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# Conformational analysis of the riboflavin-responsive ETF:QO-p.Pro456Leu variant associated with mild multiple acyl-CoA dehydrogenase deficiency

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ABSTRACT

Multiple-CoA dehydrogenase deficiency (MADD) is an inborn disorder of fatty acid and amino acid metabolism caused by mutations in the genes encoding for human electron transfer flavoprotein (ETF) and its partner electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO). Albeit a rare disease, extensive newborn screening programs contributed to a wider coverage of MADD genotypes. However, the impact of non-lethal mutations on ETF:QO function remains scarcely understood from a structural perspective. To this end, we here revisit the relatively common MADD mutation ETF:QO-p.Pro456Leu, in order to clarify how it affects enzyme structure and folding. Given the limitation in recombinant expression of human ETF:QO, we resort to its bacterial homologue from *Rhodobacter sphaeroides (Rs)*, in which the corresponding mutation (p.Pro389Leu) was inserted. The *in vitro* biochemical and biophysical investigations of the *Rs* ETF:QO-p.Pro389Leu variant showed that, while the mutation does not significantly affect the protein α/β fold, it introduces some plasticity on the tertiary structure and within flavin interactions. Indeed, in the p.Pro389Leu variant, FAD exhibits a higher thermolability during thermal denaturation and a faster rate of release in temperature-induced dissociation experiments, in comparison to the wild type. Therefore, although this clinical mutation occurs in the ubiquinone domain, its effect likely propagates to the nearby FAD binding domain, probably influencing electron transfer and redox potentials. Overall, our results provide a molecular rational for the decreased enzyme activity observed in patients and suggest that compromised FAD interactions in ETF:QO might account for the known riboflavin responsiveness of this mutation.

## 1. Introduction

Multiple acyl-CoA dehydrogenase deficiency (MADD, OMIM #231680), also known as glutaric aciduria type II (GA-II), is a human autosomal recessive inherited inborn error of fatty acid, amino acid and choline metabolism caused by defects in *ETFA, ETFB* and *ETFDH* genes [1,2]. The clinical features of MADD patients are rather heterogeneous, ranging from lethal cases with neonatal anomalies to mildly affected individuals, presenting in childhood or adulthood with hypoglycemic, encephalopathy and/or myopathy [3]. A large number of distinct genotypes has been identified in MADD patients, especially in the *ETFDH* gene [4], but only a limited number has been characterized. Mounting evidence shows that the severity of MADD phenotypes depends on the location and the nature of mutations, with null mutations being associated with lethal disease; and missense mutations, resulting in electron transfer flavoprotein (ETF) or electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO) proteins with some residual enzyme activity, being associated with milder clinical forms [5–7]. Despite these evidences, the molecular genetic basis and functional characterization of MADD genotypes remains elusive.

ETF:QO, is a 64 kDa monomeric protein bound to the inner mitochondrial membrane possibly *via* an amphipathic helix, which is rather structurally complex harboring three different functional domains [8]. The domains are packed in close proximity and share some structural elements, each one binding a different redox active cofactor (flavin adenine dinucleotide (FAD), iron-sulfur cluster ([4Fe4S]) and ubiquinone (UQ)). The FAD-binding domain and the iron-sulfur cluster domain face the mitochondrial matrix and the UQ-binding domain

*Abbreviations:* CD, Circular dichroism; DDM, n-Dodecyl-β-maltoside; ETF, Electron transfer flavoprotein; ETF:QO, Electron transfer flavoprotein:ubiquinone oxidoreductase; FAD, Flavin adenine dinucleotide; Fe-S, Iron Sulfur cluster; GA-II, Glutaric aciduria type II; IPTG, Isopropyl-1-thio-β-D-galactopyranoside; MADD, Multiple acyl-CoA dehydrogenase deficiency; MCAD, Medium chain acyl-CoA dehydrogenase; PDB, Protein Data Bank; PMSF, Phenylmethylsulfonyl fluoride; *Rs*, *Rhodobacter sphaeroides*; $T_m$, Temperature midpoint transition; UQ, Ubiquinone; WT, wild type

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anchors the protein to the inner mitochondrial membrane. The membrane bound ETF:QO and its partner, ETF in the mitochondrial matrix, are critical enzymes in a series of mitochondrial metabolic pathways since they transfer electrons derived from the oxidation of fatty acids and some amino acids to the mitochondrial respiratory chain [9]. Electrons derived from at least 11 dehydrogenases are transferred to the FAD cofactor in ETF and then to the Fe−S and FAD cofactors within ETF:QO [10], resulting in reduction of membrane-embedded UQ, thus mediating the transfer of reducing equivalents to the respiratory chain for subsequent ATP production.

This study is focused on the human ETF:QO-p.Pro456Leu variant, one of the several missense mutations that has been repeatedly identified in MADD patients with mild phenotypes, both in heterozygous and homozygous forms [11–17]. The expression of recombinant human ETF:QO has so far only been achieved in Sf9 insect cells using a baculovirus vector [18] and it is extremely challenging to obtain protein in amounts enough for structural biophysical studies. Indeed, even using multiple constructs of human ETF:QO with and without a diversity of tags, its recombinant expression in *E. coli* has been revealed to be unsuccessful regarding obtaining enough amounts of properly folded protein (our own unpublished data). Therefore, we resorted to the ETF:QO homologue from *Rhodobacter sphaeroides* (*Rs*), which is 67% identical to the human protein, as a proxy for the human protein. Henceforth the *Rs* ETF:QO-p.Pro389Leu was investigated in lieu of the human ETF:QO-p.Pro456Leu variant. In particular, the mutation on interest occurs in a conserved position across different species [19] which further validates the approach undertaken. This mutation is located in the UQ-binding domain, in a loop between two α-helices, one from the FAD domain and the other from the membrane domain [8]. In this work we combine biochemical and spectroscopic approaches to determine the influence of this mutation on the stability and folding of the protein and thus contribute to establish more genotype-structure-function relationships that may help in determining disease-severity and functional limitations in patients.

## 2. Materials and methods

### 2.1. Chemicals

All reagents were of the highest purity grade commercially available. FAD, riboflavin, octanoyl-CoA, coenzyme Q1, ammonium iron-citrate and 8-hydroxiquinoline were purchased from Sigma. Isopropyl-β-D-thiogalactopyranoside (IPTG) and Bacterial Cell Lysis Buffer were purchased from Nzytech.

### 2.2. Protein expression and purification

The expression an purification of *Rs* ETF:QO was performed as previously described [20]. Briefly, *E. coli* C43 (DE3) cells (Lucigen) transformed with the respective plasmid, ETF:QO-wild type (ETF:QO-WT) or ETF:QO-p.Pro389Leu variant (a kind gift from Dr. Peter Bross, Arhus University), were grown in LB medium (10 g of Bacto Tryptone, 5 g of Bacto Yeast extract and 10 g of NaCl) supplemented with 100 μg.ml$^{-1}$ ampicillin, 2 μM riboflavin and 40 μM $Fe^{3+}$ complexed with 8-hydroxyquinoline, at 37 °C in a shaking incubator until an $OD_{600nm}$ between 0.6 and 1.0 was reached. The cells were then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and grown overnight at 30 °C. Cells were harvested by centrifugation, resuspended in a proportion of 1:4 (m/v) in 20 mM Tris pH 7.4, 50 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol (DTT) and 0.5 mM PMSF (Roth) in presence of DNase (PVL), and disrupted in a French press apparatus. Membrane fraction was obtained by centrifugation at 150,000 × *g* for 90 min and diluted to a concentration of 20 mg protein.ml$^{-1}$. A n-Dodecyl-β-maltoside (DDM) solution was prepared in the same buffer and added to the resuspended membranes in a ratio of 2.5:1 (DDM:protein) over 5–10 min. The membrane proteins were extracted for 90 min at 4 °C under stirring, and then obtained by centrifugation at 100,000 *g* for 1 h. The soluble solution was applied on a Q-Sepharose High Performance column (GE Healthcare, 1 ml) equilibrated in buffer A (20 mM Tris pH 7.4, 150 mM NaCl, 0.1% DDM, 0.1 mM DTT, 0.5 mM PMSF). The column was washed with 7 volumes of buffer A containing 250 mM NaCl, and bound proteins were eluted by a linear gradient between 250 and 500 mM NaCl in buffer A. ETF:QO pure proteins eluted at a salt concentration around 340 mM. Purity was confirmed by SDS-PAGE and by the characteristic visible absorption spectrum, which denotes spectral features compatible with the presence of the FeS and FAD cofactors, as in [20]. Protein concentration was determined using the Bradford assay, and flavin content was determined using the molar extinction coefficient reported for FAD bound in ETF:QO, $\varepsilon_{430\ nm}$ = 24.0 mM$^{-1}$.cm$^{-1}$ in [20]. Pure fractions were fast-frozen using liquid nitrogen and stored at −80 °C. Recombinant human proteins, ETF and medium-chain acyl-CoA dehydrogenase (MCAD), were expressed and purified as reported elsewhere [21,22].

### 2.3. Spectroscopic methods

UV–visible spectra were recorded in a Shimadzu UVPC-1601 spectrometer with cell stirring, at room temperature. Fluorescence spectroscopy was performed using a Jasco FP-8200 spectrofluorometer, with Peltier temperature control. Tryptophan emission studies were performed using an excitation wavelength of 280 nm, and flavin emission followed using an excitation wavelength of 450 nm, in both cases excitation and emission slits were set to 5 nm. Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-1500 CD spectropolarimeter with Peltier temperature control. A quartz polarized 1 mm path length cuvette (Hellma) was used. Typical protein concentration in fluorescence and CD studies was 0.1 mg.ml$^{-1}$.

### 2.4. Enzyme activity assays

ETF:QO enzyme activity was measured following coenzyme Q1 reduction at 275 nm ($\Delta\varepsilon$ = 7400 M$^{-1}$.cm$^{-1}$) in a coupled assay using recombinant human MCAD and ETF proteins, and octanoyl-CoA were employed as described in [18]. Typically, assays were performed at room temperature, in 10 mM hepes pH 7.4, and coenzyme Q1 concentration never exceeded 4% (*v*/v) on the assay mixture. One unit of catalytic activity is defined as nmol of coenzyme Q1 reduced per minute in the conditions used in the assay. All specific activities reported are based on total flavin content. Activity value determined represent the mean of at least five independent experiments.

### 2.5. Thermal stability

Protein unfolding, with a linear temperature increase from 20 to 90 °C, was followed using circular dichroism (ellipticity variation at 222 nm) and fluorescence spectroscopy - tryptophan emission ($\lambda_{ex}$ = 280 nm; $\lambda_{em}$ = 340 nm), FAD emission ($\lambda_{ex}$ = 450 nm; $\lambda_{em}$ = 530 nm) and Trp-FAD FRET ($\lambda_{ex}$ = 280 nm; $\lambda_{em}$ = 530 nm). In all experiments, a heating rate of 1 °C.min$^{-1}$ was used. Protein concentration was 0.1 mg.ml$^{-1}$ in 20 mM tris pH 7.4, 150 mM NaCl, 0.1 mM DTT, 0.1% DDM. At least three replicates were performed for each technique. Data were analyzed according to a two-state model and fitted using CD Pal [23]. The unfolding transitions were irreversible, impeding further thermodynamic analysis, and only the apparent midpoint unfolding temperature ($T_m^{app}$) is reported.

### 2.6. Kinetics of flavin release

ETF:QO proteins (0.1 mg.ml$^{-1}$) were incubated at 39 °C for up to 60 min in 20 mM Tris pH 7.4, 150 mM NaCl, 0.1 mM DTT, 0.1% DDM. The kinetics of flavin release were monitored from the increase of the 530 nm emission peak arising from free FAD upon excitation at 450 nm.

After 1-h incubation, the samples were boiled for 5 min to release the remaining FAD and a spectrum was collected at 39 °C. The fraction of bound FAD was determined at each point in respect to the point of 100% release of FAD.

### 2.7. Trypsin limited proteolysis

The susceptibility toward degradation of ETF:QO was performed by incubating wild type and p.Pro389Leu variant proteins with trypsin (bovine pancreas trypsin, Applichem), at 37 °C in 100 mM Tris-HCl pH 8.5, in a 10-fold excess over the protease. As a control, identical samples without addition of trypsin were also subjected to the same procedure. Aliquots with 0.05 nmol of protein were sampled at different time points up to 5 h, and the reaction was stopped by the addition of SDS-PAGE loading buffer (2% SDS and 5% β-mercaptoethanol). The products of the proteolysis reaction were analyzed by 12% SDS-PAGE, stained with Coomassie.

### 2.8. Structural analysis and modelling

Molecular modelling of human ETF:QO was carried out in Swiss-Model based on the porcine ETF:QO crystallographic structure (PDB: 2gmh). Insertion of mutation was done using the WhatIF server [24] and visualization and structural analysis was carried out in Pymol (Schrödinger, LLC).

## 3. Results

### 3.1. Structure and conformational properties of Rs ETF:QO-p.Pro389Leu

Previous cellular studies have shown that, in respect to the wild type, the ETF:QO-p.Pro456Leu variant has compromised activity and longer chaperone association before membrane-insertion [16]. Since no structure-function relationships are established, we studied the effects of this mutation on protein folding and conformation. To analyze protein folding and secondary structure we employed far-UV circular dichroism. We observed that the CD spectrum of the ETF:QO-p.Pro389Leu variant protein is dominated by features at 208 and 222 nm (Fig. 1A), which are typical for α-helix containing proteins, in agreement with the known α/β fold of ETF:QO. A comparison with the spectrum of ETF:QO-WT reveals that the mutation does not cause a major change in the protein secondary structure. Tertiary structure changes were then evaluated using protein intrinsic fluorescence, taking advantage of the fact that mature ETF:QO contains twelve tryptophan residues, which are relatively accessible to the solvent and are thus sensitive conformational probes. A comparison of the emission spectra of the two proteins upon Trp excitation at 280 nm reveals subtle, yet relevant differences (Fig. 1B). While the emission maximum of ETF:QO-WT is centered at ~334 nm, that of ETF:QO-p.Pro389Leu is slightly red shifted to about ~337 nm which indicates an overall higher solvent exposure of Trp moieties, suggesting a slightly less compact fold. In agreement, the observed differences in emission intensity are compatible with a higher fluorescence yield in the mutant *versus* the wild type.

We then analyzed the spectroscopic characteristics of the FAD cofactor, whose visible absorption fingerprint and fluorescence emission are very sensitive to changes in the protein-cofactor H-bonding network (Fig. 1C). The two characteristic flavin absorption bands with maxima at 380 and 430 nm, superimposing the underlying iron-sulfur cluster absorption, are observed in both the mutant and wild type, indicating that FAD is inserted in similar protein moieties. This is also confirmed by the similar $Abs_{380}/Abs_{430}$ ratios determined for the two proteins, which provide evidence for similar [4Fe−4S] contents. The $Abs_{275}/Abs_{380}$ ratios for the wild type ($r$ = 7.5) and mutant ($r$ = 6.6) are identical to those from the literature ($r$ = 6.0 in [18]) and also confirm that the Pro389Leu change does not impar FAD binding. However, there is a notorious difference between the two proteins upon comparison of their flavin fluorescence, which is more sensitive than visible absorption to subtle conformational changes. While FAD emission is essentially quenched in wild type ETF:QO, in agreement with a compact insertion within the binding pocket [25], that of ETF:QO-p.Pro389Leu variant is substantially higher, evidencing that in this case the cofactor may be more loosely attached to the protein, not so tightly H-bonded and more accessible to solvent interactions (Fig. 1D). Overall, these results indicate that the ETF:QO-p.Pro389Leu variant retains the overall protein fold although it introduces some plasticity on the tertiary structure and FAD binding.

### 3.2. Effect of Pro389Leu mutation on conformational stability

We have further investigated the effect of the mutation on the protein conformational stability from thermal melting experiments using different spectroscopic techniques (Fig. 2), a common approach to inspect how different structural elements contribute to the thermal stability of a protein [26–28]. The thermal denaturation of ETF:QO was found to be an irreversible process, as expected from a very complex multi cofactor protein. Therefore, thermodynamic analysis of the thermal unfolding transitions is not possible, and we hereby report apparent midpoint unfolding temperatures ($T_m^{app}$) (Table 1), which can be reliably used to compare irreversible thermal unfolding profiles obtained under similar concentrations and experimental conditions [29,30].

The comparison of the thermal stability of ETF:QO-WT probed by far-UV CD (secondary structure, Fig. 2A), Trp emission (tertiary structure, not shown) and FAD emission (cofactor structure, Fig. 2B) reveals similar $T_m^{app}$ between 49 and 51 °C. However, although the stability of the secondary structure of the ETF:QO-p.Pro389Leu variant is comparable to that of the wild type, flavin is less stable by nearly 4 °C (Fig. 2B, Table 1), in agreement with previous the spectroscopic data.

### 3.3. Flavin dissociation on ETF:QO-p.Pro389Leu variant

RR-MADD patients commonly develop symptoms associated with catabolic stress or/and illness periods, like during fever episodes. These patients improve their symptoms under riboflavin therapeutic doses. The molecular rational for this response has been clarified, and results from an increase of flavin content in the cells which favors protein functional state [21,31]. As our results suggest that the ETF:QO-p.Pro389Leu variant is slightly destabilized, we further investigated the effect of the mutation on flavin lability by following the kinetics of FAD thermal dissociation at 39 °C, a near physiological yet stress-inducing temperature (Fig. 3).

Considering that the intensity of the fluorescence emission spectrum for free FAD is higher than that of bound FAD, the release of flavin could be determined monitoring the increase in flavin fluorescence emission (experimental details in materials and methods). The results in Fig. 3 show that indeed the ETF:QO-p.Pro389Leu variant differs in kinetic stability, as the rates of FAD release during thermal perturbation were always higher than those observed for wild type protein. This effect becomes particularly evident under mild thermal stress conditions closer to the apparent melting temperature, as it translates the differences between thermal stabilities of the two proteins, as monitored by FAD release. Overall, FAD in ETF:QO-p.Pro389Leu presents a higher thermolability as evidenced in this temperature-induced dissociation experiments, as well as by a lower apparent melting temperature determined when unfolding is monitored through FAD emission (Table 1).

### 3.4. ETF:QO-p.Pro389Leu proteolytic susceptibility

Missense mutations often lead to misfolded variants with decreased stability and increased propensity to proteolytic degradation [32,33].

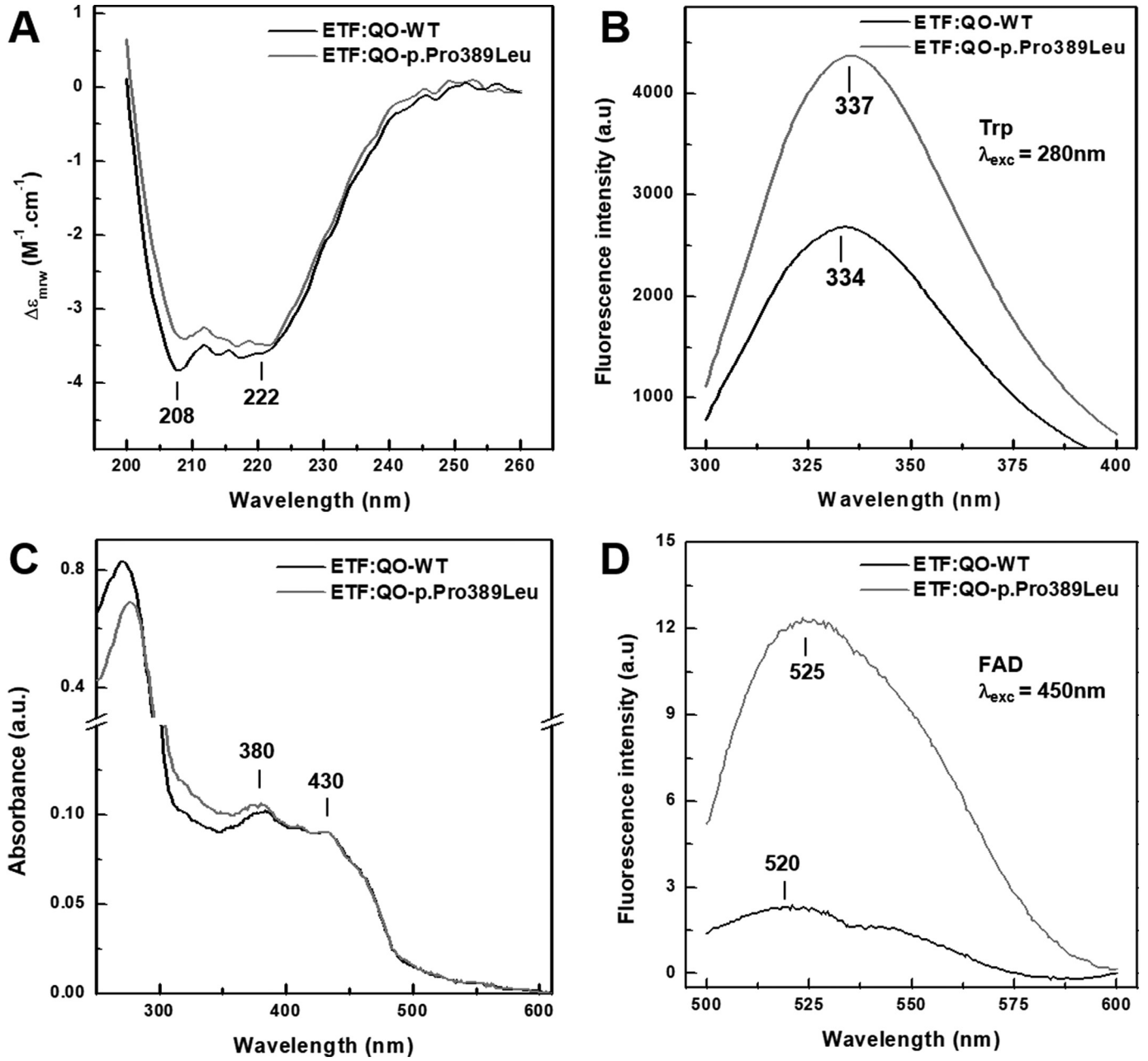


**Fig. 1.** Spectroscopic analysis of the ETF:QO-p.Pro389Leu variant. Far-UV CD (A), tryptophan fluorescence emission (B), visible absorption (C), and FAD fluorescence emission spectra (D) all at 1.6 μM (except C, 3.8 μM) in 20 mM Tris pH 7.4, 150 mM NaCl, 0.1 mM DTT, 0.1% DDM. See materials and methods for details.

Having shown that the flavin thermolability and ring exposure differs in ETF:QO-p.Pro389Leu, we then tested if the increased lability of the FAD in the variant would render the protein more prone to proteolysis. To test this, we monitored the progression of trypsin-mediated proteolysis, considering that destabilized conformations will have more accessible cleavage sites for digestion, as a result of an increased flexibility of the polypeptide chain, and will undergo faster proteolysis [34]. The digestion was carried out incubating for 5 h wild type or Pro389Leu variant with trypsin, at 37 °C. Control experiments in absence of trypsin were also performed. Fig. 4, a representative SDS-PAGE analysis of the trypsin-mediated digestion, shows a decrease in protein amount during the 5 h- incubation in the presence of trypsin for the ETF:QO-Pro389Leu variant.

Indeed, these results indicate a higher propensity for degradation of the protein harboring the clinical variant, thus supporting our hypothesis. Interestingly, during heterologous protein expression we have observed that the ETF:QO-p.Pro389Leu variant is slightly less expressed in comparison to wild type protein (not shown). Assessment of these early folding events suggests that the mutation may have an impact on the efficiency of protein folding, making it rather susceptible to be misfolded and degraded. These two observations, trypsin digestion and protein expression, permit us to infer that ETF:QO-p.Pro389Leu presumably has a higher propensity for *in vivo* degradation.

### 3.5. Functional deficiency of the RR-MADD associated mutant

The Pro389Leu mutation is located in the UQ-binding domain, and Cornelius et al. [35] have observed, measuring tryptophan fluorescence quenching by CoQ10 pseudo-substrate (Q10Br), a weaker binding of the coenzyme Q10 cofactor. In our study, we have already showed that ETF:QO-p.Pro389Leu variant had a higher rate of FAD release and a decreased thermal stability following FAD fluorescence emission

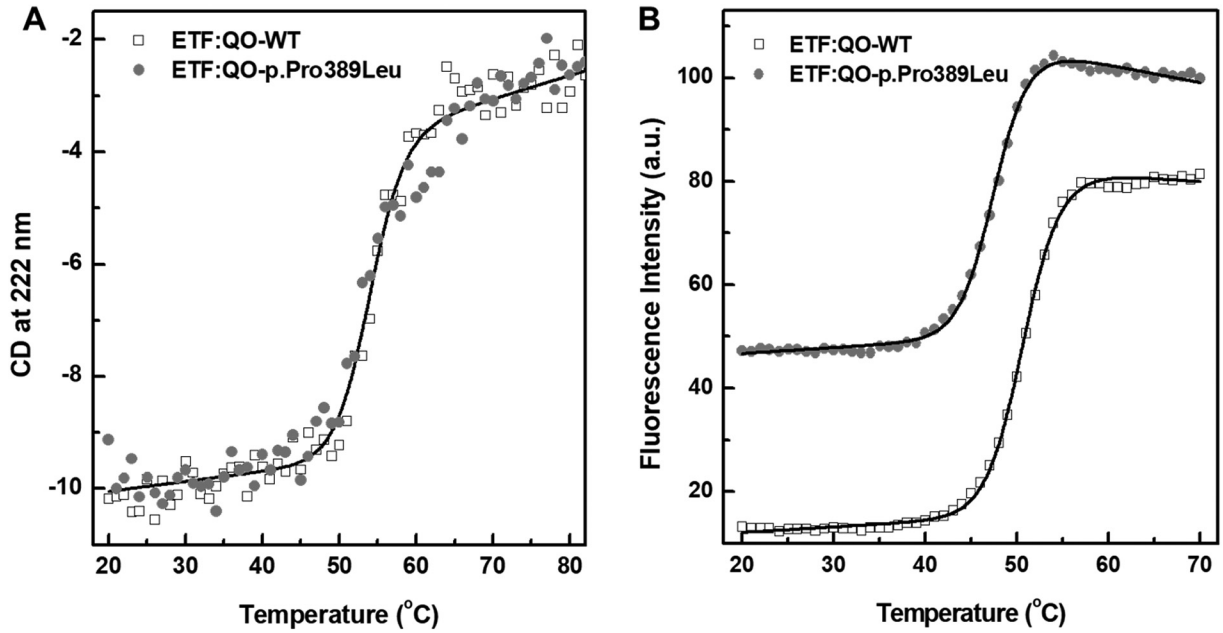


**Fig. 2.** Thermal denaturation profiles of ETF:QO-WT and ETF:QO-p.Pro389Leu. Representative thermal denaturation curve of ETF:QO-WT (open squares) and ETF:QO-Pro389Leu (filled circles) followed by far-UV CD (A) and FAD fluorescence emission (B). The solid lines represent a fit to a two-state model profile from which the apparent melting temperature ($T_m^{app}$) was determined ($n = 3$). In A, a single representative fit is shown, for simplicity. See Table 1 for details.

**Table 1**
Thermal stability of wild type and mutant *Rs* ETF:QO.

| ETF:QO | $T_m^{app}$ (°C) | | |
|---|---|---|---|
| | Tertiary structure (Trp emission) | Flavin cofactor (FAD emission) | Secondary structure (far-UV CD) |
| Wild type | 49.3 ± 1.5 | 50.0 ± 1.0 | 51.8 ± 1.7 |
| Pro389Leu | 47.7 ± 1.5 | 46.3 ± 1.5 | 51.3 ± 2.1 |

The results are the mean ± S.D. values of 3 independent experiments

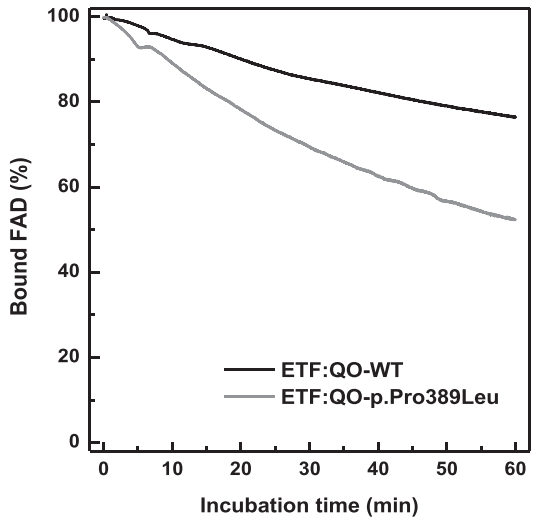


**Fig. 3.** Kinetics of flavin release. ETF:QO-p.Pro389Leu (—) and ETF:QO-WT (···) were incubated at 39 °C for 1 h, and flavin release was monitored as a function of time, following the increase in fluorescence emission at 530 nm, upon excitation at 450 nm, due to FAD release. See text for details.

indicating that the FAD binding is also perturbed in this variant. Since the ETF:QO enzyme requires three redox active cofactors properly bound for correct function we expect that this mutation will cause

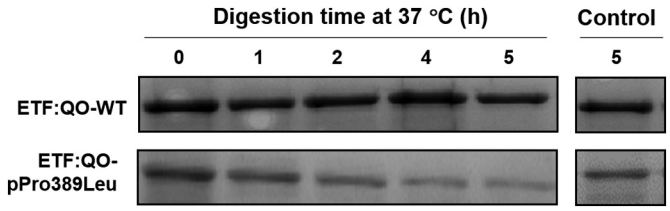


**Fig. 4.** Effects of Pro389Leu mutation in ETF:QO proteolytic susceptibility. Representative SDS-PAGE of a time course of trypsin digestion at 37 °C. The profile of wild type ETF:QO is included for comparison. Control represents protein, variant and WT respectively, treated in the same conditions in absence of trypsin.

enzyme deficiency. To determine exactly the effect of the mutation on ETF:QO activity we here employed a coupled enzymatic assay that uses the full chain of physiological electron donors/acceptors. The ETF:QO activity is determined from the efficiency of reduction of coenzyme Q1, through oxidation of octanoyl-CoA mediated by MCAD and ETF: octanoyl-CoA → MCAD → ETF → ETF:QO → Q1. Using this sophisticated assay, which was only made possible by our expertise in simultaneously producing highly purified and homogenous preparations of three proteins from the fatty acid oxidation (FAO) pathway, we determined the specific activity of the purified ETF:QO-p.Pro389Leu variant to be 4745 ± 537 $U.mg^{-1}$ ($n = 4$), which is around ~70% of wild type protein 6602 ± 681 $U.mg^{-1}$ ($n = 5$).

The lower activity can be framed in the context of the ETF:QO structure. The Pro389Leu mutation, although located in the UQ-domain, is part of a loop connecting the FAD and membrane domains (Fig. 5).

The fact that we are dealing with an homology model impairs a detailed structural analysis; however, given the known characteristics of prolines that introduce sharp kinks in protein structure, its replacement will certainly impact on the organization of the loop connecting one helix from the FAD domain to another one from the UQ domain. This local disorganization is certainly changing the dynamics of that region of the protein, which is also likely influencing the FAD and UQ moieties. It is well established that even discrete changes in the H-binding network around flavin cofactors induce changes in protein-

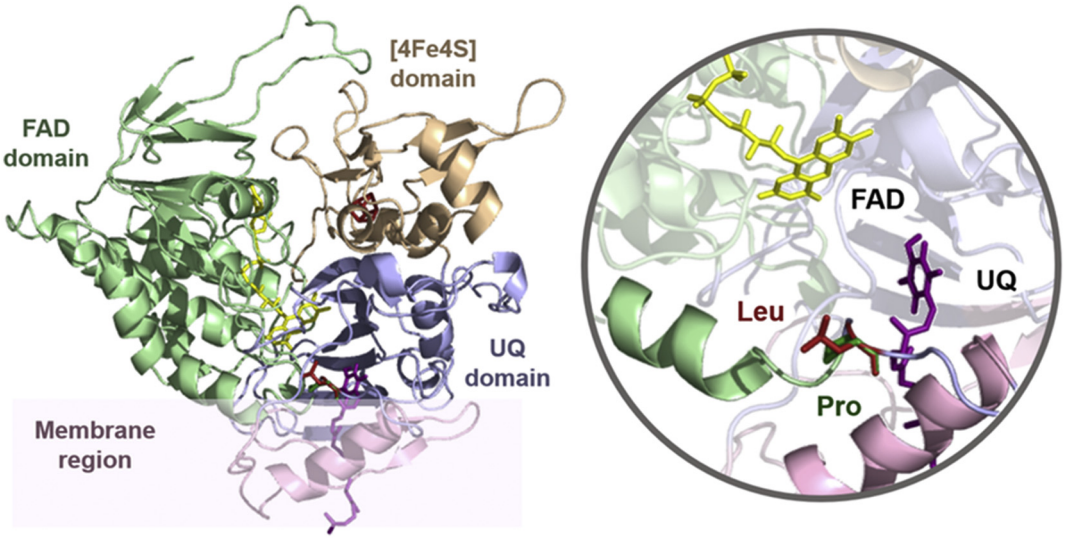


**Fig. 5.** Structural model of human ETF:QO highlighting the position of Pro456Leu in respect to FAD and UQ cofactors. The different structural domains are color coded and the putative membrane-binding region is also represented. The zoomed area highlights the position under analysis. Prepared using PyMol.

cofactor interactions [31,36], compatible to the ones we report leading to increased kinetic instability and thermolability of the cofactor. It is also conceivable that such changes have an impact on electron transfer due to changes in cofactor distances, as well as in the redox properties of the FAD, with a direct impact on the catalytic activity. Indeed, increased kinetic instability of FAD may be the underlying cause for riboflavin responsiveness in patients with this mutation: riboflavin supplementation will increase FAD levels in cells [37], disfavoring FAD lability from the ETF:QO-p.Pro456Leu variant, and contributing to activity above a critical disease-causing threshold.

## 4. Discussion

More than 160 missense mutations, distributed all over the protein structure, have been identified on ETF:QO protein, and for several the effect on protein expression and enzyme function has been described. Nevertheless, molecular studies to clarify effects on protein structure and stability are scarce. Therefore, clinicians have difficulty predicting disease progression and riboflavin response when a new mutation is identified. To elucidate the molecular aspects underlying MADD and riboflavin supplementation, we have here used as model a bacterial ETF:QO-p.Pro389Leu variant, highly homologous to its human counterpart, and proceeded with an investigation of the effect of this missense mutation on enzyme function, structure and overall fold. Our detailed analysis showed that the mutation introduces some plasticity on the tertiary structure of FAD binding region although does not negatively impact the overall protein fold. In agreement with this observation, this variant presented a decreased stability regarding thermal denaturation following FAD fluorescence emission, indicating that the mutation perturbs the FAD moiety. Furthermore, during mild thermal stress (1 h at 39 °C), the rate of flavin release is higher for the RR-MADD associated variant, denoting increased instability and thermolability. Thermal stress has been considered as an important modulator of disease in mitochondrial β-oxidation disorders, and point mutations that affect protein stability or activity are particularly susceptible to this effect [5,38–40]. This is the case in the studied variant, for which our *in vitro* results fully agree with the likely *in vivo* situation, for which disease symptoms are associated to stress episodes. Indeed, increased cellular temperature (such as fever), genetic factors or physiologic stressors like pregnancy, restricted diet and viral infections have been known to decreased cellular FAD content [16,41,42], and may push ETF:QO variants, like the Pro389Leu, toward a non-productive folding pathway developing a clinical phenotype. Moreover, our results revealed that although this mutation is in the UQ-domain, it also affects the FAD domain possibly due to short range effects due to the proximity of the different domains and respective cofactors. The three different domains cannot be isolated structurally, and due to their importance for enzyme function, they must interact in perfect conditions. The ETF:QO-p.Pro389Leu variant presented 70% of wild type activity in agreement with our investigated conformational impairment. A possible mechanistic insight for the mild disease phenotype may arise, as previously suggested, from a weaker ubiquinone binding as suggested by others [35] to which we add compromised FAD interactions. This provides a molecular rational for riboflavin responsiveness, since during flavin oral supplementation, cellular FAD levels increase, promoting ligand-induced stabilization effects, as reviewed elsewhere [43–45].

## Declaration of Competing Interest

Tânia Lucas, Bárbara Henriques and Cláudio Gomes declare that they have no conflict of interest.

## Acknowledgements

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