

Acidic residues of yeast frataxin have an essential role in Fe–S cluster assembly

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Friedreich ataxia is caused by decreased levels of frataxin, a mitochondrial acidic protein that is assumed to act as chaperone in the assembly of Fe–S clusters on the scaffold Isu protein. Frataxin has the *in vitro* capacity to form iron-loaded multimers, which also suggests an iron storage function. It has been reported that alanine substitution of residues in an acidic ridge of yeast frataxin (Yfh1) elicits loss of iron binding *in vitro* but has no effect on Fe–S cluster synthesis *in vivo*. Here, we show that a marked change in the electrostatic properties of a specific region of Yfh1 surface—by substituting two or four acidic residues by lysine or alanine, respectively—impairs Fe–S cluster assembly, weakens the interaction between Yfh1 and Isu1, and increases oxidative damage. Therefore, the acidic ridge is essential for the Yfh1 function and is likely to be involved in iron-mediated protein–protein interactions.

Keywords: acidic ridge; Fe–S cluster; frataxin; Isu1; mitochondria; acidic residue

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INTRODUCTION

Friedreich ataxia, a progressive recessive neurodegenerative disease, is caused by a deficit in the levels of frataxin, a small acidic protein localized to mitochondria in eukaryotes (Campuzano *et al*, 1996). Frataxin has a role in the biosynthesis of Fe–S clusters, as suggested by severe alteration of the activity and assembly of Fe–S proteins in frataxin-deficient cells (Rötig *et al*, 1997; Duby *et al*, 2002; Mühlhoff *et al*, 2002). A link with Fe–S cluster formation was confirmed by independent lines of evidence. *In vitro* and at low iron to protein ratios, monomeric frataxins from different organisms bind both Fe²⁺ and Fe³⁺ ions with a defined stoichiometry (Adinolfi *et al*, 2002; He *et al*, 2004; Nair *et al*, 2004; Cook *et al*, 2006). Yeast frataxin forms a complex with Isu–IscU, the scaffold protein that assembles Fe–S clusters,

and with Nfs1–IscS, the cysteine desulphurase that provides the sulphur atoms (Gerber *et al*, 2003; Ramazzotti *et al*, 2004). It is thus widely accepted that frataxin is an iron chaperone, which delivers iron to Isu (Yoon & Cowan, 2003) or IscS (Layer *et al*, 2006). It has also been proposed that frataxin has an iron storage function. Defects in frataxin increase sensitivity to oxidants and *in vitro*, at low ionic strength and high iron to protein ratios, frataxins aggregate as large polymeric structures that trap numerous iron atoms (Adamec *et al*, 2000; Adinolfi *et al*, 2002).

The three-dimensional structure of frataxin is highly conserved from bacteria to humans and is characterized by a β -sheet that packs against two α -helices (Cho *et al*, 2000; Dhe-Paganon *et al*, 2000; Musco *et al*, 2000; He *et al*, 2004). The first helix and the edge of the β 1-sheet form a semi-conserved acidic ridge. The role of the acidic ridge in iron binding has been shown *in vitro* by nuclear magnetic resonance spectroscopy and mutant studies (Nair *et al*, 2004; Cook *et al*, 2006). A triple E18K/E19K/D22K mutation of CyaY leads to the complete loss of iron-promoted protein aggregation and Fe²⁺ ion binding to the monomers, indicating that these residues have the highest affinity for this cation (Adinolfi *et al*, 2002; Nair *et al*, 2004). In yeast, replacement of several acidic residues of Yfh1 by neutral residues encompassing the two presumed iron-binding sites causes the loss of iron-promoted aggregation (D86A(N)/E90A(Q)/E93A(Q), D78N/D82N/E89Q, D79N/D86N/E90Q, E93A/D97A/E103A) or ferroxidase activity (D79A/D82A). However, the yeast mutants show only a slight increase in cellular oxidative damage and no defect in the biosynthesis of Fe–S clusters (Aloria *et al*, 2004; Gakh *et al*, 2006). This has raised the puzzling question of the biological role of the conserved acidic ridge. Here, we show that replacing four of the most conserved residues of the frataxin family—D86, E89, D101 and E103—either by lysines or by alanines severely impairs the metabolism of Fe–S clusters *in vivo*.

RESULTS

Mutations D86K, E89K, D101A, D86A/E89A, D86K/E89K, D101A/E103A, D101K/E103K and D86A/E89A/D101A/E103A were obtained by site-directed mutagenesis of the *YFH1* gene (Fig 1) and introduced into yeast. These positions are exposed to the solvent in the frataxin structure and therefore should not affect the stability of the three-dimensional protein fold. In CyaY,

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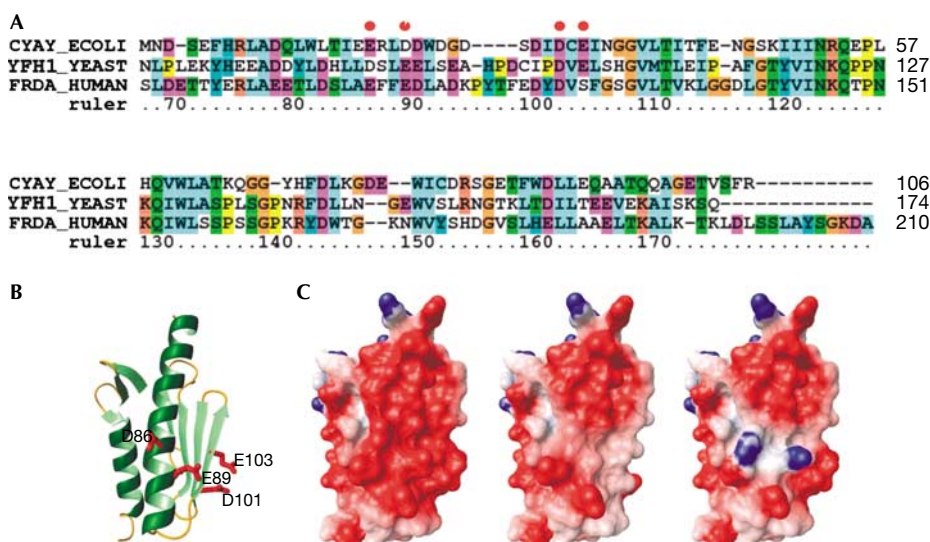


Fig 1 | Sequence and structural comparisons. (A) Sequence alignment of the conserved domain of *Escherichia coli* (CyaY), yeast (Yfh1) and human frataxins. The numbering refers to the Yfh1 sequence. (B) Ribbon representation of the conserved domain of Yfh1 (2ga5). The side chains of the residues mutated in this article are shown in red. (C) Comparison of the electrostatic surfaces of Yfh1 wild type (left), D86A/E89A/D101A/E103A (middle) and D86K/E89K (right).

lysine residues introduced at equivalent positions markedly change the electrostatic potential of the protein without affecting the tertiary structure (Adinolfi *et al*, 2002; A.P. & C. Pastore, unpublished data).

Cell sensitivity to iron

Iron is toxic to yeast cells deficient in frataxin as a consequence of the profound alteration in iron homeostasis, which leads to the accumulation of iron in mitochondria (Babcock *et al*, 1997; Foury & Cazzalini, 1997). Moreover, when $\Delta yfh1$ cells are grown in high-iron media, mitochondrial DNA is lost. On addition of 7 mM FeSO_4 in the culture medium, the cellular growth of mutants in which a single acidic residue was changed into lysine, or two residues into alanine, was not, or only very slightly, affected (Fig 2A). By contrast, the growth of the D86K/E89K, D101K/E103K and D86A/E89A/D101A/E103A *yfh1* mutants was severely inhibited in high-iron media. A high loss of mitochondrial DNA promoted by iron was also observed (Fig 2B). All mutants were more sensitive to hydrogen peroxide than wild type (data not shown), but strong oxidative damage was observed only in mutants harbouring two substitutions with lysine or four substitutions with alanine.

Frataxin levels in wild type and mutants

In a previous report, we showed that Yfh1 levels were low in several temperature-sensitive mutants (Ramazzotti *et al*, 2004). This probably results from protein degradation by the mitochondrial quality control protease machinery that has recognized folding defects (Wagner *et al*, 1994). By contrast, Yfh1 levels in all mutants of this study were similar to those of wild type (Fig 3), providing confirmation that the Yfh1 fold is unaffected by the lysines. The electrophoretic mobility of D101A and D101K/E103K Yfh1 in SDS-PAGE was only very slightly increased compared

with that of wild-type Yfh1, which migrates as a 20-kDa protein (Branda *et al*, 1999), whereas D86K, E89K, D86A/E89A, D86K/E89K and D86A/E89A/D101A/E103A Yfh1 migrated roughly as expected for a 14-kDa protein. A similar modification of the electrophoretic mobility was reported for D79A/D82A Yfh1 by Gakh *et al* (2006), who verified that the molecular weight was identical for wild-type and D79A/D82A Yfh1 by using mass spectrometry.

Defect of Fe-S cluster assembly in isolated mitochondria

Fe-S cluster assembly was followed by measuring the incorporation of a 2Fe-2S cluster into ^{35}S -radiolabelled apo-Yah1 ferredoxin, synthesized *in vitro* and imported into isolated energized mitochondria (Fig 4; Duby *et al*, 2002). The kinetics of conversion of apo- to holo-Yah1 were not affected in D86K, and only slightly affected in the E89K *yfh1* mutant. However, the conversion rate to the holo-form was substantially decreased in the D86K/E89K *yfh1* mutant. These data show the synergistic effect of the double substitution on Fe-S cluster assembly.

Aconitase activity and mitochondrial iron load

Cells were grown in a synthetic medium supplemented with raffinose, a good respiratory carbon source. When no extra iron is added to the medium, iron does not accumulate in mitochondria even in a null *yfh1* strain (Foury, 1999), and thus cellular oxidative damage is reduced to a minimal level. Under these conditions, the activity of aconitase—an enzyme requiring a 4Fe-4S cluster for its activity—was decreased only in D86K/E89K, D101K/E103K and D86A/E89A/D101A/E103A mutants (Table 1, No iron). However, when $5\ \mu\text{M}$ FeSO_4 was added to the medium (Table 1, $5\ \mu\text{M}$ iron), aconitase activity decreased even in the least severe mutants, although to different extents. Although the mean values of the mitochondrial iron concentrations from several independent

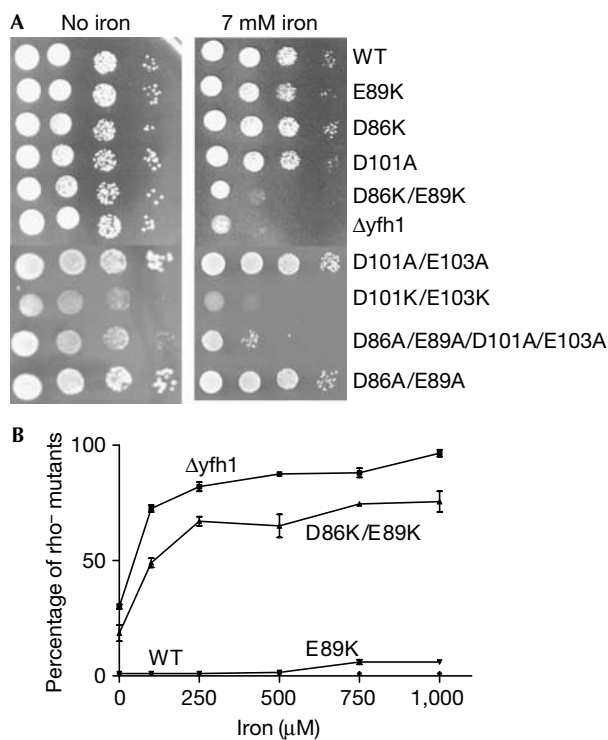


Fig 2 | Iron sensitivity of wild-type and mutant cells. (A) Serial dilutions of cell suspensions were spotted onto plates containing glucose minimum medium and different concentrations of FeSO_4 , and cells were grown at 28 °C for 3 days. (B) Cells were grown in liquid glucose-rich medium for 16 h in the presence of increasing concentrations of FeSO_4 and spread for single colonies on minimum medium. The small colonies were identified as respiratory-deficient ρ^- mutants. WT, wild type; $\Delta yfh1$, *YFH1* gene-deleted strain.

experiments, carried out with wild-type and mild mutants, were not statistically different (except for E89K), in each separate experiment taken, the iron concentration was always higher in mutants than in wild type (data not shown). This small disturbance in iron homeostasis is probably the cause of the decrease in the activity of aconitase by losing its external iron atom, which is extremely sensitive to free radicals.

Weakened association of Yfh1 and Lsu1 in mutants

We prepared soluble cell extracts from yeast strains over-expressing Yfh1 and Lsu1, and used affinity chromatography with an antiserum raised against Yfh1 to detect a physical interaction between Yfh1 and Lsu1. Wild-type Yfh1 was quantitatively bound by the Yfh1 antibody, and a substantial fraction of Lsu1 (30–50%) was also bound to the resin and co-eluted with Yfh1 at low pH (Fig 5A). When a preimmune serum was used instead of the anti-Yfh1 serum (Fig 5A), no detectable Yfh1 or Lsu1 was recovered in the bound fraction. When EDTA was added during the washing steps, Yfh1 was recovered in the bound fraction but Lsu1 was released (Fig 5B). These data show that the interaction between Lsu1 and Yfh1 requires a metal, probably iron as previously reported (Gerber et al, 2003). When the same experiment was performed with D86K/E89K, no significant Lsu1 binding was detected (Fig 5C). The fraction of bound Lsu1 was slightly higher

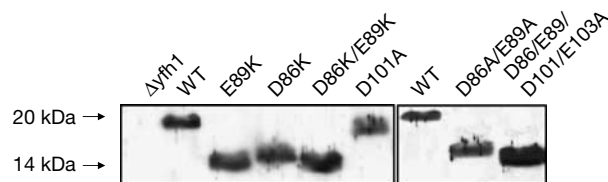


Fig 3 | Electrophoretic mobility of wild-type and mutant Yfh1 in SDS-polyacrylamide gels. A 40 μg portion of mitochondrial proteins was loaded onto 14% polyacrylamide gels. Yfh1 was detected by western blot analysis using a polyclonal Yfh1 antibody. D86/E89/D101/E103A is D86A/E89A/D101A/E103A. WT, wild type.

for the E89K and D86A/E89A/D101A/E103A mutants but remained very low compared with wild type (Fig 5C,D). These data show that the loss of negative charges at the surface of Yfh1 weakens the interaction between Lsu1 and Yfh1. Considering the rather slight defects of the E89K *yfh1* mutant in Fe-S cluster assembly, we were expecting a higher fraction of Lsu1 to be associated with Yfh1. However, the complex formed by E89K Yfh1 and Lsu1 is likely to be less stable and to dissociate during the preparation of cell extracts. It is also possible that although decreased, the levels of the E89K Yfh1 and Lsu1 complex are not significantly limiting for the synthesis of Fe-S clusters. Consistently, no decrease in Fe-S cluster assembly has been observed in cells with low levels of frataxin (Aloria et al, 2004; Ramazzotti et al, 2004).

DISCUSSION

It was shown previously that substitutions of several acidic residues in helix 1 and strand 1 of Yfh1 by alanines result in loss of the *in vitro* capacity to form multimeric structures binding iron (Aloria et al, 2004; Gakh et al, 2006). In the yeast mutants, there was only a slight increase in sensitivity to oxidative stress, but no effect on Fe-S cluster assembly. It was thus suggested that frataxin had an iron storage function relevant to anti-oxidant defence, which is distinct from the chaperone function (Gakh et al, 2006). It must be noted, however, that experiments were performed *in vitro* without the biological partners of frataxin, such as Lsu1 and Nfs1, and it can be expected that iron-binding properties within the complex formed by Nfs1, Lsu1 and Yfh1 *in vivo* are different from those of Yfh1 alone *in vitro*.

Analysing another set of mutations, we show here that the acidic ridge is crucial for Yfh1 function and is involved in Fe-S cluster assembly. Loss of function, however, requires a marked change in the electrostatic properties of the Yfh1 surface, which is obtained by replacing either two acidic residues by lysines or four residues by alanines, whereas single mutants have a mild phenotype. In the D86K/E89K, D101K/E103K and D86A/E89A/D101A/E103A *yfh1* mutants, the assembly of Fe-S clusters is severely altered, the interaction between Yfh1 and Lsu1 is markedly weakened, and the cellular sensitivity to iron and hydrogen peroxide is increased. By contrast, a mild phenotype is observed in single lysine or double alanine mutants. An appropriate acidic environment is therefore required for the function of frataxin in Fe-S cluster assembly.

Our data do not support the idea that sensitivity to oxidative damage and defects in Fe-S cluster assembly proceed

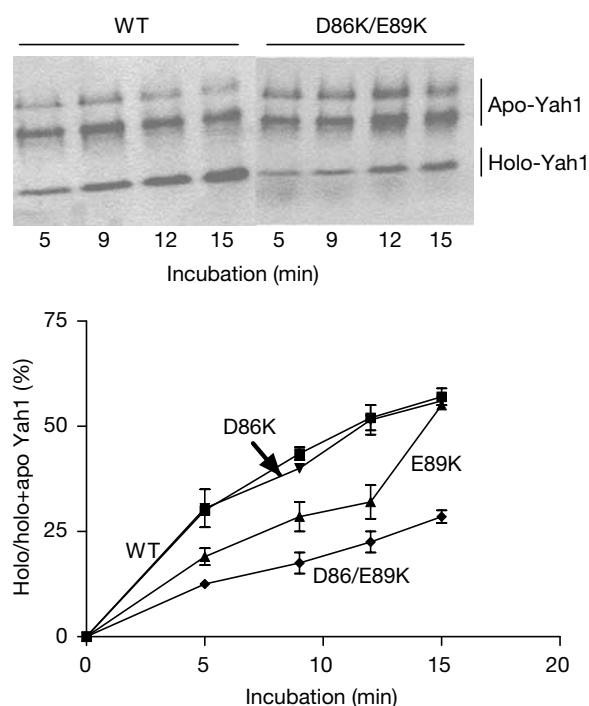


Fig 4 | Conversion of ³⁵S-radiolabelled apo- to holo-Yah1 in isolated energized mitochondria from wild-type and mutant strains. In native gel electrophoresis, the acidic mature form of Yah1 gives a fast migrating holo-form and two slowly migrating reduced and oxidized apo-forms (Leibrecht & Kessler, 1997). Exposure time of the autoradiography was 10 days. The experiment was carried out twice for each strain. WT, wild type.

independently: there is a strict correlation between the degree of sensitivity to oxidative agents and defects in Fe-S cluster assembly, D86K/E89K, D101K/E103K and D86A/E89A/D101A/E103A exhibiting both the greatest sensitivity to oxidative agents and the least efficient assembly of Fe-S clusters. We propose that the least severe mutants have a defect in Fe-S cluster synthesis that is under the detection limits of our standard assays. It must be emphasized that an *in vivo* phenotype can only be detected when the function becomes limiting for the metabolism of the cell. We, and others, have previously found that under standard culture conditions, low Yfh1 levels are not a limiting factor for Fe-S cluster synthesis in yeast (Aloria *et al*, 2004; Ramazzotti *et al*, 2004). Therefore, mild Yfh1 defects can remain 'silent' *in vivo* during Fe-S cluster synthesis. These subtle defects are, however, sensed by the cell, which responds by increasing the transcription of the genes involved in iron uptake (F.F., unpublished data). This leads to mitochondrial iron overload in iron-replete culture media and an increase in oxidative damage, in a manner that is proportional to the Fe-S cluster metabolism defect. These latter traits are commonly shared by the mutants in other components of the mitochondrial Fe-S cluster synthesis machinery. Therefore, we propose that a defect in the assembly of Fe-S clusters is the primary event in our acidic mutants, whereas increased oxidative damage might be secondary to the abnormal iron status resulting from the defect in Fe-S cluster assembly (Rouault & Tong, 2005). It

Table 1 | Aconitase activity and mitochondrial iron content

Strain	No iron*		5 μM iron*	
	Aconitase [†]	Mitochondrial iron [‡] (nmol/mg protein)	Aconitase [†]	Mitochondrial iron [‡] (nmol/mg protein)
Wild type	100	15 ± 7	100	15 ± 7
D86K	93 ± 3	27 ± 2	70 ± 7	27 ± 2
E89K	81 ± 5	49 ± 4	55 ± 8	49 ± 4
D101A	95 ± 2	21 ± 5	73 ± 8	21 ± 5
D86A/E89A	90 ± 2	22 ± 2	75 ± 3	22 ± 2
D86K/E89K	31 ± 8	82 ± 10	18 ± 6	82 ± 10
D101K/E103K	34 ± 6	75 ± 8	20 ± 5	75 ± 8
D86A/E89A/D101A/E103A	30 ± 6	81 ± 7	17 ± 6	81 ± 7
Δyfh1	19 ± 5	82 ± 8	14 ± 6	82 ± 8

*Assays were performed with mitochondrial extracts from cells grown in raffinose synthetic medium in the absence or in the presence of 5 μM FeSO₄. The data are averages obtained from several cultures of two independently isolated transformants.
[†]Aconitase activity was normalized to isocitrate dehydrogenase activity and expressed as a percentage of the control value of each experiment.
[‡]Free iron was measured using bathophenanthroline sulphonate in the presence of dithionite (Foury & Cazzalini, 1997).

must also be mentioned that in mammals the iron storage property is assumed by a distinct mitochondrial ferritin (Levi & Arosio, 2004).

The severe defects of the D86K/E89K, D101K/E103K and D86A/E89A/D101A/E103A *yfh1* mutants highlight the crucial role of the sites centred around the strongly conserved D86/E89 and D101/E103 residues, respectively. By analogy with the data obtained for the E19K/D22K CyaY mutant, which shows complete loss of Fe²⁺ ion binding to monomers whereas Fe³⁺ ion binding around sites 29–33 is retained (Nair *et al*, 2004), it might be proposed that D86 and E89—the yeast equivalent residues of E19 and D22—host the first populated iron-binding site, whereas D101 and E103—the equivalent residues of D31 and E33—might be part of a secondary site. Our data show that the four residues fulfil their role in the frataxin function cooperatively, as neutralization of all four acidic residues is required to abrogate this function. On the basis of the role of Yfh1 carboxylate groups in iron binding *in vitro* (Cook *et al*, 2006), the observation that loss of two negative charges is not deleterious was unexpected. However, there are examples of metal-binding domains that can tolerate a change of acidic residues to alanine or glycine. A comparison of the calcium-binding sites of the EF-hand protein family shows that loss of the carboxyl oxygen-providing aspartates at positions 1, 3 or 12 abolishes calcium binding, whereas substitutions at positions 5 and 9 of the EF loop by glycine or alanine residues are tolerated (Kawasaki *et al*, 1998). In certain cases, these small residues allow metal coordination by a water molecule. By contrast, bulky basic residues, which drastically change the surface properties of a protein, are never tolerated (Kawasaki *et al*, 1998). In our *yfh1* mutants, positive charges at one site (D86/E89 or D101/E103) are likely to prevent access of Yfh1 ligands such as iron and/or Isu1 or Nfs1 to both sites.

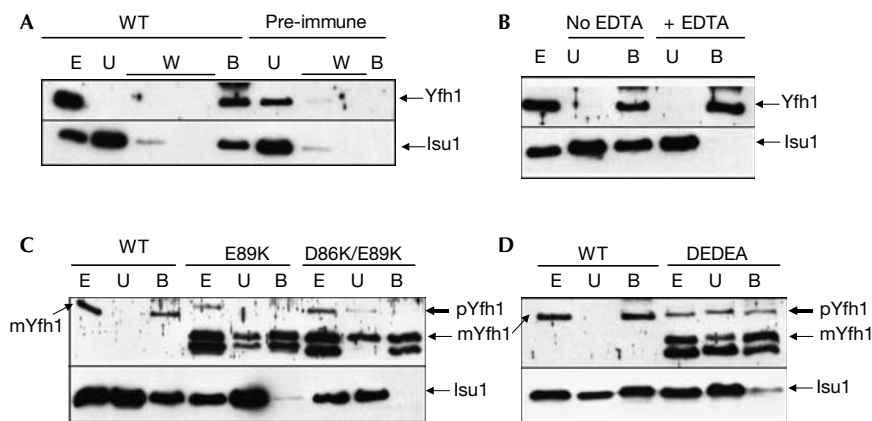


Fig 5 | Physical association of Yfh1 and Isu1 in wild-type and mutant cell extracts, using immunoaffinity chromatography with a polyclonal antibody raised against Yfh1. Cell extracts (E), unbound (U) and bound (B) fractions were analysed by SDS–polyacrylamide gel electrophoresis and western blotting using antibodies against Yfh1 and Isu1. (A) Interaction between Yfh1 and Isu1 (wild type) and replacement of Yfh1 antibody by the pre-immune serum (pre-immune). (B) EDTA (5 mM) was added during the washing steps. (C, D) The interaction between Yfh1 and Isu1 is weak in *yfh1* mutants. pYfh1 (precursor) and mYfh1 (mature) are shown for the mutants and only mYfh1 is shown for the wild type. In the mutants, the lower band corresponds to a degradation product of Yfh1. DEDEA, D86A/E89A/D101A/E103A; W, washing step fractions; WT, wild type.

In conclusion, our *in vivo* approach has identified four acidic residues in two regions of the acidic ridge that act cooperatively in the interaction between Yfh1 and Isu1. A three-dimensional structure of the complex is now required to understand more precisely the mechanisms mediating iron binding and Yfh1/Isu1 interaction.

METHODS

Strains, plasmids and media. The parental strain was W303-1BΔ*yfh1* (*MATalpha ade2 ura3 his3 leu2 trp1 yfh1Δ::KanMX4 rho+*; Foury & Cazzallini, 1997). This strain was transformed with the pFL39 centromeric plasmid harbouring wild-type *YFH1* or mutated *yfh1* genes in a DNA fragment encompassing the two *HindIII* sites, upstream and downstream of *YFH1*, respectively. For analysis of Yfh1 and Isu1 interactions, W303-1BΔ*yfh1* was co-transformed with two multi-copy plasmids as follows. The *ISU1* gene was harboured by the pRS425 plasmid, and wild-type or mutated *yfh1* genes were expressed under the control of the *GAL1* promoter in the Yeplac195 plasmid (pGAL195). *GAL1* promoter was inserted into the *EcoRI* and *BamHI* sites of the polylinker of Yeplac195, and the *YFH1* (*yfh1*) open-reading frames were amplified by PCR using the oligonucleotides CTAAGCGAGAAGATCTAGTGTAGCAATGA and GTTCAGCTGAGTTTCTTTATAGATGACGTTG, and cloned into the *BamHI* and *Sall* sites of pGAL195 using artificial *BglII* and *Sall* sites created in the oligonucleotides.

Incorporation of a 2Fe–2S cluster in apo-Yah1 in isolated energized mitochondria. Mitochondria were prepared from raffinose-grown cells collected at the end of the exponential phase. *In vitro* synthesis of ³⁵S-labelled apo Yah1, import into mitochondria, proteinase K treatment, lysis of mitochondria by Triton X-100, native polyacrylamide gel electrophoresis and autoradiography were carried out as reported previously (Duby et al, 2002).

Co-purification of Yfh1 and Isu1. Nab protein A Spin Chromatography kit (PIERCE; Perbio Science; UK Ltd, Northumberland, UK)

was used for co-purification. Raffinose-grown cells harbouring pGAL195-YFH1 and pRS425-ISU1 plasmids were inoculated in 2% galactose-rich medium and incubated at 28 °C for 5 h. Two grams of cells were lysed in 4 ml of buffer A (20 mM Hepes pH 7.4, 50 mM NaCl and 2 mM phenylmethyl sulphonyl fluoride (PMSF)) by vortexing for 3 min with 4 g of glass beads. After centrifugation at low speed to eliminate cell debris, the supernatant was centrifuged at 100,000g for 45 min. The soluble extract (200 μl) was diluted twofold with 200 μl of buffer B (50 mM sodium phosphate and 75 mM NaCl, pH 7.4) and mixed on a rocker at 4 °C for 3 h with 100 μl of the anti-Yfh1 antibody affinity resin. The bound material was washed twice with 400 μl of buffer B and eluted with 400 μl of 0.1 M glycine pH 2.5, which releases immunoglobulins from the resin-immobilized protein A. The eluate was neutralized with 40 μl of 1 M Tris–HCl pH 8.0.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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