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Molecular Cloning and Kinetic Characterization of a Flavin-Containing Monooxygenase from *Saccharomyces cerevisiae*

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An open reading frame from yeast coding for a homologue of flavin containing monooxygenase (FMO) has been cloned into several *Escherichia coli* expression vectors. A His_{10} peptide attached to the amino terminus produced a high yield of soluble protein when coexpressed with GroEL and GroES. The protein was purified on an affinity column and characterized. The protein binds one mole per mole of flavin but the binding is relatively weak and 50 μM exogenous FAD is used to maintain full occupancy. The yeast enzyme, like mammalian enzymes, exhibits NADPH oxidase activity. The enzyme does not catalyze the oxidation of amines, but thiols, including glutathione, cysteine, and cysteamine, show substrate activity. The K_m values for these are 7.0, 9.9, and 1.3 mM, respectively; k_{cat} values are 94, 246, and 94 per min, respectively. The enzyme apparently does not accept xenobiotic compounds but may be involved in maintaining cellular reducing potential, probably through its action on cysteamine. This activity may represent the initial role of the FMO family of enzymes, giving rise to the multigene family of drug metabolizing enzymes seen in modern mammals. © 1996 Academic Press, Inc.

Key Words: **kinetic analysis; bacterial expression; thiol metabolism.**

The oxygenation of small molecules, including xenobiotics, is vital to the integrity of living cells. Oxygenation helps in the detoxification of many foreign compounds and often increases their solubility and aids in their excretion. Perhaps the most well-known class of oxygenase is cytochrome P450 (1, 2). Most vertebrates contain 30 to 50 genes for these hydroxylases, some of which are found in the endoplasmic reticulum and catalyze hydroxylation of a very wide range of xenobiotics. Another major class of oxygenation enzymes, known to function in detoxification of xenobiotics, is the flavin-containing monooxygenase or FMO group. The chemistry, structure, and physiology of these enzymes has been reviewed recently (3, 4).

FMOs are found in all mammals and down through the eucaryotic organisms to marine invertebrates (5). Within mammals there are multiple isozyme forms with varying activity and substrate specificity (6). The isoforms each show a range of tissue distribution which can vary with development and physiologic state of the animal (7).

The FMO reaction requires molecular O_2, NAD(P)H, H^+, and an oxygenatable substrate. Based on extensive experimental work the ordered reaction sequence is as follows (6, 8): The FAD-containing FMO binds NADPH which reduces the flavin to $FADH_2$; molecular O_2 then reacts with the enzyme bound $FADH_2$ to form a stable 4α-hydroperoxyflavin, FAD-OOH; an organic substrate (S) with an oxygenatable nucleophile, typically a nitrogen or sulfur, interacts with the enzyme bound 4α-hydroperoxyflavin yielding the monooxygenated product (SO) and an FAD-OH (hydroxyflavin) complex; and water and $NAD(P)^+$ are then released, leaving the FAD, fully oxidized, form of the enzyme.

There are usually multiple isozymes of FMO in a given organism or tissue, but each isozyme has a broad range of substrates it can oxygenate. For example, a compilation by Poulsen (9) shows that the hog-liver FMO can attack nitrogen containing compounds such as primary, secondary, and tertiary amines, as well as primary and secondary hydroxylamines and hydrazines. It can also attack thiols, disulfides, sulfoxides, thiocarbamides, sufenic acids, and thioamides. Kinetic

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parameters were first measured for the pig-liver FMO using methimazole as the oxygenatable substrate. The K_m for NADPH is 5.3 μM, the K_m for O_2 is 21 μM, the K_m for the methimazole substrate is 5.2 μM, and k_{cat} = 35 min^{-1} (10). Because of the nature of the ordered reaction the binding of NADPH and O_2 is essentially independent of the substrate. However, the K_m for the organic substrates can vary widely from low μM to mM ranges (9). The k_{cat} values for these reactions do not vary as widely, because the rate limiting step in the reaction sequence is either the dehydration of the flavin or release of $NADP^+$.

FMOs are generally bound in the microsomal membranes and have a molecular mass of about 60 kDa. The amino acid sequence for a number of FMOs have been proposed based on gene sequences, including those for the well-characterized hog-liver (11) and medically important human enzyme (12). Based on these sequences a unifying nomenclature for the FMOs has been proposed (13). There are five mammalian FMO families, FMO1 through FMO5. Within a given family the amino acid sequences (orthologs) tend to show over 80% amino acid sequence identities, while the similarity between the isozyme families is between 50 and 60%. The hog liver FMO is a member of the FMO1 family.

Recently, the total gene sequence for *Saccharomyces cerevisiae* chromosome VIII (14) revealed an open reading with a high amino acid sequence similarity to the FMOs. This open reading frame (ORF) consists of 1,119 bases encoding a 373 amino acid protein of molecular mass 42,437 daltons. In this paper we report the cloning of the gene, its expression in *Escherichia coli* as a $(His)_{10}$ fusion protein, and the kinetic characterization of the enzyme.

MATERIALS AND METHODS

Oligonucleotides for PCR were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). *Taq* DNA polymerase was obtained from Perkin–Elmer (Norwalk, CT). FAD, $NADP^+$, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO).

Cloning of the yeast FMO-like gene. The *S. cerevisiae* ORF suggestive of mammalian FMOs does not have any introns. Yeast genomic DNA was isolated from *S. cervisiae* strain X2180 and amplified by using a sense strand primer (5′-GCAAGATAATCCTGTAGCCTC-3′) complementary to DNA 150 bp upstream from the putative start codon (5′ untranslated region) and an antisense primer (5′-CGATCA-GTCTATCATCCCATAC-3′) hybridizing 20 bp downstream from the stop codon (3′ untranslated region). The PCR error rate with *Taq* DNA polymerase is a function of $MgCl_2$ concentration (15). Tests showed that in this case, 1.5 mM $MgCl_2$ was optimal for amplification.

The anticipated 1.35-kb-amplified DNA product was cloned into the TA cloning vector pCRII (Invitrogen, San Diego, CA). Ligations were performed at 16°C for 16 h by incubating, in a 10-μl reaction solution, 2 ml of the PCR products, 1 μl of 10× ligation buffer, 50 ng of linearized T/A cloning vector, pCRII, and 4 units of T4 DNA ligase. *E. coli* was transformed according to the Invitrogen protocols supplied with the One Shot competent cells (INVαF′). Various amounts of each transformation were plated on 2× YT medium supplemented with 0.1 mg/ml ampicillin and 0.1 mg/ml X-gal. The plates were incubated overnight at 37°C.

The transformed colonies were screened by blue-white screening and white colonies were selected. Recombinant plasmids were purified from liquid cultures by the alkaline-lysis method (16) and the recombinants were confirmed by restriction analysis and sequencing.

Subcloning of yeast FMO homologue gene into the expression vector. For bacterial expression of the yeast FMO homologue, five different constructs were made: (1) normal yeast FMO in pET21d, (2) yeast FMO with C-terminal His tag in pET21a abbreviated as FMO-His_6, (3) FMO-His_6 coexpressed with bacterial GroES and GroEL, (4) yeast FMO with N-terminal His tag in pET16b abbreviated as His_{10}-FMO, and (5) His_{10}-FMO coexpressed GroEL and GroES. PCR was used to introduce the yeast FMO coding sequences into the pET vectors (Novagen, Madison, WI). The PCR reactions were carried out under the same conditions as those for the yeast FMO gene. The amplified DNAs were digested with appropriate restriction enzymes and then ligated into the expression vectors digested with the same or compatible enzymes. *E. coli* strain BL21(DE3) (Novagen) was transformed and selected for expression. To coexpress GroEL and GroES, pREP4-GroESL (17) was cotransformed with the FMO construct. Transformants were plated on YT medium containing 100 μg/ml ampicillin and 30 μg/ml kanamycin.

Expression of the recombinant FMO protein with His-Tag from E. coli. A single colony expressing recombinant FMO was inoculated into 10 ml of 2× YT medium. This culture was grown overnight at 37°C and added to 1 liter of 2× YT supplemented with appropriate antibiotics. The culture was grown at 37°C for 4–5 h with shaking. IPTG and FAD were added to a final concentration of 0.5–1 mM and 10 μM, respectively, to induce expression. To coexpress GroEL and GroES with FMO, ATP was added to a final concentration of 1 mM. The culture was grown at 30°C after 3 h more and the cells were harvested.

The cell pellet was resuspended in 1/20 vol of lysis buffer (50 mM K-phosphate, pH 8.0, 10 μM FAD) and the cells were broken in a precooled French Pressure cell (SLM Aminco, Urbana, IL) at 20,000 psi, twice. The lysed cells were centrifuged at 3,000*g* for 20 min. The supernatant was saved and subjected to ultracentrifugation in a Ti 60 rotor (Beckman) at 4°C for 40 min at 100,000*g*. The supernatant (S100) was saved for the soluble fraction and the pellet (P100) was resuspended in the lysis buffer for the membrane fraction. Fractions of all constructs were analyzed for FMO activity.

Isolation of the recombinant FMO protein with His-Tag from E. coli. To purify the soluble forms of His-tagged FMO, cells were broken in a French Pressure cell and centrifuged. Incorporation of the prosthetic group, FAD, is necessary for active yeast FMO; to obtain the flavin-bound form of the enzyme during purification, FAD concentration of all buffers, except dialysis buffer, was kept at 50 μM and salts and detergents were not used. The supernatant fraction was applied to a 5-ml Ni-NTA column, previously equilibrated in lysis buffer with 5 mM imidazole. After loading, the column was washed with same buffer and with wash buffer (50 mM K-phosphate, pH 8.0, 10 μM FAD, and 60 mM imidazole) until the A_{280} of the flow through is less than 0.05. The bound proteins were eluted with 0.5 M imidazole in wash buffer. Fractions containing FMO were pooled and dialyzed into storage buffer (50 mM K-phosphate, pH 8.0, 10% glycerol); protein concentration was determined by the Bradford method.

Absorbance spectra. UV/visible spectra of the yeast FMO were recorded using a Hewlett–Packard diode array spectrometer with a temperature controlled (15°C) 3-ml cuvette. The contents of the cells were continuously stirred. The spectrum of the oxidized enzyme was made by placing purified His_{10}-FMO (2 μM) in 0.1 M K-phosphate

TABLE I

Localization of Yeast FMO Activities from Five Plasmid Constructs in *E. coli*

Construct	Total activity in nmol oxygen/min (%)		
	Cell extract	Soluble fraction[a]	Membrane fraction[a]
Vector only	0	0	0
FMO	5,324	584	4,740
	(100)	(11)	(89)
FMO-His_6	6,474	440	6,034
	(100)	(7)	(93)
FMO-His_6 (GroEL/GroES)	12,292	5,371	6,921
	(100)	(44)	(54)
His_{10}-FMO	7,257	5,333	1,924
	(100)	(73)	(27)
His_{10}-FMO (GroEL/GroES)	114,464	105,156	9,308
	(100)	(92)	(8)

[a] Cell extracts were centrifugated for 40 min at 100,000*g*; the soluble fraction is the supernatants and pellets resuspended in 50 mM potassium phosphate buffer (pH 8.0) are the membrane fractions.

buffer, pH 8.2. The reduced form of the enzyme was obtained by anaerobically reducing the enzyme in a stoppered cuvette, at 15°C, using an NADPH generating system, and the spectrum was read. The generator consisted of 1 unit of glucose-6-phosphate dehydrogenase, 0.25 mM NADPH, and 1.25 mM glucose-6-phosphate in a 0.1 M K-phosphate buffer, pH 8.2. The hydroperoxyflavin form of the enzyme was created by introducing air into the cuvette, the spectrum was read, and the absorbance of NADPH and reduced enzyme were subtracted to obtain the spectrum of the hydroperoxyflavin.

Enzymatic assay of recombinant FMO. Enzymatic activities were measured by substrate-dependent oxygen uptake at 37°C (10) in mixtures containing 0.25 mM $NADP^+$, 1.25 mM glucose 6-phosphate, 1.0 IU of glucose-6-phosphate dehydrogenase, 50 μM FAD, and 0.1 M potassium phosphate, pH 8.2 or 6.1, in final volume of 2.0 ml. Oxygen uptake was determined in a 2-ml thermostated oxygraph vessel (Gibson Medical Electronics) fitted with a Clark-type electrode. The signal from the electrode was recorded with a EU-200-02 DC offset module. After 3–4 min of temperature equilibration, the enzyme was added through the capillary access port and substrate-independent oxygen uptake was recorded for at least 2 min. The substrate dissolved in water was added in the access port and oxygen uptake recorded for an additional 2–4 min.

Kinetic analysis. Kinetic constants were calculated from initial reaction velocities using a range of substrate concentrations running 10-fold above and below K_m. K_m and V_{max} values were determined by a least squares fit to the kinetic equation using SigmaPlot 2.0 for Windows (Jandel Scientific) and from Lineweaver–Burk plots; the methods gave essentially identical results. Substrate-dependent activities were calculated by subtracting the NADPH oxidase rates from the total rate in the presence of substrate.

RESULTS

The yeast FMO-like ORF (14) consists of 373 amino acids and there is no information about possible post-translational processing of this gene product; we have assumed the protein functions in an unprocessed form. The amino acid sequence of yeast FMO is 18–23% identical with the mammalian FMO sequences and shows limited similarity to other enzymes which also use both FAD and NADPH. In particular, FMO residues 12–23, IGGGPGGLAAAR, are 83% identical to the glutathione reductase sequence IGGGSGGLASAR, a sheet and turn region forming the floor of the FAD binding site (18). This strong conservation suggests that little of the amino terminus of FMO can be posttranslationally removed without damaging the cofactor binding site and justifies expression of the complete FMO ORF. Other less-definitive similarities between FMO and the FAD and NADPH binding motifs of other proteins are also apparent but are statistically less impressive.

The expression levels from the five FMO constructs are summarized in Table I. It shows that total cellular expression of FMO is roughly the same for native enzyme or when tagged on either the N or C terminus with a poly His tail. However, wild-type and C-tailed FMO tend to accumulate in the membrane fraction (89 and 93%, respectively), whereas the N-tailed construct was largely found in the soluble fraction (73%). When the tailed constructs were coexpressed with GroES and GroEL there was a significant increase in FMO activity. Expression of the C-tailed protein roughly doubled, and 44% was soluble. Coexpression with the N-tailed protein led to a 16-fold increase in activity and 92% of the protein was soluble. This His_{10}-FMO construct was used for all subsequent work.

After disrupting the cells and dialyzing the supernatant as described under Materials and Methods, the FMO containing extract was loaded on a Ni-NTA column eluted with 0–1.0 M gradient of imidazole; yeast FMO eluted at 0.2 M imidazole. The yield of FMO was 91% and the purification factor was 16.5. Figure 1 shows the purity of the FMO, based on polyacrylamide gels. Applying this purification protocol, 21 mg of

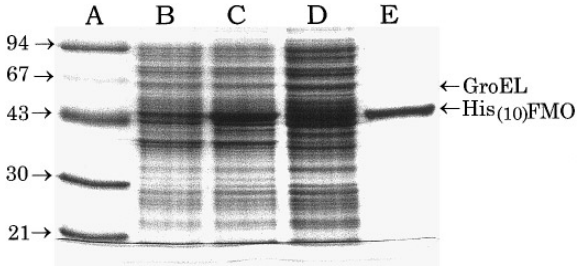

FIG. 1. Isolation of His_{10}-FMO, coexpressed with GroEL and GroES, as monitored by gel electrophoresis. Lane A, protein molecular weight standards; lane B, total protein from uninduced *E. coli;* lane C, total protein after IPTG induction; lane D, the soluble fraction from induced cells; lane E, eluent from the nickel column.

TABLE II

Purification of Recombinant Yeast His_{10}-FMO from *E. coli*

Fractions	Volume (ml)	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min/mg)	Purification factor	Yield (%)
Soluble fraction	22	381	105,156	276	1	100
Nickel affinity	6.7	21	95,613	4,553	16.5	91

highly purified, active recombinant yeast FMO was obtained from a 1-liter culture (8 g wet weight of *E. coli* pellet) as summarized in Table II.

During purification the protein exhibited a yellow color, indicating a bound flavin. Figure 2 shows that optical absorption spectra (solid line) of His_{10}-FMO with absorption peaks at 378 and 450 nm, and a shoulder at 480 nm in the oxidized form (pH 8.2), typical of oxidized flavoproteins. In the presence of oxygen and NADPH the spectrum obtained was similar to that of the flavin hydroperoxide observed with bacterial luciferase or synthetic isoalloxazine hydroperoxides (19, 10). The flavin content of purified yeast FMO was 16 to 22 nmol/mg protein; using a molecular weight 44,824, the FAD content is 0.85 to 0.99 mol/mol. Upon dilution FAD appeared to dissociate from the protein as shown by the low activity in the absence of added FAD.

FMOs are known to have NADPH oxidase activity; that is they can reduce O_2 in the absence of an organic substrate. NADPH oxidase activity was measured for the purified yeast His_{10}-FMO with the oxygraph during a titration with 0–0.1 mM of exogenous flavin (Fig. 3). Enzyme activity was saturated at 50 μM exogenous flavin, and this concentration was maintained in subsequent kinetic studies to assure complete FAD binding.

Figure 4a shows the pH profile for NADPH oxidase activity of purified yeast His_{10}-FMO. There were two pH optima at pHs 6.1 and 7.9, which is unique to this enzyme compared to other FMOs. The peak activities are 480 and 290 nmol/min/mg (21 and 13 mol/mol/min), respectively. The ratio of NADPH to oxygen consumption is 0.85; oxidation of NADPH appears to result in the formation of H_2O_2, because incubation with catalase reduces O_2 uptake by about one-half. Oxygen consumption was unaffected by a 10-fold excess of NADH to NADPH, indicating the enzyme is specific for NADPH. *n*-octylamine, a known activator of hog FMO, did not affect endogenous NADPH oxidase activity of purified yeast FMO.

Mammalian FMOs are known to accept a wide range of organic substrates, consistent with their role in the

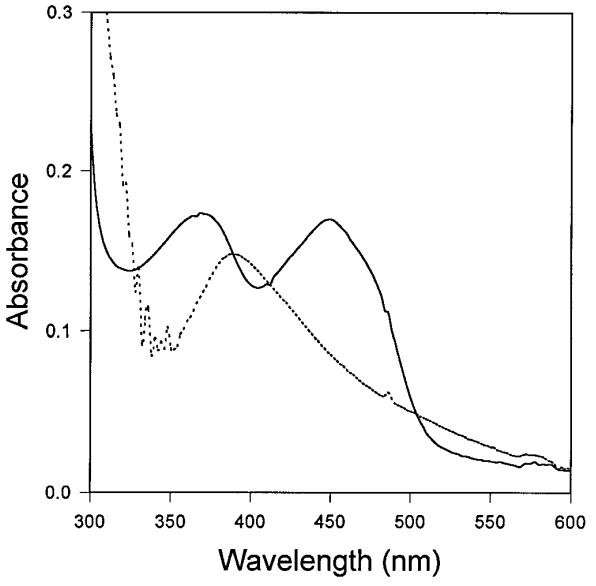

FIG. 2. Absorbance spectra of yeast His_{10}FMO. The solid line represents the fully oxidized form of the enzyme; the dotted line is the hydroperoxyflavin form produced by anaerobic reduction as described under Materials and Methods.

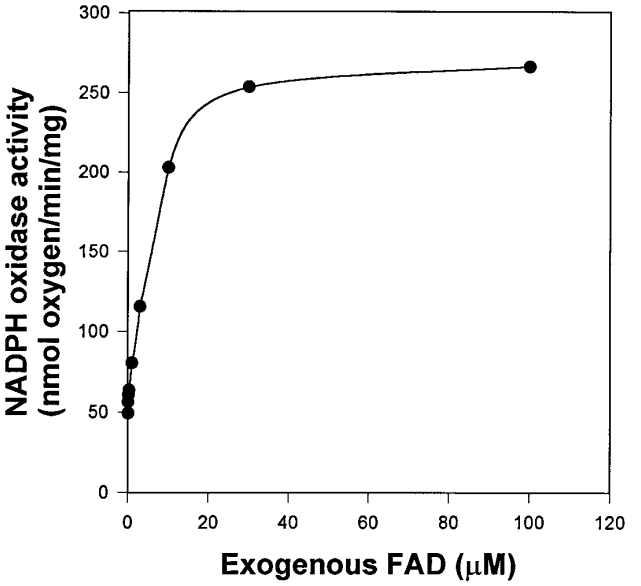

FIG. 3. Yeast FMO NADPH oxidase activity as a function of exogenous FAD.

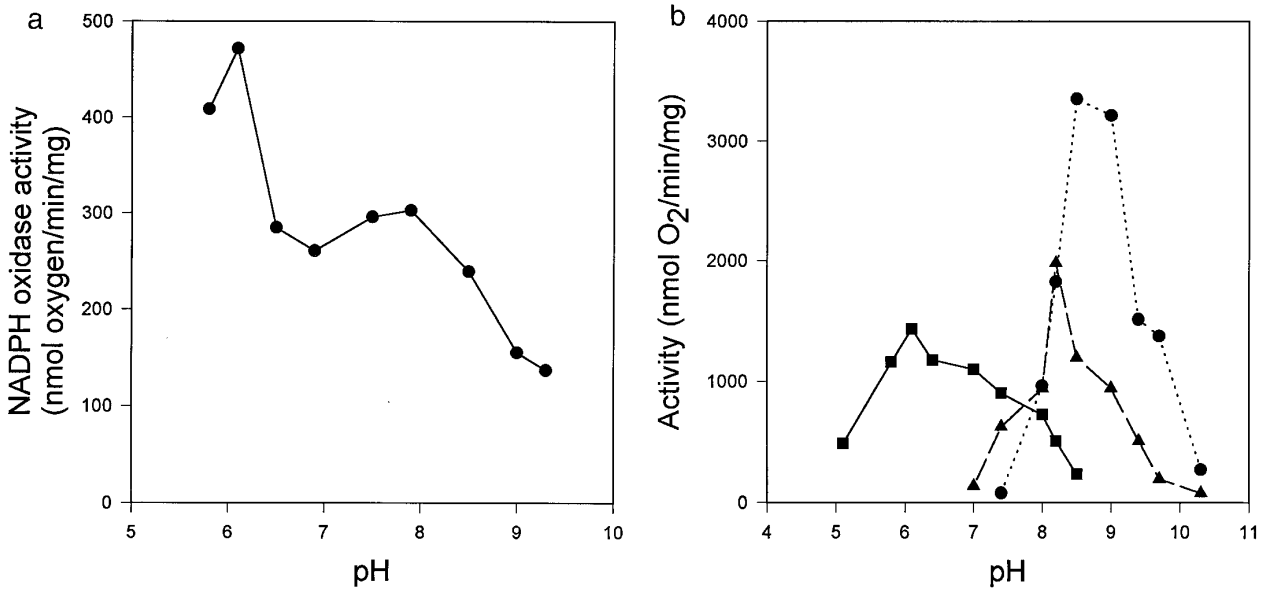

FIG. 4. The pH-dependent activity of yeast His_{10}-FMO. (a) NADPH oxidase activity. (b) Substrate-dependent activity. Circles represent cysteine, squares represent cysteamine, and triangles represent GSH.

metabolism of xenobiotic compounds. To explore the role of yeast FMO, oxygen consumption was measured in the presence of a variety of amine and thiol compounds. None of the amine containing compounds tested showed substrate activity; that is, their presence did not stimulate O_2 consumption above the base NADPH oxidase rate. Candidates included lysine, *n*-octylamine, *N*,*N*-dimethylaniline, trimethylamine, and prochlorperazine. Although methionine was not a substrate, cysteamine, cysteine, and glutathione (GSH) stimulated O_2 reduction catalyzed by yeast His_{10}-FMO.

The pH-dependent activity of His_{10}-FMO was tested against saturating concentrations of GSH, cysteamine, and cysteine (Fig. 4b). Cysteine and GSH show a single maximum at 8.5 and 8.2, respectively, while cysteamine has a maximum at pH 6.1.

With GSH, cysteamine, and cysteine as substrates in the presence of saturating NADPH, initial rates followed saturation kinetics. Double reciprocal plots (Fig. 5) were linear and the kinetic constants for these substrates are summarized in Table III.

DISCUSSION

The yeast genome appears to contain a single gene coding for a homologue of FMOs found in a variety of species, including mammals. FMOs are usually membrane bound and catalyze the oxidation of a wide range of compounds, including xenobiotics, bearing soft nitrogen or sulfur nucleophiles (20). The yeast gene has been cloned into *E. coli* and can be expressed in a soluble form, when a His tag is placed on the amino terminus. It may be that the presence of this polar tail disrupts normal membrane binding signals and allows folding in the cytoplasm. The folding appears to be productive in that (1) the bacterially expressed protein binds stoichiometric levels of FAD which exhibit typical spectra; the fully oxidized and hydroperoxyflavin show ab-

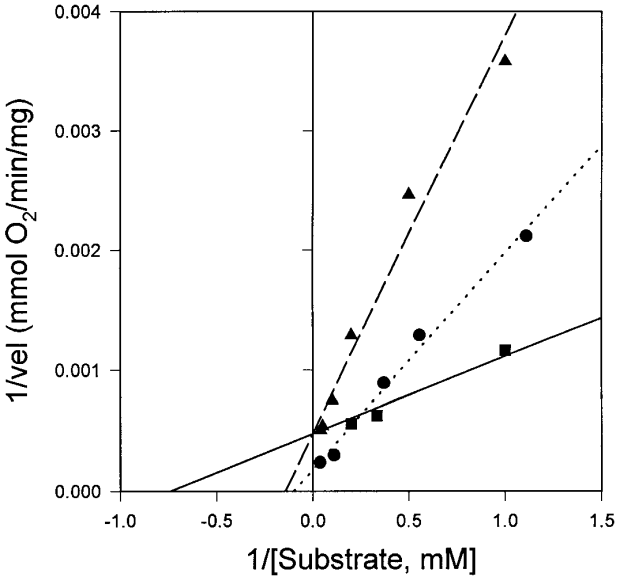

FIG. 5. Double reciprocal plots of initial rate data for His_{10}-FMO against thiol-containing substrates. Circles represent cysteine, squares represent cysteamine, and triangles represent GSH.

TABLE III

Kinetic Parameters for Recombinant Yeast His_{10}-FMO Using Various Thiol Substrates

Substrate	K_m (mM)	V_{max} (nmol/min/mg)	k_{cat} (min^{-1})	pH optimum
Cysteine[a]	9.9	5488	246	8.5
Cysteamine[b]	1.3	2105	94.4	6.1
Glutathione[a]	7.0	2092	93.8	8.2

[a] Based on initial rates at pH 8.2.
[b] Based on initial rates at pH 6.1.

sorbance profiles essentially identical to the hog-liver FMO (8); (2) the folded enzyme carries out an NADPH oxidase reaction with a turnover rate comparable to that measured for mammalian FMOs (10); and (3) the bacterially produced soluble enzyme shows FMO-like activity against thiol containing compounds, with kinetic parameters comparable to, and generally significantly greater than, those seen for other established FMOs. The ability to express an active FMO in soluble form suggests that the native protein is probably not an integral membrane protein, but one which is anchored to the membrane as the result of an undefined signal sequence that may be disrupted by the polyHis tail.

The biological function of the yeast FMO is not clear. It does not appear to have the very broad range of organic substrates which can be attacked by mammalian FMO and it does not appear to catalyze the oxidation of amines. This suggests yeast FMO may not have evolved to deal with xenobiotic compounds, consistent with the observation that there is only one isoform in the genome, whereas most mammals have five or more. The fact that yeast FMO oxidizes thiol containing compounds suggests that it may be involved in the maintenance or regulation of intracellular reducing potential. Ziegler and Poulsen (21) proposed that mammalian FMOs might be involved in maintaining a low level of disulfides in the cell. Although the cytoplasm is generally reducing, with a great excess of GSH over GSSG, some cellular disulfides are required for optimum folding of proteins containing disulfides. They observed that cysteamine is a good substrate for mammalian FMO and was oxidized only to cystamine disulfide. Since FMO is normally localized at the membrane of the endoplasmic reticulum (ER), it seems reasonable that the enzyme may be involved in producing the local concentrations of disulfides which optimize folding of proteins exported through the ER—proteins most likely to contain disulfide bonds.

The cytoplasmic pH of yeast has been measured as 6.5 (22). Although the yeast enzyme catalyzes the NADPH oxidation of GSH and cysteine above pH 8, at values closer to the internal pH of yeast, the enzyme appears rather specific for cysteamine (Fig. 4b). It may be that the main function of yeast FMO is the maintenance of optimum intercellular disulfide concentrations in the form of cystamine, by the efficient oxidization of cysteamine. It is known from a search of the complete genomic sequence of *S. cerevisiae*, and confirmed by our Southern blot experiments, that there is only one FMO gene in the yeast cell. Since this FMO appears to be involved in regulating the redox level of the cytoplasm it may well be that this is the initial function of mammalian FMOs as well. The function of oxidizing xenobiotic compounds in a substrate- and tissue-specific way probably arose later as a multigene family from this original monooxygenase.

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