

## Yeast flavin-containing monooxygenase generates oxidizing equivalents that control protein folding in the endoplasmic reticulum

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**ABSTRACT** The flavin-containing monooxygenase from yeast (yFMO) catalyzes the O<sub>2</sub>- and NADPH-dependent oxidations of biological thiols, including oxidation of glutathione to glutathione disulfide (GSSG). Glutathione and GSSG form the principle redox buffering system in the cell, with the endoplasmic reticulum (ER) being more oxidizing than the cytoplasm. Proper folding of disulfide-bonded proteins in the ER depends on an optimum redox buffer ratio. Here we show that yFMO is localized to the cytoplasmic side of the ER membrane. We used a gene knockout strain and expression vectors to show that yFMO has a major effect on the generation of GSSG transported into the ER. The enzyme is required for the proper folding, in the ER, of test proteins with disulfide bonds, whereas those without disulfide bonds are properly folded independently of yFMO in the ER or in the cytoplasm.

Protein folding is essential for cell function and is a specialized and compartmentalized activity (1). The cytoplasm of eukaryotic cells is generally a reducing environment. The proper redox potential is buffered by glutathione (GSH) and glutathione disulfide (GSSG) at a ratio of about 100:1 and a total concentration  $\approx 10$  mM. The endoplasmic reticulum (ER) of eukaryotes is a more oxidizing environment, with a GSH/GSSG ratio of 1:1–3:1 and a total concentration likely to be  $\approx 1$  mM (2). This redox potential is similar to that shown *in vitro* to be optimal for refolding of proteins with disulfide bonds (3). However, yeast mutants unable to synthesize GSH can grow if supplied with exogenous reducing agents such as GSH or DTT (4). It has also been shown that such mutants can properly fold, in the ER, disulfide-containing proteins, suggesting that although GSSG may be the principal form of oxidizing equivalents in the ER, it can be replaced by other chemicals (5).

The ER is specialized for maturation of secretory and membrane proteins and is the site of disulfide-bond formation. For these purposes, the ER contains molecular chaperones (6, 7), peptidyl-proline isomerase (8), and protein disulfide isomerase (9). It has recently been shown that the lumen of the ER contains a 65-kDa glycoprotein called ERO1, which is essential for oxidative folding of proteins with disulfide bonds (5, 10). Deletion of the gene renders cells hypersensitive to exogenous DTT, and overexpression confers resistance to the reducing agent. ERO1 is thought to be part of the redox machinery of the ER and to help maintain its oxidizing potential. There are no data to suggest how ERO1 is itself oxidized or to suggest that it generates oxidizing potential.

Cytoplasmic GSH is maintained in its reduced form through the action of the ubiquitous enzyme GSH reductase, which catalyzes the reaction:  $\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+$ . Until now it has not been clear how the necessary

oxidizing equivalents are generated or how the oxidizing environment of the ER is created and maintained. It has been suggested that there may be a preferential transport of GSSG from the cytoplasm to the ER, although the apparent  $K_m$  for such transport, 9 mM, may be too high to be physiologically meaningful (2). It has also been suggested that flavin-containing monooxygenases (FMOs), known to oxidize certain thiols, may be involved in maintaining the cellular redox balance, perhaps through the disulfide exchange with cysteamine (11). We recently showed that the lone FMO in yeast (yFMO) can catalyze the O<sub>2</sub>- and NADPH-dependent oxidation of a range of biological thiols, including GSH, cysteine, and cysteamine (12). Here we show that the enzyme is required for proper folding of disulfide-containing proteins and has a direct effect on generating the oxidizing environment of the ER.

### MATERIALS AND METHODS

**Media and General Methods.** Complete (YPD) and synthetic minimal (SC) media are described in ref. 13. To induce *GAL1* promoter-dependent gene expression, 2% galactose was added in early logarithmic phase ( $\text{OD}_{600} \approx 0.6$ –1.0). Yeast transformation was performed by using lithium acetate procedures (14). yFMO activity was measured as substrate-dependent oxygen uptake as described (12).

**Strain and Plasmid Construction.** The chromosomal yFMO gene was disrupted by homologous recombination. An FMO PCR fragment including 5' and 3' untranslated regions was first cloned into TA cloning vector and named p5'FMO3'. The coding sequence of yFMO was replaced with the *URA3* gene from pJR-URA3 (15). The resulting plasmid (p5'FMO3'-URA3) was restricted with *EcoRI*, and a linear fragment containing the *URA3* marker gene, flanked by the yFMO gene fragments, was used for transformation of *Saccharomyces cerevisiae* DBY1827 or DBY1829. To delete the integrated *URA3* gene, the deleted strain was transformed with pHM54 having site-specific recombinase under the control of *GAL1* promoter. The transformants were cultured in the medium containing 2% galactose. The *URA3* gene-deleted clone was selected by using PCR analysis and named SKY1.

To construct a yFMO expression vector, the 2.7-kb fragment from (p5'FMO3') containing the FMO coding sequence and the 5' and 3' untranslated regions was cloned into pYX123 (R&D Systems), a centromeric yeast vector having a *HIS3* marker. The resulting plasmid was designated pY5'FMO3'. SKY1 strain was transformed with pY5'FMO3' and named SKY2. For His-tagged yFMO construct having *GAL1* promoter, the His-tagged FMO sequences from pHis-FMO (12) were cloned into pYES2 vector.

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Abbreviations: ER, endoplasmic reticulum; GSH, glutathione; GSSG, glutathione disulfide; yFMO, flavin-containing monooxygenase from yeast; HA, hemagglutinin.

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To make a reporter gene expression vector for yeast chitinase (pYCHIT), the coding sequence of yeast chitinase (including the signal sequence) was amplified by using *Pfu* DNA polymerase (Stratagene) and cloned into pYES2 vector. To construct hemagglutinin (HA)-tagged yeast chitinase, the coding sequence of yeast chitinase was cloned into pYX223 (R&D Systems), and the resulting plasmid was designated pYHCIT-HA. For the  $\beta$ -glucuronidase expression vector (pYGLU), the coding sequence of  $\beta$ -glucuronidase from pGUS N358  $\rightarrow$  S (CLONTECH) was cloned behind the signal sequences of yeast chitinase from pYCHIT. The galactosidase expression vector under control of *GAL1* promoter was obtained from R&D Systems, and YE<sub>p</sub>FLAGBAP having pre- $\alpha$  leader sequence and *ADH2* promoter was purchased from Scientific Imaging Systems (Rochester, NY).

**DTT Sensitivity Assay.** Early logarithmic-phase cultures of DBY1827, SKY1, and SKY2 were transferred to YMD containing 0 mM or 5 mM DTT. At the indicated times, the growth was determined by measuring OD<sub>600</sub>.

**Microsome Preparation.** Yeast microsomes were prepared by a modification of the method described in ref. 16. Briefly, DBY1827 was grown in SC medium containing 2% dextrose to a final OD<sub>600</sub> of 2. SKY1/YES and SKY1/FMO were grown in SC medium having 2% raffinose to OD<sub>600</sub> of 1 and 2, respectively. Galactose was added to induce the expression of yFMO. Cells were harvested, washed with water, and resuspended at 0.2 g/ml 10 mM Tris-HCl (pH 7.5) containing 1.2 M sorbitol and 10 mM DTT. Zymolase (Zymo Research, Orange, CA) was added at 15 units/ml, and the solution was incubated for 2 hours at 30°C. The spheroplasts were underlaid with 10 ml of solution containing 0.8 M sucrose and 1.5% Ficoll in 20 mM Hepes (pH 7.4) and harvested at 3,000  $\times$  g for 15 min at 4°C. The spheroplast pellet was resuspended at 0.5 g/ml in lysis buffer containing 0.2 M sorbitol, 2 mM EDTA, and 50 mM potassium acetate in 20 mM Hepes (pH 7.4), and cells were lysed with 10–15 strokes of a Dounce homogenizer. The homogenates were transferred to Corex tubes containing equal volumes of 0.5 M sucrose, 50 mM potassium acetate, and 2 mM EDTA in 20 mM Hepes (pH 7.4). The homogenates were underlaid with the same buffer, but with 1 M sucrose, and centrifuged at 8,000 rpm for 15 minutes at 4°C in a Sorval HB-4 rotor. The supernatant was saved and centrifuged at 15,000 rpm for 15 minutes at 4°C in a Beckman Ti 45 rotor. The membrane pellet was washed and resuspended in membrane buffer (0.25 M sucrose/50 mM potassium acetate/20 mM Hepes, pH 7.4). The membrane pellet was loaded onto 2 ml of 1.2 M/1.5 M sucrose step gradients. Gradients were centrifuged at 40,000 rpm for 1.5 hours in an SW50.1 rotor. The microsome band that sedimented at the 1.2 M/1.5 M sucrose interface was collected and washed with membrane buffer, and the final microsome pellet was resuspended in membrane buffer to 30 OD<sub>280</sub> units/ml. To test for localization of FMO on the ER membranes, microsomal fractions were treated with 0.2 mg/ml proteinase K for 90 minutes.

**Western Blot Analysis.** To localize yFMO and Kar2p, microsomes from yeast cells were extracted with increasingly strong agents, 0.5 M NaCl, 0.1 M Na<sub>2</sub>CO<sub>3</sub> at pH 11, 2 mM glucose 6-phosphate, and 1% Triton X-100. The extracted microsomes were centrifuged at 100,000  $\times$  g for 45 minutes at 4°C, and the supernatants from each extraction were analyzed by using SDS/PAGE and Western blot analysis using anti-His<sub>6</sub> (CLONTECH) and anti-Kar2p (7) antibodies. For Western blot analysis for HA-tagged yeast chitinase, cells were washed with water, resuspended with 1 $\times$  PBS containing 0.1% Triton X-100, disrupted by glass beads, and boiled in SDS/PAGE sample buffer. Samples were analyzed by using SDS/PAGE and Western blot analysis using the 12CA5 antibody conjugated with horseradish peroxidase (Boehringer Mannheim). For the control, anti-actin (clone C4) was purchased from

**GSH Oxidation and GSSG Transport Assay.** Microsomes were quantified by measuring the A<sub>280</sub> of SDS-extractable protein; one unit (U) is defined as the amount of microsomes having 1 OD of protein. Two units of microsomes was incubated in 0.1 ml of the transport buffer (TB) with various amounts of GSH, 1 mM NADPH, 4 mM ATP, 1 mM DTT, and 50  $\mu$ M of FAD for various time intervals, and the samples were subjected to centrifugation at 100,000  $\times$  g. Supernatants were discarded, and microsomal pellets were washed with TB. The microsomes were resuspended with 0.2 ml of 1 $\times$  Mes buffer (0.2 M Mes/0.1 M phosphate/2 mM EDTA, pH 6.0) containing 1% Triton X-100, and 0.2 ml of 1% metaphosphoric acid was added to precipitate proteins; GSH was quenched by the addition of 10 mM 2-vinylpyridine. The samples were centrifuged at over 2,000  $\times$  g for 5 minutes, and the supernatants were collected for GSSG determination. GSSG concentrations were determined by using the Enzymatic Recycling method adapted by Griffith (17).

**Reporter Gene Assay.** The reporter gene constructs for  $\beta$ -galactosidase,  $\beta$ -glucuronidase, and chitinase were grown to mid-logarithmic phase, and 2% galactose was added to the cultures to induce protein expression. After 6 hours, cells were harvested and broken, and the cell extracts were used for the appropriate assay. For the alkaline phosphatase assay, yeast cells were grown to late logarithmic phase, the cells were separated by centrifuging, and the activity of secreted bacterial alkaline phosphatase in the medium was determined.

$\beta$ -Galactosidase and alkaline phosphatase activity were measured according to the manufacturer's instructions (CLONTECH). Yeast chitinase activity was determined as described (18).  $\beta$ -glucuronidase activity was determined according to the method described in ref. 19.

## RESULTS

**yFMO Localization.** Wild-type yeast extracts were fractionated into soluble and membrane fractions. Assays with GSH substrate showed that 96% of activity was in the membrane and only 4% was in the soluble fraction. Treatment with proteinase K destroyed 86% of the activity, suggesting that wild-type yFMO is localized to the outer membrane of the ER (data not shown).

To examine the role of yFMO in cellular processes, we constructed a yeast strain with the *FMO* gene deleted (SKY1). Shuttle vector pYES2 was engineered to express the His-tagged yFMO gene under control of the *GAL1* promoter. SKY1 cells transformed with the parent vector are denoted SKY1/YES and those expressing yFMO from the plasmid are SKY1/FMO.

The transformed strains were grown in galactose-containing media, and microsomes were isolated from each culture. The isolated microsomes were then tested for yFMO (GSH oxidase) activity. As shown in Fig. 1a, the SKY1/FMO microsomes had about the same activity as the wild-type (DBY1827) cells and  $\approx$ 50-fold greater activity than those from SKY1/YES microsomes. Fig. 1b shows that expressed yFMO physically accumulated in the microsomes of SKY1/FMO cells but not in the control SKY1/YES cells. No FMO was observed in the cytoplasm (data not shown). Both strains showed roughly equivalent levels of the ER chaperone protein Kar2p (used as a marker). Together, these data show that the plasmid-borne yFMO is ER-associated and functional. The plasmid-expressed yFMO is associated with the outer membrane surface of the microsomes. The protein cannot be extracted with NaCl, indicating it is not weakly associated, but can be extracted with carbonate or glucose 6-phosphate, known to remove membrane bound proteins. More vigorous treatment with the detergent Triton X-100 removes yFMO and also Kar2p, a protein known to reside in the ER lumen (data not shown).

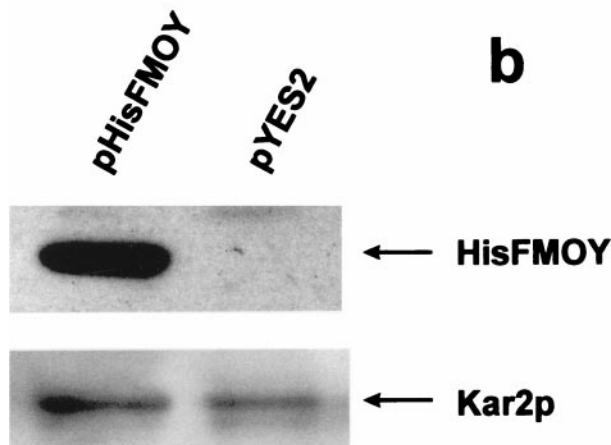
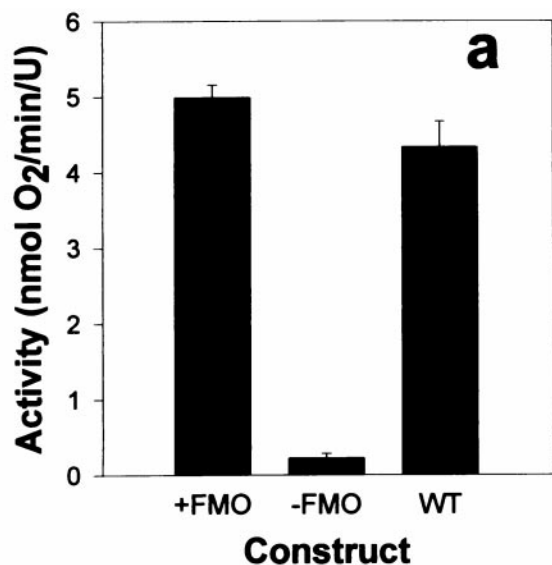


FIG. 1. yFMO activity from isolated microsomes. (a) FMO activity with GSH was measured at pH 7.4 by using the strains SYK1/YES (-FMO) and SYK1/FMO (+FMO) and DBY1827 (wild-type). (b) The localization of His-tagged yFMO and the ER marker protein Kar2p is shown for microsomes isolated from SYK1/YES and SYK1/FMO. His-tagged yFMO was detected by using anti-His<sub>6</sub> antibody, and yeast BiP was detected by using anti-Kar2p antibodies.

**yFMO and Cellular Redox Potential.** Fig. 2 shows the effect of FMO deletion on yeast cell growth. The deletion has no effect in minimal media but retards growth in the presence of a reducing agent, DTT. Plasmid-borne FMO can rescue the wild-type response to DTT. To assess oxidation and ER transport of GSSG, microsomes were isolated from SKY1/FMO cells and exposed to varying concentrations of GSH. After 100 minutes, the microsomes were centrifuged down, rinsed, lysed, and the GSSG concentrations were measured. As seen in Fig. 3a, GSSG production and transport into the ER is saturable, and the  $K_m$  for this process, 10 mM, is close to the physiological concentration of GSH. The transport is also time-dependent, as shown in Fig. 3b. Fig. 3b also shows that the formation and transport of GSSG is NADPH-dependent. In the absence of added NADPH, transport is reduced 80%, and measured GSSG is presumably caused by endogenous cofactor or autooxidation of GSH by oxygen. These results suggest that yeast microsomes have the capacity to convert GSH to GSSG and transport it in an NADPH-dependent manner to the ER.

yFMO activity also is dependent on NADPH, with a  $K_m$  of 30  $\mu$ M (12), so it was reasonable to test whether yFMO might

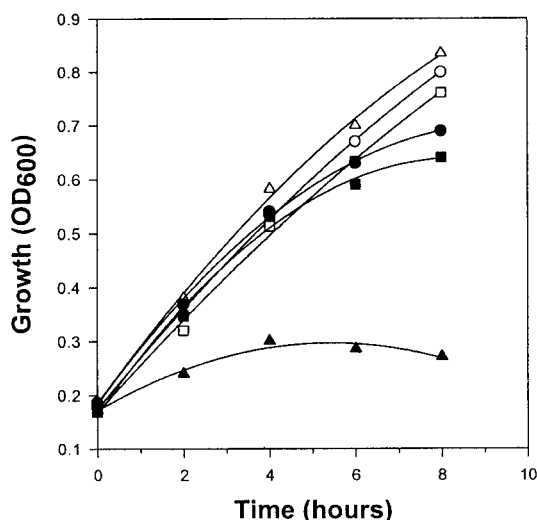


FIG. 2. The effect of DTT on growth of yeast strains. Open symbols correspond to growth on minimal media in the absence of DTT; filled symbols include 5 mM DTT. Circles correspond to wild-type yeast, triangles to the FMO-deletion strain, and squares to the deletion strain rescued with plasmid-borne FMO.

participate in the observed oxidation and transport of GSH. To test this hypothesis, we examined the uptake of GSSG by microsomes from SKY1/YES, wild-type DBY1827, and SKY1/FMO. As shown in Fig. 4a, this activity was essentially the same in all three cell lines, and the transport itself appears to be independent of yFMO activity. Next, the isolated microsomes were incubated with roughly physiologic concentration (4 mM) GSH, and the GSSG contents were assayed as a function of time. As shown in Fig. 4b, GSSG increased in the SKY1/FMO and saturated at around 26 nmol/unit microsomal protein, essentially the same activity as wild-type microsomes. However, the GSSG content of SKY1/YES microsomes saturated at <7 nmol/unit (25% of the FMO value). These results show that the GSSG concentration in yeast microsomes strongly depends on the extent of GSH  $\rightarrow$  GSSG conversion, catalyzed by O<sub>2</sub>- and NADPH-dependent yFMO activity.

**yFMO and Protein Folding.** Given its role in generating GSSG and the subsequent vectorial transport into the ER, it is reasonable to assume that yFMO activity may affect the synthesis of secretory proteins containing disulfide bonds. To examine this role, we constructed expression vectors for a variety of test proteins.  $\beta$ -Galactosidase has no disulfide bonds and no leader to direct it to the ER; it should be expressed in the cytoplasm.  $\beta$ -Glucuronidase has no disulfide bonds but was engineered with a leader that directs it to the ER. Yeast chitinase has three disulfide bonds and its own secretion leader, whereas bacterial alkaline phosphatase (BAP), which has two disulfide bonds, was placed behind a pre-pro- $\alpha$  leader to direct it to the ER. These constructs were expressed in wild-type (DBY1827) and FMO-deleted strain (SKY1) and one strain in which the FMO gene was carried into the SKY1 strain on a centromeric plasmid compatible with the test plasmids (SKY2). The expression levels for these constructs is shown in Fig. 5.

Fig. 5a shows that  $\beta$ -galactosidase was expressed at essentially identical levels in the cytoplasm of each strain. Clearly, FMO activity does not influence the cytoplasmic expression of this protein, which lacks disulfide bonds. Fig. 5b shows that the ER expression of a protein lacking disulfide bonds ( $\beta$ -glucuronidase) is also not significantly affected by FMO activity. The FMO-deletion strain produces about half the activity of wild-type or the FMO-rescued strain. Fig. 5c and

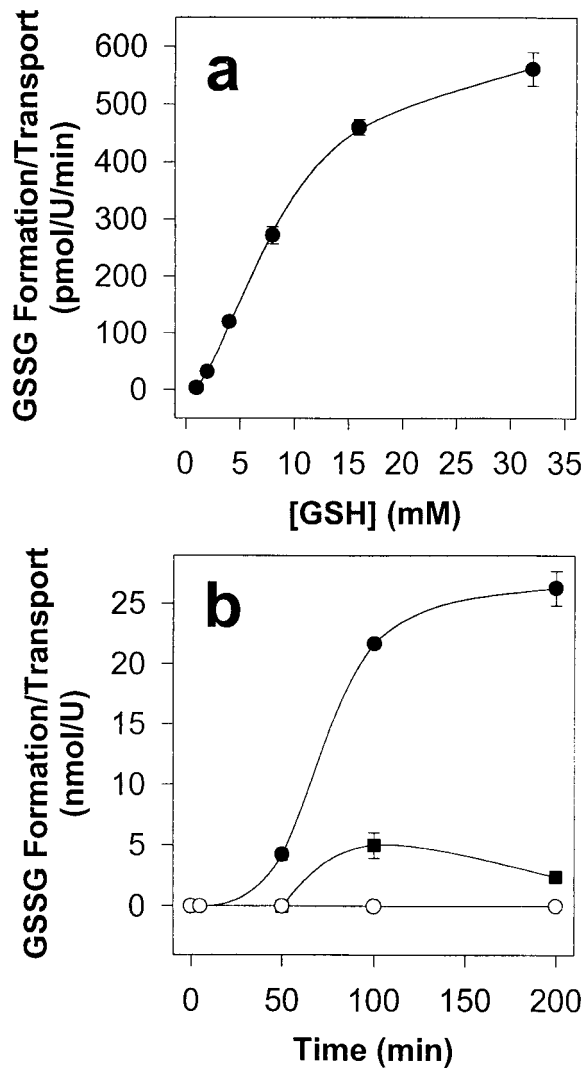


FIG. 3. Oxidation of GSH and transport of GSSG into yeast SKY1/FMO microsomes. (a) Oxidation and transport in 100 minutes as a function of external GSH concentration. (b) Oxidation and transport into microsomes as a function of time. Microsomes were incubated in the presence of 1 mM NADPH and 4 mM GSH (●), in the absence of NADPH (■), or in the absence of GSH (○). A unit of microsomes is defined as the amount containing 1 OD<sub>280</sub> of SDS-extractable protein.

*d* shows the ER expression of proteins containing disulfide bonds. In both cases, the wild-type and FMO-rescued strain show equal expression levels. In strong contrast, the FMO-deletion strain shows essentially no active expression of either test protein.

Fig. 6 is a Western blot showing that the chitinase peptide chain is expressed at reduced levels, about 20%, in the FMO-deletion strain compared with the wild-type strain.

## DISCUSSION

Our results demonstrate that yFMO is localized to the cytoplasmic surface of the ER membrane. The enzyme uses molecular oxygen and NADPH to oxidize a variety of thiols, including GSH, cysteine, and cysteamine. The enzyme is shown to be a vital component of the redox machinery of the cell in that deletion of the gene renders the cell sensitive to excess reducing agents and prevents the proper folding of disulfide bond-containing proteins in the ER. This phenotype is very similar to that recently described for deletion of the ER

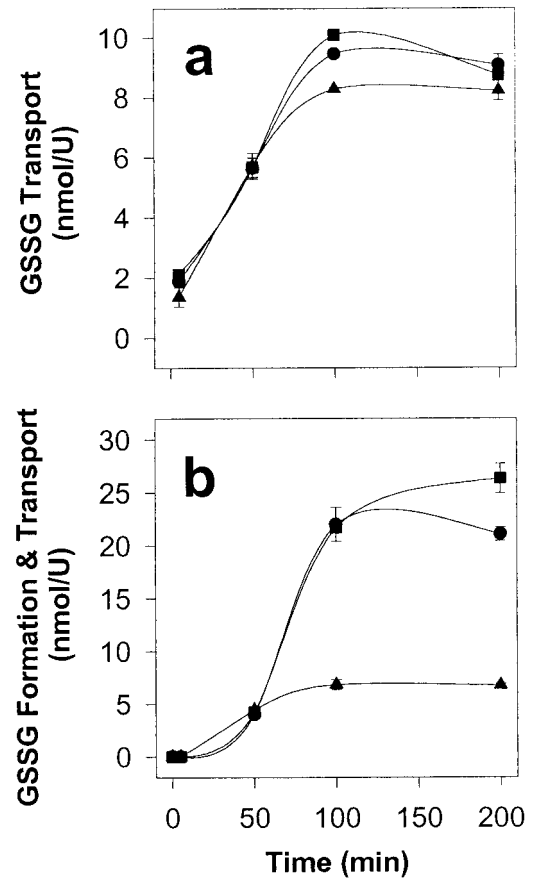


FIG. 4. Oxidation of GSH and transport of GSSG as a function of yFMO. (a) Time course of GSSG transport into DBY1827 microsomes (●), SYK1/FMO microsomes (■), and SKY1/YES microsomes (▲) against 40  $\mu$ M external GSSG, roughly physiological concentration. (b) Oxidation of GSH and transport of GSSG into microsomes of the strains from *a* in the presence of 4 mM external GSH, roughly physiological concentration.

lumen protein ERO1 (5, 10). Presumably, each protein is a key link in the redox chain.

It is reasonable to assume that yFMO generates oxidizing equivalents, in the form of GSSG, cystine, and cystamine, in an

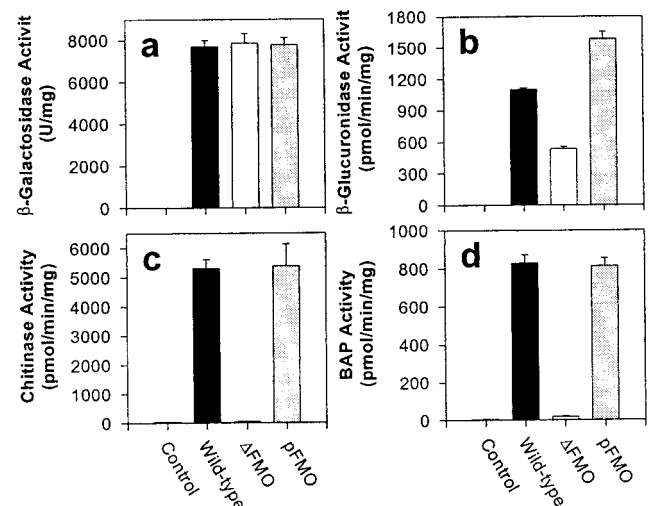


FIG. 5. Expressions of reporter constructs in the wild-type, FMO-deleted ( $\Delta$ FMO), and FMO-rescued (FMO) strains of yeast. The wild-type strain (DBY1827) transformed with parent vector was used as the control.

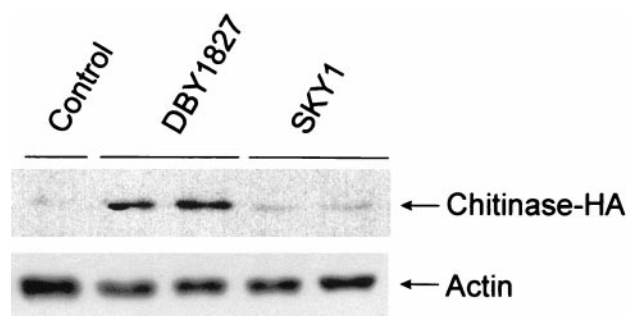


FIG. 6. Western blots of HA-tagged yeast chitinase expressed in yeast strains. The extracts from two independent colonies each of DBY1827 and SKY1 having pYCHIT-HA were analyzed by Western blot analysis using the 12CA5 antibody and anti-actin antibody and compared with the control cell having the vector (pYX223).

energy-dependent step at the ER membrane surface. GSSG and cystine are transported to the lumen of the ER, at  $\approx 10$ -fold the rate of their reduced counterparts, by as-yet unknown mechanisms (2, 20); we have shown that this transport of oxidized molecules is independent of their formation by FMO. The oxidized disulfides are likely the ultimate source of oxidizing potential in the ER. In the ER, they may participate in disulfide-exchange reactions with ER-resident proteins (2). This reaction may include exchanges catalyzed by protein disulfide isomerase, Erp72, and other oxidoreductases in assembling proteins with a correct disulfide-bond pattern (21, 22). The newly discovered ERO1 protein may act as a carrier of oxidizing potential within the lumen of the ER and may be directly involved with the oxidative folding of proteins. Without yFMO activity, the ER is unable to maintain the oxidizing environment optimal for the proper folding of disulfide bond-containing proteins. Fig. 5 shows that disulfide bond-containing proteins, like chitinase and BAP, are not folded into an active form in the absence of yFMO. Fig. 6 shows that chitinase protein is synthesized in the FMO-deletion strain, but the fact that it is completely inactive suggests that the protein is misfolded. It may also be that the reduced level of protein results from rapid proteolytic digestion of the misfolded protein in the ER. Proteins that lack disulfide bonds (such as  $\beta$ -glucuronidase) but are imported to the ER may be affected to a much lesser extent. Only 50% of activity was observed compared with wild-type cells (Fig. 5b), presumably because the ER environment has been changed in an unspecified way by the deficiency of imported oxidizing equivalents. In any case, our findings are consistent with the observation that exogenous reducing agents, such as DTT and 2-mercaptoethanol, also induced accumulation of unfolded proteins (23), with improperly matched disulfide bonds, in the ER (24).

It is well known that GSH reductase catalyzes the NADPH-dependent reduction of GSSG to GSH (2), and that this activity is primarily responsible for maintaining the high redox

potential in the cytoplasm. It now appears that the NADPH-dependent yFMO is its counterweight, producing oxidizing equivalents at the cytoplasmic surface of the ER. GSH reductase and yFMO may be used to help maintain the cytoplasmic GSH/GSSG ratio, but they are also imported to the lumen of the ER. It is important to stress that yFMO generates a variety of oxidized disulfides, not just GSSG. Yeast mutants unable to synthesize GSH can still oxidatively fold proteins in the ER (5). Redox buffering, like many vital cell functions, appears to have protection in depth, and other small molecules can substitute for GSSG. For example, cystine is also produced by yFMO and is transported into the ER as readily as GSSG (2).

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