Frataxin interacts with Isu1 through a conserved tryptophan in its β-sheet

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Friedreich’s ataxia is a neurodegenerative disease caused by the low expression of frataxin, a mitochondrial iron-binding protein which plays an important, but non-essential, role in the formation of iron–sulfur (Fe/S) clusters. It has been shown that Yfh1, the yeast frataxin homologue, interacts functionally and physically with Isu1, the scaffold protein on which the Fe/S clusters are assembled. The large β-sheet platform of frataxin is a good ligand candidate for this interaction. We have generated 12 yeast mutants in conserved residues of the β-sheet protruding at the surface or buried in the protein core. The Q129A, I130A, W131A(F) and R141A mutations, which reside in surface exposed residues of the fourth and fifth β-strands, result in severe cell growth inhibition on high-iron media and low aconitase activity, indicating that Fe/S cluster biosynthesis is impaired. The null phenotype of the I130A mutant results from the high instability of the protein, pointing that this buried residue is essential for folding. In contrast, Gln-129, Trp-131 and Arg-141 residues which are spatially closely clustered define a patch important for protein function. Co-immunoprecipitation experiments using cell extracts show that W131A, unlike W131F, is the sole mutation that strongly decreases the interaction with Isu1. Therefore, Trp-131, which is the only strictly conserved frataxin residue in all sequenced species, appears as a major contributor to the interaction with Isu1 through its surface-exposed aromatic side chain.

INTRODUCTION

Friedreich’s ataxia (FA), the most frequent hereditary ataxia in Caucasians (1,2) is caused by the insufficient expression of frataxin, a small protein of the mitochondrial matrix (3). The disease is characterized by a progressive neurodegeneration affecting, among others, spinocerebellar tracts and large sensory neurons from the dorsal root ganglia. Neurological tissue insults are often coupled to cardiac hypertrophy and less frequently to diabetes and/or skeletal abnormalities. At the cellular level, deficiencies in iron–sulfur (Fe/S) cluster-containing enzymes, iron deposits, reduced ATP synthesis and increased oxidative stress have been observed (4–9). The low expression of frataxin in FA patients is due to a homozygous GAA triplet repeat expansion in the first intron of the gene leading to transcription inhibition (3). However, in a small fraction of the patients (around 4%) the disease is caused by the presence of a pathogenic GAA repeat in one allele and a micromutation (point mutation, deletion, frame-shift, splicing-defect, stop mutation) in the other (10). The presence of point mutations in one allele and the length of the shortest GAA repeat are critical factors affecting the severity of the symptoms (3,11,12). Although the involvement of frataxin in FA is well-documented, the exact function of the protein remains controversial.

Consistent with the deficiencies observed in FA patient cells, studies in Saccharomyces cerevisiae and in other model organisms have revealed that frataxin (Yfh1 in yeast) is involved in the biosynthesis of Fe/S clusters (13–15). However, frataxin deficiency leads also to decreased heme biosynthesis (16), respiratory chain dysfunction (17), altered iron homeostasis (18,19) and increased oxidative damage (20,21). Some of these defects are probably secondary consequences of Fe/S cluster assembly deficiency and currently, a role in Fe/S cluster assembly is the most widely accepted function for frataxin. Basically, Fe/S cluster assembly consists
in the de novo synthesis of an Fe/S cluster on a scaffold protein (Isu in eukaryotes, IscU in bacteria) using the sulfur provided by a cysteine desulfurase (Nfs1 in eukaryotes, IscS in bacteria), which is assisted by Isd11 in eukaryotes (22–24). It has been proposed that frataxin is a chaperone furnishing iron. However, a quite different mechanism has been proposed very recently (25), using the bacterial frataxin CyaY as a model enzyme. At high iron to protein ratios CyaY has been shown to act as a negative regulator of the formation of the Fe/S cluster through its binding to the cysteine desulfurase. Noteworthy, Yfh1 is important but not essential (26) in the formation of the Fe/S cluster.

The three-dimensional structure of several frataxin orthologues has revealed a novel protein fold strongly conserved through evolution and characterized by a flat β-sheet platform supported by two parallel α-helices (27–31). Two hallmarks of the protein structure are (i) the presence of numerous acidic residues in the first α-helix (α-1) and β-strand (β-1) aligned in a ridge at the surface of the protein, and (ii) a flat β-sheet that probably allows the docking of protein partner(s) on frataxin.

Monomers of frataxin bind iron through the line of acidic residues mentioned above (30–36). Furthermore it has been reported that, in vitro, human frataxin is able to transfer iron to ISU (36). In aerobiosis and under low salt concentration, frataxin assembles in vitro into large oligomers sequestering high amounts of iron, suggesting a role in iron storage similar to that of ferritin (37–41). As shown by the mild phenotype of yeast mutants deficient in oligomerization of Yfh1 (42), the physiological role of these oligomers remains limited so far to a decrease in iron toxicity and oxidative stress (43).

Many partners have been reported for frataxin in vivo and/or in vitro, these include Isu, Isd11, Nfs1 (IscS), ferrochelatase, aconitase, subunits of succinate dehydrogenase and several chaperones (36,44–48). With regards to Fe/S cluster assembly, data suggest that frataxin, Isu and Isd11 are assembled in a transient molecular complex that could engage multiple interactions. The β-sheet platform is an excellent candidate for these interactions and it has recently been shown that, in yeast, amino acids at the end of the third β-strand play a role in the physical association with Isu1 (48). To determine more precisely the site of interaction between Yfh1 and Isu1, we have constructed a collection of mutants in the more precisely the site of interaction between Yfh1 and Isu1 (48). To determine more precisely the site of interaction between Yfh1 and Isu1, we have constructed a collection of mutants in the Fe/S cluster through its binding to the cysteine desulfurase. Noteworthy, Yfh1 is important but not essential (26) in the formation of the Fe/S cluster.

The efficiency of Fe/S cluster biosynthesis was assessed using two different methods: First, we measured the activity of aconitase (Aco1 in yeast), a Krebs cycle enzyme containing a [4Fe–4S] cluster required for its activity, and, secondly, we monitored the incorporation rate of the [2Fe–2S] cluster into Yah1 ferredoxin.

Mitochondria were isolated from cells grown in synthetic medium with raffinose, a good respiratory carbon source. In agreement with the absence of in vivo phenotype, the aconitase activity of T110A, T118A, V120A, N122A, Q124A, L132A and S137A was not, or only slightly, affected. On the other hand, cellular growth of the Q129A, I130A, W131A, W131F and R141A mutants was markedly inhibited on high-iron media. As observed for other frataxin-deficient strains (49) copper was also inhibitory (data not shown).

**RESULTS**

**Design of β-sheet mutants**

We have generated a collection of mutations in the β-sheet platform using site-directed mutagenesis of the YFH1 gene and we have introduced them in S. cerevisiae using the centromeric pFL39 plasmid. Nine residues are exposed at the surface of the protein (Fig. 1 and Supplementary Material, Fig. S1), Thr-110 and Thr-118 are conserved in animals, Val-120 is present in most species, Asn-122 is strongly conserved except in protozoa, Gln-124 is conserved in all species except Neisseria gonorrhoeae, Gln-129 present in most species can be replaced by a glutamate residue, Trp-131 is strictly conserved in all species sequenced, Ser-137 which is strongly conserved is replaced by threonine or glycine residues in some bacteria and protozoa, Arg-141 is strongly conserved in animals, yeasts and plants but is replaced by a histidine residue in bacteria and by lysine or tyrosine residues in protozoa. Ile-130 and Ile-132, buried in the protein core on each side of Trp-131, are strongly conserved (Fig. 1), although in some species they are replaced by another similar hydrophobic residue. All these residues were changed to alanine, and Trp-131 was also changed to phenylalanine. It must be mentioned that Asn-122 and Gln-124 have already been included in the work published by Wang and Craig (48).

**Cellular growth on high-iron media**

Yeast cells deficient in Fe/S cluster assembly machinery components are sensitive to high-iron concentrations, due to markedly altered iron homeostasis which leads to toxic iron accumulation inside the mitochondria (19,49). Therefore, we assessed the cellular growth of the mutants on minimum medium supplemented with 7 mM FeSO4 (Fig. 2A). On one hand, the growth of several mutants, namely T110A, T118A, V120A, N122A, Q124A, L132A and S137A was not, or only slightly, affected. On the other hand, cellular growth of the Q129A, I130A, W131A, W131F and R141A mutants was markedly inhibited on high-iron media. As observed for other frataxin-deficient strains (49) copper was also inhibitory (data not shown).

**Fe/S cluster metabolism defects**

The efficiency of Fe/S cluster biosynthesis was assessed using two different methods: First, we measured the activity of aconitase (Aco1 in yeast), a Krebs cycle enzyme containing a [4Fe–4S] cluster required for its activity, and, secondly, we monitored the incorporation rate of the [2Fe–2S] cluster into Yah1 ferredoxin.

Mitochondria were isolated from cells grown in synthetic medium with raffinose, a good respiratory carbon source. In agreement with the absence of in vivo phenotype, the aconitase activity of T110A, T118A, V120A and Q124A mutants was similar to that of the wild-type strain (wt-like area, Fig. 2B). Even though the values obtained for N122A and L132A mutants were not statistically significant based on the average values from several independent mitochondria isolations, we observed that, in each separate experiment, aconitase activities of N122A and L132A mutant strains were invariably below that of the wild-type strain, suggesting a mild but real defect in these mutants. All mutants whose cellular growth was decreased on high-iron media (Q129A, I130A, W131A and R141A) showed aconitase activities similar to that of the yfh1Δ strain (yfh1Δ-like area, Fig. 2B). More curiously, even though the S137A mutant did not show growth defect on high-iron media, its aconitase activity was substantially decreased (yfh1Δ-like area, Fig. 2B).
It has been shown that the [4Fe–4S] cluster ofaconitase is sensitive to free radicals and that Aco1 harboring a damaged cluster is prone to degradation by the mitochondrial quality control protease machinery (50). Except for the I130A mutant strains, Aco1 levels estimated by western blot (Fig. 2C) were not substantially decreased compared with wild-type, suggesting that the low aconitase activity was not a consequence of aconitase degradation.

In a second approach, we measured the incorporation rate of the [2Fe–2S] cluster into 35S-radiolabelled apo-ferredoxin (Yah1) synthesized in vitro and imported into isolated mitochondria. As previously reported (26,51), the conversion rate of apo-Yah1 into the holoform was decreased in the yfh1Δ strain (Fig. 3A and B). Unexpectedly, only a slight decrease was observed for the W131A mutant, whereas conversion rates were similar to wild-type for the Q124A and R141A mutants. The absence of discrimination between wild-type and R141A strains probably results from the poor sensitivity of this method. Indeed, the amounts of apo-Yah1 synthesized in vitro and imported into mitochondria are so tiny that the apo-Yah1 molecules are rapidly saturated with an Fe/S cluster even with a poorly efficient Fe/S cluster synthesis machinery. Nevertheless, these data show that W131A and R141A are not null mutants.

Frataxin stability is decreased in several beta-sheet mutants

In a previous study, we have reported yfh1 mutations that reduced protein levels (45). This is probably due to the low stability of yeast frataxin (52) coupled to the high capacity of the mitochondrial quality control machinery to recognize structural defects in the proteins and degrade them (53). Western blot analysis performed with mitochondrial extracts revealed that Yfh1 levels were substantially decreased in T110A, T118A, Q129A, W131A and W131F mutants, and hardly detectable in V120A and L132A mutants (Fig. 4A). Furthermore, Yfh1 levels were below the detection threshold of the antibody in mitochondria isolated from I130A and S137A mutant strains, pointing to the high instability of the
proteins and suggesting a structural defect. Even though it has been shown that low amounts of functional Yfh1 are able to sustain a normal—or almost normal—maturation of the Fe/S clusters [(45,48), and in this work, T110A and T118A], the respective contributions of loss of function or low frataxin amounts to the phenotype were ambiguous for mutants Q129A, W131A, W131F and S137A. To solve this problem, the YFH1 gene was overexpressed in wild-type and mutant strains.

Overexpression of mutant frataxin

Yfh1 genes harboring mutations Q129A, I130A, W131A, W131F, S137A and R141A were overexpressed using a 2μ-based multicopy vector (YEplac112). Overexpression of the wild-type YFH1 gene is not toxic for the cell. Western blot analysis of the mitochondrial proteins showed that in all mutants, except I130A and S137A, the presence of a high copy number of the yfh1 gene increased Yfh1 levels to values above that of wild-type Yfh1 expressed from the centromeric vector (Fig. 4B). I130A and S137A Yfh1 levels remained below the detection threshold of the antibody. In order to avoid a possible degradation of Yfh1 during mitochondria preparation, proteins were directly extracted from cells by sodium hydroxide treatment in the presence of β-mercaptoethanol. Under these conditions, a small amount of S137A Yfh1 was detected, whereas I130A Yfh1 remained undetectable (Fig. 4C). These data emphasize the essential role of Ile-130 in the folding of the protein.

Frataxin overexpression does not restore wild-type phenotype

High copy number of the yfh1 gene did not restore mutant cellular growth on high-iron media except for W131F (Fig. 5A). In this mutant, partial recovery of the growth was observed. Similar results were obtained on high-copper media (data not shown).

Comparing the aconitase activity of the strains overexpressing Yfh1 showed no improvement (W131A and R141A) or only partial recovery (Q129A, W131F) of this activity (Fig. 5B), an observation suggesting that in these mutants the defects mainly result from a loss of function. In contrast, the good recovery of the aconitase activity observed when the amount of S137A frataxin was slightly increased (Fig. 4C) suggests that the aconitase defect observed in the S137A mutant essentially results from an insufficient amount of this highly unstable frataxin (Fig. 4B). The absence of any restoration of the aconitase activity in the overexpressing I130A strain is in agreement with the total absence of detectable frataxin.
The W131A mutation weakens the association between Isu1 and Yfh1

It has been reported by several groups (44–46,48) that in yeast Yfh1, Isu1 and Nfs1/Isd11 are members of the complex that is responsible for the formation of the Fe/S cluster on Isu1. In the absence of a three dimensional structure of this complex and without knowledge of the sequence of the different steps, it is delicate to distinguish between direct and indirect physical interactions among complex members. Nevertheless, we have shown in a previous work that residues located in the acidic patch are involved in the association between Yfh1, Isu1 and Nfs1. It has been reported by several groups (44–46,48) that in yeast Yfh1, Isu1 and Nfs1 were in the same range in the different extracts (Fig. 6A). The amount of Yfh1 produced in the strain harboring the chromosomal YFH1 gene only was ~5% of that in the overproducing strain (Fig. 6A). Thus, when a mutant Yfh1 is strongly overproduced, the contribution of the unique wild-type chromosomal YFH1 copy is negligible. All mutant frataxins were highly overproduced except Q129 Yfh1.

The low amount of antibody-bound Yfh1 expressed from the chromosome was sufficient to detect Isu1 in the co-immunoprecipitate (Fig. 6C), however, the Isu1 signal was observed only upon ISU1 overexpression (data not shown). This is not surprising since interactions between the Yfh1 and Isu1 proteins extracted from cells are transient, fragile and easily disrupted. Our data are in agreement with previous reports (44,48) showing that in co-immunoprecipitation and pull-down experiments high levels of Isu1 increase the fraction of Isu1 bound to Yfh1. As expected from the wild-type phenotype of the Q124A yfh1 mutant, amounts of Isu1 co-immunoprecipitated with wild-type and Q124A frataxins were similar (Fig. 6D and E). On the other hand, although the Fe/S cluster metabolism was severely altered in the mutants, a substantial amount of Isu1 was also co-immunoprecipitated with W131F or R141A frataxins (Fig. 6H and I). In contrast, the Isu1 signal obtained with W131A Yfh1 was markedly decreased (Fig. 6G). This weak Isu1 signal cannot be explained on the sole bases of decreased levels of either Isu1 in the extracts, which are quite similar (Fig. 6A) or immunoprecipitated W131A Yfh1. Indeed, the amount of immunoprecipitated W131A Yfh1 reached 70% of that of W131F Yfh1, whereas the amount of Isu1 co-immunoprecipitated with W131A Yfh1 was less than 20% of that co-immunoprecipitated with W131F Yfh1 (Fig. 6G and H). This strongly suggests that the amount of W131A Yfh1 is not a major limiting factor in the co-immunoprecipitation, but that a modification of the Yfh1 properties by the W131A mutation prevents Isu1 co-immunoprecipitation. Since phenylalanine can substitute for tryptophan, the aromatic side chain should play a crucial role in the interaction mechanism.

The weak intensity of the Isu1 signal obtained with Q129A Yfh1 (Fig. 6F) correlates with the low amount of frataxin present in the cell extract, but interpretation of this result is ambiguous, since due to the low amounts of Q129A Yfh1, the chromosomal wild-type and plasmidic mutant frataxins compete for anti-Yfh1 antibody binding. Thus, either the signal corresponds to a real interaction between Q129 Yfh1 and Isu1, or more likely, the interaction between Q129A and Isu1 is decreased and the signal corresponds to an interaction between Isu1 and the wild-type chromosomal Yfh1 copy. This ambiguity could not be solved by overexpressing the mutated yfh1 genes in a yfh1Δ strain because in this strain the short induction time in galactose medium required to observe the physical interaction between Yfh1 and Isu1 did not allow expression of Yfh1 and Isu1 at sufficient levels.
It was also of interest to determine whether a mutated form of Isu1 had lost its capacity of interacting with Yfh1. We have previously reported (45) that the G60D/M141V isu1 mutation does not rescue the synthetically lethal phenotype of a yfh1Δ isu1Δ strain. Moreover in Yfh1-depleted cells, the phenotype produced on high-iron media by the G60D/M141V isu1 mutation is even more severe than in an isu1Δ strain suggesting that it is dominant negative. Gly-60 localized on the first β-strand with its side chain inside the protein core and Met-141 at the surface of the protein are strongly conserved residues, however, only the G60D mutation is deleterious, probably by modifying the protein structure. Figure 6I shows that G60D/M141V Isu1 fails to interact with Yfh1. Altogether, these data suggest that the well expressed G60D/M141V Isu1 prevents its orthologue Isu2, usually able to complement an Isu1 deficiency (45), to fulfill its function efficiently.

**DISCUSSION**

A role for frataxin in Fe/S cluster assembly is now widely accepted (14,15,26,51). It has been shown that frataxin is a physical partner of Nfs (IscS), Isd11 and Isu (IscU), the proteins responsible for the de novo assembly of Fe/S clusters (44,45,54,55). Whether frataxin is an iron chaperone involved in the delivery of iron to the complex formed by Nfs1/Isd11/Isu1 (56), or a fine regulator of the cluster formation through its binding to the cysteine desulfurase (25) remains a matter of debate. It has recently been shown that the N122K and N122A/K123T/Q124A Yfh1 mutations, localized at the end of the third β-strand, weaken the physical interaction between frataxin and Isu1, supporting the idea that this strand plays an important role in the interaction (48).

In order to get a more precise view of the residues of the β-sheet involved in the interaction with Isu1, we have performed a systematic functional analysis of mutations changing conserved residues exposed at the surface of frataxin to alanine. Several yfh1 mutations did not alter the wild-type phenotype; they are localized in moderately conserved residues except Gln-124 which is conserved in all species sequenced except N. gonorrhoeae which possesses a histidine residue. Moreover, the Q124A mutation did not produce synergistic defects in combination with W131A or R141A (data not shown). If we do not consider mutations I130A and S137A which most likely contribute here to the phenotype through structural defects resulting in high protein instability, only mutations Q129A, W131A, W131F in the fourth β-strand and R141A in the fifth β-strand result in severe defects of the Fe/S cluster metabolism. The R141A protein reaches wild-type levels, whereas the stability of Q129A, W131A and W131F Yfh1 is decreased. The absence of restoration to wild-type phenotype when Q129A and W131A frataxins are overexpressed to wild-type levels indicates that the decreased stability of these proteins is not sufficient to explain the severe in vivo phenotypes. In contrast, W131F Yfh1 overexpression partially compensates for the defects suggesting that the substitution of tryptophan by phenylalanine affects frataxin function slightly only.

Among mutations Q129A, W131A, W131F and R141A only W131A Yfh1 markedly decreases the amount of co-immunoprecipitated Isu1. It has been reported that frataxin interacts with Isu1, Nfs1 and Isd11 within the complex that is responsible for the formation of the Fe/S cluster on Isu1 (44–48). Therefore, in the absence of a three dimensional structure of this complex, it cannot be excluded that the interaction between Trp-131 and Isu1 is mediated through Nfs1/Isd11. In bacteria IscS (Nfs1) appears as the privileged partner of
CyaY (bacterial frataxin) in pull-down and in vitro reconstitution experiments (25,55). However, titration data in pull-down experiments have shown that when Asn-122 which is the closest non-contiguous neighbor of Trp-131 is changed to lysine the interaction between Yfh1 and Isu1 is the most affected (48), indicating that Asn-122 is involved primarily in this interaction. This strongly suggests that Trp-131 also interacts with Isu1 directly.

It could be argued that the loss of the interaction between W131A Yfh1 and Isu1 is in fact caused by a modification of the fold of Yfh1 produced by the loss of the stabilizing role of π interactions between Trp-131 and the nearby Arg-141. It is known that the change of this tryptophan to arginine which is associated to a severe form of FA (57) modifies the fold of human frataxin (58), resulting in slightly reduced thermodynamic stability, higher tendency to aggregation and proteolysis. The existence of a protein folding defect in mitochondria is often suggested by decreased levels of this protein which is partially degraded by the mitochondrial quality control protease machinery which has recognized the defect (53).

And indeed, W131A Yfh1 levels are decreased; however, W131F Yfh1 levels are also decreased, though to a lesser extent, suggesting a fold defect in both frataxins. In addition, the change of Arg-141 to alanine which should also elicit the loss of the π interactions with Trp-131 and thus destabilize the protein fold does not reduce R141A Yfh1 levels and does not modify the interaction between Yfh1 and Isu1.

Altogether our data make it unlikely that the fold defect of W131A Yfh1 is sufficient to explain the loss of the interaction between Yfh1 and Isu1. Since exposed and conserved tryptophan residues are known to be preferential protein–protein interaction hot spots (59), we feel that the most reasonable explanation is that Trp-131 is directly involved in this interaction. Indeed, based on multiple amino acid alignment of frataxins from more than 30 organisms including the highly divergent protozoa class, Trp-131 is the only strictly conserved residue of the β-sheet platform (Supplementary Material, Fig. S1), and it is exposed at the surface of the protein. It is thus an excellent candidate for a hot spot of binding energy (59). Under our conditions, the replacement...
W131F prevents, directly or indirectly, the interaction between Yfh1 and Isu1. It is not excluded that cell extracts may have masked some weakness in the physiological amounts of Isu1 and Yfh1 contained in the co-immunoprecipitation experiments and the high non-tryptophan (59). In fact, the qualitative character of the residue is not a preferential hot spot of interactions as is alanine residue reduces Yfh1 stability and secondly, this contains a phenylalanine residue, the latter is certainly not a preferred residue in hot spots (59). As discussed earlier, the contribution of Gln-129 to the interaction with Isu1 is unclear. Glutamine is not a preferred hot spot of energy, but Gln-129 is in close vicinity to Trp-131 and could contribute to a favorable environment for the interaction. In contrast, the interaction between R141A Yfh1 and Isu1 is clearly similar to wild-type indicating that though spatially close to Trp-131 (Fig. 7A), Arg-141 on the fifth β-strand fulfills a different function. Arg-141 is conserved in the majority of eukaryotes; however, it is replaced by a histidine residue in prokaryotes and in Schizosaccharomyces pombe, and by a lysine or tyrosine residue in a few organisms. Arg-141 might be involved in a functional interaction with Nfs1/Isd11.

Wang and Craig (48) were the first to demonstrate a role for the β-sheet in the interaction with Isu1. Pull-down and co-immunoprecipitation experiments showed that residues at the end of the third strand, and in particular Asn-122, the non-contiguous residue which is the closest to Trp-131 in the three dimensional structure (Fig. 7B), are important for this interaction in a specific manner since neither Nfs1 nor Isd11 binding is significantly affected by the N122K mutation. However, as shown in the present work, the change of Asn-122 to alanine (N122A) had no substantial deleterious effect on the interaction with Isu1, whereas the more drastic change to lysine (N122K) inhibited cell growth only when frataxin and Isu levels were lower than normal (48), suggesting that Asn-122 belongs to the hot spot of binding energy but is a minor contributor compared with Trp-131, in agreement with the fact that asparagine is not a preferred residue in hot spots (59).

In conclusion, this work confirms and extends the findings reported by Wang and Craig that the β-sheet platform interacts with Isu1. Altogether data suggest that Isu1 interacts with Yfh1 through a subdomain defined by residues at the end of the third β-strand and in the fourth β-strand. Trp-131, through its aromatic side chain, is the major contributor to the binding energy. If we consider that the acidic ridge plays also a role in the association between Yfh1 and Isu1 (46), our work supports the recent hypothesis (33) of a dipole interaction through the acidic ridge and the beta-sheet platform.

**MATERIALS AND METHODS**

**Strains, plasmids and media**

The parental strain W303-1B yfh1Δ (MATa leu2-3, 112 trpl-1 ade2-1 his3-11, 15 ura3-1 yfh1Δ::KanMX4 rho+) was transformed with the empty centromeric pFL39 plasmid or with pFL39 containing either wild-type YFHI or mutated yfh1 genes.
cloned using the two HindIII sites on each side of the gene. Strains overexpressing frataxin were obtained by transformation of the W303-1B yfh1Δ strain with the empty multicopy YEpplac12 plasmid or YEpplac12 containing either the wild-type YFH1 or mutated yfh1 genes inserted into the HindIII sites. Interaction studies were performed with W303-1B strain co-transformed with two multicopy 2μ-based plasmids: pRS425 (LEU2) and pGAL195 (URA3). The pGAL195 plasmid was obtained by introducing the GAL1 promoter into the YEpplac195 vector using the EcoRI and BamHI polylinker restriction sites. Wild-type YFH1 or mutated yfh1 open reading frames were inserted downstream of the GAL1 promoter into the BamHI and HindIII sites of pGAL195, using an artificial BglII site created in the CTAAGCGAGAAGATCTAGTGACAAATGA primer. Wild-type ISU1 or mutated isu1G60D/M141V open reading frames were cloned into the pRS425 vector using artificial BamHI and HindIII sites added by PCR amplification using the GGATCCCCGTGCCATGATGATTGCATC and AAGCTTCTTGTTCTTGTCCCGTTATC primers, respectively.

Strains were grown either in rich-medium containing 3% glycerol (or 2% galactose) and 2% yeast extract KAT, or in synthetic minimum medium containing 2% glucose (or 2% raffinose), 0.7% yeast nitrogen base without amino acids (Difco), 0.5% ammonium sulfate, a mixture of amino and the required auxotrophic elements. FeSO₄ solubilized in 0.1 N HCl was added at the indicated concentration and solid media were obtained by adding 2% agar.

Mitochondria isolation, enzyme activities, western blot analysis and incorporation of Fe/S cluster into Yah1 ferredoxin

Mitochondria were prepared from raffinose-grown cells in the late exponential phase of growth as described previously (60). Aconitase and isocitrate dehydrogenase activities were measured by standard procedures. Levels of Aco1, Nfs1, Ssq1, Yfh1 and Isu1 apoproteins were determined by western blot analysis using polyclonal anti-rabbit antibodies. Anti-Isu1 antibody was a generous gift from Roland Lill (Institut für Zytobiologie and Zytopathologie, Philippus-Universität, Marburg, Germany). In vitro synthesis and mitochondrial import of 35S-radiolabeled Yah1 was performed as described previously (26). Apo- and holo-Yah1 were separated on native acrylamide gels and dried before autoradiography of the gels. The signals were quantified using Kodak Image Station 4000 R.

Co-isolation of Yfh1 and Isu1

Raffinose-grown cells overexpressing YFH1 and ISU1 genes were inoculated in rich galactose medium and incubated for 5 h at 28°C. Three grams of cells were collected and lysed in 3 ml of a buffer containing 20 mM Tris–HCl pH 8.0, 50 mM NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF) by vortexing for 3 min with 6 g of glass beads (acid washed) at 4°C. After eliminating cellular debris by low-speed centrifugation, a soluble extract was obtained by a 45 min ultracentrifugation at 100 000g. Two hundred microliters of this extract were diluted 2-fold in a phosphate buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) and mixed on a rocker for 3 h at 4°C with 100 μl of the anti-Yfh1 affinity resin. This resin was obtained by mixing 200 μl of anti-Yfh1 antibodies with 100 μl of the resin from the Nab Protein A Spin Purification Kit (Pierce). Bound material was washed twice with 400 μl of phosphate buffer, eluted with 400 μl of 0.1 M glycine pH 2.5 and immediately neutralized with 40 μl of 1 M Tris–HCl pH 8.0.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
Conflicts of Interest statement. None declared.

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