CHAPTER 4

REVIEW ON HUMAN FMO3: TOPOLOGY AND RECOMBINANT PRODUCTION IN *ESCHERICHIA COLI*

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Chapter 4

ABSTRACT

Human flavin-containing monooxygenase isoform 3 (hFMO3) is a microsomal enzyme that plays an important role in human oxidative metabolism. It converts xenobiotics containing heteroatoms (e.g. S or N atoms) into easy to secrete oxides. The efficient production of the human enzyme in a recombinant manner is desirable as it could be used for the preparation of hFMO3-related metabolites. It would also enable in-depth biochemical studies, contributing to improved insights into human metabolism and diseases related to malfunctioning of hFMO3. Nevertheless, it has been proven to be extremely difficult to produce and isolate recombinantly expressed hFMO3. As a result, many molecular aspects concerning hFMO3, such as its structure, remain unknown.

This chapter reviews previous studies that focused on obtaining soluble expression of functionally active hFMO3 in *Escherichia coli*. Also the current status of knowledge about the sequence and structural properties of hFMO3 is discussed. Our bioinformatic analysis has revealed two new sequence features. Multiple sequence alignment analysis and inspection of structures of sequence related enzymes suggest that a specific region in hFMO3 (residues 232-250) may form an additional membrane association region besides the hydrophobic C-terminus. Also a new FMO-specific sequence fingerprint was identified: (A/F)a(I/V)Gxxb (a: hydrophobic; b: charged). The respective residues are predicted to be crucial for interactions with the FAD and NADPH cofactors.

The experimental part of this chapter presents the work performed on trying to express hFMO3 as a C-terminally truncated form and/or fused to phosphite dehydrogenase (PTDH). The tested expression conditions did not result in successful overexpression and isolation of soluble hFMO3 variants, again confirming the resilience of this human enzymes towards heterologous expression and subsequent purification. A previously reported approach of functional overexpression of soluble hFMO3 by a specific C-terminal truncation could not be reproduced. Nevertheless, we could show that PTDH-hFMO3 can be expressed in a functional form in a bacterial host.

1. INTRODUCTION

Human flavin-containing monooxygenases (hFMOs, EC 1.14.13.8) are membrane-associated, O₂ and NADPH-dependent, and FAD-containing enzymes encoded by five different genes (Cashman and Zhang 2006). Each of these genes encodes a different FMO isoform, and they share 52-58 % sequence identity between each other (Table 1) (Cashman et al., 1995). Human FMOs are responsible for 10 % of the oxidative, phase I metabolism. The remaining 90 % is catalysed by so-called P450 monooxygenases, which are heme-containing enzymes (Krueger and Williams, 2005). While both monooxygenase families share some catalytic properties (e.g. dependence on NAD[P]H), hFMOs differ from P450 monooxygenases in many ways. For example, they employ a different catalytic
mechanism, can catalyse different reactions, show no significant sequence homology and use different prosthetic groups. Human FMOs catalyse substrate oxygenations via formation of a stable peroxyflavin intermediate, ready to react with a suitable substrate as soon as it enters the active site. The reactive peroxyflavin enzyme intermediate is formed before any substrate is bound to the enzyme.
Therefore, formation of this enzyme intermediate seems unregulated and therefore FMOs are often described as “loaded guns” (Eswaramoorthy et al., 2006; Orru et al., 2010). This catalytic strategy has been observed for mammalian FMOs, microbial FMOs, and other sequence-related flavoprotein monooxygenases. The activities of the human FMO and P450 monooxygenase families are usually described as complementary. Human P450 monooxygenases typically perform hydroxylations, demethylations and aminations which supplements the catalytic repertoire of hFMOs. Yet, there is also a large degree of catalytic overlap as various compounds have been found to be targeted by both P450 monooxygenases and hFMOs (Cashman et al., 1995; Krueger and Williams, 2005).
Metabolites generated by hFMOs have significantly increased polarity versus the original drugs, which allows for their rapid excretion from the human body. Most of the research efforts related to hFMOs have focussed on the isoform 3 (hFMO3), which is predominantly expressed in human liver (Cashman and Zhang, 2006). hFMO3 is active toward drugs of huge medical importance. Examples are albendazole (anti-parasite agent), cimetidine (anti-ulcer agent) or recently developed anticancer drugs with a kinase inhibition activity (Ballard, Prueksaritanont and Tang, 2007). Mutations in the hFMO3-encoding gene that lead to inactive or poorly active enzyme result in trimethylaminuria, also called “fish odor syndrome” (Yamazaki et al., 2007). There are numerous mutations known that cause this metabolic disorder which result in the accumulation of trimethylamine leading to a strong and unpleasant body odor. Human FMO3 has been described as a labile enzyme, with relatively low activity (Krueger and Williams, 2005). Even though the monooxygenase is rather inefficient as isolated enzyme, its high regio-, chemo- and enantioselectivity is difficult to be replaced with chemical synthesis. In fact, it is a challenge to generate hFMO3-related metabolites using alternative (bio)catalysts (Gul et al., 2016). Up to now, several systems for generating hFMO3-dependent metabolites have been used. One way is the use of liver tissue or isolated microsomal fractions (Störmer, Roots and Brockmöller, 2000). This approach is rather expensive, due to limited accessibility of the material, its high lability and low activity. Additionally, hFMO3-catalyzed metabolism with such preparations requires inhibition of alternative metabolic enzymes, such as P450 monooxygenases. Alternatively, one can use recombinantly produced hFMO3. It has been shown that hFMO3 can be produced by exploiting the Baculovirus expression system. The microsomes prepared from such recombinant insect cells were very useful to perform drug conversions. While hFMOs in insect microsomal fractions are commercially available, in-house preparation of Baculovirus membranes reduces costs and such preparations yield expression of the enzyme in the range of nanograms per milligram of material (Haining et al., 1997). However, it is still an expensive approach
due to laborious procedures, low yield, and costly materials (media components and fermenters). Therefore, for larger scale production of hFMO3-related metabolites, Baculovirus expression of the membrane-associated enzyme is not a convenient solution.

Altogether, producing hFMO3 in an efficient manner still remains challenging. While eukaryotic expression has been shown to be successful in producing the enzyme, the amounts and stability of the generated enzyme samples are far from ideal. In recent years, attempts of using bacterial expression for generating easy-to-use hFMO3 or other hFMOs have been reported. Ideally, these approaches result in the production of a soluble enzyme, free from surfactants, which allows for flexibility in buffer composition and opens new avenues for more advanced applications. Based on the observation that hFMO3 and its isoforms have a highly hydrophobic C-terminus, it has been suggested that expression of soluble hFMO3 requires truncation of this part of the protein. Alternatively, fusion constructs with highly soluble fusion protein partners have been explored (Brunelle et al., 1997; Hanlon et al., 2012). Yet, these approaches would benefit from insight into the structural features of hFMO3: what part of the protein determines its membrane association behaviour? This chapter provides a thorough analysis of the hFMO3 sequence and reports on our attempts on expressing functional hFMO3 in E. coli.

1. Bioinformatic Analysis of hFMO3

Sequence Homology

hFMO3 is highly preserved among mammals reaching 80-95% of sequence identity. At the same time, the sequence similarity with the other four human FMO isoforms is significantly lower (Table 1). Currently, no crystal structure is available for any hFMO or mammalian FMO. The closest FMO homologue for which the crystal structure has been elucidated shares only 29% of sequence identity with hFMO3 and comes from a marine bacterium: Methylophaga sp. (Table 2) (Alfieri et al., 2008; Orru et al., 2010). This enzyme has been shown to exhibit similar oxygenating activities when compared with hFMOs. It is capable of performing S- and N-oxygenations, and was found to be able to produce indigo blue from indole (Rioz-martinez et al., 2011). Also, other sequence-related microbial flavoprotein monooxygenases have been shown to exhibit similar oxygenation activities. Recently, we have shown that such enzymes can be used as hFMO mimics for the generation of FMO-generated drug metabolites (Gul et al., 2016). An overview of some flavoprotein monooxygenases with similar activity to hFMO3 is presented in Table 2. Such microbial FMOs can be attractive alternative biocatalysts to mimic hFMOs as they are often easy to be produced recombinantly, and for several of these monooxygenases the crystal structure has been elucidated. The latter would enable enzyme engineering approaches to tune the respective FMO. Yet, the microbial enzymes are clearly different from the mammalian FMOs. Except for a moderate to poor sequence similarity, they exhibit
different catalytic efficiencies and substrate specificities. Therefore, an effective recombinant hFMO3 production strategy is still highly desired.

Table 1. Sequence homology and recombinant expression of hFMO3 and some other mammalian FMOs.

<table>
<thead>
<tr>
<th>FMO</th>
<th>Organism</th>
<th>Uniprot entry</th>
<th>Seq. id. with hFMO3 [%]</th>
<th>Remarks:</th>
</tr>
</thead>
<tbody>
<tr>
<td>hFMO1</td>
<td>Homo sapiens</td>
<td>Q01740</td>
<td>54</td>
<td>No data</td>
</tr>
</tbody>
</table>
| hFMO2 | Homo sapiens         | Q99518        | 58                      | 2a. Truncation of 64 aa at the C-terminus results in inactive protein (Dolphin et al., 1992, 1998)  
   |         |                |                          | 2b. Truncation up to 45 residues did not result in cytosolic expression, but activity was retained (Geier et al., 2015)  |
| hFMO3 | Homo sapiens         | P31513        | 100                     | 1. hFMO3 with N-terminal MBP purified using Triton-X100 (Brunelle et al., 1997; Lattard et al., 2003; Cashman and Lomri, 2004)  
   |         |                |                          | 2a. 17 aa truncated hFMO3 was reported to give the same cellular localization as the wild type and resulted in inactive enzyme (Yamazaki et al., 2007; Shimizu, Kobayashi and Yamazaki, 2012)  
   |         |                |                          | 2b. P510Stop truncation yielded inactive enzyme (Yamazaki et al., 2007; Shimizu, Kobayashi and Yamazaki, 2012)  
   |         |                |                          | 2c. His tags at C- and N-terminus with C-terminal 17 aa truncation was reported to be expressed as cytosolic protein (Catucci et al., 2012)  
   |         |                |                          | 2d. Truncation of 17 aa at C-terminus retained enzyme activity in whole cells, but did not relocate protein into soluble fraction. Fusing PTDH to the N-terminus gives the same result (this thesis)  |
| hFMO4 | Homo sapiens         | P31512        | 52                      | No data                                                                                     |
| hFMO5 | Homo sapiens         | P49326        | 54                      | 1a. Expression with His-SUMO tag at N-terminus: the enzyme could be purified using Triton-X-100, and the tag could be removed (Fiorentini et al., 2016)  
   |         |                |                          | 1b. Active hFMO5 was obtained using E. coli JM 109 (White and Atta-asafo-adjei, 2011)  |
| FMO2  | Oryctolagus cuniculus| P17635        | 58                      | 2a. Truncation of 26 aa at C-terminus did not result expression of soluble protein in Baculovirus, but increased cytosolic fraction upon addition of high salt (Krueger et al., 2006)  
   |         |                |                          | 2b. Truncation of 26 aa at C-terminus did not change the subcellular localization (Lawton and Philpot, 1993)  |
| FMO2  | Rhesus macaque       | Q28505        | 57                      | 2. 64 aa truncation did not change subcellular distribution (Krueger et al., 2001)  |
Table 2. Some microbial flavin-containing monooxygenases and their similarity with hFMO3.

<table>
<thead>
<tr>
<th>Monooxygenase</th>
<th>Organism</th>
<th>PDB entry</th>
<th>Identity with hFMO3 [%]</th>
<th>Activity</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAMO</td>
<td><em>Thermobifida fusca</em></td>
<td>1W4X</td>
<td>15</td>
<td>Baeyer-Villiger oxidations, N- and S-oxygenations</td>
<td>de Gonzalo et al. 2005</td>
</tr>
<tr>
<td>FMO</td>
<td><em>Schizosaccharomyces pombe</em></td>
<td>1VQW</td>
<td>25</td>
<td>N-oxygenations</td>
<td>Eswaramoorthy et al. 2006</td>
</tr>
<tr>
<td>FMO</td>
<td><em>Methylophaga sp.</em></td>
<td>2VQ7</td>
<td>29</td>
<td>N-oxygenations</td>
<td>Orru et al. 2010</td>
</tr>
<tr>
<td>BVMO&lt;sub&gt;R24&lt;/sub&gt;</td>
<td><em>Rhodococcus jostii</em></td>
<td>-</td>
<td>16</td>
<td>N-oxygenation of lidocaine</td>
<td>Gul et al. 2016</td>
</tr>
<tr>
<td>FMO</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>-</td>
<td>27</td>
<td>S-oxygenations</td>
<td>Zhang and Robertus 2002</td>
</tr>
<tr>
<td>FMO</td>
<td><em>Pseudomonas stutzeri</em></td>
<td>-</td>
<td>22</td>
<td>Enantioselective S-oxygenations</td>
<td>Jensen et al. 2014</td>
</tr>
</tbody>
</table>

SEQUENCE MOTIFS

Although no protein structure is available for hFMOs, a thorough sequence analysis may provide clues concerning the structural organisation of these enzymes. For our analysis, we focused on the hFMO3 protein sequence (Uniprot entry P31513) which entails 532 amino acids. The predicted protein mass is 60,033 Da (without its FAD cofactor bound) and the predicted pI is 7.9. For mammalian FMOs, multiple sites of phosphorylation have been suggested. In fact, the PhosNet server, which detects possible phosphorylation sites in eukaryotic proteins, suggests around 80 of them in hFMO3 (www.phosphosite.org). Nevertheless, for hFMO3 only a few sites have been experimentally confirmed. The strongest evidence for a phosphorylation site is at Tyr90. Also Ser159 and the C-terminal Thr532 have been found to be phosphorylated. The NetNGlyc server predicts only one putative glycosylation site for hFMO3: Asn61 (www.cbs.dtu.dk/services/NetNGlyc). This residue was found to be highly conserved within eukaryotic FMOs and is crucial for catalysis (Koukouritaki et al., 2007). Based on studies on enzymes mutated at this position and inspection of the structures of related monooxygenases, one can conclude that Asn61 is part of the active site of hFMOs and is not a candidate for glycosylation. In fact, posttranslational modifications do not seem to be required for hFMO3 enzyme activity. One piece of evidence comes from our own work and work by other groups (Geier et al. 2015; Hanlon et al. 2012b) showing that active hFMO3 can be produced in E. coli (vide...
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Additionally, when expressed in Baculovirus, glycosylation of hFMO3 was excluded based on mass spectroscopy analysis of the recombinant protein (Haining et al., 1997).

**Table 3.** Sequence features of hFMO3 – occurrence of conserved residues, motifs and regions. The PRINTS database was used for identification of the majority of the motifs (Attwood et al. 2012). The occurrence of sequence motifs in FMOs has also been reviewed by Krueger and Williams (2005).

<table>
<thead>
<tr>
<th>Residues</th>
<th>Sequence characteristic</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-20</td>
<td>Highly hydrophobic, sometimes suggested to contain a signal sequence</td>
<td>Highly conserved in all species, gives negative result using the tested signal peptide recognition tools</td>
<td><a href="http://www.csbio.sjtu.edu.cn">www.csbio.sjtu.edu.cn</a> <a href="http://www.cbs.dtu.dk/services/SignalP-4.1">www.cbs.dtu.dk/services/SignalP-4.1</a></td>
</tr>
<tr>
<td>9-14</td>
<td>GXGXXG Rossmann fold sequence motif</td>
<td>Part or the FAD binding domain</td>
<td>(Krueger and Williams, 2005)</td>
</tr>
<tr>
<td>60 (Ser)</td>
<td>Conserved residue</td>
<td>Conserved in FMOs, predicted to be part of the active site</td>
<td>(White and Atta-asafo-adjei, 2011)</td>
</tr>
<tr>
<td>61 (Asn)</td>
<td>Conserved residue</td>
<td>Conserved in FMOs, predicted to have a catalytic role, point mutations cause loss of activity for all substrates</td>
<td>(Lomri, Gu and Cashman, 1995)</td>
</tr>
<tr>
<td>66-78</td>
<td>Low complexity</td>
<td>Highly conserved, mutation of M66 leads to loss of FAD</td>
<td>pfam.xfam.org (Treacy et al., 1998)</td>
</tr>
<tr>
<td>150-157</td>
<td>VMVSGHH</td>
<td>Highly conserved in mammalian FMOs, predicted to be linking NADPH and FAD binding domains</td>
<td>(Lomri, Gu and Cashman, 1995)</td>
</tr>
<tr>
<td>165-171</td>
<td>FXGXXXHXXXYK</td>
<td>Preserved in FMOs and described as contributing to the NADPH binding. A very similar motif is present in BVMOs</td>
<td>(Fraaije et al., 2002; Krueger and Williams, 2005)</td>
</tr>
<tr>
<td>191-196</td>
<td>GXGXXG Rossmann fold sequence motif</td>
<td>Part or the NADPH binding domain</td>
<td>(Lomri, Gu and Cashman, 1995)</td>
</tr>
<tr>
<td>327-331</td>
<td>FATGY</td>
<td>Part of the NADPH binding domain</td>
<td>(Fraaije et al., 2002; Krueger and Williams, 2005)</td>
</tr>
<tr>
<td>364-373</td>
<td>(A/F)a(I/V)Gxxb; a – hydrophobic residue, b – polar residue</td>
<td>Conserved within all mammalian and bacterial FMOs. Potentially involved in interactions with both cofactors</td>
<td>Vide infra</td>
</tr>
</tbody>
</table>

Table 3 shows an overview of sequence motifs that can be found in the hFMO3 sequence. Their specific locations are also marked in the sequence alignment (Figure 1). Several canonical flavoenzyme sequence motifs, like Rossmann fold sequence motifs, are fully preserved in hFMO3. Besides that, some FMO-specific motifs could be identified. For example, the FxGxxxHxxxYK motif is also present. This motif is highly specific for FMOs, and can be used for identification of FMO sequences in predicted proteomes and discrimination of FMOs from other sequence-related
flavoprotein monooxygenases (Riebel, de Gonzalo and Fraaije, 2013). When inspecting the multiple sequence alignment we also observed strong conservation for all FMOs in the 360–380 region of hFMO3. Interestingly, the conservation in this region is not present in BVMOs. The highest conservation concentrates on a few residues, based on which a motif can be defined as \((A/F)a(I/V)Gxxb\), where \(a\) represents a hydrophobic residue, and \(b\) is a polar residue. From the sequence alone it is impossible to deduce the molecular basis for the observed conservation. The motif was not noticed before, and to verify its role, it would be interesting to study the effect of mutating one or more of the conserved residues. In the next paragraph the possible structural role of the motif is discussed.

As mentioned before, no crystal structure is available neither for hFMO3 nor any other mammalian FMO. When looking for proteins homologous to hFMO3 in the PDB database, the best hit is a bacterial FMO. This protein shares 29% identity with hFMO3 and comes from the marine bacterium *Methylophaga sp*. The respective bacterium was isolated from sea and the FMO is supposed to facilitate catabolism of amines. Several crystal structures of this enzyme complexed with both cofactors, FAD and NADP\(^+\), have been elucidated (i.e. PDB entry 2XLP (Orru *et al.*, 2010)). These structures provide insight into the architecture of this specific enzyme but also of the sequence-related mammalian FMOs, including hFMO3. For example, many hFMO3 mutations known to cause trimethylaminuria can now be located in the bacterial FMO structure (Orru *et al.*, 2010). The roles of some of these residues in catalysis and/or structural integrity could be inferred from the structure. For example, it provided clear evidence for an important role in catalysis for Asn61 as it is located next to the reactive part of the flavin cofactor and the bound NADP\(^+\) cofactor. Apart from that, the newly identified motif \((A/F)a(I/V)Gxxb\) is present in the bacterial FMO as FYIGxxD and is part of the binding pocket around the pyrophosphate moiety of the FAD cofactor while the aspartate (Asp322) is close to the bound NADP\(^+\) cofactor (Figure 2). In fact, it is within H-bond distance from the 3-OH of the ribose moiety of the bound NADPH, and assures efficient reduction of the FAD cofactor. A multiple sequence alignment of bacterial and mammalian FMOs (116 sequences, data not shown) revealed that this specific residues is highly conserved, and mostly an Asp or Gln. Both amino acids can form hydrogen bounds suggesting a conserved role. Interestingly, in mammalian FMOs a dependency between the type of residue \(b\) and substrate specificity is observed. Asp and Gln are present in most hFMOs (hFMO2, hFMO3, and hFMO5) which are active on various amines. At the same time hFMO1, with Lys as residue \(b\), oxidises primarily tertiary amines (Cashman, 2000) and for hFMO4, with a Gly as \(b\), no clear substrate have been identified (Krueger and Williams, 2005). It would be interesting to verify whether this correlation is true by enzyme engineering studies. Very recently, a similar suggestion regarding an important role of this residue in a newly described bacterial FMO marine was suggested, based on its elucidated crystal structure (Li *et al.*, 2017). Again, the corresponding aspartate in the crystal structure

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of this FMO from *Roseovarius nubinhibens* interacts with the bound NADP*. The latter enzyme shares 65% sequence identity with the FMO from *Methylophaga* and 20% with hFMO3. Overall, the newly discovered motif [(A/F)a(I/V)Gxxb] reflects an important structural element of FMOs from both, bacteria and animals.
hFMO3 is as microsomal, membrane-associated enzyme. However, the exact molecular basis for the interaction with the lipid bilayer it is not completely clear till now. The C-terminus of hFMOs typically contain a relatively large number of hydrophobic amino acids and is often described as a membrane anchoring domain (Krueger et al., 2006). However, while highly identical to hFMO3, hFMO2 is shorter and lacks a hydrophobic C terminus while it is membrane associated (Geier et al., 2015). It has also been demonstrated that the C-terminus may not be the only membrane association segment in mammalian FMOs (Cashman and Lomri, 2004; Krueger et al., 2006). For rabbit FMO2, membrane association has been found to be based on more complex interactions (Krueger et al. 2006).
**Table 4.** Membrane binding regions in hFMO3 predicted by computational tools. The TOPCONS server combines the outcome of several tools (indicated in grey) to generate a consensus prediction.

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Putative membrane associated regions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMHMM v2.0</td>
<td>509-531 (E)</td>
<td><a href="http://www.cbs.dtu.dk/services/TMHMM">www.cbs.dtu.dk/services/TMHMM</a></td>
</tr>
<tr>
<td>HMMTOP</td>
<td>321-338 (C); 365-382 (D); 524-531 (E)</td>
<td><a href="http://www.enzim.hu/hmmtop">www.enzim.hu/hmmtop</a> (Tusnády and Simon, 2001)</td>
</tr>
<tr>
<td>MPex software</td>
<td>232-250 (B); 325-343 (C); 362-370 (D); 510-530 (E)</td>
<td>blanco.biomol.uci.edu/mpex</td>
</tr>
<tr>
<td>OCTOPUS</td>
<td>315-335 (C); 360-380 (D); 511-531 (E)</td>
<td>topcons.cbr.su.se (Viklund and Elofsson, 2008)</td>
</tr>
<tr>
<td>SCAMPI</td>
<td>2-22 (A); 364-384 (D); 510-530 (E)</td>
<td>topcons.cbr.su.se (Bernsel et al., 2008)</td>
</tr>
<tr>
<td>Philius</td>
<td>1-21 (A) signal peptide 510-531 (E)</td>
<td>topcons.cbr.su.se (Reynolds et al., 2008)</td>
</tr>
<tr>
<td>Polypophious</td>
<td>1-21 (A) signal peptide 513-531 (E)</td>
<td>topcons.cbr.su.se (Käll, Krogh and Sonnhammer, 2005)</td>
</tr>
<tr>
<td>SPOCTOPUS</td>
<td>1-17 (A) signal peptide 360-380 (D); 511-531 (E)</td>
<td>topcons.cbr.su.se (Viklund et al. 2008)</td>
</tr>
<tr>
<td>TOPCONS</td>
<td>1-22 (A) signal peptide 360-380 (D); 511-531 (E)</td>
<td>topcons.cbr.su.se (Tsirigos et al. 2015)</td>
</tr>
</tbody>
</table>

**Figure 3.** Output of the TOPCONS server when analyzing hFMO3.

Using computational tools, we analysed which parts of the hFMO3 sequence are predicted to anchor the enzyme in the membrane. Computational predictions concerning the topology of hFMO3 are shown in Table 4 and Figure 3. In total, 5 regions are indicated as potential transmembrane or signal sequences (Table 4). The suggested function of the 20 N-terminal amino acids as signal peptide can be dismissed because this part of the sequence contains the typifying Rossmann fold sequence motif (GxGxxG) which is always preceded by a short stretch of hydrophobic residues. While this sequence segment displays characteristics of being a signal peptide (hydrophobic residues followed by some small residues), it is clearly part of the FAD binding domain and cannot make extensive interactions with a membrane. Except for the consistent prediction of a C-terminal transmembrane segment, none
of the other predicted transmembrane segments are predicted by all computational protocol. Only one tool (MPex) suggests region 232-250 to represent a transmembrane segment. By inspection of sequence alignments we have found that this part of the sequence is lacking in the sequence of the soluble bacterial and yeast FMOs (alignment not shown). The crystal structures of the bacterial FMOs suggests that this segment can form an excursion from the NADPH-binding domain. Therefore, this part of hFMO3 may indeed interact with a membrane. The segments 321-338 and 365-382 are suggested as candidates for transmembrane segments by some of the computational tools. However, both sequence regions overlap with sequence motifs that were also in Table 3. Based on the analysis of the structures of the bacterial FMOs, it appears that these segments are integral parts of the NADPH binding domain and are unlikely to play a role in the interaction with the membrane. The C-terminal segment 511-531 is consistently predicted to represent a transmembrane segment. This suggests that hFMO3, and most other mammalian FMOs, are anchored to the membrane via their C-terminus.

**Figure 4.** Hydropathy plot generated by the Membrane Protein Explorer tool using default parameters (Jayasinghe, Hristova and White, 2001). Red lines indicate predicted membrane association segments while the blue cross indicates a segment of interest as discussed in the text (residues 232-250).

An additional approach to identify sequence segments that may play a role in membrane association, a hydropathy analysis was performed (Figure 4). This approach identified most of the regions mentioned in Table 4. The C-terminus stands out in the hydropathy plot, and, interestingly, also the 232-250
region is again identified as potential membrane association segment. Importantly, the membrane binding part does not need to be based only on hydrophobic regions. A protein might also interact with the membrane through electrostatic attractions. Such interactions were observed for rabbit FMO2 (Krueger et al., 2006). It was shown that a significant solubility increase of rabbit FMO2 can be achieved by using high final salt concentrations.

Concluding, there are strong indications that the C-terminus of hFMO3 plays an important role in the membrane association of the enzyme. Yet, also other parts of the enzyme may play a role in its affinity towards the membrane. The lack of more detailed structural information, for example a crystal structure of a more closely related FMO, hampers accurate predictions on which residues of hFMO3 participate in membrane interactions. We could identify a region (residues 232-250) that may responsible for such interactions. Future mutagenesis studies would reveal whether this part of hFMO3 is indeed involved in the membrane anchoring. Clearly, elucidation of the structure of hFMO3 would solve the above issues.

2. TOWARD RECOMBINANT EXPRESSION OF SOLUBLE hFMO3

PREVIOUS WORK

For a better understanding of the enzyme properties of hFMO3 and its use as biocatalyst, it is essential to be able to isolate this enzyme. As isolation from human tissue is cumbersome and inefficient, it is highly attractive to develop a recombinant expression system. The first trials for FMO expression in microbial hosts were reported in the early 90s. Already at that time, the issue of membrane association was addressed by attempts to express a C-terminally truncated mutant (Lawton and Philpot, 1993). Table 1 provides an overview of the reports on hFMO3 expression trials. Various attempts which aimed at producing C-terminally truncated variants failed in obtaining soluble enzyme. It is worth mentioning that Krueger et al (Krueger et al., 2006) were able to increase the amount of soluble cytosolic enzyme by a combination of producing a truncated mutant and employing high salt concentration, while using Baculovirus as expression host. A similar experiment resulted in no subcellular relocalization, even when a high salt concentration was used, when yeast or E. coli JM 109 was used as expression host (Lawton and Philpot, 1993). This suggests that the successful expression of a significant part of the enzyme as soluble protein may be due to the use of the specific Baculovirus expression system. In 1993 Lomri et al published on the successful cloning of cDNA from human liver in the expression vector pTrc for enzyme production in E. coli NM522. This study described that the monooxygenase could be obtained in a soluble fraction when using detergents (Triton X-100 and phosphatidylcholine). Remarkably, activity of the purified enzyme was found to be limited to tertiary amines and no S-oxygenation activity was detected (Lomri, Yang and Cashman, 1993). Recently, another research group reported on the expression of hFMO3 as a cytosolic and active enzyme in E.
coli JM109 using the pJL2 vector (Catucci et al., 2012). For this expression construct, the gene had been truncated in such a way that 17 C-terminal amino acids were not part of the recombinant protein. Furthermore, the truncated hFMO3 was decorated with poly-histidine tags at both termini. Interestingly, similar hFMO3 expression experiments reported before failed to result in expression of soluble enzyme. In 2006, cDNA from Japanese patients was used for creating pTrc99A vector-based expression constructs for producing truncated hFMO3 (truncated by 7 or 33 C-terminal amino acids). Using E. coli JM109, the recombinant hFMO3 was still membrane associated or insoluble and displayed no more than 10% of the expected activity. In addition, over two decades ago the same host in combination with another expression vector (pKKHC) was employed for the expression of a 26 amino acid C-terminally truncated FMO2 from rabbit (Lawton and Philpot, 1993). This experiment revealed that the engineered monoxygenase also remains attached to the membrane.

The findings discussed above and the data summarized in Table 1 suggest that recombinant production of soluble and active hFMO3 is feasible with the use of detergents. Yet, several studies contradict such conclusion. In fact, in the studies that report on soluble and active (truncated) hFMO3, often the isolated amount or measured monoxygenase activity is rather low or not reported (Catucci et al., 2012). From the latter report, it is also difficult to retrieve all required experimental details for reproducing the expression and purification. For example, the paper of Catucci et al (2012) contains a rather cryptic section describing the experimental procedures and lacks vital details regarding centrifugation steps, used temperature and buffers. Besides that, remarkably, the presented UV-Vis spectra of the wild type and truncated hFMO3 appear fully identical, which is rather unexpected (see Figs. 2C and 2D in Catucci et al, 2012). Also, a quartz crystal microbalance experiment to test the membrane association of the truncated enzyme purified from the cytosolic fraction and the wild type enzyme obtained from the membrane fraction lacks a negative control. This makes it difficult to judge whether remaining membrane particles could have resulted in a false positive result for the wild type enzyme sample. Interestingly, Shimizu et al (2012), in a letter to the editor, put forward their findings that in their hands an identically truncated hFMO3, while expressed in the same E. coli strain, did not end up on the soluble fraction (Shimizu, Kobayashi and Yamazaki, 2012). The response of the Gilardi group to this letter (Enna, 2012) does not convincingly take away worries concerning the potential flaws. Additionally, the group has shown reluctance to share the expression vector with others which makes it impossible to reproduce the reported results.

Several studies have demonstrated that the recombinant expression of hFMO3 fused to a protein partner can be successful. Maltose binding protein (MBP) has been a popular fusion protein and is typically fused to the N-terminus of hFMO3 (Brunelle et al., 1997; Lattard et al., 2003; Reddy et al., 2010). For this fusion, a rather peculiar linker with a repeat of ten asparagines has been used. This approach was also successfully applied for another FMO isoforms (Table 1). The purification of MBP fusion proteins is relatively simple because affinity chromatography can be used, exploiting the
affinity of MBP towards amylose column material. Purification of MBP-hFMO3 yields around 2-5 mg of protein per 1 L of bacterial culture (Reddy et al., 2010). Though MBP often improves the solubility of the fusion partner protein, the created MBP-hFMO3 fusion still required surfactants in the purification procedure. Typically, 0.5 % Triton X-100 is used (Motika et al., 2009; Reddy et al., 2010). It has been reported that the MBP-hFMO3 fusion displays a slower turnover rate compared to native hFMO3 (Reddy et al., 2010). Probably the interaction between MBP and the monooxygenase influences the accessibility and/or architecture of the active site of hFMO3. The effect of detergents on activity are similar for the fusion and the non-fused enzyme (Reddy et al., 2010). Interestingly, it has been observed that fusing hFMO3 to MBP increases its thermostability and resistance towards proteolysis (Brunelle et al., 1997).

All in all, active MBP-hFMO3 fusions can be expressed in E. coli and the recombinant enzyme can be purified and used for in vitro research on human metabolism (Reddy et al., 2010). The fact that, using detergents, the fusion enzyme can be obtained as active enzyme demonstrates that membrane binding is not required for hFMO3 activity. Encouraged by this, we set out to develop a new recombinant expression vector to produce hFMO3 that could be easily isolated and used.

A NEW APPROACH: EXPRESSION OF (TRUNCATED) hFMO3 FUSED TO PHOSPHITE DEHYDROGENASE

In 2008 it was shown that various Baeyer-Villiger monooxygenases (BVMOs) can be overexpressed in E. coli when fused to phosphite dehydrogenase (PTDH) (Pazmino et al. 2008). Such fusion enzymes have been described as self-sufficient BVMOs as they can regenerate NADPH at the expense of (cheap) phosphite. The approach of fusing PTDH was also successfully shown for various other flavoprotein monooxygenases and a P450 monooxygenase (Torres Pazmiño et al., 2009; Beyer et al., 2016). For expression of these latter fusion proteins a dedicated pBAD-based expression vector (pCRE) containing a codon-optimized gene encoding for an engineered PTDH that is thermostable, solvent tolerant His-tagged, and active with both NAD⁺ and NADP⁺, was developed (Torres Pazmiño et al., 2009). Except for optimizing the PTDH-encoding gene, also the linker that fused the two enzymes was optimized (Riebel et al. 2009). By equipping the target enzyme with an effective coenzyme recycling activity, which facilitates the use of these enzyme in a cost-effective manner, the fusion with PTDH was also found to promote higher expression of soluble protein. This effect has previously been described when using maltose-binding protein (MBP) or the so-called Small Ubiquitin-like Modifier protein (SUMO) as fusion tags. Because FMOs are sequence related to BVMOs, we decided to use the pCRE expression vector to express hFMO3 fused to His-tagged PTDH (as N-terminal tag). Except for expressing a fusion protein, we also generated pBAD-based expression plasmids for the expression of N-terminally His-tagged hFMO3, C-terminally truncated hFMO3, and C-terminally truncated hFMO3 with a C-terminal His-tag (see Table 7).
Upon testing different culture conditions and subsequent SDS-PAGE analysis, it was found that none of the non-fused hFMO3 expression plasmids resulted in soluble or solubilized hFMO3. Irrespective of the used temperature or arabinose concentration, for the N-terminally His-tagged hFMO3 no significant overexpression was observed in the prepared crude cell extracts. Overexpression of C-terminally truncated hFMO3 was observed at all tested growth temperatures as evidenced by a clear protein band at around 63 kDa upon SDS-PAGE analysis. Yet, the overexpressed protein always was found to be insoluble. A similar result was found for the His-tagged version of the truncated hFMO3. Importantly, analysis of the crude cell extract of cells containing the expression plasmid for expression of the PTDH-fused hFMO3 revealed clear overexpression of a protein with molecular mass of around 100 kDa (predicted mass is 103 kDa) (Figure 5). By subcellular fractionation, it became clear that in this case the recombinant protein is in the membrane fraction after the first mild fractionation step (Figure 6). Unfortunately, the protein could not be solubilized with the tested detergents. This suggests that the fusion protein is membrane associated and that solubilisation from the membrane is difficult or that the protein tends to aggregate during the fractionation procedure.

Using Ni-Sepharose affinity chromatography, it was attempted to isolate the recombinant PTDH-hFMO3 from crude cell extracts. Unfortunately, SDS-PAGE analysis revealed that none of the tested elution buffers contained a protein with the expected molecular mass. In fact, it was found that the fusion protein eluted in the flow-through fraction. The fusion protein could also not be purified from a detergent-enriched crude extract. There are many plausible reasons for the resilience towards purification. It could be that the used detergent did not result in solubilisation or did induce aggregation of the protein. Another possible reason can be a shielded His-tag (possible covered by the fused hFMO3), not accessible to bind to the Ni-Sepharose.
Motivated by the observation that the PTDH-fused hFMO3 is expressed differently when compared with the other hFMO3 expression constructs, we set out to use recombinant cells for testing a hFMO3-catalyzed oxidation reaction. Cells were grown for expressing all four different recombinant proteins using conditions for which expression of the protein had been confirmed (Table 5). As test reaction, the sulfoxidation of albendazole was analysed. The highest sulfoxidation activity was observed for the cells that had expressed PTDH-hFMO3. These cells displayed 2-3 fold higher activity when compared with cells containing no plasmid or another hFMO3 expression plasmid. The observed hFMO3 activity is line with the expression of the PTDH-hFMO3 as membrane-associated protein in E. coli. It is also another confirmation that hFMO3 can be functionally expressed in a bacterial expression host.

Table 5. Expression conditions and activity of recombinant E. coli cells expressing all different hFMO3 variants.

<table>
<thead>
<tr>
<th>construct</th>
<th>expression conditions</th>
<th>albendazole sulfoxide formation [%]a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>temperature [°C]</td>
<td>arabinose concentration [%]</td>
</tr>
<tr>
<td>His-PTDH-hFMO3T</td>
<td>24</td>
<td>0.002</td>
</tr>
<tr>
<td>His-hFMO3</td>
<td>30</td>
<td>0.02</td>
</tr>
<tr>
<td>hFMO3T</td>
<td>24</td>
<td>0.02</td>
</tr>
<tr>
<td>hFMO3T-His</td>
<td>24</td>
<td>0.02</td>
</tr>
<tr>
<td>no plasmid</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

4. CONCLUSIONS

Human FMO3 plays a crucial role in the oxidative metabolism. So far, no crystal structure has been solved for this membrane-associated flavoenzyme and the molecular basis for its interactions with the membrane remain mysterious. It appears that the C-terminus acts as membrane anchor, but at the same time it does not seem to be the only membrane-binding protein segment. In this chapter, based on a bioinformatics analysis, a candidate region (residues 232-250) residue has been identified that may play a role in membrane-binding. Future experimental work may confirm such a structural role. It may help to engineer a hFMO3 variant which can be expressed as a soluble and functional monooxygenase. Besides the finding described above, inspection of a multiple sequence alignments revealed a new FMO-specific sequence motif: (A/F)a(I/V)Gxxb, where a is a hydrophobic and b is a polar amino acid. Upon analysis of related flavoprotein structures, it can be concluded that the conserved (I/V)G residues are part of a structural motif that allows proper FAD cofactor - protein interactions. The
conservation of the polar residue seems to be involved in an interaction with the NADPH cofactor. This shows that the motif is at the core of the protein structure, involved in interactions with both FMO cofactors. It would be interesting to experimentally verify whether the type of amino acid at position b directly affects enzyme activity.

In the experimental part of this chapter, we tested expression of several engineered hFMO3 variants in E. coli. For that, we prepared expression plasmids for producing hFMO3 with or without PTDH as fusion partner (Torres Pazmiño et al., 2009), with or without a C-terminal truncation, and with or without a His-tag. Unfortunately, none of the constructs resulted in expression of hFMO3 that could be purified as soluble or solubilized protein. Gratifyingly, the expression of the PTDH-hFMO3 fusion revealed significant monooxygenase activity when using whole cells. This is in line with the observation that this was the only recombinant protein that did end up in the membrane fraction. All other constructs led to no expression or expression as insoluble protein. The successful expression of PTDH-hFMO3 may provide a lead to develop an effective expression system that can be used to perform hFMO3 metabolite synthesis and analysis. The fusion partner may help in efficient regeneration of NADPH that is needed for hFMO3 activity.

**Materials and Methods**

*Bioinformatics* - The multiple sequence alignment (using ClustalW) and visualization was performed using Geneious 8.0.5. Visualisation and inspection of enzyme structures has been performed using the PyMOL software. All other employed bioinformatics tools are indicated in the text.

*Materials* - All chemicals were obtained from ACROS Organics, Sigma-Aldrich, PROMEGA, Qiagen, Roche Applied Sciences, New England or ThermoScience and used without further purification. Oligonucleotide primers for PCR reactions were obtained from Sigma and DNA sequencing was done at the GATC. Amplifications of inserts and vectors were performed with the Expand Long Range dNTPack kit from Roche. Digestion and ligation buffers, if not indicated, were purchased from the New England. Three vectors were used: pBAD/myc-HisA, pCRE (Torres Pazmiño et al., 2009; Mascotti et al., 2013) and pPTDH (a pBAD vector with a gene encoding a 18x PTDH mutant, see(Dudek et al., 2013). The *E. coli* codon-optimized gene for hFMO3 expression (UniProt entry P13531) was purchased from GenScript (the sequence is shown in the supplementary material).

*Cloning* - The Sequence and Ligation Independent Cloning approach (SLIC) was taken to generate the desired expression plasmids. For this, the general procedure reported before (Li and Elledge, 2012) was slightly modified. For the amplification of the insert, primers of 26 base pairs with an additional 15 base pairs complementary to the vector overhangs were used. Primers for vector amplification had a similar length and blunt ends. PCRs conditions are described in Table 6, and all the used primers are
listed in Error! Reference source not found.. In 10 µL water supplemented with 1 µL of buffer 4, 50 ng of amplified vector was incubated for 22 min at 22 °C with 0.025 U of polymerase T4. The reaction was stopped by the addition of 1 mM dCTP. Subsequent ligation was performed in Promega ligation buffer for 30 min at 37 °C. For the ligation, 1:2 insert to vector molar ratio was used.

**Table 6. PCR conditions for amplification for SLIC.**

<table>
<thead>
<tr>
<th>Temp [°C]</th>
<th>Time [min : sec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>0:29</td>
</tr>
<tr>
<td>94</td>
<td>0:10</td>
</tr>
<tr>
<td>50-64 for pCRE</td>
<td>0:15</td>
</tr>
<tr>
<td>54-67 for pBAD and pPTDH</td>
<td>4:10 (for vector)</td>
</tr>
<tr>
<td>50-67 for hFMO3</td>
<td>3:00 (for insert)</td>
</tr>
<tr>
<td>68</td>
<td>10:00</td>
</tr>
<tr>
<td>8</td>
<td>∞</td>
</tr>
</tbody>
</table>

**Table 7. Primers used for vector and insert amplifications.** For the two last constructs the stop codon was introduced already in the primer and an extra QuickChange reaction was not needed. The position of “His” in the names indicates whether a His-tag is present as N- or C-terminal tag. The “T” in the names indicates a C-terminal truncation.

<table>
<thead>
<tr>
<th>Name</th>
<th>Truncation at C-terminus</th>
<th>Vector</th>
<th>Vector amplification primers</th>
<th>Insert amplification primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>hFMO3T-His</td>
<td>16</td>
<td>pBAD</td>
<td>FW: ATGTAATTCCTCCTGTTAGCCCAAAAAAC&lt;br&gt;RW: CTGCAGCTGGTACCATATCGGAATTC</td>
<td>FW: CAGGAGGAATTACATCATATGGGTAAGAAAGTTGCTATCATTGGTGCTG&lt;br&gt;RW: TGTACACGCTGCAATTTTCAGCACCAGGAAGAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FW: CAGGAGGAATTACATCATATGGGTAAGAAAGTTGCTATCATTGGTGCTG&lt;br&gt;RW: TGTACACGCTGCAATTTTCAGCACCAGGAAGAGC</td>
<td></td>
</tr>
<tr>
<td>His-hFMO3</td>
<td>-</td>
<td>pPTDH</td>
<td>FW: GGGCGCGAACAAAAACTCATCTCAGAAGAGGATC&lt;br&gt;RW: GCGGGATGGCGAACAGTTACAGCCAATGAAAGAAAAAGCACG</td>
<td>FW: CTGCCGCGCGGACTGGTACATATGGGTAAGAAAGTTGCTATCATTGGTGCTG&lt;br&gt;RW: TTGGGCCCGAACAAAAACTCATC</td>
</tr>
<tr>
<td>hFMO3T</td>
<td>17</td>
<td>pPTDH</td>
<td>FW: ATGTAATTCCTCCTGTTAGCCCAAAAAAC&lt;br&gt;RW: TTGGGCCCGAACAAAAACTCATC</td>
<td>FW: CTGCCGCGCGGACTGGTACATATGGGTAAGAAAGTTGCTATCATTGGTGCTG&lt;br&gt;RW: TTGGGCCCGAACAAAAACTCATC</td>
</tr>
<tr>
<td>His-PTDH-hFMO3</td>
<td>17</td>
<td>pCRE</td>
<td>FW: ACCAGCTCCAGCACATCTACTGAG&lt;br&gt;RW: TTGGGCCGGCACTGGTACATATGGGTAAGAAAGTTGCTATCATTGGTGCTG&lt;br&gt;RW: TTGGGCCCGAACAAAAACTCATC</td>
<td>FW: GATCTTGCTGGTACATATGGGTAAGAAAGTTGCTATCATTGGTGCTG&lt;br&gt;RW: TTGGGCCCGAACAAAAACTCATC</td>
</tr>
</tbody>
</table>

The DNA was transformed into chemically competent *E. coli* TOP10 cells and the sequences of the obtained plasmids were verified by DNA sequencing. Some constructs required a QuickChange reaction for introducing or removing a STOP codon (for creating a C-terminal His-tag). By this approach, the hFMO3 gene was inserted into the pCRE vector, while in case of pPTDH, the PTDH gene was replaced with hFMO3. The QuickChange reactions were performed using the MasterMix kit.
Review on human FMO3: topology and recombinant production in *E. coli*

from Thermo Scientific using the conditions recommended by the manufacturer. The final constructs were named as indicated in Table 7.

**ENZYME EXPRESSION OPTIMIZATION AND SUBCELLULAR LOCALIZATION**

*E. coli* TOP10 cells were transformed with the prepared plasmids and grown overnight at 37 °C in 5 mL of Luria–Bertani medium (LB) supplemented with 50 μg/mL ampicillin. The next day, 5 mL of LB with the same concentration of ampicillin and 0.02% or 0.002% (w/v) L-arabinose were inoculated 1:100 v/v with the cultures from the previous day. Subsequently, the cells were grown for 48 h at 37, 30, 24 °C or 72 h at 17 °C. Cells were harvested by centrifugation and analysed for expression and subcellular localisation of the recombinant protein according to the scheme shown in Figure 6. The collected fractions were analysed with SDS-PAGE.

**His-tag purification of recombinant His-PTDH-hFMO3**

Using different culture temperatures (24, 30, and 37 °C) and 0.002% arabinose, several 100 mL cultures were grown. All the following steps were performed at 4 °C. Cells of each culture were harvested and resuspended in 10 mL 50 mM Tris/HCl (pH 8) containing 100 μM FAD, 0.5% Triton X-100 and EDTA-free protease inhibitor mix from Thermo Scientific. The cell suspension was sonicated and the obtained cell lysate was fractionated according to the scheme in Figure 6. The fractions with soluble or solubilized proteins were used for protein purification. For this, each fraction was incubated with Ni-Sepharose in 50 mM Tris/HCl (pH 8). The bound protein was eluted using three different elution buffers: 5, 200, and 500 mM imidazole in 50 mM Tris/HCl, 5% glycerol (pH 8). The buffers were applied one after another. The eluted fractions were analysed by SDS-PAGE.
Figure 6. Scheme showing the protocol for preparation of cellular fractions for determining the subcellular localisation of expressed protein.

Whole cell conversions of Albendazole

For activity screening upon expression using different conditions, an enzyme activity assay was used based on the assay published before (Prasad, Girisham and Reddy, 2010). For that, sterile test tubes containing 1 mL of LB supplemented with 50 mg/L ampicillin, 4 mM β-cyclodextrin and 100 mg/L of albendazole (1% final concentration of DMSO) were inoculated 1:100 (v/v) with overnight grown cultures. To analyse the formation of the albendazole sulfoxide, cells were spun down and the supernatant was diluted with acetonitrile (ACN) 1:1 (v/v) and centrifuged at 8000 g for 4 min. The resulting supernatant was diluted with ACN containing 0.2 % formic acid and again centrifuged at 8000 g for 4 min. The resulting supernatant was supplemented with an internal standard (IS), acetaminophen and subsequently analysed by LC-MS. The apparatus (Thermo Scientific HPLC) was equipped with a C18 reversed-phase column (GraceSmart RP 18 5 μm, 2.1×150 mm; Grace Davison, Lokeren, Belgium). Solvent A (H2O with 0.1 % formic acid) and solvent B (ACN with 0.1 % formic acid) were used for elution using the following program: 5 to 50 % B over 10 min, increase to 95 % B in 2 min, and 1 min hold. The flow rate was 250 μL/min. The LC was coupled to the ESI mass spectrometer equipped with a triple quadrupole analyser from Thermo Finnigan. Analysis was performed in the positive mode (settings: spray voltage 4000 V, skimmer offset 0 V, sheath gas pressure 30, auxiliary gas pressure 15, tube lens offset 89 V, capillary temperature 300 °C, scan range m/z 50-400). For selected reaction monitoring (SRM), the following transitions were analysed: albendazole (266/234), albendazole sulfoxide (282/240), albendazole sulfone (298/266) and the IS (152/110). For each sample, the results were normalized for the internal standard and corrected for the formation of albendazole sulfoxide by the negative control sample (E. coli without a hFMO3-expression plasmid).

References


SUPPLEMENTARY DATA

DNA sequence of codon-optimised hFMO3 purchased from the GenScript

CATATGGGTAAGGAATGGCTATCATTTGGTGTGCTGATGGCCTGGCATCAGTTGGAATGTCT
GGAAGAAGGTCTGGGACCCCAGCTGCTACATAATTCTAGAGCTGCTGCTGCTTGGGAAATTTTCAG
ACCAGCAGAAGAGGCACCCAGCTGCTATCTAATAATCAGTTTTTCAAGACGCTCTAAAGTTGATG
TGTGGTGGGTTTCTCCCAGGATGACTCTCCCGGAATGACTCAACAGTACAGGCAAATCTCGAGATA
ATTTATACCTCAGCAGATTCGTCAGTTTCGAGTACTATCTTCTCCAGGCTGCTGGTGGGAAAGAAGGTA
TCTTTTCTGATGGCGATGACATGGCTGCTGCGTCAGGCAGTATCCACAGGTATCGGCTGCTAT
TCTGTACCGTGACAGGTAGTATTTTCACTCAGGCTAGTGCGCTCCTGGGTTATGAGTGCCTCTGGGA
TAATGGTTATCCGTTGGAATGCTGCTGTTTACCCGGCTTTCGGCAAGCTTTCTGAAAAACAAATCTCGCA
CCGCGATCTCCGAGTTGCTGTATGTAACAAATAAGCAGGCCGCTTTCAAAACAGAAATACGGCCTG
ATGCCCTGTAACGCGTGTCTCTGCGCGAAAGCTGATATGACGACTGAGATTTCTGAGGGCTATT
CGTTCGGCTACCCGTTTCTGGATGAAAGCATCATCAAATCTCGTAACAACGAAATCATCCTGTTCAAAGGTGT
TTTCCCGCCGCTGCTGGAAGATTTGATAGAATCGGCTTTGCGGCTTATGCGCTGCTGTTTCTGAGAG
CGGCTGATCCGGTAAACCGAAGTTAAAGAATTTACCCGAAGATGCTGCTGCTGACTGTGCAGCCCGATCTG
CGATCGTTGGAACGCTCAAGAATTTAAGGCTACTGTAGCCTGCGGTAAGCGATATGGAAC
GACAATACGAGAAAAATGGGAAAGTTTTGCGGAAACCAGAATCTTCCGGCAGAGGCAATTA
TATTGTGACATGGGCAAGTGACCTTTACGCTGCTGTTGTCTGCAGGAAATCCGTAACATCTGCTGCTGCA
CCGACTCCGAACTGGCTATGGGAAGTCTATATCTCCGGCCCGCGTCAGCAGCTTCTCCGGTCTGCTG
CCGGTCAATGGCGGCTGACGTAACGTATCTACGAGCTGAAATGGGATGCTCTCTGAAAAACCGGATG
GACGCGTGTCGGGCGCGCTGAAAACCGCTGCTTTTCTTCTTACATTGGCTGAAACTGTTGTCGACCATCC
CGATCTGCTGTGATTGGCTCTGCTCTTGATGACCTAAAGCTT