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

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Supporting Information

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SI Text

Cloning

Methylophaga. FMO gene was subcloned by PCR amplification using the following primer set:

5′-CGACGGATCC**ATG**GCAACTCGTATTGCGATACTTGGTG-3′

5′-CAGCCTCGAG**TTA**AGCTTCTTTAGCCACAGGAATTTCGCTC-3′.

where the underlined sequences correspond to BamHI and XhoI restriction sites, respectively, and the sequences in bold font correspond to the start and stop codon of the gene, respectively. The amplified product was then digested with BamHI and XhoI restriction enzymes and ligated into plasmid pET-28aTM. The plasmid pET-28aTM is a modification of the commercial vector pET-28a(+) (Novagen); it contains a TEV protease-specific cleavage site between the N-terminal purification tag and the insert. Cleavage by TEV protease removes the 23-amino acid N-terminal segment containing the purification His-tag and leaves a residual N-terminal peptide (Gly-Ala-Met-Gly-Ser). Comparison between wild-type *Methylophaga* FMO (previously expressed and purified in our laboratory from a non-His-tagged vector) and the same protein expressed in pET-28aTM and cleaved by TEV protease did not show any differences in biochemical or crystallization behavior. The plasmids pET-28aTM containing wild-type FMO gene was checked by sequencing in the region of the insert.

Expression. The protocol has been adopted from Choi *et al*. (1), with modifications. Plasmids were transformed by heat-shock into *E. coli* BL21(DE3) cells. Resulting colonies were preinoculated into LB broth containing kanamycin 50 μg/ml and grown overnight at 37°C. The following day this culture was inoculated 1:50 into LB broth added with kanamycin 50 μg/ml and powder tryptophan (2 g/liter), the latter to induce a considerable production of indigo by the cells as this works as a useful qualitative indicator of protein activity. Cultures were grown at 37°C under moderate shaking until OD $\approx$ 0.4, then they were induced with isopropyl β-D-1-thiogalactopyranoside 0.5 mM, the temperature was shifted to 25°C and the growth allowed to continue for a total time of about 24 h. A good level of activity was indicated by the dark blue color of the broth at the end of the growth, due to the accumulation of the blue dye indigo. Cells were harvested by centrifugation (5,000 $\times$ *g*, 15 min, 4°C).

To obtain selenomethionine-substituted FMO, the protocol of Doublie (2) was followed. *E. coli* BL21(DE3) cells were transformed and grown at 37°C in minimal M9 medium added with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.4% wt/vol glucose, 1.5 μM thiamine, and kanamycin 50 μg/ml until mid-log phase; then they were supplemented with Lys, Phe, Thr (100 mg/liter), Ile, Leu, Val (50 mg/liter), L-selenomethionine (60 mg/liter) and 1 mM isopropyl β-D-1-thiogalactopyranoside and grown at 25°C for additional 20 h.

Extraction and Purification. All operations were performed on ice or at 4°C. Cells were resuspended 1:7 in a lysis buffer (TrisCl 25 mM, pH 8.0, NaCl 250 mM, imidazole 40 mM, 2-mercaptoethanol 3 mM, DNAseI 5 μg/g of cell paste, phenylmethanesulphonylfluoride 1 mM) and sonicated (total time 3 min). After centrifugation (70,000 $\times$ *g*, 45 min, 4°C), supernatant was filtered, loaded onto a nickel Sepharose affinity chromatography column (HisTrap HP, GE Healthcare) preequilibrated with TrisCl 25 mM pH 8.0, NaCl 250 mM, imidazole 40 mM, 2-mercaptoethanol 3 mM, and eluted by a linear ascending gradient of the same buffer containing 500 mM imidazole. His-tagged TEV protease was then added to the pooled fractions at a concentration $\approx$20–30$\times$ lower than estimated concentration of His-tagged FMO, and the sample was dialyzed against TrisCl 25 mM pH 8.0, NaCl 250 mM overnight at 4°C to remove imidazole and allow proteolytic cleavage of the purification tag. The following day the sample was loaded onto the same nickel Sepharose affinity chromatography column (HisTrap HP, GE Healthcare) preequilibrated with TrisCl 25 mM pH 8.0, NaCl 250 mM, and recovered as wild-type protein from flow-through fractions. An additional gel filtration column (Superdex 200 HiLoad 16/60, GE Healthcare) preequilibrated with TrisCl 25 mM pH 8.0, NaCl 250 mM (storage buffer) was performed in most cases to obtain a higher degree of purification and/or evaluate the polymerization state of the proteins. DTT (1 mM) was usually added to the protein sample used for crystallization screens but not to the sample used in kinetic assays. Purification procedures for selenomethionine-substituted FMO were the same as for the native enzyme, except for the addition of 2-mercaptoethanol 4 mM through the whole process, replaced by DTT 5 mM in the final storage buffer. The protein sample was monitored from extraction to the final purification step by SDS/PAGE, kinetic assays and UV/visible spectrophotometry.

Spectroscopic Studies. UV-visible absorbance spectra were recorded in quartz cuvettes (Hellma) at 4°C with an Agilent diode-array spectrophotometer (8453 UV-Visible) equipped with a thermostatic cell compartment. FMO concentrations were calculated on the basis of the following extinction coefficients: ε_{273} = 134000 M^{-1} cm^{-1}; ε_{442} = 13350 M^{-1} cm^{-1} as determined according to ref. 3.

pH Dependence. The pH dependence of the kinetic parameters was investigated at pH values between 6.0 and 9.0 by increments of 0.5, replacing TrisCl buffer with Mes 50 mM (6<pH <7), Hepes 50 mM (7<pH <8), TAPS 50 mM (8<pH <9), using trimethylamine 2 mM as substrate and adjusting the ionic strength to 50 mM by changing the concentration of NaCl. These experiment showed that highest enzymatic activity was measured in the pH range 8.0–8.5. Therefore all kinetic assays (Table 1) were performed at pH 8.5.

Determination of K_i for $NADP^+$. Activity assays were performed at different NADPH concentations (30, 60, 90, 120 μM) including $NADP^+$ in the initial mixture. For each of the four NADPH concentrations, four different concentrations of $NADP^+$ were tested (30, 60, 90, 120 μM), and the K_i deduced by the intersection of the interpolated lines in a Dixon plot.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed on plasmid pET-28aTM containing wild-type *Methylophaga* FMO as template, by using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Eleven mutants were designed (Table S1). Plasmids of the resulting colonies were extracted and sequenced in the region of the insert to check for the presence of the mutations. Mutants were sequentially tested for expression and activity and purified. They were next subjected to crystallization trials.

1. Choi HS, *et al*. (2003) A novel flavin-containing monooxygenase from *Methylophaga* sp strain SK1 and its indigo synthesis in *Escherichia coli*. *Biochem Biophys Res Commun* 306:930–936.
2. Doublie S (1997) Preparation of selenomethionyl proteins for phase determination. *Methods Enzymol* 276:523–530.
3. Macheroux P (1999) in *Methods in Molecular Biology*, eds Chapman SK, Reid GA (Humana, Totowa, NJ), Vol. 131, pp 1–7.

Table S1. List of designed mutants of *Methylophaga* FMO

Mutant	Expressed	Purified	Crystals
E182A/K183A	Yes	Yes	Twinned
E268A/K269A	Yes	No	-
K350A/E351A	Yes	Yes	Twinned
E362A/K363A	Yes	No	-
K409A/K410A/E411A	Yes	No	-
E21A/K22A	Yes	Yes	Twinned
E158A/E159A	Yes	Yes	Nontwinned
K201A/D202A/K203A	No	-	-
K227A/K228A	No	-	-
M342S/P346K/L347K	No	-	-
P346K/L347K	No	-	-