Friedreich's ataxia protein: phylogenetic evidence for mitochondrial dysfunction

Toby J. Gibson, Eugene V. Koonin, Giovanna Musco, Annalisa Pastore and Peer Bork

Friedreich's ataxia is the most common inherited spinocerebellar ataxia. A decade of linkage and physical mapping studies have culminated in the identification of the Friedreich's ataxia gene. The presence of homologues in purple bacterial genomes, but not in other bacteria, allows us to infer a mitochondrial location for frataxin (Friedreich's ataxia protein) on the basis of bacterial phylogeny. Frataxin possesses a non-globular N-terminus domain providing a candidate mitochondrial targeting peptide. Clues to the function of frataxin are provided by the mitochondrial location, a clinically similar ataxia with vitamin E deficiency, and certain neuropathies with mitochondrial DNA instability caused by mutations in nuclear genes.

Trends Neurosci. (1996) 19, 465–468

MITOCHONDRIAL DYSFUNCTION is a major cause of both myopathy and neuropathy[1,2]. Very often, the two occur together. This linkage is due to the fact that only nerve and heart-muscle cells respire exclusively aerobically, making them totally dependent on mitochondrial oxidative phosphorylation. Although other types of skeletal muscle can respire anaerobically, the high energy requirements and build up of lactic acid under anaerobic working conditions also lead to a strong dependence on proper mitochondrial function. The majority of mitochondrial myopathies are caused by lesions in the mitochondrial genome, affecting proteins in the respiratory chain either directly or indirectly. More rarely, mutations are found in nuclear-encoded enzymes of mitochondrial intermediary metabolism. Mutations that cause disease are less common in nuclear-encoded mitochondrial proteins, in part because the mutation rate is lower than in mitochondria, but also because of the occurrence of heteroplasmy: each cell possesses many mitochondrial genomes, allowing it to carry a proportion of defective genomes that would certainly be lethal if uncomplemented.

Ataxia, meaning impaired movement due to loss of motor co-ordination, is associated with many of the mitochondrial myopathies, and in these cases is usually due to progressive neural degeneration. However, hereditary ataxias can also be caused by defects in genes that are remote from mitochondria, for example, ataxia telangiectasia, which has a defect in a protein involved in nuclear DNA splicing and repair processes[3].

Friedreich's ataxia (FRDA) is the most common hereditary ataxia with spinocerebellar degeneration, occurring with a frequency of about 1 in 50 000. It is an autosomal recessive disease characterized by a variety of symptoms, the most important being progressive ataxia, hypertrophic cardiomyopathy and, often, diabetes mellitus or carbohydrate intolerance[4–6]. The disease primarily affects neurones with very long axons, which appear to die back from the periphery[7]. Onset of the disease is typically in late childhood with mortality in early adulthood. Mapping of the gene[8] to chromosomal location 9q13–q21 has subsequently allowed several variant ataxias to be mapped to the same locus, including an ataxia with retained tendon reflexes and a milder late-onset ataxia[9,10]. However, an apparently similar ataxia, but with vitamin E deficiency, was found to be unrelated, mapping to a gene that encodes an enzyme involved in the export of vitamin E (α-tocopherol) from the liver, α-tocopherol transferase[11]. The progression of both this ataxia and a second vitamin E deficiency, due to abetalipoproteinaemia, are alleviated by treatment with vitamin E (Refs 5,12).

Recently, Campuzano *et al.* have cloned and sequenced the *FRDA* gene[13]. The gene encodes a putative 210-amino acid protein designated frataxin[13]. A shorter, 171-amino acid protein might be expressed as the result of alternative splicing. Highly significant sequence similarities between frataxin and two partially sequenced, uncharacterized putative proteins from the nematode *Caenorhabditis elegans* and from the yeast *Saccharomyces cerevisiae* were also reported. Thus, at first sight, the gene cloning has yielded disappointingly little insight into the biochemical defect underlying the disease.

Frataxin homologues in bacteria

We undertook searches of the DNA and protein sequence databases for additional frataxin homologues, using sequence comparison tools that were more sensitive than those employed in the initial report (for review, see Ref. 14). We have been able to find some clues which, while falling short of a definite functional assignment, should nevertheless be useful to experimentalists in delimiting the range of possible functions for frataxin.

The Smith–Waterman algorithm, as implemented in the SearchWise program[15], ranked CyaY protein sequences[16] from *Escherichia coli*, *Erwinia chrysanthemi* and two *Yersinia* species as the top-scoring entries in SWISS-PROT. Reciprocal searches with CyaY from *E. coli* ranked frataxin highest after the CyaYs. The significance assessment provided by the BLITZ server[17] yielded a *P*-value of 10^{-8} that the similarity between

Toby J. Gibson, Giovanna Musco, Annalisa Pastore and Peer Bork are at the European Molecular Biology Laboratory, Postfach 102209, Meyerhofstrasse 1, D-69012 Heidelberg, Germany. Eugene V. Koonin is at the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA. Peer Bork is also at the Max-Delbrück-Centre for Molecular Medicine, D-13122 Berlin-buch, Germany.

 0166 - 2236/96/$15.00 PII: S0166-2236(96)20054-2

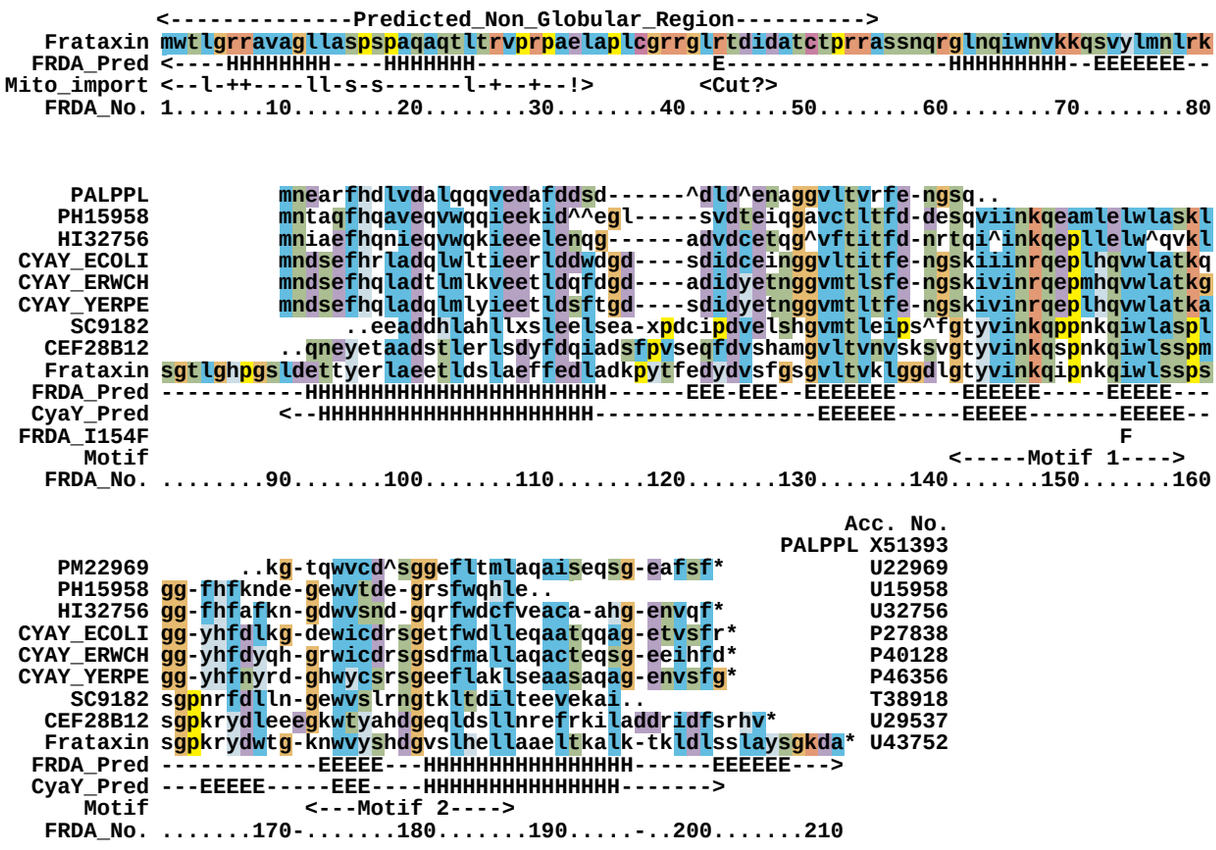

Fig. 1. ***Multiple alignment of frataxin with its eukaryotic and bacterial homologues.*** *The initial alignment was constructed using ClustalW (Ref. 19), and the partial sequences added subsequently. Frameshift errors in several sequences are indicated by ^. Amino acid residues are colour-coded by property conservation. Predictions of secondary structures[20] are shown separately for frataxin plus the nematode homologue, and for the CyaY family proteins (H indicates α-helix, E indicates β-strand). The typical features of a mitochondrial targeting peptide[26] (α-helicity, abundant Arg, Ser and Leu residues but rare Glu and Asp residues), the consensus peptide and the predicted cleavage site are indicated for frataxin. The two most conserved motifs are indicated, as is the Ile154Phe substitution in motif 1 (Ref. 13). The yeast frataxin homologue maps to chromosome IV, position 245 922 and is assigned database identifier e253043.*

frataxin and CyaY is due to chance. Comparable results were obtained with the BLAST programs[18], for example, CyaY matched the yeast frataxin homologue with a *P*-value of $<10^{-5}$. Additional six-frame translation searches[15,18] of nucleotide sequence databases with the CyaY protein sequences revealed new members of the CyaY family among unannotated (and error-prone) sequences from *Haemophilus influenzae*, *Pasteurella haemolytica*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.

Multiple alignment[19] of frataxin with the related protein sequences from eukaryotes and bacteria revealed that the complete CyaY sequences align with the C-terminus portion of frataxin, with most of the conserved amino acid residues concentrated in two distinct motifs (Fig. 1). Each of these motifs was used for pattern searches with the MoST program[21] in order to detect potential distant similarities. However, no new sequences were selected. Similarly, using these alignments for SearchWise[15] profile searches also failed to detect any additional sequences. Predictions of secondary structures, made separately for frataxin and its nematode homologue and for the CyaY proteins, using the PHD program[20] both produced similar results, indicating that the conserved domain is most likely to form a β-sheet with two long flanking α-helices at the N- and C-termini (Fig. 1). On the basis of these analyses we conclude that frataxin and CyaY comprise a new conserved protein family. It is noteworthy that one of the deleterious *FRDA* point mutations[13] is located in the most conserved motif at a conserved aliphatic residue – likely to be an essential core residue (Fig. 1).

The evidence for a mitochondrial location

The expression of the *CyaY* gene in *E. coli* has recently been demonstrated[22], although its function is still unknown. So far, CyaY has been detected in eight prokaryotic species, all within the γ subdivision of purple bacteria. A careful search of the complete genome sequence of *Mycoplasma genitalium*[23] and of the *Bacillus subtilis* sequences (comprising about 50% of the genome: A. Danchin, pers. commun.) did not reveal any related sequences. Therefore, CyaY appears not to be present in Gram-positive bacteria. Phylogenetic trees constructed from aligned ribosomal RNAs show purple bacteria to be the closest living relatives to mitochondria[24]. Therefore the bacterial phylogeny implies that the *FRDA* gene evolved from a *CyaY* gene present in the mitochondrial ancestor, as illustrated in Fig. 2.

Nuclear-encoded mitochondrial proteins possess N-terminus targeting peptides for import. Frataxin possesses an additional N-terminus domain, not shared by the CyaY homologues. Segmentation analysis using the SEG program[25] (with settings optimized for delineation of non-globular domains: window = 45; trigger complexity = 3.4; extension complexity = 3.75), indicates that residues 1–55 form a non-globular domain. The properties of this extra region of frataxin are compatible with a mitochondrial targeting peptide[26],

as summarized in Fig. 1. Thus, frataxin might be a nuclear-encoded mitochondrial protein and, accordingly, mitochondrial dysfunction would then be the primary cause of neurone degeneration in FRDA patients. In order to confirm this, it is essential to obtain experimental demonstration of the location of frataxin by methods such as cell fractionation, *in vitro* mitochondrial import assays and the classic (if unreliable) cell staining with an anti-frataxin antibody.

Implications for frataxin function

Mitochondrial dysfunction has long been suggested to underlie Friedreich's Ataxia (summarized by Cedarbaum and Blass[7]), although since 1988, when the *FRDA* gene was first mapped to chromosome 9, the theory appears to have been placed in suspended animation. Mitochondria are the seat of aerobic energy metabolism and have special relevance to the three most affected tissues in the ataxia: heart muscle respires aerobically, comprising 40% mitochondria; neurones respire aerobically, using glucose as their sole energy source; pancreatic islet cells regulate the level of blood glucose and diabetes is a frequent complication of various mitochondrial myopathies. Yet little or no support has been forthcoming experimentally with, for example, conflicting and inconclusive results for mitochondrial enzyme assays from several laboratories[7]. These assays have often shown moderately reduced enzyme activity, yet there has been no consistency between the affected enzymes. At best therefore, such results are thought to show a secondary effect, indicative of mitochondrial damage[7].

Therefore, frataxin lesions would seem to have somewhat subtle effects, not related directly to the primary metabolic functions of the organelle. Owing to its small size (100 residues), the C-terminus domain of frataxin is highly unlikely to be an enzyme. More-typical roles for small soluble proteins include acting as regulators, adaptors or, as in the case of acyl carrier protein, metabolite carriers. Ataxia with vitamin E deficiency is thought likely to provide an important clue to the function of frataxin, assuming that the similar symptoms indicate a common biochemical dysfunction[12]. Vitamin E is a lipid-soluble antioxidant and free-radical scavenger found in cell membranes. It is most abundant in the nuclear and mitochondrial membranes[27]. In the mitochondria, the vitamin protects unsaturated fatty acids and ubiquinone from peroxidation, the latter being part of the electron transport chain. It has also been shown that peroxidation can damage mitochondrial DNA and that here too vitamin E has a protective effect[28]. Chronic damage to the DNA will lead to reduced numbers and activity of mitochondria by death or by failure to multiply. In axons, mitochondria at the paranodal regions provide energy for the fast axoplasmic transport necessary for neural function[7]. Disruption of this transport by toxins of oxidative metabolism causes dying-back neuropathy. It seems likely therefore that the vitamin E defect leads to chronic damage of the mitochondrial genomes, resulting ultimately in reduced respiration and subsequent pathophysiological consequences.

As frataxin lesions have the same consequences as the vitamin E deficiency, the role of frataxin in mitochondria is likely to be bounded by vitamin E antioxidative metabolism and the repair or replication of DNA. At one extreme frataxin might bind the vitamin itself, while at the other it might be a DNA-binding protein regulating

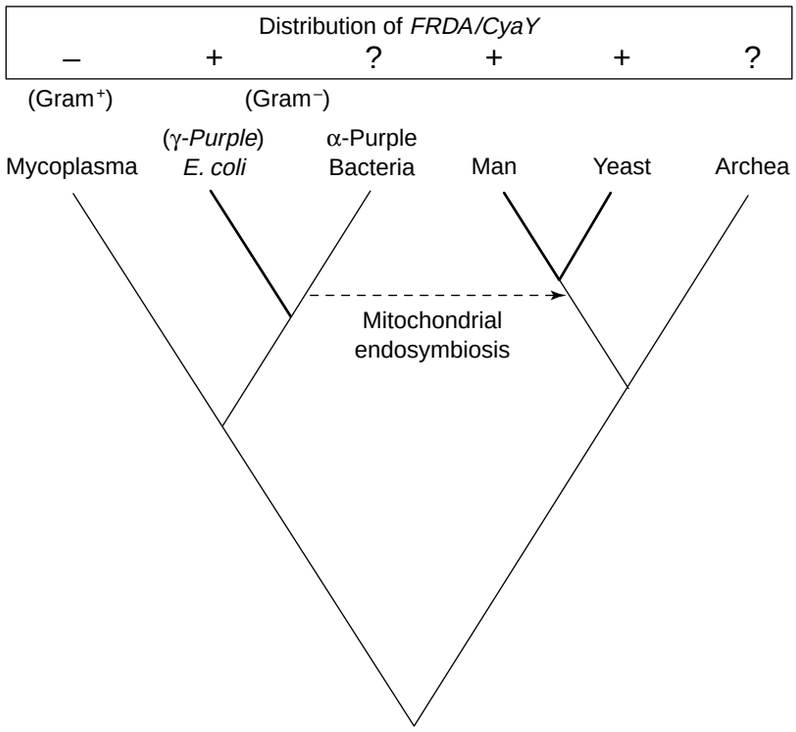

Fig. 2. ***Superposition of the* FRDA/CyaY *family members upon the phylogenetic tree, showing the parsimony implicit in a mitochondrial origin for* FRDA.** *Thick branches lead to organisms known to possess* FRDA/CyaY. *Phylogenetic tree topology is adapted from Ref. 24.*

DNA replication or repair. Therefore peroxidative damage to DNA and genome maintenance would appear to be the main areas to probe in elucidating the function of frataxin. Mitochondrial DNA repair mechanisms are known to exist[29], but are not well understood. It is noteworthy that two dominant hereditary diseases are known in which nuclear gene defects lead either to mitochondrial DNA instability or depletion[2]. Thus, in the late-onset disorder mitochondrial myopathy with multiple deletions of mitochondria DNA, mitochondrial function is degraded gradually by the proliferation of DNA deletions[30]. In yeast, disruptions of the *MHR1* gene exhibit a recessive phenotype with mitochondrial DNA instability and increased induction of UV-induced mutations in mitochondrial DNA[31].

Given the possibility that frataxin mutations affect mitochondrial DNA repair or replication, mitochondrial DNA from patients with Friedreich's ataxia needs to be examined for copy number and for evidence of increased accumulation of substitution mutations as well as deletions or other rearrangements. If frataxin is involved in DNA maintenance, then the progression of the ataxia is presumably accelerated by exposure to environmental mutagens. This would be of considerable medical significance.

To conclude, there is every reason to expect good progress in elucidating the function of frataxin. The presence of homologues in the model organisms yeast and *E. coli*, with their powerful genetics, should be useful in these forthcoming endeavours.

Note added in proof

The complete sequence of the archaeon *Methanococcus jannaschii* has now been reported[32]. Consistent with the proposed mitochondrial location of frataxin (see Fig. 2), we could not detect any similarity in the archael genome with the *FRDA*/*CyaY* sequences.

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LETTERS TO THE EDITOR

Feedback models of gamma rhythms

Jefferys *et al.*[1] in the May issue of *TINS* reviewed the use of neuronal network analysis for modeling oscillations in the gamma range, particularly to distinguish between intrinsic oscillations from neurons that entrain others and interneuronal oscillations that arise from feedback between excitatory and inhibitory populations. They write (Ref. 1, p. 204) that in the olfactory system, 'Freeman *et al.*[2] predicted that inhibitory cells should lag behind the excitatory cells by a quarter cycle (6.5 ms at 40 Hz). …In contrast, hippocampal interneurones recorded during the gamma EEG fire in phase with the pyramidal cells', citing Bragin *et al.*[3] They concluded that interactive models from olfactory systems might not hold for the hippocampus.

Bragin *et al.*[3] wrote that units and waves from pyramidal cells and interneurons were 'synchronized', but showed (their Fig. 5, p. 52) that 'All three hilar units discharged phase-locked to the ascending portion of the local gamma waves'. Although Bragin *et al.* did not report measurements of phase differences nor their standard errors in radians or degrees, this quarter-cycle lag on visual inspection is fully consistent with interneuronal feedback. In other words, phase-locking is not the same as synchrony. Prior evidence for the interneuronal hypothesis in the hippocampus has been published by Horowitz *et al.*[4] and Leung[5].

My olfactory model of interactive oscillations was based on fitting a curve in one step to data from sets of multiple averaged elicited potentials and poststimulus time histograms, not to single traces. I used a single variable in turn to fit each dose–response relationship between a stimulus parameter or a pharmacological agent and the global pattern shift[6]. The successful prediction of phase relations between putative excitatory and inhibitory populations was a minor spin-off. Nothing of comparable scope has yet been performed for the hippocampus.

In reviewing recent developments Jefferys *et al.* did not note or exemplify some fundamentals of neuronal systems analysis: (1) The feedforward and feedback loops must be opened by any of several chemical and surgical methods, and the mean and standard error of the open-loop time constants must be measured; (2) Measurements must then be made to determine whether or how the open-loop parameters change on loop closure; (3) Paired-shock stimulation in conjunction with the superposition principle must be made to find linear and piecewise linear domains. This is especially important in invoking 'background' input to explain sustained oscillations; (4) Closed-loop gain coefficients must be evaluated by fitting simulated responses to observed wave forms. Changes in gains must be reduced to a scalar value that can be related one-on-one to the putative action of a drug at synapses and trigger zones by means of root locus techniques; (5) Stability analysis should be undertaken using input perturbations and parameter variations[6].

It is good to see increasing interest in nonlinear systems analysis among neurobiologists, but the invocation of '40 Hz' for 'gamma' oscillations suggests that the researchers have not advanced beyond point and limit cycle attractors. Modeling aperiodic attractors[7] (so-called 'chaos') will require more-thorough grounding in basics than has commonly been demonstrated. Confusing synchrony with phase-locking is a case in point.

Walter J. Freeman
Dept of Molecular and Cell Biology,
University of California at Berkeley,
CA 94720, USA.

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Recurrent inhibition model of hippocampal CA1 *in vivo*

In a recent article[1], Jefferys *et al.* showed that a metabotropic glutamate agonist, in the presence of NMDA and AMPA glutamate antagonists, induced a 40 Hz (gamma) rhythm in CA1 pyramidal cells *in vitro*. Jefferys *et al.*[1,2] proposed that an interneuronal network, without the involvement of pyramidal cells, was responsible for the gamma rhythm *in vivo*.

I believe that more caution should be exercised in inferring the importance of this interneuronal network *in vivo*. An alternative hypothesis, specifically that of