Published in final edited form as:

Arch Biochem Biophys. 2007 August 15; 464(2): 251–259.

Functional Characterization of Genetic Variants of Human FMO3 Associated with Trimethylaminuria

Catherine K. Yeung^{a,†}, Elinor T. Adman^b, and Allan E. Rettie^{a,*}

^aUniversity of Washington School of Pharmacy, Department of Medicinal Chemistry, Seattle, Washington 98195

^bUniversity of Washington Department of Biological Structure, Seattle, Washington 98195

Abstract

Impaired conversion of trimethylamine to trimethylamine *N*-oxide by human flavin containing monooxygenase 3 (FMO3) is strongly associated with primary trimethylaminuria, also known as ‘fish-odor’ syndrome. Numerous non-synonymous mutations in *FMO3* have been identified in patients suffering from this metabolic disorder (e.g. N61S, M66I, P153L, R492W), but the molecular mechanism(s) underlying the functional deficit attributed to these alleles has not been elucidated. The purpose of the present study was to determine the impact of these disease-associated genetic variants on FMO3 holoenzyme formation and on steady-state kinetic parameters for metabolism of several substrates, including trimethylamine. For comparative purposes, several common allelic variants not associated with primary trimethylaminuria (i.e. E158K, V257M, E308G and the E158K/E308G haplotype) were also analyzed. When recombinantly expressed in insect cells, only the M66I and R492W mutants failed to incorporate/retain the FAD cofactor. Of the remaining mutant proteins P153L and N61S displayed substantially reduced (<10%) catalytic efficiencies for trimethylamine *N*-oxygenation relative to the wild-type enzyme. For N61S, reduced catalytic efficiency was solely a consequence of an increased K_m , whereas for P153L, both K_m and k_{cat} were altered. Similar results were obtained when benzydamine *N*-oxygenation was monitored. A homology model for FMO3 was constructed based on the crystal structure for yeast FMO which places the N61 residue alone, of the mutants analyzed here, in close proximity to the FAD catalytic center. These data demonstrate that primary trimethylaminuria is multifactorial in origin in that enzyme dysfunction can result from kinetic incompetencies as well as impaired assembly of holoprotein.

Keywords

FMO; human; polymorphism; kinetics; structure; model

Introduction

Mammalian microsomal flavin-containing monooxygenase (FMO) enzymes (EC 1.14.13.8) catalyze biotransformation reactions at nucleophilic heteroatom centers that are relevant to drug, xenobiotic and endogenous substrate metabolism [1]. FMO3 is a major form of the enzyme present in adult human liver, where it plays an important role in the metabolism of

* Corresponding author University of Washington School of Pharmacy, Department of Medicinal Chemistry, Box 357610, Seattle, WA 98195, Phone: (206) 685-0615, Fax: (206) 685-3252

[†]Current address: University of Michigan College of Pharmacy, Department of Medicinal Chemistry, Ann Arbor, Michigan 48109

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

trimethylamine (TMA). TMA is derived *in vivo* primarily from bacterial degradation of dietary choline, carnitine, and lecithin [2-4] which are found in foods such as egg yolk, liver, kidney, legumes, soybeans, peas, shellfish, and salt-water fish [4]. TMA is thought to be the primary endogenous substrate for FMO3, although it is possible that other biogenic amines such as tyramine [5] and phenylethylamine [6] may also be physiologically relevant substrates. TMA is metabolized exclusively to trimethylamine *N*-oxide (TMAO) in humans, primarily by FMO3, although other FMO isoforms are capable of oxidizing TMA under non-physiologic conditions [7].

Normally, TMA metabolism in humans is an efficient process and individuals will typically excrete more than 18 μmol of TMA/ μmol creatinine daily in the urine with a TMA:TMAO ratio less than 5:95 [8,9]. However, in cases of the metabolic disorder, trimethylaminuria (TMAU), greater than 46 μmol urinary TMA/ μmol creatinine is excreted daily [10], and the TMA: TMAO ratio changes dramatically to greater than 80:20 [11]. Distressingly for TMAU sufferers, the unmetabolized trimethylamine is excreted in the sweat and exhaled breath, resulting in a characteristic fish-odor [12]. While 'transient' or secondary TMAU can be caused by a variety of factors including dietary abnormalities and hormonal effects around puberty, primary, unremitting TMAU is the result of genetic polymorphisms in the *FMO3* gene that reduce the metabolic competency of the human enzyme [13,14].

An international database has been created that catalogs novel single nucleotide polymorphisms (SNPs) of *FMO3* (available at http://human-fmo3.biochem.ucl.ac.uk/Human_FMO3/) [15], and dozens of allelic variants of FMO3 are now known. Four of the earliest reported disease-associated mutations in FMO3 were P153L [13], M66I and R492W [10], and N61S [16]. Heterologous expression confirmed that metabolism of TMA by these mutant forms was abrogated [13,16,17]. The most commonly occurring allelic variants, E158K and E308G, are not associated with symptomatic TMAU, although the 158K/308G haplotype has been linked with transient TMAU [18]. Additionally, multiple upstream haplotype variants have been described that are predicted to modulate FMO3 gene function [19].

Despite growing knowledge of genetic variation in human *FMO3*, little is known about the molecular mechanisms underlying the loss of function for disease-associated variants. This knowledge gap has been exacerbated by the lack of any detailed structural information for the microsomal FMOs. However, recently the crystal structure of a yeast FMO was solved [20] providing, for the first time, a highly useful template for construction of homology models for human FMO3. Therefore, the goal of the present study was to express disease-related genetic variants of FMO3, assess proper folding of the enzymes through analysis of FAD incorporation/retention, and measure steady-state kinetic parameters for oxidation of the probe substrates trimethylamine, benzydamine, methyl *p*-tolyl sulfide (Figure 1). Results from these experiments are discussed in the context of a homology model for human FMO3 based on the yeast FMO structure.

Materials and Methods

Chemicals

Restriction enzymes and T4 ligases were purchased from New England Biolabs, Inc. (Beverly, MA). PfuTurbo DNA polymerase was purchased from Stratagene (La Jolla, CA). The pFastBac vector, Bac-to-Bac expression system and custom oligonucleotides were purchased from Invitrogen (Carlsbad, CA). Ex-Cell 420 insect cell culture medium was from JRH Biosciences (Lenexa, KS). Antibiotics and fetal calf serum for insect cell culture, NADPH, benzydamine, methyl *p*-tolyl sulfide and (R)- and (S)-sulfoxides were purchased from Sigma-Aldrich (St. Louis, MO). Benzydamine *N*-oxide maleate and the dazidamine internal standard were kind gifts from Dr. M. Byron Kneller and Dr. Dieter H. Lang, respectively. ^{14}C -Radiolabeled

trimethylamine was obtained from Sigma Radiochemicals. DNA sequencing was performed at the University of Washington Department of Pharmaceutics DNA Sequencing Facility. Solvents for HPLC were obtained from the Fisher Chemical Co. All other chemicals and reagents were obtained from Sigma Chemical Co.

Instrumentation

HPLC-UV and fluorescence analyses were performed on a Hewlett Packard 1050 series instrument (Agilent Technologies, Palo Alto, CA) consisting of a quaternary pump, in-line degasser, autosampler, variable wavelength detector, and a fluorescence detector. Data were analyzed with the corresponding HP1050 ChemStation software. Radiometric analysis was performed on a Shimadzu instrument consisting of two LC-10ADvp pumps, an SPD-10Avp UV-Vis detector, an SCL-10Avp controller, SIL-10ADvp auto-injector, and Packard 500TR series flow scintillation analyzer. Data were collected using ADI instruments (v2.1.3, Castle Hill, NSW, Australia) PowerChrom software and Packard Flo-One software.

FMO3 protein expression

Recombinant human FMO3 was expressed in *Spodoptera frugiperda* IPLB-Sf21-AE (Sf9) insect cells as previously described by Haining et al. [21]. The original cDNA construct was modified by PCR to include a C-terminal hexa-histidine tag connected to the FMO3 sequence by a serine-threonine linker sequence using the following oligonucleotide primers:

(forward) 5'-
GCTAGTCTAGAATGGGGAAGAAAGTGGCCATCATTGGAGCTGGTGTGAGTGGC-3'
(reverse) 5'-GGCGGCAAGCTTTCAATGATGATGATGATGATGGGTGGAGG-3'

The forward 5'-flanking primer for overlap extension incorporated an XbaI restriction site and the reverse 3'-flanking primer incorporated the linker and hexa-histidine tag followed by a HindIII restriction site. Despite the affinity tag, FMO3 purification according to standard protocols was of limited success, and only small amounts sufficient for use as standards for immunoquantitation were obtained. Accordingly, insect cell membranes were used to determine kinetic parameters for FMO3 variants.

Amino acid mutations in the wild type FMO3 sequence were produced using either PCR overlap-extension or Quikchange II XL (Stratagene, La Jolla, CA) protocols with the primers listed in Table 1. DNA sequences were verified using the services of the University of Washington Department of Pharmaceutics Gene Sequencing Facility following ligation into the pFastBac vector as well as during enzyme expression from DNA isolated from infected insect cells. Cell pellets from a minimum of three independent 500 ml expression cultures were combined for each variant enzyme and membrane fractions prepared by cell disruption and ultracentrifugation as described previously [21].

Characterization of FMO3 containing insect cell membranes

Insect cell membrane preparations were characterized for total protein content, FAD content and immunoreactive FMO3 content. Total protein was assayed using the method described by Lowry et al. [22] with a bovine serum albumin standard. FAD was quantitated as previously described by Lang et al. [7]. Immunoreactive FMO3 was quantitated using Western blotting analysis as previously described [23] following separation of protein bands on a 9% SDS-polyacrylamide gel [24].

Microsomal incubations

Incubation mixtures contained 0.1 M phosphate buffer (pH 7.4) and 0.5 mM NADPH. Twenty picomoles of holo-FMO3 were used in initial studies with incubation times ranging from 5-20

minutes. Metabolic reactions with enzymes that exhibited low substrate turnover (N61S, M66I, P153L, and R492W) were repeated using 50-100 pmol of FMO for 20 minutes to facilitate product quantitation. All reactions were performed in triplicate. Following a preincubation of 3 minutes at 37° C, the reaction was initiated with the addition of substrate and allowed to continue with gentle agitation for 5-20 minutes. Substrate concentration ranges were 5-1000 μ M, 20-5000 μ M, and 10-1500 μ M for benzydamine, methyl p-tolyl sulfide, and TMA, respectively. For benzydamine, the reaction was quenched with an equal volume of acetonitrile containing 0.1 mg/ml dazidamine internal standard. Methyl p-tolyl sulfide and TMA incubations were terminated by the addition of HClO₄ to a final concentration of 1%. Incubations were then placed on ice for 5 minutes to allow for full protein precipitation. The protein was pelleted by centrifugation (14,000 rpm $\times$ 5 minutes) and the supernatant subjected to HPLC analysis.

HPLC Analyses

Benzydamine, its *N*-oxide, and the internal standard, dazidamine were analyzed on an Agilent Hypersil ODS C18 (5mm/4.0 $\times$ 250mm) column using a protocol modified from a published procedure [25]. Mobile phase A consisted of methanol-water-25% NH₄OH (50:40:10:0.5 v/v), and mobile phase B was composed of nanopure H₂O. The mobile phase components were mixed at a fixed ratio of 92% A + 8% B, at a flow rate of 1.0 ml/min. The effluent was monitored fluorometrically with an excitation wavelength of 307 nm and an emission wavelength of 377 nm. Approximate retention times for benzydamine *N*-oxide, dazidamine, benzydamine, and norbenzydamine were 2,3,4, and 10 minutes respectively. Norbenzydamine was not detected in any incubations.

Methyl p-tolyl sulfide, and the (R)- and (S)- sulfoxides were separated on a Regis (R,R)-Whelk-O 1 (250 mm $\times$ 4.6 mm) column. Mobile phase A was composed of acetonitrile, and mobile phase B was composed of 0.05% acetic acid in water at a pH of 4.7. The mobile phase components were mixed at a fixed ratio of 25% A + 75% B at a flow rate of 1.0 ml/min. The effluent was monitored at 254 nm. The retention times of the (R)- and (S)- enantiomers were approximately 22 and 23 minutes respectively, these retention times were verified with commercially available enantiomerically pure standards.

¹⁴C-TMA and ¹⁴C-TMAO were separated on a Biorad Aminex HPX-72 O (300 mm $\times$ 7.8 mm) column with a mobile phase of 0.01 M NaOH at a flow rate of 1.0 ml/min as described previously [7]. Commercially available radiolabeled TMA (1.7 μ Ci/mmol) was diluted to working concentrations without addition of non-labeled TMA. Prior to analysis, the acidic supernatants of the incubation mixtures were adjusted to pH 7-8 with NaOH. ¹⁴C-TMA and ¹⁴C-TMAO were monitored by radiometric detection and eluted at 5.5 and 7.5 minutes, respectively.

Data analysis method

K_m and k_{cat} were obtained by Eadie-Hofstee transformations and linear regression methods using Microsoft Excel (2000).

Structural modeling

When the structure of FMO from *Schizosaccharomyces pombe* became available (PDB ID codes 2GVC (with FAD and substrate bound) and 2GV8 (with FAD and NADP bound) [20], the sequences of human FMO1, FMO2, and FMO3 (SwissProt Data Bank accessed via the ExPASy web site (<http://www.expasy.org>) [26] were added to the previously published alignment of *S. pombe* FMO, *Saccharomyces cerevisiae* FMO1 and *Drosophila* FMO2 (see Fig 8 of [20]). This alignment was used to guide construction of a homology model for FMO3 using the Molecular Operating Environment software, (MOE, available from the Chemical

Computing Group, Montreal (<http://www.chemcomp.com>), by iteratively building and readjusting the alignment according to where insertions and deletions were deemed most likely to occur relative to yeast FMO. The C-terminal portion of FMO3, which extends beyond the yeast structure, was built on a mostly helical backbone (selected as significantly likely by MOE from a sequence-based structure search of the PDB), from PDB file 1M56 (cytochrome C oxidase from *Rhodobacter sphaeroides*). This segment was attached to the FMO3 model using the interactive graphics features of MOE. Finally, when the insertions were satisfactorily incorporated (final alignment used is shown in Figure 4), the model was rebuilt using the 2GV8 coordinates, which contains both FAD and NADPH, and energy minimized. Key residues in the neighborhood of the NADPH and FAD cofactors were evaluated and found to have satisfactory counterparts in both models. While a number of problematic dihedral angles remained in the FMO3 model, especially in regions which differ most from the 2GV8 structure, these were not in locations likely to affect the conclusions of this paper. Drawings were rendered using MOE.

Results

Expression and Characterization of Wild Type and Mutant FMO3 enzymes

Expression levels of wild type FMO3 on the order of 200 nmol of protein per liter of culture were achieved. Mutants at amino acid positions 61, 66, 153, 158, 308, 158/308, and 492 all expressed well, such that immunochemically determined FMO content for all preparations was in the 1-2 nmol/mg range (Table 2, Figure 2). Approximately 50% of wild type FMO3 existed as holoprotein with the remainder apparently lacking the FAD cofactor. Most FMO3 mutants retained significant levels of FAD (>20%) in microsomal preparation with the exception of M66I and R492W, which were comprised of 1 and 7% holoprotein respectively (Table 2).

Effects on k_{cat} for FMO3 Variant Catalyzed Trimethylamine, Benzydamine and Methyl p-Tolyl Sulfide Oxidation

All functional enzymes exhibited hyperbolic Michaelis-Menten kinetics with the three substrates investigated (data not shown). Eadie-Hofstee transformations for TMAO formation by wild-type FMO3 and the N61S and P153L disease-associated mutants are shown in Figure 3. Data in Table 3 show k_{cat} values for substrate oxidation grouped according to the magnitude of the effect relative to wild-type FMO3. The E158K, V257M, E308G and E158K/E308G mutants metabolized both TMA and benzydamine with k_{cat} values ($\sim 20\text{-}30\text{ min}^{-1}$), similar to wild-type FMO3. In contrast, the N61S and P153L mutants exhibited lower k_{cat} values of 2.0 and 5.5 min^{-1} respectively for both substrates. Qualitatively similar k_{cat} values were obtained for most of the mutant enzymes towards methyl p-tolyl sulfide. Data presented in Table 3 represent total p-tolyl methyl sulfoxide product formed because all functional enzymes generated the (R)- and (S)- methyl p-tolyl sulfoxide enantiomers in a 60:40 ratio. Interestingly, the P153L mutant exhibited a relatively high k_{cat} for sulfoxidation, and the V257M mutant a low k_{cat} for this reaction, relative to the N-oxygenation pathways analyzed.

Effects on K_{m} for FMO3 Variant catalyzed Trimethylamine, Benzydamine and Methyl p-Tolyl Sulfide Oxidation

As shown in Table 4, most functional enzymes catalyzed metabolism of TMA with apparent K_{m} values in the region of 20-30 μM . The exception was the N61S variant which exhibited almost a ten-fold increase in K_{m} (205 μM) for this reaction. A different pattern emerged for the much larger tertiary amine, benzydamine, where K_{m} values for the N61S, P153L and V257M mutants were all increased relative to wild-type. Apparent K_{m} values for FMO3-catalyzed p-tolyl methyl sulfoxidation were much higher (in the 200-300 μM region) and did not differ across any of the functional enzymes examined, including N61S and P153L.

Effects on Intrinsic Catalytic Efficiency (k_{cat} / K_m) for N- and S-Oxygenation Catalyzed by FMO3 variants

Relative k_{cat} / K_m values for each FMO3 variant, normalized to 100% for the wild-type enzyme, are summarized in Table 5. Only N61S demonstrated a consistently large decrease in catalytic efficiency (>90%) towards these three substrates. A very prominent substrate-dependent change in catalytic efficiency was evident for the P153L mutant, which lost N-oxygenation capacity, but retained a significant amount of S-oxidation activity. More modest substrate-dependent changes were exhibited by the V257M mutant. No large differences in catalytic efficiency, relative to wild-type FMO3, were observed for the E158K, E308G, or the E158K/E308G mutants across the three substrates examined.

Features of a yeast FMO-based structural model for human FMO3

A structural model for human FMO3 based on the crystal structure coordinates for yeast FMO is shown in Figure 5. The conserved nucleotide binding regions are shown as yellow ribbons. Near the NAD site, residues S223 (mc), D226, Y176 and Q89 (mc) interact with NAD(P)H. In the FMO3 model, S195, D198, H150 and F59 provide satisfactory interactions. Residues near the FAD in 2GV8 (V46, W47, P16, R39, V138, T92) also have satisfactory spatial counterparts in the model (L40, W41, V12, K33, V110 and S62). A new feature in the FMO3 model is the favorable interaction between the long insertion at residues 217-295 and the extended C-terminus of FMO3, the blue and red regions marked 'insert' and 'C-ter', respectively. Most significantly, yeast N91, found in 2GV8 to bind the reactive oxygen, is the equivalent of N61 in the present FMO3 model. A one residue Gly insertion in a loop (368-379) apposing the loop containing N61 causes the local geometry of the model to differ from the yeast structure. It is possible that this could have some bearing on enzyme reactivity, as this loop contains the reportedly [20] characteristic FMO identifying sequence, FxGxxxHxxxY/F, which in human FMO3 appears as VxGxxxSxGxxI.

Mapping of FMO3 allelic variants

The seven mutation sites examined here were mapped onto this homology model as shown in Figure 5. P153 and E158 are situated on a putative access channel to the catalytic center, with P153 closer to the FAD. Val 257 is located further around on the access channel, about the same distance out from the FAD as E158. E308 is located on the periphery of the protein. M66 lies at the interface between the FAD binding domain and the extended C-terminal domain. R492 lies in this C-terminal domain, at the interface with the inserted residues between 219–296. The only amino acid residue that is located in close proximity to the FAD catalytic center is N61.

Discussion

The present study sought to discriminate between mechanisms responsible for obviating the catalytic activity of rare FMO3 mutations closely associated with primary TMAU. To provide some control for non-specific mutational effects, we also examined three common allelic variants that are known not to cause phenotypic TMAU. Another reason for focusing on these common variants is that conflicting data have been presented on the extent to which these common polymorphisms alter FMO3 function [17,27-29]. We measured the ability of each enzyme variant to bind its FAD cofactor, as well as kinetic parameters for the oxidation of multiple substrates of varying size and ease of oxidation. The experimental design also included two substrates in addition to TMA, benzydamine and p-tolyl sulfide, to evaluate possible substrate-dependent effects on metabolism. Finally, we developed a new homology model for human FMO3 and compared experimental and *in silico* observations.

All of the recombinant proteins expressed well in the baculovirus mediated insect cell expression system, which suggests that both the mRNA transcripts and the subsequently translated (apo)proteins are relatively stable. However, it is well recognized that human FMO3 activity is quite thermolabile. While the precise nature of the interaction between the FMO3 apoprotein and its FAD cofactor is not known, it is certainly non-covalent, and the lack of heat tolerance of FMO3 is often attributed to the facile loss of FAD from the FMO3 cofactor-binding domain. A significant amount of heterologously expressed wild type FMO3 is not coupled with a stoichiometric amount of FAD cofactor, implying that the FAD cofactor is either easily dislodged or incompletely inserted in many cases. It seems likely that the dissociation of the FAD cofactor (or inability of the apoprotein to bind FAD during post-translational processing) could be exacerbated by even a minor alteration of the FMO3 structure. Our data show that all of the commonly occurring genetic variants (WT, E158K, V257M, E308G, 158K/308G), as well as TMAU variants, P153L and N61S, comprised >20% holoprotein in insect cell membrane preparations. In contrast, the M66I and R492 variants exhibited very low levels of holoprotein, despite robust expression of the apoprotein (Table 2). Moreover, rates of metabolism of each of the three probe substrates were below detectable limits for these variants when comparable amounts of membrane protein were used in the incubations. Therefore, the experimental data suggest that TMAU attributable to the M66I and R492W variants is likely due to low incorporation or poor retention of FAD in the translated protein.

We attempted to rationalize the effects of FMO3 polymorphisms within a homology model for the enzyme based on the crystal structure of yeast FMO [20]. To evaluate the homology model, a number of presumably highly conserved features of the 2GV8 structure were examined. Notably, key residues in the neighborhood of the FAD and NADPH cofactors were satisfactorily represented by their counterparts in the FMO3 model. In our homology model, R492 is located at some distance from the FAD but at the interface between the C-terminal domain and the long insertion not found in yeast FMO. M66 lies at a different interface between the C-terminal loop and the FAD nucleotide binding domain (Figure 5). It may be that optimal relative orientations of these fragments are required to adequately bind FAD. Consideration of the V257M mutant (see below) suggests that while direct interaction between the inserted loop and the C-terminus is suggested by the homology model, the exact nature of that interaction may not yet be well modeled.

The common allelic variants E158K, E308G and V257M caused little change in the catalytic efficiency of TMA metabolism. This finding parallels *in vivo* studies on TMAO excretion conducted with healthy volunteers who had been genotyped for the various haplotype combinations at these three allelic sites [29]. Neither E158K nor E308G mutations significantly reduce catalytic efficiency towards benzydamine or p-tolyl methyl sulfide metabolism. Their putative location on the exterior of the FMO3 homology model is not inconsistent with these minimal effects. The V257M variant did exhibit ~60% reduction in catalytic efficiency towards benzydamine and p-tolyl methyl sulfide. While V257 is not an immediate neighbor of the active site locale, it appears to be situated in the middle of a long loop that protrudes from the NAD binding domain and extends over to the FAD domain, forming a lid. This feature, (“insert” in Figure 5), is not present in the yeast FMO structure. That the K_m for the V257M mutant with the largest substrate used, benzydamine, (see Table 3) changes the most suggests that the model could be improved by closing down the interacting loop and C-terminal domains around the substrate access channel.

In contrast, the other partly functional TMAU-associated alleles, N61S and P153L exhibited grossly diminished catalytic efficiencies towards TMA, which mirrors the phenotype. For P153L, this was very largely a consequence of reduced k_{cat} , with little or no effect on K_m for any of the substrates examined. These observations seem to fit reasonably well with the location of P153 on the putative access channel to the active site, suggested by the homology model.

Mutations at this site might reasonably be expected to impede ingress/egress of substrates and/or cofactors without greatly influencing substrate binding or dynamics within the active site of the enzyme. Another speculative possibility arises from the observation that P153 appears to lie on a strand adjacent to the tyrosine in the iconic “FATGY” sequence of FMO3 (Figure 5). The tyrosine in this stretch forms part of the binding wall of the isoalloxazine ring of FAD. Inasmuch as the integrity of this surface could be modified by a larger, more flexible adjacent amino acid, the optimal interaction of substrate and the active FAD hydroperoxy species could conceivably be affected.

The N61S mutant was the only FMO3 variant examined that showed greatly reduced catalytic efficiencies for all three substrates. As well as substantial effects on k_{cat} , large increases in K_{m} were observed for both of the amine substrates. An alignment of all mammalian FMOs, yeast FMO and four putative *Caenorhabditis elegans* FMOs shows this asparagine to be conserved in all FMOs [16]. The recently solved crystal structure for yeast FMO localizes the N91 residue close to the FAD catalytic center, as does the homology model for human FMO3. Moreover, yeast N91 was bonded to a dioxygen molecule in all three crystal forms analyzed, prompting speculation that this residue plays a key role in delivering oxygen to the reduced FAD [20]. If N61 were to play a similar role in the generation of the activated C4a-hydroperoxide in FMO3, the global effect of dramatically reduced catalytic efficiency, independent of substrate, would be readily rationalized.

In summary, the disease-associated M66I and R492W mutants are inactive, due likely to the absence of a protein-associated FAD cofactor. TMAU secondary to the FMO3 gene mutations N61S and P153L is caused, at least partially, by a decrease in k_{cat} . Changes in kinetic parameters of substrate oxidation for these two allelic variants may reflect the location of the mutated amino acids at two ends of the the FAD active site, as tentatively suggested by homology modeling. Regardless, the experimental data demonstrate that primary TMAU is multifactorial in origin, in that enzyme dysfunction can result from kinetic incompetencies as well as impaired assembly of FAD-bound holoprotein.

Acknowledgements

This work was supported in part by NIH Grants RO1GM43511 and NIEHS P30ES07033. CKY was supported in part by NIH Training Grant GM07750.

References

1. Krueger SK, Williams DE. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol Ther* 2005;106:357–387. [PubMed: 15922018]
2. Holmes HC, Burns SP, Michelakakis H, Kordon V, Bain MD, Chalmers RA, Rafter JE, Iles RA. Choline and L-carnitine as precursors of trimethylamine. *Biochem Soc Trans* 1997;25:96S. [PubMed: 9056994]
3. Tjoa S, Fennessey P. The identification of trimethylamine excess in man: quantitative analysis and biochemical origins. *Anal Biochem* 1991;197:77–82. [PubMed: 1952078]
4. Ruocco V, Florio M, Filioli FG, Guerrera V, Prota G. An unusual case of trimethylaminuria. *Br J Dermatol* 1989;120:459–461. [PubMed: 2713263]
5. Lin J, Cashman JR. Detoxication of tyramine by the flavin-containing monooxygenase: stereoselective formation of the trans oxime. *Chem Res Toxicol* 1997;10:842–852. [PubMed: 9282832]
6. Lin J, Cashman JR. N-oxygenation of phenethylamine to the trans-oxime by adult human liver flavin-containing monooxygenase and retroreduction of phenethylamine hydroxylamine by human liver microsomes. *J Pharmacol Exp Ther* 1997;282:1269–1279. [PubMed: 9316835]
7. Lang DH, Yeung CK, Peter RM, Ibarra C, Gasser R, Itagaki K, Philpot RM, Rettie AE. Isoform specificity of trimethylamine N-oxygenation by human flavin-containing monooxygenase (FMO) and

- P450 enzymes: selective catalysis by FMO3. *Biochem Pharmacol* 1998;56:1005–1012. [PubMed: 9776311]
8. Treacy E, Johnson D, Pitt JJ, Danks DM. Trimethylaminuria, fish odour syndrome: a new method of detection and response to treatment with metronidazole. *J Inher Metab Dis* 1995;18:306–312. [PubMed: 7474897]
 9. Zhang AQ, Mitchell S, Smith R. Fish odour syndrome: verification of carrier detection test. *J Inher Metab Dis* 1995;18:669–674. [PubMed: 8750603]
 10. Akerman BR, Lemass H, Chow LM, Lambert DM, Greenberg C, Bibeau C, Mamer OA, Treacy EP. Trimethylaminuria is caused by mutations of the FMO3 gene in a North American cohort. *Mol Genet Metab* 1999;68:24–31. [PubMed: 10479479]
 11. Hadidi HF, Cholerton S, Atkinson S, Irshaid YM, Rawashdeh NM, Idle JR. The N-oxidation of trimethylamine in a Jordanian population. *Br J Clin Pharmacol* 1995;39:179–181. [PubMed: 7742158]
 12. Cashman JR, Camp K, Fakharzadeh SS, Fennessey PV, Hines RN, Mamer OA, Mitchell SC, Nguyen GP, Schlenk D, Smith RL, Tjoa SS, Williams DE, Yannicelli S. Biochemical and clinical aspects of the human flavin-containing monooxygenase form 3 (FMO3) related to trimethylaminuria. *Curr Drug Metab* 2003;4:151–170. [PubMed: 12678693]
 13. Dolphin CT, Janmohamed A, Smith RL, Shephard EA, Phillips IR. Missense mutation in flavin-containing mono-oxygenase 3 gene, FMO3, underlies fish-odour syndrome. *Nat Genet* 1997;17:491–494. [PubMed: 9398858]
 14. Treacy EP, Akerman BR, Chow LM, Youil R, Bibeau C, Lin J, Bruce AG, Knight M, Danks DM, Cashman JR, Forrest SM. Mutations of the flavin-containing monooxygenase gene (FMO3) cause trimethylaminuria, a defect in detoxication. *Hum Mol Genet* 1998;7:839–845. [PubMed: 9536088]
 15. Hernandez D, Addou S, Lee D, Orenge C, Shephard EA, Phillips IR. Trimethylaminuria and a human FMO3 mutation database. *Hum Mutat* 2003;22:209–213. [PubMed: 12938085]
 16. Dolphin CT, Janmohamed A, Smith RL, Shephard EA, Phillips IR. Compound heterozygosity for missense mutations in the flavin-containing monooxygenase 3 (FMO3) gene in patients with fish-odour syndrome. *Pharmacogenetics* 2000;10:799–807. [PubMed: 11191884]
 17. Cashman JR, Bi YA, Lin J, Youil R, Knight M, Forrest S, Treacy E. Human flavin-containing monooxygenase form 3: cDNA expression of the enzymes containing amino acid substitutions observed in individuals with trimethylaminuria. *Chem Res Toxicol* 1997;10:837–841. [PubMed: 9282831]
 18. Mayatepek E, Flock B, Zschocke J. Benzydamine metabolism in vivo is impaired in patients with deficiency of flavin-containing monooxygenase 3. *Pharmacogenetics* 2004;14:775–777. [PubMed: 15564885]
 19. Koukouritaki SB, Poch MT, Cabacungan ET, McCarver DG, Hines RN. Discovery of novel flavin-containing monooxygenase 3 (FMO3) single nucleotide polymorphisms and functional analysis of upstream haplotype variants. *Mol Pharmacol* 2005;68:383–392. [PubMed: 15858076]
 20. Eswaramoorthy S, Bonanno JB, Burley SK, Swaminathan S. Mechanism of action of a flavin-containing monooxygenase. *Proc Natl Acad Sci U S A* 2006;103:9832–9837. [PubMed: 16777962]
 21. Haining RL, Hunter AP, Sadeque AJ, Philpot RM, Rettie AE. Baculovirus-mediated expression and purification of human FMO3: catalytic, immunochemical, and structural characterization. *Drug Metab Dispos* 1997;25:790–797. [PubMed: 9224773]
 22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275. [PubMed: 14907713]
 23. Yeung CK, Lang DH, Thummel KE, Rettie AE. Immunoquantitation of FMO1 in human liver, kidney, and intestine. *Drug Metab Dispos* 2000;28:1107–1111. [PubMed: 10950857]
 24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
 25. Kawaji A, Ohara K, Takabatake E. An assay of flavin-containing monooxygenase activity with benzydamine N- oxidation. *Anal Biochem* 1993;214:409–412. [PubMed: 8109727]
 26. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* 2003;31:3784–3788. [PubMed: 12824418]

27. Stormer E, Roots I, Brockmoller J. Benzydamine N-oxidation as an index reaction reflecting FMO activity in human liver microsomes and impact of FMO3 polymorphisms on enzyme activity. *Br J Clin Pharmacol* 2000;50:553–561. [PubMed: 11136294]
28. Cashman JR. Human flavin-containing monooxygenase: substrate specificity and role in drug metabolism. *Curr Drug Metab* 2000;1:181–191. [PubMed: 11465082]
29. Lambert DM, Mamer OA, Akerman BR, Choiniere L, Gaudet D, Hamet P, Treacy EP. In vivo variability of TMA oxidation is partially mediated by polymorphisms of the FMO3 gene. *Mol Genet Metab* 2001;73:224–229. [PubMed: 11461189]

Figure 1. Reaction schematic for FMO3 probe substrates. (A) Methyl p-tolyl sulfide, (R)- and (S)- enantiomers of methyl p-tolyl sulfoxide are indicated. (B) Benzydamine. (C) Trimethylamine.

Figure 2.

(A) Coomassie-stained SDS-PAGE gel (B) Western Blot of wild type and mutant FMO3-expressing insect cell membranes. 10 μ g of insect cell membranes were loaded in each lane of a 9% acrylamide resolving gel. Lane 1, molecular weight markers in kDa; lanes 2 and 12, purified FMO3 standard; lane 3, uninfected insect cell membranes; lane 4, wild type FMO3; lane 5, N61S; lane 6, M66I; lane 7, P153L; lane 8, E158K; lane 9, E308G; lane 10, 158K/308G; lane 11, R492W.

Figure 3.
Eadie-Hofstee plots for wild-type FMO3 (♦), P153L (▲) and N61S (●) catalyzed TMA N-oxygenation.

Figure 4. Sequence alignment used to build FMO3 homology model prepared using ESript 2.2 from Expsay Web server [26]. FMO3 sequence is repeated top and bottom for ease of comparison with structural template (24% homology with 2Gv8, at top) and with yeast, drosophila, and other human FMOs (26%, 27%, 55%, 57% and 57% homology) (at bottom). Conserved residues are both in black, and starred underneath. Boxed residues are semi-conserved. Motifs are indicated, as are mutated residues characterized in this study.

Figure 5.

FMO3 structural model based on yeast FMO. Model is shown with FAD cofactor and O₂ from yeast FMO structure, and benzydamine (green) superposed on substrate (methimazole) found in yeast FMO structure. Sites of mutation are designated by colored spheres at alpha carbons, pink for those not significantly affecting reactivity, yellow for those which cannot bind FAD, and magenta for those which show significant change in reactivity. White ribbon depicts regions most similar to yeast FMO. Blue, insert at residues 217-295. Red, additional residues at C-terminus. Other motifs depicted in color (cyan, FATGY; yellow, nucleotide binding motifs; purple, FxGxxxHxGxxI/F)

Table 1

Oligonucleotide primers used in site directed mutagenesis of FMO3

Mutation	Oligonucleotide primers
N61S	5 -CAGTCTTTTCC AGC TCTTCC-3 3 -GTCAGAAAAGG TCG AGAAGG-5
M66I	5 -CCAAAGAG ATT ATGTGTTTCCC-3 3 -GGTTTCTC TAA TACACAAAGG-5
P153L	5 -GGACATCATGTGTAT CTC AACCTACC-3 3 -CCTGTAGTACACATAG AGT TGGATGG-5
E158K	5 -CAACCTACCAAAA AAG TCCTTTCC-3 3 -GTTGGATGGAAAA TT CAGGAAAGG-5
E308G	5 -CGTGAAGGAATTCACAG GG ACCTCGGCC-3 3 -GCACTTCCTTAAGTGT CCCT GGAGCCGG-5
158K/308G	5 -CAACCTACCAAAA AAG TCCTTTCC-3 3 -GTTGGATGGAAAA TT CAGGAAAGG-5 5 -CGTGAAGGAATTCACAG GG ACCTCGGCC-3 3 -GCACTTCCTTAAGTGT CCCT GGAGCCGG-5
R492W	5 -GGGACT TGG TCGTTGAAAC-3 3 -CCCTG ACC AGCAACTTTG-5

Table 2

Characterization of FMO3 content in insect cell membrane preparations

FMO3 Variant	Immunoreactive FMO3 content (nmol/mg)	FAD content (nmol/mg)	Fraction Holoprotein[*]
Wild Type	1.36 ± 0.15	0.67 ± 0.01	0.49
N61S	1.52 ± 0.31	0.75 ± 0.01	0.49
M66I	2.30 ± 0.57	0.03 ± 0.01	0.01
P153L	0.74 ± 0.18	0.16 ± 0.01	0.21
E158K	2.19 ± 0.40	0.45 ± 0.01	0.21
V257M	1.72 ± 0.32	0.82 ± 0.04	0.48
E308G	2.53 ± 0.32	0.67 ± 0.02	0.27
158K/308G	1.12 ± 0.05	0.65 ± 0.01	0.58
R492W	0.80 ± 0.15	0.06 ± 0.01	0.07

^{*} FAD content/immunoreactive FMO

Table 3

k_{cat} values (min^{-1}) for trimethylamine, benzydamine and methyl p-tolyl sulfide metabolism by FMO3 allelic variants

FMO3 Variant	Trimethylamine	Benzydamine	Methyl p-tolyl Sulfide
Wild Type	23 ± 1	26 ± 3	14 ± 1
E158K	27 ± 4	29 ± 4	18 ± 1
V257M	33 ± 1	26 ± 2	5 ± 0.1
E308G	18 ± 1	33 ± 9	18 ± 3
158K/308G	30 ± 2	36 ± 4	12 ± 0.5
N61S	5.5 ± 0.6	2.7 ± 2	1.1 ± 0.2
P153L	2.0 ± 0.1	3.4 ± 0.2	9 ± 2
M66I	NA	NA	NA
R492W	NA	NA	NA

NA: No activity detected

Table 4

K_m values (μM) for trimethylamine, benzydamine and methyl p-tolyl sulfide metabolism by FMO3 allelic variants.

FMO3 Variant	Trimethylamine	Benzydamine	Methyl p-tolyl Sulfide
Wild Type	31 $\pm$ 3	43 $\pm$ 4	248 $\pm$ 28
E158K	27 $\pm$ 3	33 $\pm$ 2	181 $\pm$ 23
V257M	27 $\pm$ 8	121 $\pm$ 24	207 $\pm$ 5
E308G	29 $\pm$ 2	39 $\pm$ 5	219 $\pm$ 39
158K/308G	22 $\pm$ 1	47 $\pm$ 5	301 $\pm$ 42
N61S	205 $\pm$ 31	335 $\pm$ 31	264 $\pm$ 16
P153L	30 $\pm$ 6	73 $\pm$ 10	242 $\pm$ 36
M66I	NA	NA	NA
R492W	NA	NA	NA

NA: No activity detected

Table 5

$k_{\text{cat}} / K_{\text{m}}$ for trimethylamine, benzydamine and methyl p-tolyl sulfide metabolism by FMO3 allelic variants normalized to wild type FMO3.

FMO3 Variant	Trimethylamine	Benzydamine	Methyl p-tolyl Sulfide
Wild Type	100	100	100
E158K	135	145	171
V257M	165	36	43
E308G	84	140	145
158K/308G	183	127	71
N61S	4	1	7
P153L	9	8	66
M66I	NA	NA	NA
R492W	NA	NA	NA

NA: No activity detected