Revealing the moonlighting role of NADP in the structure of a flavin-containing monooxygenase

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Flavin-containing monooxygenases (FMOs) are, after cytochromes P450, the most important monooxygenase system in humans and are involved in xenobiotics metabolism and variability in drug response. The x-ray structure of a soluble prokaryotic FMO from Methylophaga sp. strain SK1 has been solved at 2.6-Å resolution and is now the protein of known structure with the highest sequence similarity to human FMOs. The structure possesses a two-domain architecture, with both FAD and NADP+ well defined by the electron density maps. Biochemical analysis shows that the prokaryotic enzyme shares many functional properties with mammalian FMOs, including substrate specificity and the ability to stabilize the hydroperoxyflavin intermediate that is crucial in substrate oxygenation. On the basis of their location in the structure, the nicotinamide ring and the adjacent ribose of NADP+ turn out to be an integral part of the catalytic site being actively engaged in the stabilization of the oxygenating intermediate. This feature suggests that NADP(H) has a moonlighting role, in that it adopts two binding modes that allow it to function in both flavin reduction and oxygen reactivity modulation, respectively. We hypothesize that a relative domain rotation is needed to bring NADP(H) to these distinct positions inside the active site. Localization of mutations in human FMO3 that are known to cause trimethylaminuria (fish-odor syndrome) in the elucidated FMO structure provides a structural explanation for their biological effects.

drug metabolism | trimethylaminuria | oxygen

Two effective families of enzyme systems have evolved in eukaryotes to oxygenate xenobiotics: cytochromes P450 and flavin-containing monooxygenases (FMOs). They both help organisms to deal with potentially toxic exogenous compounds from natural sources and, most notably in humans, represent the enzyme systems involved in the metabolism of drugs and pollutants (1). FMOs belong to the flavoenzyme class of single-component flavoprotein monooxygenases (2); they use equivalents from NADPH to reduce the FAD cofactor, which, in turn, accounts for an efficient reduction of the flavin cofactor. Like the one most abundantly expressed in adult liver and largely involved in drug metabolism (11, 12), all isoforms display ~60% sequence identity, a moderate level of polymorphism (13–15), and the presence of tissue- and age-specific splice variants (16). Several mutations in hFMO3 have been reported to cause the inheritable disease known as trimethylaminuria (TMAU) or “fish-odor syndrome,” because of accumulation of substrate trimethylamine in body fluids (17). All mammalian FMOs have a strong membrane association and poor water solubility (9), and no structure of a mammalian FMO is currently available.

In recent years, several plant (18) and prokaryotic enzymes have been annotated as FMOs. The first discovered bacterial FMO was identified in the methylothrophic bacterium Methylophaga sp. strain SK1 (mFMO) for the purpose of biocatalytic production of the blue dye indigo (19). mFMO is not membrane-associated. Its sequence (456 aa) scores ~31% identity with hFMO3 and contains two dinucleotide-binding signatures (Rossmann folds) for FAD and NADP and the FMO-identifying motif FXGXXXHXXX(Y/F) (20). mFMO shares 27% sequence identity with Schizosaccharomyces pombe FMO, whose crystal structure has been recently reported (21). The sequences of Methylophaga, human, and S. pombe FMOs are aligned (22, 23) in Fig. 1B.

Here, we present the biochemical characterization of mFMO with kinetic data on a number of substrates and the 2.6-Å resolution structure obtained by x-ray crystallography. In addition to the interest raised by the homology with the hFMOs, the crystal structure of the bacterial enzyme highlights the direct involvement of NADP+ in the stabilization of the crucial C4a-hydroperoxyFAD intermediate, a unique feature of these enzymes.

Results

Biochemical Properties. mFMO is a 2 × 53-kDa dimeric protein containing one molecule of noncovalently tightly bound FAD per monomer. The spectrum of the fully oxidized enzyme exhibits typical maxima at 273, 372, and 442 nm, a shoulder at 462 nm, and a ratio Abs273/Abs442 of 9.9 (Fig. 2, trace A). Addition of NADPH rapidly bleaches the protein solution, accounting for an efficient reduction of the flavin cofactor. Like commonly observed in mammalian enzymes (24), in the absence

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of an organic substrate, mFMO shows a slight NADPH oxidase activity ($K_m$,NADPH = 13 μM, $k_{cat}$ = 0.06 s$^{-1}$ measured in steady-state conditions) producing hydrogen peroxide (Fig. 1A). The ability to stabilize the C4a-hydroperoxyflavin intermediate, well documented in the early works on pig FMO (4, 6, 7), can also be demonstrated in mFMO. Mixing the enzyme with an equimolar amount of aerated NADPH (in the absence of an organic substrate) leads to the immediate formation of a C4a-hydroperoxyflavin intermediate characterized by the typical absorbance spectrum with a peak at 360 nm (refs. 6, 25–27; Fig. 2, trace B). Over ≈30 min, the spectrum slowly reacquires the characteristic 442-nm peak of the oxidized flavin (Fig. 2, traces C to G). Conversely, addition of excess substrate immediately brings the spectrum back to that of the fully oxidized flavin.

Steady-state kinetic assays performed at a fixed concentration of NADPH with typical FMO substrates showed Michaelis–Menten behavior (Table 1). Substrates were selected on the basis of the following considerations: trimethylamine is a common endogenous substrate for hFMO3; methimazole and (S)-(-)-nicotine are of pharmacological relevance (9); methimazole and N,N-dimethylaniline has been extensively exploited to test mammalian FMO activity in literature; indole is the putative substrate of mFMO in the pathway for the production of indigo (19). All these compounds are good substrates, with trimethylamine exhibiting the highest catalytic efficiency (Table 1). An enzymatic activity has also been unexpectedly appreciated on dimethyl sulfoxide initially used for dissolving some substrates, confirming that mFMO accepts a broad range of N- and S-containing molecules largely overlapping with the substrates of the first Rossmann fold for FAD binding, the fingerprint sequence for FMOs (20), and the second Rossmann fold for NADP binding, respectively. The green asterisks mark residues that are part of the active site, whereas the red asterisks indicate residues that are mutated in patients affected by TMAU. The alignment was performed with ClustalW2 (22) and ESPript (23).
mammalian FMOs. Moreover, as observed in steady-state kinetics of mammalian enzymes (4), NADP+ was competitive (Ki of 30 μM) with NADPH. Taken together, these observations suggest that, similar to the mammalian FMOs, the bacterial enzyme behaves like a “cocked gun,” spending most of the time in the form of the hydroperoxyflavin intermediate. Any heteroatom-containing compound gaining access to the stabilized intermediate is monoxygenated, in accordance with the striking substrate promiscuity of FMOs.

Overall Three-Dimensional Structure. Wild-type mFMO crystalized readily, but all crystals invariably suffered from severe twinning. This problem was addressed by exploiting a number of techniques, among which surface mutagenesis proved to be successful (28). We targeted sets of two/three consecutive charged residues (mainly Glu and Lys) supposed to be solvent-exposed based on alignment with homologous proteins [supporting information (SI) Table S1]. The mutant E158A/E159A gave crystals that turned out to be free from twinning (the mutant has biochemical properties virtually identical to those of wild type; Table 1). Addition of NADP+ was essential for crystallization. mFMO structure was solved by single-wavelength anomalous dispersion method at 2.6-Å resolution (Tables 2 and 3) (29, 30). Protein molecules are arranged to give two identical homodimers (Fig. 3) in the asymmetric unit, which is consistent with the dimeric form determined by size-exclusion chromatography for both wild-type and E158A/E159A mFMOs. However, each of the active sites is exclusively built up by residues belonging to a single subunit, rmsd between equivalent Ca atoms of the monomers are <0.1 Å.

The protein is made up of two distinct domains (Fig. 3A), a larger FAD-binding domain (residues 1–169 and 281–461) and a smaller NADP-binding domain (residues 170–280). Both domains display a typical dinucleotide-binding fold and are connected through a double linker (around residues 170 and 280) that may act as a flexible hinge (Fig. 3A). There are three long stretches interposed between the two domains; they lack secondary structure elements (residues 44–80, 166–186, 276–306), suggesting the absence of any rigid block opposing the reciprocal movement of the domains. A relatively similar overall conformation (rmsd of 2.3 Å for 343 Ca atoms of the flavin N5 (C4-N5 distance of 5.1 Å) so that the NADP+ amide group is within hydrogen-bond distances from the flavin O4 and N5. These potential hydrogen-bond interactions led us to tentatively assign the orientation of the NADP+ carboxamide group as shown in Fig. 4B. Spanning in proximity of the flavin ring, a 161-Å3 cavity was detected by the program VOIDOO (31) by using a 1.4-Å radius sphere as probe (Fig. 4C). Its limits are defined by the isoalloxazine, the NADP+ ribose and several protein residues (Asn-78, Gly-79, Pro-80, Cys-83, Leu-84, Phe-402, Trp-405, Gln-323, Trp-324, Tyr-325, Ser-326, Tyr-212). The role of Tyr-212 is remarkable, because its side chain together with the ribose of NADP+ shields the cavity from the outside, forming the only barrier to the solvent (Fig. 4).

After obtaining the native dataset at 2.6-Å resolution, several attempts were made to bind substrate molecules inside the crystals by either soaking or cocryrstallization but none of them were successful. Nevertheless, in the electron density map calculated from soaked crystals (we refer to the experiment with N,N-dimethylaniline hereafter named “dataset from soaking”; Tables 2 and 3), a well defined ellipsoidal peak of density was found in proximity to the catalytic site inside the above-described cavity, halfway between Tyr-212 and the pyrimidine ring of the flavin (Fig. 4C). Alternative refinements with several candidate molecules and atoms only yielded reasonable B-factors when dioxygen or monoatomic ions of comparable electron density were fitted inside. Based on these observations, we assigned the orphan electron densities to dioxygen, which is included in the coordinate file deposited in the Protein Data Bank (32). However, we do not rule out that, instead of molecular oxygen, a superoxoide molecule, possibly generated upon the x-ray exposure of the crystal, is bound at this position. Likewise, the presence of a monoatomic ion (especially if positively charged, 

Table 1. Data from steady-state kinetic assays on mFMO

<table>
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<tr>
<th>Substrate</th>
<th>Km, μM</th>
<th>kcat, s−1</th>
<th>kcat/Km, M−1 s−1</th>
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</thead>
<tbody>
<tr>
<td>Trimethylamine</td>
<td>7.3 ± 0.6</td>
<td>6.1 ± 0.1</td>
<td>8.4 × 10² ± 0.7</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>7.1 ± 1</td>
<td>6.4 ± 0.3</td>
<td>9.0 × 10² ± 1</td>
</tr>
<tr>
<td>(E158A/E159A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td>130 ± 30</td>
<td>3.0 ± 0.2</td>
<td>2.3 × 10² ± 0.6</td>
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<tr>
<td>Methimazole</td>
<td>66 ± 7</td>
<td>1.0 ± 0.02</td>
<td>1.5 × 10² ± 0.2</td>
</tr>
<tr>
<td>N,N-dimethylaniline</td>
<td>232 ± 28</td>
<td>1.8 ± 0.4</td>
<td>7.8 × 10² ± 2</td>
</tr>
<tr>
<td>Indole</td>
<td>90 ± 14</td>
<td>0.7 ± 0.03</td>
<td>7.8 × 10¹ ± 1</td>
</tr>
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</table>

Table 2. Data collection statistics

<table>
<thead>
<tr>
<th></th>
<th>SeMet*</th>
<th>Native</th>
<th>Soaking†</th>
</tr>
</thead>
<tbody>
<tr>
<td>a, c Å*</td>
<td>219.2, 126.7</td>
<td>219.6, 133.6</td>
<td>218.7, 130.7</td>
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<tr>
<td>Resolution, Å</td>
<td>3.0</td>
<td>2.6</td>
<td>2.8</td>
</tr>
<tr>
<td>Rsym%</td>
<td>0.12 (0.51)</td>
<td>0.12 (0.45)</td>
<td>0.08 (0.43)</td>
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<tr>
<td>Completeness, %</td>
<td>99.0 (97.2)</td>
<td>97.3 (86.1)</td>
<td>99.7 (100.0)</td>
</tr>
<tr>
<td>Redundancy‰</td>
<td>5.1 (5.0)</td>
<td>3.0 (2.4)</td>
<td>3.2 (3.1)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>11.7 (2.1)</td>
<td>8.0 (2.3)</td>
<td>13.1 (2.1)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>69,172</td>
<td>110,675</td>
<td>86,940</td>
</tr>
</tbody>
</table>

*Phasing was performed with the program SHARP (41). The overall figure of merit for centric and acentric and reflections was 0.2 and 0.4, respectively.
†The dataset was measured from a crystal soaked for 30 min at 4°C in a solution containing N,N-dimethylaniline 5 mM, NADP+ 1 mM, PEG4000 25% (wt/vol), NaHepes 0.1 M, pH 7.5.
‡The crystals were obtained with the mutant E158A/E159A. They belong to space group P61, and their asymmetric unit contains four mFMO monomers.
§Rsym = Σ|hkl|−|f(hkl)|/Σ|f(hkl)|.
¶Data for the highest-resolution shell are given in parentheses.

Table 3. Refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
<th>Soaking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution, Å</td>
<td>2.6</td>
<td>2.8</td>
</tr>
<tr>
<td>R factor (Rwp)*</td>
<td>0.248 (0.269)</td>
<td>0.225 (0.242)</td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>14,569</td>
<td>14,552</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>124</td>
<td>0</td>
</tr>
<tr>
<td>No. of FAD and NADP+ atoms</td>
<td>404</td>
<td>404</td>
</tr>
<tr>
<td>rsmd bond length, Å‡</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>rsmd bond angle, °‡</td>
<td>1.42</td>
<td>1.43</td>
</tr>
<tr>
<td>Ramachandran plot‡</td>
<td>89.2</td>
<td>88.2</td>
</tr>
<tr>
<td>Most favorable region, %</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Disallowed regions, %</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Rwp = Σ|Fobs|−|Fcalf|/Σ|Fobs|. Rwp is the Rwp value for 5% of the reflections excluded from the refinement.
†No electron density has been detected for residues 1–7 and 451–461.
‡Rmsd from ideal values calculated with REFMACS (29).
§Figures from PROCHECK (30).
such as K⁺) cannot be excluded. A structural superposition of the two sets of atomic coordinates (native dataset and dataset from soaking) reveals that the accommodation of putative dioxygen does not induce any significant shift in the position of protein residues and cofactors.

**Location of hFMO3 Mutations and Polymorphisms.** The remarkable degree of sequence identity makes it interesting to locate mFMO residues corresponding to hFMO3 positions affected by patho-

ological mutations and polymorphisms (11, 17). All classical TMAU-causing mutations target residues that are located either in the active site or in structurally relevant regions (Figs. 1B and 3A). Examples are the human mutation Asn61Ser that targets the catalytically crucial Asn-78 in mFMO and the Pro153Leu mutation (homologous to mFMO Pro-173) that targets a residue of the domain linker. Conversely, hFMO3 polymorphisms commonly observed in human populations concern positions that in mFMO structure fall in solvent-exposed areas far apart from the active site (Fig. 3A).

**Discussion**

We investigated mFMO for its ability to work as a good model for the study of human enzymes. Biochemical analysis revealed that this prokaryotic enzyme shares many functional properties with mammalian FMOs, including the ability to stabilize C4a-hydroperoxyFAD for minutes. The well known role of NADPH coenzyme in the catalytic cycle of FMOs is that of providing FAD with reducing equivalents (Fig. 1A). The chemistry of NADPH requires that reduction of the flavin cofactor is brought about through a hydride transfer from the C4 atom of the nicotinamide ring to the flavin N5 atom (Fig. 1B). The stereochemistry observed in mFMO structure does not reveal a close proximity between these two crucial atoms and thus is clearly not compatible with a hydride transfer. This implies that the structure in the crystals does not correspond to the conformation in which NADPH is able to reduce the flavin. A modeling experiment (Fig. 3A) in which the hypothetical coordinates of the C4a-hydroperoxyflavin are superimposed onto the flavin coordinates of the mFMO structure places the two additional oxygen atoms of the C4a-adduct within hydrogen-bond distance from O₂⁻ atom of NADP⁺ ribose, which then would be perfectly positioned to properly stabilize this oxygenating intermediate. The positioning of the two additional oxygens would require only a small (1–2 Å) shift of the Asn-78 side chain, which in turn could contribute to their stabilization through polar interactions. Consistently, the homologous Asn-61 of hFMO3 is essential for activity (refs. 11 and 17; Fig. 3A). These considerations suggest that the conformation in mFMO crystals is likely to correspond to that promoting the stabilization of C4a-hydroperoxyFAD. This feature is known to depend on the presence of NADP⁺ that was proposed to act as a “gate keeper,” preventing the decay of the intermediate possibly by protecting the flavin N5 from solvent attack (6). Our structure helps to explain these functional properties and is a clear observation of a second fascinating functional property of NADP⁺ in FMO catalytic cycle, i.e., the stabilization of C4a-hydroperoxyflavin. NADP⁺ shields the active site and provides a proper H-bonding environment that can prolong the intermediate half-life. Therefore, NADP⁺ is an integral part of the enzyme catalytic machinery that promotes intermediate stabilization and substrate monooxygenation. The approach of a substrate to the intermediate may be promoted by a displacement of both the loop of Tyr-212 (NADP-binding domain) and the stretch of residues 407–415 at the end of a α-helix (FAD-binding domain, Fig. 3A), as suggested by the higher B-factors of this protein region.

The crystal structure of mFMO is similar to that of S. pombe FMO (21). In particular, most of the catalytic residues are identical in the two enzymes (Fig. 1B), which display similar active sites and, most importantly, essentially the same conformation of bound NADP⁺ (Fig. 5B). In their original article, Eswaramoorthy et al. (21) explained the observed NADP⁺-binding mode as indicative of a flavin-reduction mechanism involving hydride transfer from the nicotinamide C2 atom (rather than C4) to the flavin. This interpretation has been subsequently revised as not compatible with known NADP(H) chemistry without any further comment (33). We believe that, in view of their structural similarity, Methylophaga and S. pombe FMOs are very likely to have a similar catalytic mechanism with NADP⁺ being actively involved in the stabilization of the C4a-hydroperoxyflavin intermediate as described above. In this context, we remark that also sequence-related Baeyer–Villiger monooxygenases, e.g., cyclohexanone monooxygenase and phenylacetone monooxygenase (34, 35), probably use the same catalytic strategy. Indeed, for phenylacetone monooxygenase it has been shown that NADP⁺ is a prerequisite for efficient and
enantioselective catalysis (36) and its crystal structure predicts a NADP\(^+\)-binding mode identical to that found in FMOs, suggesting a similar mechanism of intermediate stabilization (37).

To bring about the first of its two roles (reduction of FAD by hydride transfer; Fig. 1A), NADPH coenzyme must occupy a position different from that observed in our structure. A shift of the ribose ring of NADP\(^+\) coenzyme might be enough to move the nicotinamide ring to a position productive for hydride transfer. However, our repeated observation that soaking mFMO crystals in a solution of fresh NADPH rapidly bleaches and cracks them rather suggests that a relative domain rotation could be involved in promoting FAD reduction by NADPH. A similar movement has been hypothesized for proteins with similar topology (38) and in the catalytic mechanism of phenylacetone monooxygenase (37).

In conclusion, in FMOs and related monooxygenases, NADP(H) has two binding modes likely to be associated to distinct protein conformational states; the first binding mode along the catalytic cycle promotes FAD reduction through hydride transfer, whereas the second mode exerts stabilization of the crucial catalytic intermediate, a sort of moonlighting activity of NADP(H).

Materials and Methods

Crystallization and Structure Determination. Gene cloning and protein expression and purification were carried out by using standard protocols as described in SI Text. Wild-type mFMO crystallized in many conditions using low-molecular-weight PEGs as precipitant. However, these crystals were affected by twinning. Conversely, mutant FMO E158A/E159A (SI Text) gave crystals of a different form in microbatch technique at 4°C by mixing equal volumes of 8 mg of protein/ml in Tris\(\cdot\)HCl 25 mM, pH 8.0; NaCl 250 mM; NADP\(^+\) 1 mM and of PEG4000 20% (wt/vol) in Na/Hepes 0.1 M, pH 7.5. These crystals were not affected by twinning and could be used for structure determination.

Data were collected at the European Synchrotron Radiation Facility (Grenoble, France) and processed with the CCP4 package (39). The structure was solved by the single-wavelength anomalous diffraction method (Table 2) by using SHELXD (40) and SHARP (41). The resulting electron density map was position different from that observed in our structure. A shift of the ribose ring of NADP\(^+\) coenzyme might be enough to move the nicotinamide ring to a position productive for hydride transfer. However, our repeated observation that soaking mFMO crystals in a solution of fresh NADPH rapidly bleaches and cracks them rather suggests that a relative domain rotation could be involved in promoting FAD reduction by NADPH. A similar movement has been hypothesized for proteins with similar topology (38) and in the catalytic mechanism of phenylacetone monooxygenase (37).

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Data were collected at the European Synchrotron Radiation Facility (Grenoble, France) and processed with the CCP4 package (39). The structure was solved by the single-wavelength anomalous diffraction method (Table 2) by using SHELXD (40) and SHARP (41). The resulting electron density map was
used in molecular replacement calculations to solve the structure of the native (nonselenomethionine-substituted) enzyme. Phases were further improved by multiple-crystal density averaging with DMMULTI (39). All subsequent model-building and refinement calculations were done by using data from the native crystals. The initial model was traced with ARPwARP (42). The programs COOT (43) and Refmac5 (29) were used for model rebuilding and refinement (Table 3). Figures were generated by using PyMOL (DeLano Scientific; www.pymol.org).

Kinetic Assays. Kinetic assays were performed in duplicate at 25°C on a Varian spectrophotometer (Cary 100 Bio) equipped with a thermostatic cell compartment. A 1-ml mixture containing mFMO 0.1–0.2 min; the reaction was triggered by addition of substrate and followed by decrease in absorbance at 340 nm (εmax = 6.22 M−1 cm−1) for NADPH. All substrates (Table 1) were dissolved in water except N,N-dimethylaniline (dissolved in ethanol). Km for NADPH was measured by using a fixed trimethylamine concentration (2 mM) and varying NADPH concentrations (0–230 μM). To generate the C4a-hydroperoxoflavin intermediate (Fig. 2, trace B), an aerated solution of NADPH 7 μM was added to a solution of mFMO (7 μM in KPi 100 mM, pH 7.4) in a final volume of 1 ml at 4°C.

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