The FRE1 Ferric Reductase of Saccharomyces cerevisiae Is a Cytochrome b Similar to That of NADPH Oxidase*

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Plasma membrane preparations from strains of the yeast Saccharomyces cerevisiae gave a reduced minus oxidized spectrum characteristic of a b-type cytochrome and very similar to the spectrum of flavocytochrome b558 of human neutrophils. The magnitude of the signal correlated with the level of ferric reductase activity and the copy number of the FRE1 gene, indicating that the FRE1 protein is a cytochrome b. Sequence similarities with the flavin binding site of flavocytochrome b558 and other members of the ferredoxin-NADP reductase family, together with higher levels of noncovalently bound FAD and iodonitrotetrazolium violet reductase activity in membranes from a yeast strain overexpressing ferric reductase, suggested that the FRE1 protein may also carry a flavin group. Potentiometric titrations indicated that FRE1, like neutrophil NADPH oxidase, has an unusually low redox potential, in the region of −250 mV, and binds CO.

Iron uptake in Saccharomyces cerevisiae is a two-step process. An externally directed plasma membrane ferric reductase converts insoluble, environmental ferric (Fe3+) iron to the soluble ferrous (Fe2+) form which is transported across the membrane by an iron transport complex (Stearman et al., 1996). Reduction of Fe3+ is primarily attributable to the FRE1 protein (Dancis et al., 1990, 1992), although in its absence low levels of residual activity are detectable, due largely to a second reductase, FRE2 (Georgatos and Alexandraki, 1994). The FRE1 gene encodes a protein 686 amino acids in length, with a calculated molecular mass of 78.8 kDa. It has an apparent 22-amino acid membrane insertion leader peptide and hydrophobic analysis (Fig. 1B) reveals multiple hydrophobic regions consistent with membrane spanning domains, thus indicating that the FRE1 gene product is a membrane bound structural component of the reductase. This view is supported by its homology with the large subunit, gp91phox, of NADPH oxidase from human phagocytic cells (Dancis et al., 1992; Roman et al., 1993). NADPH oxidase requires the assembly of gp91phox with a smaller subunit, p22phox, creating the flavocytochrome b558. This flavocytochrome is located in the plasma membrane and membrane of the specific granules, and becomes incorporated into the wall of the phagocytic vacuole. It takes electrons from NADPH in the cytoplasm and passes them across the membrane via FAD and heme to molecular oxygen, generating superoxide that is expelled into the lumen of the vacuole (Wientjes and Segal, 1995).

The C-terminal 402 amino acids of FRE1 show 18% identity and 62% similarity with gp91phox. In addition there are several clusters of much higher identity. These include an HPFXXXS motif which is believed to function in FAD binding in the respiratory burst oxidase and a glycine-rich motif and cysteine-glycine couplet, which represent peptide loops thought to be involved in NADPH binding (Fig. 1A) (Taylor et al., 1993). The hydropathic profiles of the two proteins when aligned from the C terminus also show some resemblance (Fig. 1B). Given that both proteins are electron transporters, the similarity in structure suggested that FRE1 might also be a membrane bound flavocytochrome. Further evidence for this hypothesis came from Lesuisse and Labbe (1989) who reported that heme deficient yeast strains lack ferric reductase activity. In this report we present evidence that the yeast FRE1 protein is a cytochrome b and quite probably a flavocytochrome, with properties similar to those of flavocytochrome b558 of the human NADPH oxidase.

MATERIALS AND METHODS

Strains and Media—A parental strain H1085 (MATα ura3-52 leu2-3, 112) and two derivative strains of S. cerevisiae were used for these experiments. To create strain Δfre1::LEU2, the FRE1 locus of H1085 was replaced with a LEU2 marker gene by double homologous recombination. The replacement of the genomic sequences lying between flanking Clal sites of the FRE1 locus was verified by Southern blotting. To generate strain 352-FRE1, the 4.2-kilobase pair genomic fragment of FRE1 was subcloned into the vector YEp352, and this high copy number plasmid (approximately 40 copies per cell) was used to transform strain H1085 to uracil prototrophy.

Cells were grown to a high density (A600 approximately 1.5) in 6.7 g/liter yeast nitrogen base lacking iron and copper (BIO 101 Inc., 20 g/liter dextrose and 20 μg/mL uracil and/or 33 μg/mL -leucine as appropriate, at 30 °C on an orbital shaker. They were then diluted back to an A600 of 0.2 into YPD (1% yeast extract, 1% peptone, 2% dextrose) with 100 μg/mL bacitracin and grown for 5 h prior to harvesting (A600 approximately 0.5–0.6).

Isolation of Plasma Membranes— Cultures were harvested and the cells washed once in 0.4 M sucrose in buffer A (2 mM EDTA, 25 mM imidazole, pH 7.0, with protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 100 μM N-tosyl-L-phenylalanine chloromethyl ketone, 2 μM pepstatin A). They were then disrupted by vortexing with glass beads, diluted 3-fold in 0.4 M sucrose in buffer A and spun at 530 × g. The supernatant was centrifuged at 22,000 × g, and the pellet, which included the plasma membranes and mitochondria, was resuspended in buffer A and loaded onto a discontinuous sucrose gradient comprising 2.25 M, 1.65 M, and 1.1 M sucrose in buffer A. After overnight centrifugation at 80,000 × g, the essentially pure plasma membranes were removed from the 2.25 M/1.65 M interface, diluted 4-fold, and pelleted at 30,000 × g. Membranes were resuspended in 0.1 M EDTA, 25 mM imidazole·HCl, pH 7.0, 50% glycerol and stored at −20 °C. The absence of significant mitochondrial contamination in membrane preparations produced by this technique was demonstrated by measuring the level of

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Yeast FRE1 Ferric Reductase, a Cytochrome b

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TABLE I

Ferric reductase activity for cells at the time of harvesting, and heme and FAD concentrations for membrane preparations isolated subsequently

| Yeast strain | Ferric reductase activity | Heme concentration | FAD concentration |
|--------------|---------------------------|--------------------|------------------|
| Δfre1::LEU2  | 0.4                       | 54.5               | 16.6 ± 2.2       |
| H1085        | 8.4                       | 94.0               | 15.4 ± 1.0       |
| 352-FRE1     | 107.3                     | 571.0              | 44.2 ± 1.0       |

**RESULTS**

Three strains of *S. cerevisiae* were used in this study: H1085, a wild-type strain, Δfre1::LEU2, a mutant derived from H1085 by deletion of the FRE1 gene and 352-FRE1, the wild-type strain transformed with a high copy number plasmid carrying the FRE1 gene. The cells were grown under conditions that facilitated a high level of ferric reductase activity. Reduction of Fe(III) was measured at the time of harvesting and shown to be negligible in the deletion mutant and substantially raised in 352-FRE1 relative to the parental strain (Table I).

Plasma membranes were isolated from these cultures on a sucrose density gradient and their dithionite-reduced minus oxidized spectra determined over the wavelength range 650-400 nm. These spectra are shown together with a spectrum for pure neutrophil flavocytochrome b$_{558}$ in Fig. 2. The similarities are striking. H1085 and 352-FRE1 both gave spectra characteristic of a b-type cytochrome. There is an a peak at 558 nm, a β peak at 528 nm, and a large γ or heme peak at 428 nm. Importantly, the magnitude of the peaks increases with the level of ferric reductase activity in the yeast cells (Table I) and with the FRE1 copy number indicating that FRE1 is the plasma membrane cytochrome b. A very small heme peak can be seen in the spectrum for the deletion mutant, Δfre1::LEU2 (Fig. 2); this may be due either to another plasma membrane

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**Note:** The text contains a table with data on ferric reductase activity and heme and FAD concentrations for different yeast strains. The table is captioned as follows: Yeast strain Ferric reductase activity, Heme concentration, FAD concentration.

**Results:**

Three strains were used: H1085 (wild-type), Δfre1::LEU2 (mutant), and 352-FRE1 (wild-type transformed with a high copy number plasmid). Ferric reductase activity was measured at the time of harvesting. H1085 and 352-FRE1 showed similarities in spectra, indicating a b-type cytochrome. Δfre1::LEU2 showed a small heme peak.

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reductase, possibly FRE2 (the level of FRE2 expression varies according to the strain background and the growth phase of the cells, and was minimal under the conditions of these experiments), or to a very low level of mitochondrial cross-contamination.

To confirm the nature of the cytochrome, the dithionite-reduced minus air-oxidized difference spectrum of the pyridine hemochrome was recorded for detergent-solubilized plasma membranes from strain 352-FRE1. The concentration of protoheme was calculated using

\[ \text{Extinction coefficient} = \frac{\text{D}_{557-541}}{20.7 \text{ mM}^{-1} \text{ cm}^{-1}} \]

Porra and Jones, 1963) for the reduced minus oxidized protoheme pyridine hemochrome, and this in turn was used to derive the extinction coefficients from the reduced minus oxidized difference spectrum of the hemoprotein in aqueous buffer. The calculated extinction coefficients for the ferric reductase hemoprotein are shown in Fig. 3. The calculated values of the principal spectral features are summarized in Table II. Of interest is the unusually low absorbance of the \( \beta \)-band in the reduced minus oxidized difference spectrum, a feature that is shared with neutrophil flavocytochrome \( b_{558} \). The relatively small extinction coefficient is primarily a result of the large absorbance of the oxidized cytochrome in this spectral region.

Attempts to determine the midpoint potential for FRE1 by potentiometric titration of the hemoprotein in solubilized plasma membrane fractions from strain 352-FRE1 did not yield optimal titrations, due to the apparent instability of the ferrous form of the heme which resulted in progressive loss of the absorbance spectrum. However, little reduction was observed at potentials above \(-200 \text{ mV}\) and reduction was virtually complete at \(-300 \text{ mV}\) (data not shown). Thus, the midpoint potential was estimated to be around \(-250 \text{ mV}\). This low redox potential is remarkably similar to that of flavocytochrome \( b_{558} \) which has two heme centers with closely spaced midpoint potentials of \(-225 \text{ mV}\) and \(-265 \text{ mV}\) (Cross et al., 1995b). The low potential in the neutrophil system is necessary to catalyze the production of \( O_2 \) from molecular \( O_2 \).

Neutrophil flavocytochrome \( b_{558} \) forms a low affinity complex with CO (Cross et al., 1982). Although the cytochrome is thought to transfer electrons to \( O_2 \) from the heme edge rather than by direct ligation of \( O_2 \) to heme iron, the ability to bind CO is often taken as a sign of oxygen reactivity among hemoproteins. This ability is shared by the ferric reductase heme protein as shown in Fig. 4. Assuming the extinction coefficient of the ferrous-CO complex is similar to that of the ferric heme protein, the ferric reductase is fully complexed to CO after a 180-s exposure of the ferrous form to CO and thus has a somewhat higher affinity for CO than cytochrome \( b_{558} \). Approximately 40% of the latter forms a CO complex at room temperature and 1 atm CO (Cross et al., 1982).

One possibility is that \( S. \) cerevisiae exploits the rapid reaction of \( O_2 \) with ferric iron as a mechanism for releasing environmental iron. Plasma membranes from strains \( \Delta \)fre1::LEU2 and 352-FRE1 were tested for superoxide generation by meas-

**TABLE II**

| Absorbance band                  | Extinction coefficient | \( \text{mM}^{-1} \text{ cm}^{-1} \) |
|----------------------------------|------------------------|-------------------------------|
| \( \alpha_{\text{red}} \) (558)  |                        | 29.9                          |
| \( \alpha_{\text{ox}} \) (558)  |                        | 10.8                          |
| \( \alpha_{\text{r-o}} \) (558) |                        | 19.1                          |
| \( \beta_{\text{r-o}} \) (528)  |                        | 5.9                           |
| Soret\( \text{red} \) (426)      |                        | 196                           |
| Soret\( \text{ox} \) (412)       |                        | 157                           |
| Soret\( \text{r-o} \) (427.5)    |                        | 129                           |
| Soret\( \text{peak-trough} \) (427.5–411) | | 202                           |

**Fig. 2.** Reduced minus oxidized spectra for plasma membrane preparations from wild-type \( S. \) cerevisiae, H1085 (B), a mutant defective in ferric reductase activity, \( \Delta \)fre1::LEU2 (A), and a strain in which FRE1 was overexpressed, 352-FRE1 (C), as compared with that of purified neutrophil flavocytochrome \( b_{558} \) (D). The results shown are from a single representative experiment.

**Fig. 3.** Extinction coefficients of the FRE1 hemoprotein. The concentration of the hemoprotein was derived from the pyridine hemochrome spectrum as described under "Materials and Methods." The spectrum shown is that of the dithionite-reduced minus air-oxidized difference spectrum.
Cross production by the yeast membranes was not detected in a cell free assay. This may reflect the lability of a critical cofactor during membrane purification may provide a more satisfactory explanation for the nonstoichiometric increase in FAD with heme in strain 352-FRE1. Alternatively the flavin group may reside within a separate, loosely associated membrane protein.

The very low midpoint potential and the apparent oxygen reactivity of the FRE1 protein suggested a mechanism whereby Fe$^{3+}$ is reduced to the ferrous form by O$_2$. A mechanism of iron reduction utilizing a small intermediate would explain the ability of the FRE1 reductase to reduce chemically varied substrates (ferric citrate, ferric-EDTA, ferricyanide, ferrioxamine B, Cu$^{2+}$, cytochrome c, nitro blue tetrazolium, resazurin c) (Lesuisse and Labbe, 1994). However, O$_2$ was not detected in the cell free assay. This may reflect the liability of a critical cofactor that was lost during membrane purification. Alternatively, the absence of O$_2$ production by the yeast membranes could result from the lack of one or more essential cytosolic proteins. Generation of O$_2$ by NADPH oxidase in a cell free assay requires three cytosolic factors, p47$^{	ext{phox}}$, p67$^{	ext{phox}}$, and p21$^{	ext{ras}}$, together with an amphipathic activating reagent such as SDS or arachidonic acid.

NADPH is the most probable electron donor for the FRE1 ferric reductase. It donated electrons in the INT reductase assay and in potentiometric titrations, was a good reductant at higher potentials although it failed to drive the potential below about −225 mV, apparently because of the inherent instability of the protein. Furthermore, motifs likely to be involved in NADP(H) binding have been identified at positions analogous to the NADPH binding sites of neutrophil NADPH oxidase (Fig. 1).

Although the human NADPH oxidase is a heterodimer comprising both $\alpha$ and $\beta$ subunits, both the NADPH and FAD binding sites, together with at least one of the hemes, are accommodated entirely within the $\beta$ subunit, suggesting that the $\alpha$ subunit may have a regulatory function. For the yeast FRE1 reductase, regulation occurs at the level of control of transcription of the FRE1 gene, and a regulatory subunit may not, therefore, be required. Evidence regarding the presence of a second protein subunit for the FRE1 reductase has been equivocal. Expression of the FRE1 genomic clone on a high copy number plasmid leads to increased surface ferric reductase, indicating that a second subunit, if present, is not limiting for reductase activity. Searches for reductase deficient mutants have led to repeated identification of mutant alleles of FRE1.
However, a single isolate of a mutant in the UTR1 gene (Swissprot accession no. P21373) was noted to be deficient in reductase. This gene product was not membrane associated and the sequence bore no resemblance to other sequences in the database. Thus the role of the UTR1 protein in the FRE1 reductase system remains unclear.

FRE1 shows homology not only to gp91phox but also to FRE2 and to the plasma membrane ferric reductase of the evolutionarily distant yeast Schizosaccharomyces pombe, Frp1 (Roman et al., 1993). FRE1, FRE2, and Frp1 demonstrate functional and regulatory similarities and, furthermore, share with gp91phox a similar hydropathic profile and clusters of amino acid identities at analogous positions. It is possible, therefore, that these three proteins represent a distinct family of membrane bound flavocytochromes capable of transporting electrons across the cell membrane.

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