Evidence for the Saccharomyces cerevisiae Ferrireductase System Being a Multicomponent Electron Transport Chain

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We have studied the relationships between in vivo (whole cells) and in vitro (plasma membranes) ferrireductase activity in Saccharomyces cerevisiae. Isolated plasma membranes were enriched in the product of the FRE1 gene and had NADPH dehydrogenase activity that was increased when the cells were grown in iron/copper-deprived medium. The diaphorase activity was, however, independent of Fre1p, and Fre1p itself had no ferrireductase activity in vitro. There were striking similarities between the yeast ferrireductase system and the neutrophil NADPH oxidase: oxygen could act as an electron acceptor in the ferrireductase system, and Fre1p, like gp91, is a glycosylated hemoprotein with a b-type cytochrome spectrum. The ferrireductase system was sensitive to the NADPH oxidase inhibitor diphenylene iodonium (DPI). DPI inhibition proceeded with two apparent Kᵢ values (high and low affinity binding) in whole wild-type and Δfre1 cells and with one apparent Kᵢ in Δfre1 cells (high affinity binding) and in plasma membranes (low affinity binding). These results suggest that the Fre1-dependent ferrireductase system involves at least two components (Fre1p and an NADPH dehydrogenase component) differing in their sensitivities to DPI, as in the neutrophil NADPH oxidase. A third component, the product of the UTR1 gene, was shown to act synergistically with Fre1p to increase the cell ferrireductase activity.

The ferrireductase system of Saccharomyces cerevisiae is a plasma membrane electron transfer system which reduces extracellular ferric chelates to their ferrous counterparts (1–3). This reduction step thermodynamically favors the dissociation of iron from its ligands, which seems to be a prerequisite for iron uptake. Free ferrous ions, which are far more soluble than ferric ions, are then taken up by an unidentified high affinity transport system (4). Translocation of iron into the cells could be accompanied by a second oxidation step catalyzed by Fe3p (5).

The ferrireductase activity of cells is greatly increased when the cells are grown in iron-deficient conditions (1–3). Reciprocally, the reductase system is repressed by excess iron in the growth medium, but also by copper (6) and by cadmium ions (7). Copper could be taken up via the same reductase system (8).

Dancis et al. (9) identified the gene FRE1 as essential for the cell ferrireductase activity. The product of this gene is expected to be a plasma membrane protein with several transmembrane domains. Interestingly, Fre1p has significant homologies with gp91phox, a component of the neutrophil cytochrome b₅₅₈ (9). Cytochrome b₅₅₈, together with other plasma membrane and cytosolic proteins, constitute the NADPH oxidase complex which, in response to various stimuli, uses intracellular NADPH to reduce extracellular oxygen to superoxide anions (reviewed in Ref. 10). Strains lacking the FRE1 gene still have a residual activity (about 10% as described by Dufour et al. (11) which is completely lost when a second gene, FRE2, is deleted (11). The product of the FRE2 gene presents significant homologies with Fre1p (24.5% identity) and with gp91phox (11). Transcription of FRE1 and FRE2 are regulated differently, and their products could be components of separate reductase systems with different physiological roles.

We partially purified a NADPH flavocytochrome whose activity in plasma membranes from iron-deprived cells was higher than that of iron-rich cells (12). However, we also showed that the NAD(P)H-dependent ferrireductase activity associated with isolated plasma membranes did not reflect the ferrireductase activity of whole cells (13). First, the increase in ferrireductase activity upon iron/copper starvation was considerably less in purified plasma membranes than in intact cells. Second, the reductase activities in plasma membranes isolated from various strains (wild-type, fre1, hem1, or ras1 mutants), which had very different ferrireductase activities in vivo, were similar. It is possible that Fre1p and/or Fre2p are lost during plasma membrane purification, or the measured NAD(P)H-dependent reductase activities are due to mitochondrial and/or endoplasmic reticulum contamination. We therefore re-examined the question of the link between in vivo and in vitro ferrireductase activity and provide evidence against the model of Fre1p/Fre2p as a transmembrane NAD(P)H dehydrogenase.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—The reference strain used was S150-2B (ρ, his3, leu2, trp1, ura3). The following strains, provided by D. Alexandraki (Institute of Molecular Biology and Biotechnology, University of Crete), were used in some experiments: S288C (wild-type), S288CΔfre1, S288CΔfre2, S288CΔfre1Δfre2. Cells were grown as described previously (6) except when cultures were used for plasma membrane isolation. For the latter, cells were preincubated in 50 ml of YNB-glucose medium for 24 h and then inoculated to a final A₆₀₀ of 0.005 in 2.5 liters of the same medium. When the A₆₀₀ reached 1.2, the medium was diluted 2-fold with medium containing 4% glucose, 2% yeast extract, 2% peptone, and either 200 µM BPS1 (iron/copper-deprived medium) or 25 µM CuSO₄ (iron/copper-rich medium). Cells were harvested by centrifugation when the A₆₀₀ reached 1.5.

Isolation of Plasma Membranes—Cell fractionation and plasma membrane purification were done as described by Dufour et al. (14), using disruption of cells with glass beads followed by differential cen-
trifugation and acid precipitation (acetic acid) of mitochondrial membranes. Aliquots of plasma membranes were suspended at about 5 mg/mL in 10 mM Tris acetate buffer (pH 7.5) and frozen. Low temperature spectra (−191 °C) of plasma membrane suspensions were obtained as described previously (15), with an optical path length of 1 mm with one sheet of wet filter paper in the reference path. Spectra were corrected for the baseline shift.

Enzyme Assays—Whole-cell ferrireductase activity was measured as described previously (1) with 360 μM Fe(II)-citrate (1:20) as substrates. The cell suspension was about 1 mg (wet weight) per ml. When resazurin was used as the electron acceptor, the reductase activity was recorded by the appearance of resorufin at 30 °C with a J obin Yvon Y 3 D spectrophotofluorimeter (λex 560 nm, λem 585 nm, slit widths of 2 nm for both excitation and emission). The incubation mixture in 50 mM sodium citrate buffer (pH 6.5) containing 10 μM resazurin was magnetically stirred. Activity was quantified from a resorufin calibration curve prepared under the conditions of the enzymatic assay. The reductase activity of plasma membranes (10–50 μg/mL) was measured in the same way, using 500 μM Fe(II)-EDTA, or 10 μM resazurin and 100 μM NAD(P)H as substrates. When DPI was added, the cells or plasma membranes were preincubated for 10 min with the inhibitor (whole cells) and NAD(P)H (membranes) before adding the electron acceptor. Cytochrome c reduction was followed spectrophotometrically at 550 nm. Protoporphyrinogen oxidase, used as a mitochondrial marker, was assayed as described by Camadro et al. (16).

Plasmids and DNA Constructions—Disruption by TRP1 of the FRE1 gene was performed as described previously (7). For overexpression, FRE1, FRE2, and UTR1 were cloned in YEp351 and YEp352. The FRE1-lacZ gene fusion was constructed as described previously (7). For the UTR1-lacZ construction, a DNA fragment containing 600 base pairs of the UTR1 promoter and the first 135 base pairs of the coding sequence was cloned in the multicopy vector YEp33 (17). For epitope tagging, FRE1 was cloned in YEp352. New BamHI restriction sites were created at different points in the FRE1 gene by site-directed mutagenesis (18). In each case, it was verified that the mutations did not affect the activity of the resulting Fre1p. Double-stranded synthetic DNA encoding the c-myc epitope and flanking pentaglycine linkers (GGGGGMEQQKLISEEDNGGGG) were then cloned in-frame into the new BamHI sites.

Electrophoresis and Immunoblotting—Native PAGE of plasma membrane proteins and in situ detection of reductase activity were performed using 3–20% polyacrylamide gradient gels containing 0.1% Triton X-100 (1.5 mm thick) (19). Gels were electrophoresed for 16 h at 4 °C (30 mA per gel, 50 mM Tris-glycine, pH 9) and then incubated at 30 °C in 50 mM citrate buffer (pH 6.5) containing 100 μM NAD(P)H and either 0.5 mM NBT or 200 μM Fe(III)-EDTA plus 1 mM BPS. Similar results were obtained with either NBT or Fe(III)-EDTA as electron acceptors, but bands of NADPH dehydrogenase activity were enhanced with NBT. SDS-polyacrylamide gels and transfer to nitrocellulose were done using standard procedures. Immunodetection was with anti-myc monoclonal antibody (immunodetection of Fre1p-Myc), or with antisera specific to Sss1p (20) and employed horseradish peroxidase-conjugated anti-rabbit IgG second antibody after SDS-PAGE and Western blotting of 20 μg of protein from the whole homogenate (1), cell debris (2), cytosol (3), crude membranes (4), mitochondria (5), and plasma membranes (6).

RESULTS

Isolated Plasma Membranes Are Enriched in Fre1p and Show Specific NADPH-dependent Ferrireductase Activity—The FRE1 gene was engineered with an epitope tag from c-myc at the carboxy-terminal domain of the protein to allow Fre1p to be monitored during cell fractionation, but this led to complete loss of Fer1p activity (data not shown). A DNA fragment encoding the c-myc epitope and flanking pentaglycine linkers was then introduced at different positions in the FRE1 gene in a multicopy plasmid. Insertion sites (positions 137–138, 246–247, and 314–315 of the protein) were in regions encoding putative hydrophilic loops of the protein. A ΔFre1 strain then was transformed with the recombinant genes. Ferrireductase activity was retained when the c-myc epitope was inserted at positions 137–138 and 246–247, but not at position 314–315. Subcellular fractions were then prepared from transformants grown under iron/copper starvation, and the Fre1p-Myc protein was immunodetected in the plasma membrane fraction (Fig. 1). In some cases, a doublet was apparent for Fre1p (Fig. 1) which could correspond to a partial degradation of the protein.

Mitochondrial contamination of the plasma membrane fraction was monitored by assaying protoporphyrinogen oxidase, an enzyme of the inner mitochondrial membrane. The extent of mitochondrial contamination of the plasma membrane preparation was estimated to be 3–8%. Contamination of the plasma membrane fraction with endoplasmic reticulum was estimated by immunodetection of Sss1p, an endoplasmic reticulum protein involved in protein secretion (20). Sss1p (75%) was mostly associated with the mitochondrial fraction (Fig. 2).

NADH-dependent ferrireductase activity (99 nmol/min/mg of protein) was higher than NADPH-dependent ferrireductase activity (14 nmol/min/mg of protein) in the mitochondrial fraction. The opposite was true in the plasma membrane fraction (69 nmol/min/mg of protein for NADH-dependent activity and 186 nmol/min/mg of protein for NADPH-dependent activity in one representative experiment). Intact mitochondria prepared by osmotic lysis of protoplasts showed no NADPH-dependent ferrireductase (12). Therefore, the activity in the mitochondrial fraction was probably due to contamination by endoplasmic reticulum and plasma membrane. Reciprocally, the NADPH ferrireductase activity of the plasma membrane fraction could not result from contamination with either mitochondria or...
Cells (S288C and isogenic mutants) were grown in iron/copper-deprived medium to induce the cell ferrireductase activity. Whole cell ferrireductase activities (mean ± S.D. from 3 experiments) were expressed in nanomoles/min/mg of total protein to allow direct comparison with the in vivo values. It was assumed that 100 mg wet weight of cells/ml (corresponding to an A600 of 75) = 10 mg of total protein/ml. In vitro ferrireductase activities were measured with NADPH (100 μM) as electron donor. Results are for two experiments (wild-type, △fre1) or one experiment (△fre2, △fre1△fre2).

| Strain   | Wild type | △fre1 | △fre2 | △fre1△fre2 |
|----------|-----------|-------|-------|------------|
| In vivo  | 9 ± 0.8   | 0.75 ± 0.3 | 12 ± 1.5 | 0          |
| In vitro | 102, 145  | 138, 158 | 96     | 164        |

**Fig. 3. Immunodetection of Fre1(c-Myc) in the insoluble (A) and soluble (B) fractions of plasma membranes treated with various detergents.** Plasma membrane proteins (5 mg/ml) were incubated for 1 h at 4°C with 0.3% (w/v) (lanes 1–4) or 0.9% (lanes 5–8) of the following detergents: CHAPS (1, 5), Triton X-100 (2, 6), octyl glucoside (3, 7), Zwittergent 3-14 (4, 8), and centrifuged for 30 min at 40,000 × g. Ferrireductase activity in the pellet and the supernatant was then measured. For lanes 1–8, respectively, 82%, 94%, 64%, 98%, 87%, 96%, 99%, and 99% of the total ferrireductase activity was associated with the insoluble fractions.

**Fig. 4. Detection of the NADPH dehydrogenase activity associated with different subcellular fractions on native PAGE and immunodetection of Fre1(c-Myc).** Cells (S150-2B△fre1 transformed by FRE1(c-myc) in a multicopy plasmid) were grown in iron/copper-deficient medium. 100 μg of protein of the plasma membranes (1, 4), mitochondria (2, 5), or the whole homogenate (3, 6) were separated by native PAGE, and the NADPH dehydrogenase activity was revealed in situ with NBST as the electron acceptor (lanes 1–3). The proteins on the gel were then denatured (16-h incubation with 1% SDS in 50 mM Tris-glycine buffer, pH 8.3) and blotted onto nitrocellulose for immunodetection of Fre1(c-Myc) (lanes 4–6).

**Table I**

| Strain | Wild type | △fre1 | △fre2 | △fre1△fre2 |
|--------|-----------|-------|-------|------------|
| In vivo | 9 ± 0.8   | 0.75 ± 0.3 | 12 ± 1.5 | 0          |
| In vitro| 102, 145  | 138, 158 | 96     | 164        |
However, the ferrireductase activity of double disruptant cells that overexpressed FER1 was only 2-fold higher than when FER2 was overexpressed in the same conditions.

**Cellular Production of Superoxide—Cytochrome c** was slowly reduced by resting cell suspensions. This was fully inhibited by adding superoxide dismutase to the reaction medium (data not shown), indicating that superoxide production was involved.

The rate of superoxide generation was low (20–100 pmol min⁻¹ mg⁻¹), which explains why there was no observable effect of superoxide dismutase on the rate of femtomed. (see above) and which was not sensitive to DPI (data not shown). Resazurin may be able to bypass

**Effects of DPI and Detergents on the Reduction of Resazurin and Ferric Citrate by Whole Cells and Isolated Plasma Membrane**—Whole cells of *S. cerevisiae* can reduce a variety of physiological and nonphysiological electron acceptor via the ferrireductase system (22). The nonpermeate dye resazurin is reduced to its highly fluorescent derivative resorufin, providing a very sensitive assay for the cell reductase activity. The reduction of resazurin was increased when cells were grown under iron/copper limitation and was strongly decreased in a Δfre1 mutant (data not shown). However, the residual reductase activity of a Δfre1 mutant (compared to the wild-type) varied, depending on the substrate used: the rate of femtomed. reduction by Δfre1 cells was 10–13% (iron/copper-deprived cells) or 3–4% (iron/copper-rich cells) of the reduction rate by the wild-type grown in the same conditions. When resazurin was used as electron acceptor, these values were 25–27% and 20–21%, respectively (data not shown). In addition, a Δfre1 Δfre2 strain showed no residual activity with ferric citrate as substrate and 10–15% residual activity with resazurin as electron acceptor. This activity was still inducible since it was increased 2–3-fold when cells were grown under iron/copper deprivation (data not shown). These observations suggest that a component other than Fre1p and Fre2p is able to transfer electrons to resazurin but not to ferric citrate. Reduction of both substrates by resting cells was strongly inhibited by DPI, a powerful inhibitor of the neutrophil NADPH oxidase (21). However, while ferric citrate reduction was completely inhibited at DPI concentrations of 5–10 μM, there was always a residual activity (15–20%) when resazurin was the electron acceptor (Fig. 6A). This difference was even more pronounced when Δfre1 was used instead of the wild-type (Fig. 6A).

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**Fig. 5.** Low temperature absorbance spectra of purified yeast plasma membranes. S150-2B Δfre1 was transformed by FRE1-c-myc in a multicopy plasmid (YEp352) and grown under iron/copper deprivation. The plasma membranes were purified, reduced with a few crystals of dithionite, and the main spectrum was obtained with 18 mg of protein/ml. Inset A, absorbance spectra of plasma membranes isolated from cells grown in either iron/copper-rich medium (dotted line) or iron/copper-deficient medium (continuous line) (about 15 mg of protein/ml in both cases). Inset B, absorbance spectra of plasma membranes isolated from S288C Δfre1 Δfre2 overexpressing FRE1 (dashed line, 8 mg of protein/ml), S288C Δfre1 Δfre2 overexpressing FRE2 (dashed line, 20 mg of protein/ml), S288C Δfre1 Δfre2 overexpressing FRE1 (continuous line, 13 mg of protein/ml); cells were grown under iron/copper deprivation. The spectra in dashed/dotted lines have a shoulder at 551 nm and a small peak at 558 nm, which probably correspond to the α bands of contaminant mitochondrial b and c₁ cytochromes.
the DPI-sensitive electron flow of the reductase system, similar to 2,6-dichloroindophenol with the neutrophil NADPH oxidase (21). Resazurin would then be able to accept electrons from Fre1p (Fre2p) in a DPI-sensitive way and from another component, possibly the NADPH dehydrogenase component, upstream of the DPI-sensitive sites. This would also account for the complex effects of detergents on the cell reductase activity. We previously reported the strong inhibitory effect of some ionic detergents on the ferrireductase activity of whole cells (22). Deoxycholate has a similar effect on the neutrophil NADPH oxidase, and it was suggested that the detergent could abrogate interactions between two components of the oxidase system (21). Working with whole cells (wild-type or Dfre2 cells) we found that the detergent Zwittergent 3-16 (6–8 mM) inhibited the reduction of ferric citrate by 85–100% and the reduction of resazurin by 50–65%. The same detergent had no effect on the NADPH-dependent reductase activity associated with isolated plasma membranes (data not shown). These observations suggest that, as in the neutrophil oxidase, the detergent could act by weakening interactions between two components of the reductase system. Resazurin reduction would be less sensitive to this effect because of its capacity to partly bypass the electron flow from NADPH to Fre1p. Fig. 7 is a model of the system.

Utr1p, a Cytosolic Factor Involved in Ferrireductase Activity of the Cells—In their search for ferrireductase-deficient mutants, Anderson et al. (24) identified a strain which was mutated in the UTR1 gene encoding a protein of unknown function (25). The product of the UTR1 gene was predicted to be cytosolic, was not involved in the regulation of FRE1 expression, and transcription of this gene was not regulated by iron. When our wild-type strain S150-2B was transformed by a multicopy plasmid bearing either the UTR1 gene or the FRE1 gene, the ferrireductase activity of the cells was only slightly increased (by less than 20%). However, when cells were transformed by both genes on multicopy plasmids, the resulting transformants showed ferrireductase activity that is 5–7-fold higher than in wild-type grown under iron/copper-deficient conditions and up to 20-fold higher than in wild-type grown under iron/copper-rich conditions (data not shown). No significant increase in ferrireductase activity was observed when FRE2, and not FRE1, was overexpressed together with UTR1. In contrast to FRE1, transcription of the UTR1 gene was not affected by iron/copper deprivation (data not shown). However, our results clearly show that the FRE1 and UTR1 gene products act synergistically on the cell ferrireductase activity. Utr1p should therefore be considered to be a true component of the ferrireductase system, and we propose to rename the UTR1 gene FRE5 (it was originally named FRE2 by Anderson et al. (24) but this name is now devoted to the gene described by Georgatsou and Alexandraki (11); we do not suggest "FRE3" since the sequences of two new genes homologous to FRE1/FRE2 are available in data banks). The effect of manipulating the FRE1 and UTR1 copy number on ferrireductase activity was strongly dependent on the yeast strain used. For example, wild-type cells of W303-1B showed a 2–3-fold lower reductase activity.

Fig. 6. Effect of DPI concentration on the inhibition of cell and plasma membrane reductase activities. A, reduction of resazurin (squares) or Fe" (circles) by S288C (wild-type, closed symbols) or S288CΔfre1 (open symbols); B, reduction of resazurin (○) or Fe" (●) by S288C; C, reduction of resazurin (○) or Fe" (●) by S288CΔfre1; D, reduction of resazurin (○) or Fe" (●) by isolated plasma membranes (NADPH, 100 μM as electron donor).

Fig. 7. Model of the Fre1-dependent reductase system in vivo (whole cells) and in vitro (isolated plasma membranes). Electrons are transferred in vivo from NADPH to iron or resazurin via two redox centers, both of which are inhibited by DPI. Resazurin can partly bypass the electron flow upstream to the DPI-sensitive sites. In vitro, both resazurin and iron are reduced via the first redox center acting as a diaphorase.
than S150-2B, but this activity was increased (about 3-fold and 2-fold, respectively) when either FRE1 alone or UTR1 alone was overexpressed. The effect of overexpressing both genes was less pronounced in W303-1B (4-fold increase in redox activity) than in S150-2B (data not shown).

The predicted product of the UTR1 gene is a 59.4-kDa protein of 530 amino acids which has significant homologies with the product of POS5, a gene recently described whose disruption causes cells to become more sensitive to hydrogen peroxide (26). It is tempting to speculate that the UTR1/FRE5 gene product could play a role in the yeast ferrireductase system similar to that of the cytosolic factors (p47 and p67) in the neutrophil NADPH oxidase. Sequence alignments using Clustal w (version 1.5) multiple sequence analysis software did not allow to show any overall homology between the product of UTR1/FRE5 and the neutrophil cytosolic factors p47 and p67. However, a short segment of Utr1p (amino acids 324–354) showed 30% identity with amino acids 161–189 of p47, and 19% identity with amino acids 245–273 of p67 (data not shown). In p47 and p67, these segments are part of larger motifs (50 amino acids) similar to a motif found in the noncatalytic domain of src-related tyrosine kinases (27).

**DISCUSSION**

Genetic studies have indicated that the ferrireductase activity of *S. cerevisiae* depends on two transmembrane reductases, the products of the FRE1 and FRE2 genes, which are believed to transfer electrons directly from intracellular NAD(P)H to extracellular ferric chelates (28). However, enzymological and biochemical data do not fit with this simple model. The increase in ferrireductase activity of whole cells subjected to iron/copper deprivation does not correlate with the increase in FRE1 transcription, and cells bearing FRE1 on a multicopy plasmid do not always show, depending on the strain used, a significant increase in ferrireductase activity over that of cells with a single copy of the gene, suggesting that other component(s) could be limiting. A study on reduction of different substrates by whole cells and isolated plasma membranes led us to suggest that the yeast plasma membrane redox system is actually a complex chain of electron carriers (22). The present study demonstrates that, at least in vitro, Fre1p as such has no reductase activity and that a plasma membrane NADPH dehydrogenase is responsible for the ferrireductase activity in vitro and is probably also involved in transmembrane electron transfer in vivo. In isolated plasma membranes, electrons are directly transferred from this component, acting as a diaphorase, to the electron acceptor(s). Thus, the electron acceptors have direct access to this component in isolated membranes, but this is probably not the case in vivo, except for hydrophobic substrates like resazurin. It also seems probable that the electron flow from NADPH to Fe(d) in vivo needs the transplasma membrane potential (Δψ) to be preserved (22).

Our results show that there are striking similarities between the ferrireductase system of yeast and the neutrophil NADPH oxidase. The latter is composed of two plasma membrane-bound redox centers, a NADPH flavodoxidoreductase and a low-potential cytochrome b559, both of which are sensitive to DPI, plus additional proteins which are located in the cytosol of dormant cells and are translocated to the plasma membrane during activation (reviewed in Ref. 10). One of these proteins (p47) is phosphorylated during neutrophil activation. The yeast ferrireductase system can use oxygen as electron acceptor to produce superoxide radicals, and the Fre1p has the spectral characteristics like the neutrophil cytochrome b559. In addition, there is a NADPH flavodoxidoreductase (12) distinct from Fre1p in the yeast plasma membranes. Its activity is increased under the same conditions that cause whole cell ferrireductase activity to be induced. A protein kinase A-dependent phosphorylation step is also likely to occur during "activation" of the ferrireductase system, since full derepression under iron/copper deprivation needs the integrity of the Ras/cAMP pathway (29). Such a phosphorylation step may affect the putative cytosolic factor Utr1p/Fre5p. The ferrireductase activity is probably regulated at several levels, both transcriptional and post-transcriptional. For example, a peak of ferrireductase activity occurs in the late exponential growth phase even in iron-rich medium (12), i.e. when FRE1 transcription is repressed while UTR1/FRE5 transcription remains unaffected. Finally, it was shown recently that gp91, the main component of cytochrome b559, can act as a proton pump (30), which also parallels our previous findings that ferricyanide reduction by the cells is accompanied by a measurable decrease in the extracellular pH (22) and that ferrireductase-deficient mutants are also affected in their capacity to acidify the surrounding medium (31). Fre1p involvement in proton translocation is currently being examined, and we are also working on the nature of Utr1p/Fre5p and its role in regulating ferrireductase activity.

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