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Redox Regulation of Yeast Flavin-Containing Monooxygenase

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The flavin-dependent monooxygenase from yeast (yFMO) oxidizes biological thiols such as cysteine, cysteamine, and glutathione. The enzyme makes a major contribution to the pools of oxidized thiols that, together with reduced glutathione from glutathione reductase, create the optimum cellular redox environment. We show that the activity of yFMO, as a soluble enzyme or in association with the ER membrane of microsomal fractions, is correlated with the redox potential. The enzyme is active under conditions normally found in the cytoplasm, but is inhibited as GSSG accumulates to give a redox potential similar to that found in the lumen of the ER. Site-directed mutations show that Cys 353 and Cys 339 participate in the redox regulation. Cys 353 is the principal residue in the redox-sensitive switch. We hypothesize that it may initiate formation of a mixed disulfide that is partially inhibitory to yFMO. The mixed disulfide may exchange with Cys 339 to form an intramolecular disulfide bond that is fully inhibitory. © 2000 Academic Press

Key Words: **yFMO; flavo-enzyme; redox regulation; mutagenesis.**

Flavin-containing monooxygenases (FMOs)[2] are found in all mammals and down through the eucaryotic organisms to marine invertebrates (1). They catalyze the oxidation of an exceptional range of xenobiotics, including pesticides, drugs, and carcinogens (2). For example, hog-liver FMO catalyzes oxidation of nitrogen-containing compounds such as secondary and tertiary amines, as well as primary and secondary hydroxylamines and hydrazines; it can also oxidize a number of thiol compounds, but not glutathione (γ-glutamyl-cysteinyl-glycine, abbreviated as GSH) (3). Within mammals, there are at least five FMO families (4). These isozymes have differing activity and substrate specificity and show a range of tissue distribution which can vary with development and physiologic state of the animal (5).

In contrast to the mammalian FMOs, the *Saccharomyces cerevisiae* genome appears to contain a single gene, *fmo1,* coding for a homologue of FMOs (6). The recombinant yeast enzyme, yFMO, lacks the very broad range of organic substrates attacked by some mammalian FMOs, and it does not catalyze the oxidation of amines (6). Instead, it uses molecular O_2 and NADPH to oxidize important biological thiols including cysteine, cysteamine, and GSH; this suggests that yFMO may be involved in the maintenance or regulation of intracellular reducing potential (6). A knockout mutant was used to demonstrate that yFMO is required for the proper folding of plasmid-encoded proteins that contain disulfide bonds but not for those lacking such bonds (7). Additional experiments revealed that the *fmo1* gene is expressed at low levels in unstressed cells and makes a major contribution to maintaining the redox potential necessary to fold the endogenous disulfide bond-containing proteins. The gene is strongly induced by reductive stress, and the induction is required for cell growth under these conditions. The induction is mediated by the transcription factor hac1p, which is the main signal for the unfolded protein response, or UPR (8).

The expression of mammalian FMOs is affected by exogenous factors, such as diet and disease, and endogenous factors including, age, gender, and hormonal status (9). The FMO enzyme activities are also modulated by several factors. For example, *n*-octylamine appears to accelerate the rate of some FMO-mediated

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[2] Abbreviations used: FMO, flavin-containing monooxygenase; UPR, unfolded protein response; GSH, γ-glutamyl-cysteinyl-glycine; BME, β-mercaptoethanol; PCR, polymerase chain reaction.

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substrate oxygenations (10) but may also decrease FMO-mediated product formation and thus serve as a competitive inhibitor (11). *N*-Octylamine has no affect on yFMO activity (6), but in this paper we report that enzymatic activity of yFMO is modulated by redox regulation through at least one of its cysteine residues.

MATERIALS AND METHODS

Materials. Oligonucleotides for polymerase chain reaction (PCR) were purchased from Life Technologies, Inc. (Gaithersburg, MD). All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). TA Cloning Kit was purchased from Invitrogen Corp. (Carlsbad, CA). *Taq* DNA polymerase was from Perkin–Elmer (Norwalk, CT). The Ni-NTA resin was purchased from Qiagen Inc. (Santa Clarita, CA). FAD, NAD, $NADP^+$, ATP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and cysteamine were purchased from Sigma Chemical Co. (St. Louis, MO).

Bacterial strains and plasmids. Escherichia coli strains INVαF′ (Invitrogen Corp) (F′ endA1 recA1 hsdR17(r_k^-, m_k^-) supE44 thi-1 gyrA96 relA1 f80lacZ15D(lacZYA-argF)u169) and BL21(DE3) (12) (Novagen Inc., Madison, WI) (F^- ompT hadS_B (r_b^-, m_b^-) gal dcm(DE3)) were used for cloning and expression experiments. The expression plasmid pET16b was purchased from Novagen Inc. (Madison, WI), and pREP4groESL (13) was provided by Dr. M. Stieger (Hoffman-La Roche, Basel, Switzerland).

Site-directed mutagenesis and construction of expression vectors. Site-directed mutagenesis was carried out to convert cysteines 183, 339, and 353 to Ser on a His-tagged yFMO platform called His_{10}-FMO (6); plasmids pHis_{10}-FMO and pYFMO-B (6) were used as templates for polymerase chain reaction (PCR). The C183S mutagenic oligonucleotide, 5′-TCACGGGCAAACTCGGAGTTTT-TGAAATCC-3′, corresponded to codons 179 to 183 and contained one mismatch at codon 183. The C339S mutagenic oligonucleotide, 5′-TATCGGTAAGCTTTTAGAGAATACTTCGAC-3′, corresponded to codons 335 to 344 and contained one mismatch at codon 339 of the yeast FMO gene. The C353S mutagenic oligonucleotides, 5′-TGTGCGTTCCGGAGGCATTAGAGTCGAA-3′ corresponded to codons 348 to 362 and contained one mismatch at codon 353 of the yeast FMO gene. Site-directed mutagenesis was carried out using a modified megaprimer method as previously described (14, 15). The first round of PCR was performed with pHis_{10}-FMO (6) as the template and two primers: the mutagenic primer and the T7 promoter primer. The second round of PCR was done using the first PCR product and *Bsp*HI cut pYFMO-B as the templates with the T7 promoter primer. The final PCR products were cloned into T/A cloning vector, pCRII. Once a recombinant mutant clone was identified, the mutated gene was subcloned into the expression vector, pET16b, as previously described (6). To construct the double mutant, C339S/C353S (CdmS), the mutagenized plasmid of C353S was used as a template and the first round of PCR was done with the mutagenic primer for the C339S and the T7 promoter primer.

Expression and purification of yeast FMOs from E. coli. Expression and purification of yeast FMOs were done as previously described (6). Briefly, cells from overnight cultures were added to 1 L of 2 × YT media supplemented with appropriate antibiotics. The culture was grown at 37°C for 4–5 h with shaking. IPTG, FAD, and ATP were added to final concentrations of 1 mM, 10 μM, and 1 mM, respectively, to induce expression. The culture was grown at 30°C for 3 h more, and the cells were harvested. The cell pellet was resuspended in lysis buffer (50 mM K-phosphate, pH 8.0, 10 mM 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 subjected to ultracentrifugation in a Ti 60 rotor (Beckman) at 4°C for 1 h at 100,000*g*. The S100 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 lysis buffer and with lysis buffer containing 60 mM imidazole. The bound proteins were eluted with 0.5 M imidazole in lysis buffer. The eluted proteins were collected in 2-ml fractions. Fractions containing FMO were pooled and dialyzed against storage buffer (50 mM K-phosphate, pH 8.0, 10% glycerol). The protein concentration was determined by the Bradford method (16). The FAD content of purified yFMOs was determined spectroscopically by the method of Faeder and Siegel (17); an SLM8000 fluorescence spectrophotometer was used to excite at 450 nm, and emission was measured at 535 nm.

Enzymatic assay of yeast FMO. Enzymatic activities were measured by substrate-dependent oxygen uptake at 37°C (18) in a mixture containing 0.25 mM $NADP^+$, 1.25 mM glucose-6-phosphate, 1.0 IU of glucose-6-phophate dehydrogenase, 50 μM FAD, and 0.1 M K-phosphate buffer, pH 8.0, in a 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 an EU-200-02 DC offset module. Various substrates, with or without inhibitors, were added through the capillary access port. After 2–3 min of temperature equilibration, the enzyme was added through the access port and oxygen uptake was recorded for an additional 2–4 min; the velocity was determined from the initial slope of the O_2 uptake, usually within the first minute after the addition of enzyme. Like all FMOs, yFMO has an NADPH oxidase activity that generates a blank rate that must be removed in order to assess substrate rates. This oxidase rate, 10/mol/mol/min, is about 5% of the 250/mol/mol/min rate generated by Cys oxidation (6).

To measure the effect of chemical inhibitors, 50 μM of purified enzyme was pretreated with 2 mM inhibitory reagents, methimazole (1-methylimidazole-2-thiol), DTNB, iodoacetamide, diamide and H_2O_2 at room temperature for 1 h, and then the treated samples were dialyzed against the storage buffer and assayed as above.

Light scattering. Protein solutions (0.5 mM) were filtered through a syringe having a 0.1-μm ceramic alumina oxide filter disk. The filtered protein was then injected into the cuvette. Light scattering of protein solutions was measured using a DynaPro-801 Dynamic Light Scattering Instrument (Protein Solution Inc., Charlottesville, VA). The molecular weight of yFMO was calculated using the measurement program DYNAMICS (Protein Solution Inc., Charlottesville, VA).

Determination of yFMO redox status. The enzyme activity of yFMO was determined by measuring the enzyme activity under various redox conditions. In particular, yFMO activity was measured against varying concentrations of GSH and glutathione disulfide, GSSG, such that the total concentration of glutathione tripeptide was 5 mM; that is $[GSH] + \frac{1}{2}[GSSG] = 5$ mM. In these assays, GSH itself was the substrate. Enzyme activity at each concentration of GSH and GSSG was compared to activity with the same concentration of GSH and no GSSG. The % activity values were plotted against various redox status as measured by the ratio of [GSH] to [GSSG].

To assess the physical structure of yFMO under various redox potentials, protein was electrophoresed through non-reducing SDS–PAGE. Purified yFMO was incubated with various concentrations of GSSG in the presence of 0.1 mM FAD (to assure saturation (6)) and 1 mM NADPH for 30 min. The samples were incubated in 1% SDS solution containing 5 mM iodoacetamide by agitating at room temperature for 30 min, followed by incubation at 37°C for 10 min. Proteins were then separated by 8% SDS–PAGE without using any reducing agent.

Microsomes. Yeast microsomes were isolated as previously described (6). yFMO enzyme activity associated with the microsomal fraction was assessed as a function of redox status in the same way as was the purified soluble enzyme described above.

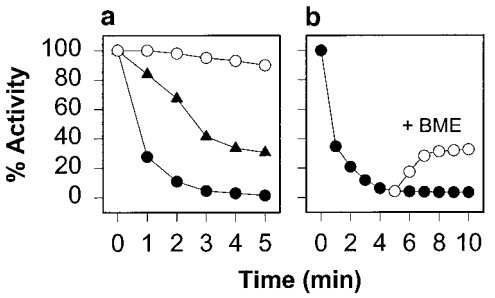

FIG. 1. Methimazole inhibition of yFMO activity. (a) Rate of oxidation of 50 mM Cys substrate, by yFMO, is shown as a function of time, after addition of enzyme, by the open circles. yFMO activity against 2.5 mM methimazole is shown by closed circles. Activity against 2.5 mM methimazole is also shown in the presence of 5 mM BME (closed triangles). The absolute initial rates are about 250/min for Cys oxidation and 180/min for methimazole. (b) Oxidation of methimazole in the absence of reducing agents is shown by the closed circles and in the presence of 5 mM BME (open circles), added after 5 min.

RESULTS

Effects of Chemical Agents on yFMO Activity

yFMO expressed in *E. coli* oxidizes cellular thiols, cysteine, cysteamine, and glutathione to the disulfide form (6). However, when methimazole, a well-known substrate for mammalian FMOs, was used as a substrate, the enzyme activity was inhibited over time. Figure 1a shows the enzymatic activity of yFMO for cysteine and methimazole substrates as a function of time. With cysteine as substrate, there was no substantial decrease in activity over a 5-min incubation; that is, the enzyme retained its initial ability to oxidize substrate and consume O_2. However, with methimazole, the initial rate of activity was dramatically decreased in 1 min and no enzyme activity, above blank rate, was detectable after 5 min. These results show that methimazole is both a substrate and also an inhibitor for yFMO. It is known that mammalian FMOs oxidize thiocarbamides, like methimazole, primarily to sulfenic acids (19), and that sulfenic acids are quite reactive in oxidizing thiols to disulfides (20). As a consequence we tested if reducing agents could counteract the inhibition.

β-Mercaptoethanol (BME) retards the rate of inactivation by 40% (Fig. 1a), presumably due to its ability to reduce sulfenic acids back to thiols (20). After 2 min, yFMO retained 70% activity in the presence of 1 mM BME compared with only 20% in the absence of the reducing agent. In the same vein, Fig. 1b shows that the inactivation of yFMO by methimazole oxidation can be partially reversed by BME. The enzyme is completely inactivated by 5 min of turnover of methimazole. Addition of either 5 mM BME or 20 mM GSH (data not shown) then recovers about 40% activity, the same level attained in the presence of BME shown in Fig. 1a.

Expression of Wild-Type and Mutant Yeast FMO in E. coli

Three cysteine to serine mutants (C183S, C339S, and C353S), a double mutant in which C339 and C353 were converted to serines (CdmS), and the wild-type proteins were each expressed in *E. coli* and purified using Ni-NTA resin; milligram quantities of highly purified mutant and wild-type yFMO were obtained. The mobilities of the mutant proteins were identical to that of the recombinant wild type, based on polyacrylamide gel electrophoresis (data not shown). During purification, the mutant proteins exhibited a yellow color, indicating a bound flavin; the flavin contents of purified mutants was 0.90 to 1.13 mol/mol, identical to the wild type (6).

Steady-state kinetic parameters for wild-type yFMO have been determined for several substrates (6). For Cys, the K_M is 9.9 mM and the k_{cat} is 246/min. The various mutant proteins in this study were characterized only for activity (k_{cat}/K_M) using Cys substrate in the presence of saturating NADPH. Compared to wild-type, the C353S mutant protein exhibited 70% activity, the C339S protein 54% activity, and the C183S protein 5% activity.

Light-scattering data for native yFMO, under reducing conditions, suggested a monodisperse system with a mean radius of 4.6 nm and a molecular mass of approximately 114 kDa. Since the molecular weight of the yFMO monomer is 45 kDa (6), it appears that yFMO exists as a dimer.

Effect of Cys Mutations on Inhibition of yFMO

The inhibition of the various yFMO proteins by a variety of reagents that can react with thiols was investigated. These include methimazole, the thiol exchange reagent DTNB, the thiol-alkylating agent iodoacetamide, and two thiol-oxidizing agents, H_2O_2 (21) and diamide (22). The results are summarized in Fig. 2. All the reagents inhibit wild-type yFMO, under these assay conditions, by roughly 70%. Changing Cys 183 to serine (C183S) results in no change in activity from the wild-type.

The C353S mutant shows a more dramatic difference from the wild type. Methimazole, DTNB, and iodoacetamide exhibit only 15% inhibition, while the two oxidizing agents, H_2O_2 and diamide, exhibit only about 30% inhibition. The double mutant behaves in essentially the same way as the C353S protein.

Regulation of yFMO Activity by Cellular Redox Potential

yFMO, which is known to play a key role in generating the redox potential in the cell, is regulated by the UPR (6, 8). It is therefore reasonable to ask if the

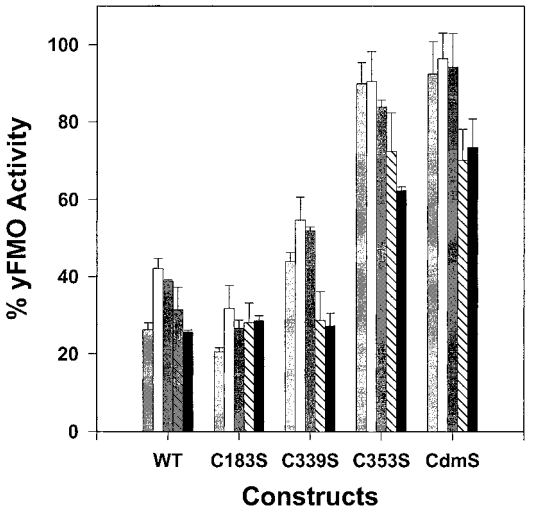

FIG. 2. Chemical inhibition of yFMO strains. Each strain of yFMO protein was incubated with 2 mM inhibiting agent for 60 min prior to measuring activity. Enzyme activity was then measured using 50 mM Cys as a substrate. Inhibition was observed for methimazole (light gray bar), DTNB (white), iodoacetamide (dark gray), H_2O_2 (hatch), and diamide (black).

enzyme activity itself is also regulated by the redox potential of the cell. A plot of yFMO activity under a range of redox potentials, that is, buffering mixtures of oxidized and reduced glutathione, is shown in Fig. 3. The standard state redox potential for glutathione is −240 mV (23), and this potential will obtain when the ratio of $[GSH]^2/[GSSG]$ is 1. The Nernst equation $E = E_0 + 2.303(RT/nF)\log([GSH]^2/[GSSG])$ describes how the potential increases about 30 mV for each 10-fold decrease in that ratio. For convenience, these values are shown along the top of Fig. 3. In eucaryotic cells, the range of normal cytoplasmic redox potentials appears to be −221 to −236 mV (24); this range is shown in Fig. 3 as the transparent gray bar on the right. The redox potential for the ER has been estimated to range from −133 to −165 mV, assuming a total glutathione concentration of about 1 mM, in contrast to the roughly 8 mM concentration in the cytoplasm (24); this range is indicated as the gray bar on the left side of the figure.

Figure 3 shows that wild-type yFMO is fully active under the reducing potential expected for the cytoplasm of the cell. Increasing oxidation potential (that is, increasing the concentration of GSSG) decreases the activity to about 20% of the full activity under conditions expected in the ER. A very similar pattern is seen for microsomal fractions isolated from wild-type yeast. The microsomal fractions contain yFMO bound to the cytoplasmic surface of the ER membrane (7); this activity is inhibited by GSSG in the same way as that for the soluble enzyme expressed in *E. coli*. The measurements in Fig. 3 reflect initial enzyme rates in the presence of great substrate excess, so the diminished enzyme activity does not reflect simple product inhibition.

The C339S mutant did not show the full wild-type range of redox sensitivity. It has wild-type activity under cellular conditions but drops to only about 60% activity at the ER potential. The C353S mutant showed full activity at all redox potentials—that is, it was not inhibited by increasing oxidation potential.

We examined the effect of GSSG inactivation of yFMO on the protein conformation. Wild-type and C353S protein were reacted with buffers of differing ratios of [GSH]/[GSSG] identical to those used in the redox titration of Fig. 3. Free thiols were then blocked with iodoacetamide, and the alkylated proteins were electrophoresed in non-reducing SDS–PAGE. The gels, not shown, gave no indication of intermolecular crosslinking.

DISCUSSION

It is interesting to ask if yFMO has a biochemical regulation in addition to its transcriptional regulation. An observation that enzyme activity was inhibited by oxidation led us to examine the role of the thiols of yFMO in this process. We converted all three Cys residues of yFMO to Ser. The C353S and C339S mutations had very little effect on enzyme activity showing 70% and 54% activity compared to wild-type. Although

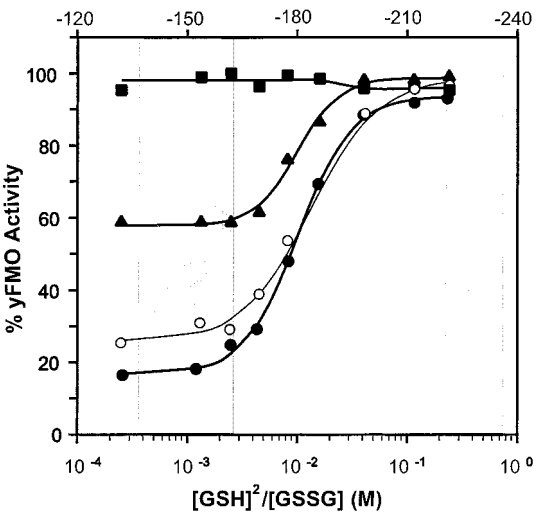

FIG. 3. Activity of yFMO as a function of redox potential. Enzyme activity is shown as a function of the ratio of $[GSH]^2/[GSSG]$. In these experiments the total glutathione concentration, $[GSH] + \frac{1}{2}[GSSG]$, = 5 mM. The redox potential, as determined by the glutathione buffer, is listed across the top of the figure in mV. The wild-type enzyme is shown in solid circles, the C339S mutant as solid triangles, and the C353S mutant as solid squares. The activity of yFMO in the microsomal fraction of wild-type yeast is shown as open circles. The gray bar at the left indicates the range of redox potentials found in the ER, and the bar at the right indicates the redox potentials found in the cytoplasm (24).

these cysteines are clearly not crucial to enzyme catalysis, the data presented in this paper indicate that C353 is involved in regulation of yFMO by the local redox potential. The C183S mutant was reduced 20-fold in enzyme activity, but this is too small an effect to implicate it as a key part of the catalytic mechanism. For example if C183 formed a transient disulfide bond with the substrate as part of the mechanism, one might expect the activity of the mutant to be virtually eliminated. In the absence of an X-ray structure, it is difficult to rationalize the observed inhibition of yFMO activity due to alteration of C183.

In analyzing the regulation of yFMO, it is important to recall that the cytoplasm of eucaryotic cells is reducing, governed by a roughly 30- to 100-fold excess of GSH to GSSG (24). Cytoplasmic GSH is generated by glutathione reductase, but the thermodynamic equilibrium for that reaction is on the order of 10^{14}, suggesting that the observed cellular [GSH]/[GSSG] ratio is not at equilibrium and requires a source of GSSG. It has been speculated that in mammals this might be one role for an FMO isozyme (25). One known source of GSSG is glutathione peroxidase (26), a cytoplasmic and mitochondrial enzyme that uses H_2O_2 to oxidize GSH. However, it is likely that the main source of GSSG, at least in yeast, is yFMO (8).

yFMO is anchored to the cytoplasmic surface of the ER where it uses molecular O_2 and NADPH to generate oxidized products like GSSG, cystine, or cystamine (6). At this time, it is unclear how the ER establishes its oxidizing environment that leads to a [GSH]/[GSSG] ratio near 1:1 (24). There is evidence that GSSG can permeate the ER membrane (7, 24) as well as evidence that it cannot (27). It has also been suggested that GSH is transported into and oxidized within the lumen of the ER by Ero1. However, the oxidant for Ero1 has not been identified (28).

Because of its effect on the forming disulfide bonds in proteins, it seems reasonable that yFMO is strongly involved in generating the cellular redox potential. Because it uses molecular O_2, it may even be the ultimate source of the oxidizing equivalents for the ER. The local potential generated by yFMO at the cytoplasmic surface of the ER may pass directly through the ER membrane if GSSG transport models are correct, or its equivalents may be channeled through the membrane by exchange among integral membrane proteins. That is, yFMO may also be able to oxidize as yet undefined proteins in the ER membrane that could transfer oxidizing equivalents into the lumen of the ER to oxidize proteins such as Ero1. In any case, if oxidizing equivalents generated at the ER cytoplasmic surface are not removed fast enough, they will accumulate and inhibit yFMO. It is clear from the data presented here, especially in Fig. 3, that Cys 353 plays a major role in the inhibition of yFMO. yFMO inhibition is observed for both the soluble enzyme expressed from a clone in *E. coli*, and also for the native yFMO enzyme attached to the ER membrane in microsomal fractions.

It is difficult, in the absence of an X-ray structure, to propose a detailed chemical mechanism for the redox inhibition of yFMO. However, it is plausible that as GSSG accumulates in the cell due to yFMO action, a mixed disulfide is initially formed with C353. This disulfide bond may then exchange with C339 to form an intramolecular disulfide that partially blocks substrate access to the active site and reduces enzyme activity. If C339 is not available, as in the C339S protein, then the putative mixed disulfide initially formed between C353 and glutathione partially blocks the active site, again reducing observed enzyme activity, although to a lesser extent than the proposed disulfide-bonded form (Fig. 3).

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