The essential role of Glu-185 and Tyr-354 residues in the ferroxidase activity of *Saccharomyces cerevisiae* Fet3

Maria Carmela Bonaccorsi di Patti[a], Maria Rosa Felice[b], Angela Pia Camuti[b], Amalia Lania[b], Giovanni Musci[b,*]

[a] *Department of Biochemical Sciences, University of Rome La Sapienza, Rome 00185, Italy*
[b] *Department of Organic and Biological Chemistry, University of Messina, Salita Sperone 31, S. Agata Messina 98166, Italy*

Received 25 February 2000

Edited by Hans Eklund

Abstract The structural determinants required for ferroxidase activity by the yeast multicopper oxidase Fet3 have been partially clarified by site-directed mutagenesis based on homology modeling. Glu-185 and Tyr-354 were substituted with Ala and Phe, respectively. Fet3 E185A retained ca. 5% residual ferroxidase catalytic efficiency, and almost 40% oxidase efficiency. On the other hand, Fet3 Y354F exhibited 50% residual efficiency as a ferroxidase and more than 70% as an oxidase. These results provide new insights in the mechanism of iron binding and oxidation by Fet3, establishing the essential role of Glu-185 and Tyr-354, and allowing to dissect ferroxidase from non-iron oxidase activity.

Key words: Fet3; Ferroxidase; Iron homeostasis; Site-directed mutagenesis

1. Introduction

The safe handling of iron is of paramount importance to living cells which have to cope with their requirement for this essential trace metal and with the unpleasantness of iron chemistry. A picture is now emerging that links redox reactions to the control of the vectorial transport of iron across biological membranes [1]. Two members of a very exclusive family of enzymes appear to be involved in iron homeostasis in eukaryotes as distantly related as yeast and vertebrates: the multicopper oxidases Fet3 and ceruloplasmin, which accomplish the oxidation of Fe(II) to Fe(III). Membrane-bound Fet3 is involved in high affinity iron uptake together with the permease Ftr1 [2], while the role of ceruloplasmin is less clear since evidence has been reported both in favor of its participation in uptake [3,4] and in efflux of the metal from cells [5,6]. The requirement for a multicopper oxidase is dictated by the ability of these enzymes to couple the one-electron oxidation of substrate(s) to full reduction of molecular oxygen to water. This ability is provided by a functional unit formed by three types of copper binding sites with different spectroscopic and functional properties [7]. Type 1 blue copper is the primary electron acceptor from the substrate, while a trinuclear cluster formed by type 2 copper and binuclear type 3 copper is the oxygen binding and reduction site. Intramolecular electron transfer from reduced type 1 copper to the cluster would take place through a conserved branched His-Cys-His motif which is typical of the blue oxidases and links the type 1 copper Cys ligand to two of the cluster His ligands [8]. Fet3 and ceruloplasmin are the only two multicopper oxidases endowed with ferroxidase activity, however, iron is not the sole substrate of these enzymes as also a variety of other substances can be oxidized. As a matter of fact, aromatic diamines such as *p*-phenylenediamine (*p*PD) or *o*-dianisidine (*o*DA) are routinely employed for detection of Fet3 and ceruloplasmin through oxidase activity and furthermore, ceruloplasmin is able to oxidize also biogenic amines [9,10], with possible implications in neuropathophysiology. The structural determinants required for this multisubstrate recognition have just begun to be investigated. The functional unit in the active site of Fet3 includes the trinuclear copper cluster and one type 1 blue copper atom, while ceruloplasmin is more complex since it possesses two additional type 1 copper sites. Recent structural data on ceruloplasmin demonstrate that different substrates bind to different sites on the protein. In particular, iron binding sites were identified close to the two type 1 copper atoms in domains 4 and 6 [11], while aromatic diamines such as *p*PD appear to be located in the crystal structure at the bottom of domain 4, quite far away from the mononuclear copper present in this domain [12]. Consistent with this view, anions have been found to selectively modulate the oxidase activity of ceruloplasmin, while being essentially ineffective on iron oxidation [13].

Structural information on Fet3 has been derived by homology modeling and the predicted structure is in line with the general features of the blue multicopper oxidases [14]. A potential iron binding site was identified in a position close to the type 1 copper atom homologous to that found in domain 6 of ceruloplasmin; the iron binding site was proposed to be formed by residues Glu-185, Tyr-354, Asp-409 and the type 1 copper ligand His-489. On the basis of this model we have performed site-directed mutagenesis of Fet3 and we have expressed the recombinant protein in the methylotrophic yeast *Pichia pastoris* with the purpose of defining the residues which are essential for the ferroxidase activity of Fet3.

2. Materials and methods

2.1. Strains

The following yeast strains were supplied by ATCC: *Saccharomyces cerevisiae* YPH252 (*MAT*α, *ura3–52, lys2–801, ade2–101, trp1*-Δ*1,*

*Corresponding author. Fax: (39)-90-393895.
E-mail: musci@isengard.unime.it

PII: S0014-5793(00)01435-6

his3-Δ200, leu2-Δ1). *P. pastoris* GS115 (*his4*) and *Escherichia coli* Top10F′ were from Invitrogen.

2.2. Fet3 cloning and mutagenesis

S. cerevisiae YPH252 genomic DNA was prepared according to established procedures [15] and employed for amplification of the Fet3 coding sequence by PCR with the expand high fidelity PCR system (Boehringer) with the following primers: upstream alpha: 5′GGAATTCATGACTAAGGCTTTGCT3′ and downstream omega: 5′GGAATTCTTAGAAGAACCGTTTGGC3′. The single PCR product was purified and cloned in pBluescript KS II after restriction with *Eco*RI (pBSFet3). Site-directed mutagenesis was performed by the PCR megaprimer method [16]. The first round of PCR was carried out with primer alpha and the mutagenic primer E185A: 5′TGGGATGGGCGCAGCACC3′ or with primer omega and the mutagenic primer Y354F: 5′GTGAATTTCGCCTTCTTC3′ with pBSFet3 as the template; the megaprimers were purified and employed in the second round of PCR, alpha-E185A with primer omega, and Y354F-omega with primer alpha to obtain the full-length mutated Fet3. Restriction with *Bst*EII and *Bam*HI was carried out to obtain a cassette containing the desired mutation which was inserted in pHIL-D2Fet3 and verified by sequencing. Automated DNA sequencing was performed at the Laboratorio Genoma Vegetale (ENEA) facility.

2.3. Fet3 expression

For heterologous expression in *P. pastoris* GS115, wild-type Fet3 DNA was cloned in the expression vector pHIL-D2 via the *Eco*RI cloning site and the correct orientation of the insert was verified by restriction analysis. pHIL-D2Fet3 was digested with *Not*I to obtain the expression cassette which was used to transform GS115 cells by electroporation according to the *Pichia* expression system manual. This expression cassette should direct integration of the foreign gene to replace the *Pichia* alcohol oxidase gene, giving rise to yeast colonies which grow very slowly in the presence of methanol as the sole carbon source. Positive clones were identified by screening for slow growth in the presence of methanol followed by screening for expression of recombinant Fet3 according to the *Pichia* expression system manual. The conditions chosen for production of recombinant Fet3 were: growth in BMGY medium to saturation ($OD_{600} \sim 20$) followed by induction in 1/5 volume BMMY medium for 3 days with addition of 0.5% methanol after the second day.

2.4. Fet3 purification and analysis

Total membrane extracts from *P. pastoris* GS115 cells were obtained as described [17] except that MOPS was used instead of Tris buffer and membranes were collected by ultracentrifugation at $100\,000 \times g$ for 50 min. Recombinant Fet3 was partially purified on ConA-Sepharose. Briefly, 15–20 ml membrane extracts were passed on 4 ml resin equilibrated in MOPS 25 mM pH 7.4/NaCl 500 mM/Triton 0.5%, the resin was washed with 10 volumes of the same buffer and bound proteins were eluted with two volumes of MOPS/Triton buffer containing NaCl 1 M and α-methylmannoside 1 M at room temperature for 60 min, repeated twice. Protein samples were then dialyzed against MOPS/Triton/NaCl 100 mM overnight and concentrated with Centricon 10 devices (Amicon).

2.5. Fet3 activity assays

Ferroxidase activity was measured by following iron oxidation at 315 nm in sodium acetate 100 mM pH 5 at 30°C with ferrous ammonium sulphate as the substrate. Oxidase activity was measured at 540 nm in sodium acetate 100 mM pH 5 at 30°C with *p*PD as the substrate.

2.6. Miscellaneous

Non-denaturing SDS–PAGE was carried out as previously described [17]. Total protein was determined by the microBCA assay (Pierce). Copper content was measured by atomic absorption spectroscopy on a Perkin Elmer 3030 instrument equipped with a graphite furnace. Fet3 three-dimensional model was visualized using GRASP [18]

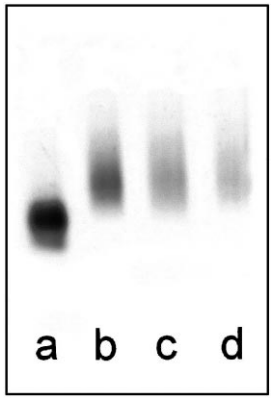

Fig. 1. Non-denaturing SDS–PAGE analysis of recombinant *S. cerevisiae* Fet3 wild-type (lane b), E185A (lane c) and Y354F (lane d) after ConA-Sepharose and concentration. Lane a is a membrane extract from the parent *P. pastoris* GS115 strain grown in YPD supplemented with the iron chelator BPS (80 μM) to induce endogenous Fet3. 20 μg of total protein were loaded and the gel was stained for oxidase activity with *o*-dianisidine.

3. Results and discussion

Recombinant *S. cerevisiae* Fet3 has been expressed in the methylotrophic yeast *P. pastoris* under the control of the alcohol oxidase promoter. Since *P. pastoris* produces its own Fet3 [17], which can be easily recognized on the basis of its different electrophoretic mobility, the absence of the endogenous *P. pastoris* Fet3 under the conditions employed for expression of the recombinant *S. cerevisiae* Fet3 (i.e. high cell density in the presence of methanol as the sole carbon source) was verified by non-denaturing SDS–PAGE. As shown in Fig. 1, the endogenous ferroxidase was undetectable in membrane extracts of methanol-induced *P. pastoris* expressing recombinant Fet3 (cf. lanes a, b). As a further control, the N-terminal sequence of the whole oxidase-active band of lane b was verified, and the sequence turned out to be that expected for *S. cerevisiae* Fet3, with no detectable contamination from *P. pastoris* Fet3.

Table 1
Catalytic parameters of wild-type and mutant Fet3

Ferroxidase activity	V_{max} (μM Fe/min)	K_m (μM)	V_{max}/K_m	Activity (%)
Wild-type	4.54	2.71	1.675	100
E185A	1.76	16.59	0.106	6.3
Y354F	3.59	4.17	0.861	51.4
Oxidase activity	V_{max} (ΔA_{540nm}/min)	K_m (mM)	V_{max}/K_m	Activity (%)
Wild-type	0.0179	0.30	0.060	100
E185A	0.0086	0.37	0.023	38.7
Y354F	0.0135	0.31	0.044	73.1

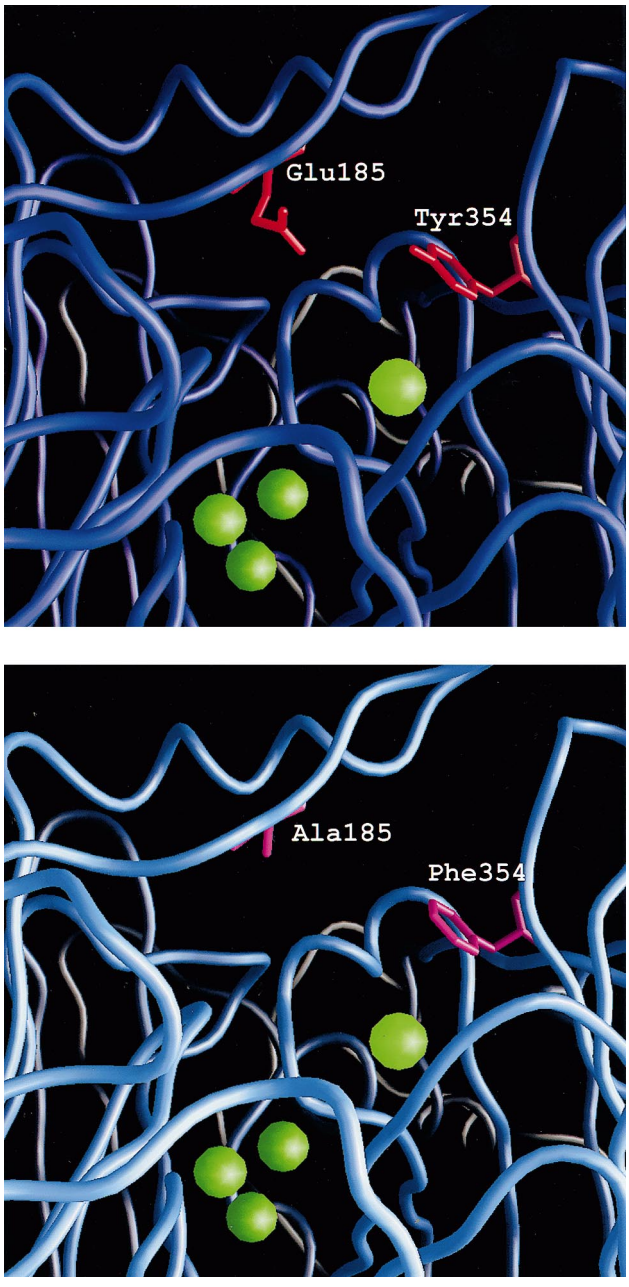

Fig. 2. Backbone worm representation of wild-type (top panel) and mutant (bottom panel) Fet3. For sake of clarity, the only residues shown are those mutated in the present study. Green spheres represent the copper ions.

S. cerevisiae Fet3 was mutated either at Glu-185 or at Tyr-354, which were changed to Ala (E185A) and Phe (Y354F) respectively, in order to investigate the role of these two residues in the catalytic activity of the protein. Visual inspection of the three-dimensional model (Fig. 2) revealed that either mutation removed a possible ligand for Fe(II). Wild-type and mutant *S. cerevisiae* Fet3 were partially purified by passage of membrane extracts on ConA-Sepharose as detailed in Section 2, and the amount of recombinant protein obtained from 140 ml of induction culture was estimated to be about 1 mg, based on copper content of samples. SDS–PAGE analysis shows that recombinant *S. cerevisiae* Fet3, both wild-type and mutant, appears to be heterogeneous (Fig. 1, lanes b–d), possibly due to heavy glycosylation in line with previous reports on this protein [19,20].

The catalytic activity of wild-type and E185A and Y354F mutant Fet3 towards iron and aromatic amines was measured and the results are reported in Fig. 3 and Table 1. All data are normalized for the copper content of Fet3 samples, on the assumption that all the copper present is bound to Fet3 with a stoichiometry of 4 Cu/protein. It has been reported that Fet3 has a tendency to loose copper during isolation [20]; we have noted that storage and/or freeze-thawing of the recombinant protein can cause loss of activity, as demonstrated by an increase in activity following reconstitution with $CuSO_4$/ascorbate (data not shown). This behavior was common to both wild-type and mutant Fet3; thus, manipulations were kept to a minimum and samples were purified and concentrated avoiding freeze-thawing throughout the whole procedure. Treatment with $CuSO_4$ and ascorbate did not increase the activity of such samples, suggesting that Fet3 was still fully loaded with copper. The two mutations have different effects on the catalytic parameters of Fet3, both with respect to ferroxidase activity and oxidase activity. Fet3 E185A reveals a decrease in V_{max} together with a dramatic increase in K_m for iron, resulting in a protein with 6% residual catalytic efficiency compared to wild-type, given as V_{max}/K_m ratio. On the other hand, the oxidase activity towards *p*PD appears to be less affected by this mutation, since the protein retains about 40% catalytic efficiency (Table 1). Fet3 Y354F behaves differently: the decrease in ferroxidase efficiency is less pronounced and it is paralleled by a similar decrease in oxidase catalytic efficiency (50% vs. 70% residual activity, respectively).

Fet3 is an excellent model system to investigate the struc-

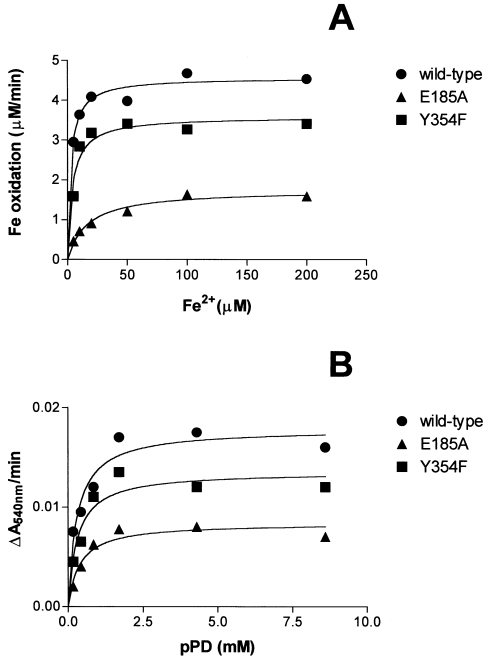

Fig. 3. Ferroxidase and oxidase activity of recombinant *S. cerevisiae* Fet3. Ferroxidase activity and oxidase activity vs. *p*PD were assayed as described in Section 2. The protein was 0.33 μM in copper content (corresponding to 82.5 nM Fet3, see Section 3) in a final volume of 0.5 ml. Data are representatives of four independent experiments.

tural determinants required for the catalytic activity towards different substrates exhibited also by ceruloplasmin. This work clearly demonstrates the role of Glu-185 and Tyr-354 in the ferroxidase activity of Fet3, as predicted by homology modeling [14]. Mutation of Glu-185 to Ala was expected to alter significantly the iron binding properties of Fet3, affecting especially the K_m value for iron. While steric effects should be negligible, the aliphatic side chain of Ala is unable to coordinate iron and the polarity of the iron binding site is also changed by substituting a charged residue such as Glu with a hydrophobic residue like Ala. As a matter of fact, the Fet3 mutant E185A showed an increase in K_m for iron and also a notable decrease in V_{max} with an overall residual catalytic efficiency of much less than 10%. The effect on the reaction velocity is more difficult to explain, since one would expect that once substrate iron is bound, catalysis can proceed through reduction of type 1 copper, possibly via the His-489 ligand. However, it is well known that electron transfer can be modulated by subtle changes at the copper sites [7] and due to the close proximity of the iron binding site to type 1 copper, it might be postulated that mutation E185A can affect also electron transfer rates either from substrate iron to type 1 copper or from type 1 copper to the trinuclear cluster. Another interesting feature of Fet3 E185A is the possibility to separate ferroxidase activity from oxidase activity, as this protein still retains significant activity towards *p*PD. This finding clearly indicates that iron and *p*PD bind at different sites on Fet3, as demonstrated by the almost unaltered K_m for *p*PD, and is in line with the recent report of a selective modulation by anions of the catalytic activity of the closely related oxidase ceruloplasmin [13]. Again, the decrease in V_{max} might be attributed to the introduction of the Ala residue close to type 1 copper.

Substitution of Tyr-354 with Phe in Fet3 Y354F is a more conservative change from a structural point of view. The mutation introduced is expected to be less dramatic than E185A, since maintaining the aromatic ring should not strongly perturb the overall hydrophobicity and geometry of the iron binding site, as only the hydroxyl group of Tyr would be missing. Glu-185, Asp-409 and His-489 should be sufficiently strong ligands for iron to ensure that the protein is still able to bind the metal with moderate to high affinity. Thus, the catalytic properties of the mutant Fet3 Y354F are perfectly in line with these assumptions: the decrease to 50% in ferroxidase catalytic efficiency is less severe but still remarkable, and oxidase activity is moderately affected by mutation of Tyr-354 to Phe, confirming that iron and *p*PD binding sites are different.

The results reported in this paper experimentally demonstrate for the first time the structural determinants required for ferroxidase activity of the multicopper oxidase Fet3. The role of predicted ligands for substrate iron has been demonstrated by site-directed mutagenesis allowing also to dissect ferroxidase activity from oxidase activity. Due to essential conservation of the location of the substrate binding site close to type 1 copper also in other multicopper oxidases, information gained on Fet3 will be useful in studies concerning the more complex ferroxidase ceruloplasmin.

Acknowledgements: We acknowledge Dr. E. Schininà for performing N-terminal sequence, and the University of Messina ('ex60%' funds) for financial support. This work was in partial fulfillment of the requirements for M.R.F.'s Ph.D. thesis.

References

[1] Wessling-Resnick, M. (1999) Crit Rev. Biochem. Mol. Biol. 34, 285–314.
[2] Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D. and Dancis, A. (1996) Science 271, 1552–1557.
[3] Mukhopadhyay, C.K., Attieh, Z.K. and Fox, P.L. (1998) Science 279, 714–717.
[4] Attieh, Z.K., Mukhopadhyay, C.K., Seshadri, V., Tripoulas, N.A. and Fox, P.L. (1999) J. Biol. Chem. 274, 1116–1123.
[5] Harris, Z.L., Durley, A.P., Man, T.K. and Gitlin, J.D. (1999) Proc. Natl. Acad. Sci. USA 96, 10812–10817.
[6] Richardson, D.R. (1999) J. Lab. Clin. Med. 134, 454–465.
[7] Solomon, E.I., Sundaram, U.M. and Machonkin, T.E. (1996) Chem. Rev. 96, 2563–2605.
[8] Messerschmidt, A. and Huber, R. (1990) Eur. J. Biochem. 187, 341–352.
[9] Frieden, E. (1979) in: Copper in the Environment, Part II (Nriagu, J.O., Ed.), pp. 241–284, Wiley, New York.
[10] Medda, R., Calabrese, L., Musci, G., Padiglia, A. and Floris, G. (1996) Biochem. Mol. Biol. Int. 38, 721–728.
[11] Lindley, P.F., Card, G., Zaitseva, I., Zaitsev, V.N., Reinhammar, B., Selin-Lindgren, E. and Yoshida, K. (1997) J. Biol. Inorg. Chem. 2, 454–463.
[12] Zaitsev, V.N., Zaitseva, I., Papiz, M. and Lindley, P.F. (1999) J. Biol. Inorg. Chem. 4, 579–587.
[13] Musci, G., Bellenchi, G.C. and Calabrese, L. (1999) Eur. J. Biochem. 265, 589–597.
[14] Bonaccorsi di Patti, M.C., Pascarella, S., Catalucci, D. and Calabrese, L. (1999) Protein Eng. 12, 895–897.
[15] Hoffman, C.S. (1994) in: Current Protocols in Molecular Biology (F.M. Ausubel et al., Eds.) 13.11, pp. 2–4, Wiley, New York.
[16] Sarkar, G. and Sommer, S.S. (1990) Biotechniques 8, 404–407.
[17] Bonaccorsi di Patti, M.C., Bellenchi, G.C., Bielli, P. and Calabrese, L. (1999) Arch. Biochem. Biophys. 372, 295–299.
[18] Nicholls, A., Sharp, K.A. and Honig, B. (1991) Proteins 11, 281–296.
[19] Hassett, R.F., Yuan, D.S. and Kosman, D.J. (1998) J. Biol. Chem. 273, 23274–23282.
[20] de Silva, D., Davis-Kaplan, S., Fergestad, J. and Kaplan, J. (1997) J. Biol. Chem. 272, 14208–14213.