Site-directed Mutagenesis of the Yeast Multicopper Oxidase Fet3p

(Candice C. Askwith‡ and Jerry Kaplan§)

From the Division of Immunology and Cell Biology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132

High affinity iron transport in yeast is mediated by two proteins, Fet3p and Ftr1p. The multicopper oxidase Fet3p is thought to convert extracellular ferrous iron to ferric iron, which then crosses the plasma membrane through the permease Ftr1p. Fet3p is capable of oxidizing other substrates, such as p-phenylenediamine, and there is still a question of whether it is the ferroxidase activity that is essential for iron transport. Fet3p is also required for Ftr1p localization to the cell surface, making it difficult to prove a direct role for Fet3p oxidase in high affinity iron transport. In an attempt to generate Fet3p specifically lacking ferroxidase activity, we used site-directed mutagenesis to alter residues within Fet3p that had been suggested to impart iron oxidase activity. These substitutions resulted in either a loss or retention of both p-phenylenediamine and ferroxidase activities, indicating that the ability of Fet3p to act as a ferroxidase involves other amino acids. Inactive Fet3p, however, did mediate Ftr1p localization to the cell surface but did not mediate high affinity iron transport. These observations indicate that the ferroxidase activity of Fet3p is intrinsically required for high affinity iron transport.

The high affinity iron transport system of Saccharomyces cerevisiae is specific for iron and is transcriptionally regulated by iron need (1). This transport system is composed of two proteins, Ftr1p and Fet3p (2, 3). Expression of both proteins is necessary and sufficient for high affinity iron transport (3, 4). Ftr1p, the iron permease, has six transmembrane regions and a potential iron binding motif present within the third transmembrane domain (3). Fet3p is a copper-requiring enzyme with a classical leader sequence and one transmembrane domain near the carboxyl terminus (2, 5). Both Fet3p and Ftr1p are dependent upon one another for maturation through the secretory pathway. When Fet3p is absent, Ftr1p fails to localize to the plasma membrane. Without Ftr1p, Fet3p is not loaded with copper and does not become enzymatically active (3).

Fet3p is a member of the multicopper oxidase family of proteins. It has been proposed that high affinity iron transport occurs when Fet3p oxidizes extracellular ferrous iron, which is then transported into the cell through the permease. This hypothesis is supported by the fact that Fet3p is responsible for an iron-dependent oxygen consumption in intact cells, and iron is the best substrate yet tested for Fet3p in vitro (5, 6). Most multicopper oxidases, including Fet3p, can oxidize a variety of organic substrates. Because Fet3p is able to act on substrates other than iron, the specific role of the ferroxidase activity in iron transport has yet to be directly demonstrated. In this study, we set out to use site-directed mutagenesis to alter the substrate specificity of Fet3p in order to generate a Fet3p possessing p-phenylenediamine (pPD) oxidase activity but lacking iron oxidase activity. We reasoned that such a protein would help to determine the importance of Fet3p ferroxidase activity.

Of all the multicopper oxidases, only Fet3p and ceruloplasmin have been reported to oxidize iron (6–8). Specific regions and amino acids in these enzymes have been hypothesized to impart ferroxidase activity (9, 10). Ligands involved in type I copper binding are thought to define the ability of a multicopper oxidase to oxidize iron (9). These ligands are a P1 histidine, P2 cysteine, P3 histidine, and a P4 ligand that varies between members of the multicopper oxidase family. Ceruloplasmin has two multicopper oxidase domains; one with a P4 leucine that may be critical for ferroxidase activity. Consistent with this theory, Fet3p, the only other multicopper oxidase reported to oxidize iron, possesses a leucine in the P4 position (2). To test this theory, we used site-specific mutagenesis to alter the P4 position in Fet3p. We also tested a second set of predictions that suggested that domains in Fet3p dissimilar to non-iron-oxidizing multicopper oxidases, such as ascorbate oxidase and laccase, might be involved in conferring ferroxidase activity (10). Our data suggests that neither of these predictions define amino acids, which determine iron oxidase activity. We did generate mutated Fet3p, however, devoid of catalytic activity that was used to further define the role of Fet3p activity in high affinity iron transport.

MATERIALS AND METHODS

Construction of Site-directed Mutant Vectors—Site-directed mutagenesis primers: pSDMET, 5'-aaagattcctcagagaacaagcattgctaaccaaatg-3'; pSDVAL, 5'-aaagattcctcagagaacaagcattgctcaaccataccttg-3'; pSDDPH, 5'-aaagattcctcagagaacaagcattgctcaaccataccttg-3'; pSDDLys, 5'-aaagattcctcagagaacaagcattgctcaaccataccttg-3'; pSDILE, 5'-aaagattcctcagagaacaagcattgctcaaccataccttg-3'; pSDA- LA, 5'-aaagattcctcagagaacaagcattgctcaaccataccttg-3'; pSDHE489, 5'-aaagattcctcagagaacaagcattgctcaaccataccttg-3'; pSDAVRII, 5'-tgtaggtgccttaggtgaag-3'; pCHECK1, 5'-caggaacctgaccatctc-3'; pCHECK2, 5'-cttactgtgcagacaggac-3'; pSDE227A, 5'-cctgatagtgaagcattgctcaaccataccttg-3'; pCHECK4, 5'-tgaagcaccatatcag-3'.

The abbreviations used are: pPD, p-phenylenediamine; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
To generate the type I P4 mutant proteins, PCR primers were made that spanned the P4 codon and a unique BanHI site. Within these primers, the P4 codon was altered to produce the desired amino acid. A second PCR primer was generated that spanned a unique BamHI site within the P4 codon and a unique BstEII site. Within these primers were produced from this construct whether cells were grown in iron-replete or iron-depleted galactose conditions. To generate the second set of mutants, we took advantage of unique AI sites within Fet3. PCR products were made with newly generated primer pairs. The resulting clones were sequenced using pCHECK1 and pCHECK2.

**Fet3p Detection**—Cells were grown overnight in CM-URA-galactose media and then incubated in CM-URA-galactose bathophenanthroline disulfonic acid for 8 h. The cells were harvested, and membrane preparations were made using previously established procedures (11). Thirty µg of total membrane protein was run on a 4–20% SDS-PAGE gel before being transferred onto polyvinylidene difluoride membrane (Gelman Sciences). The blot was washed, incubated with a 1:10,000 dilution of rabbit antibody, and then further incubated with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories) for 1 h at 70 degrees and developed with ECL chemiluminescent detection solution (Amersham Pharmacia Biotech).

**Ferrooxidase assays** were done by altering the FET3 captoethanol and heat denaturation. The gels were incubated in 10% glycerol, 0.05% Triton X-100 solution for 1 h at room temperature. The gels were then incubated under dark and humid conditions to allow PPD to oxidize. The blot was washed, incubated with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories) for 1 h at 70 degrees and developed with ECL chemiluminescent detection solution (Amersham Pharmacia Biotech).

**Immunofluorescence of Ftr1p**—Cells harboring each of the SD constructs were trants of a Pet1p-Myc construct generously supplied by A. Dancis (3). Transformed cells were grown overnight in CM-URA-LEU galactose media and then placed into CM-URA-LEU galactose bathophenanthroline disulfonic acid for 6 h to induce the expression of Pet1p-Myc. Immunofluorescence was performed as described previously (3). Images were captured using a Nikon microscope with the Openlab software using a Macintosh work station.

**pPD Oxidase Assay**—Thirty µg of total membrane protein in 1% SDS was run onto 4–10% SDS-PAGE gradient gels in the absence of β-mercaptoethanol and heat denaturation. The gels were incubated in 10% glycerol, 0.05% Triton X-100 solution for 1 h and then incubated in 3 mM pPD dihydrochloride in 100 mM sodium acetate buffer (pH 5.7) for 1 h at room temperature as described previously (11). The gels were then incubated under dark and humid conditions to allow PPD to oxidize.

**Ferroxidase Assays**—Ferroxidase assays were done by altering the PPD oxidase assay protocol. Six µg of membrane protein were prepared and run onto 4–20% SDS-PAGE gradient gels as before. The gels were treated in 10% glycerol, 0.05% Triton X-100 solution for 1 h and then incubated in 200 mM ferrous ammonium sulfate, added from a fresh 200 mM stock, in 100 mM sodium acetate buffer (pH 5.7) for 1 h at room temperature. The gels were then incubated as above and then overlaid with cellophane rehydrated in a 15 mM ferrozine stock solution made in water with the pH adjusted to 7.0 with sodium hydroxide.

**Iron Transport Assays**—Cells grown overnight in CM-URA-galactose media and then incubated in CM-URA-galactose bathophenanthroline disulfonic acid media for 8 h. Iron transport assays were done in the presence of ascorbate as described previously (4).
RESULTS

Mutagenesis—The multicopper oxidase type I copper P4 has been hypothesized to impact substrate specificity. The identity of the P4 amino acid has been suggested to alter the redox potential of the type I copper, affecting the ability of the protein to oxidize various substrates (9). Specifically, a P4 amino acid that is able to act as a copper ligand, such as phenylalanine or methionine, has been predicted to oxidize pPD but not iron. A leucine at the P4 position was predicted to be unable to act as a copper ligand, resulting in a type I copper with a redox potential high enough to oxidize iron (9). This theory is consistent with the observation that the two multicopper oxidases reported to oxidize iron, Fet3p and ceruloplasmin, have a P4 leucine. In addition, the P4 leucine is conserved between Fet3p and the Schizosaccharomyces pombe Fet3p homolog, Fio1p (Fig. 1). To test this hypothesis, the P4 leucine of Fet3p (Leu-494) was altered using site-directed mutagenesis. Leucine 494 was changed to either valine (L494V), alanine (L494A), or isoleucine (L494I). These conservative alterations were not expected to affect enzyme activity. Additionally, leucine 494 was changed to a lysine (L494K), and the invariant P3 histidine 489 was changed to a phenylalanine (H489F). These changes were expected to abolish Fet3p enzyme activity.

Expression of the mutant genes was normalized by placing the FET3 gene under the control of an iron-independent pro-
 requires approximately 3 ng of total membrane protein (10% of the amount used in these blots) from cells containing wild type Fet3p. Thus, any mutation that decreases Fet3p activity by 10-fold may not appear active in this assay.

**Iron Transport Activity of P4 Mutant Fet3p—Functional activity of the mutated Fet3ps was tested by measuring high affinity iron transport. Cells were grown in low iron galactose medium and then assayed with various concentrations of radiolabeled iron. Expression of wild type Fet3p (Leu-494) showed characteristic high affinity iron transport, and cells with vector alone showed no high affinity iron transport (Fig. 3). The L494I, L494K, or H489F proteins showed no evidence of any high affinity iron transport. The L494A and L494V showed some iron transport activity although at a significantly reduced rate. Those substitutions that severely disrupted iron transport activity also resulted in a loss of pPD and iron oxidase activity. The L494F substitution showed a modest level of iron transport activity in which the estimated $K_m$ for iron transport is relatively intact, but the $V_{max}$ is markedly altered. The L494M substitution shows levels of high affinity iron transport activity comparable with wild type Fet3p. Although the approximate $V_{max}$ for iron uptake is unchanged, the $K_m$ of the L494M protein is approximately 2-fold higher than wild type.

**Immunofluorescence of Ftr1p in Cells Expression the P4 Mutants—** Many of the mutated Fet3ps displayed neither wild type oxidase or high affinity iron transport activity. Reduction in high affinity iron transport may result directly from loss of Fet3p oxidase activity or from a failure of inactive Fet3p to mediate Ftr1p cell surface localization. We visualized Ftr1p by indirect immunofluorescence in cells expressing mutated Fet3ps to determine whether these proteins could mediate Ftr1p cell surface localization (3). Cells without the Ftr1p:Myc plasmid, GAL10. A Fet3 disruption strain was transformed with the plasmids, and Western analysis was used to visualize Fet3p. All the mutant proteins demonstrated the same mobility as wild type Fet3p (Fig. 2A). Most mutated proteins also were expressed equivalently except L494V, L494F, and L494A, which sometimes showed lower levels of protein on a Western.

**pPD Oxidase Activity of P4 Mutant Fet3p—** To determine whether our substitutions affected Fet3p multicopper oxidase function, pPD oxidase activity was measured. Membrane extracts from cells expressing mutated proteins were run on an SDS-PAGE gel, and pPD oxidase activity was visualized by the color change when pPD becomes oxidized (11). This assay, although not quantitative, is highly specific for Fet3p activity. Wild type extracts (Leu-494) showed pPD oxidase activity, whereas extracts from vector alone (Vect) produced no pPD oxidase activity (Fig. 2B). As expected, substitutions of the type I copper ligands that were predicted to disrupt enzyme activity (L494K and H489F) did not show any pPD oxidase activity. Of the P4 amino acid substitutions found in other multicopper enzymes, pPD oxidase activity was observed only in L494M. The predicted conservative substitutions such as L494A, L494I, and L494V also did not show any pPD oxidase activity. The lack of observable activity in the P4 mutants does not indicate that these enzymes are completely inactive. This assay requires approximately 3 ng of total membrane protein (10% of the amount used in these blots) from cells containing wild type Fet3p. Thus, any mutation that decreases Fet3p activity by 10-fold may not appear active in this assay.

**Ferroxidase Activity of the P4 Mutant Fet3p—** To determine whether the mutant Fet3p constructs had lost or retained iron oxidase activity, ferroxidase assays were performed using crude membrane preparations in a gel-based assay similar to pPD activity assays. Gels were incubated in the presence of ferrous iron and developed by the addition of ferrozine, which changes color when bound to ferrous iron. Areas that remained color-free corresponded to the location of Fet3p or the multicopper oxidase ceruloplasmin. This assay has the advantage of being highly specific for Fet3p activity, with no confounding effects from nonspecific ferroxidase activity. Heat-denatured Fet3p and ceruloplasmin did not show ferroxidase activity. Ferroxidase assays of the mutant constructs showed that only L494M possessed detectable ferroxidase activity (Fig. 2C). This is consistent with the results obtained for pPD oxidase activity, indicating that the mutations made to the P4 copper ligand have not resulted in a detectable change of specificity but an overall affect on enzyme activity.
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construct showed no specific fluorescence (Fig. 4A). In the presence of Fet3p, the primary localization of Ftr1p was at the plasma membrane, with some remaining within intracellular vesicles (Fig. 4B). Not all cells possessing the Ftr1p:Myc construct exhibited fluorescence. The variability of immunofluorescence may be due to the Ftr1p induction time (6 h), production of endogenous Ftr1p rather than the plasmid-borne Ftr1p: Myc, or cellular variability of Ftr1p production. Cells that do not express Fet3p, however, displayed little Ftr1p:Myc fluorescence (Fig. 4C), and the signal detected was present only within intracellular vesicles. All cells expressing the P4 mutant proteins showed detectable cell surface Ftr1p immunofluorescence (Fig. 4, D–J). These results indicate that all the mutant Fet3p proteins were capable of mediating the localization of Ftr1p to the cell surface.

Examination of Other Potential Ferroxidase-specific Regions—Residues within domains of Fet3p that are not found in non-iron-oxidizing multicopper oxidases also have been hypothesized to determine ferroxidase activity (10). Of the domains and residues predicted to confer ferroxidase activity, only one domain was highly conserved between Fet3p and the Fet3p S. pombe homologue Fio1p (4). This protein has also been shown to effect high affinity iron transport with a homologous S. pombe permease. We predicted that any domain important for ferroxidase activity should be conserved between these two homologous proteins since they function similarly. The conserved domain in Fet3p contained three residues hypothesized to be important for ferroxidase activity, glutamic acid 228, aspartic acid 228, and glutamic acid 330. To assess their importance, these three residues were changed to an alanine.

Mutant Fet3ps E227A, D228A, and E330A were expressed at normal levels and demonstrated the same mobility on SDS-PAGE gels as wild type Fet3p (Fig. 5A). All substitutions resulted in normal pPD and iron oxidase activity (Fig. 5, B and C). Thus, mutations of these residues did not affect protein function and did not alter the substrate specificity of Fet3p in vitro. These substitutions also did not substantially affect high affinity iron transport (Fig. 6), although D228A consistently showed slightly lower levels of high affinity iron transport.

Discussion

Data obtained in vivo and in vitro suggest that Fet3p acts as a ferroxidase to effect iron transport, converting ferrous to ferric iron, which is then transported through the permease Ftr1p (3, 5). This activity presents a conundrum because yeast require the action of a cell surface ferrireductase to convert extracellular ferric iron to ferrous, which is then recognized by the high affinity iron transport system (12, 13). We have hypothesized that the ferrireductase serves to solubilize the iron and free it from extracellular chelates. The ferroxidase activity of Fet3p may serve to impart specificity to the high affinity iron transport system (12, 13). We have shown that Fet3p lacking oxidase activity does mediate Ftr1p localization to the cell surface. This result indicates that Fet3p, but not Fet3p activity, is required for Ftr1p localization. Fet3p oxidase activity, however, is required for high affinity iron transport, since cells harboring enzymatically inactive mutant Fet3p are unable to transport ferrous iron even though Ftr1p is on the cell surface. These findings establish that Fet3p oxidase activity is directly required for high affinity iron transport.

Acknowledgments—We thank Dr. Andrew Dancis for supplying the FTR1:Myc construct for the immunofluorescence studies and Dr. Diane Ward for technical assistance with the fluorescent imaging. We also thank Dr. James P. Kushner, Dr. Rich Ajoka, and the members of the Kaplan lab for editorial comments regarding this manuscript.

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