Revealing the moonlighting role of NADP in the structure of a flavin-containing monooxygenase
Alfieri, Andrea; Malito, Enrico; Orru, Roberto; Fraaije, Marco; Mattevi, Andrea

Published in:
Proceedings of the National Academy of Sciences of the United States of America

DOI:
10.1073/pnas.0800859105

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2008

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Supporting Information

**Alfieri et al. 10.1073/pnas.0800859105**

**SI Text**

**Cloning**

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

\[ 5'-\text{CGACGGATCCATGGCACAATCGTATTGCGATCATTTGCGTGG-3'} \]

\[ 5'-\text{CAAGCTTCCGAGTAAGCTTCTTTAGCCACAGGAA-}
\quad \text{TTCGCCCT-3'} \]

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 tag and the insert. The wild-type FMO gene was then inserted into pET-28aTM containing wild-type FMO gene was checked for differences in biochemical or crystallization behavior. The plasmid 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 preincubated 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 \( \beta \)-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 Doublic (2) was followed. *E. coli* BL21 (DE3) cells were transformed and grown 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 mM thiamine, and kanamycin 50 µg/ml until mid-log phase; then they were supplemented with Lys, Phe, Thr (100 mg/ml), Ile, Leu, Val (50 mg/liter), L-selenomethionine (60 mg/liter) and 1 mM isopropyl \( \beta \)-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/ml 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:

- \( e_{273} = 134000 \text{ M}^{-1} \text{ cm}^{-1} \)
- \( e_{442} = 13350 \text{ M}^{-1} \text{ cm}^{-1} \)

**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 (pH \( < 7 \)), Heps 50 mM (pH \( > 7 \)), TAPS 50 mM (pH \( < 9 \)), using trimethylamine 2 mM as substrate and adjusting the ionic strength to 50 mM by changing the concentration of NaCl. These experiments 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 concentrations (30, 60, 90, 120 \( \mu \)M) including NADP\(^+\) in the initial mixture. For each of the four NADPH concentrations, four different concentrations of NADP\(^+\) were tested (30, 60, 90, 120 \( \mu \)M), 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 QuickChange 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.
Table S1. List of designed mutants of *Methylophaga* FMO

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