Effect of genetic variants of the human flavin-containing monooxygenase 3 (FMO3) on N- and S-oxygenation activities

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Running title: **N- and S-oxygenation by recombinant FMO3 variants**

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Abstract

The decreased capacity of the flavin-containing monooxygenase 3 (FMO3) to oxygenate xenobiotics including trimethylamine is believed to contribute to metabolic disorders. The aim of this study was to functionally characterize FMO3 variants recently found in a Japanese population and compare them with selective functional activity of other FMO3 variants. Recombinant Glu158Lys and Glu158Lys-Glu308Gly FMO3 expressed in Escherichia coli membranes showed slightly decreased N-oxygenation of benzydamine and trimethylamine. Selective functional S-oxygenations of methyl p-tolyl sulfide or sulindac sulfide of these variants were comparable with those of wild type FMO3. The Glu158Lys-Thr201Lys-Glu308Gly and Val257Met-Met260Val variants showed significantly decreased oxygenation of typical FMO3 substrates (i.e., approximately one-tenth of the V_max/K_m values). Val257Met FMO3 had a lower catalytic efficiency for methyl p-tolyl sulfide and sulindac sulfide S-oxygenation. However, compared with wild type FMO3, Val257Met FMO3 showed a similar catalytic efficiency for N-oxygenation of benzydamine and trimethylamine. The catalytic efficiency for benzydamine and trimethylamine N-oxygenation by Arg205Cys FMO3 was only moderately decreased but it possessed decreased sulindac sulfide S-oxygenation activity. Kinetic analysis showed that Arg205Cys FMO3 was inhibited by sulindac in a substrate dependent manner, presumably because of selective interaction between the variant enzyme and the substrate. The results suggest that the effects of genetic variation of human FMO3 could operate at the functional level for N- and S-oxygenations for typical FMO3 substrates. Genetic polymorphism in the human FMO3 gene might lead to unexpected changes of catalytic efficiency for N- and S-oxygenations of xenobiotics and endogenous materials.
Introduction

The flavin-containing monooxygenase (FMO, EC 1.14.13.8) is an NADPH-dependent enzyme that catalyzes the oxygenation of a wide variety of compounds containing nitrogen, sulfur or other heteroatoms (Cashman and Zhang, 2006; Krueger and Williams, 2005). FMO3 is considered a prominent form expressed in adult human liver (Lomri, et al., 1992) and plays a role in processing nucleophilic drugs such as the anticancer drug tamoxifen, the pain medication codeine, the antifungal drug ketoconazole, the addictive chemical nicotine found in tobacco, and the diet-derived chemical trimethylamine (Cashman, et al., 2000; Ziegler, 2002).

Due to its strong linkage with the genetic disorder trimethylaminuria, or fish-like odor syndrome, considerable work has been done to relate coding region polymorphisms of the \textit{FMO3} gene to inter-individual differences in FMO3 phenotype (Cashman, 2002; 2004; Dolphin, et al., 1997a). According to the list of \textit{FMO3} gene mutations summarized in a systematic and trivial name Web-database, Glu158Lys, Val257Met, and Glu158Lys-Glu308Gly FMO3 forms are common genetic FMO3 polymorphisms (Hernandez, et al., 2003). There is considerable genetic variation reported among different ethnic groups (Cashman et al., 2001). In the course of identification of novel mutations of \textit{FMO3} and/or haplotypes of the \textit{FMO3} gene in Japanese individuals suffering from trimethylaminuria (Yamazaki, et al., 2004), we recently reported some variants of the \textit{FMO3} gene using genomic DNA from individuals that showed low FMO3 metabolic capacity (Yamazaki, et al., 2005; 2006). In our preliminary reports (Fujieda, et al., 2003; Shimizu, et al., 2006), there were some FMO3 variants such as Thr201Lys, Arg205Cys or Met260Val that were thus far only observed only in a Japanese population. Interestingly, Thr201Lys and Met260Val variants also existed together with the well-known Glu158Lys-Glu308Gly and Val257Met
mutations, respectively, in the FMO3 gene (Shimizu, et al., 2006). However, the complete characterization of these FMO3 variants has not been examined in detail.

Herein we report data showing low catalytic efficiency of three novel FMO3 variants expressed in bacteria that are causative of abnormal N- and S-oxygenation of typical FMO3 substrates including benzydamine, trimethylamine, methyl p-tolyl sulfdie, and sulindac sulfide (Fig. 1) and compare the data from wild type enzyme with FMO3 mutants including Glu158Lys, Val257Met, and Glu158Lys-Glu308Gly. The effect of Arg205Cys and Val257Met FMO3 mutations on the rates of N- and S-oxygenation of model substrates are also reported.

Materials and methods

Chemicals

Benzydamine hydrochloride, trimethylamine N-oxide, methyl p-tolyl sulfdie, methyl p-tolyl sulfoxide, sulindac sulfoxide (Z-5-fluro-2-methyl-1-[p-(methylsulfinyl)benzylidiene]indene-3-acetic acid), and sulindac sulfide were purchased from Sigma-Aldrich (St Louis, MO). Trimethylamine hydrochloride was obtained from Wako Pure Chemicals (Osaka, Japan). Benzydamine N-oxide (> 99.0% of purity; Yeung and Rettie, 2006) was a generous gift from Dr. A.E. Rettie (University of Washington, Seattle, WA). The other chemicals and reagents used were obtained in the highest grade commercially available.

Recombinant FMO3 protein preparations

The procedure for preparation of wild type FMO3 and Arg205Cys FMO3 cDNA was previously reported (Yamazaki, et al., 2006). To produce Glu158Lys, Glu158Lys-Glu308Gly, Glu158Lys-Thr201Lys-Glu308Gly, Val257Met, Val257Met-Met260Val FMO3, site directed mutagenesis was done using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla,
CA). The wild type and modified FMO3 cDNAs were introduced into the pTrc99A expression vector (Pharmacia Biotechnology, Milwaukee, WI) and then transformed into *Escherichia coli* strain JM109 as described previously (Yamazaki, et al., 2006). The entire coding regions of the wild type and mutagenized FMO3 cDNAs including the mutated sites were verified by re-sequencing both strands. From bacterial pellets, membrane FMO3 fractions were prepared by a series of fractionations and high-speed centrifugation steps. The amount of recombinant FMO3 (0.025-0.10 nmol FMO3/mg bacterial protein) was determined by immunoquantification using an anti-FMO3 antibody (BD Gentest, Woburn, MA) by comparison with a standard of recombinant human FMO3 (BD Gentest) according to manufacture’s data sheet (Yamazaki, et al., 2006).

**Enzyme Assays**

Rates of *N*-oxygenation of benzydamine (Yeung and Rettie, 2006) and *S*-oxygenation of methyl *p*-tolyl sulfide (Stevens, et al., 2003) and sulindac sulfide (Hamman, et al., 2000) were determined using HPLC methods described previously with minor modifications. Briefly, a typical incubation mixture consisted of 50 mM potassium phosphate buffer (pH 8.4), an NADPH-generating system (0.25 mM NADP⁺, 2.5 mM glucose 6-phosphate and 0.25 units/mL glucose 6-phosphate dehydrogenase), a substrate, and bacterial membranes (5.0 pmol of FMO3) in a final volume of 0.20 mL. Incubations were carried out at 37 °C for 10 min: the linearity of product formation was confirmed with recombinant FMO3 preparations for 30 min. The incubation was terminated by adding 0.20 mL of ice cold acetonitrile for benzydamine and methyl *p*-tolyl sulfide and by adding 50 µL of 50% phosphoric acid and 1.0 mL of ethyl acetate for sulindac sulfide. For sulindac sulfide, after centrifugation at 2,000 g for 10 min, the organic phase was transferred to a clean tube and evaporated to dryness at 40 °C. The residue was dissolved in 0.20 mL of mobile phase (50%
acetonitrile in 25 mM potassium phosphate buffer (pH 3.0)) and introduced onto the HPLC. For benzydamine and methyl p-tolyl sulfide, the aqueous supernatant was centrifuged at 2,000 g for 10 min and was subjected to HPLC equipped with an analytical C18 column (4.6 × 150 mm, 5 µm). The formation of methyl p-tolyl sulfoxide and sulindac sulfoxide was monitored at wavelengths of 237 nm (Stevens, et al., 2003) and of 360 nm (Hamman, et al., 2000), respectively. Benzydamine N-oxide was fluorometrically determined (Yeung and Rettie, 2006).

Rates of N-oxygenation of trimethylamine were determined by gas chromatography as described previously (Yamazaki, et al., 2006). The kinetic analysis of N- or S-oxygenation was done using a nonlinear regression analysis program (KaleidaGraph, Synergy Software, Reading, PA). When substrate inhibition was observed, an equation of \( v = V_{\text{max}} \cdot \frac{[S]}{(K_m + [S] + [S]^2/K_s)} \) was used instead of the Michaelis-Menten equation, and [S] and K_s were defined as substrate concentration and substrate inhibition constant, respectively.

Results and Discussion

To investigate the effects of novel FMO3 variants found in Japanese and to compare them with other FMO3 variants, recombinant wild type, Glu158Lys, Glu158Lys-Glu308Gly, Glu158Lys-Thr201Lys-Glu308Gly, Val257Met, Val257Met-Met260Val, and Arg205Cys FMO3 were expressed in E. coli membranes. Kinetic parameters for N-oxygenation of benzydamine and trimethylamine activities were determined by nonlinear regression analysis for six variant FMO3s and were compared with those of wild type FMO3 expressed in bacterial membranes (Table 1). Apparent \( V_{\text{max}} \) values of Glu158Lys-Thr201Lys-Glu308Gly and Val257Met-Met260Val FMO3 for benzydamine N-oxygenations were approximately one-tenth and one-twentieth, respectively, that of wild type enzyme, but apparent K_m values
were not different. Apparent catalytic efficiency (\(V_{\text{max}} / K_m\)) of Glu158Lys, Glu158Lys-Glu308Gly, and Arg205Cys FMO3 were slightly decreased compared with wild type FMO3, however, catalytic function of Val257Met was similar to that of the wild type FMO3. A similar series of results were obtained using trimethylamine \(N\)-oxygenation as a marker reaction catalyzed by wild type and variant FMO3 enzymes in terms of effects of amino acid substitutions (Table 1).

Kinetic parameters for \(S\)-oxygenation of methyl \(p\)-tolyl sulfide and sulindac sulfide activities were also determined and compared with those of wild type FMO3 expressed in bacterial membranes (Table 1). Apparent \(V_{\text{max}} / K_m\) values for methyl \(p\)-tolyl sulfide and sulindac sulfide \(S\)-oxygenations of Val257Met FMO3 as well as Glu158Lys, Glu158Lys-Glu308Gly, Glu158Lys-Thr201Lys-Glu308Gly, Val257Met-Met260Val, and Arg205Cys FMO3 were decreased in compared with wild type FMO3. In contrast to the \(N\)-oxygenations described above, apparent \(K_m\) and \(V_{\text{max}}\) values of Glu158Lys-Thr201Lys-Glu308Gly and Val257Met-Met260Val-FMO3 for methyl \(p\)-tolyl sulfide \(S\)-oxygenations were increased and decreased, respectively, compared with wild type FMO3. A similar series of results were obtained using sulindac sulfide \(S\)-oxygenation catalyzed by these same variants FMO3 (Table 1), except for the case of Arg205Cys FMO3, that showed a unique substrate-velocity curve that resembled substrate inhibition, as shown in Fig. 2. Because wild type FMO3 has no free cysteine residues in the native form, this inhibition might be caused by selective interaction between Arg205Cys-FMO3 and sulindac sulfide or the \(S\)-oxide, sulindac. However, because most of the reported FMO3 inhibition is generally considered due to competition with NADP/H (Cashman and Zhang, 2006), further studies are needed to interpret this interesting phenomenon. Apparent instability of the enzyme or substrate inhibition by methyl \(p\)-tolyl sulfide on its \(S\)-oxygenation was not observed under the present conditions (data not shown).
The structural organization of the human FMO3 gene has been reported (Dolphin, et al., 1997b) and because the functional FAD- and NADP+-binding domains of the human FMO3 are encoded by exon 2 and exon 5, respectively (Dolphin, et al., 1997b), it is possible that mutation(s) located near the N-terminus of FMO3 could be responsible for decreased expression of the gene and/or abnormal functional activity or instability of the encoded protein. Previously, inactive variants of FMO3 at positions in exon 2 and exon 5 have been reported (Hernandez, et al., 2003 Zhang, et al., 2003). The present results collectively suggest that novel Glu158Lys-Thr201Lys-Glu308Gly and Val257Met-Met260Val FMO3 possessed little functional activity for N- and S-oxygenations, suggesting additional changes in specific regions of exon 5 and exon 6 of the FMO3 gene was required for functional activity. Consistently, individuals harboring Val257Met-Met260Val/Arg500stop and Glu158Lys-Thr201Lys-Glu308Gly/Arg205Cys FMO3 genotypes showed low FMO3 metabolic capacity (< 40 %) as phenotyped by measuring trimethylamine and trimethylamine N-oxide excreted in urines (Shimizu, et al., 2006; Yamazaki, et al., 2006).

In genotype-phenotype studies of individuals with trimethylaminuria, the Val257Met FMO3 mutation showed no significant changes in trimethylamine N-oxygenation (Cashman, 2002; 2004; Dolphin, et al., 1997a). However, the Val257Met amino acid substitution in FMO3 could decrease the S-oxygenations of some xenobiotics while not changing the extent of N-oxygenation (Table 1). In the present study, the Arg205Cys substitution of FMO3, which was found with low allele frequency in a Japanese self-reported cohort of trimethylaminuria (Yamazaki, et al., 2006), was the most representative example for showing different effects between FMO3 substrate N- and S-oxygenations (Fig. 2 and Table 1). In conclusion, effects of genetic variation of the human FMO3 could lead to unexpected changes in catalytic efficiency of N- and S-oxygenations for typical FMO3 substrates and this
may have clinical consequences.

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References


Footnote

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Legend for figures

Fig. 1. Chemical structures of FMO3 substrates used in this study. Arrows indicate the positions of N- or S-oxygenation.

Fig. 2 Effects of substrate concentrations on the formation of sulindac sulfoxide catalyzed by wild FMO3 (○) and Arg205Cys (●) FMO3 cDNA expressed in bacteria.

Kinetic analysis was done using nonlinear regression analysis employing the Michaelis-Menten equation, \( v = \frac{V_{\text{max}} \cdot [S]}{(K_m + [S])} \), or \( v = \frac{V_{\text{max}} \cdot [S]}{(K_m + [S] + [S]^2/K_s)} \) for substrate inhibition.
Table 1

*N- and S-Oxygenation of wild type and variant FMO3 expressed in E. coli membranes*

| Variant   | Benzodamine |  | Trimethylamine |  |  |  | Methyl p-tolyl sulfide |  | Sulindac sulfide |  |
|-----------|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|           | $K_m$, µM   | $V_{max}$, min$^{-1}$ | $V_{max} / K_m$ | $K_m$, µM   | $V_{max}$, min$^{-1}$ | $V_{max} / K_m$ | $K_m$, µM   | $V_{max}$, min$^{-1}$ | $V_{max} / K_m$ |
| Wild      | 51 ± 19     | 300 ± 27        | 5.9(100)        | 47 ± 6    | 78 ± 12        | 1.7(100)        | 45 ± 9    | 180 ± 5        | 4.0(100)        |
| 158K      | 54 ± 17     | 230 ± 18        | 4.3 (73)        | 57 ± 12   | 74 ± 8    | 1.3 (77)        | 78 ± 7    | 130 ± 2        | 1.7 (42)        |
| 158K/308G | 61 ± 9      | 215 ± 62        | 3.5 (59)        | 53 ± 7    | 62 ± 4    | 1.2 (71)        | 69 ± 5    | 120 ± 2        | 1.7 (42)        |
| 158K/201K/308G | 43 ± 8 | 29 ± 1        | 0.67(11)        | 52 ± 15   | 10 ± 2 | 0.18(12)        | 120 ± 17  | 19 ± 1 | 0.16(4) | 40 ± 12 | 12 ± 4 | 0.30(14) |
| 257M      | 48 ± 11     | 290 ± 17        | 6.0(102)        | 48 ± 10   | 74 ± 8    | 1.5 (88)        | 48 ± 8    | 140 ± 4        | 2.9 (73)        |
| 257M/260V | 44 ± 11     | 15 ± 1          | 0.34 (6)        | 45 ± 15   | 7 ± 2    | 0.16 (9)        | 170 ± 59  | 11 ± 1 | 0.06 (2) | 44 ± 10 | 7 ± 4 | 0.16 (7) |
| 205C      | 44 ± 24     | 180 ± 22        | 4.1 (69)        | 47 ± 13   | 52 ± 7    | 1.1 (64)        | 55 ± 15   | 87 ± 5        | 1.6 (40)        |

Each substrate (0-1000 µM benzydamine, 0-500 µM trimethylamine, 0-1000 µM methyl p-tolyl sulfide and 0-300 µM sulindac sulfide) was incubated with recombinant FMO3 (5-50 pmols equivalent) at 37 °C for 10-30 min in the presence of an NADPH-generating system. Kinetic parameters were calculated from a fitted curve by non-linear regression (mean ± SE).

* Shows apparent substrate-inhibition with a $K_s$ value of 60 ±10 µM (mean ± SE, see Fig. 2 in detail).
Fig. 1

- **Benzydamine**
- **Trimethylamine**
- **Methyl p-tolyl sulfide**
- **Sulindac sulfide**
Fig. 2

Sulindac sulfide S-oxygenation (v, nmol product/min/nmol FMO3) vs. [S], µM

- Wild FMO3
- Arg205Cys FMO3