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Human flavin-containing monooxygenase 2.1 catalyzes oxygenation of the antitubercular drugs thiacetazone and ethionamide

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Running Title: Thiacetazone and ethionamide metabolism by FMO2.1

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Abbreviations: ETA, ethionamide; FMO, flavin-containing monooxygenase; TAZ, thiacetazone; TB, tuberculosis; GSH, glutathione.

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Abstract

The second-line antitubercular drugs thiacetazone (TAZ) and ethionamide (ETA) are bioactivated by the mycobacterial enzyme EtaA. We report here that human FMO2.1, which is expressed predominantly in the lung, catalyzes oxygenation of TAZ. The metabolites generated, the sulfenic acid, sulfinic acid and carbodiimide derivatives, are the same as those produced by EtaA and by human FMO1 and FMO3. Two of the metabolites, the sulfenic acid and carbodiimide, are known to be harmful to mammalian cells. FMO2.1 also catalyzes oxygenation of ETA, producing the *S*-oxide. We have developed a novel spectrophotometric assay for TAZ oxygenation. The assay was used to determine kinetic parameters for TAZ oxygenation catalyzed by human FMO1, FMO2.1 and FMO3 and by EtaA. Although the K_M values for the four enzyme-catalyzed reactions are similar, k_{cat} and, consequently, k_{cat}/K_M (the specificity constant) for FMO2.1-catalyzed TAZ oxygenation are much higher than those of FMO1, FMO3 or EtaA. This indicates that FMO2.1 is more effective in catalyzing TAZ oxygenation than are the other three enzymes and is thus likely to contribute substantially to the metabolism of TAZ, decreasing the availability of the prodrug to mycobacteria and producing toxic metabolites. Because of a genetic polymorphism, Europeans and Asians lack FMO2.1. However, in sub-Saharan Africa, a region in which tuberculosis is a major health problem, a substantial proportion of individuals express FMO2.1. Our results may thus explain some of the observed interindividual differences in response to TAZ and ETA and have implications for the treatment of tuberculosis in sub-Saharan Africa.

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Introduction

Pulmonary tuberculosis (TB) is a serious respiratory disease caused by the opportunistic bacterium *Mycobacterium tuberculosis*. The World Health Organization estimated 9.2 million new cases of TB infection worldwide in 2006, of which 31% were in Africa. The appearance of strains of *M. tuberculosis* that are resistant to more than one first-line antitubercular drug has required the use of second-line drugs (Peloquin, 1993) such as the thiourea thiacetazaone (TAZ, 4'-formylacetanilide thiosemicarbazone) and the thioamide ethionamide (ETA, 2-ethylpyridine-4-carbothioamide). TAZ has been widely used in the developing world (Brown, 1992). Although an effective treatment for multidrug-resistant TB, it can produce adverse effects such as liver toxicity, gastrointestinal disturbances and life-threatening skin reactions, particularly in HIV patients (Teklu, 1976; Brown, 1992; Peloquin, 1993; Ipuge et al., 1995), and, consequently, its use has been discontinued in several countries (Brown, 1992). ETA continues to be prescribed in both developed and developing countries.

Both TAZ and ETA are prodrugs that are converted to their active forms by the mycobacterial enzyme EtaA (Baulard et al., 2000; DeBarber et al., 2000; Qian and Ortiz de Montellano, 2006), a flavin-containing monooxygenase (Vannelli et al., 2002). EtaA activates TAZ by two sequential oxidation steps to form a sulfinic acid and a carbodiimide, via a postulated sulfenic acid intermediate (Qian and Ortiz de Montellano, 2006). TAZ treatment affects mycolic acid biogenesis in mycobacteria (Dover et al., 2007; Alahari et al., 2007), and this may be the mechanism by which the drug exerts its antimicrobial effect.

The flavin-contaning monooxygenases (FMOs; EC 1.14.13.8) of mammals catalyze the oxidative metabolism of numerous xenobiotics, including pesticides,

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fertilizers and therapeutic drugs (Krueger and Williams, 2005; Cashman and Zhang, 2006; Phillips et al., 2007; Phillips and Shephard, 2008). Humans express five functional FMOs, FMOs 1-5 (Phillips et al., 1995; Hernandez et al., 2004). FMO1, FMO2 and FMO3 can bioactivate thiourea-based drugs (Smith and Crespi, 2002; Henderson et al., 2004; Onderwater et al., 2006) and FMO1 and FMO3 have been shown to catalyze oxygenation of TAZ *in vitro*, forming the same products as EtaA (Qian and Ortiz de Montellano, 2006).

A genetic polymorphism of the *FMO2* gene, g.23238C>T (Q472X), gives rise to an allele, *FMO2*2*, which encodes a truncated, non-functional protein (FMO2.2) (Dolphin et al., 1998). Essentially all Europeans and Asians are homozygous for *FMO2*2* and thus do not express functional FMO2 (Dolphin et al., 1998; Whetstine et al., 2000). However, in sub-Saharan Africa, and in populations recently descended from this region, a substantial proportion of individuals possess at least one copy of the ancestral *FMO2*1* allele, which encodes a full-length functional protein (FMO2.1) (Dolphin et al., 1998; Whetstine et al., 2000; Veeramah et al., 2008). In contrast to *FMO1* and *FMO3*, which, in the adult human, are expressed primarily in kidney and liver, respectively (Dolphin et al., 1996; Yeung et al., 2000; Hernandez et al., 2004; Cashman and Zhang, 2006), the main site of expression of *FMO2* is the lung (Dolphin et al., 1998; Krueger et al., 2002; Hernandez et al., 2004; Cashman and Zhang, 2006). Expression of functional FMO2.1 has been confirmed in lung microsomes from an individual heterozygous for the *FMO2*1* allele (Krueger et al., 2002).

Because TAZ and ETA act against mycobacteria in the lung, we investigated the ability of human FMO2.1 to catalyze the oxygenation of these antitubercular drugs. In this paper, we demonstrate that the protein encoded by the *FMO2*1* allele,

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FMO2.1, catalyzes oxygenation of both TAZ and ETA, forming the same metabolites as those produced by human FMO1 and FMO3 and by the mycobacterial enzyme EtaA. Furthermore, we show that the specificity constant of FMO2.1 for TAZ is higher than that of any of the other three enzymes. Our results provide a potential explanation for some of the observed interindividual differences in the efficacy of and response to TAZ and ETA and have implications for the treatment of TB in sub-Saharan Africa and in individuals of recent African descent.

Methods

Materials. Chemical reagents, enzymes and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Plasticware for insect cell culture was obtained from VWR (West Chester, PA). Reagents for HPLC were obtained from Fischer Scientific (Hampton, NH) and were of HPLC grade.

Protein expression. Recombinant bacmids encoding full-length human FMO2 (FMO2Q472; FMO2.1) and FMO3 were as described previously (Dolphin et al., 1997; Dolphin et al., 1998). A recombinant bacmid encoding human FMO1 was prepared from a FMO1 cDNA (Dolphin et al., 1991) (accession number: Q01740) via site-specific transposition in *E. coli,* using the Bac-to-Bac system (Invitrogen, Carlsbad, CA) as described previously (Janmohamed et al., 2006). Bacmid DNAs were isolated using a modified alkaline lysis method. Production of baculovirus and expression of FMOs in *Spodoptera frugiperda* (*Sf*) 9 cells were as described previously (Janmohamed et al., 2006) except that, after infection with recombinant baculovirus, cells were cultured for 96 h before harvesting. EtaA was expressed and purified as previously described (Vannelli et al., 2002).

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Isolation of microsomal membranes. *Sf*9 cells were harvested and resuspended in HEPES buffer (0.154 M KCl, 10 mM HEPES (pH 7.4), 1 mM EDTA, 20% (v/v) glycerol). Cells were lysed by three 12-s bursts of sonication on ice. Cell lysates were centrifuged at 1000 x *g* for 10 min at 4 ˚C. The resulting supernatant was centrifuged at 100,000 x *g* for 1 h at 4˚C. The pellet was resuspended in HEPES buffer by hand, using a glass-glass homogenizer placed on ice, and stored in aliquots at -80 ˚C. Protein concentration was determined using the method of Lowry (D_C Protein Assay kit, Bio-Rad, Hercules, CA) and bovine serum albumin (Bio-Rad, Hercules, CA) as a standard.

Quantification of FMOs. Antibodies to FMO1 and FMO2 and FMO3 were a gift from Dr. R. Philpot. Heterologously expressed FMOs were quantified by western blotting essentially as described previously (Dolphin et al., 1997; Dolphin et al., 1998). Blots were incubated with goat anti-(rabbit FMO1), goat anti-(rabbit FMO2) or goat anti-(rabbit FMO3) serum (1 in 3000 dilution), then with a rabbit anti-(goat immunoglobulin G)-alkaline phosphatase conjugate (1 in 30,000 dilution). Antigen was visualized through the use of a colour development kit (AP Conjugate Substrate Kit, Bio-Rad, Hercules, CA). The concentration of each expressed FMO was determined by scanning densitometry and Image Gauge software, version 4.2.1 (Science Lab , FujiFilm, Tokyo) using a standard curve of authentic rabbit FMO1 and FMO2 or human FMO3 (which were gifts from Dr. R. Philpot). As a comparison, the relative abundance of heterologously expressed FMOs was estimated from scans of Coomassie Blue-stained SDS-polyacrylamide gels of a range of amounts of microsomal protein isolated from *Sf*9 cells infected with recombinant baculoviruses

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(data not shown). The results corresponded well with those obtained by western blotting, indicating that there was no appreciable difference in cross-reactivity among the antibodies.

Enzyme incubations with TAZ or ETA. *Sf*9 insect cell microsomes containing heterologously expressed human FMO1, FMO2.1 or FMO3 (at a final concentration of 500 nM) or purified EtaA (1 µM final concentration) were incubated with TAZ (100 µM final concentration) at 37 ˚C for 90 min in the buffer described previously (Qian and Ortiz de Montellano, 2006). Reactions were initiated by the addition of enzyme. Reactions were stopped by addition of an equal volume of ice-cold CH_3CN. Mixtures were centrifuged at 10,000 x *g* for 5 min at 4 ˚C and analysed by HPLC as described below.

*Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 were incubated with ETA (final concentration 100 µM) for 60 min as described previously (Qian and Ortiz de Montellano, 2006). Reactions were analysed by Liquid Chromatography-Mass Spectroscopy (LC-MS) as described below.

HPLC

The supernatants were diluted to a final concentration of 5% CH_3CN and then analysed by HPLC on a reverse-phase C18 column (Waters, 3.5 µm particle size, 4.6 mm i.d x 150 mm, Symmetry) employing two buffers: A, H_2O and 0.1% formic acid (FA); and B, CH_3CN and 0.1% FA. The solvent flow rate was 0.2 mL/min and the eluent was spectrophotometrically monitored using two bandwidths [330 +/- 60 nm and 260 +/- 4 nm]. The column was eluted from 0 to 25 min with a linear gradient

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from 5 to 20% buffer B. For spectral analysis of metabolites eluent peaks were monitored between 200 and 500 nm.

Liquid chromatography-mass spectroscopy (LC-MS) analyses. For analysis of TAZ metabolites LC-MS was performed as described previously (Qian and Ortiz de Montellano, 2006), except for the following modifications. The reverse-phase column was eluted with a flow rate of 0.2 mL/min (buffer A, H_2O and 0.1% FA; and buffer B, CH_3CN and 0.1% FA) with the following protocol: 0 - 16 min, 5-30% buffer B (linear gradient). The eluent was monitored at 310 nm. The mass spectrometer settings were as described previously (Qian and Ortiz de Montellano, 2006).

LC-MS analysis of ETA metabolites produced by FMO2.1 was performed as described above, with the following modifications. The column was eluted at a flow rate of 0.2 mL/min (buffer A, H_2O and 0.1% FA; and buffer B, CH_3CN and 0.1% FA) with the following protocol: 0-15 min with 1% buffer B (isocratic). The eluent was monitored at 350 nm.

Determination of kinetic parameters by spectrophotometric analysis. The molar extinction coefficient of TAZ was determined as follows. Two cuvettes containing 1 mL Tris-HCl (pH 8.5), 1mM EDTA were placed in a Varian Cary 100 dual-beam spectrophotometer (Palo Alto, CA). The spectrophotometer was set to blank correction mode. TAZ (in DMSO) was added to the sample cuvette and DMSO to the reference cuvette. The final organic solvent concentration in each cuvette was held at 0.1% (v/v). Using the Varian Scan application, samples were scanned from 200 to 500 nm over a range of TAZ concentrations between 1 and 20 μM. These measurements were carried out in triplicate using three independently prepared stock solutions of

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TAZ (20 µM) that were diluted accordingly in DMSO. The absorbance of TAZ, measured at its λ_{max} (328 nm) was plotted against concentration. The molar extinction coefficient of TAZ in DMSO was determined from the gradient of this graph and the Beer-Lambert equation.

Enzyme-catalyzed oxidation of TAZ was monitored by measuring the rate of decrease in the absorbance of TAZ at 328 nm, using a dual-beam spectrophotometer (Varian Cary 100 (kinetics module)). The pH optima for human FMOs and EtaA were determined using the following reaction buffers: 0.1 mM potassium phosphate (pH 7.5), 1 mM EDTA; 0.1 M Tris-HCl (pH 8.5) 1mM EDTA and 0.1 M Tricine-OH (pH 9.5), 1 mM EDTA. Immediately before use buffers were aerated at 37 ˚C for 30 min in a shaking water bath. Assays were performed in a volume of 1 mL in reaction buffer containing 0.1 mM NADPH and either *Sf*9 cell microsomes containing human FMO1 (320 nM), FMO2.1 (5 nM) or FMO3 (230 nM), or purified EtaA (1 µM). Reaction mixtures were allowed to equilibrate for 1 min at 37 ˚C. Reactions were initiated by the addition of TAZ in DMSO (to a final TAZ concentration of 10 µM) to the sample cuvette and DMSO to the reference cuvette. Initial rates were recorded between 1 and 5 min.

For determination of kinetic parameters assays were performed on triplicate preparations of enzymes at concentrations of TAZ ranging from 1 to 20 µM. The final organic solvent concentration was held at 0.1% (v/v). Assays were carried out in triplicate as described above in the optimum pH buffer for each enzyme: pH8.5 for FMO1 and FMO3 and 9.5 for FMO2.1 and EtaA.

K_M and *Vmax* values were determined from vi versus [S]o data using non-linear regression and the kinetics module 3.1 of Sigmaplot version 10. For calculation of k_{cat} values enzyme concentration was determined as described above.

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Determination of kinetic parameters by HPLC analysis. *Sf*9 cell microsomes containing heterologously expressed human FMO2.1 were incubated in 0.1 M Tricine-OH (pH 9.5), 1 mM EDTA, 0.1 mM NADPH and TAZ (concentrations ranged from 1 - 50 μM in DMSO). A duplicate set of samples was prepared but without the addition of enzyme. The final organic solvent concentration was held at 0.1% (v/v). Mixtures were incubated at 37 ˚C for 5 min and reactions were quenched with an equal volume of ice-cold CH_3CN. ETA was added as an internal standard at a final concentration of 100 μM (in DMSO) and mixtures were prepared for HPLC analysis as described above. A standard curve was generated by plotting the ratio of the integrated HPLC peak areas of TAZ and ETA (from the sample set without added enzyme), against the range of TAZ concentrations used. The ratio of the integrated HPLC peak areas of TAZ and ETA, in the sample set with added enzyme, was calculated and the amount of unmetabolized TAZ determined from the standard curve. This value was subtracted from the input concentration of TAZ to calculate the amount of TAZ metabolized by the enzyme. V_{max}, K_M and k_{cat} were determined as above.

Enzyme incubations with methimazole. *Sf*9 insect cell microsomes containing heterologously expressed human FMO1, FMO2.1 or FMO3 were assayed for activity towards methimazole by the method of Dixit and Roche (Dixit and Roche, 1984) as described previously (Dolphin et al., 1998). Assays for FMO1 and FMO3 activity were carried out at pH 8.5 and those for FMO2.1 at pH 9.5.

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Results

Catalytic oxidation of TAZ by human FMO2.1. Incubation of TAZ with *Sf9* insect cell microsomes containing heterologously expressed human FMO2.1, in the presence of NADPH, resulted in the formation of three major metabolites with reverse-phase retention times of 7.5 min (**M1**), 11.4 min (**M2**) and 14.6 min (**M3**) (Fig. 1). No products were observed when TAZ was incubated with microsomes isolated from non-infected *Sf9* cells or when NADPH was omitted (results not shown). UV spectral analysis of the metabolites (Fig. 2) showed that **M1** had a maximal absorption peak at 325 nm and a smaller peak at approximately 230 nm. **M2** had a similar spectrum, with peaks at 320 and 220 nm. The absorption spectrum of **M3** exhibited a main peak at 295 nm and a secondary peak at 220 nm.

To identify the three metabolites, **M1**, **M2**, and **M3,** formed from TAZ by the action of human FMO2.1, they were analysed by LC-MS. The mass spectrum of **M1** had a molecular ion $[M + H]^+$ at *m/z* 269.07, with fragment ions at *m/z* 205.14 and 163.12 (Fig. 3a). The mass of the molecular ion of **M1** is 32 atomic mass units more than that of the molecular ion of TAZ (237), suggesting a structure in which TAZ has incorporated two oxygen atoms (Fig. 3a) and hence supports identification of the metabolite as the sulfinic acid derivative. The mass spectrum of **M2** had a molecular ion $[M + H]^+$ at *m/z* 253, with fragment ions at *m/z* 235 and 193 (Fig. 3b). The mass of the molecular ion of **M2** is in accord with a structure in which TAZ has incorporated a single oxygen atom, and thus supports identification of **M2** as the monooxygenated, sulfenic acid derivative. The metabolite **M3** has a molecular ion $[M + H]^+$ at *m/z* 203.13, which suggests that it is the carbodiimide generated by elimination from **M2** of the oxidized sulfur atom (Fig. 3c). Previously synthesized authentic standards of TAZ – sulfinic acid and TAZ – carbodiimide (Qian and Ortiz

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de Montellano, 2006) were used to confirm the identity of **M1** and **M3**. Comparison of their HPLC elution times, UV spectra and MS spectra with those of the synthetic standards unambiguously identified the metabolites **M1** and **M3** as the sulfinic acid and carbodiimide derivatives of TAZ, respectively.

The same three metabolites (the sulfinic acid, sulfenic acid and carbodiimide derivatives) were also produced when TAZ was incubated with purified EtaA or with *Sf9* cell microsomes containing heterologously expressed human FMO1 or FMO3 (data not shown). Previous work identified **M1** and **M3** as products formed by the action of these enzymes on TAZ (Qian and Ortiz de Montellano, 2006). Although the sulfenic acid derivative (**M2)** was not detected, it was postulated as an intermediate in the enzyme-catalyzed metabolism of TAZ (Qian and Ortiz de Montellano, 2006). To confirm this, incubations of TAZ with heterologously expressed human FMO2.1 were quenched at different time points (Fig. 4). It is clear from the results that formation and accumulation of **M2** precedes that of **M1** and **M3**, indicating that **M2** is an intermediate in the formation of the latter two metabolites.

Catalytic oxidation of ETA by human FMO2.1. Incubation of ETA with *Sf9* microsomes containing heterologously expressed human FMO2.1, in the presence of NADPH, resulted in the formation of a major product, **P1**, with LC retention time of 6.2 min (Fig. 5a). The mass spectrum of **P1** had a molecular ion $[M + H]^+$ at *m/z* 183, with fragment ions at *m/z* 151 and 133 (Fig. 5b). The mass of the molecular ion of **P1** is 16 atomic mass units more than that of the molecular ion of ETA (166), suggesting a structure in which ETA has incorporated one oxygen atom (Fig. 5b). This supports identification of **P1** as the *S*-oxide of ETA. The retention time and mass spectrum of P1 are identical to those of authentic ETA *S*-oxide (Vannelli et al., 2002), therefore

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unambiguously identifying this metabolite as the *S*-oxide of ETA. A mass spectrum of the broad peak with a LC retention time of approx. 4 min (Fig. 5a) could not be obtained.

Development of a spectrophotometric assay of TAZ oxidation. A UV absorption spectrum of TAZ revealed an absorption maximum at 328 nm and a smaller peak at approx. 220 nm (data not shown). The absorbance of TAZ at 328 nm was linear between 1 and 20 µM (data not shown) and at this wavelength the molar extinction coefficient of TAZ in DMSO was determined as 38,300 ± 2,320 $M^{-1}cm^{-1}$. Incubation of TAZ, in the presence of NADPH, with *Sf*9 cell microsomes containing heterologously expressed human FMO1, FMO2.1 or FMO3, or with purified EtaA, resulted in a decrease in TAZ absorbance at 328 nm that was linear over time (Fig. 6 and data not shown) and with respect to enzyme concentration. No decrease in TAZ absorbance was observed in the absence of EtaA or human FMOs, or when microsomes prepared from non-infected *Sf*9 cells were used. Omission of NADPH from reaction mixtures containing heterologously expressed human FMOs resulted in a very small (<1% of that observed in the presence of NADPH) and short-lived (<2 min.) decrease in TAZ absorbance. This is due to the presence of endogenous NADPH in the insect cell microsomes. This spectrophotometric assay was used to determine the pH optima and kinetic parameters of enzyme-catalyzed oxidation of TAZ.

Effect of pH on TAZ oxidation catalyzed by human FMOs and EtaA. TAZ was incubated with purified EtaA or with heterologously expressed human FMO1, FMO2.1 or FMO3 in buffers of pH 7.5, 8.5 or 9.5. Oxidation of TAZ was measured

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spectrophotometrically. The pH optimum for the oxidation of TAZ at a concentration of 10µM was 8.5 for human FMO1 and FMO3, and 9.5 for human FMO2.1 and EtaA (Fig. 7).

Kinetics of TAZ oxidation by human FMOs and EtaA. The kinetics of TAZ oxidation catalyzed by EtaA or by heterologously expressed human FMO1, FMO2.1 or FMO3 were evaluated by determining the initial rates of TAZ oxidation, measured spectrophotometrically, over a range of TAZ concentrations (Fig. 8, and data not shown). Assays were performed at the optimum pH determined for each enzyme as described above. Steady-state kinetic parameters were determined from vi versus [S] data by non-linear regression.

The K_M values for TAZ oxidation catalyzed by human FMO1, FMO2.1 and FMO3 are very similar to each other and slightly lower than that of the EtaA-catalyzed reaction (Table 1). The k_{cat} of the FMO2.1-catalyzed reaction is much higher than that of reactions catalyzed by human FMO1, FMO3 or EtaA (Table 1). Consequently, k_{cat}/K_M (the specificity constant) for TAZ is much higher for human FMO2.1 than for the other three enzymes (Table 1). Very similar kinetic parameters for FMO2.1-catalyzed TAZ oxygenation were obtained through the use of an HPLC-based assay, thus validating the spectrophotometric assay (data not shown).

As a comparison, we determined kinetic parameters for FMO1-, FMO2.1- and FMO3-catalyzed oxygenation of methimazole, a prototypic substrate for FMOs (Table 1). The k_{cat} of each enzyme was similar for TAZ and methimazole. However, for FMO2.1 the K_M for methimazole was 100-fold greater than that for TAZ. Consequently, for methimazole oxygenation, the k_{cat}/K_M of FMO2.1 is considerably less than that of FMO1 and slightly less than that of FMO3.

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Discussion

Our results demonstrate that full-length, functional FMO2 of human (FMO2.1) catalyzes oxygenation of the second-line thiourea antitubercular drug TAZ *in vitro*. The metabolites generated, the sulfenic acid, sulfinic acid and carbodiiimide derivatives, are the same as those produced by the action of human FMO1, human FMO3 or the mycobacterial enzyme EtaA. The sulfinic acid and carbodiimide derivatives have been identified previously as products of TAZ oxygenation catalyzed by FMO1, FMO3 or EtaA (Qian and Ortiz de Montellano, 2006). Although these authors did not detect the sulfenic acid derivative, it was postulated to be an intermediate. Our results confirm that, in reactions catalyzed by all three of the human FMOs and by EtaA, TAZ sulfenic acid is indeed an intermediate in the formation of the sulfinic acid and carbodiimide metabolites.

Human FMO2.1 also catalyzes the oxygenation of ETA, a thioamide second-line antitubercular. The single metabolite identified, the *S*-oxide, has been shown to be the product of EtaA-catalyzed metabolism of the drug (Vannelli et al., 2002). However, the second product identified by these authors, the amide of ETA, was not detected in our study.

Our kinetic analyses reveal that k_{cat}/K_M (the specificity constant) for FMO2.1-catalyzed TAZ oxygenation is much higher than that of FMO1, FMO3 or the mycobacterial enzyme EtaA, indicating that FMO2.1 is more effective in catalyzing TAZ oxygenation than are the other three enzymes. In contrast, FMO2.1 is less effective than FMO1 or FMO3 in catalyzing the oxygenation of methimazole. Although kinetic analyses were done at the pH optimum for each enzyme, even at a more physiological pH (7.5), as shown in Fig. 7, FMO2.1 is the most effective of the

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enzymes studied in catalyzing TAZ oxygenation. The higher value of the specificity constant of FMO2.1 for TAZ is a consequence of the higher k_{cat} value rather than a lower K_M value, which is similar to those of the other enzymes with lower k_{cat} values (Table 1). Similar high k_{cat} values have been reported for human FMO2.1-catalyzed oxygenation of other thioureas, including thiourea and ethylene-thiourea (Henderson et al., 2004). The relatively low k_{cat} values determined for TAZ oxygenation catalyzed by FMO1 and FMO3 are comparable to those reported for oxygenation of a panel of thioureas by these enzymes (Onderwater et al., 2006). The high values of K_M and k_{cat} determined for FMO2.1-catalyzed oxygenation of methimazole are similar to the values reported for purified or heterologously expressed FMO2 of rabbit (Lawton et al., 1991).

Spectrophotometric and kinetic studies indicate that the rate-limiting step for FMO-catalyzed reactions occurs after substrate oxygenation (reviewed in Ziegler, 2002) and thus is independent of oxidizable substrate. Consequently, for a particular FMO, the value of k_{cat} would be expected to be similar for all its substrates. Although our results conform to this expectation, they indicate that the k_{cat} of human FMO2.1 is higher than that of FMO1 or FMO3, suggesting that FMO2.1 is more effective in catalyzing the rate-limiting step of the reaction, the elimination of H_2O, than is either of the other two FMOs investigated.

Substrate oxygenation by FMOs is usually a detoxification process. However, in the case of thioureas the products of FMO-catalyzed oxygenation are typically more toxic than the parent compound (Smith and Crespi, 2002; Henderson et al., 2004; Onderwater et al., 2004, 2006). TAZ sulfenic acid is an electrophile that can react with glutathione (GSH) (Qian and Ortiz de Montellano, 2006) to regenerate the parent compound and convert GSH to its oxidized form (GSSG). In the presence of

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GSH reductase, a redox cycle may be established that depletes GSH, thus causing oxidative stress and cellular injury (Krieter et al., 1984; Henderson et al., 2004; Onderwater et al., 2004). Sulfenic acid metabolites of thioureas can react covalently with other thiol-containing molecules, such as cysteine residues in proteins, and thus directly perturb protein function (Decker and Doerge, 1992). The TAZ carbodiimide, too, has the potential to form covalent products with cysteine residues in proteins. Thus, each of the human FMOs that we have investigated, FMO1, FMO2.1, FMO3, is able to catalyze oxygenation of TAZ, producing metabolites that are known to be harmful to mammalian cells.

The FMO-catalyzed production of TAZ metabolites that are known to be harmful to mammalian cells may be the basis for the adverse clinical reactions associated with this drug (Teklu, 1976; Brown, 1992; Peloquin, 1993; Ipuge et al., 1995). This is supported by the fact that all of the adverse effects associated with TAZ occur in tissues in which FMOs are expressed: hepatotoxicity, FMO3 in the liver (Dolphin et al., 1996; Hernandez et al., 2004); gastrointestinal problems, FMO1 in the small intestine (Yeung et al., 2000); and skin rashes, FMO1 and FMO3 in skin (Janmohamed et al., 2001).

Human FMO1 and FMO3 are expressed primarily in the kidney and liver, respectively, and thus may contribute to the extra-pulmonary metabolism of TAZ. FMO1 displays interindividual variation in its expression (Yeung et al., 2000; Koukouritaki et al., 2002) and several nonsynonymous polymorphic variants of FMO3 have been identified (reviewed in Phillips et al., 2007; Phillips and Shephard, 2008)) one of which, L360P, increases enzyme activity (Lattard et al., 2003), whereas others, such as E158K and E308G, when present together in *cis*, decrease enzyme activity (reviewed in Phillips et al., 2007; Phillips and Shephard, 2008). Promoter

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variants that affect transcription have also been identified (Koukouritaki et al., 2005). Therefore individuals who have lower expression or activity of FMO1 or FMO3 would be less able to metabolise TAZ in extra-pulmonary tissues, whereas those with higher expression or activity would metabolize TAZ more effectively, thus reducing the amount of prodrug reaching the lung and increasing the amounts of metabolites toxic to the host. Polymorphic variants in *FMO3* have been shown to influence the metabolism and therapeutic outcomes of drugs such as benzydamine and sulindac (Stormer et al., 2000; Hisamuddin et al., 2004).

TAZ and ETA are prodrugs that are converted to their active forms in mycobacteria. Therefore, for treatment to be effective, a sufficient amount of unmetabolized drug must reach mycobacteria in the lung. Although Europeans and Asians lack functional FMO2, a substantial proportion of sub-Saharan Africans and individuals of recent African descent possess an ancestral *FMO2*1* allele (Whetstine et al., 2000; Veeramah et al., 2008) and would thus be expected to express functional FMO2 in the lung (Krueger et al., 2002). The relatively high specificity constant for FMO2.1-catalyzed TAZ oxygenation suggests that, when present in lung, FMO2.1 is likely to contribute substantially to the metabolism of TAZ in this tissue, thus decreasing the availability of the prodrug to mycobacteria and producing metabolites toxic to the host. Therefore patients with multidrug-resistant TB who express FMO2.1 may respond less well to treatment with second-line antitubercular drugs such as TAZ and ETA, and may be more prone to adverse clinical reactions to these drugs. The relatively high frequency of the *FMO2*1* allele in sub-Saharan Africa, a region in which TB is a major health problem, has implications for the efficacy of and response to antitubercular drugs, such as TAZ and ETA, that are substrates for FMO2.1.

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Footnote

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Figure Legends

Figure 1. UV-HPLC chromatogram of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH. Reactions were carried out at pH 9.5 for 90 min at 37 °C.

Figure 2. UV-absorption spectra of the three metabolites produced from the incubation of TAZ with heterologously expressed human FMO2.1 and NADPH. Dotted line, **M1**; solid line **M2**; and dashed line, **M3**.

Figure 3. Mass spectra and structures of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH in 100 mM tricine buffer, pH 9.5, for 90 min at 37 °C. (A) **M1,** identified as the sulfinic acid, has a molecular ion [M + H]+ = *m/z* 269.07, with fragment ions at *m/z* 205.14 and 163.12. (B) **M2**, identified as the sulfenic acid, has a molecular ion $[M + H]^+$ = *m/z* 253, with fragment ions at *m/z* 235 and 193, and (C) **M3**, identified as the carbodiimide, has a molecular ion [M + H]+ = *m/z* 203.13, with a fragment ion at *m/z* 161.11.

Figure 4. UV-HPLC chromatograms of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH. Reactions were carried out at pH 9.5 and 37 °C for 0 min (A), 10 min (B) or 20 min (C).

Figure 5. Analysis of the products from incubation of ETA with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH in 100

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mM potassium phosphate buffer (pH 7.5) for 60 min at 37 °C. (A) LC chromatogram (350 nm). (B) The mass spectrum and structure of the product **P1**. **P1** has a molecular ion $[M + H]^+$ at *m/z* 183, with fragment ions at *m/z* 151 and 133.

Figure 6. Linearity of FMO2.1- and NADPH-dependent decrease in TAZ absorbance over time. TAZ (20 µM) was incubated with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH in tricine buffer (pH 9.5) at 37 °C, and absorbance at 328 nm was monitored over time.

Figure 7. Effect of pH on the rate of TAZ oxygenation catalyzed by purified EtaA or by heterologously expressed human FMO1, FMO2.1 or FMO3. The initial concentration of TAZ was 10 µM. The decrease in TAZ concentration over time was measured at 328 nm in the presence of NADPH in buffers at pH 7.5, 8.5 or 9.5.

Figure 8. (A) Michaelis-Menten plot of TAZ oxygenation catalyzed by heterologously expressed human FMO2.1 in the presence of NADPH at pH 9.5. (B) Linear transform of Michaelis-Menten data using Hanes-Woolf regression (r2 = 0.961).

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Table 1. Kinetic parameters of enzyme-catalyzed oxygenation of TAZ and methimazole

	thiacetazone			**methimazole**		
Enzyme	K_M (μM)	*k*cat (min^{-1})	*k*cat/ K_M ($min^{-1}M^{-1}$) ($x10^5$)	K_M (μM)	*k*cat (min^{-1})	*k*cat/ K_M ($min^{-1}M^{-1}$) ($x10^4$)
FMO1	6.30 ± 0.80	5.08 ± 0.46	7.94 ± 1.99	8.08 ± 2.35	2.27 ± 0.30	28.11 ± 7.95
FMO2.1	5.80 ± 0.55	80.10 ± 4.42	142.56 ± 18.30	575.75 ± 60.02	31.50 ± 2.11	5.48 ± 0.68
FMO3	7.01 ± 0.53	1.37 ± 0.20	1.96 ± 0.39	29.30 ± 4.06	2.60 ± 0.38	8.97 ± 1.81
EtaA	9.05 ± 0.57	3.02 ± 0.30	3.29 ± 0.81	nd	nd	nd

TAZ assays were carried out in triplicate on batches of microsomes isolated from three independent infections of *Sf*9 cells (i.e., 9 measurements per FMO). Methimazole assays were carried out in triplicate on a single batch of microsomes for each heterologously expressed human FMO. EtaA assays were carried out in triplicate on purified protein. Kinetic parameters are reported as mean ± standard error; nd, not determined.

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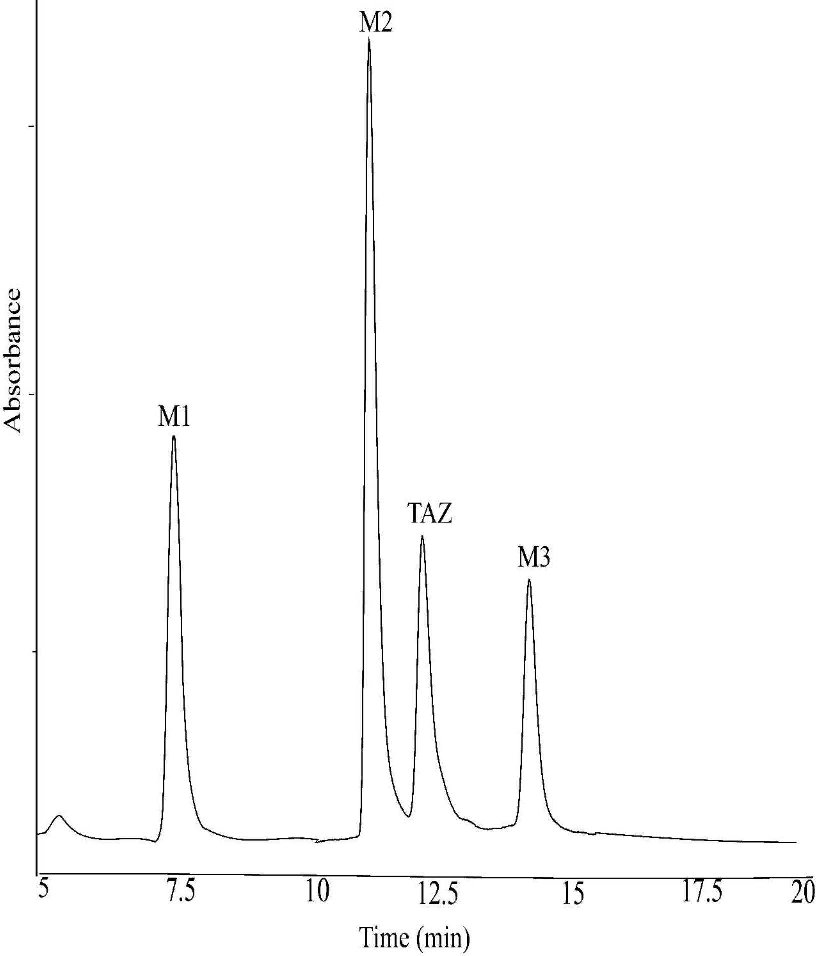
FIGURE 1



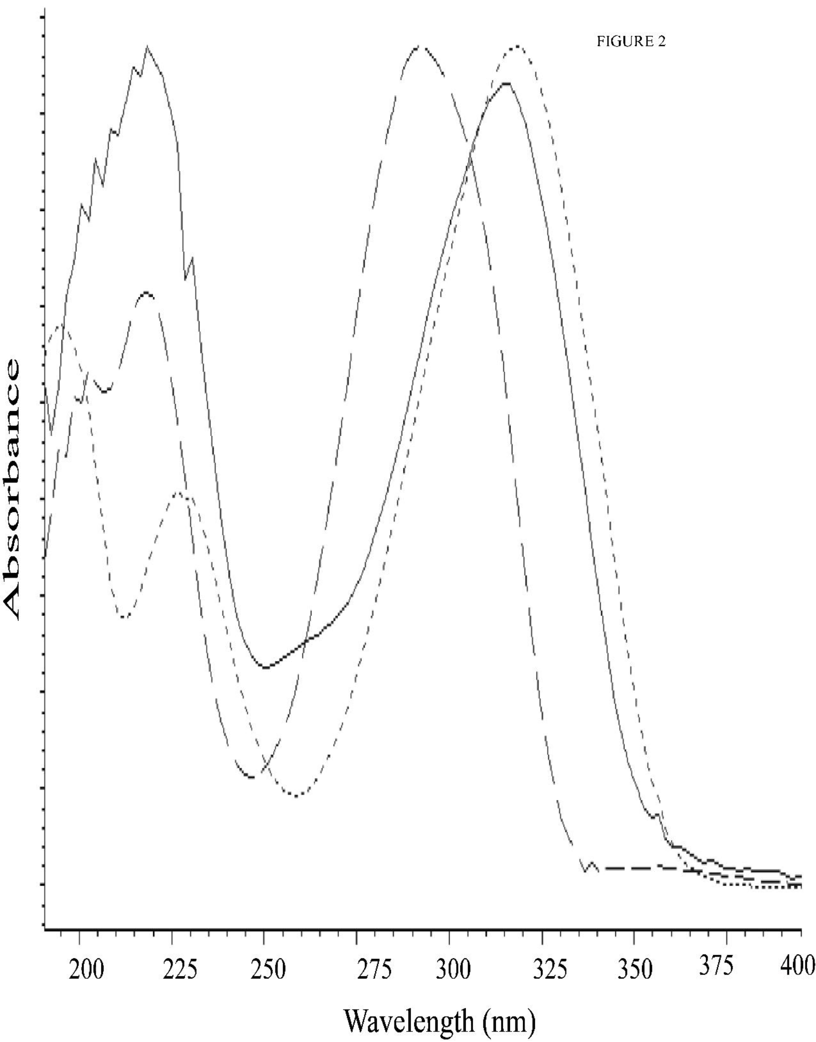


FIGURE 2
Absorbance
200
225
250
275
300
325
350
375
400
Wavelength (nm)

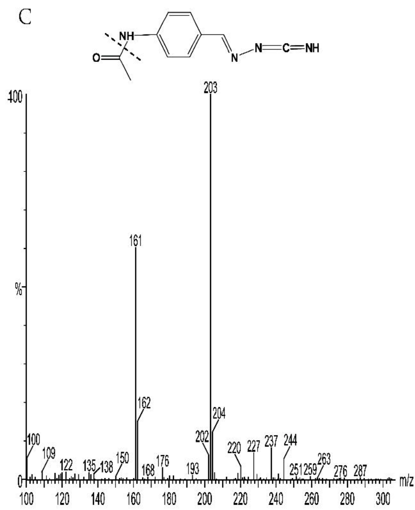


FIGURE 3

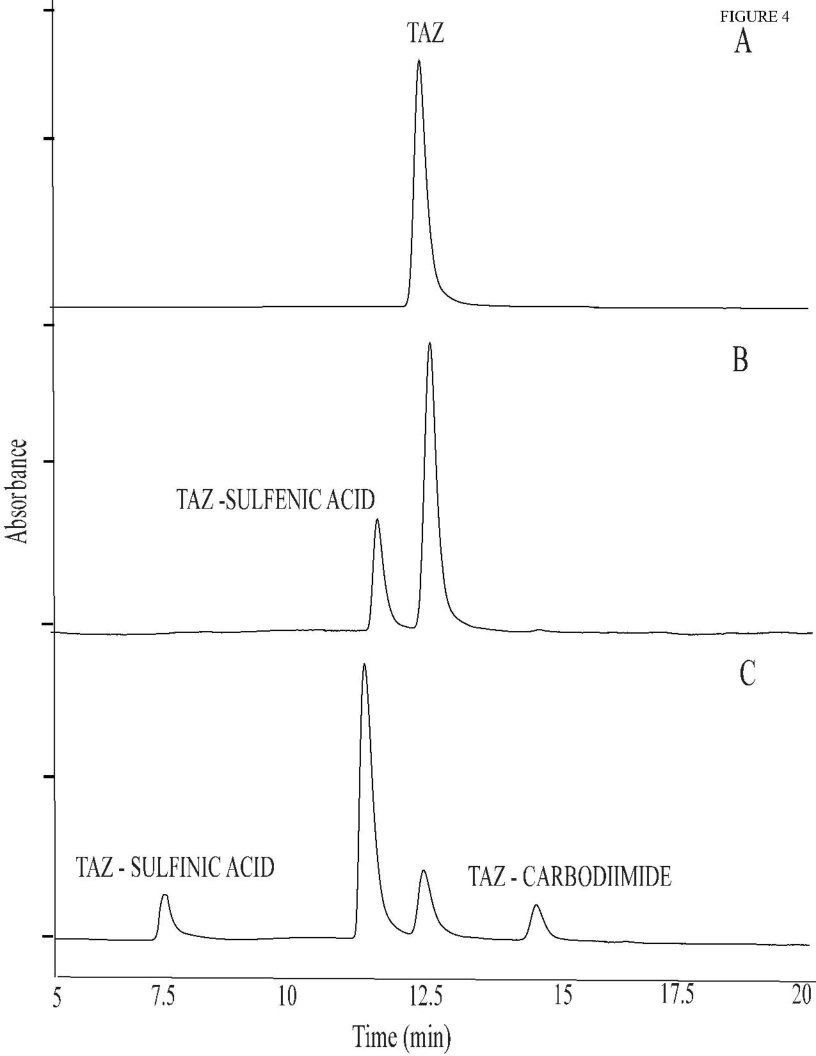


FIGURE 4
TAZ
A
B
TAZ -SULFENIC ACID
C
TAZ - SULFINIC ACID
TAZ - CARBODIIMIDE
Absorbance
5
7.5
10
12.5
15
17.5
20
Time (min)

FIGURE 5

A

100

%

0

P1

ETA

3 4 5 6 7 8

Column Retention Time (min)

B

100

%

0

183

151

184

133

134

152

185

56 69 73 85 88 97 107 115 118 139 167 168 186 205 224 229 241 245 257 269 280 287

m/z

60 80 100 120 140 160 180 200 220 240 260 280 300

^{-}O S^{+} NH_2

N CH_2CH_3

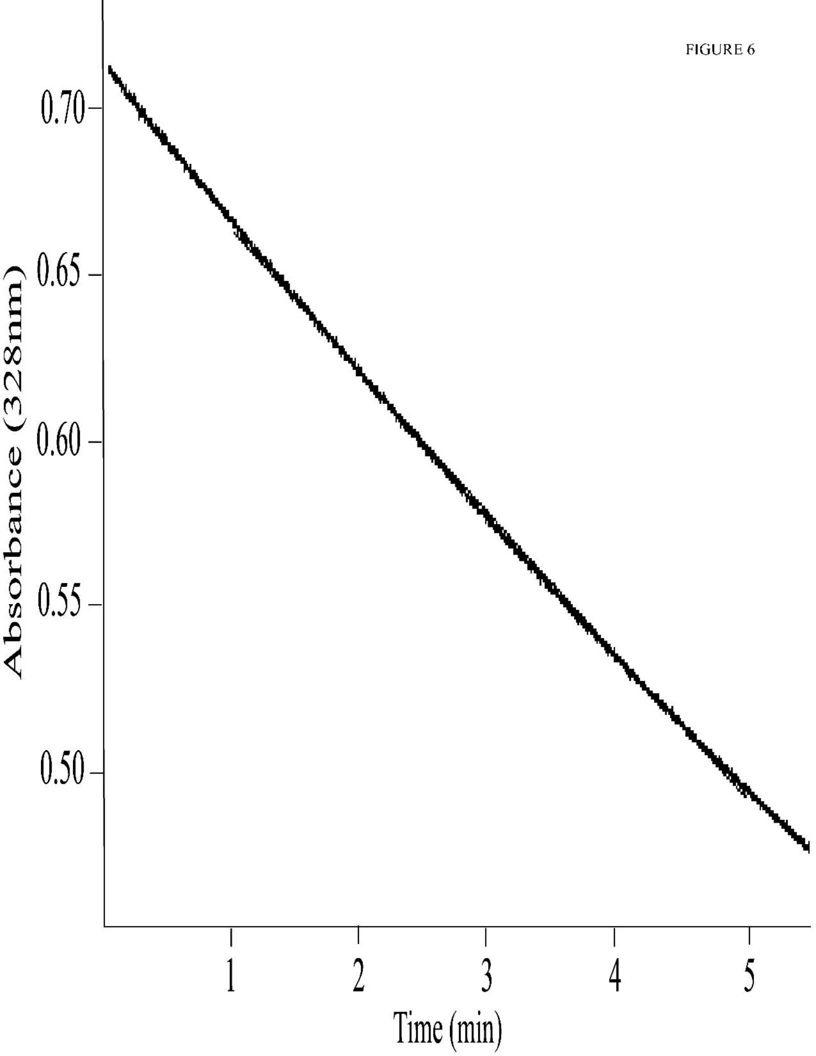


FIGURE 6
Absorbance (328nm)
0.70
0.65
0.60
0.55
0.50
1
2
3
4
5
Time (min)

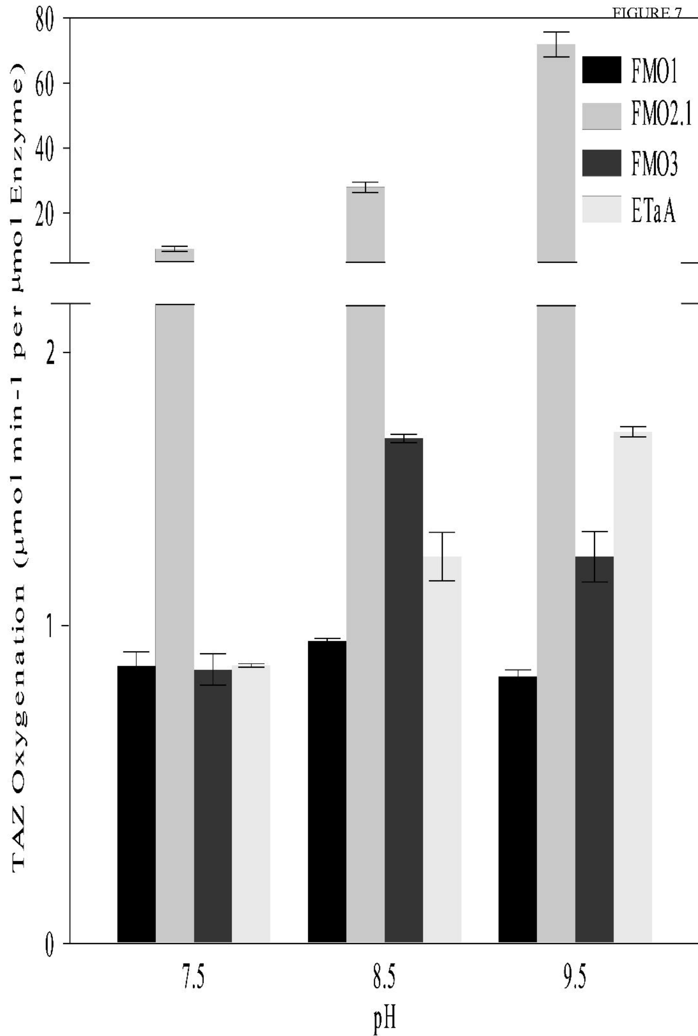


FIGURE 7
TAZ Oxygenation (μmol min-1 per μmol Enzyme)
80
60
40
20
2
1
0
FMO1
FMO2.1
FMO3
ETaA
7.5
8.5
9.5
pH

A

FIGURE 8

TAZ OXYGENATION (μM/min) (x10-7)

0
1
2
3
4

0 5 10 15 20 25

[TAZ] (μM)

B

[TAZ] (μM)/TAZ OXYGENATION (μM/min) (x10^7)

2
4
6

-10 -5 0 5 10 15 20

[TAZ] (μM)