Molecular mechanisms of riboflavin responsiveness in patients with ETF-QO variations and multiple acyl-CoA dehydrogenation deficiency

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Riboflavin-responsive forms of multiple acyl-CoA dehydrogenation deficiency (RR-MADD) have been known for years, but with presumed defects in the formation of the flavin adenine dinucleotide (FAD) co-factor rather than genetic defects of electron transfer flavoprotein (ETF) or electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO). It was only recently established that a number of RR-MADD patients carry genetic defects in ETF-QO and that the well-documented clinical efficacy of riboflavin treatment may be based on a chaperone effect that can compensate for inherited folding defects of ETF-QO. In the present study, we investigate the molecular mechanisms and the genotype–phenotype relationships for the riboflavin responsiveness in MADD, using a human HEK-293 cell expression system. We studied the influence of riboflavin and temperature on the steady-state level and the activity of variant ETF-QO proteins identified in patients with RR-MADD, or non- and partially responsive MADD. Our results showed that variant ETF-QO proteins associated with non- and partially responsive MADD caused severe misfolding of ETF-QO variant proteins when cultured in media with supplemented concentrations of riboflavin. In contrast, variant ETF-QO proteins associated with RR-MADD caused milder folding defects when cultured at the same conditions. Decreased thermal stability of the variants showed that FAD does not completely correct the structural defects induced by the variation. This may cause leakage of electrons and increased reactive oxygen species, as reflected by increased amounts of cellular peroxide production in HEK-293 cells expressing the variant ETF-QO proteins. Finally, we found indications of prolonged association of variant ETF-QO protein with the Hsp60 chaperonin in the mitochondrial matrix, supporting indications of folding defects in the variant ETF-QO proteins.

INTRODUCTION

Multiple acyl-CoA dehydrogenation deficiency (MADD), also known as glutaric aciduria type II (GAII) (OMIM #231680), is an autosomal recessively inherited disorder. In most cases, MADD is caused by variations in one of two genes encoding electron transfer flavoprotein (ETF) or electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO). The ETF/ETF-QO proteins make up the electron transport chain in the mitochondria that links dehydrogenation reactions of 12 flavoprotein dehydrogenases to oxidative phosphorylation and thereby production of cellular energy in the inner mitochondrial membrane (1,2). The clinical features of MADD are highly heterogeneous and have been classified into lethal neonatal onset forms, often with severe congenital anomalies (severe MADD or S-MADD) and milder forms (milder MADD or M-MADD), typically with later onset and a large spectrum of symptoms ranging from hypoglycemic encephalopathy, sometimes combined with cardiomyopathy and childhood death, to patients presenting with an isolated and potentially progressive muscle myopathy as adults (2,3). A genotype–phenotype relationship has been demonstrated in

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MADD, with S-MADD patients being homozygous or compound heterozygous for functional null variations of ETF/ETF-QO, and M-MADD patients have at least one missense variation, which may potentially give rise to some enzyme activity. Thus, M-MADD shows moderately decreased fatty acid oxidation flux (14–52% of controls) in cultured M-MADD fibroblasts (3–6), whereas cultured S-MADD fibroblasts have fatty acid oxidation flux of 3–12% of controls (3,5). There is no known treatment for S-MADD patients. In contrast, early dietary treatment (protein and fatty acid restriction) combined with various diet supplements like riboflavin, carnitine and/or co-enzyme Q10 has been tried with varying success in patients with M-MADD (6–13). A sub-group of M-MADD patients show a significant response to treatment with riboflavin, resulting in near-normalized biochemical and clinical parameters after high doses (100–400 mg/day) of oral riboflavin supplementation. Such patients are referred to as riboflavin-responsive MADD (RR-MADD) patients (14,15). Fibroblasts from RR-MADD patients show fatty acid oxidation flux of 32–129% of controls when cultured in standard riboflavin-supplemented media, and disease prognosis is good when the patients are diagnosed and treated early (8,13). Therefore, it seems that S-MADD can be categorized as riboflavin-non-responsive, whereas M-MADD is either partially responsive or responsive, the latter with the label RR-MADD. To substantiate this notion, there is a clinical and scientific interest in elucidating the molecular mechanisms of the response to riboflavin observed in MADD patients.

For several years, it was believed that the phenotype of RR-MADD patients was due to a defect in the formation of the co-factor flavin adenine dinucleotide (FAD) from riboflavin (vitamin B2) (9,14,16,17). Gene defects of riboflavin metabolism or transport were only recently identified in a few RR-MADD patients (18–21). Rather, most RR-MADD patients reported to date have variations in the ETFDH gene encoding ETF-QO. This was revealed in 2007, when we and others reported ETFDH variations in 16 unrelated RR-MADD patients (8,13). Since then an increasing number of reports of ETFDH variations in RR-MADD have been published (7,22–28).

ETF-QO, which is a 64 kDa monomeric protein, is synthesized as a 67 kDa precursor protein. It is processed to the mature form in the course of translocation into the mitochondria (29). The mature form of ETF-QO contains one FAD cluster and one iron–sulfur ([4Fe4S]2+ ·1) cluster (2). Electrons derived from the various dehydrogenation reactions are transferred from ETF to ETF-QO at the iron–sulfur cluster and subsequently to the FAD co-factor. FAD is the electron donor to ubiquinone (UQ) (Q10) in the inner mitochondrial membrane (30–32). The ETF-QO protein has a three-domain structure, with the FAD-binding domain and the iron–sulfur cluster domain facing the mitochondrial matrix and the UQ-binding domain anchoring the protein to the inner mitochondrial membrane (31).

The mutational spectrum of RR-MADD is large with more than 50 different ETFDH variations reported to date (7,8,13,22–28). All published RR-MADD patients have at least one missense variation. Some of these missense variations (p.Ser82Pro, p.Ala84Thr, p.Tyr257Cys, p.Leu377Pro, p.Leu409Phe, p.Val451Leu, p.Pro456Leu and p.Pro483Leu) have repeatedly been identified in RR-MADD patients (8,13,22,24), suggesting that the nature of the variations is responsible for riboflavin responsiveness. It is well known that missense variations often lead to misfolding of the variant protein with consequently increased proteolytic degradation and decreased stability (33). FAD binding has been demonstrated to promote chaperone-mediated folding, oligomerization and/or conformational stabilization of a number of flavoenzymes (34–38), and FAD deficiency in rat liver mitochondria results in the instability and rapid degradation of ETF and a number of acyl-CoA dehydrogenases (39). In this context, it could be speculated that increasing the availability of riboflavin, which is converted to the FAD, promotes folding and/or stabilizes the native conformation of certain types of ETF-QO variant proteins. Such a mechanism of action was recently indicated for the p.Asp128Asn ETF-β variant protein, where the presence of FAD improved conformational and proteolytic stability as well as enzyme activity (40).

In the present study, we aim to test this hypothesis by using two different expression systems to study the in vitro influence of riboflavin and temperature on the folding/stability and catalytic activities of selected variant ETF-QO proteins. We relate our experimental results to structural data and knowledge about the influence of the variant proteins on clinical severity and riboflavin responsiveness in vivo to propose a model for FAD-assisted folding of ETF-QO in mitochondria.

RESULTS

The effect of riboflavin on the steady-state level and the activity of variant ETF-QO proteins

Missense variations associated with RR-MADD have been assigned to the FAD-binding domain or the UQ-binding domain of ETF-QO, although none of the affected amino acids makes direct contact with the FAD co-factor (41). ETF-QO variant proteins with amino acid changes located in the FAD-binding domain (c.1285G>C/p.Pro456Leu, c.1448C>T/p.Pro483Leu) (13) were selected for the current study and the underlying molecular mechanisms of riboflavin responsiveness were studied in relation to one ETF-QO variant protein (c.413T>G/p.Leu38Arg) identified in patients with M-MADD (6) and two variant ETF-QO proteins (c.1001T>C/p.Leu334Pro and c.1414G>A/p.Gly472Arg) identified in S-MADD patients (3,42).

To test, in a cellular system, whether the steady-state level and the activity of the selected variant ETF-QO proteins respond to variations in cellular riboflavin concentrations and correlate to phenotype, we expressed human wild-type ETF-QO and the six different variant ETF-QO proteins in HEK-293 cells cultured in media containing 16, 28.5 or 530 nmol/l of riboflavin (see Materials and Methods).

ETF-QO activity was determined on extracted membrane fractions, using an assay which measures the integrity of both redox prosthetic groups ([4Fe4S]+2 ·1 and FAD), to be able to measure the whole electron pathway (Fig. 1A). The ETF-QO protein amount was detected by western blot analysis of whole cell lysates (Fig. 1B). The identity of the mature

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64 kDa gel band as human ETF-QO was confirmed by mass spectrometry analysis (see Materials and Methods).

All ETFDH variations tested compromise the ETF-QO enzyme amount and activity to various extent and show different dependency on riboflavin availability. At supplemented riboflavin concentrations (530 nmol/l), at which wild-type ETF-QO is expected to be fully flavinylated with 100% activity, the activities of variant ETF-QO proteins associated with an RR-MADD phenotype (p.Pro456Leu, p.Pro483Leu and p.Gly429Arg) were 70–80% of those of wild-type. When riboflavin was moderately (28.5 nmol/l) or severely (16 nmol/l) depleted, activities of these variant ETF-QO proteins were decreased to ~40 and 30%, respectively, of those of fully flavinylated and active wild-type protein (Fig. 1A). In contrast to this, activities of variant ETF-QO proteins, identified in patients with S-MADD (p.Gly472Arg and p.Leu334Pro), did not respond significantly to changes in riboflavin concentrations, and they reached a maximum enzyme activity of only ~5–20% of that of wild-type when cultured at supplemented riboflavin concentrations (Fig. 1A). Activities of the variant p.Leu138Arg protein (M-MADD) showed a slight but significant ($P < 0.05$) response to changes in riboflavin concentrations, but surprisingly enzyme activity could be rescued to only 15% of that of wild-type levels at

Figure 1. Expression of wild-type and variant ETF-QO proteins in transient transfected HEK-293 cells cultured at 37 °C at supplemented (530 nmol/l), moderate deficient (28.5 nmol/l) or severe deficient (16 nmol/l) concentrations of riboflavin. (A) The activity of wild-type and variant ETF-QO proteins was measured as described in Materials and Methods. The values are expressed relative to wild-type cultivated in the presence of 530 nmol/l riboflavin. (B) Steady-state levels of wild-type and variant ETF-QO proteins were analyzed by western blot on SDS–PAGE gels immunostained with ETF-QO and VDAC antibodies. The amount of ETF-QO protein was quantified relative to VDAC, using the Vision WorksLS Image Acquisition software. All values are expressed relative to wild-type cultured in the presence of 530 nmol/l riboflavin. Bars in (A) and (B) represent mean values of three independent experiments ± SEM. A filled triangle represents all samples, $P < 0.05$; two filled triangles represent samples between 530 and 16 nmol/l, $P < 0.05$; three filled triangles represent samples between 530 and 28.5 nmol/l and between 530 and 16 nmol/l, $P < 0.05$. 
supplemented riboflavin concentrations. Wild-type ETF-QO activity was also affected by riboflavin depletion, but to a lesser extent than the variant ETF-QO proteins.

Four ETF-QO variant proteins (p.Pro483Leu, p.Gly429Arg, p.Leu138Arg and p.Leu334Pro) showed decreased steady-state protein levels with proportional reduced enzyme activities when compared with wild-type, indicating that the variants mainly cause decreased proteolytic stability with only minor or no alteration of specific enzyme activity (Fig. 1A and B). In contrast, the p.Pro456Leu and p.Gly472Arg variant proteins showed disproportionately less residual enzyme activity (30 and 70%, respectively) than the protein amount (60 and 90%, respectively) when compared with that of wild-type protein at supplemented riboflavin concentrations. This indicates that high riboflavin concentration increases the proteolytic stability of p.Pro456Leu and p.Gly472Arg variant proteins. Despite this increase, none of the two variant proteins reach full activity.

Riboflavin dependency during heat stress

Since proteins unfold at elevated temperatures, the proteolytic stability and enzymatic function of variant proteins with impaired folding or stability properties are often more compromised at elevated temperatures in vitro with consequent increased risk of metabolic decompensation during fever in vivo (33). Patients with milder forms of MADD (M-MADD and RR-MADD) often get symptoms in connection with feverish infection (3,13). Therefore, we investigated the relative steady-state level of wild-type and variant ETF-QO proteins in HEK-293 cells at an increased temperature (40°C), and at the three different levels of riboflavin. As shown in Figure 2, the relative amount of all variant ETF-QO proteins were clearly affected by heat stress. For most ETF-QO variant proteins, heat stress at 40°C seems to cause too severe folding/stability defects to allow any significant modulation of the protein amount by changes in the riboflavin levels. Only p.Pro456Leu and p.Gly429Arg showed significant responses to riboflavin during heat stress. However, they could be rescued to only ~50 and ~35% of that of wild-type levels, respectively. In contrast, at 37°C, the same two variant proteins could be rescued to 90 and 70% of wild-type levels, respectively, when cultured at supplemented riboflavin concentrations (Figs 1C and 2). Since the increased temperature (40°C) was so detrimental to the protein, the activity was not relevant to measure under these conditions.

Overall, our findings that the six ETFDH variations result in decreased steady-state level of the ETF-QO protein amount to various extents in response to riboflavin depletion and elevated temperature show that the variations mainly affect protein biogenesis and are consistent with the hypothesis that FAD co-factors can act as chemical chaperones, resulting in increased stability and/or reduced turnover of ETF-QO proteins. The intrinsic capabilities of the variant proteins to fold and reach a stable and active conformation determine the extent to which increased riboflavin (FAD) levels and decreased temperature can increase the protein amount and rescue the enzyme function. Variant p.Pro456Leu, p.Pro483Leu and p.Gly429Arg ETF-QO proteins show clearly a milder impact on overall protein folding/stability than the p.Leu138Arg, p.Leu334Pro and p.Gly472Arg ETF-QO proteins.

Thermal stability of mature ETF-QO proteins

For nuclear encoded mitochondrial membrane proteins, like ETF-QO proteins, loss of steady-state protein amount and activity can occur during ETFDH cDNA expression/translation, mitochondrial import and maturation to an active membrane-bound enzyme, or as a consequence of a destabilized native enzyme once it is formed. To gain further insights into the mechanisms by which ETFDH variations cause protein dysfunction and RR-MADD, the catalytic stabilities of extracted membrane fractions of p.Pro456Leu, p.Pro483Leu and p.Gly429Arg variant ETF-QO proteins, cultured at 37°C and at supplemented riboflavin concentrations (530 nmol/l), were determined and compared with that of wild-type ETF-QO by monitoring the loss of activity at 42°C as a function of time.
Initial experiments were carried out at 40°C. However, the activities of variant proteins were unchanged at this temperature (results not shown). As no new ETF-QO protein is synthesized during the experiment, the experiment allows us to evaluate whether the variations affect overall conformational stability and activity once the native monomer has been formed in the membrane. As seen in Figure 3, wild-type ETF-QO, which is expected to be fully flavinylated at supplemented riboflavin concentrations, was very stable for at least 2 h at 42°C. In contrast, the activities of the variant proteins were severely affected at these conditions. As soon as after 20 min of incubation, the p.Pro456Leu variant protein was reduced to ≏50% of its initial activity. The p.Pro483Leu and p.Gly429Arg variant proteins had lost 50% of their initial activities after about 60 min. The protein concentrations in the extracts were ≏1 mg/ml, so the differences observed in heat stability do not reflect altered stability due to differences in protein concentrations. These data show that for the proportion of the variant proteins, which acquires the native membrane-bound conformation, which is more temperature sensitive, the catalytic stability is compromised when compared with that of wild-type. Thus, although FAD allows the variant proteins to fold and reach their mature membrane forms, FAD cannot correct native structure. Western blot analysis revealed that wild-type and variant proteins were stable during incubation at 42°C for 2 h (data not shown). This does not exclude that increased degradation of variant proteins does take place in vivo as most proteases most likely are inactive in the extracted membrane fractions.

H₂O₂ production in HEK-293 cells expressing variant ETF-QO proteins

Conformational changes in the final structures may allow an increased flow of electrons from the variant ETF-QO enzymes to oxygen and thereby give rise to reactive oxygen species (ROS), such as superoxide. Superoxide produced from mitochondrial oxygen is rapidly converted to H₂O₂ by mitochondrial superoxide dismutase (mnSOD or SOD2). The peroxide crosses the mitochondrial membranes and moves to the cytosol (43). To investigate whether misfolded ETF-QO proteins may leak electrons and thus give rise to increased cellular peroxide production, HEK-293 cells expressing the p.Pro456Leu variant were cultured at 37°C in supplemented (530 nmol/l) or in severely depleted (16 nmol/l) riboflavin media and treated with the peroxide-sensitive fluorescent probe CM-H₂DCFDA, followed by quantification of fluorescence signals by flow cytometry. For comparison, H₂O₂ production was analyzed also in HEK-293 cells expressing a severe stability variant (p.Leu138Arg) and in HEK-293 cells expressing a relatively stable variant, but with severely decreased activity (p.Gly472Arg). When cultured in supplemented riboflavin medium, HEK-293 cells expressing p.Pro456Leu protein had increased peroxide production when compared with HEK-293 cells expressing wild-type ETF-QO. Peroxide production in HEK-293 cells expressing p.Leu138Arg or p.Gly472Arg was less pronounced than in p.Pro456Leu, relative to HEK-293 cells expressing wild-type ETF-QO (Fig. 4). At these conditions, the steady-state amount and the activity of the variant proteins relative to those of wild-type protein are expected to be 90 and 70%, respectively, for p.Pro456Leu, 15 and 10%, respectively, for p.Leu138Arg and 60 and 30%, respectively, for p.Gly472Arg (Fig. 1A and C). The capacity of the same cells to produce H₂O₂ did not change significantly when the cells were cultured in depleted riboflavin medium (Fig. 4), even though the steady-state amount of active variant proteins are expected to be significantly affected during riboflavin deficiency, where the amount and the activity of the variant proteins relative to those of wild-type protein are 30 and 30%, respectively, for p.Pro456Leu, 10 and 0%, respectively, for p.Leu138Arg and 20 and 20%, respectively, for p.Gly472Arg (Fig. 1A and C). This most likely reflects that the capacity of the HEK-293 cells to produce H₂O₂ is saturated with the low amount of expressed variant ETF-QO proteins produced during riboflavin deficiency. H₂O₂ production in HEK-293 cells transfected with wild-type ETFDH was comparable with...
that of HEK-293 cells transfected with vector alone, reflecting that the production of variant ETF-QO proteins and not transfection is responsible for the increased ROS production (data not shown). The results clearly show an increase in the ROS level when HEK-293 cells were transfected with ETF-QO folding variant proteins compared with ETF-QO activity variant proteins and wild-type protein.

**Mitochondrial import and maturation of the p.Pro456Leu variant protein**

Expression analysis of the ETF-QO variant protein in HEK-293 cells demonstrated that RR-MADD variant ETF-QO proteins have decreased thermal stability when located in the inner mitochondrial membrane. To further elucidate the molecular mechanisms by which RR-MADD ETFDH gene variations cause decreased steady-state level of the ETF-QO enzyme, it was of interest to determine whether the intra-mitochondrial folding of the variant proteins is also affected. An in vitro mitochondrial system allowed the characterization of the import pathway as well as the investigation of possible chaperone interactions during import. The import experiments were performed in mitochondria from rats fed a normal diet.

As a proof of principle, we investigated the import of the in vitro-translated ETF-QO variant protein, p.Pro456Leu, into isolated rat mitochondria for up to 180 min at 26 and 37°C. The import pathway of ETF-QO has not yet been characterized. Therefore, we included an analysis of the mitochondrial matrix to investigate whether the ETF-QO protein is imported through the matrix rather than being directly inserted into the inner mitochondrial membrane. Aliquots withdrawn at different time intervals were divided into three fractions (for details, see Materials and Methods): a membrane fraction, a matrix fraction and an insoluble fraction. All fractions were analyzed by SDS–PAGE. As seen from Figure 5A, wild-type ETF-QO protein and p.Pro456Leu variant protein behaved similarly during import at 26°C, with a continuous import into the membrane and a continual increase of the insoluble fraction over time. A difference is observed in the matrix fraction, where an increase is present in the p.Pro456Leu variant protein compared with the wild-type (after 60 min import, the matrix fraction of wild-type had increased less than 2-fold, compared with a 3-fold increase in the matrix fraction of p.Pro456Leu), which suggests that the variant protein is more difficult to fold, and thus is associated for a longer period of time with chaperones before insertion into the membrane.

When the temperature was increased to 37°C (Fig. 5B), the apparent rate of import was faster for both wild-type and p.Pro456Leu, where maximum import after 30 and 60 min, respectively, is detected, and thereafter there is a turnover of the membrane fraction. A clear difference is seen in the amount of wild-type and p.Pro456Leu proteins in the membrane fractions, with almost 25% more wild-type protein imported into the membrane after 15 min. Moreover, the p.Pro456Leu variant protein reaches the turnover point after 60 min compared with 30 min for the wild-type protein. The insoluble fraction for the wild-type and the p.Pro456Leu is similar and has a more than 4-fold increase over the import period. As also seen at 26°C, a larger fraction of protein in the matrix fraction for the variant protein compared with the wild-type protein was identified.

In order to investigate whether the ETF-QO protein found in the matrix fraction is associated with other proteins, such as chaperones, the matrix fraction of protein was loaded onto native gels (Fig. 5C). Variant matrix proteins, having difficulties with folding, are often found in association with molecular chaperones like Hsp60 (44,45). As seen in Figure 5C, the gel showed two bands of larger size (~120 and ~480 kDa) than native ETF-QO (64 kDa). The intensity of the 120 kDa band is constant over time, whereas the band of 480 kDa for the wild-type increased in intensity up to 30 min and then decreased. For the variant p.Pro456Leu protein, there is a
constant and more pronounced increase in amounts of this 480 kDa complex over time until 180 min, followed by a small decrease at 240 min. The 120 kDa band might be a dimer of ETF-QO as the electrophoresis was performed in the absence of a detergent. A band of similar size was seen when human ETF-QO proteins were overexpressed in Sf9.

Figure 5. Import and maturation of the p.Pro456Leu ETF-QO precursor into isolated rat liver mitochondria. (A) Experiments were performed at 26°C (A) and 37°C (B). The wild-type protein is imported in parallel with the variant protein. Samples are collected at four different times: 15, 30, 60 and 180 min. As described in detail in the Materials and Methods section, the samples are separated into three fractions: a membrane fraction, a matrix fraction and an insoluble fraction. The samples were analyzed by SDS–PAGE, followed by radiography. Each sample (wild-type or variant) was normalized to its respective wild-type sample taken at 30 min. Data are representative of three independent sets of experiments. (C) Native gel electrophoresis of the matrix fraction. The matrix fraction loaded on the native gel is the fraction dissolved in lysis buffer without a detergent. Samples were taken over a time period from 0 to 240 min. The gel shows a strong band with a molecular mass of ~480 kDa and a weaker band with a molecular mass of ~120 kDa. No bands corresponding to 64 kDa could be detected.

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insect cells and analyzed in their native structure, using perfluoro-octanoic acid PAGE (46). The 480 kDa band is very interesting since it is dynamic over time and the size of the band correlates with ETF-QO in complex with an Hsp60 chaperone complex. Heptameric, functional Hsp60 migrates in native PAGE as a 420 kDa complex corresponding to a heptamer, as previously documented for another misfolded protein (44). If ETF-QO forms a complex with the Hsp60 heptamer, this would give an expected band size of \( \approx 484 \) kDa, which correlated with the band we identify at \( \approx 480 \) kDa on the native gel. All together, the mitochondrial import experiments demonstrate that the variant p.Pro456Leu is a mild folding variant with increased chaperone-mediated folding and holding in the matrix. Finally, the experiments show, for the first time, that the ETF-QO protein is imported through the matrix and then inserted into the inner membrane corresponding to a heptamer (44).

**DISCUSSION**

In this paper, we demonstrate that three variant ETF-QO proteins (p.Pro456Leu, p.Pro483Leu and p.Gly429Arg) identified in RR-MADD patients respond to increasing concentrations of riboflavin in the cultivate medium when expressed in HEK-293 cells. In contrast, ETF-QO variant proteins (p.Leu334Pro, p.Gly472Arg and p.Leu138Arg) identified in patients who do not respond, or respond poorly, to riboflavin therapy *in vivo* do not respond in the cellular system. Thus, a clear genotype–phenotype relationship for the observed riboflavin responsiveness in patients with genetic defects of ETF-QO is emerging. Our data are also consistent with the hypothesis that the riboflavin response results from the ability of FAD to promote the folding of the variant proteins or otherwise stabilize their mature proteins or folding intermediates. This was inferred from (i) the negative effect of temperature on the steady-state level of ETF-QO in cells, which must process and translocate the polypeptides, incorporate two redox centers and insert the proteins into the inner mitochondrial membrane and (ii) the thermal stability of the partially purified riboflavin-responsive proteins, which suggests that these variant proteins can be stabilized by riboflavin in spite of structural differences from the wild-type protein.

The effect of riboflavin on expression of ETF-QO proteins with variations in the FAD domain, like the p.Gly429Arg, can be reasonably explained by FAD binding, which affects the kinetics and/or thermodynamics of the folding of that FAD domain, limiting the number of favorable conformations leading to a near-native protein structure, as shown by Er et al. (47). Gly429 is located in a loop between the two helices \( \alpha 6 \) and \( \alpha 7 \), but far from the active FAD-binding site (Fig. 6). In the early folding pathway, glycine residues play a critical role in loop structure by maintaining flexibility (48). Substitution to arginine with a large cationic side chain into this 14

![Figure 6. Location of the investigated ETF-QO variations and structures surrounding the variations. The ribbon representation of ETF-QO is based on the crystal structure of porcine ETF-QO (31). The FAD-binding domain is in blue; the 4Fe4S cluster domain is in red; the UQ-binding domain is in dark green; and the membrane spanning part of the UQ-binding domain is in light green. The three redox centers are shown as stick models: FAD in orange, 4Fe4S in pink and UQ in purple. The mutated amino acids investigated in the present study are shown in yellow space filling. Magnifications of the ETF-QO structural regions in which the variations are located are shown in gray overlaying the variation (in red) and the original residues (in green). Structures were prepared using PyMOL.](image-url)
amino acid loop that emerges from the surface of ETF-QO is an obstacle to maintaining flexibility and likely presents barrier, which may be partially overcome by binding FAD to an intermediate in the folding pathway, decreasing the ensemble of pathways open to the final structure. The other two RR-MADD amino acid variations investigated here, and several identified by others (8,13,22), are not located in the flavin domain, but are located in the UQ-binding domain (Fig. 6). The FAD and UQ structural domains are not isolated in the primary structure of ETF-QO, although the functional domains are closely packed and share structural elements. The interspersed, non-contiguous elements of the FAD and UQ domains in the primary structure (see Fig. 6 and reference 31) suggest that a variation in a UQ functional domain may well be influenced by FAD interaction with the folding of the protein in the flavin domain. The p.Pro456Leu amino acid variation is located in the UQ domain in a loop between helix α8 in the FAD domain and α9 in the membrane domain of the tertiary structure (31). Loop and turn formation is critical early in the folding pathway because these elements permit sampling of more energetically favorable structures that lead to chain compaction (48). Proline imparts rigidity to a polypeptide and therefore, the entropy of the unfolded protein would increase in the p.Pro456Leu and p.Pro483Leu variants by substitution of a leucine residue. p.Pro483Leu, which is located four residues to the C terminus of the membrane-associated region of the protein in the middle of a rather structure-less region (Fig. 6) so that p.Pro483 would also limit the number of conformations of this region as the protein folds (31). FAD binding to folding intermediates could counteract the resulting increase in entropy and increase the ensemble of unfolded structures. Previous work by Wittung-Stafshede (38) on the in vitro unfolding and refolding of desulfovibrio desulfuricans flavodoxin, a small flavoprotein, showed that the flavin co-factor, FMN, can bind to the unfolded protein and accelerate its folding. It is conceivable that an increase in the concentration of intra-mitochondrial FAD in a similar way may affect both the kinetics and thermodynamics of the folding of the variant p.Pro456Leu and p.Pro483Leu ETF-QO proteins. Another indication of early defective folding associated with these ETF-QO variants was the result from native gel analyses of in vitro-translated and mitochondria-imported ETF-QO proteins (Fig. 5C), where we found indications of prolonged association of the p.Pro456Leu variant protein with the Hsp60 chaperonin system in the mitochondrial matrix compared with wild-type protein. Although FAD can assist in overcoming misfolding and allow the RR-MADD variant ETF-QO proteins to be inserted into the inner mitochondrial membrane, it does not correct the mutation-induced structural defects as reflected by the decreased thermal catalytic stability of the partially purified variant proteins (Fig. 3) along with the increased tendency of the p.Pro456Leu variant to produce ROS when expressed at supplemented riboflavin concentrations (Fig. 4).

The p.Leu138Arg variant has profoundly reduced the protein amount and activity in the cell system, with a slight but significant response to increasing concentrations of riboflavin in the culture media (Fig. 1A and B). A patient, compound heterozygous for the p.Leu138Arg variation and a splice variation (IVS3 +3A>T) causing degradation of the corresponding ETFDH transcripts, showed a partial clinical response to treatment with riboflavin and a remarkably mild fatty acid oxidation flux in fibroblasts (24–52% of those of controls) when cultured in riboflavin-supplemented media. The patient died at 14 years of age despite intensive treatment. Other patients reported with this variation have died, probably resulting from not as intensive care as the patient reported by Olsen et al. (6). Leu138 is buried in the structure of the protein between the α2 helix in the flavin domain and the β3 sheet of the UQ domain (Fig. 6). Substitution of the cationic arginine residue in the p.Leu138Arg variant would be energetically unfavorable and likely too structurally distorted to be overcome by riboflavin.

The p.Leu334Pro and p.Gly472Arg variants have been reported in the homozygous state in S-MADD patients with neonatal death and very impaired the ETF-QO enzyme function in fibroblasts cultured in riboflavin-supplemented media (3,42,49,50). Expression studies showed that the p.Leu334Pro and the p.Gly472Arg variant ETF-QO proteins profoundly reduced the protein amount and activity, with no significant response to increasing concentrations of riboflavin in the culture media (Fig. 1A and C). The p.Leu334Pro variant is located at the N + 1 position relative to the α5 helix which is located in the UQ domain (Fig. 6). Substitution of a proline residue could twist the helix, altering the orientation of the amino acid side chains in the helix. Gly472 is located in the helical membrane domain (α9) that forms part of the UQ entry channel and it is not unreasonable that the p.Gly472Arg variant would be unstable with less activity because the cationic arginine side chain would extend toward the lipid phase. These mutations likely cause too severe structural distortion to be stimulated by riboflavin.

In summary, all mutations investigated in this study showed significant abnormalities in molecular phenotype ranging from mild to severe and correlated with clinical phenotype. Decreased proteolytic stability seems to be their major molecular mechanism of action. All variant proteins studied were affected by increased temperature, but only milder variants responded to changes in riboflavin concentrations and only those associated with RR-MADD could be rescued to significant levels when expressed at supplemented concentrations of riboflavin. The results from experimental analyses and presumed structural effects of the variants are consistent with the hypothesis that the riboflavin response results from the ability of FAD to act as a chemical chaperone that can promote folding of secondary elements already early in the folding pathway of ETF-QO or at later steps by stabilizing folding intermediates or mature membrane-inserted proteins. The ability of riboflavin and temperature to modulate the molecular and clinical phenotypes depends on the inherited folding properties of the variant ETF-QO proteins as illustrated in our proposed model for the mitochondrial folding pathway of ETF-QO variants (Fig. 7). According to this model, increased cellular temperature (such as fever) or genetic factors or physiologic stressors like pregnancy, restricted diet and viral infections that are known to decrease cellular FAD content (51–53) may push milder variant ETF-QO proteins, like the p.Gly429Arg, p.Pro456Leu and p.Pro483Leu proteins or even wild-type ETF-QO, toward a non-productive
folding pathway (red arrows), giving rise to significant accumulation of MADD metabolites and clinical disease.

These gene–environmental interactions present a challenge for the diagnostic evaluation of RR-MADD patients, and compiled knowledge gained by documentation of riboflavin responsiveness in relation to the clinical and biochemical course and precise knowledge on the pathogenic nature of the specific genotypes are needed to further improve our understanding of genotype–phenotype relations, and gene–environmental interaction in MADD.
**MATERIALS AND METHODS**

**Plasmid constructs**

The region from position −29 to +40 in the 3′UTR of a human wild-type *ETFDH* cDNA was cloned into the Srf I cloning site of the pCR-Script™ Amp SK(+) expression vector (Stratagene), using the pCR-Script™ Amp SK(+) Cloning Kit (Stratagene). The c.1367C>T/p.Pro456Leu, c.1448C>T/p.Pro483Leu, c.1285G>C/p.Gly429Arg, c.413T>G/p.Leu138Arg, c.1414G>A/p.Gly472Arg and c.1001T>C/p.Leu334Pro variations were each introduced into the pPCR-Script-ETFDH wild-type plasmid by use of the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The sequences of the mutagenic insertions were confirmed by DNA sequencing and subcloned into the HindIII and Not I cloning sites of the pcDNA3.1(+)-expression vector (Invitrogen). Correct orientation and sequences of the final expression vectors were confirmed by DNA sequencing. All references to nucleotides or amino acids in the text are based on the cDNA sequence of human *ETFDH* (NM_004453). The initiating ATG codon is numbered as bp 1_3, and the initiator methionine is numbered as amino acid 1.

**Cell culture**

Unless otherwise stated, human embryonic kidney (HEK-293) cells (cat. no. CRL-1573, American Type Culture Collection) were maintained in RPMI-1640 medium (Lonza) with 10% fetal bovine serum (Sigma-Aldrich), 0.06 mg/ml penicillin (FarmaPlus), 0.1 mg/ml streptomycin and 0.29 mg/ml glutamine (both Sigma-Aldrich). Cells were cultivated in tissue culture flasks or plates (TPP Techno Plastic Products AG) at 37°C with 5% (v/v) CO₂ as previously described (54). Transient transfection of cells was performed using FuGENE 6 transfection reagent (Roche Diagnostics) according to Corydon et al. (54). To test differences in riboflavin responsiveness among various variant ETF-QO proteins, transfected HEK-293 cells were cultured in RPMI-1640 media containing 16, 28.5 or 530 nmol/l of riboflavin. These riboflavin concentrations were established in a pilot study where changes in the steady-state amount of expressed wild-type and variant p.Pro456Leu ETF-QO protein were measured by western blot analysis in response to changes in riboflavin concentrations of culture media (data not shown): 16 nmol/l (severe deficient media) is the riboflavin concentration, at which the amount of both variant and wild-type ETF-QO protein is profoundly decreased and to a similar level; 28.5 nmol/l (moderate deficient media) is the riboflavin concentration, at which the amount of p.Pro456Leu variant ETF-QO protein is significantly more decreased than that of wild-type ETF-QO; 530 nmol/l (supplemented) is the riboflavin concentration in standard RPMI-1640 media, at which no significant changes in the wild-type and variant ETF-QO protein amounts are observed. Deficient media were made by adding the appropriate amount of riboflavin (Sigma) to riboflavin-depleted RPMI-1640 media (custom-made by PromoCell) supplemented with 10% dialyzed fetal calf serum (Sigma-Aldrich). For the experiments conducted at 37°C, the cells were cultivated for 6 days in media with the different concentrations of riboflavin before harvest. For the experiments performed at 40°C, the cells were initially cultivated at 37°C. Twelve hours after transfection, the cells were transferred to 40°C and maintained at this temperature until harvest.

**Western blot analysis**

Protein was extracted in a lysis buffer [50 mM Tris–HCl, pH 7.8, 5 mM EDTA, pH 8.0, 1 mM dithiothreitol (DTT), 10 µg/ml aprotinin (Sigma-Aldrich), 1 mg/ml trypsin inhibitor (Bie & Berntsen), one tablet of protease inhibitors (Roche) in 10 ml and 1% Triton X-100] according to Schmidt et al. (55). Following centrifugation, 25 µg of the lysate was analyzed by SDS–PAGE on a 12.5% Tris–HCl Criterion Gels (Bio-Rad), Western blot analysis was performed according to Schmidt et al. (55) with polyclonal porcine anti-ETFQO, a kind gift from Frank Freeman (29), and VDAC (Abcam) antibodies, followed by incubation with a secondary goat anti-rabbit-HRP antibody (Dako), using the ECL plus Western Blotting Detection System (Amersham Biosciences). Detection was done using the Chemidoc-It™ Imaging System (UVI); the intensities of bands were quantified using Vision-WorksLs Image Acquisition (UVI).

**Mass spectrometry analysis to confirm the identity of the 64 kDa gel band as human ETF-QO**

Twenty-five micrograms of proteins from HEK-293 cells, expressing wild-type ETF-QO, were extracted and separated by SDS–PAGE on a 12.5% Tris–HCl Criterion Gel (Bio-Rad) as described above. The gel was stained with Coomassie Blue, and a gel slice containing 45–75 kDa proteins was removed and de-stained. Subsequently, proteins in the gel were digested by trypsin, and the resulting peptides were extracted and purified essentially as described in Pedersen et al. (56). The peptide mixture was analyzed by nano-liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) on an Easy nLC (Proxeon, Odense, Denmark) coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA, USA) (57). For the identification of peptides and proteins, the MS data were searched against IPI human database version 3.45 (71 983 sequences, released 6/10/2008) using the MudPIT scoring algorithm in the Mascot software (Matrix Science, version 2.2.04, London, UK). Peptide mass tolerance and fragment mass tolerance were set to 0.006 as peptides originating from human ETF-QO protein (UniProt #Q16134). The eleven peptides covered 25% of the protein sequence.

**ETF-QO enzymatic assay**

Cell pellets were suspended in 0.7 ml of 10 mM Tris–HCl, pH 7.4, and sonicated in an ice-salt bath using a Branson 450 sonifier at 30% duty and a power setting of 2. The cells were sonicated with eight cycles of five bursts and were kept on ice for 1 min between cycles. The disrupted cells were centrifuged at 100 000g for 30 min at 4°C and the sedimented membranes were suspended in 0.15 ml of 10 mM Tris–HCl, pH 7.4, containing 1% dodecyl maltoside and homogenized with 10 strokes in a glass homogenizer. The preparations were then
incubated on ice for 1 h, homogenized and centrifuged as described above. The resulting supernatant was assayed for ETF-QO activity. ETF-QO activity was assayed spectrophotometrically at 30°C in reaction mixtures containing 20 mM Hepes (K+), pH 7.4, 30 μM glutaryl-CoA, 0.5 μM glutaryl-CoA dehydrogenase, 1.5 μM ETF, 0.01% dodecyl maltoside and 100 μM UQ homolog, Q1 (46,58). Q1 reduction was monitored at 275 nm, using Δε = 7.5 mM⁻¹ cm⁻¹. Protein concentrations were determined with the Bradford reagent kit (Bio-Rad). Human glutaryl-CoA dehydrogenase and human ETF were expressed in Escherichia coli and purified as previously described (59,60). Q1 was obtained from Sigma and dodecyl maltoside was obtained from Anatrace.

The thermal stability of the ETF-QO variant proteins and the wild type protein was assayed by measuring ETF-QO enzymatic activity after incubation at an elevated temperature (42°C). Cellular membranes were extracted from cells cultured at 37°C, and at supplemented riboflavin concentrations. The solubilized proteins were incubated at 42°C. Aliquots were removed at 0, 10, 20, 30, 60 and 120 min and the activity measured as described above.

**Measurements of intra-cellular hydrogen peroxide production**

HEK-293 cells expressing wild-type or variant ETF-QO proteins were cultured in standard RPMI-1640 media at 37°C as described above. To measure H₂O₂, ~80% confluent cells were incubated for 20 min at 37°C in phosphate buffered saline (PBS) containing 10 μmol/l of the redox-sensitive dye 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Invitrogen). The cells were washed three times in PBS, harvested and resuspended in RPMI-1640 media before fluorescence-activated cell sorting (FACS) analysis. A minimum of 1 x 10⁴ detached cells were counted per sample and analyzed on a FACS Aria II flow cytometer with the FACSDiva software v.5.0.3 (Beckton-Dickinson). Propidium iodide staining was used for the exclusion of dead cells.

**In vitro rat mitochondria import assay**

*In vitro* transcription and translation of wild-type or c.1367C>T *ETFDH* cDNA inserted in pcDNA3.1(+) expression vectors (see above) were performed in the presence of [³⁵S]methionine, and the translation products were imported to isolated rat liver mitochondria as described in Pedersen et al. (44). Experiments were carried out at 26 and 37°C, and intra-mitochondrial biogenesis of ETF-QO protein was followed by withdrawing samples after 15, 30, 60 and 180 min of import. The samples were treated with trypsin to remove non-imported protein, and mitochondrial pellets were isolated as described in Pedersen et al. (44). The pellets were kept at −80°C and resuspended in 20 μl of lysis buffer [50 mmol/l Tris–HCL, pH 7.8, 5 mmol/l EDTA, 1 mmol/l DTT, 10 μg/ml aprotinin, 1 mg/ml soybean-trypsin inhibitor, 250 mmol/l sucrose]. Samples were sonicated for 20 s followed by centrifugation at 14 800g for 20 min at 4°C. The supernatant (matrix and inter-membrane space) was carefully removed and analyzed by native PAGE on 4–15% Tris–HCL Criterion gels (Bio-Rad) and by SDS–PAGE on 12.5% Tris–HCL Criterion gels (Bio-Rad). The pellet was resuspended in the lysis buffer containing 1% Triton X-100 (see western blot analysis) and centrifuged at 14 800g for 20 min at 4°C. The supernatant (membrane fraction) was analyzed by SDS–PAGE on 12.5% Tris–HCL Criterion gels (Bio-Rad). The final pellet (insoluble fraction) was resuspended in SDS sample buffer (350 mmol/l Tris–HCL, 6 mmol/l DTT, 10% SDS, 30% glycerol, 0.12% bromphenol blue) and analyzed on 12.5% Tris–HCL Criterion gels (Bio-Rad). The gels were dried on a slab gel dryer (Drygelsr, model SE1160, Hoefer Scientific Instruments, CA, USA), and radio-labeled ETF-QO proteins were visualized on an Amersham Biosciences PhosphorImager (STORM 840) and quantified using the ImageQuant™ software.

**Statistical analysis**

Indicated data are expressed as a mean ± SEM of triplicate experiments. Two-tailed Student’s *t*-test was used to compare the mean between groups.

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**Conflict of Interest statement.** None declared.

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