human renal tissue and human kidney microsomes from three different patients were provided by Dr. R. Tynes, NOVARTIS. Human intestine microsomes were provided by Dr. K. Thummel, University of Washington. Microsomal preparations of eleven heterologously expressed human P450 isoforms, P4501A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 4A11, respectively, with coexpressed oxidoreductase except for P4501A1, 1A2, 2B6, and 2C19, were purchased from the GENTEST Corp. Control activities of these preparations have been confirmed as previously described [15]. Rabbit lung FMO2 was purified as previously described [16]. Isoforms of human FMO1,* FMO4t (truncated) [17], and FMO5 [10] were expressed in the baculovirus expression system, and membranes from infected insect cells were prepared according to the procedures detailed previously for human FMO3 [18]. FMO content of these membrane preparations varied from 0.6 to 2.4 nmol/mg of protein.

Microsomal Incubations

The incubation mixtures contained 0.1 M phosphate buffer (pH 7.4), 0.2 to 0.6 mg of microsomal protein or 50 to 200 pmol heterologously expressed FMO or 25 pmol recombinant P450, 1000 IU of catalase, and an NADPH-generating system consisting of 1 mM $NADP^+$, 6 mM glucose-6-phosphate and 2.8 IU of glucose-6-phosphate dehydrogenase. After a preincubation of 2 min at 37°, the reaction was initiated by the addition of substrate, and was allowed to continue at 37° for 5–60 min. Incubations with TMA were terminated with the addition of $HClO_4$ to a final concentration of 1%, whereas incubations with BA were quenched with an equal volume of acetonitrile containing 0.1 mg/mL of dazidamine as internal standard. Protein was precipitated by centrifugation, and the supernatants were subjected to HPLC analysis.

HPLC Analyses

A modification of a previously described HPLC method [19] was used to analyze [^{14}C]TMA and [^{14}C]TMAO. Separation was achieved on an Aminex® HPX-72 O (300 mm × 7.8 mm) column from Bio-Rad with 0.01 M sodium hydroxide as eluent at a flow rate of 1 mL/min. Prior to analysis, the acidic supernatants of the incubation mixtures were adjusted to pH 7–8 with diluted NaOH. [^{14}C]TMAO and [^{14}C]TMA, monitored by radiochemical detection, eluted at 5.5 and 7.5 min, respectively. Quantitation of enzymatically derived [^{14}C]TMAO was based on a calibration curve obtained from synthesized standards. Under the same analytical conditions, dimethylamine eluted at 6.8 min as determined with the unlabeled standard and UV detection at 214 nm [19].

BA, its *N*-oxide, and the internal standard, dazidamine, were analyzed on a ZORBAX 3.5 μm CN column (150 × 3 mm). The effluent consisted of 60% acetonitrile and 40% 20 mM phosphate buffer, pH 7.0, at a flow rate of 0.7 mL/min. The effluent was monitored fluorometrically with an excitation wavelength of 307 nm and emission wavelength of 377 nm [20].

For the analysis of FAD and FMN, 50–100 pmol recombinant FMO (30–100 μg insect cell membranes) was boiled for 2 min in 200 μL of 5 mM $(NH_4)_2HPO_4$ buffer (pH 5.5), and immediately cooled on ice; 2.5 μg of lumazine was added as internal standard. The protein was precipitated at 3000 *g* for 5 min, and the supernatant was subjected to HPLC analysis. Separation of FAD and FMN was achieved by a previously described method [21] with some modifications. A reversed-phase 3 μm C18-column (4.0 × 125 mm) and an eluent consisting of 11% acetonitrile and 89% aqueous phosphoric acid (0.7%, w/v) at a flow rate of 0.8 mL/min was used. Lumazine, FAD, and FMN, detected fluorometrically with an excitation wavelength of 447 nm and an emission wavelength of 520 nm, eluted at 1.8, 3.6, and 5.7 min, respectively.

Other Analyses

Oxygen consumption was analyzed polarographically at 37° using a Micro system with a 0.6 mL reaction chamber from Yellow Springs Instruments. Incubations containing 0.1 M phosphate buffer (pH 7.4), recombinant FMO, 1000 IU catalase, and an NADPH-generating system were monitored for several minutes to assess endogeneous oxygen consumption, prior to initiating the reaction with substrate.

* Human FMO1 cDNA cloned from human kidney exhibited an identical sequence as reported in the Gen Bank Libraries under Accession No. M64082 (human FMO1).

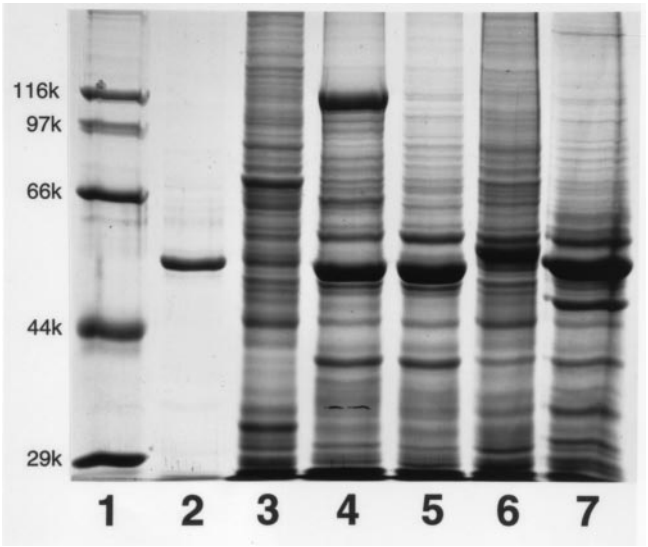

FIG. 1. Expression of human FMO isoforms. Proteins were separated on a 9% SDS-polyacrylamide gel and stained with Coomassie Blue. Lane 1, markers; lane 2, 7.5 pmol purified recombinant FMO3; lane 3, 10 μg uninfected insect cell membrane protein; lane 4, 10 μg FMO1 membrane protein; lane 5, 7.5 μg FMO3 membrane protein; lane 6, 10 μg FMO4t membrane protein; and lane 7, 12.5 μg FMO5 membrane protein. Expression levels ranged from 0.6 to 2.4 nmol FMO/mg membrane protein.

NADPH oxidation was measured spectrophotometrically at 340 nm. Incubations consisted of 0.1 M phosphate buffer (pH 7.4), recombinant FMO, 1000 IU catalase, and 100 μM NADPH. Calculations are based on an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Kinetic parameters for TMAO formation, as analyzed by NADPH oxidation and polarographic methods, were determined from direct plots using the k.cat program (Biometallics) for Macintosh. Kinetic parameters derived from the radiometric analyses of substrate depletion were obtained according to Segel [22]. Regression coefficients were >0.90.

RESULTS

Expression of Human FMOs

Membrane fractions were prepared from *Trichoplusia ni* cell cultures infected with recombinant baculovirus containing a sequence for either human FMO1, FMO3, FMO4t, or FMO5, or from uninfected insect cell cultures. Membrane proteins and purified recombinant FMO3 were separated on a 9% SDS polyacrylamide gel and stained with Coomassie Blue. Infected cultures and purified human FMO3 showed intense bands in the relevant (50–60 kDa) region (Fig. 1). Expression levels for FMO based on FAD content were corrected for low, endogeneous FAD levels in uninfected insect cell membranes and ranged from 0.6 to 2.4 nmol FMO/mg membrane protein. Enzymatic FMO activity was confirmed with benzydamine *N*-oxidation, a specific, isoform-independent, FMO-mediated reaction. Rates for human FMO1, FMO3, FMO4t membrane preparations, and purified rabbit FMO2, at 500 μM substrate concentration, were 45, 37, 9, and 6 min^{-1}, respectively. FMO5 exhibited a low catalytic activity only for sulfoxidation of methyl *p*-tolyl sulfide.

Analysis of TMAO Formation

Indirect determination of TMAO formation, by monitoring either oxygen consumption or NADPH oxidation could be achieved only with insect cell membrane preparations, because of very low endogenous NADPH-oxidase activity. In contrast, microsomal preparations from human tissues exhibited substantial endogenous NADPH-dependent oxygen consumption. Therefore, a specific and sensitive HPLC method employing [^{14}C]TMA was developed. Representative chromatograms for NADPH-dependent [^{14}C]TMAO formation by human liver microsomes and recombinant human FMO3 are shown in Fig. 2. The limit of detection for this assay was about 20 pmol of [^{14}C]TMAO.

TMA Metabolism in Human Tissues

Microsomal preparations of five adult human liver, three fetal human liver, three human intestine, and three adult human kidney samples were used for the measurement of TMA oxidation under physiological conditions. Adult human liver microsomes exhibited readily detected levels of enzyme activity (2.9 to 9.1 nmol TMAO/mg protein/min) with a substrate concentration of 500 μM at pH 7.4 (Fig. 3). However, activity in fetal liver and kidney microsomes was close to the limit of detection (<0.05 nmol TMAO/mg protein/min), and no detectable metabolite was generated by human intestine microsomes. The lack of TMAO formation in human fetal liver, adult kidney, and intestine is not likely due to heat lability of the FMOs, since all tissues were frozen rapidly after procurement. Moreover, positive controls for FMO activity in these preparations were obtained with benzydamine, a general probe for FMO catalysis (Fig. 3).

N-Oxides can be reduced back to the parent compound in microsomal preparations, particularly in the presence of Fe-EDTA and NADPH or NADH [23]. However, incubation of [^{14}C]TMA with human liver microsomes or recombinant P450s did not result in any detectable reduction to [^{14}C]TMA. Also, no *N*-demethylation to form dimethylamine was detectable in the tissues or expressed P450s investigated.

TMA Metabolism in Heterologously Expressed Monooxygenases

[^{14}C]TMA (500 μM) was incubated at pH 7.4 with recombinant human P4501A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 4A11, as well as expressed human FMO1, FMO3, FMO4t, and FMO5 and rabbit FMO2. Only FMO3 exhibited substantial enzyme activity—30 nmol [^{14}C]TMAO/nmol FMO3/min. The rate of

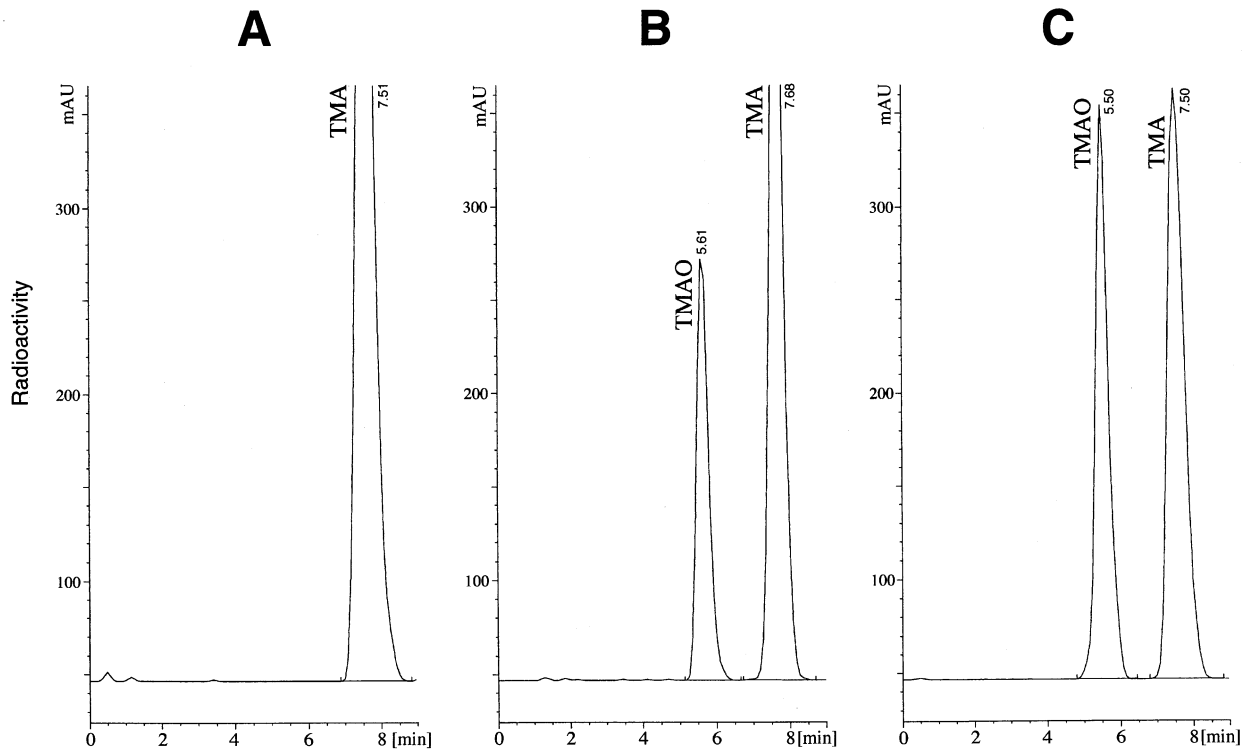

FIG. 2. HPLC analysis of incubations with [^{14}C]TMA. Representative chromatograms are shown for the incubation of [^{14}C]TMA (500 μM) at pH 7.4 for 10 min with (A) 200 pmol FMO3 without NADPH, (B) 0.4 mg human liver microsomes with NADPH, and (C) 200 pmol FMO3 with NADPH.

TMAO formation catalyzed by rabbit FMO2 was 1 nmol [^{14}C]TMAO/nmol FMO2/min, whereas for FMO1 and FMO4t it was 0.1 nmol [^{14}C]TMAO/nmol FMO/min. For FMO5, and all P450 isoforms tested, no TMAO formation was detectable (Fig. 4). At the pH optimum of 9 for recombinant human FMO3, the rate of TMAO formation was 173 nmol TMAO/nmol FMO3/min (Fig. 5). At this elevated pH, or with 5 mM TMA at pH 7.4, human FMO1

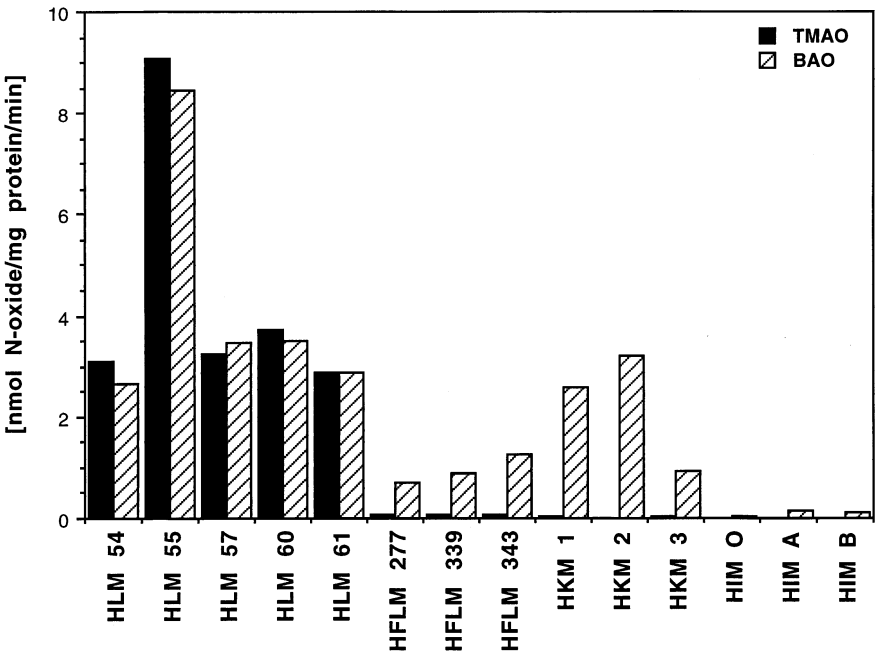

FIG. 3. Metabolism of [^{14}C]TMA and benzydamine (BA) in human tissues. [^{14}C]TMA (0.5 mM) or BA (0.5 mM) was incubated at pH 7.4 for 10 to 20 min with 0.1 to 0.6 mg of microsomal protein. HLM = adult human liver microsomes, HFLM = human fetal liver microsomes, HKM = adult human kidney microsomes, and HIM = human intestine microsomes. Data represent the means of duplicate determinations.

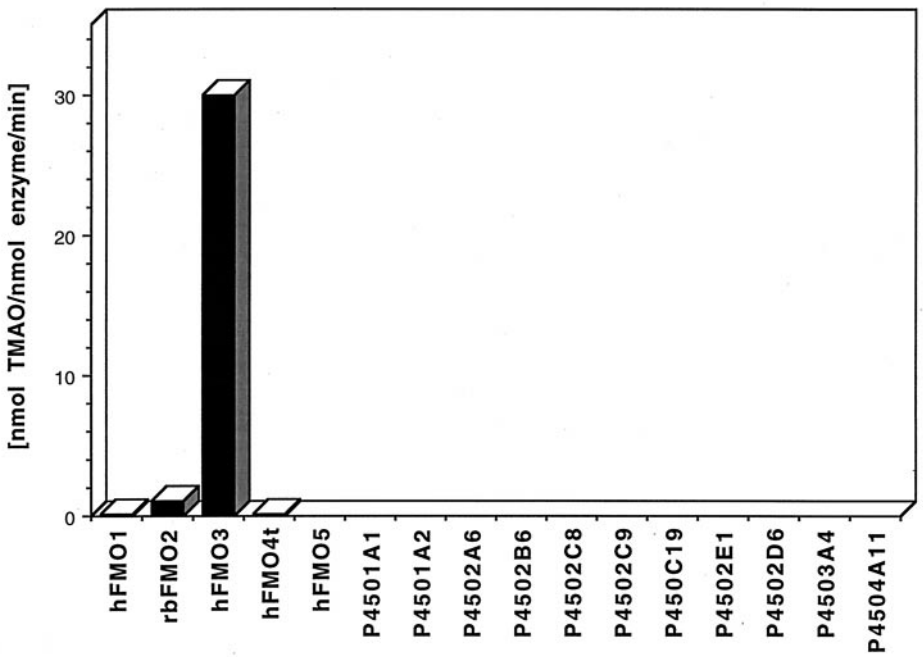

FIG. 4. Metabolism of [^{14}C]TMA by recombinant monooxygenases. [^{14}C]TMA (0.5 mM) was incubated with 200 pmol FMO for 10 min, or 25 pmol P450 for 60 min at pH 7.4. Data represent the means of duplicate determinations.

also catalyzed TMAO formation with turnover numbers of 5.7 or 4.7 min^{-1} respectively.

Kinetics of TMA Metabolism

Recombinant FMO3 and microsomal preparations from three human liver samples were used to obtain kinetic parameters (apparent K_m and apparent V_{max}) for TMAO formation. Three different analytical approaches, oxygen consumption, NADPH oxidation, or radiometric HPLC analysis, were taken for the measurement of TMA kinetics, but only for recombinant enzyme, since liver microsomes exhibited substantial endogenous NADPH-dependent oxygen uptake. Apparent kinetic parameters K_m and V_{max} were determined either by the analysis of substrate depletion [22] from an initial TMA concentration of 100 μM, or by the analysis of initial velocities over a substrate concentration range of 10–200 μM. At saturating substrate concentration, TMAO formation was linear for up to 40 min with up to 2 mg protein/mL. A plot for the determination of K_m and V_{max} by max analysis of substrate depletion is shown in Fig 6. The values of apparent K_m and apparent V_{max} are summarized in Table 1. K_m and V_{max} values

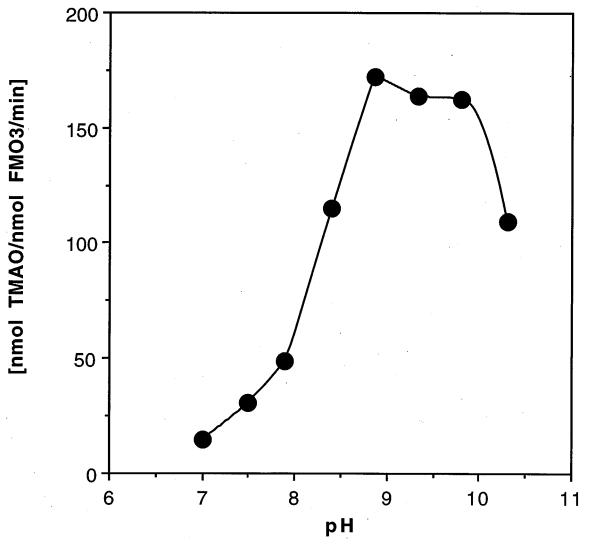

FIG. 5. pH profile for [^{14}C]TMAO formation by human FMO3. Data represent the means of duplicate determinations.

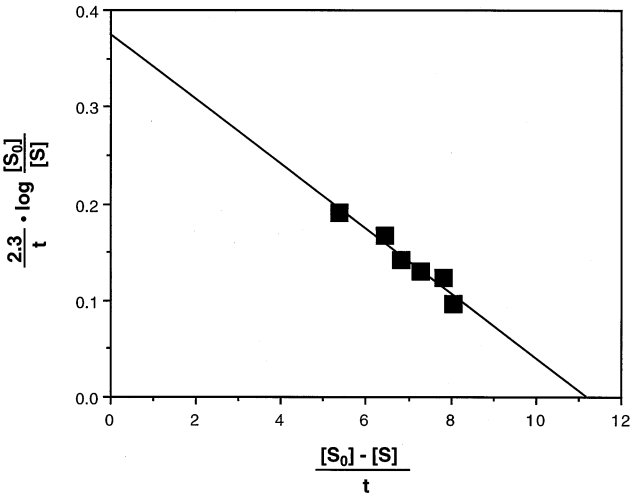

FIG. 6. Kinetic plot for the determination of kinetic parameters by substrate depletion. [^{14}C]TMA (100 μM) was incubated with 100 pmol FMO3, and [^{14}C]TMA turnover, as a function of time, was analyzed by HPLC. Data represent the means of duplicate determinations.

TABLE 1. Apparent kinetic parameters for TMAO formation in human liver microsomes and recombinant human FMO3

Enzyme	Analysis	K_m (μM)	V_{max}
			(nmol/nmol/min)
FMO3*	Polarographic	29.1 ± 8.6	32.2 ± 1.4
	NADPH oxidation	27.1 ± 2.3	33.8 ± 7.3
	Radiometric	27.8 ± 1.7	42.8 ± 3.0
			(nmol/mg/min)
HLM 54†	Radiometric	54.8	3.1
HLM 55	Radiometric	13.0	9.2
HLM 60	Radiometric	33.7	3.5

Kinetic parameters were determined at pH 7.4, as described in Materials and Methods.

*Data for FMO3 are presented as the means of two independent experiments (conducted in duplicate) ± range from the mean.

†Kinetic parameters for each human liver microsomal preparation were derived from a single experiment.

obtained for recombinant FMO3 with different analytical methods ranged from 27.1 to 29.1 μM and 32.2 to 42.8 nmol TMAO/nmol FMO3/min, respectively. In human liver microsomes K_m and V_{max}, determined with radiolabeled TMA, ranged from 13.0 to 54.8 μM and 3.1 to 9.2 nmol TMAO/mg protein/min, respectively.

DISCUSSION

The main objective of this study was to investigate the contribution of different human FMO and P450 isoforms to TMA *N*-oxygenation. We used the baculovirus expression system previously to achieve high overexpression of human FMO3 [18], and this system was used in the present study to obtain preparations of human FMO1, FMO4t, and FMO5 protein. Membrane preparations from virus-infected cells were used directly for catalytic studies because the endogenous insect cell membrane activities interfered minimally with the analytical methods employed. A cDNA encoding human FMO2 was not available to us, and so catalytic comparisons were made with the orthologous isoform purified from rabbit lung.

In studies with FMOs, it has been common to evaluate enzyme activity indirectly by analyzing NADPH oxidation and/or oxygen consumption. Since these methods are not very specific and relatively insensitive, we established an HPLC method with radiochemical detection in order to measure product formation from TMA directly. The only metabolite detected was the *N*-oxide of TMA, as anticipated from a previous study in humans showing that *in vivo* more than 95% TMA is converted to TMAO [5]. This methodology also allowed us to look for reduction of TMAO to TMA *in vitro*, which is known to occur *in vivo* [11]. It is also reported that P450 enzymes and liver xanthine oxidase are capable of catalyzing the anaerobic reduction of *N*-oxides of, for example, imipramine, *N*,*N*-dimethylaniline, and benzydamine [24–27]. However, recombinant P450 enzymes or human liver microsomes incubated with 500 μM [^{14}C]TMAO exhibited no detectable reduction to TMA.

With the exception of FMO5, which has poor catalytic activity for classical FMO substrates [10], the viability of all enzyme preparations was confirmed by measuring BAO formation, a versatile and sensitive probe for FMO activity. With regard to the human tissues examined, the order of microsomal benzydamine *N*-oxygenase activity decreased in the order: adult human liver > human kidney > fetal liver > human intestine. In contrast, significant rates of TMAO formation were obtained only from adult human liver microsomes where K_m and V_{max} values ranged from 13 to 54.8 μM and from 3.1 to 9.2 nmol TMAO/mg protein/min, respectively. Parallel experiments conducted with cDNA-expressed human FMOs demonstrated that only FMO3 formed substantial quantities of TMAO at physiological pH. Kinetic studies confirmed FMO3 to be a highly efficient TMA-oxygenase at physiological pH 7.4, with a K_m of 28 ± 1 μM and a V_{max} of 36.3 ± 5.7 nmol TMAO/nmol FMO3/min. These findings are in good agreement with the tissue-selective expression of FMO3 in adult human liver [9]. In addition, eleven heterologously expressed P450 enzymes were examined for their capacity to metabolize TMA, but neither *N*-demethylation nor *N*-oxide formation could be detected. Therefore, human FMO3 is a very specific TMA *N*-oxygenase under physiological conditions.

Two previous studies have also reported kinetic parameters for TMA oxidation by human monooxygenases. K_m values of 170–1600 μM and a V_{max} range of 0 to 0.56 nmol TMAO/mg protein/min, were found at pH 8.1 in human liver homogenates of biopsy and mainly autopsy samples [28]. More recently, catalysis by a soluble maltose fusion protein of human FMO3 was studied at pH 8.5, and a K_m of 724 μM but no k_{cat} was reported [29]. These studies with autopsy material or a fusion protein examined at nonphysiological conditions are unlikely to reflect the native situation in humans. The excellent congruence between the K_m values obtained in human liver microsomes and with the recombinant protein in the present study demonstrate that FMO3 is a highly efficient TMA *N*-oxygenase. It has also been reported that the pig ortholog of FMO1 can catalyze TMA oxidation. Polarographic analysis conducted at pH 7.4 provided K_m and k_{cat} values ranging from 3200 to 4200 μM and 18.5 to 33 min^{-1} [6, 30, 31], respectively. Although human FMO1 can be coaxed to catalyze TMAO formation at very high TMA concentrations (5 mM at pH 7.4) or at a higher pH (8.7), these conditions are not relevant for TMA clearance *in vivo* in humans.

The pH optimum for TMAO formation catalyzed by human FMO3 is between 9 and 9.5. The turnover number approached 200 min^{-1} at the pH optimum, which is the highest velocity reported for an FMO3-dependent reaction. It was speculated previously [32] that, for FMO3, the high pH optimum for tertiary amines could derive from providing more nonprotonated substrate at higher pH. Based on the pK_a of 9.8 for TMA [33], the amount of free base is 0.4% at pH 7.4 and 13.7% at pH 9, respectively. This represents a 35-fold increase of free amine, but only a 5-fold

increase in the rate of FMO3-dependent TMA *N*-oxygenation over the same pH range. Moreover, we have found that sulfoxidation of sulindac sulfide, a non-amine substrate for FMO3, provided an identical pH profile and a 5-fold increase of catalytic activity (data not shown). Collectively, these data suggest that enhanced TMA *N*-oxygenation by human FMO3 at higher pH is not due solely to the increased concentration of free base. Since at physiological pH 99.6% of TMA is protonated, we speculate that the selectivity of FMO3 for TMA may reside in the ability of the isoform to accept the protonated form of this relatively hydrophilic substrate and/or to deprotonate it prior to oxygen transfer.

In conclusion, several FMO isoforms are capable of oxidizing TMA under non-physiological conditions. However, at pH 7.4 and at low substrate concentrations, only human FMO3 catalyzes TMA oxidation efficiently. Under these conditions TMAO formation may be used as a specific *in vitro* probe for FMO3-mediated catalysis in human tissues.

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