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

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Flavin-containing monoxygenases (FMOs) are, after cytochromes P450, the most important monoxygenase 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 reactant 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 trimethylammonium (fish-odor syndrome) in the elucidated FMO structure provides a structural explanation for their biological effects.

Two effective families of enzyme systems have evolved in eukaryotes to oxygenate xenobiotics: cytochromes P450 and flavin-containing monoxygenases (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 monoxygenases (2); they use equivalents (3). The first FMO isolated from pig liver was described (4), 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 reactant 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 trimethylammonium (fish-odor syndrome) in the elucidated FMO structure provides a structural explanation for their biological effects.

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 NADP(H) 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 \( \left( K_{m, \text{NADPH}} = 13 \, \mu \text{M}, k_{\text{cat}} = 0.06 \, \text{s}^{-1} \right) \) measured in steady-state conditions) producing hydrogen peroxide (Fig. 1A).

The ability to stabilize the C4a-hydroperoxoflavin 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-hydroperoxoflavin intermediate characterized by the typical absorbance spectrum with a peak at \( \lambda = 360 \, \text{nm} \) (refs. 6, 25–27; Fig. 2, trace B). Over \( \sim 30 \, \text{min} \), 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 monooxygenated, in accordance with the striking substrate promiscuity of FMOs.

Overall Three-Dimensional Structure. Wild-type mFMO crystallized readily, but all crystals invariably suffered from severe twinning. This problem was addressed by exploiting a number of strategies for both wild-type and E158A/E159A mFMOs. However, 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) has been observed in the structure of S. pombe FMO in complex with NADP⁺ (21).

The Active Site. Inspection of the active site reveals the existence of a cleft leading to the NADP⁺ molecule at the interface between the two protein domains (Fig. 4A). NADP⁺ occupies the cleft, blocking the access to the flavin and the catalytic core. The nicotinamide ring is sandwiched between the re side of the flavin and the single α-helix of the NADP-binding domain and is roughly coplanar with isosalaloxazine at a distance of 3.0–4.0 Å. Importantly, the nicotinamide orients its reactive C4 away from 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 isosalloxazine, 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 is bound to the interface between the ribose of NADP⁺ and the flavin molecule at the interface between the two subunits, rmsd between equivalent Ca atoms of the monomers are <0.1 Å.

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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 pathological 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 promote 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. 3B). 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, *Methylobacterium* 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-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

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**Fig. 4.** The active site of mFMO. (A) Surface representation of the enzyme with the catalytic site viewed from outside using the coloring scheme and approximately the same orientation of Fig. 3B. (B) Binding of the NADP⁺ nicotinamide ring. Only crucial residues are displayed. Oxygen is red, nitrogen is blue, carbon is differently colored depending on its belonging to FAD (yellow), NADP⁺ (cyan), FAD-binding domain (orange), NADP-binding domain (green). For the sake of clarity, only the backbone atoms of Trp-76 are shown. The reactive C4a and N5 atoms of the flavin and C4 of NADP⁺ nicotinamide are labeled. (C) Stereoscopic view of the active site of mFMO in approximately the same orientation as Fig. 4B and with the same color code. The red-colored electron density (2Fo-Fc, contoured at 1.5σ) was found in the dataset resulting from a soaking experiment (Table 2) and tentatively assigned to a dioxygen bound inside the cavity located in front of the flavin (here shown as a light blue transparent surface).

**Fig. 5.** The role of NADP⁺ in the stabilization of C4a-hydroperoxyflavin intermediate. (A) Modeling experiment in which the hypothetical structure of C4a-hydroperoxyflavin was superimposed to the flavin in mFMO structure. The color code is the same as in Fig. 4B: Hypothetical hydrogen bonds involving the hydroperoxyflavin atoms are shown as blue dashed lines. The accommodation of the additional oxygen atoms of the C4a-adduct would require a shift of ~1.5 Å of Asn-78 side chain (whose conformation in the native structure is shown as thin black stick). (B) Comparison of the NADP⁺-binding mode in S. pombe (Protein Data Bank ID code 2gv8) and Methylophaga FMOs. The picture was obtained by superimposing the Cα atoms of the two proteins and shows the FAD (yellow) and NADP⁺ (blue) molecules of mFMO together with FAD and NADP⁺ of the S. pombe enzyme (red). The N5 and C4a atoms of the flavin and C4 and C2 atoms of NADP⁺ are labeled.
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 ARPUWARP (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 reaction mixture contained 1–0.2 μM Tris-HCl 25 mM, pH 8.5; NaCl 35 mM; 0.1 mM NADPH (dissolved in NaOH 20 mM) was equilibrated for 2 min; the reaction was triggered by addition of substrate and followed by decrease in absorbance at 340 nm (εmax = 6.22 mM−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-hydroperoxyflavin 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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