The *in vivo* mitochondrial two-step maturation of human frataxin

Stéphane Schmucker\(^1\), Manuela Argentini\(^1\), Nâdège Carelle-Calmels\(^1\), Alain Martelli\(^1\) and Hélène Puccio\(^1\),

\(^1\)IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), 1 rue Laurent Fries, BP 10142, Illkirch F-67400, France, \(^2\)INSERM, U596, Illkirch F-67400, France, \(^3\)CNRS, UMR7104, Illkirch F-67400, France, \(^4\)Université Louis Pasteur, Strasbourg F-67000, France and \(^5\)Collège de France, Chaire de génétique humaine, Illkirch F-67400, France

Received July 28, 2008; Revised and Accepted August 12, 2008

Deficiency in the nuclear-encoded mitochondrial protein frataxin causes Friedreich ataxia (FRDA), a progressive neurodegenerative disorder associating spinocerebellar ataxia and cardiomyopathy. Although the exact function of frataxin is still a matter of debate, it is widely accepted that frataxin is a mitochondrial iron chaperone involved in iron–sulfur cluster and heme biosynthesis. Frataxin is synthesized as a precursor polypeptide, directed to the mitochondrial matrix where it is proteolytically cleaved by the mitochondrial processing peptidase to the mature form via a processing intermediate. The mature form was initially reported to be encoded by amino acids 56–210 (m\(^{56}\)-FXN). However, two independent reports have challenged these studies describing two different forms encoded by amino acids 78–210 (m\(^{78}\)-FXN) and 81–210 (m\(^{81}\)-FXN). Here, we provide evidence that mature human frataxin corresponds to m\(^{81}\)-FXN, and can rescue the lethal phenotype of fibroblasts completely deleted for frataxin. Furthermore, our data demonstrate that the migration profile of frataxin depends on the experimental conditions, a behavior which most likely contributed to the confusion concerning the endogenous mature frataxin. Interestingly, we show that m\(^{56}\)-FXN and m\(^{78}\)-FXN can be generated when the normal maturation process of frataxin is impaired, although the physiological relevance is not clear. Furthermore, we determine that the d-FXN form, previously reported to be a degradation product, corresponds to m\(^{78}\)-FXN. Finally, we demonstrate that all frataxin isoforms are generated and localized within the mitochondria. The clear identification of the N-terminus of mature FXN is an important step for designing therapeutic approaches for FRDA based on frataxin replacement.

INTRODUCTION

Friedreich ataxia (FRDA), an autosomal recessive neurodegenerative disease characterized by gait and limb ataxia, loss of proprioception, hypertrophic cardiomyopathy and increased incidence of diabetes, is due to deficiency of the mitochondrial protein frataxin (FXN) (1). The common disease-causing mutation is a (GAA)\(_n\) expansion within the first intron of the FXN gene (2). Two non-exclusive hypotheses provide the mechanism by which this (GAA)\(_n\) expansion leads to a strongly reduced expression of frataxin: the formation of a triple helix which interferes with transcriptional elongation (3) or epigenetic changes leading to heterochromatin formation thereby impairing gene transcription (4). Frataxin deficiency in FRDA patients, mouse and yeast models is associated with deficiency in iron–sulfur cluster (Fe–S) enzyme activities (5–8), mitochondrial iron deposits (7,9,10), reduced oxidative phosphorylation (11) and impaired antioxidant defense (12). While frataxin seems likely to serve diverse cellular roles, several of these functions appear to involve iron delivery (13,14). Although the extent of the primary functions of frataxin is still a matter of debate, frataxin is clearly involved in mitochondrial iron homeostasis with a direct binding of frataxin to iron ions (Fe\(^{2+}\) or Fe\(^{3+}\)) in a micromolar range (15–18). While relatively weak by physiological standards, the interaction of frataxin with its partners, such as the Fe–S
cluster scaffold protein IscU is iron-dependent (19–21). The current commonly accepted hypothesis confers a role for frataxin as an iron-chaperone involved in Fe–S biogenesis and heme biosynthesis (13,19–22). However, its functions as an iron storage molecule (23), an antioxidant (24,25) or a tumor suppressor (26,27) protein is more controversial.

There is a high degree of structural homology in frataxin proteins, from bacteria to humans, with a compact globular structure maintained by a hydrophobic network and composed of two α-helices and seven β-sheets (28,29). An anionic surface defined by a dozen acidic residues lies on a surface localized on the first α-helix and the β1-sheet that is solvent-exposed. This region has been demonstrated in biochemical and structural studies in yeast, bacteria and human frataxins to be crucial for its function and to be implicated in the iron binding of frataxin as well as in the ferrochelatase binding and ferroxidase activity (13,16,17,20,30,31).

The nuclear-encoded human frataxin protein is synthesized as a 210 amino acid precursor (23 kDa) with a N-terminal mitochondrial-targeting sequence (Fig. 1) previously shown to be removed by an unusual two-step maturation process by the mitochondrial processing peptidase (MPP) (32). The β-subunit of MPP was shown to directly interact with the frataxin precursor by yeast two-hybrid and GST pull-down experiments (33). In vitro processing assays using recombinant MPP demonstrated an initial cleavage generating the intermediate form (i-FXN; 19 kDa) between Gly41 and Leu42 for the human protein followed by a second cleavage (26,27) protein is more controversial.

In the present article, we demonstrate that the major endogenous form of mature frataxin results from a cleavage between Lys80 and Ser81, thus confirming the site described by Testi and colleagues. Besides, we provide additional evidence that m56-FXN is not found in normal physiological conditions and that the reported degraded form of frataxin (d-FXN) corresponds to m78-FXN. Finally, we show that the complete maturation process of frataxin is exclusively localized within the mitochondria.

### RESULTS

**Neither FXN56–210 nor FXN79–210 correspond to endogenous human mature frataxin**

In a first attempt to identify the potential final maturation site of endogenous FXN, we compared the migration profile under different conditions (see Materials and Methods) of two N-terminal truncated frataxins corresponding to either FXN56–210 or FXN81–210, further reinforcing the confusion as to the size of mature frataxin.

We have previously seen that endogenous frataxin migrates with an apparent mobility of <15 kDa on a 16% SDS–Tricine–PAGE. Other groups have reported a migration on standard SDS–Glycine–PAGE around 15–17 kDa (34). This abnormal mobility on SDS–PAGE for yeast, human and murine frataxin is attributed to the acidic nature of the protein (31,37). It is interesting to note that endogenous human frataxin was found by different groups to co-migrate with markers corresponding to either FXN56–210 or FXN81–210, which would serve to inhibit the iron-promoted cleavage of m78-FXN to the Fe–S cluster assembly scaffold partner protein, IscU (36). Interestingly, both the m78-FXN and the m56-FXN forms have been reported not only in recombinant human frataxin purification, but also in human heart extracts as degradation products (d-FXN) (15,20,29,34). Finally, bioinformatic analysis identifies each of the three sites (between residues 41 and 42, 55 and 56 and around amino acid 81) as putative MPP cleavage sites.

In order to begin at amino acid 78 (m78-FXN; 14.5 kDa). The authors propose a structural gating role for the N-terminal residues of m56-FXN, which would serve to inhibit the iron-promoted binding of m78-FXN to the Fe–S cluster assembly scaffold partner protein, IscU (36). Interestingly, both the m78-FXN and the m56-FXN forms have been reported not only in recombinant human frataxin purification, but also in human heart extracts as degradation products (d-FXN) (15,20,29,34). Finally, bioinformatic analysis identifies each of the three sites (between residues 41 and 42, 55 and 56 and around amino acid 81) as putative MPP cleavage sites.

We have previously seen that endogenous frataxin migrates with an apparent mobility of <15 kDa on a 16% SDS–Tricine–PAGE. Other groups have reported a migration on standard SDS–Glycine–PAGE around 15–17 kDa (34). This abnormal mobility on SDS–PAGE for yeast, human and murine frataxin is attributed to the acidic nature of the protein (31,37). It is interesting to note that endogenous human frataxin was found by different groups to co-migrate with markers corresponding to either FXN56–210 or FXN81–210, which would serve to inhibit the iron-promoted cleavage of m78-FXN to the Fe–S cluster assembly scaffold partner protein, IscU (36). Interestingly, both the m78-FXN and the m56-FXN forms have been reported not only in recombinant human frataxin purification, but also in human heart extracts as degradation products (d-FXN) (15,20,29,34). Finally, bioinformatic analysis identifies each of the three sites (between residues 41 and 42, 55 and 56 and around amino acid 81) as putative MPP cleavage sites.

We have previously seen that endogenous frataxin migrates with an apparent mobility of <15 kDa on a 16% SDS–Tricine–PAGE. Other groups have reported a migration on standard SDS–Glycine–PAGE around 15–17 kDa (34). This abnormal mobility on SDS–PAGE for yeast, human and murine frataxin is attributed to the acidic nature of the protein (31,37). It is interesting to note that endogenous human frataxin was found by different groups to co-migrate with markers corresponding to either FXN56–210 or FXN81–210, which would serve to inhibit the iron-promoted cleavage of m78-FXN to the Fe–S cluster assembly scaffold partner protein, IscU (36). Interestingly, both the m78-FXN and the m56-FXN forms have been reported not only in recombinant human frataxin purification, but also in human heart extracts as degradation products (d-FXN) (15,20,29,34). Finally, bioinformatic analysis identifies each of the three sites (between residues 41 and 42, 55 and 56 and around amino acid 81) as putative MPP cleavage sites.

We have previously seen that endogenous frataxin migrates with an apparent mobility of <15 kDa on a 16% SDS–Tricine–PAGE. Other groups have reported a migration on standard SDS–Glycine–PAGE around 15–17 kDa (34). This abnormal mobility on SDS–PAGE for yeast, human and murine frataxin is attributed to the acidic nature of the protein (31,37). It is interesting to note that endogenous human frataxin was found by different groups to co-migrate with markers corresponding to either FXN56–210 or FXN81–210, which would serve to inhibit the iron-promoted cleavage of m78-FXN to the Fe–S cluster assembly scaffold partner protein, IscU (36). Interestingly, both the m78-FXN and the m56-FXN forms have been reported not only in recombinant human frataxin purification, but also in human heart extracts as degradation products (d-FXN) (15,20,29,34). Finally, bioinformatic analysis identifies each of the three sites (between residues 41 and 42, 55 and 56 and around amino acid 81) as putative MPP cleavage sites.
Mature frataxin corresponds to m81-FXN

To identify the N-terminus of the mature form, frataxin was immunopurified from COS-1 cells overexpressing the recombinant FXN<sup>1–210</sup>. Overexpression of a recombinant precursor of FXN<sup>1–210</sup> in COS-1 cells leads to the expression of three forms of frataxin: precursor, intermediate and mature forms. Each form of human frataxin (precursor, intermediate and mature) was analyzed by MALDI-PMF after enzymatic digestion. Protein sequence coverage ranged from 63 to 70% after trypsin digestion and 44 to 69% after Asp-N digestion (Supplementary Material, Table S2). The presence of C-terminal peptides in all isoforms demonstrated that the immunoprecipitated frataxins were not degraded in their C-terminal regions (Supplementary Material, Table S2). Hence the different migration between endogenous m-FXN and FXN<sup>79–210</sup> or FXN<sup>56–210</sup> is not due to C-terminal degradation. While trypsin digestion was not appropriate to detect the N-terminal peptide of the m-FXN because of the presence of arginine and lysine residues near the predicted maturation site, Asp-N digestion (preferential cleavage: N-terminal of aspartine) uncovered a specific peak (m/z = 925 498) in the mass spectrum of the mature form (Fig. 3). This diagnostic peak, that was absent from both the precursor and the intermediate mass spectra, matches the 81–90 peptide (sequence = SGTLGHPGSL/D), indicating that mature frataxin begins at the Ser81. Subsequent LC/MS/MS analysis unambiguously demonstrated that the m/z = 925 498 peak corresponds to the predicted sequence of the human frataxin 81–90 peptide (data not shown). Our results thus confirm that mature frataxin corresponds to the m<sub>81</sub>-FXN form, as previously reported (35).

Figure 2. Neither FXN<sup>56–210</sup> or FXN<sup>79–210</sup> correspond to endogenous human mature frataxin. Protein extracts were analyzed by SDS–Glycine–PAGE at different acrylamide percentages (12, 13, 14 and 15%) or by 16% SDS–Tricine–PAGE. Western blots were performed with the anti-frataxin R1270 antibody. Endogenous frataxin was detected in human fibroblasts, HeLa cells, skeletal muscle, and in non-transfected COS-1 cells (NT). COS-1 cells were transiently transfected with WT FXN<sup>1–210</sup> (WT), FXN<sup>56–210</sup> (56–210) and FXN<sup>79–210</sup> (79–210). Asterisks indicate a nonspecific band recognized by the primary antibody in HeLa cells.

Indeed, at the lowest tested concentration (12%), endogenous frataxin migrates very close to FXN<sup>56–210</sup> while at higher percentage of acrylamide (15%) or in SDS–Tricine (16%), endogenous frataxin co-migrates with the second marker FXN<sup>79–210</sup> (Fig. 2). This unusual behavior of frataxin may explain the contradictory reports on the identification of mature frataxin based on co-migration with different frataxin constructs (34,35). To determine whether endogenous mouse frataxin presents a similar profile, extracts from C2C12 myoblast cells and heart muscle tissue were compared with the two corresponding predicted forms of mature mouse frataxin (Fx<sup>54–207</sup> and Fxn<sup>76–207</sup>). Similarly to human frataxin, the endogenous murine frataxin migrated between both size markers in a 14% acrylamide gel (Supplementary Material, Fig. S1). Together, these results suggest that the endogenous mature form of frataxin is different from the previously described m<sub>56</sub>-FXN as well as m<sub>79</sub>-FXN.

Mature frataxin displays an unusual migration profile on SDS–PAGE

The identification of the starting residue of mature frataxin at residue 81 was surprising, since endogenous frataxin migrates higher than the theoretical molecular weight of FXN<sup>81–210</sup> (14.2 kDa) and higher than the recombinant FXN<sup>79–210</sup> (14.5 kDa). To further refine the migration profile of frataxin, different N-terminal truncated recombinant frataxins (beginning at amino acids 56, 64, 71, 76, 79 or 81) were used as protein ladder and compared with endogenous m-FXN (Fig. 4A). Each truncated recombinant protein migrated according to its theoretical molecular weight, except FXN<sup>81–210</sup>. Indeed, similarly to endogenous m-FXN, the recombinant FXN<sup>81–210</sup> migrated slower in the gel and co-migrated perfectly with the endogenous m-FXN, further confirming the site of maturation at Ser81. To exclude a post-translational modification that could explain an unusual migration profile, FXN<sup>79–210</sup> and FXN<sup>81–210</sup> were expressed using an in vitro transcription/translation assay, a method that significantly reduces post-translational modifications. The in vitro translated FXN<sup>81–210</sup> migrated similarly to endogenous frataxin (Fig. 4B). Bacterially expressed FXN<sup>81–210</sup> also co-migrated with endogenous m-FXN (data not shown), further indicating that the abnormal migration of the mature frataxin cannot be attributed to specific post-translational modification. Moreover, in vitro dephosphorylation and N-deglycosylation assays excluded that the abnormal migration was due to these post-translational modifications (data not shown).

Previous studies using yeast and bacterial frataxin have suggested that the abnormal migration of frataxin was due to the specific acidic nature of the protein, more specifically of its first α-helix and the β1-sheet. Mutations of some negatively charged residues (aspartates and glutamates) in the yeast
Frataxin significantly changed the migration on SDS–PAGE (31,37). We hypothesized that some of these N-terminal residues in human frataxin could confer the abnormal migration either by electrostatic change or by being implicated in a stable secondary structure of the protein. To verify this hypothesis, we performed site-directed mutagenesis of specific N-terminal residues of FXN81–210 (Fig. 4C). Several mutated residues (L90, Y95, D91, E92 and D104) cause a significant modification in the migration profile. Mutants in Leu90 and Tyr95 present a slower migration than the m-FXN (Fig. 4C). Interestingly, mutations in conserved acidic residues (D91, E92 and D104) lead to a migration consistent with the theoretical molecular weight of m81-FXN (14.2 kDa), but mutation in the acidic residue D91 of FXN79–210 did not modify its migration in gel (data not shown). These results demonstrate that the abnormal migration profile of mature human frataxin is directly linked to the acidic nature of its N-terminal α-helix.

Maturation of human frataxin is a two-step process

In order to better characterize the maturation process of human frataxin and to determine which of the previously reported sites are required for proper maturation, we generated mutants by site-directed mutagenesis on critical residues for the i-FXN, m56-FXN and m81-FXN cleavage sites (Fig. 1). It was previously shown that the maturation of frataxin occurs in a two-step process mediated by MPP (34). As MPP requires basic residues at position -2 or -3 from the cleavage site, we mutated to glycine the arginines at positions 53 and 54 (mut 53–54) or the Leu78 and the Arg79 (mut 78–79) to avoid formation of m-FXN56–210 or m-FXN 81–210, respectively (Fig. 1). To determine whether the maturation process necessitates the formation of the intermediate frataxin, the arginines at position 39 and 40 were mutated to glycine (mut 39–40). Finally, the different double and triple mutants were generated by combination of the above single mutations.
requires the presence of Arg79, a highly conserved residue cleavage at amino acid 81, generating the m-FXN. From our 42, which gives rise to the i-FXN, followed by a second of frataxin occurs by a first cleavage of the precursor at residue 79–210(see below). Therefore, the maturation a strong band migrating faster than mature frataxin and Material, Fig. S2). Interestingly, for mut 78–79, there is an also prevents the formation of mature frataxin (Supplementary results. An alternative construct mutated at Arg79 and Lys80 position 81 in agreement with the above mass spectrometry directly implicated in the final maturation step of frataxin at physiological relevant site for proper maturation of frataxin in vivo. Furthermore, the mut 78–79 mutation eliminates the presence of mature form (Table 1; 4), indicating that 78–79 residues are directly implicated in the final maturation step of frataxin at position 81 in agreement with the above mass spectrometry results. An alternative construct mutated at Arg79 and Lys80 also prevents the formation of mature frataxin (Supplementary Material, Fig. S2). Interestingly, for mut 78–79, there is an increase in the intermediate form as well as the presence of a strong band migrating faster than mature frataxin and close to the FXN79–210 (see below). Therefore, the maturation of frataxin occurs by a first cleavage of the precursor at residue 42, which gives rise to the i-FXN, followed by a second cleavage at amino acid 81, generating the m-FXN. From our mutational analysis, we can infer that the second cleavage requires the presence of Arg79, a highly conserved residue in mammalian frataxins.

The m56-FXN was never detected in endogenous conditions neither in cell cultures nor in tissues (data not shown). Interestingly, when the protein is mutated at residues 78–79, thereby impairing the normal maturation of frataxin, a band co-migrating with m56-FXN is detectable (Fig. 5 and Table 1; 4). This band is also detected when the intermediate site is disrupted (mut 39–40) (Fig. 5 and Table 1; 2). These results suggest that the m56-FXN is processed only when the normal maturation is impaired. Moreover, in the mut 39–40/78–79, the presence of m56-FXN at high level indicates that this isoform can be directly generated from the precursor (Fig. 5 and Table 1; 6). In situation with high expression of WT frataxin (over 20-fold), a band co-migrating with the FXN56–210 forms can be detected (data not shown), suggesting that upon saturation of the maturation machinery, and therefore accumulation of the precursor, this forms can be generated in cells. Our data, however, do not exclude that m56-FXN is generated from the i-FXN. Furthermore, although we have no evidence for the presence of endogenous m56-FXN, our results do not completely exclude the possibility of its presence in very low level or under specific conditions.

Each time the normal maturation process of frataxin was disrupted, a supplementary product was detected around 14 kDa and migrated faster than the mature protein. This band is not present when WT frataxin is overexpressed or in the mut 53–54, suggesting that it can be a degradation product (d-FXN) of the different forms generated, when the mature form cannot be produced. The presence of this d-FXN 14-kDa product in the triple (mut 39–40/53–54/78–79) and double mut 53–54/78–79 and mut 39–40/78–79 mutants shows that it can be generated either from the precursor, the intermediate or the m56-FXN.

The MALDI-PMF analysis of this 14-kDa product after Asp-N digestion of mut 53–54/78–79 allows to detect a diagnostic peak corresponding to human frataxin 78–90 peptide (sequence=GGKSGTLGHPGSL/D, data not shown). Despite the presence of glycine residues instead of leucine and arginine at positions 78–79 of frataxin in the identified peptide, the co-migration of this d-FXN 14-kDa product in other mutants not mutated at residues 78 and 79 (i.e. mut 39–40) indicates that m56-FXN can be produced in a cellular context.

The presence of m78-FXN in the mut 39–40 mutant (Table 1; 2) further reinforces that the m56-FXN is a marginal site for the maturation of frataxin under physiological conditions in living cells. Interestingly, mut 39–40 abolishes the maturation through intermediate FXN and leads to the formation of m56-FXN and a larger amount of mature FXN compared with non-transfected cells, suggesting that m56-FXN can serve as an alternative intermediate form. On the contrary, the mut 39–40/53–54 mutation (Table 1; 5) did not generate this increased level of mature form, further supporting the view that the formation of the mature protein is a two-step process. Altogether, these data confirm that mature frataxin is processed at amino acid 81, but also show that m56-FXN and m78-FXN can be produced in a cellular environment when the normal maturation process is impaired.

**m81-FXN rescues frataxin deletion in murine fibroblast**

To determine if the different identified forms of frataxin (m81-FXN, m56-FXN and m78-FXN) are functional, we used a cellular assay based on the conditional frataxin allele and a fluorescent recombinase to sort frataxin deleted cells by
With this system, we have shown that complete absence of frataxin in fibroblasts inhibits cell division and leads to cell death (N. Carelle-Calmels, submitted for publication). Furthermore, stable expression of a transgenic murine or human frataxin in these cells rescued the deleterious effect of the deletion of endogenous frataxin expression. We therefore used this new tool to determine the ability of the different frataxin forms to rescue the cell survival of cells deleted for endogenous frataxin. Fibroblasts expressing the conditional frataxin allele (FrdaL3/L2) were stably transfected with different constructs containing either WT frataxin or the single or double maturation mutants (Fig. 6A). After a second transient transfection with the fluorescent recombinase pEGFP-Cre, the cells were sorted by FACS and isolated one cell per well and cultured for 7–10 days. As expected, no clones were able to grow when transfected with an empty vector (Fig. 6B), confirming the lethality of total frataxin deficiency in these cells, while 30 clones with stable expression of WT frataxin were obtained (Fig. 6B). In contrast, for cells transfected with the mutant mut 78–79, expressing no WT mature frataxin but rather the i-FXN, m56-FXN and m78-FXN, 14 clones were identified. For the mutant mut 39–40/78–79, expressing both the m56-FXN and m78-FXN, and the mutant mut 39–40/53–54/78–79, expressing only m78-FXN, the number of growing clones were nine and two, respectively (Fig. 6B). For each construct tested, the same number of EGFP-positive cells was isolated. The number of healthy clones was fewer with the mutant constructs than with WT frataxin suggesting that m81-FXN is more efficient and functional than m56-FXN and m78-FXN. However, the isolation of several clones with mutant constructs suggests that m56-FXN and m78-FXN could be functional to rescue cell survival, although we cannot exclude that the mutant constructs do not completely abolish the normal cleavage of the mature frataxin resulting in an undetectable pool of mature frataxin in these cells.

Maturation of frataxin occurs within the mitochondria in cells

The existence of an extramitochondrial pool of frataxin has recently been suggested by several groups (25,38,39). In contrary,
Frataxin is a nuclear-encoded protein, synthesized in the cytosol as a 210 amino acid precursor, which is targeted to the mitochondria and undergoes proteolytic processing to generate the functional mature form (m-FXN) (23,34,40). The initial characterization demonstrated that the endogenous m-FXN was encoded by amino acids 56–210 (m56–FXN). However, two recent reports have challenged these initial studies, and have described two different forms of mature frataxin m78–FXN and m81–FXN, encoded by amino acids 78–210 and 81–210, respectively (35,36). A third recent report which describes a lateral-flow immunoassay as a novel tool to measure the frataxin protein level in FRDA patients and carriers, reaffirms that the endogenous mature frataxin is m56–FXN (41). In the present paper, we provide clear evidence that endogenous mature frataxin corresponds to m56–FXN and demonstrate that transgenic expression of m56–FXN rescues the survival of murine fibroblasts totally deficient for endogenous frataxin. Further supporting our results is the recent report by Li et al. (42) suggesting, by metabolic methionine radiolabeling experiments, that Met76 is not present in the mature protein. Our analysis of site-directed maturation mutants independently or in combination confirms a two-step maturation process. Indeed, no direct cleavage from the precursor to the mature protein is observed while the internal cleavage at the intermediate step (amino acid 41) is crucial to have an efficient normal maturation. Furthermore, we show that the abnormal migration of m56–FXN could be restored by mutagenesis of three acidic residues in the N-terminal (D91, E92 and D104). The abnormal migration profile of frataxin has most likely contributed to the contradictions reported for the N-terminal sequence of endogenous mature frataxin. Specifically, we show that the previously reported degraded form of frataxin (d-FXN) (15,29,34) corresponds to m78–FXN, despite a faster migration than the mature m56–FXN. Thus, contrary to previous suggestions, the d-FXN form is not a degradation product of the mature form of frataxin. It is interesting to note that when the sites of normal maturation (amino acids 39–40 and 78–79) are mutated, two cryptic maturation products are generated that co-migrate with FXN56–210 and FXN78–210. Mass spectrometry analysis of these two cryptic products and mutagenesis experiments demonstrated that they correspond to m56–FXN and m78–FXN, respectively. Furthermore, our mutational analysis suggests that m56–FXN is directly generated from the precursor, since high expression of m56–FXN is seen in the 39–40/78–79 double mutant frataxin, which does not generate intermediate form. Of interest, cytoplasmic expression of a FXN56–210 form has been shown to be able to rescue frataxin-defective cells derived from FRDA patients from exogenous oxidative stress (25). We therefore hypothesized that m56–FXN, as it is directly generated from the precursor, might be processed within the cytosol. However, our fractionation experiments clearly show that when the normal maturation process is impaired, m56–FXN is generated within the mitochondria, similar to the normal maturation process of WT frataxin. Although our experiments do not exclude the possibility that a cytosolic m56–FXN form could be processed under specific conditions endogenously, we have never detected the m56–FXN form in any tested cell lines and murine tissues.
Different groups have reported the existence of shorter forms of frataxin (d-FXN) occurring from ‘spontaneous’ proteolysis, with the major degradation form corresponding to m56-FXN (15,23,43). In a recent report, the m78-FXN form was proposed to be generated by an iron-mediated self cleavage of the m56-FXN form (36). The authors propose that this post-translational modification by autoproteolysis has a regulatory role, possibly in synchronizing iron influx into the matrix with delivery to target proteins, by generating the functional form of frataxin capable of iron delivering to IscU and ferrochelatase. On the other hand, another report suggests that dihydrolipoamide dehydrogenase (DLD) has a ‘moonlighting’ protease activity converting the m56-FXN form of frataxin to shorter forms of ≈14 kDa, the major one corresponding to m77-FXN (44). As biochemical experiments have demonstrated that the d-FXN is unable to assemble and detoxify iron in vitro (43), the authors suggest that this cleavage by DLD is a mechanism to regulate the level of frataxin within the cell. Although our results do not directly address the proteolytic mechanism that leads to m77-FXN, it is clear that under our experimental conditions, the m78-FXN form can be generated either from the precursor, the intermediate form or the m56-FXN form when normal maturation is impaired, but not from the endogenous mature frataxin. Furthermore, although our functional test clearly shows that transgenic expression of a WT frataxin giving rise to the m81-FXN can rescue the cell death linked to endogenous frataxin deletion, m56-FXN and m78-FXN appear to also be functional, although less efficiently than m81-FXN. Particularly, m78-FXN appears to be far less efficient than m81-FXN and even m56-FXN. However, we cannot exclude that mutation of residues around each cleavage site do not completely abolish the normal cleavage, generating a small undetectable level of mature frataxin that can rescue endogenous frataxin deletion. Interestingly, the expression of a frataxin construct carrying mutation in residues 53–54 and 79–80, which therefore express only m78-FXN (this paper), failed to restore the aconitase activity deficiency in patients fibroblasts compared with WT frataxin or m56-FXN (35).

Although the abnormal migration of frataxin has been recognized for a long time, our results show that only the mature m81-FXN presents this abnormal migration profile on SDS–PAGE. Indeed, different deletion constructs of FXN going from 56–210 to 79–210 migrate according to their theoretical molecular weights (Fig. 4), while m81-FXN migrates in between the 71–210 and 64–210 constructs. Furthermore, we show that depending on the acrylamide concentration, m81-FXN can co-migrate either with the m78-FXN or close to the m56-FXN. We exclude that this abnormal migration profile is due to a putative post-translational modification. The abnormal migration profile of frataxin has been attributed to the specific acidic nature of the protein due to the presence of a dozen acidic residues, involved in iron binding, localized at the surface of the protein. Mutations of some of these residues has been shown to modify the migration profile for yeast frataxin (31,37), potentially through a change in the electrostatic potential of the protein. In agreement, we demonstrate that point mutations in some N-terminal acidic residues of human frataxin can change its migration in gel, notably to a migration according to its calculated molecular weight. It is interesting to note that two independent reports suggest that the N-terminal residues (residues 75–90), which has a 310-helix, lies toward the α1-helix and the β1-sheet of the core structure of frataxin (residues 91–210) (16), and may through multiple electrostatic contacts block access to the anionic surface. The property of such secondary structure of the N-terminus of frataxin might be responsible for the abnormal migration profile. It is tempting to speculate, based on our results as well as published results (17,37), that acidic residues are important for the proper folding of the N-terminus of m81-FXN, while the presence of residues L78, R79 and K80, not normally present in the mature FXN, might prevent it.

In conclusion, we have shown that the major endogenous form of mature human frataxin is m81-FXN, in agreement with one of the recently reported mature forms of frataxin (35). Furthermore, we show that m81-FXN is fully functional for cell survival, and that the two-step maturation process occurs entirely within the mitochondria. Both crystallographic data and solution NMR structures suggest that the core of frataxin (residues 90–210) is rigid (28,29). The identification of the final maturation step at amino acid 81 indicates that this core structure covers most of the mature frataxin, and that the resulting size of m81-FXN (130 amino acids), is very comparable in size with the mature yeast and bacterial protein. Recently, in yeast, the N-terminal part of the mature protein which has a 310 helix was suggested to stabilize a trimer conformation (45), suggested to be the functional unit of frataxin giving iron to its partners and the building blocks for frataxin oligomerization for iron storage and iron detoxification (46). Although the m78-FXN form has been shown to be unable to oligomerize (47), it will be of interest to determine whether the m81-FXN is capable of forming trimers and oligomers. In the future, the clear determination of the structure and biochemical properties of the mature human m81-FXN is important not only to determine the in vivo role of human frataxin in Fe–S and heme biosynthesis as well as in iron storage and detoxification, but also as it will provide essential tools and knowledge to envisage new therapeutical approaches for FRDA based on protein stabilization or replacement therapies.

**MATERIALS AND METHODS**

**Plasmids constructions and mutagenesis**

Mutations in frataxin were introduced by primer-directed PCR mutagenesis (Supplementary Material, Table S1) using 10 ng of pcDNA3.1-hFXN vector, which contains human frataxin cDNA and Pfu DNA polymerase (10 U) in the presence of 2 ng/μl of primers, 200 μM of dNTP. Each mutation corresponds to the replacement of the indicated residue by glycine. Frataxin N-terminal truncated mutants were amplified from pcDNA3.1-hFXN by classical PCR (Supplementary Material, Table S1) and cloned using EcoRI/Xhol restriction sites into a pENTR1A Gateway entry vector (Invitrogen) and recombined in a pSG5 vector using the GATEWAY technology (Invitrogen) according to the manufacturer’s protocol. All constructs were verified by sequencing.
**Cell culture and transfections**

COS-1 and HeLa cells were grown at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium with 1 g/l glucose supplemented with 5% fetal calf serum (FCS). Immortalized mouse fibroblasts established from Frda<sup>L3/L</sup> mice (7) (N. Carelle-Calmels, submitted for publication) were grown in the same conditions with 10% FCS.

Cell lines were transiently transfected with the different frataxin constructs using Eugene Reagent 6 (Roche Diagnostic) as recommended by the manufacturer. Cells were harvested 24 h after transfection. Stable transfection was obtained by antibiotic selection (250 μg/ml of zeocin) for a minimum of 3 weeks. The expression of the different constructs was checked by western blot analysis.

For functional survival test, immortalized mouse Frda<sup>L3/L</sup> fibroblasts stably transfected with empty vector or constructs expressing frataxin (WT or mutant) were transiently transfected with a pEGFP-Cre vector (N. Carelle-Calmels, submitted for publication). Two days post-transfection, cells were harvested twice with PBS. Cells were resuspended in PBS and filtered through a 50 μm sterile mesh (BD Biosciences). The EGFP-positive cells were sorted on a FACSDiVa Vantage (Becton-Dickinson). Positive cells were either isolated one cell per well in 96-well plate or collected into growth medium in 6-well plate. Growing clones were selected for further analysis. Genotyping was performed as previously described (7).

**Total protein extraction and subcellular fractionation**

Cells were washed twice with PBS and then scrapped in Tris–HCl 100 mm, pH 7.5, 10% glycerol, complete protease inhibitor cocktail (Roche). Total extracts were obtained by resuspending cells in the same buffer supplemented with 0.2% Triton X-100 and incubating on ice for 30 min. Suspensions were centrifuged 10 000g, 10 min, 4°C and the protein concentration of supernatants was determined using Bradford reagent.

For the subcellular fractionation, cells were washed twice with PBS and then scrapped in Buffer A. The cell pellet was washed once with the same buffer before adding 0.014% digitonin for 10 min at 4°C. The suspension was centrifuged at 10 000 g, 4°C for 10 min. The supernatant was further centrifuged at 25 000g, 4°C for 30 min to get the soluble cytosolic fraction, while the pellet, corresponding to the enriched mitochondrial fraction, was re-suspended in buffer A using a 25-G syringe and centrifuge at 5000 g, 4°C for 5 min to eliminate membranes and nuclear fraction. The mitochondrial fraction was obtained by centrifugation at 10 000g, 4°C for 10 min and washed twice with buffer A. The mitochondrial pellet was re-suspended in buffer A containing 0.2% Triton X-100 and incubated for 30 min at 4°C to get, after centrifugation, mitochondrial extracts.

**Western blotting and in vitro transcription/translation assay**

Two different protein electrophoresis systems were used (SDS–Glycine–PAGE with 12, 13, 14 or 15% acrylamide and SDS–Tricine–PAGE 16%) and western blot were carried out as previously described (7). Note that we used 14% acrylamide SDS–Glycine–PAGE, when it is not precise. Antibodies were diluted as follow: anti-frataxin 1/1000 (R1270, directed against peptide TLDPSSLDE-TAYERLAETL) (7) or monoclonal 1G2 (Mitoscience); anti-B-tubulin 1/5000 (MAB3408, Chemicon International); anti-MnSOD 1/5000 (SOD-110, StressGen). HRP-coupled secondary antibodies were diluted at 1/5000. In vitro transcription/translation of recombinant frataxin protein was produced according to the manufacturer’s protocol (Promega) using the TnT® quick-coupled transcription/translation systems and then analyzed by western blot.

**Immunopurification and staining of mature frataxin**

The immunoprecipitating monoclonal 1G2 frataxin antibody was cross-linked to G-sephrose beads using dimethyl pimelimidate (DMP, Sigma). Briefly, antibody was incubated with the beads and then washed twice with borate buffer 0.15 M, pH 9.0. Beads were incubated twice with 20 μg M DMP for 30 min. Cross-linking reaction was stopped by 2 h incubation in ethanalamine 0.2 M, pH 8.0. Beads were washed once with glycine 1 M, pH 2.5, to eliminate uncross-linked antibody and twice with PBS to eliminate glycine.

Cell total extracts were prepared from HeLa cells transiently transfected with WT frataxin. Immunoprecipitation was carried out in Tris–HCl 100 mm, pH 7.5, 0.1% NP40 containing protease inhibitors (Roche) using 1 mg of total proteins and the cross-linked 1G2 antibody, overnight at 4°C.

Beads were pelleted by centrifugation at 5000g, 5 min, 4°C and washed four times with Tris–HCl 100 mm, pH 7.5, containing 250 μmol NaCl, 0.1% NP40 and protease inhibitors. Two washes were done without any salt. Beads were resuspended in glycine 1 M, pH 2.5, to elute immunoprecipitated frataxin and prevent contamination by the cross-linked antibody. pH of the sample was neutralized by adding a small volume of Tris 1 M buffer, pH 8.8. Several immunoprecipitation were pooled together and concentrated in a small volume with the Vivaspin system (Vivascience). Loading buffer was added and samples were boiled 10 min before analysis on 14% SDS–Tris–Glycine–PAGE. Immunoprecipitated frataxin was detected by classical silver nitrate staining (48). Slices from silver nitrate stained gel were excised and subjected to MALDI-TOF spectrometry analysis.

**Mass spectrometry analyses**

In gel digestion. Enzymatic digestion of excised bands was performed as previously described (49). Depending on the gel volume, 5–10 μl of 10 ng/μl Porcine Trypsin (V511A, Promega) or of 10 ng/μl Pseudomonas fragi Asp-N (Roche) were added. Each enzyme was freshly diluted in 25 mM NH₄HCO₃. The digestion was performed overnight at room temperature (RT). Five microliters of 40% H₂O/60% acetonitrile/0.1% TFA were added, and samples were incubated for 3 h at RT. After 2 min sonication, 0.5 μl of peptide extracts was used for MALDI analyses.
MALDI measurements. Peptide extracts (0.5 μl) were mixed with an equal volume of 2,5-dihydroxybenzoic acid (DHB, 10 mg/ml) purchased from Sigma (Sigma-Aldrich). DHB was dissolved in 20% acetonitrile and crystals were obtained using the dried droplet method. MALDI MS measurements were carried out on a Bruker Reflex IV MALDI-TOF spectrometer, at a maximum accelerating potential of 20 kV and in positive reflectron mode. The acquisition range was set to m/z 800–3000 with low mass gate at m/z 700. Approximately 300 mass spectra were averaged per spot to optimize the signal-to-noise ratio. Monoisotopic peptide masses were assigned using the FlexAnalysis software (Bruker Daltonics). For positive mode measurements after enzymic digestions, internal calibrations were performed using peaks corresponding to autolytic peptides of the enzymes: m/z = 842.509 and 2211.104 for trypsin; m/z = 1022.521, 1365.622, 1917.062 for Asp-N.

Tools for interpretation of MALDI-PMF data

Peak lists from recombinant proteins were submitted to the FindMod and FindPep software (http://www.expasy.org/ tools) using the following parameters: enzymatic digestion trypsin (cleavage at the C-terminus of Arg and Lys residues even before Pro) or Asp-N (cleavage at the N-terminus of Asp and Glu residues); iodoacetamide treatment on cysteines was considered complete and methionine oxidation only partial; tolerance 50 ppm. Sequence coverage percentages have been manually calculated. The submitted sequence for the analyses was human frataxin (Q16595).

FUNDING

This work was funded by the French National Agency for Research (ANR-05-MRAR-013-01) and the French Medical Research Foundation ‘Equipe FRM 2005’ (DEQ-1205774).

ACKNOWLEDGEMENTS

We thank M. Koenig, J.L. Mandel and all members of the Human genetics group for fruitful discussions, L. Reutenauser, N. Vaucamps and F. Ruffenach for technical assistance.

Conflict of Interest statement. None declared.

REFERENCES


