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# Tryptophan-47 in the active site of *Methylophaga sp.* strain SK1 flavin-monooxygenase is important for hydride transfer

Andre Han [a], Reeder M. Robinson [a], Somayesadat Badieyan [a], Jacob Ellerbrock [a], Pablo Sobrado [a,b,c,*]

[a] Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061, USA
[b] Virginia Tech Center for Drug Discovery, Virginia Tech, Blacksburg, VA 24061, USA
[c] Fralin Life Science Institute, Virginia Tech, Blacksburg, VA 24061, USA

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ABSTRACT

Flavin-dependent monooxygenase (FMO) from *Methylophaga sp.* strain SK1 catalyzes the NADPH- and oxygen-dependent hydroxylation of a number of xenobiotics. Reduction of the flavin cofactor by NADPH is required for activation of molecular oxygen. The role of a conserved tryptophan at position 47 was probed by site-directed mutagenesis. FMOW47A resulted in an insoluble inactive protein; in contrast, FMOW47F was soluble and active. The spectrum of the flavin in the mutant enzyme was redshifted, indicating a change in the flavin environment. The $k_{cat}$ values for NADPH, trimethylamine, and methimazole, decreased 5-8-fold. Primary kinetic isotope effect values were higher, indicating that hydride transfer is more rate-limiting in the mutant enzyme. This is supported by a decrease in the rate constant for flavin reduction and in the solvent kinetic isotope effect values. Results from molecular dynamics simulations show reduced flexibility in active site residues and, in particular, the nicotinamide moiety of $NADP^+$ in FMOW47F. This was supported by thermal denaturation experiments. Together, the data suggests that W47 plays a role in maintaining the overall protein flexibility that is required for conformational changes important in hydride transfer.

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Flavin-dependent monooxygenase from *Methylophaga sp.* strain SK1 (FMO) catalyzes the NADPH- and oxygen-dependent hydroxylation of several xenobiotics (Scheme 1) [1]. The reaction mechanism can be divided into oxidative and reductive half-reactions. In the reductive half-reaction, FMO reacts with NADPH leading to the formation of reduced flavin and $NADP^+$ (Scheme 1a and b). In the oxidative half-reaction, the reduced flavin reacts with molecular oxygen to form the C4a-hydroperoxyflavin, which is the hydroxylating species (Scheme 1c) [1,2]. In the presence of a substrate containing a soft-nucleophilic heteroatom, the enzyme is capable of transferring a single oxygen atom to the nucleophilic site, thereby oxygenating the substrate (Scheme 1d) [3].

Site-directed mutagenesis and structural studies have shown that the C4a-hydroperoxyflavin is stabilized by $NADP^+$, which remains bound throughout the catalytic cycle [4–7]. If this intermediate is not stabilized it decays to hydrogen peroxide and oxidized flavin. Thus, stabilization of this intermediate is essential for preventing uncoupling of the reaction. It has been shown that interactions of several residues with $NADP^+$ are important for placing the coenzyme in a position optimal for intermediate stabilization [7]. The active site of FMO contains a conserved tryptophan residue that is in a site opposite of the NADP(H) and xenobiotic binding sites [6]. This residue is conserved in all known FMO sequences and in other related Class B flavin-monooxygenases (Fig. 1). The work presented here describes the biochemical characterization and analysis of the effect of mutating W47 to probe the role of this residue in catalysis.

## Materials and methods

### Materials

Methimazole, glutathione, and trimethylamine hydrochloride were obtained from Sigma–Aldrich. pVP55A was obtained from the Center for Eukaryotic Structural Genomics, University of Wisconsin, Madison [8]. *Escherichia coli* BL21-TI$^R$ cells were from Invitrogen. AccuPrime *Pfx* DNA Polymerase was from Invitrogen (Carlsbad, CA). The oligonucleotide primers used in the mutagenesis reactions were obtained from Integrated DNA Technology. Nucleotide sequencing was performed at the Virginia Bioinformatics Institute DNA Sequencing facility.

* Corresponding author at: Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061, USA. Fax: +1 540 231 9070.
*E-mail address:* psobrado@vt.edu (P. Sobrado).

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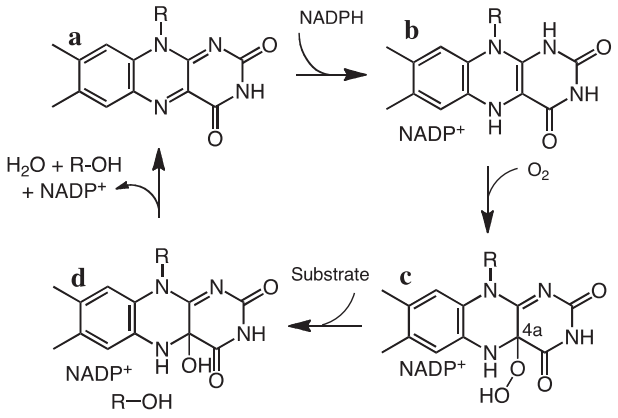


**Scheme 1.** Reaction catalyzed by FMO. The oxidized flavin (a) reacts with NADPH to form the reduced flavin and oxidized $NADP^+$ complex (b). The reduced enzyme is then able to react with molecular oxygen to form the C4a-hydroperoxyflavin intermediate (c). The stability of this intermediate depends on the presence of $NADP^+$ in the active site. After substrate binding, hydroxylation occurs and the hydroxylated flavin is formed (d). After flavin dehydration, release of $NADP^+$ and hydroxylated product, the oxidized flavin is formed for the next catalytic cycle.

### *Cloning and mutagenesis*

The gene encoding for FMO was synthesized and codon optimized for expression in *E. coli* (GenScript, NJ). The nucleotide sequence recognized by the enzymes *Sgf*I and *Pme*I were added at the 5′ and 3′ ends, respectively. The gene, digested with *Pme*I and *Sgf*I restriction endonucleases, was ligated into the pVP55A plasmid, which was previously digested with the same enzymes. Mutation of W47 in FMO to F or A was performed using the QuikChange method (Agilent Technologies, Santa Clara, CA). Primers used are shown in Table 1S.

### *Protein expression and purification*

The gene coding for FMO was cloned into the pVP55A plasmid such that the recombinant protein was expressed as an *N*-terminal 8xHis fusion. A single colony of BL21-$TI^R$ cells transformed with pVP55Afmo was used to inoculate 50 mL Luria–Bertani (LB) medium (100 μg/mL ampicillin), and incubated at 37 °C. The next day, six fernbach flasks containing 1.5 L LB medium (100 μg/mL ampicillin) were each inoculated with 10 mL of the overnight culture. After reaching an optical density value of ~0.6 at 600 nm ($OD_{600}$), isopropyl β-D-1-thiogalactopyranoside was added at 100 μM final concentration and the temperature lowered to 15 °C overnight. The cells were harvested via centrifugation at 8,000*g* and the resulting pellet (~40 g) was stored at −80 °C. For purification, the cell pellet was thawed and resuspended in 150 mL of buffer A (25 mM HEPES, 125 mM NaCl, 5 mM imidazole) and 25 μg/mL of DNase, RNase, and lysozyme were added. The solution was stirred for 40 min at 4 °C. Cells were then lysed by sonication for

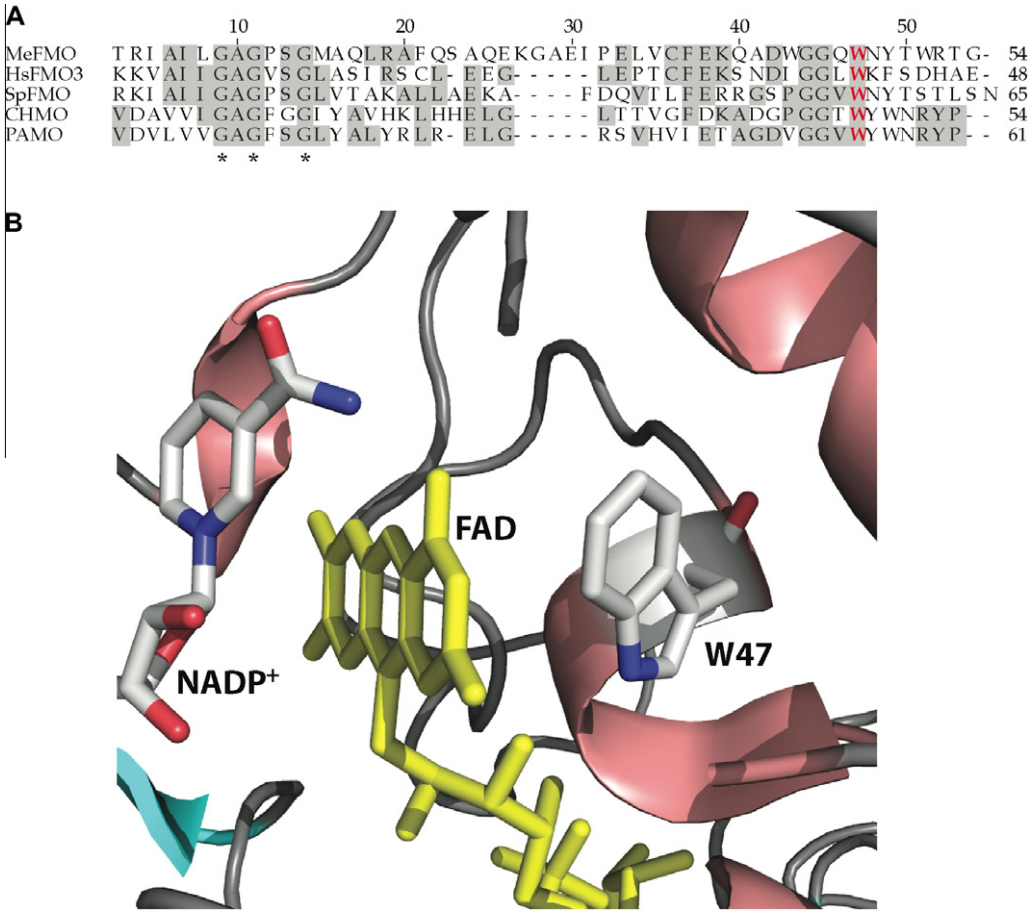


**Fig. 1.** (A) Amino acid sequence alignment of members of the Class B flavin-dependent monooxygenases (MeFMO, flavin monooxygenase from *Methylophaga sp.* strain SK1; HsFMO3, *Homo sapiens* flavin monooxygenase isoform 3; SpFMO, *Schizosacchromyces pombe* flavin monooxygenase; CHMO, cyclohexanone monooxygenase; PAMO, phenylacetone monooxygenase). The flavin binding motif (GXGXXG) is shown with asterisks. The conserved tryptophan corresponding to W47 in FMO is shown in red. The alignment was performed using Clustal W. (B) Position of W47 in the active site of *Methylophaga sp.* strain SK1 FMO. The figure was made using Pymol (PDB code 2vq7). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)