Formation of a Quinoneimine Intermediate of 4-Fluoro-*N*-methylaniline by FMO1: Carbon Oxidation Plus Defluorination

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Here, we report on the mechanism by which flavin-containing monooxygenase 1 (FMO1) mediates the formation of a reactive intermediate of 4-fluoro-*N*-methylaniline. FMO1 catalyzed a carbon oxidation reaction coupled with defluorination that led to the formation of 4-*N*-methylaminophenol, which was a reaction first reported by Boersma et al. (Boersma et al. (1993) *Drug Metab. Dispos. 21*, 218−230). We propose that a labile 1-fluoro-4-(methylimino)cyclohexa-2,5-dienol intermediate was formed leading to an electrophilic quinoneimine intermediate. The identification of *N*-acetylcysteine adducts by LC-MS/MS and NMR further supports the formation of a quinoneimine intermediate. Incubations containing stable labeled oxygen ($H_2{}^{18}O$ or $^{18}O_2$) and ab initio calculations were performed to support the proposed reaction mechanism.

4-Hydroxylanilines and analogues are known to convert to quinoneimine intermediates, either chemically or enzymatically (*1*), that can lead to organ toxicity. For example, amodiaquine undergoes bioactivation to an electrophilic quinoneimine metabolite that results in oxidative stress or conjugation to cysteinyl sulfydryl groups on proteins that elicits toxicity by either cytotoxic or immunological mechanisms (*2*−*5*). In the clinical setting, the use of amodiaquine has been limited due to severe adverse reactions, including hepatotoxicity (*6*−*10*). Replacing the *para* hydroxyl group in the case of amodiaquine with a fluorine atom diminishes such toxicity (*2, 11*). The *p*-fluoroaniline analogue of amodiaquine blocks the oxidation to the reactive quinoneimine that results in the depletion of intracellular glutathione and covalent binding to proteins (*11, 12*). The rationales are that the *p*-fluoro substitution decreased the electron density in the benzene ring and that the fluoro group could block the metabolically labile site at the *para* position (*13*). There are several reports where the *para* substituted fluorine in aniline containing compounds are oxidized to form a phenol, and it is known that these reactions involve cytochrome P450 or peroxidases (*14, 15*). Boersma et al. (*16*) demonstrated that in the case of 4-fluoro-*N*-methylaniline (**1**), FMO also catalyzes this reaction to form 4-(methylamino)phenol (**2**) in purified rat liver FMO. The focus of this work was to understand the underlying mechanism of the oxidative defluorination of **1** by FMO.

Incubation of **1** (1 mM) with rat liver microsomes (0.5 mg/mL in 0.5 mL of 100 mM potassium phosphate buffer) and NADPH (1 mM) led to the formation of **2** in an NADPH- and time-dependent manner. In the presence of an inactivator of cytochrome P450 (preincubation with rat liver microsomes, NADPH, and 1 mM 1-aminobenzotriazole (ABT) for 15 min) or FMO inhibitor (coincubation with 0.1 mM methimazole), only methimazole inhibited the formation of **2**, which indicated that FMO was the major enzyme system responsible for the formation of this metabolite in rat liver microsomes. The identification of **2** as 4-(methylamino)phenol was confirmed by comparing the LC retention time (3.4 min), the product ion fragmentation pattern (*m*/*z* 53, 80, 109), and high resolution mass spectra ($[MH]^+ = 124.07535$ within 3 ppm of the exact mass of $C_7H_{10}NO^+$) to the authentic standard.

Furthermore, incubations with recombinant human FMO (rFMO) enzymes were performed. Of the three isoforms tested (rFMO1, 3, and 5), only rFMO1 was responsible for the formation of **2**. In incubations with rFMO1 (0.5 mg/mL), NADPH, and **1**, high resolution LC-MS was used to identify major metabolites, which included 4-(methylamino)phenol, 4-aminophenol, *N*-(4-fluorophenyl)-*N*-methylhydroxylamine, *N*-(4-fluorophenyl)hydroxylamine, and 4-fluoroaniline. Compound **2** was the major metabolite on the basis of LC-MS peak area, and its formation rate was calculated to be 1.34 nmol/min/mg (20.1 μM in 30 min) using a standard curve for its quantification. Although Boersma et al. used purified rat liver FMO (*16*) and we used recombinant human FMO1, the same metabolites were identified and presented in Figure 1.

To further understand the mechanism leading to the oxidative defluorination of **1** mediated by FMO1, we investigated the source of the oxygen atom that was incorporated in **2** by using either stable labeled $H_2{}^{18}O$ or $^{18}O_2$ in the incubation. In the reaction mixtures containing $^{18}O_2$, rFMO1, NADPH, and **1**, the incorporation of ^{18}O in **2** was observed. The incorporation of ^{18}O was calculated to be 91% when compared to the amount of ^{18}O incorporated into the *N*-hydroxylation of **1** in the same incubation, which was a known FMO catalyzed reaction (*16*). The incorporation of ^{18}O into **2** was confirmed by high resolution mass spectra which had an exact mass of *m*/*z* 126.07950 (within 4 ppm of $C_7H_{10}N^{18}O^+$). In the reaction mixtures containing $H_2{}^{18}O$, rFMO1, NADPH, and **1**, no ^{18}O labeled **2** was detected.

Additional incubations were performed to ensure that the oxidative defluorination was not formed due to a side reaction or a nonenzymatic process such as release of superoxide and hydrogen peroxide which FMO has been shown to produce (*17*). To assess this, incubations (**1** (1 mM), rFMO1 (0.5 mg/mL), and NADPH (1 mM)) were performed in the presence of superoxide dismutase (500 units) or catalase (2000 units),

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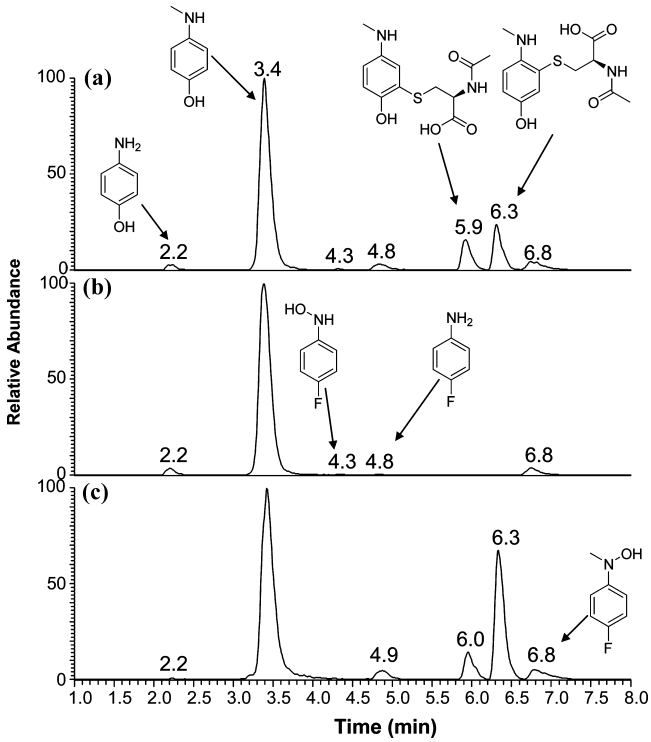
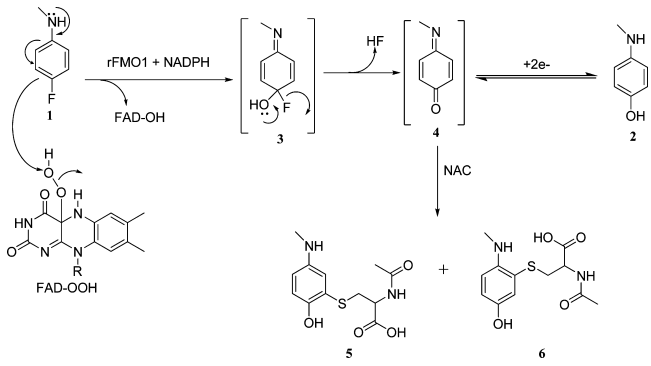


Figure 1. Composite extracted ion chromatograms of the metabolites of **1** incubated with rFMO after 30 min. (a) (+) NADPH and (+) NAC, (b) (+) NADPH and (−)NAC, and (c) (+) NADPH, (+) NAC, and superoxide dismutase (500 units). The metabolites include 4-aminophenol (*m/z* 110.06004, *RT* = 2.2), 4-(methylamino)phenol (*m/z* 124.07569, *RT* = 3.4), *N*-(4-fluorophenyl)hydroxylamine (*m/z* 128.05062, *RT* = 4.3), 4-fluoroaniline (*m/z* 112.05570, *RT* = 4.8), *N*-(4-fluorophenyl)-*N*-methylhydroxylamine (*m/z* 142.06627, *RT* = 6.8), and *N*-acetylcysteine conjugates (*m/z* 285.09035, *RT* = 5.9 and 6.3).

Scheme 1



respectively. Under these conditions, the formation of **2** was not inhibited, which further supports the fact that the oxidative defluorination was an enzymatic process.

The FMO-mediated reaction to form 4-(methylamino)phenol is notable because it includes carbon oxidation and defluorination as well as the formation of a reactive quinoneimine intermediate. FMO enzymes are well documented as catalyzing the oxidation of soft nucleophilic heteroatoms such as sulfur and nitrogen (*18*). In the catalytic mechanism of the enzyme, incorporation of an oxygen atom into the substrate originates from molecular oxygen (O_2) that exists as a peroxyflavin group (FAD-OOH) located in the active site of the enzyme (*19*). We proposed that the lone pair of electrons from the aniline nitrogen could delocalize and facilitate the *para* position carbon to attack the distal oxygen on the FAD-OOH (Scheme 1). From this, a 1-fluoro-4-(methylimino)cyclohexa-2,5-dienol intermediate (**3**) would be formed that could then eliminate HF to form a reactive quinoneimine (**4**). The quinoneimine was then reduced to generate the final product **2**. This reaction mechanism, depicted in Scheme 1, is consistent with reported FMO reactions and is reasonable for nucleophilic moieties.

To test our hypothesis for the formation of the reactive intermediate **4**, incubations with rFMO1, NADPH (1 mM), and **1** (1 mM) were carried out in the presence of a nucleophilic thiol, *N*-acetylcysteine (NAC; 5 mM). If **4** was formed and has a reasonable half-life, it should form the NAC conjugates **5** and **6** (similar to NAPQI in the case of acetaminophen (*20*)). In fact, we detected two NAC conjugates that correspond to **5** and **6** within 2 ppm by high resolution mass spectrometry (*m/z* 285.08987). The formation of these conjugates was further confirmed by high resolution product ion scans and was within 4 ppm of the expected mass accuracy. The same product ions were detected for **5** and **6**, and the high resolution mass spectra are found in Supporting Information. When glutathione (GSH) was used as the trapping agent instead of NAC, only trace amounts of GSH conjugates were detected. This was consistent with a previous finding that GSH cannot access the active site of FMO (*21*). Therefore, we proposed that this may be the reason why GSH does not react with **4**. Perhaps this is a protective feature of the active site of FMO from futile cycling of GSH oxidation. Further investigation is needed to prove this hypothesis. The trace amounts that were found could be due to GSH reacting with **2** after it was released from the active site.

To characterize the regiochemistry of the conjugates, synthetic **2** (1 mM in 3 mL of 100 mM potassium phosphate buffer) was prepared by chemical reaction of NAC (5 mM) under basic conditions (pH 8) to facilitate the formation of **4** for 24 h (*22*). The samples were analyzed by LC-MS, and peaks containing NAC adducts at *m/z* 285 were fraction collected. The resulting fractions were dried under vacuum, dissolved in DMSO-d_6 (D, 99.8%, Cambridge Isotope) containing 0.05% V/V TMS as an internal chemical shift reference standard, and transferred to 3 mm NMR tubes (Norell, S-3-600-SC-7), purged with nitrogen, and sealed. A combination of many NMR methods (NOE and HMBC) were performed to confirm the regiochemistry of the two NAC conjugates using a Bruker Avance 3, 600 MHz spectrometer equipped with a 5 mm, TCI, Z gradient CryoProbe (see Supporting Information). Interestingly, when GSH was used instead of NAC under the same conditions, two GSH adducts were detected by mass spectrometry, which further supports our hypothesis that GSH does not access the active site of FMO1 to interact with **4**.

Various computational studies on the behavior of peroxy groups, including the FAD-OOH group, have been published to confirm the proposed viability or mechanism of reactions. These computational studies have been carried out using semiempirical, ab initio, and density functional theory regarding the reactivity, transition state geometry, and activation barrier of FAD reacting with various substrates (*23*). To further support our mechanism in Scheme 1, an ab initio study of the frontier molecular orbital analysis (*24, 25*) was performed on *p*-fluoro-*N*-methylaniline. The mechanism of nucleophilic substitution is consistent with the frontier molecular orbital analysis, which showed the fluorine having a strong inductive effect in extracting electron density from the aromatic ring and the directly attached *ipso* carbon bearing the largest positive calculated atomic electrostatic potential (ESP) charge of all the carbons in the phenyl ring. The frontier molecular orbital analysis showed that the carbon with the highest HOMO (highest occupied molecular orbital) coefficient (Figure 2) being consistent with the *para*-carbon initiating the nucleophilic attack. These calculations were

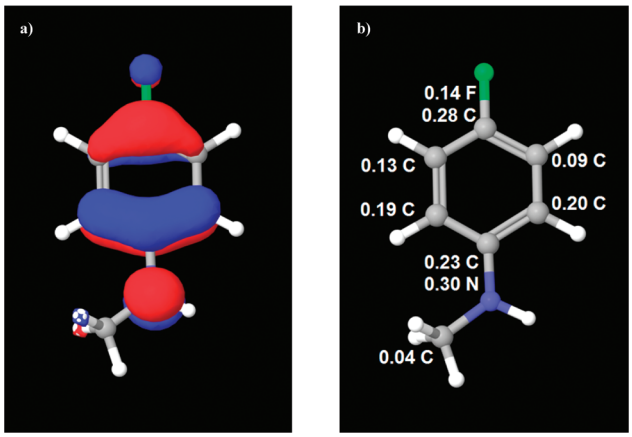


Figure 2. Molecular representations HOMO orbitals and coefficients. Calculations were performed at the HF/6-31+G** level using the Gaussian 03 program. (a) Representation of the HOMO orbitals. The tightly packed orbital around the fluorine atom indicates the lesser conjugation of that atom to the ring, while the nitrogen shows strong conjugation with the π-ring system. The blue and red color scheme shows the molecular orbital wave function sign. (b) Representation of the calculated HOMO orbital coefficients. The carbon next to the fluorine bears the largest coefficient on the phenyl ring consistent with it initiating the nucleophilic attack.

in line with what O'Hagan (*26*) discusses in his review of the carbon−fluorine bond. He states that the three lone pairs of electrons on fluorine are held tightly and that the electrons in the C−F bond are reluctant to get involved in any resonance structures due to its high electronegativity.

The electron donation from the nitrogen to the ring has an effect on lowering the HOMO and lowest unoccupied molecular orbital (LUMO) energy gap, given that the simple fluoro phenyl HOMO−LUMO energy gap was larger (0.42 eV vs 0.35 eV for the *p*-fluoroaniline). It was also noteworthy that the HOMO−LUMO gap for the *p*-fluoro-*N*-methylanaline was larger (0.28 eV vs 0.35 eV) than that of *p*-fluoroaniline. This was consistent with our observation that no carbon oxidation was observed from an incubation of *p*-fluoroaniline and rFMO1.

In summary, we demonstrated an FMO-based carbon oxidation plus defluorination reaction. Consistent with FMO reactions, one oxygen atom was incorporated from O_2. We propose that the lone pair of electrons from the nitrogen facilitates the nucleophilic attack by the C-1 carbon to the distal oxygen from FAD-OOH followed by the loss of HF and the formation of **3**. The formation of the quinoneimine intermediate was confirmed by trapping it with NAC and the detection of **5** and **6**. To our knowledge, this is the first time FMO has been shown to cause the formation of a quinoneimine intermediate.

Supporting Information Available: NMR data and NAC adduct spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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