Mutational analysis of the iron binding site of *Saccharomyces cerevisiae* ferroxidase Fet3. An in vivo study

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Abstract The role of residues predicted to be involved in the binding of iron by the yeast ferroxidase Fet3 has been studied by site-directed mutagenesis. The effect of Fet3 mutations E185A, E185Q, Y354F, D409V and H489D has been investigated in vivo by kinetic analyses of high affinity iron uptake. Our results indicate that Glu-185 is critical for the binding of iron, since substitution of this residue with Ala or Gln strongly affects both growth and the kinetic parameters of high affinity iron uptake, greatly increasing K_m. Mutations Y354F and D409V result in less severe alteration of high affinity iron uptake, while mutant H489D is unable to grow under conditions of iron limitation. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Fet3; Ferroxidase; High affinity iron uptake; Site-directed mutagenesis

1. Introduction

Iron is essential for living cells, however it can become extremely toxic if its intracellular levels are too high. Thus, cells have evolved sophisticated systems to control iron uptake. In the yeast *Saccharomyces cerevisiae* a low affinity and a high affinity iron uptake system have been recently discovered [1]. The ferroxidase Fet3 is a key component of the yeast high affinity iron uptake system together with the iron permease Ftr1 [1,2]. It has been proposed that extracellular Fe(III) is reduced by metalloreductases belonging to the Fre family of proteins, subsequently Fet3 oxidizes Fe(II) to Fe(III), which is then transported into the cell through Ftr1. Fet3 belongs to the family of the blue multicopper oxidases, which are able to couple the one-electron oxidation of substrates to full reduction of molecular oxygen to water, avoiding release of reactive partially reduced oxygen species. This ability is provided by the presence of type 1 blue copper, which is the primary electron acceptor from the reducing substrate, and of a trinuclear copper cluster, formed by type 2 and binuclear type 3 copper, which constitutes the oxygen binding and reduction site [3]. Thus, electrons flow from reduced type 1 copper to the trinuclear copper cluster through a branched His–Cys–His pathway, where the type 1 copper Cys ligand is flanked by two of the trinuclear cluster His ligands. In the multicopper oxidases, substrate binding is predicted to take place close to type 1 copper; in particular, in Fet3 an iron binding site has been proposed to be formed by residues Glu-185, Tyr-354, Asp-409 and possibly the type 1 copper ligand His-489, on the basis of homology modeling [4]. We have clearly established the role of Glu-185 and Tyr-354 by site-directed mutagenesis and in vitro assay of the ferroxidase activity of recombinant Fet3 E185A and Y354F [5].

To further investigate the functional properties of Fet3, we have produced different Fet3 mutants in which each of the residues proposed to be required for binding of iron has been substituted by site-directed mutagenesis. The mutant proteins have been expressed in a *S. cerevisiae* strain lacking endogenous Fet3 and the functional relevance of the mutations introduced has been assayed in vivo by complementation of the growth defect of the Δfet3 strain under conditions of iron limitation and by estimation of the kinetic parameters of high affinity iron transport.

2. Materials and methods

2.1. Yeast strains and culture media

S. cerevisiae strains DY150 (*MATa*, *ura3-52, leu2-3, 112, trp1-1, his3-11, ade2-1, can1-100(oc)*) and DEY1397-6A (*MATα*, *fet3::HIS3, ade2, can1, his3, leu2, trp1, ura3*) were a generous gift of Professor J. Kaplan. Yeast cells were grown at 30°C in minimal medium (0.67% yeast nitrogen base without amino acids, with the necessary auxotrophic supplements) with 2% glucose (MD) or 2% galactose (MGal). The medium was buffered to pH 6 with 100 mM potassium phosphate buffer and made iron-limited by addition of the iron chelator bathophenanthrolinedisulfonate (BPS) 80 μM. The amount of iron in buffered MGal was estimated to be 1–1.2 μM by colorimetric determination with BPS (ε_{535} 22 400 M^{-1} cm^{-1}) [6].

2.2. Site-directed mutagenesis of Fet3

Site-directed mutagenesis was performed by the polymerase chain reaction (PCR) megaprimer method [7]. Fet3 mutants E185A and Y354F have already been described [5]. Mutants E185Q, D409V and H489D were produced with the following mutagenic primers: E185Qrev, 5′-tgggatgggctgagcacc-3′; D409Vfwd, 5′-aaccaggtcacaggtacc-3′; H489Dfwd, 5′-gaatgggatttgttgcaagg-3′. These primers were used in the first round of PCR with the external primers α 5′-ggaattcatgactaaggctttgct-3′ or ω 5′-ggaattcttagaagaaccgtttggc-3′ (α-E185Q, D409V-ω, H489D-ω) to produce the megaprimers, with pBSFet3 wild type as template. The megaprimers were purified and employed in the second round of PCR with the same template, α-E185Q with primer ω, D409V-ω with primer α and H489D-ω with primer α to obtain the full-length mutated Fet3 which were cloned in pBluescript. The presence of the desired mutations was verified by automated DNA sequencing at the Biogen-ENEA facility.

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2.3. Fet3 expression and analysis

The multicopy vector pYeDP1/8-2 [8] was employed for expression of recombinant wild type and mutant Fet3 under control of the inducible Gal10 promoter. Wild type Fet3 and mutants E185A and Y354F were cloned in pYeDP via the *Eco*RI cloning site (pYFet3) and the correct orientation of the insert was verified by restriction analysis. Restriction of pBSFet3 E185Q, D409V or H489D with *Eco*72I and *Eco*91I was carried out to obtain a cassette containing the desired mutation which was then inserted in pYFet3. The yeast Δfet3 strain DEY1397-6A was transformed by electroporation according to standard procedures [9] with wild type and each mutant pYFet3 and with the vector alone. Cells were grown in MGal medium without uracil and total membrane extracts were obtained following lysis of cells with glass beads, as described [5]. Total protein content was determined with the microBCA assay (Pierce). Non-denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and staining for Fet3 oxidase activity with *o*-dianisidine were performed as described [5]. Immunodetection of Fet3 was performed with a polyclonal rabbit antibody directed against a peptide spanning Fet3 residues 164–178, obtained from Primm (Milan, Italy). The antibody was affinity purified using peptide coupled to CNBr-Sepharose (Sigma) according to established methods. SDS–PAGE on a 7.5% gel was performed under denaturing and reducing conditions, and was followed by transfer onto nitrocellulose. The blot was incubated with a 1:1000 dilution of the affinity purified antibody overnight at 4°C and developed with the BM Chemiluminescence Western blotting kit (Roche).

2.4. Iron uptake assay

Cells were grown overnight in MGal medium, inoculated in buffered MGal for 8–10 h and 80 μM BPS was added for further 15 h. Cells were harvested at OD_{600} 0.5–1 and iron uptake was performed with $^{55}FeCl_3$ (13.6 mCi/mg, NEN Life Sciences), essentially according to published procedures [10]. ^{55}Fe uptake was measured at 30°C for 10 min in 50 mM MES pH 6.1, containing 2% galactose in the presence of 1 mM ascorbate. The assay samples were chilled on ice, vacuum filtered through Whatman GF/C glass filters and washed with 10 ml of ice-cold 20 mM citrate pH 5.5, containing 5 mM EDTA. When used above 0.2 μM, radioactive iron was diluted with cold iron and specific activity was adjusted accordingly. Background levels due to non-specific uptake were obtained on parallel samples kept on ice and were subtracted before calculation of uptake rates. Cell-associated radioactivity was measured by liquid scintillation counting with an LKB 1211 Rackbeta counter.

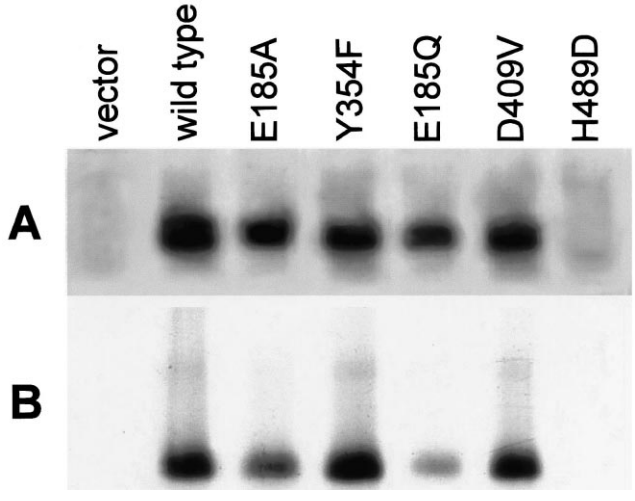

Fig. 1. SDS–PAGE analysis of recombinant Fet3. A: Western blot. Yeast cells were grown in buffered MGal medium to OD_{600} 1.5 and membrane extracts were obtained as described in Section 2. 30 μg total membrane proteins were loaded in each lane following heat denaturation in the presence of DTT. A polyclonal anti-Fet3 peptide-antibody was used and the blot was developed with a chemiluminescent detection system. B: Oxidase activity. 30 μg total membrane proteins were loaded in each lane and the gel was stained for oxidase activity with *o*-dianisidine.

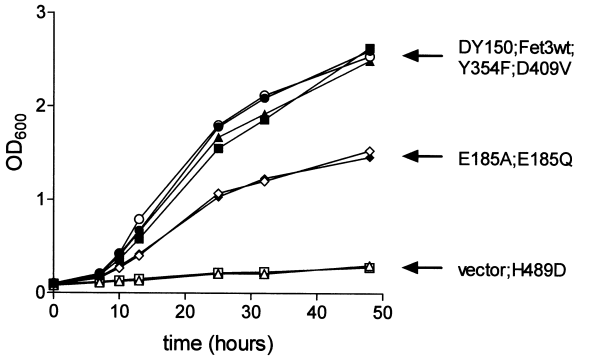

Fig. 2. Representative growth of cells containing native or recombinant Fet3. Cells were grown to OD_{600} ca. 3 in buffered MGal, inoculated to OD_{600} 0.1 in buffered MGal supplemented with BPS 80 μM and grown for further 48 h. See Fig. 3 for specific legend.

3. Results

3.1. Expression of Fet3 mutants and effect on growth

The four residues which have been proposed to constitute the iron binding site of Fet3 have been substituted by site-directed mutagenesis to generate Fet3 E185A, Fet3 E185Q, Fet3 Y354F, Fet3 D409V and Fet3 H489D. The recombinant protein has been expressed under galactose-driven induction in a *S. cerevisiae* strain lacking endogenous Fet3. Expression levels were assayed by Western blot (Fig. 1A) and by non-denaturing SDS–PAGE followed by oxidase activity staining (Fig. 1B) of membrane extracts from the recombinant strains. All mutants showed comparable levels of expression and produced oxidase-active band similar to that of the wild type protein, with the notable exception of Fet3 H489D. This latter mutant produced a very faint immunopositive band with altered electrophoretic mobility, and did not have a detectable enzymatic activity. Expression of all proteins was found to be dependent on the presence of galactose in the culture medium (data not shown).

The ability of recombinant Fet3 to complement the growth defect of the Δfet3 strain under conditions of iron limitation (buffered MGal supplemented with BPS 80 μM) was investigated (Fig. 2). Growth of the parental strain DY150 was taken as control. It can be clearly seen that while the Δfet3 strain transformed with the expression vector alone is unable

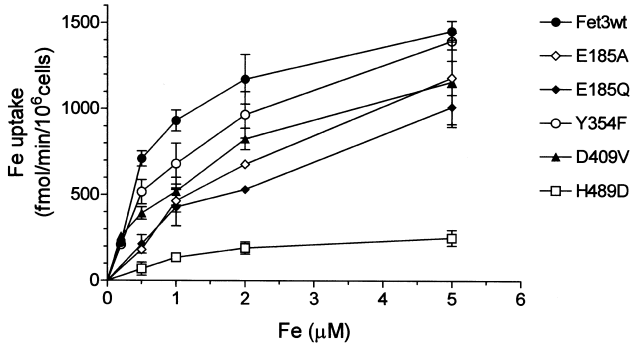

Fig. 3. High-affinity iron uptake assay of cells containing recombinant wild type or mutant Fet3. Yeast cells were grown in buffered MGal supplemented with BPS 80 μM to OD_{600} 0.5–1 and iron transport was assayed as described in Section 2. Data represent the mean of at least two independent experiments.

Table 1
Fe(II) K_m values for high affinity iron uptake of *S. cerevisiae* Δfet3 expressing recombinant Fet3

Fet3	K_m (μM)
Wild type	0.80 ± 0.15
E185A	4.34 ± 1.23
E185Q	4.25 ± 1.74
Y354F	1.28 ± 0.08
D409V	1.49 ± 0.41
H489D	N.D.[a]

[a]N.D., not determined.

to grow under these conditions, Fet3 wild type, Y354F and D409V restore growth rates similar to those of DY150. Mutations E185A and E185Q result in a partial restoration of growth, albeit at slower rate, while Fet3 H489D is unable to complement the growth defect of the Δfet3 strain under these conditions of iron limitation. Similar results were obtained when the medium was supplemented with BPS 40 μM (data not shown).

3.2. Kinetic parameters for high affinity iron uptake

To study the functional effect of mutant Fet3 on high affinity iron uptake, yeast cells were grown in the presence of galactose and of the iron chelator BPS 80 μM. The presence of BPS is required to induce the synthesis of the endogenous iron permease Ftr1, which is essential to observe high affinity iron uptake [11]. High affinity iron uptake assays were carried out in the presence of ascorbate in order to supply iron as Fe(II). Fig. 3 presents the iron transport rates obtained for strains expressing wild type or mutant Fet3. The K_m for high affinity iron uptake varied essentially in line with the observed effect of mutant Fet3 on growth. The estimation of K_m values for iron is reported in Table 1.

4. Discussion

The ferroxidase Fet3 is an essential component of the yeast high affinity iron transport system. Binding of ferrous iron by this enzyme is the first event in the process, followed by oxidation of the metal and translocation to the iron permease Ftr1, which enables iron to cross the plasma membrane and safely enter the cell. The structural requirements for substrate binding by Fet3 have recently begun to be elucidated by site-directed mutagenesis studies based on homology modeling and in vitro partial characterization of the protein [5]. We have extended our previous studies in order to gain further insight on the functional properties of Fet3. To this end, each of the residues predicted to be involved in binding of iron has been replaced to generate a protein with impaired affinity for its substrate. Expression of mutant Fet3 in a Δfet3 strain has allowed us to monitor directly in vivo the properties of the high affinity iron transport system constituted by the endogenous iron permease Ftr1 and the recombinant ferroxidase Fet3.

Expression levels of recombinant Fet3 wild type, E185A, E185Q, Y354F and D409V appeared to be comparable, as judged by Western blot analysis and by the oxidase-active band detected by non-denaturing SDS–PAGE. The functional effect of the different mutations was studied in vivo under conditions of iron limitation by monitoring cell growth and high affinity iron uptake rates. Recombinant Fet3 wild type fully complements the growth defect of the Δfet3 strain. On the other hand, the mutant Fet3 can be divided in two groups: substitution of Glu-185 either by Ala or Gln has a severe impact on growth rate, while replacement of Tyr-354 or Asp-409 apparently restores growth rates similar to those obtained with the wild type protein. In line with this observation, high affinity iron uptake by the strains expressing the different mutant Fet3 exhibits this same trend. While variations in V_{max} can be difficult to interpret, as they can be due also to slight differences in Fet3 and/or Ftr1 expression levels, the K_m value for iron, which will not depend on enzyme levels at substrate concentrations much higher than those of the enzyme, is also the kinetic parameter which is expected to be affected most by the mutations introduced on Fet3. As a matter of fact, the results reported in Fig. 3 and Table 1 show that K_m for iron is increased for mutant Fet3 compared to the wild type protein. Substitution of Glu-185 in Fet3 E185A and E185Q results in a similar ca. five-fold increase in K_m, while Fet3 Y354F and D409V exhibit a ca. two-fold increase in K_m. It is interesting to point out that these results compare well with the K_m values obtained in vitro on Fet3 E185A and Y354F, which showed a ca. 6-fold and 1.5-fold increase, with respect to Fet3 wild type [5]. Moreover, it is worth noting that, as previously reported [10,12], the K_m value for iron of the high affinity uptake system is lower than that of purified Fet3. This appears to be a general feature as also the high affinity system of the yeast *Pichia pastoris* shows a lower K_m compared to purified Fet3, both K_m values being higher than those for *S. cerevisiae* [13]. This finding suggests that interactions of Fet3 with other component(s), such as the permease Ftr1, might modulate the overall affinity of the system for iron. However, it is clear from the results reported in this paper that the ferroxidase reaction catalyzed by Fet3 may be the rate limiting step in the high affinity iron transport system of *S. cerevisiae*, at least under physiological conditions, when the machinery presumably does not work at V_{max}. In other words, a decrease of the binding affinity of Fet3 for iron is critical in that it leads to an increase of the overall K_m and thus to a diminished efficiency of the system.

The results obtained for Fet3 H489D deserve further comment: this mutation is particularly critical since His-489 is also a ligand to type 1 copper. Thus, it is possible that the mutant protein could be lacking this particular copper atom and for this reason expression levels are very low (if any), no oxidase-active band is detected by non-denaturing SDS–PAGE, and no functional complementation is evident as both growth and high affinity iron uptake are concerned.

In conclusion, the findings reported in this paper contribute to defining the role of the ferroxidase activity of Fet3 in high affinity iron uptake of *S. cerevisiae*, as probed by site-directed mutagenesis. Substitution of each of the four residues predicted to take part in binding of iron demonstrates that in all cases K_m for high affinity iron uptake is altered. In particular, a critical role for Glu-185 is evident since replacement of this residue produces the most severe effects on growth and K_m for iron.

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