Molecular cloning and characterization of a full-length flavin-dependent monooxygenase from yeast

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Abstract

Eucaryotes contain a class of enzymes called flavin-dependent monooxygenases (FMOs). Unlike mammals, yeast have only a single isoform—yFMO. Deletion mutants suggested that yFMO may play a role in folding proteins which contain disulfide bonds. Recently we detected two nucleotide errors in the GenBank sequences attributed to the yFMO gene. This previously led us to express and characterize a 373-residue catalytically active protein instead of the correct 432-residue enzyme. Here we report the sequencing, expression, and enzyme characterization of the full-length form of yFMO. Comparison of the two forms of yFMO showed similar pH profiles and $K_m$, $k_{cat}$, and $V_{max}$ values using glutathione as a substrate. These results indicate that the full-length yeast FMO has biochemical and catalytic properties similar to those of the truncated protein. Therefore, it is likely that the hypotheses concerning the enzyme’s function proposed earlier are still valid.

Keywords: Flavin-dependent oxygenase; Kinetic parameters; Sequence error; Protein expression

In the past three decades, a group of flavin-containing monooxygenases (FMOs) have been identified in many mammals, including humans [1], pigs [2], and rabbits [3,4]. At least five common isozymes have also been discovered in various organs and tissues, including lung, liver, and kidney [5]. Studies on pig liver FMO [6] have provided a model of the catalytic mechanism and substrate specificity for FMOs. The major catalytic steps are summarized in Fig. 1 [5]: NADPH reduces the FAD bound in FMO to FADH$_2$; molecular oxygen then oxidizes the FADH$_2$ to 4a-hydroperoxyflavin, FAD-HOOH. Incoming substrate (SH) is oxygenated to SOH, while the enzyme-bound peroxide is reduced to hydroxyflavin, FADHOH, and, finally, water and NADP$^+$ are released. The intermediate FADO$_2$H$_2$ is quite stable, giving a characteristic spectrum [7,8]. Another interesting feature of pig liver FMO is the broad substrate specificity. The enzyme recognizes many secondary and tertiary amines, hydrazines, and organic sulfur compounds [6]. Even though other mammalian FMOs differ in substrate specificity, they generally can recognize a range of substrates. Given the fact that many substrates of mammalian FMOs are xenobiotics and the high concentration of the enzymes in liver, it was suggested that the physiological function of mammalian FMOs was to oxidize xenobiotic nucleophiles [5].

In recent years, proteins sharing sequence homology with mammalian FMOs were identified from several nonmammalian eukaryotes and assigned different physiological functions [9–11]. For example, YUCCA from Arabidopsis contains putative FAD and NADPH binding regions and was found to be involved in auxin biosynthesis, catalyzing the crucial step of N-oxygenation of tryptamine [12].

Based on the known genomic sequence of Saccharomyces cerevisiae [13], there is only one FMO-like protein in yeast, yFMO. We used the genomic sequence to clone and express that open reading frame (ORF) in Escherichia coli [14]. The purified yeast enzyme differed from the mammalian FMOs in that it recognized only certain biological thiols as substrates; these included glutathione, cysteine, and cysteamine. Subsequent gene knockout and other experiments suggested that the yeast FMO-like protein was important for the correct folding of proteins which contained disulfide bonds. It was
proposed that it functioned by generating oxidizing equivalents that ultimately influenced the environment in the endoplasmic reticulum [15–17].

In continuing our studies, we found that there is an error in the original yeast FMO-like ORF sequence in GenBank. This error, when incorporated into our cloning strategies, caused the expression of a modified yFMO, which we will refer to as the truncated form, or yFMOt. That protein lacks 38 amino acids at the C terminus. Also, due to an open reading frame shift, caused by an extra G at position 1080, the presumed stop codon was read through, adding 36 vector amino acids to the protein.

In this paper, we report the cloning of the true full-length yFMO cDNA, its expression in E. coli, and the comparison of substrate specificity and kinetic parameters to the previously reported truncated enzyme.

Material and methods

Yeast strains and growth conditions

The S. cerevisiae strains used were X2180 and TR2. They were grown at 28°C in YPD medium, which contains 1% yeast extract, 2% Bactopeptone, and 2% glucose.

DNA sequencing and cloning of the yFMO gene

Genomic DNA was isolated from S. cerevisiae strains X2180 and TR2 using Dnasey Tissue kit (Qiagen). In all PCR amplifications, Pfu DNA polymerase (Stratagene) was used to minimize the error rate. To facilitate DNA sequencing, the fmo gene, including upstream and downstream regions, was amplified. The forward primer was (5’-TACAAGTACATTCTTTTTAC G-3’). The 1.5-kb PCR product included 163-bp upstream and 24-bp downstream non-translation regions and the 1.3-kb coding region. The PCR product was recovered from DNA agarose gel using QIAquick Gel Extraction kit (Qiagen) and then sequenced.

To clone only the coding region of yFMO gene, another pair of primers was used: 5’-GAAGTAACCTACATATGACAGTGAATGAC-3’ and 5’-CATGAGGAAACGCTGAGTAAGCGGCAC-3’. That PCR product was recovered, digested with Ndel and XhoI, and inserted into pET16b vector (Novagen). The sequence of the recombinant construct, pET16b-fyFMO, was verified by restriction digests and DNA sequencing. DNA autosequencing was performed at the ICMB facility center at the University of Texas at Austin.

Expression of the recombinant N-terminal His-tag yFMO protein in E. coli

The plasmid pET16b-fyFMO was transformed into E. coli strain HMS174(DE3) cells. A single transformant colony containing the plasmid was first grown overnight in 10 ml LB media (containing 10% NaCl, 10% tryptone, 5% yeast extract) with 100 μg/ml ampicillin at 37°C. The overnight cell culture was then diluted into 1 liter fresh LB media, containing 100 μg/ml ampicillin, and grown at 37°C. When the cell culture reached an A600 of 0.5, it was induced with 1.0 mM isopropyl- β-D-thiogalactopyranoside (Gold Biotechnology). After growth at 30°C for 4 h, the cells were harvested by centrifugation at 7000 g for 5 min at 4°C. Cell pellets were stored at −20°C.

Purification of the recombinant N-terminal His-tag yeast FMO protein

The purification process was undertaken at 4°C. Fresh or frozen cell pellets (about 12 g wet weight) were resuspended in 20 ml cold lysis buffer (100 mM sodium phosphate, pH 7.8, 1 mM phenylmethylsulfonyl fluoride) and lysed twice in a precooled French Press cell (SLM Aminco, Urbana, IL) at 700 psi. The broken cells were subjected to centrifugation at 70,000 g for 40 min at 4°C, and the supernatant was loaded onto a 3-ml Ni-NTA agarose (Novagen) column equilibrated with cold lysis buffer. Once the sample was loaded, the column was run in the dark. It was washed with lysis buffer and wash buffer (lysis buffer with 80 mM imidazole) gradient. Elution fractions were collected and analyzed on 10% SDS-PAGE. Fractions containing His-tagged yeast FMO protein were pooled and dialyzed into storage buffer (50 mM sodium phosphate, 10% glycerol, pH 7.0).
Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

**Preparation of apo-enzyme**

About 5 mg of purified His-tagged yFMO protein in 10 ml volume was dialyzed into 2 liters of semidenaturing buffer (50 mM sodium phosphate, pH 7.0, 2 M urea, 2 M KBr) at 4 °C, for 12–24 h; the dialysis buffer was changed twice during this interval. Then the protein was dialyzed into 2 liters storage buffer (50 mM sodium phosphate, 10% glycerol, pH 7.0) at 4 °C for 12–24 h, again using two changes of buffer.

**Measurement of FAD concentration in holoenzyme**

Five hundred microliters of yFMO protein sample was incubated with 50 µl 3 M trichloroacetic acid, on ice, in the dark, for 5 min. The mixture was centrifuged at 12,000 rpm for 10 min at room temperature, and 100 µl 1 M KOH were added to the supernatant. The OD_{450} was measured before and after reduction with sodium dithionite to reduce FAD. To obtain the concentration of FAD bound in the holoenzyme, the difference of OD_{450} was divided by the extinction coefficient of FAD, 11.3 mM⁻¹.

**Oxygen uptake assay**

The recombinant yFMO activity was measured by substrate-dependent oxygen uptake assay [18] at 37 °C. The assay was performed in a 2-ml thermostated oxygen chamber (Gibson Medical Electronics) with a Clark-type oxygen electrode (Yellow Springs Inc.). The signals were recorded by an EU-200-02 DC offset module. The reaction mixture contained 0.2 M sodium phosphate buffer with 0.5 mM EDTA, 50 µM FAD, and the NADPH regenerating system (0.2 mM NADP⁺, 1.5 mM glucose-6-phosphate, and 1 U glucose-6-phosphate dehydrogenase) in a 2 ml volume at pH 6.4. After a 2 min incubation, the enzyme was added, and the substrate-independent oxygen uptake was recorded for about 2 min. (This measures the “blank rate” oxidase activity.) Then the organic substrate was added and the substrate-dependent oxygen uptake was recorded for about 4 min. To measure the pH profile of NADPH oxidase activity, assays were performed at pH 5.5, 5.7, 7.0, 7.5, and 8.0. To measure the Km values for NADPH and FAD, the NADPH oxidase activity was monitored at pH 6.4 in the absence of any substrate. Apo-enzyme was used in the assay, with a range of FAD concentrations, to measure the Km for FAD. In the assay to determine the Km for NADPH, 50 µM FAD was introduced and the usual NADPH regenerating system was substituted by a range of NADPH concentrations. To calculate substrate-dependent oxygen uptake, in addition to the substrate-independent NADPH oxidase activity, the background from the substrate itself was subtracted from the total rate.

**FPLC Superdex 200 gel filtration analysis**

After the Superdex 200 (Amersham-Pharmacia Biotech.) column was equilibrated with 10 column volumes of the column buffer (0.1 M sodium phosphate, 0.25 M sucrose, pH 6.5) at room temperature, purified yFMO protein was loaded onto the column. The column was run at a flow rate of 0.5 ml/min and 0.5-ml fractions were collected. Fractions were analyzed on 10% SDS–PAGE. The standard curve for the Superdex 200 was calculated from the elution profile of the protein standards thyroglobulin, IgG 158, ovalbumin, myoglobin, and cyanocobalamin (Bio-Rad) with molecular weights of 670, 158, 44, 17, and 1.35 kDa, respectively. Protein elution was monitored by absorbance at 280 nm.

**Results**

**Nucleotide sequence analysis**

The DNA sequence of *S. cerevisiae* FMO-like cDNA is located on chromosome VIII, nucleotides 454227 to 455348 (GenBank NC_001140). When manual sequencing was performed during the previous work, no difference between the GenBank sequence and the locally cloned cDNA was detected [14]. In the process of cloning the yeast FMO cDNA into other *E. coli* expression vectors, one difference was observed at position 1080 from the automatic sequencing of the cDNA clone, as shown in Fig. 2. Our locally cloned yFMO cDNA had an extra G base compared to the GenBank sequence (NC_001140, position 455306 of *S. cerevisiae* chromosome VIII). To verify that this difference was not introduced by the cloning process, genomic DNA from two *S. cerevisiae* strains, X2180 and TR2, was used as the template to amplify the FMO-like gene by PCR. Both PCR products had the same sequence as the newly

![Fig. 2. Partial sequence alignment of the locally sequenced yeast FMO cDNA and the GenBank sequence. The differences between them are shaded; stop codons are underlined.](image-url)
determined yeast FMO cDNA clone. Further sequencing found that a C base was missing from the GenBank sequence at position 1277 (position 455503 of S. cerevisiae chromosome VIII). This C base is after the originally proposed stop codon in the GenBank sequence corresponding to the FMO open reading frame. As a consequence of these changes, the correct S. cerevisiae FMO cDNA was found to contain 1299bp nucleotides, encoding 432 amino acids rather than 373 amino acids as originally indicated by the GenBank sequence.

The newly determined protein sequence is aligned with the original yFMOt sequence and with human and pig FMO protein sequences in Fig. 3. The full-length yFMO and yFMOt are identical for the first 394 amino terminal residues. The last identical residue is marked by a shaded bar in Fig. 3 at absolute position 467. All conserved domains and motifs found previously [14], including the hypothetical FAD signature sequence (absolute position 13–18), the NADP+ signature sequence (absolute position 198–203), the “FATGY” sequence (absolute position 334–338), and the “D/QELL” sequence (absolute position 444–447) are retained. After yFMO residue 394, however, the new and old yFMO sequences diverge.

**Expression and purification of His-tagged full-length yeast FMO (His10-yFMO) protein**

Since kinetic measurements of yFMOt involved a His-tagged protein, a similar construction was made for the new full-length yFMO. pET16b-fyFMO was chosen to express the His10-fyFMO protein in E. coli. Unlike yFMOt, the full-length clone does not require plasmid containing GroEL and GroES to be expressed as soluble fractions in E. coli [14]. The purification process was performed in darkness to minimize the photoreduction of free FAD. About 20mg of purified His10-yFMO protein could be obtained from 1 liter of cell culture, whereas about 10mg purified truncated (His10-yFMOt) protein was obtained under the same condition. As shown in Fig. 4, the molecular weight of the His10-yFMO is estimated as 50kDa, close to the molecular weight predicted from the true ORF. While the His10-yFMOt proteins tended to precipitate after being purified, the full-length His10-yFMO remained soluble.

During the purification process, it was observed that the cell lysate with the full-length FMO protein had a bright yellow color in contrast to the lysate containing yFMOt. The full-length protein retained its yellow color even after elution from the Ni–NTA column, whereas the yFMOt protein required 50 μM exogenous FAD to regain activity. This indicates that the His10-fyFMO protein was able to bind FAD more tightly than the yFMOt form, in E. coli and throughout the purification protocol.

**Comparisons of enzymatic features in the full-length and truncated yeast FMOs**

In the absence of organic substrates, FMOs are known to have an NADPH oxidase activity, as indi-
cated in Fig. 1. Rates vary with pH but are generally around 20 mol/mol/min [14]. With fixed concentrations of NADPH (0.2 mM) and FAD \((50 \mu M)\), the pH profiles of NADPH oxidase activity of both proteins were obtained. Both protein forms had higher NADPH oxidase activity at lower pH, suggesting that protons are reactants, as shown below.

\[
\text{O}_2 + \text{NADPH} + H^+ \xrightarrow{\text{FMO/FAD}} \text{H}_2\text{O}_2 + \text{NADP}^+ 
\]

To measure the \(K_m\) for FAD, apo-protein of both forms was prepared at pH 6.4, the cytoplasmic pH in yeast cells [19]. Exogenous FAD was titrated in at \(0–100 \mu M\), and the NADPH oxidase activity was measured (data not shown). The \(K_m\) for FAD of the full-length \(\text{His}_{10}\)-fyFMO was 12.9 \(\mu M\) and the \(K_m\) for FAD of the truncated \(\text{His}_{10}\)-yFMOt was 17.8 \(\mu M\).

As reported previously [14], \(\text{His}_{10}\)-yFMOt recognizes only certain biological thiols as substrates and, unlike many mammalian FMO isozymes, was not active against nitrogen-containing organic molecules. To find out whether the full-length \(\text{His}_{10}\)-fyFMO has the same substrate specificity, a few key compounds were tested. These included \(N, N\)-dimethylaniline and trimethylamine, typical nitrogen-containing substrates for mammalian FMOs [5], and tryptamine, a substrate for \textit{Arabidopsis} FMO-like protein [12]. None of the N-containing compounds was recognized as a substrate by the full-length \(\text{His}_{10}\)-fyFMO protein. However, the thiol-containing molecules, cysteamine and glutathione, were good substrates, as they were for yFMOt. Therefore, we conclude that the full-length yeast FMO shares the same type of substrates as the truncated enzyme.

Glutathione is very concentrated in cellular cytoplasm and is likely to be oxidized by yFMO. We therefore focused our kinetic studies on glutathione. The pH profile of activity against glutathione was measured. The profile for the full-length yFMO is similar to that of the previously measured yFMOt (data not shown). The optimal pH was about pH 6.4, which is very close to the cytoplasmic pH in yeast cells. Initial rate data for both yFMO and yFMOt were measured. The kinetic constants are very similar, as summarized in Table 1.

### Yeast FMO protein exists as monomer

The active form of pig liver FMO was known to exist as dimers in solution (personal communication with Dr. D.M. Ziegler). To determine in what forms the yeast FMO exists, size exclusion chromatography was performed. At 2 mg/ml, in 0.1 M sodium phosphate and 0.25 M sucrose, at pH 6.5, both the full-length and the truncated yeast FMO proteins existed as monomers.

### Discussion

Our studies show that the yeast genomic sequence reported in GenBank contains two nucleotide errors in the gene coding for the protein called yFMO. Using the published GenBank sequence to design PCR primers caused us to amplify a truncated, and C-terminally modified, form of this enzyme, which we now call yFMOt. The initial clone of yFMOt, based on the GenBank sequence, was thought to contain 1119 bases encoding 373 amino acids, a stop codon, and 60 non-coding bases downstream of the stop codon. Due to the extra G base at position 1080, however, the stop codon was altered and the open reading frame continued about 110 bp nucleotides further downstream until it reached a stop codon in the expression vector pET16. As a consequence, rather than expressing the anticipated

### Table 1

<table>
<thead>
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<th>Enzyme</th>
<th>pH optimum</th>
<th>(K_m) (mM)</th>
<th>(V_{max}) ((\mu M/\text{min}))</th>
<th>(k_{cat}) (min(^{-1}))</th>
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<td>(\text{His}_{10})-yFMO</td>
<td>6.4</td>
<td>16.7</td>
<td>15.6</td>
<td>42</td>
</tr>
<tr>
<td>(\text{His}_{10})-yFMOt</td>
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<td>22.7</td>
<td>18.2</td>
<td>48</td>
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</tbody>
</table>

Fig. 4. Expression and isolation of \(\text{His}_{10}\)-yFMO analyzed by SDS–PAGE. Lane 1, total protein from uninduced \textit{E. coli} with pET16b-\(\text{yFMO}\); lane 2, total protein from \textit{E. coli} with pET16b-\(\text{yFMO}\) after induction with IPTG; lane 3, the soluble fraction from \textit{E. coli} with pET16b-\(\text{yFMO}\) induced by IPTG; lane 4, protein molecular weight standards (94, 67, 43, 30, 21 kDa); lane 5, purified full-length \(\text{His}_{10}\)-\(\text{yFMO}\) protein from the Ni–NTA column (20 \(\mu g\) loaded); lane 6, purified truncate protein \(\text{His}_{10}\)-\(\text{yFMOt}\) from the Ni–NTA column.
Affects the folding of disulfide-bonded proteins, its exact role of yFMO in folding disulfide-bonded proteins seems to be even more effective in this regard. After identifying the error in the gene sequence, we amplified the full-length yFMO gene, cloned it, and expressed it as a His-tagged protein. The most noticeable physical difference between the full-length and the truncated FMO proteins is the expression level and solubility of the proteins. Full-length yFMO is expressed at higher levels and remains soluble longer than the yFMOt. This is indicative of a more stable protein fold for the intact protein. A second and probably related difference is their affinity for FAD. The full-length yFMO is apparently more stable and has a superior FAD binding domain, even though the amino acid sequence suggests that FAD is bound in the N-terminal part of the structure conserved in both forms. It may be that the altered C-terminal regions affect the global folding in a subtle way that weakens the affinity for flavin. This may be due to a lack of native residues that normally participate in cofactor binding, or it may reflect some deleterious interactions with the incorrect C-terminal residues of yFMOt.

Having previously done an extensive enzymological characterization of yFMOt [14–17], we were interested to know how the full-length yFMO compared with it. It appears that the two forms are very similar in enzymatic activity. Both enzymes carry out the substrate-independent oxidase activity, at about the same rate. More importantly, neither form appears to oxidize nitrogen-containing molecules, which are the major substrates of several mammalian and nonmammalian FMO isoforms. Both forms of yFMO, instead, oxidize biological thiols, including cysteamine and glutathione. A steady state kinetic analysis shows that the two forms have similar \( K_m \) and \( k_{cat} \) values for glutathione (Fig. 5 and Table 1). This suggests that the active site is largely unperturbed in the truncated form, at least as far as the oxidation of small thiols is concerned.

Deleting the yFMO gene from yeast allowed us to make a number of phenotypic observations, mainly that the deletion strains could not fold endogenous [16] or exogenous [15] proteins which contained disulfide bonds. This observation depended only on deleting the gene, but it is very reasonable to assume that it would be even more effective in this regard.

Although we have presumptive evidence that yFMO affects the folding of disulfide-bonded proteins, its exact cellular function remains unclear. Previously, we suggested that by oxidizing glutathione to glutathione disulfide, yFMO could regulate the redox potential in the ER. Consistent with this idea we observed that wild-type yFMO and the plasmid-expressed yFMOt are feedback-inhibited by increasing redox potential [17]. Recently, it was reported [20] that yeast ErOlp binds FAD and suggested that Ero1p and PDI-mediated oxidative protein folding depends on the FAD level in the cell. The ultimate source of oxidizing potential, however, is still unclear. Therefore, it may be that yFMO, which uses molecular \( \text{O}_2 \) as its ultimate oxidizing source, may communicate with those proteins in the ER lumen by as yet unknown mechanisms. It may be that the true substrate for yFMO is an ER protein that can shuttle oxidizing equivalents into the ER. It could also be that the full-length protein, with an intact C-terminal domain, may allow us to identify a wider range of substrates, including protein targets.

FMOs are well known to associate with microsomal membranes, and we showed that wild-type yFMO is also membrane associated [15]. It has been suggested that the C terminus of FMOs may be involved in membrane binding, but this was disproven by deletion of the 26 C-terminal residues of the rabbit FMO 1B1, which showed no effect on cellular localization [21]. Our earlier work is consistent with this finding, because we showed that yFMOt, with its shortened and modified C terminus, also associated with the cytoplasmic surface of the ER [15].

Because yeast contain only a single FMO, we proposed that yFMO represents the original ancestral activity of this family of enzymes [14]. In contrast to yeast, mammals contain as many as five FMO isoymes [22]. The best-characterized forms, like the FMO1s shown in Fig. 3, play important roles in the metabolism of both nitrogen-containing and sulfur-containing xenobiotic compounds [5,23,24]. These mammalian enzymes are substantially larger than yFMO, having about 100 additional amino acids. These are largely found as major insertions, such as the 43-residue insertion at absolute position 275 in Fig. 3. It may be that this or other differences form part of the substrate recognition site which governs the substrate specificity of the mammalian enzymes.

Acknowledgments

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References