DEFECTIVE PROTEIN FOLDING AND FUNCTION IN METABOLIC DISORDERS

STUDIES ON THE MITOCHONDRIAL FLAVOENZYME ETF

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FOREWORD

This dissertation describes the work performed under the supervision of Prof. Cláudio M. Gomes, in the Protein Biochemistry Folding and Stability Laboratory, Instituto de Tecnologia Química e Biológica from October 2006 to June 2010.

The studies here presented aim to contribute to a better understanding of human electron transfer flavoprotein (ETF) folding and stability, towards the elucidation of the molecular rationale of multiple acyl-CoA dehydrogenase deficiency (MADD). First, ETF disease causing missense mutations and the impact of three of those mutations on the protein folding and stability is overviewed. Subsequently, the role of flavinylation on a mutant variant resulting in a mild phenotype was addressed in order to gain a better understanding on the molecular rationale for riboflavin supplementation. Further, two polymorphic ETF variants were analyzed to explore their effects on the protein folding and function and to investigate possible implication in MADD.

This thesis is organised in three parts. The first part is an introduction comprising two chapters: the first describing the state of knowledge on the protein folding problem and protein homeostasis, and the second presenting an overview on mitochondrial fatty acid β-oxidation (FAO) enzymes (particularly ETF) and on FAO associated disorders. In addition, a brief description on riboflavin as a therapeutic agent is also presented in the second chapter. The second part of the thesis is organized in three chapters describing the experimental results obtained. The third and last part consists of a general discussion integrating the described results.

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THESIS PUBLICATIONS

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3. Henriques, B. J., Olsen, R. K., Bross, P., Gomes C. M.
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Other publications not included in this thesis

5. Henriques, B. J., Saraiva, L. M., Gomes, C. M.,
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6. Henriques, B. J., Saraiva, L. M., Gomes, C. M.,
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Bioch. Bioph. Res. Comm. **2005**, *333*: 839-844

DISSERTATION ABSTRACT

The work presented in this dissertation concerns the study of the electron transfer flavoprotein (ETF), a protein involved in mitochondrial β-oxidation whose deficiency is associated to multiple acyl-CoA dehydrogenase deficiency (MADD). The thesis will focus on establishing the functional, cellular and molecular consequences of the genetic variability in ETF, and in particular it aims to clarify the basis for the effect of heat stress on disease progression. Moreover, the beneficial effects of vitamin B2 supplementation will be addressed.

MADD, which is an autosomal recessively inherited disorder of fatty acid, amino acid, and choline metabolisms, results from deficiencies in any of the following genes: *ETFA*, *ETFB* or *ETFDH*. *ETFA* and *ETFB* genes encode for the α and β subunits of ETF, whereas *ETFDH* encodes for electron transfer flavoprotein ubiquinone oxidoreductase (ETF-QO). ETF is a key enzyme in a series of mitochondrial metabolic pathways, mediating electron transfer from at least 12 dehydrogenases to the membrane-bound ETF-QO, thus funneling reducing power to the respiratory chain for subsequent ATP production. The clinical features of patients with MADD are heterogeneous and fall into 3 classes: a neonatal-onset form with congenital anomalies (type I), a neonatal-onset form without congenital anomalies (Type II), and a late-onset form (Type III). This variety of effects presumably depends on the location and nature of the intragenic lesion. Although over 55 distinct genotypes have been identified on MADD patients, with approximately one third corresponding to missense mutations in ETF, only a limited number of genotypes have been characterised. The available data is, in fact, highly suggestive of a direct correlation between the genotype and clinical

phenotype. Accordingly, while null mutations result in severe phenotypes, missense mutations in the above mentioned genes result in proteins with a lower enzymatic activity leading to cellular functional deficiency and thus milder phenotypes. In these cases, the amino acid alterations are likely to cause conformational changes in the expressed gene products which account for disease. This effect is mostly evident in late-onset MADD patients, in which the disease is often intermittent and only becomes evident during periods of illness or catabolic stress. Even so, the molecular genetic basis and functional characterisation of MADD genotypes remains elusive, thus a detailed *in vitro* investigation will contribute to a better functional understanding of the clinical heterogeneity in MADD.

An extensive *in silico* analysis was carried out on 18 disease associated missense mutations found in ETF. The analysis revealed that known mutations fall essentially in two groups: 1) mutations affecting protein folding and assembly; 2) mutations impairing catalytic activity and interactions with partner dehydrogenases. We have focused our studies on three of these mutations, ETFβ Cys42Arg, Asp128Asn and Arg191Cys, which typify different clinical phenotypes. This part of the investigation involved the study of expressed ETF variants, aiming at evaluating the impact of mutagenesis on ETF intrinsic conformational stability, enzymatic activity, protein assembly and interaction with functionally relevant enzymes.

The ETFβ-Cys42Arg mutation, a severe MADD mutation, affects directly the AMP binding site and the intersubunit contacts impairing protein folding. *In vivo* assays following the recombinant expression of the protein in an *E. coli* system have shown that the protein is expressed as insoluble aggregates. Upon co-expression with GroEL/GroES and dnaK/dnaJ/GrpE an increase in soluble protein production was noted, although no enzymatic activity was

restored, probably due to a defective insertion of the AMP and FAD cofactors.

Proteins harboring the other two mutations, ETFβ-Asp128Asn and ETFβ-Arg191Cys, which are associated to mild MADD, were purified in the soluble form after heterologous expression. We have used a combination of biophysical and biochemical methods to address the impact of these last two mutations on ETF folding, conformational stability and efficiency in mediating electron transfer. The two mutant variants had an overall α/β fold topology identical to native ETF, but both have substantially decreased enzymatic activity and conformational stability. The ETFβ-Asp128Asn mutation has a stronger impact on the protein conformational stability, as this mutation is near the dimer interface and the FAD binding site. On the other hand, the decreased activity observed for the ETFβ-Arg191Cys variant is likely to result from the impairment of interactions with the electron-donor dehydrogenases, as the mutated residue is within the predicted interface of the complex formation. Thus, combination of *in silico* analysis of mutations with experimental data has allowed to establish structural hotspots within ETF fold, which are useful to provide a rationale for the prediction of effects of mutations in ETF.

Mutations leading to mild clinical phenotypes, such as those selected in this study, are frequently associated to poor catalytic activity of the mutant variant and deficient FAD insertion. Moreover, the molecular consequences of riboflavin supplementation in the functional rescue of defective fatty acid β-oxidation flavoenzymes are becoming increasingly clear. This has been made possible with the combination of diverse studies made during the last decades, ranging from *in vitro* analysis of purified proteins, to proteomic analysis of patients and animal and cellular models. The immediate

consequence of riboflavin supplementation is an increase in the cellular availability of FAD, which is the key cofactor in many fatty acid β-oxidation enzymes. It is becoming clear that apart from being an essential chemical component of the active site, FAD may also play a multitude of additional roles such as assisting folding.

This aspect was investigated in detail using the ETFβ-Asp128Asn variant as a model. Clinical data reports that a homozygous patient for this mutation developed severe disease symptoms in association with a viral infection and fever. In agreement, heat inactivation of this mutant is more significant at temperatures above 37°C. We have found that cofactor insertion *in vitro* substantially improves the folding and stability of this ETF variant. Moreover, the presence of an excess of flavin in a concentration identical to that determined in the mitochondria from muscle of patients that take vitamin B2 orally, prevented proteolytic digestion by avoiding protein destabilization. To mimic a situation of fever *in vitro*, the flavinylation status was tested at 39°C: FAD improves the protein conformation yielding a more stable and active enzyme, thus acting as a pharmacological chaperone.

The results obtained provide a structural and functional framework for the role of vitamin B2 supplementation in the molecular pathogenesis of MADD and flavoprotein disorders in general.

Apart from disease causing mutations, a number of polymorphic variants have been reported in FAO enzymes. In particular, a polymorphic variation in the alpha-subunit of ETF, which leads to the incorporation of either a threonine or an isoleucine at position 171, has been identified. A previous study has indicated that patients suffering from a mild form of very long chain acyl-CoA dehydrogenase deficiency (VLCADD) displayed an overrepresentation of this ETFα-Thr171 polymorphic variant. This led to the

suggestion that this polymorphism could influence disease phenotype, by an as yet unknown molecular mechanism. The effect of the variation of the ETFα-Thr/Ile171 polymorphism on the ETF folding and dynamics under thermal stress was investigated. The results showed that the two variants have the identical thermodynamic stabilities (T_m=58°C), although the ETFα-Thr171 variant has a decreased thermal inactivation midpoint (T_m^{inact}=49°C) in comparison to that of ETFα-Ile171 (T_m^{inact}=58°C). Upon thermal stress, the ETFα-Thr171 variant is prone to faster cofactor (FAD) loss and conformational destabilization leading to an increase of a heterogeneous population of the variant. Also, this variant has higher conformational dynamics during thermal stress: upon 2 hours at 39°C, ETFα-Thr171 looses half of its activity, whereas ETFα-Ile171 activity remains at ~85%. ETFα-Thr171 can be rescued by the GroEL chaperonin, which captures and refolds the thermally destabilized variant, as observed by the retained enzymatic activity. Therefore, this polymorphic position has an impact on protein conformation and function under thermal stress. The latter is a common metabolic trigger for the manifestation of mitochondrial beta oxidation defects.

This dissertation has the purpose of contributing towards a molecular and functional understanding of MADD. The data gathered in this study were correlated to the wealth of data available on the genomics, cell biology and clinical presentations in MADD patients and permitted to add some insights on the molecular mechanisms underlying this pathology. Eventually, the results obtained will contribute for the setup of new therapeutic strategies and the improvement of diagnostic methods. The basic knowledge gained in this study has also the potential to contribute to a better understanding of many other diseases associated to missense gene mutations.

RESUMO DA DISSERTAÇÃO

O trabalho apresentado nesta dissertação centra-se no estudo da "flavoproteína de transferência electrónica" (ETF), uma proteína mitocondrial envolvida na β-oxidação dos ácidos gordos, cuja deficiência está associada à patologia "deficiência múltipla de acil-CoA desidrogenases" (MADD). A tese foca-se na avaliação das consequências funcionais, celulares e moleculares da variabilidade genética na ETF, e em particular, tem como objectivo elucidar a base do efeito de stress térmico na progressão da doença. As consequências moleculares do suplemento com vitamina B2 serão também abordadas.

MADD é uma patologia hereditária autossómica recessiva associada ao metabolismo mitocondrial dos ácidos gordos, aminoácidos e cholina. A patologia resulta de uma deficiência numa das seguintes proteínas: nas subunidades alfa (ETFA) ou beta (ETFB) da (ETF), ou na ETF-ubiquinona oxidoreductase (ETF-QO). Estas enzimas estão envolvidas no metabolismo energético da mitocôndria, actuando concertadamente na matriz mitocondrial catalisando a oxidação dos ácidos gordos de modo a obter energia.

A base genética e a caracterização funcional das mutações associadas à MADD ainda não se encontram bem estabelecidas. A expressão clínica da patologia é variável e pode ser agrupada em três classes: uma forma letal em recém-nascidos com anomalias congénitas (Tipo I), uma forma letal em recém-nascidos sem anomalias congénitas (Tipo II) e várias formas menos agressivas com expressão tardia (Tipo III). Esta variabilidade parece depender da localização e da natureza da alteração genética, ou seja, da mutação em cada caso. Até à data já foram identificados cerca de 55 mutações diferentes em doentes com MADD, dos quais cerca de metade correspondem a

alterações de um único aminoácido na ETF (A/B), mas apenas um número limitado de genótipos foi caracterizado. Os dados disponíveis na literatura sugerem uma relação directa entre o fenótipo clínico e os níveis de ETF com actividade biológica. Claramente, uma mutação nula resulta de um fenótipo severo, e uma mutação pontual no gene da ETF resulta numa menor actividade enzimática conduzindo a uma deficiência funcional ao nível celular. Neste casos, a alteração do aminoácido provavelmente determina alterações conformacionais na proteína que está relacionada com a patologia. Este efeito é mais proeminente nos pacientes com MADD de expressão tardia, nos quais a patologia não é continua, manifestando-se apenas em períodos de doença ou stress catabólico. Assim torna-se necessário um estudo para uma melhor compreensão da variabilidade da expressão clínica da MADD.

Uma ampla análise *in silico* foi realizada em 18 mutações pontuais, associadas à MADD, na ETF. A análise revelou que as mutações podem ser agrupadas essencialmente em dois grupos: 1) mutações que afectam a dobragem proteica e a estabilidade conformacional intrínseca; 2) mutações que perturbam actividade enzimática e a interacção com as desidrogenases. Três destas mutações, ETFβ Cys42Arg, Asp128Asn e Arg191Cys, que tipificam diferentes cenários no que diz respeito ao fenótipo clínico, foram alvo de um estudo detalhado. Esta parte do trabalho teve como objectivo obter as proteínas variantes da ETF de modo a testar o efeito das mutações na estrutura, dobragem, actividade enzimática, e interacção com co-enzimas funcionalmente relevantes.

A variante ETFα-Cys42Arg, associada a um fenótipo severo da patologia, afecta directamente o local de ligação do co-factor AMP, e os contactos entre as duas subunidades, logo resulta numa deficiente dobragem proteica. A variante foi heterologamente expressa em *E. coli* observando-se que a proteína

produzida se encontra na fracção insolúvel, na forma de agregados. Recorrendo à co-expressão com chaperões (GroEL/GroES e dnaK/dnaJ/GrpE)) foi possível expressar a proteína na fracção solúvel, mas não permitiu restaurar a actividade enzimática, provavelmente devido a uma inserção deficiente dos co-factores (AMP e FAD).

Duas variantes associadas com fenótipos menos severos, ETFβ Asp128Asn e ETF-Arg191Cys, foram heterologamente expressas e purificadas a partir da fracção solúvel. Usou-se uma combinação de métodos bioquímicos e biofísicos de modo a compreender o impacto das mutações na dobragem, estabilidade conformacional e na eficiência da transferência electrónica. Ambas as variantes dobram com uma estrutura semelhante à da proteína selvagem, no entanto, ambas têm a actividade enzimática diminuída assim como a estabilidade conformacional. A mutação Asp128Asn tem um maior efeito na estabilidade conformacional, uma vez que o resíduo 128 está localizado perto da interface do dímero e tem contactos com resíduos que pertencem ao local de ligação do FAD. Por outro lado, o decréscimo da actividade da variante ETFβ-Arg191Cys parece resultar de ineficiente interacção com as desidrogenases, pois a mutação está localizada na interface do complexo (ETF-desidrogenase).

A combinação da análise *in silico* das mutações com os resultados experimentais permitiu estabelecer "hotspots" estruturais na ETF, que são úteis para estabelecer uma correlação entre a variabilidade genética e o fenótipo.

Mutações que conduzem a fenótipos clínicos menos severos, como as seleccionadas neste estudo, são frequentemente associadas a actividade enzimática deficiente e reduzida inserção de FAD. O uso de suplementos de vitamina B2 no resgate funcional de flavoenzimas da β-oxidação dos ácidos gordos tem se tornado cada vez mais recorrente. A elucidação deste processo

tem sido possível devido aos diversos estudos realizados durante as últimas décadas, que variam desde a análise de proteínas purificadas, análise proteómica a células de pacientes e aos modelos animais e celulares. A consequência imediata da suplementação com riboflavina é um aumento na disponibilidade celular de FAD, que é o co-factor de várias enzimas de β-oxidação dos ácidos gordos.

Este aspecto foi investigado em detalhe usando a variante ETFβ-Asp128Asn como modelo. Os dados do relatório clínico do paciente homozigótico para esta mutação indicam que ele desenvolveu sintomas severos da doença, em associação com uma infecção viral e febre. Em concordância com os dados clínicos observou-se que a variante é inactivada termicamente para temperaturas acima de 37 ° C. Descobriu-se que a inserção de FAD *in vitro* melhora substancialmente a dobragem e estabilidade da variante. Além disso, a presença de um excesso de flavina, numa concentração idêntica à presente nas mitocôndrias de músculo de pacientes que tomam oralmente vitamina B2, impede a digestão proteolítica da variante através da estabilização proteica. Para reproduzir uma situação da febre *in vitro*, fez-se o estudo da inserção de flavina a 39 °C. A presença de FAD gera uma conformação mais estável e retém a actividade da proteína, actuando deste modo como um chaperão farmacológico.

Estes resultados fornecem uma ferramenta estrutural e funcional que pode ajudar a elucidar a importância do aumento de FAD na célula obtido pelo suplemento com vitamina B2.

Para além das mutações associadas à patologia, uma série de variantes polimórficas foram identificadas nas enzimas da β-oxidação dos ácidos gordos. Em particular, foi identificado uma variação polimórfica na subunidade alfa da ETF, que leva à incorporação de uma treonina ou uma isoleucina na posição 171. Um estudo prévio mostrou que pacientes que

sofrem de uma forma ligeira de deficiência da desidrogenase de acil-CoA de cadeia longa (VLCADD) apresentam uma sobre-representação do polimorfismo ETFα-Thr171, sugerindo que o polimorfismo pode contribuir para o fenótipo, através de um mecanismo molecular que ainda não está clarificado. Sendo assim, procedemos ao estudo do efeito da variação do polimorfismo ETFα-171Thr/Ile na dobragem e na dinâmica da ETF durante stress térmico.

Os resultados mostraram que as duas variantes têm a mesma estabilidade termodinâmica (T_m= 58 °C) embora a ETFα-Thr171 apresente uma menor temperatura de inactivação (T_m^{inact}= 49 °C) em comparação com a variante ETFα-Ile171 (T_m^{inact}=58 ° C). Sob stress térmico a variante ETFα-Thr171 é mais propensa a rápida dissociação de FAD e desestabilização conformacional, levando ao aumento de uma população heterogénea da variante. Além disso, a variante ETFα-Thr171 apresenta uma maior dinâmica conformacional durante stress térmico e ao fim de 2 horas a 39ºC perde metade da sua actividade, enquanto a variante ETFα-Ile171 consegue manter a sua actividade por volta dos 85%. O chaperão GroEL consegue reverter este efeito na ETFα-Thr171 capturando e re-arranjando conformacionalmente a variante termicamente desestabilizada, observado pela retenção da actividade enzimática. Deste modo, conclui-se que a posição polimorfica tem um impacto na conformação e função proteica durante stress térmicos, que é um estimulo metabólico comum para a manifestação destas doenças metabólicas.

A dissertação tem o propósito de contribuir para uma compreensão molecular e funcional da MADD. Os dados recolhidos neste estudo foram correlacionados com os dados disponíveis sobre a genómica, biologia celular

e relatórios clínicos de pacientes com MADD, permitido acrescentar conhecimento sobre os efeitos moleculares subjacentes a esta patologia. Eventualmente, os resultados obtidos irão contribuir para a aplicação de novas estratégias terapêuticas e melhorar os métodos de diagnóstico. O conhecimento básico adquirido neste estudo pode também contribuir para uma melhor compreensão de outras doenças relacionadas com mutações pontuais.

ABBREVIATIONS

ACDH	Acyl-CoA dehydrogenases
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate nucleotide
CD	Circular dichroism
C_m	Midpoint of chemical denaturation curve
DLS	Dynamic ligth scattering
ETF	Electron transfer flavoprotein
ETF-QO	Electron transfer flavoprotein ubiquinone oxidoreductase
FAD	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FMN	Flavin mononucleotide
FeS	Iron-sulphur
Hsp	Heat shock protein
K_D	Dissociation constant
MCAD	Medium-chain acyl-CoA dehydrogenase
MADD	Multiple acyl-CoA dehydrogenase deficiency
NAD	Nicotinamide adenine dinucleotide
PQC	Protein quality control
SCAD	Short-chain acyl-CoA dehydrogenase
SPR	Surface plasmon resonanse
T_m	Midpoint of thermal denaturation curve
VLCAD	Very long-chain acyl-CoA dehydrogenase

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1 PROTEIN FOLDING AND PROTEIN HOMEOSTASIS

1.1. The protein folding problem

Proteins are the most abundant macromolecules in biology, and are one of the key components of the cell, participating virtually in every cellular process. These macromolecules are needed in a wide diversity of process such as: catalysis, structural and mechanical functions, cell signalling and immune response.

In cells, proteins are synthesized in ribosomes as linear chains of amino acids, from information contained within cellular DNA. Following their biosynthesis, and in order to function, they have to acquire a specific three-dimensional structure; this process is called the protein folding.

During the 1960's, Anfinsen and co-workers, showed that RNase A, a 125 amino acid long protein, could fold spontaneously *in vitro*, without the involvement of any other cellular component [1]. From this observation it was postulated that the information contained in the primary sequence of a protein dictates the structure of its native conformation [1-2]. Moreover, Anfinsen proposed that the polypeptide chain folds under the driving force of a free energy gradient, until the thermodynamically most stable conformation is reached [2].

Simultaneously, Levinthal focused on the kinetics and dynamics of the folding process. He rose the question that if the folding mechanism would be a completely random sampling passing all possible conformations, a protein with 100 amino acids would take 10^{29} years to fold. Assuming that sampling of a single conformation would be as fast as single molecular vibration(10^{-13}s), is clearly that this would be inconsistent with the short time scale (milliseconds to seconds) that a protein takes to fold [3]. This is known as the Levinthal Paradox, and led the author to suggest that proteins must instead fold through specific pathways, during the folding process [4].

Over the last 50 years extensive research has been carried out in order to

understand the folding mechanism, and several models have been proposed, some of which will be briefly addressed here.

The classical nucleation model, proposed by Wetlaufer in 1973, suggested that at the early stages of the folding process, neighbouring residues would form elements of secondary structures that would act as nuclei from which the native structure would propagate, in a stepwise manner [5]. Subsequently, Karplus and Weaver proposed the diffusion–collision model, in which secondary structure elements could form independently of the tertiary structure, and would then diffuse until collide and assemble to form tertiary contacts (Fig. 1.1) [6]. A variation of the latter is the framework model [7]. The hydrophobic collapse model postulated that a protein would rapidly collapse around its hydrophobic residues and rearrange starting from that restricted conformational space (Fig. 1.1) [8-9]. The framework and the hydrophobic collapse models suggested the existence of intermediates, and at this point the presence of these species was taken as essential for the folding process to occur.

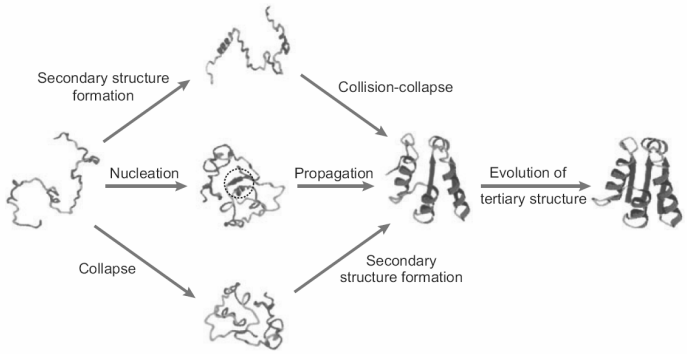

Figure 1.1: Pathways of protein folding. Diffusion-collision model: collision of pre-formed elements of secondary structure guides the formation of tertiary structure; hydrophobic collapse model: collapse around hydrophobic residues followed by a rearrange to form secondary structures; nucleation-condensation model: extended nucleus formation precedes secondary structure interactions. From [10].

However the finding that small proteins, like the 64-residue long chymotrypsin inhibitor 2, fold via a simple two-state kinetic mechanism

without formation of any intermediate at equilibrium, lead to the nucleation-condensation model (Fig. 1.1) [11]. This model combines concepts from the framework and the hydrophobic collapse models, suggesting that secondary structure is guided by native like tertiary interactions.

1.1.1. Energy landscapes

Nowadays, the majority of the authors accept the theoretical formulation of energetic funnel to illustrate the mechanism of protein folding (Fig. 1.2).

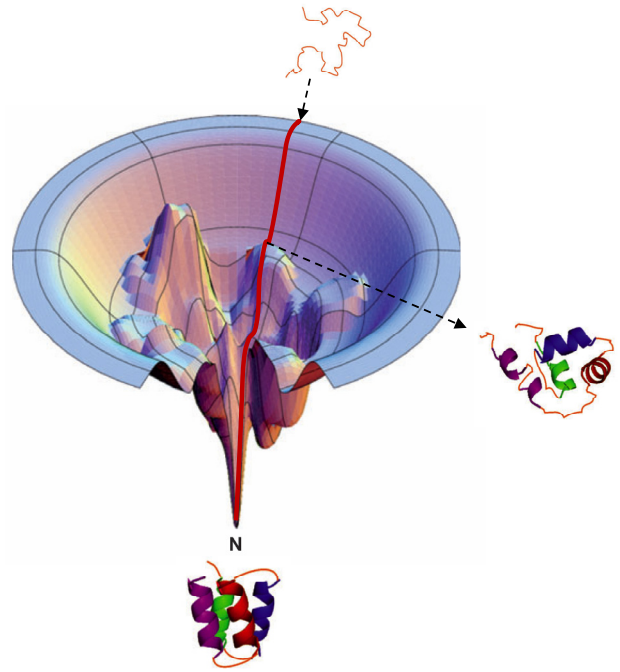

Figure 1.2: Three dimensional representation of the folding funnel. Adapted from [12].

In this view the protein folding process is analysed as a folding funnel, to illustrate that the number of possible conformations accessible to a protein becomes progressively smaller, as the number of native contacts increases [13]. The external border of the energy landscape is populated by the high energy denatured states (huge conformational entropy) that can flow through the funnel by alternative pathways until they reach, at the bottom, the native

conformation, the lowest energy state (Fig. 1.2) [12]. In general native contacts between residues are more stable than non-native interactions, so the number of possible conformations is reduced, and in principle a protein is able to find its lowest energy state [14]. In recent years, reports have described that even small proteins go down hill through the folding funnel, through structural intermediates due to the roughness of the funnel (Fig 1.2) [15-16]. The roughness of the folding funnel, which are local minima, is just a consequence of thousands of mutually supportive weak interactions that a native protein acquires during folding, that can not be satisfied simultaneously [16]. Consequently, the intermediates formed during the process can be productive for folding (on-pathway) or kinetic traps that would need major reorganization to acquire the native conformation (off- pathway) [17].

In the folding funnel model although the polypeptide chain starts to fold from a broad range of conformations at the top of the funnel, it describes the folding process for a single chain at an infinite dilution. Therefore, the model does not take in to account the complex, highly crowed, molecular environment of the cell. Several *in vivo* studies on newly synthesized polypeptides showed that aggregates, such as inclusion body, are typically formed from partially folded conformations, rather than from native states or fully denatured chains [18-19]. In order to improve the funnel folding concept, to depict more accurately protein folding and aggregation phenomena in the cell, Clark has performed a new formulation of the energy landscape, including additional deep minima (Fig. 1.3) [20]. Partially folded or misfolded states, in particular off-pathway intermediates, will have a propensity to aggregate, because these forms typically expose hydrophobic amino acid and unstructured regions [21]. The intermolecular contacts are driven by hydrophobic forces and originate amorphous structures or highly ordered fibrillar aggregates, known as amyloid (Fig. 1.3).

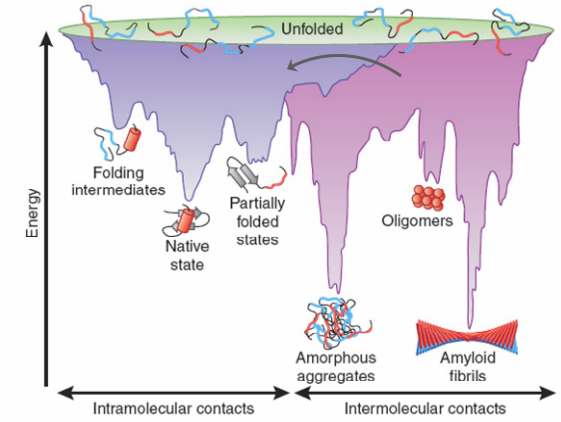

Figure 1.3: Energy landscape scheme of protein folding and aggregation. The surface shows the huge number of conformations "funneling" towards the native state via intramolecular contact formation (purple area), or toward aggregates or amyloid formation via intermolecular contacts (pink area). The arrow represents the molecular chaperone rescue. Adapted from [21].

The new areas depicted in the funnel model take into account, not only the off-pathway intermediates, but also illustrate the role of several cellular factors such as molecular chaperones and ribosomes that assist the folding process (see section 1.3) [20]. In this perspective, molecular chaperones would bind to partially folded polypeptide chains in a discrete zone near the junction between productive folding and aggregation (represented by the edge of the two colours, Fig 1.3) [20]. The interaction with molecular chaperones increases the efficiency of the folding reaction, or results in the capture of species that could otherwise go downhill into the aggregation pathway.

1.2. The native state and protein stability

The native state adopts in solution a unique, highly ordered conformation in contrast to the unfolded state that is conformationally heterogeneous and usually unstructured. To achieve the native structure a protein must overcome the forces that favour the unfolded state, mainly conformational entropy, by increasing the number of intramolecular non-covalent interactions [22]. These forces, that favour the native state, are a sum of different interaction such as:

hydrogen bounds, ion pairs, van der Walls attractions and water-mediated hydrophobic interactions (Fig 1.4) [23].

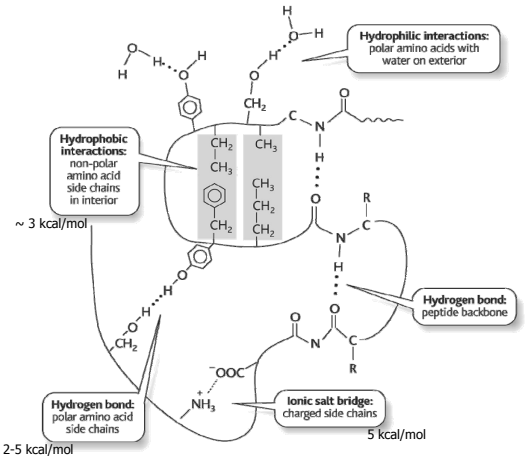

Figure 1.4: The various non-covalent forces that can operate in a protein. From [24].

In the native state the hydrophobic side chains pack in the interior of the protein, and other non-covalent interaction form in order to favour internal organization, thus lowering conformational entropy. Of particular interest are the hydrogen-bond networks formed by the polypeptide backbone that contribute to the formation of secondary structures, such as α-helix and β-sheets [25]. Covalent and coordinate chemical bounds such as the ones formed in disulfide bounds, or prosthetic groups, for example heme and iron sulfur centres, also contribute to protein stability.

Under physiological conditions the native and unfolded state of a protein are in equilibrium (Fig. 1.5), and the free energy change (ΔG) of the reaction is usually referred as conformational stability of a protein [26]. The free energy change of the equilibrium is described by the following thermodynamic equation:

$$\Delta G = \Delta H - T\Delta S$$

where ΔH and ΔS represent the enthalpic and entropic variations,

respectively, and T represents the temperature. Therefore, the native structure results from a delicate balance between large and opposing forces [22]. The folded conformation is favoured by the negative value of enthalpy due to internal interaction, and the unfolded state is favoured by a positive value of conformational entropy.

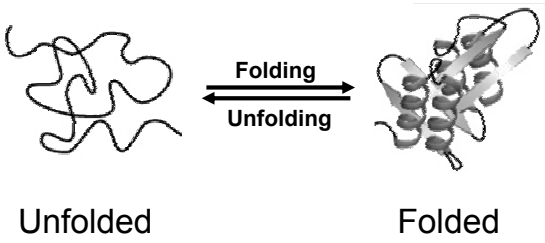

Figure 1.5: Schematic representation of a folding-unfolding reaction. The reaction represents a two-state process without the formation of intermediates.

Noteworthy, the native state is known to be only marginally stable and the difference in energy between native and unfolded states is only 5-15 kcal.mol^{-1}[27]. This difference in energy is within the magnitude of only a few non-covalent interactions.

The determinants of native state stability in aqueous solutions are the primary sequence of the protein as well as the variable conditions of pH, temperature, and concentrations of salt and ligands [28]. Manipulation of environment conditions promoting protein unfolding, such as rise in temperature, variation of pH, or addition of a chemical denaturant, will shift the equilibrium to the unfolded state. (Fig. 1.5 and 1.6) [25]. The unfolding reaction begins with small changes in the folded conformation, such as increase in flexibility and localized conformational alterations, to a point where massive alterations occur, the transition midpoint (Fig. 1.6). At the midpoint of the unfolding curve half of the protein molecules are in the unfolded state, and the ΔG is zero [25]. The abruptness of the transition is an evidence of the cooperativity of the unfolding reaction. The cooperative

effect results from the fact that although each interaction has a small stabilization effect, the disruption influences the surrounding interactions, thus lowering the equilibrium constant at each point.

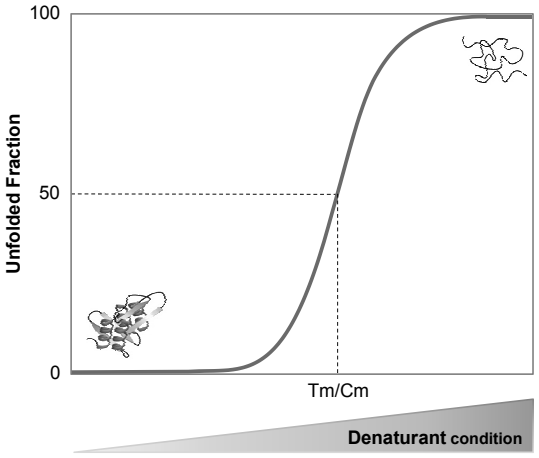

Figure 1.6: Representation of a hypothetical denaturation curve. The cartoons represent the native state (botom) and the unfolded state (Top). T_m or C_m represent temperature or chemical denaturant concentration at the midpoint of the unfolding curve.

The folded and unfolded states have different structural features therefore a diversity of biophysical methods are available to monitor the unfolding reaction. The experimental techniques, the time scale that can be monitored and the information that can be extracted from each technique is summarized in table 1.1 [16]. From the experimental data obtained by the different techniques it is possible to draw curves similar to the one represented in figure 1.6, from which thermodynamic parameters of the reaction can be obtained. The midpoint transition determined from the curves, such as temperature (T_m) or concentration of chemical denaturant (C_m), is used for comparing protein stability.

Table 1.1 Experimental techniques that have been applied to the study of protein folding [16].

Technique	Timescale	Information content	Ref
Intrinsic tryptophan fluorescence	≥ ns[a]	Environment of tryptophan (through measurement of intensity and λ_{max})	[29]
Far UV CD	≥ μs[a]	Secondary-structure content	[30]
Near UV CD	≥ μs[a]	Packing of aromatic residues	[30]
Raman spectroscopy	≥ μs[a]	Solvent accessibility, conformation of aromatic residues	[31]
Infrared spectroscopy	≥ ns[a]	Secondary-structure content	[32]
ANS	≥ μs[a]	Exposure of aromatic surface area	[29]
FRET	≥ ps[a]	Molecular ruler, dependent on the distance between two fluorophores (r^{-6} dependence assuming free rotation of the dyes)	[33]
FCS	≥ ps	Diffusion time (and hence size and shape)	[34]
Anisotropy	≥ μs[a]	Correlation time measurements provide information about shape and size of molecule	[29]
Small-angle X-ray scattering	≥ μs[a]	Radius of gyration	[35]
Absorbance	≥ ns[a]	Environment of chromophore	[36]
Real-time NMR	> min	Structural information via chemical shifts and measurement of NOEs	[37]
Native-state hydrogen exchange	h	Global stability, detection of metastable states	[38]
Pulsed H/D exchange by NMR	≥ ms	Hydrogen exchange protection of folding intermediates on a per-residue basis	[38]
Pulsed H/D exchange by ESI-MS	≥ ms	Hydrogen exchange protection of folding populations	[39]
NMR relaxation methods	~ ms	Nonrandom structure in denatured states and conformational exchange between different species	[37, 40]
Protein engineering	Depends on probe used	Role of an individual residue in determining the rate of folding and stability of a species of interest	[41]

1.3. Protein folding *in vivo*

In vivo the protein folding process can begin when the nascent polypeptide is still attached to the ribosome, in a co-translational manner [42]. Alternatively, part of the folding process may take place in the cytosol, and many proteins fold in specific compartments, such as the endoplasmic reticulum (ER) [43]. Co-translational folding restricts the conformational space, so the polypeptide chain will probably start the folding process from a more defined trajectory, preventing non-productive pathways [20]. In other cases, co-translational folding can have a negative impact, as some proteins have extensive contacts between amino acids in the native structure that are distant in primary sequence. The translational process is relatively slow (~15-75 s for a 300-residues protein) potentially leading to the formation of non-native intramolecular contacts between the N-terminal portion that is being elongated, or intermolecular ones with other molecules in the surroundings [21]. In this context, high macromolecular concentration inside the cell (300/400 $mg.ml^{-1}$) play an important role in the process [44]. To overcome the problem of molecular crowding and increase the efficiency of the folding process, nature has developed specific protein machineries - the protein quality control system (PQC)- that assists the folding process with no effect in the selection of the native structure (Fig. 1.7).

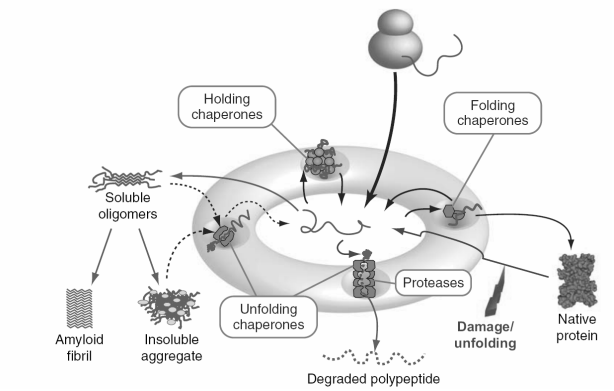

Figure 1.7: Schematic function of the protein quality control system (PQC). Folding chaperones promote folding, holding chaperones maintain solubility, unfolding chaperones unfold misfolded proteins or disaggregate aggregates and inject them into proteolytic chambers. From [45].

The PQC system has the ability to supervise folding, disable aggregation, and remove misfolded or damage polypeptide chains before they exert toxic effects (Fig. 1.7) [45]. The system is composed by molecular chaperones, specialized intracellular proteases and accessory factors that regulate the activity of chaperones and proteases or provide communication between the various components [45]. Moreover, the PQC system has the ability to operate during or after protein synthesis, or upon unfolding to facilitate proteins to achieve their correct native structure, or to be degraded. Next, a brief description on different components of the PQC will be presented.

1.3.1. Role of molecular chaperones in *de novo* folding

Chaperones that assist *de novo* protein folding in the cytosol can be divided in two classes: chaperones that stabilize nascent polypeptides on ribosomes and initiate folding, and chaperones that act downstream in completing the folding process [46-47].

Included in the first class are chaperones that bind directly to the large ribosomal subunit in close proximity to the polypeptide exit site, such as the Trigger factor in bacteria, and a specialized Hsp70 system called RAC (ribosome associated complex) and NAC (nascent chain associated complex), in eukaryotes [48-50] (Fig. 1.8). Trigger factor has an N-terminal domain that binds to the ribosome, a domain with prolyl isomerase activity and a C-terminal chaperone domain. This protein binds not only to the ribosome, but also to the nascent chain forming a complex with the elongation chain, which dissociates when synthesis is complete [51-52]. In mammals, RAC is a heterodimeric complex formed by a J-protein, the Mpp11 that associates with the ribosome, and Hsp70L1 [53-54]. NAC is also a heterodimeric protein composed of α and β subunits [55]. The β subunit binds to the ribosome and both subunits contact with nascent polypeptides [56]. NAC prevents the

association of the ribosome with the protein translocation machinery of the ER membrane. Therefore, it is believed that it regulates the destiny of the newly synthesized polypeptides, whether they are released into cytosol or are translocated to the ER [57]. This class of chaperones binds linear chain segments enriched in hydrophobic amino acids, and acts by preventing premature chain compaction, and by maintaining the elongation of the polypeptide in a non-aggregated state until the chain is able to fold correctly [21].

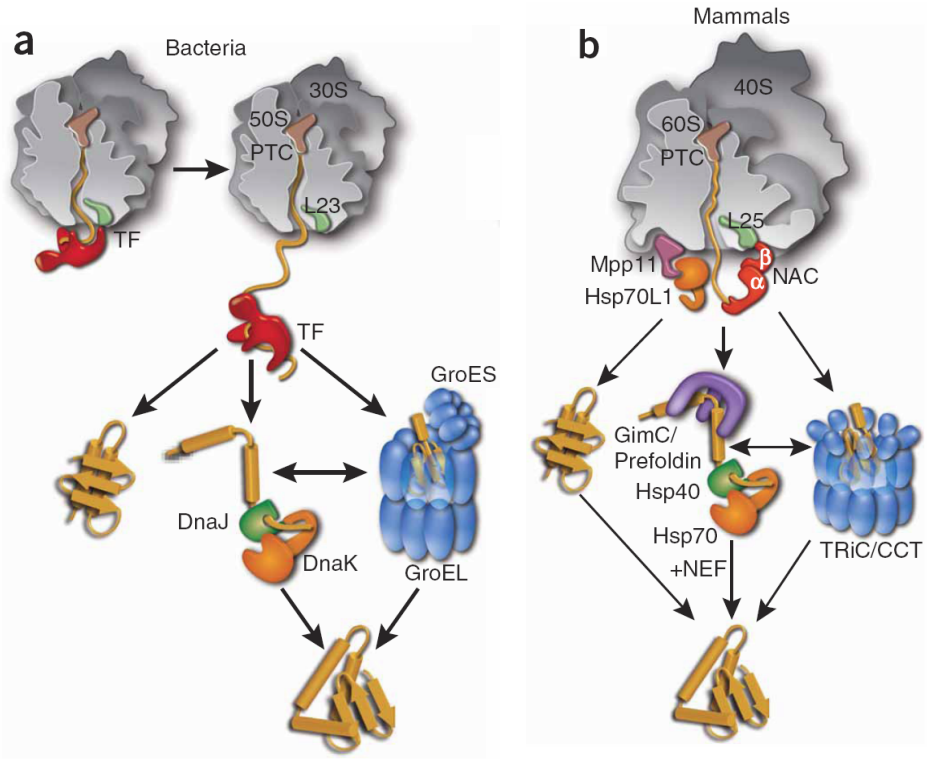

Figure 1.8: Models of molecular chaperones involved in *de novo* protein folding. a) In bacteria the Trigger factor, ribosome bounded, can move with the nascent polypeptide for a limited time promoting folding. Some proteins need further assistance by either Hsp70 chaperone system (dnaK together with its cochaperone dnaJ and the nucleotide exchange factor GrpE), or by the Hsp60 (chaperonin GroEL together with is cochaperone GroES). b) Eukaryotes have two ribosome bound systems, the NAC and the RAC (Mpp11 and Hsp70L1). As in bacteria some proteins will further be assisted by Hsp60 family of chaperonins (TriC/CCT) or the Hsp70 system (Hsp70, Hsp40 and NEF). GimC/Prefoldin is implicated in the folding of actin and tubulin. Adapted from [48].

Moreover, Brandt *et al* have recently provided new insights into the 3D organization of the bacterial polysome, demonstrating that its three dimensional organization also contributes to prevent interaction of the elongated chain with chains from adjacent ribosomes (Fig. 1.9) [58]. The pseudo helical arrangement of the polysome places the exit channels of each ribosome further apart, thus minimizing unspecific interactions between nascent chains.

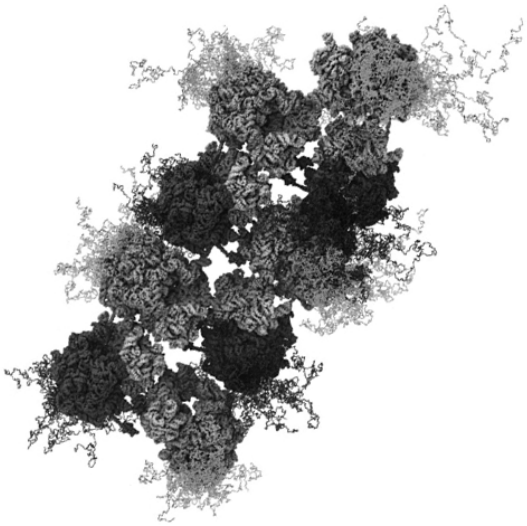

Figure 1.9: Model of nascent chain configurations in a representative polysome. From [58].

The second class of chaperones, the Hsp70 family (dnaK in bacteria and Hsc70 in mammals) acts as a second line of folding assistants (Fig. 1.8). These chaperones do not bind to the ribosome, and will interact with longer nascent chains during co- or post-translational folding through ATP regulated binding cycles. Proteins from this family have an N-terminal ATPase domain and a C-terminal peptide binding domain with high affinity to hydrophobic structural features. The Hsp70 family in general acts in conjunction with Hsp40 proteins (dnaJ in bacteria) (Fig. 1.8) [59-62]. These molecules function by presenting substrates to the chaperone, and at the same time they stimulate its ATPase activity, freezing it into an ADP-bound high affinity state. Apart

from these cofactors, a nucleotide exchange factor is also involved, Bag-1/GrpE in mammals and bacteria respectively, to stimulate chaperone activity [63-65]. The Hsp70 family is also very important because it organizes the chaperone network and distribute subsets of proteins to other downstream chaperones, such as Hsp60 (GroEL in bacteria, Hsp60 in mitochondria and TriC/CCT in cytosol in eukarya) and Hsp90 families.

1.3.2. Folding, holding and unfolding chaperones

The chaperonins, Hsp60 family, assemble into double-ring structures, functioning in an ATP dependent way, by enclosing the substrates during the folding event, so they are protected from aggregation [47, 66-67] (Fig. 1.8). These chaperonins are composed of 14 subunits arranged as two stacked rings with a sevenfold rotational symmetry. In general, GroEL and Hsp60 also need a cofactor, respectively GroES and Hsp10. In eukaryotes, the chaperonin interacts directly with Hsp70 and other upstream factors like prefoldin, increasing in this way the folding efficiency by directly recruiting nascent chains that are unable to fold with the chaperone alone [68-69].

Hsp90 acts downstream Hsp70, preventing aggregation of unfolded polypeptides [70-71]. These chaperones are proposed to play an important role in quality control, assembling into large multichaperone complexes that have a broad range of action, including interactions with a wide collection of cell-signalling molecules and transcription factors [72-73].

In the cytosol, there are also chaperones that lack ATPase activity and need to transfer their substrates to folding chaperones to achieve folding [45]. These are usually named 'holding' chaperones, and include the small heat shock proteins (sHsp). These are 12-42kDa proteins that usually form oligomers that can range from 9 to 50 subunits, and that under heat-shock conditions, bind misfolded proteins [74].

Moreover, the Hsp100/Clp family has an unfolding activity but it can also induce structural changes in its substrates, that ultimately change the substrate's biological activity [75]. This family acts on folded and assembled complexes and on improperly folded and aggregated proteins [75]. These chaperones have the ability to unfold proteins so that they can either be folded in their correct form or be otherwise degraded.

1.3.3. The Proteolytic system

Apart from the molecular chaperones, the PQC also contains the degradation machinery constituted by proteases. These proteins are restricted to specific locations in the cell, that can only be accessed by polypeptides targeted to degradation [45]. The quaternary structure of the different proteases is very similar, and resembles a barrel formed by the proteolytic subunits associated into oligomeric rings that stack up on each other [76]. The proteolytic sites are buried within the central cavity and are only accessible through narrow gates, obstructing folded proteins from entering. One of the most studied members of this family is the proteasome, a central protease in non-lysosomal ubiquitin-dependent protein degradation, which is involved in protein quality control, antigen processing, signal transduction, cell cycle control, cell differentiation and apoptosis [76].

1.3.4. Endoplasmatic reticulum (ER) chaperones

Protein folding in the ER follows analogous chaperone-mediated pathways as those described for the cytosol, with the addition that in this compartment a complex series of glycosylation and deglycosylation processes takes places, which prevent misfolded proteins from being secreted. One of the ER chaperones is BiP, a member of the Hsp70 family, that binds

transiently to newly synthesized proteins and in a more permanent way to misfolded, underglycosylated or unassembled proteins that can not be transported from the ER [77]. Other two important ER chaperones, calnexin (CNX) and calreticulin (CRT), recognize monoglucosylated glycan chains on proteins [78]. CNX is a membrane bound protein, and in addition to its chaperone activity, it participates in quality control by delaying the export of incompletely assembled proteins from the ER [79].

1.4. Regulation of protein homeostasis

To ensure protein homeostasis, the cell contains a set of pathways that make up the so called proteostasis network, which regulates protein synthesis, folding, trafficking, aggregation, disaggregation and degradation (Fig. 1.10). This network has the ability to regulate conformation, concentration, binding interactions and location of individual proteins [80]. More than 1000 general and specialized chaperones, folding enzymes, degradation and trafficking components integrate this network [81]. In normal conditions the proteostasis network ensures that each protein reaches its final target in the cell or is eliminated in order to prevent cell damage or dysfunction.

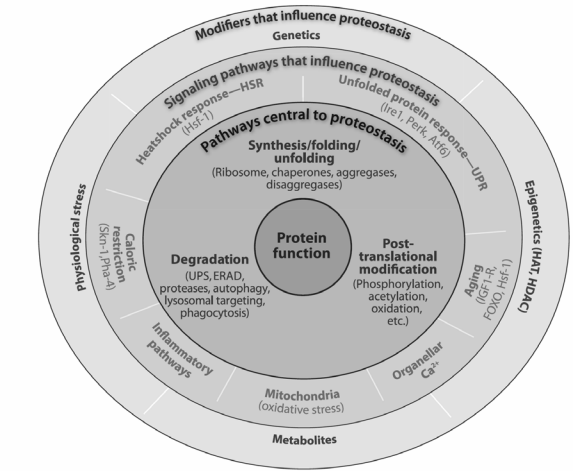

Figure 1.10: The proteostasis network. Illustration of the layers of interaction in protein homeostasis. First layer is constituted by components that operate in direct folding and pathways that select proteins for degradation. Second layer includes signalling pathways that influence the first layer. Third layer comprise factors that affect the activity of the two inner layers. From reference [81].

The control of these pathways is accomplished by signalling pathways that directly regulate the concentration, distribution and activities of the components that make up the proteostasis system. These signalling pathways include: the unfolded protein response (UPR), the heat shock response (HSR), pathways that regulate the Ca^{2+} concentration on the ER, inflammatory response and histone deacetylase (Fig. 1.10).

Several components of the first layer were object of discussion in the section above; here a brief overview on the signalling pathways will be given.

Prolonged exposure to stress can cause proteins to unfold, misfold or aggregate leading to an inefficient function of the cell, demanding an increased synthesis of the proteins from the quality control system. The heat shock response (HSR) controls the proteostasis reaction to these changes at the cytoplasmic level [80]. This signalling pathway is regulated at the transcriptional level by the activity of a family of heat shock transcription factors (HSF) [82]. Transcription of heat shock genes is activated by various acute and chronic conditions such as elevated temperatures, heavy metals, small molecules, infection, and oxidative stress [83]. Several disease states like inflammation, ischemia, tissue wounding and repair, cancer, protein mutations and neurodegenerative diseases are also associated with increase expression of proteins from the heat shock response [83]. Upon activation, an instant induction of genes encoding molecular chaperones, proteases, and other proteins associated with protection and recovery from cellular damage associated to misfolded proteins result in a rapid increase of the cellular levels of these proteins. The heat shock gene superfamily includes Hsp100/Clp, Hsp90, Hsp70, Hsp60, Hsp40, and small heat shock protein (sHsp) families, as referred above.

The HSR has been implicated in many neurodegenerative diseases because the association of theses chaperones with intracellular aggregates

formed in these types of disorders was identified. It was observed that for example the protein huntingtin aggregates transiently in association with Hsp70, and that the association/dissociation properties identified for these complexes are similar to chaperone interactions with unfolded polypeptides [84]. Also, it is important to mention that neurodegenerative disorders often occur later in life, when the heat shock genes seem to be poorly induced [85-86]. Cancer is another type of disorder where the HSR as been implicated. Tumor cells typically express higher levels of heat shock proteins compared with non-transformed cells, suggesting that the abnormal expression of chaperones is associated with the tumorigenic state [87].

The ER is extremely important to cell regulation as all proteins that enter the secretory pathway first enter in the ER, where they fold and assemble [88]. The "unfolded protein response" (UPR), is the 'supervising' pathway for the quality of the folded proteins in this organelle. Unfolded or misfolded proteins identified by the UPR are retained within the ER lumen in complex with chaperones, or are targeted for degradation through the ubiquitin-proteasome, the ER-associated degradation (ERAD) [89].

Cystic fibrosis is related to the UPR response, this is a loss-of-function disease associated to mutations on the cystic fibrosis transmembrane conductance regulator (CFTR), a transmenbranar protein that function as a cAMP-stimulated chloride channel [90] This is the most fatal genetic disease in Caucasians and is caused by mutations on CFTR, a deletion of the phenylalanine in position 508 (Δ508) is the most prevalent one [91]. This mutation causes a defect in protein maturation and on its transit to the plasma membrane, due to a change in the protein folding pathway [92]. Due to the UPR actions in the ER misfolded proteins are recognize, retained in ER and rapidly degraded by the ubiquitin-dependent proteasomal system, thus Δ508 is rapidly degraded [93].

1.5. Emerging strategies in proteostasis regulation

A decreased ability of the proteostasis network to control the diverse pathways will jeopardize the cell, and can potentially affect in a progressive way, cell, tissue, organ or the organism function. Different factors can compromise protein homeostasis such as genetic and epigenetic pathways, physiological stressors, and metabolites that affect activity of the proteostasis network components (Fig. 1.10). When the cell is no longer able to restore proteostasis, the so call loss- or gain- of function effects emerge. A loss of function disorder is characterized by misfolded proteins that have decreased function and/or are degraded rapidly resulting in a decreased steady-state amount of protein. This type of disorders comprises several inherited autosomal recessive disorders, such as cystic fibrosis, phenylketonuria, fatty acid oxidation defects, and Gaucher's disease. On the other hand, gain of-toxic function diseases are associated with intra- or extra-cellular accumulation of protein aggregates, like in amyotrophic lateral sclerosis, Alzheimer's, Parkinson's and Huntington's disease. The pathology occurs because the cell is unable to degrade misfolded proteins, leading to the formation of toxic species, in the form of oligomers, aggregates and/or amyloid fibres.

A novel approach to treat protein functional disorders is to regulate at different level the proteostasis network by manipulating the innate biology of the cell with protein replacement, protein stabilization or adaptation of proteostasis network using proteostasis regulators (Fig. 1.11).

Protein replacement is currently used for treatment in loss-of-function diseases; therapy is process injecting patients with a recombinant form of the wild type variant of the deficient protein [94-96]. The major limitations of this therapy is the fact that the injected proteins have to cross several barriers to get to the target site, and in most cases only a lower amount of the

recombinant protein reach it destination. The case of lysosomal disorders is one of these examples in which only 5 % of the protein injected enter the lysosome [97]. However, in neuropathic forms of these diseases this therapy is completely useless because recombinant proteins can not cross the blood-brain barrier [98].

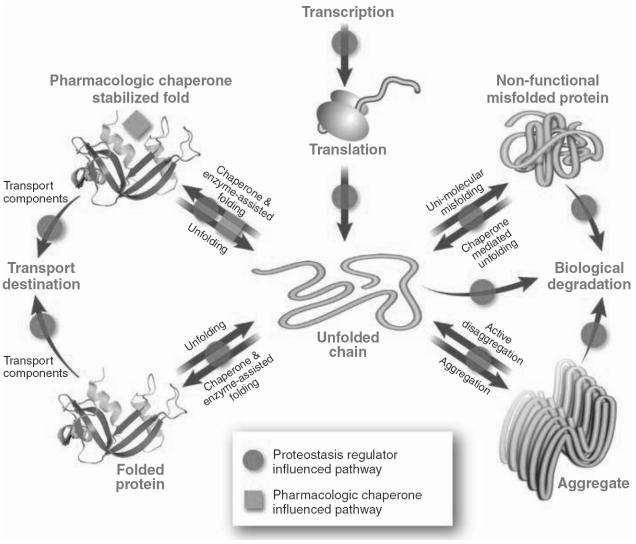

Figure 1.11: Proteostasis Regulation. Schematic representation of the different control points of protein homeostasis: red arrows represent the proteostasis pathways, magenta circles represent proteostasis regulators and green squares represent pharmacological chaperones. From [80].

Modulation of the proteostasis network by direct protein stabilization can be achieved using low molecular weight compounds that chemically-mediate protein stabilization or refolding. These compounds are called chemical chaperones, or for a more restricted group that interact with a specific protein some authors denominate them as pharmacological chaperones (Fig. 1.11) [93, 99-100]. This restricted class comprises protein cofactors, including metal ions, ligands, competitive inhibitors, and agonist/antagonist. The binding of a cofactor, by covalent or non-covalent interaction, contribute to the net of stabilizer interaction within the tertiary structure of a protein, so establish this

interaction will prevent an increase in the intrinsic breathing of the protein that could lead to protein misfolding [101]. This is the case of P-glycoprotein, an energy dependent transporter, for which as been shown that several mutants in the presence of substrates (vinblastine and capsaicin) and inhibitors (cyclosporine and verapamil) increase the presence of functional protein in cell surface, probably due to a stabilization of a folding intermediate near native conformation [102-103]. Studies on MNK a copper transporting p-type ATPase showed that the presence of copper can increase stability of the protein [104].

Moreover, cells had already developed their own chemical chaperones to deal with environmental stress; these compounds are the cellular osmolytes and comprise carbohydrates, free amino acids and methylamines. The first two groups include glycerol, sorbitol, arabitol, trehalose, glycine, alanine, proline, taurine that can accumulate to higher concentrations in the cell without affecting it normal function [101]. The methylamines such as betaine, trimethylamine N-oxide (TMAO) and glycero-phosphorycholine are produced to offset the protein denaturing effects of urea. Beside these chemical chaperones other hydrophobic compound can bound to proteins and stabilize their structure in a native like conformation. This is the case of sodium 4-phenylbutyrate (PBA), an orally bioavailable short-chain fatty acid, that can be used as an ammonia-scavenging agent in urea metabolism disorders and it also has beneficial effects in stabilizing CFTR and alpha-1 antitrypsin (AAT) [105-106].

A broader strategy consists in the use of proteostasis regulators, molecules that rather regulate the concentration, conformation and/or location of a specific protein (or family of proteins), by manipulating one or more signalling pathways in order to rebalance the whole network (Fig. 1.11). These proteostasis regulators can be small compounds or biological molecules like

small interfering RNA (siRNA), cDNA, or proteins. It is described in literature that proteostasis regulators can modulate synthesis, folding, trafficking, disaggregation, and aggregation pathways [50, 107-117]. In fact, RNA interference studies have been use with success in cellular models of gain-of function disorders, like Alzheimer and Huntington's disease. Also, small molecules like diltiazem and verapamil have been used to restore protein homeostasis in lysosomal storage disorders [98]. These two L-type voltage gate calcium channel blockers restore mutant lysosome enzyme function by modulating protein folding in the ER. Unlike the pharmacological chaperones that directly bind to the protein stabilizing the native form for trafficking to the Golgi and to lysosome, the proteostasis regulators diminish the Ca^{2+} content in the cytoplasm resulting in the increase of transcription and translation of several cytoplasmatic and ER chaperones, like BiP and Hsp40 that interact with lysosomal enzymes. One important aspect is the fact that diltiazem can cross the blood-brain barrier, in contrast to the protein replacement therapy [98]. Celastrol, a natural product derived from the Celastraceae family of plants, induced HSF1 protein expression leading to up-regulation of cytoplasmatic chaperone network [117]. Some of the molecules know to act as chemical chaperones as already mentioned can also be called proteostasis regulators once they also can regulate folding and trafficking machinery, one exemple is the use of 4-PBA and taurine-conjugated ursodeoxycholic acid to alleviated ER-stress in cells and whole animals [118].

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2 ETF, MITOCHONDRIAL FATTY ACID β- OXIDATION AND RIBOFLAVIN - AN OVERVIEW

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Mitochondria are essential cell organelles whose one of the principal functions is to produce energy through the oxidation of carbohydrates, amino acids and fatty acids. Oxidation of sugar and fatty acids yields two-carbon fragments in the form of acetyl group of acetyl-coenzyme A (acetyl-CoA), which will enter the citric acid cycle to further oxidation to CO_2. In the case of amino acids after losing the amino group, the α-keto acids will go through oxidation or, form three- and four-carbon units that will be converted to glucose. The energy produced during these oxidation cycles will be used to reduce electron carriers, like nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). These carriers are re-oxidized in the respiratory chain in a series of reactions that are coupled with the synthesis of adenosine triphosphate (ATP).

Electron transfer flavoprotein (ETF) and electron transfer flavoprotein ubiquinone oxidoreductase (ETF-QO) are two extremely important mitochondrial proteins as they are the entry point of reducing power to the respiratory chain from fatty acid β-oxidation, as well as choline and amino acid catabolism.

A brief overview of the mitochondrial fatty acid β-oxidation will be given in the first section of the chapter, since ETF is at a key point of this pathway, acting as a hub. Following the general presentation of the pathway, special attention will be given to ETF and ETF-QO. On the third section of the chapter, fatty acid oxidation (FAO) disorders will be discussed, in order to frame the reader with these diseases, in which multiple acyl-CoA dehydrogenase deficiency (MADD) is included. The last section is dedicated to riboflavin (vitamin B2), as it is widely used in the treatment of several FAO disorders.

2.1. Mitochondrial fatty acid β-oxidation enzymes

Fatty acids are carboxylic acids with long straight or branched hydrocarbon chains, and they are very important in cells as they are needed for enzymes, hormones, cell membrane and as a source of energy. In mammals fatty acid oxidation provides a major source of ATP for the heart and the skeletal muscle [1-3]. In the liver, kidney, small intestine and also white adipose tissue, β-oxidation provides the formation of ketone bodies which are used as energy source on other tissues [4]. Fatty acid oxidation is particularly important during fasting, sustained exercise, stress, and during neonatal-suckling period, when glucose supplies become limited [5].

The cell can metabolize fatty acids by three different pathways that take place in the mitochondria or in the peroxisomes. The best characterized pathway is the mitochondrial fatty acid β–oxidation (Fig. 2.1).

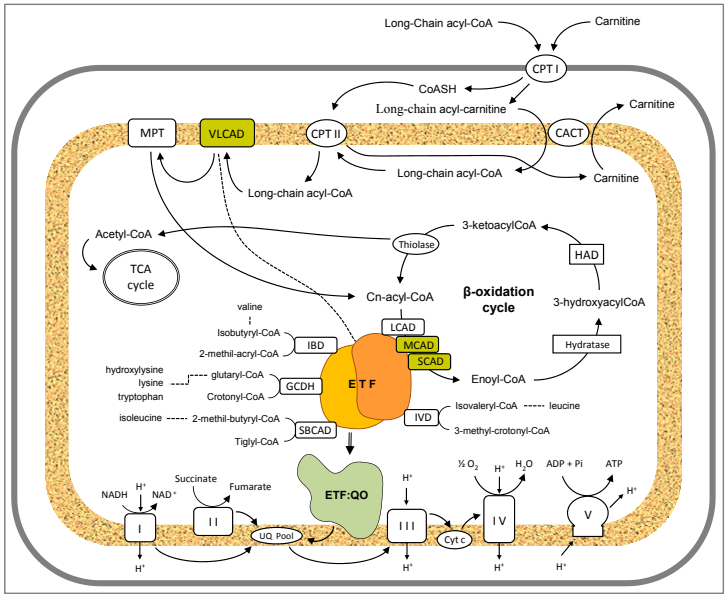

Figure 2.1: Schematic representation of the mitochondrial fatty acid β-oxidation pathway. Please see details in text.

Fatty acids are activated in the cytosol in an ATP-dependent acylation forming acyl-CoAs. The very-long-chain and long-chain fatty acids cross the inner mitochondrial membrane as carnitine-derivatives mediated by three proteins, carnitine palmitoyl transferase I (CPT I), acyl-carnitine translocase (CAT) and carnitine palmitoyl transferase II (CPT II) (Fig. 2.1). Once in the mitochondrial matrix, the acyl-CoA fatty acids undergo dehydrogenation by acyl-CoA dehydrogenases with different chain-length specificities. These enzymes are located in the matrix, with the exception of very-long chain acyl-CoA dehydrogenase that is associated with the inner membrane, and has high specificity for C12-C24 acyl-CoA fatty acids (Fig. 2.1). The subsequent steps are catalyzed by enoyl-CoA hydratase, 3-L-hydroxyacyl-CoA dehydrogenase and β-ketoacyl-CoA thiolase and lead to the formation of acetyl-CoA and fatty acyl-CoA two carbon shorter [4] (Fig. 2.1). Unsaturated fatty acids undergo the same reaction until the cis-configuration double-bound prevents the formation of a substrate for the acyl-CoA dehydrogenase and enoyl-CoA hydratase. At this point additional enzymes are required, like the 3,2 trans-enoyl-CoA isomerase and 2,4-dienoyl CoA reductase 1. Fatty acids with an odd number of carbons are degraded in the same way, but the final product has three carbons, propionyl-CoA, which is converted to succinyl-CoA to enter the citric acid cycle.

Some enzymes of the first step of the fatty acid β-oxidation will be briefly described since these enzymes are ETF substrates (Table 2.1). The acyl-CoA dehydrogenases are homo-dimers (the membrane-associated very-long-chain acyl-CoA dehydrogenase (VLCAD) and acyl-CoA dehydrogenase no 9 (ACAD-9)) or homo-tetramers (the soluble matrix enzymes short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD)). All these ACDHs carry one non-covalently bound FAD molecule per subunit and the

binding sites reside within each monomer, i.e., FAD interacts only one subunit at a time.

The ACDHs involved in fatty acid β-oxidation form a gene family with four additional acyl-CoA dehydrogenases involved in amino acid metabolism: glutaryl-CoA dehydrogenase (GCD) [6], isovaleryl-CoA dehydrogenase (IVD) [7], short-branched chain acyl-CoA dehydrogenase (SBCAD) [8] and isobutyryl-CoA dehydrogenase (IBD) [9]. These dehydrogenases also belong to the group of ETF substrates (Table 2.1).

Table 2.1: Mitochondrial flavoenzymes properties. The proteins described in the table participate in electron transfer reactions with ETF.

Protein	Structure	Preferred Substrate(s)	MIM	Gene symbol	Mutation[a]	Ref
VLCAD	3B96	C16	609575	*ACADVL*	65	[10]
LCAD	n.a.	C14	609576	*ACADL*		[11]
MCAD	1EGE	C8	607008	*ACADM*	51	[12-13]
SCAD	2VIG	C4	606885	*ACADS*	14	[14]
ACAD-9	n.a.	C16:1	611103	*ACAD9*		[15]
GCD	1SIQ	glutaryl-CoA	608801	*GCDH*	95	[16-17]
IVD	1IVH	3-methylbutyryl-CoA	607036	*IVD*	22	[18]
SBCAD	2JIF	(s)-2-methylbutyryl-CoA	600301	*ACADSB*	7	[19]
IBD	1RX0	isobutyryl-CoA	604773	*ACAD8*	18	[9]
ETF	1EFV	reduced acyl-CoA dehydrogenases	608053	*ETFA*	13	[20-21]
			130410	*ETFB*	5	
ETF-QO	2GMJ	ETF	231675	*ETFDH*	21	[22-23]

Accession numbers: Protein Data Bank
[a] Number of missense mutation, data extracted from The Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/index.php (accessed 30.08.10).
n.a. not available

2.1.1. Medium-chain acyl-CoA dehydrogenase

MCAD is the most studied enzyme of the β-oxidation, and is responsible for the oxidation of fatty acids with a chain length between 6 to 10 carbons

[24]. In 1986 Matsubara and colleagues allocate the *ACADM* gene to chromosome 1p31 [25]. As most of the mitochondrial proteins MCAD is nuclear encoded and synthesized in the cytosol as a precursor protein of 47 kDa, the N-terminal leader peptide is cleaved after the import into mitochondria producing a mature protein of 44 kDa [26-28].

Native MCAD is formed by the assembly of 4 monomers, and each monomer is folded into three domains [29]. The N- and C-terminal domains are mainly α-helices packed together and the middle domain is formed by two β-sheets (Fig. 2.2). The flavin cofactor is positioned in the cleft between the β-domain and the C-terminal domain of one subunit and the C-terminal domain of the neighboring subunit. The soluble flavoprotein has a glutamic acid in position 376 as a catalytic residue, which is responsible for the α-proton abstraction of the fatty acid [12].

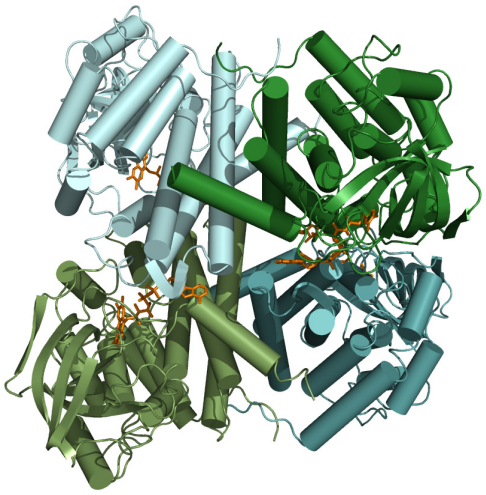

Figure 2.2: Representation of the structure of human MCAD. Protein structure is represented as cartoon with helices represented as cylinders and beta-strands as arrows, cofactors are represented as orange sticks, and each subunit is represented by a different color. The structure was obtained from the Protein Data Bank (PDB: 1ege).

2.1.2. Short-chain acyl-CoA dehydrogenase

The *SCAD* gene, located on chromosome 12q22, encodes a cytosolic precursor SCAD that is transported to mitochondria where the N-terminal

mitochondrial targeting peptide is proteolytically cleaved [30-32]. *In vitro* studies have shown that SCAD has two preferred substrates, hexanoyl- and butyryl-CoA. However, it is generally assumed that its physiological role is specific to butyryl-CoA, as *in vivo* none of the other ACDHs are active with this substrate [33].

The crystal structure of SCAD from rat was determined, and reveals an overall fold very similar with the other ACDHs [14]. A special feature of this acyl-CoA dehydrogenase is the presence of a glutamine in position 254 and threonine in position 364 that seem to shorten the substrate binding pocket, contributing to its substrate specificity [34].

2.1.3. Very long-chain acyl-CoA dehydrogenase

In the 1990's a enzyme specific for the oxidation of long-chain fatty acids was identified in rat liver [35], and one year later human VLCAD deficiency (VLCADD) (MIM 201475) was reported [36-38]. This enzyme catalyzes the first step of the β-oxidation of long-chain fatty acids with a chain length of 14 to 18 carbons [39]. *ACADVL* gene was mapped to chromosome 17p11.2-11.13 [40-41].

The crystal structure of this enzyme in complex with C14-CoA showed that the N-terminal domain has a fold similar to soluble ACDHS, and that the substrate binding pocket extends for an additional 12 Å in relation to the MCAD pocket, allowing it to bind to long chain substrates [10]. The authors also proposed that the disordered region in the crystal structure, from residues 441 to 476, is optimally positioned in the novel C-terminal domain for membrane interaction (Fig 2.3) [10].

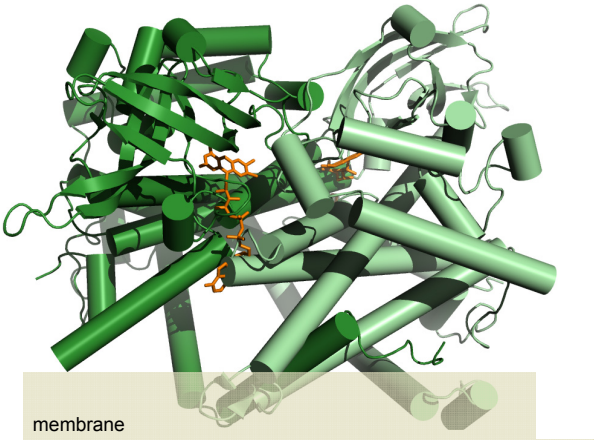

Figure 2.3: Representation of the structure of human VLCAD. Protein structure is represented as cartoon with helices represented as cylinders and beta-strands as arrows, cofactors are represented as orange sticks. The structure was obtained from the Protein Data Bank (PDB: 3b96).

2.2. The ETF and ETF-QO hub

2.2.1. Electron transfer flavoprotein (ETF)

In 1956, Crane and Beinert described a flavoprotein that was essential for oxidation of another flavoenzyme that oxidized fatty acyl derivatives of CoA [42]. At that time the authors decided to name that "new" protein electron transferring flavoprotein since it serves to transfer the electrons gained by the dehydrogenase to a conventional electron acceptor like indophenol, ferricyanide or oxygen.

In 1975 Hall and Kamin reported the purification, biochemical and spectroscopic properties of ETF and several dehydrogenases from pig liver mitochondria [43]. ETF was described as containing two subunits and two FAD mol per mol and a global molecular weight of 58 kDa [43]. Seven years later Thorpe *et al* purified ETF from pig kidney and reported for the first time that ETF binds only one FAD per dimer [44]. A subsequent report on the purification of pig liver ETF corroborated this finding establishing that in fact mammalian ETF has only one FAD per dimer [45]. Only in 1993 it was

uncovered that ETF has an additional AMP-binding site, and that this cofactor has no influence in the activity of the protein [46]. Moreover, it seems that the AMP cofactor is important for the assembly of the holo structure [47].

In parallel to this structural and biochemical characterization, it was found that both ETF subunits are nuclear encoded, the α-subunit is synthesized as a precursor protein of 35 kDa, while the β-subunit is synthesized in cytosol in a form that is indistinguishable from the mitochondrial form [48]. The α-subunit precursor sequence is cleaved after import into the mitochondria yielding a mature form with 32kDa [48]. In this work, abnormal α-ETF synthesis was for the first time described as the primary lesion causing MADD (see below). The cDNA encoding the α and β human ETF was subsequently cloned, and the genes mapped to chromosomes 15q23-q25 and 19q13.3, respectively [49-50].

The crystal structure of human ETF was solved to 2.1 Å resolution, reveling that ETF consists of three distinct domains: domain I is composed of the N-terminal portion of α-subunit; domain II, consists of C-terminal portion of α-subunit and a small C-terminal portion of the β-subunit, and; domain III is made up from the majority of the β-subunit (Fig. 2.4) [20]. The flavin cofactor is in the cleft between the two subunits, mainly interacting with the C-terminal portion of the α-subunit (Fig. 2.4). Moreover, the C7 and C8 of the methyl groups in the dimethylbenzene ring make van der Waals contact with Tyr16 and Phe41 from the β-subunit. The AMP cofactor is buried deeply within domain III, making mostly backbone interaction (Fig. 2.4). The phosphate moiety of the cofactor forms hydrogen bounds with residues Ala126, Asp29, Asn32, Gln33 and Thr34 from the β subunit.

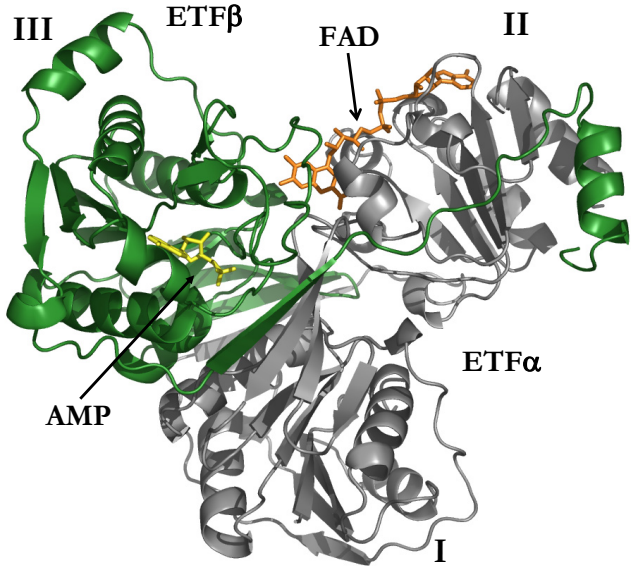

Figure 2.4: Ribbon representation of the structure of human ETF. FAD (orange) binds in a cleft formed by the α (grey) and β (green) subunits. The AMP (yellow) is located entirely within the β subunit. The structure was obtained from Protein Data Bank (PDB: 1efv).

ETF function is described in several reports has an electron acceptor for several dehydrogenases of fatty acid β-oxidation and for amino acid and choline catabolism [51]. Reduction of ETF by dehydrogenases results in the rapid formation of an anionic semiquinone, but its further reduction is slower [52-54]. The reoxidation of ETF is catalyzed by the membrane-bound iron-sulfur flavoprotein ETF-QO [55] (see below). The redox potential for human ETF are 37mV and 25mV for the oxidized/semiquinone and semiquinone/hydroquinone couple, respectively [21].

The crystal structure of ETF:MCAD complex was resolved and revealed a dual mode of protein-protein interaction, showing both specificity and promiscuity in the interaction with the different dehydrogenases [56]. The authors identified an anchor region, the "recognition loop", which is centered on the conserved residue Leu195 and docked to a hydrophobic patch on the

dehydrogenase, and a highly dynamic region on the ETF FAD domain, allowing fast interprotein electron transfer (Fig. 2.5).

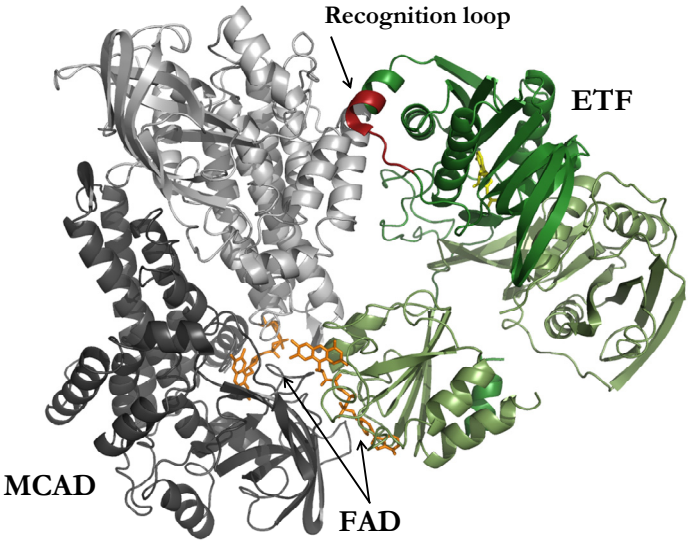

Figure 2.5: Structure of MCAD:ETFβ-Glu165Ala complex. Protein are represented by ribbon cartoon, ETF shown as green (α and β subunits shown as light and dark green, respectively), and MCAD shown as grey. Only a dimmer of MCAD and one ETF molecule were presented for clarity. The recognition loop of ETF is highlighted as red. FAD and AMP are shown as orange and yellow sticks, respectively. The structure was obtained from Protein Data Bank (PDB: 2A1T).

ETFs homologous are found in all kingdoms of life, and participate on electron transfer reactions in a variety of metabolic pathways. ETFs belong to the family of α/β-heterodimeric FAD-contaning proteins [57-59]. Based on sequence homology and functional types the members of this family can be clustered in different groups. Group I comprises mammalian and also a few bacterial ETFs, while group II contains proteins from nitrogen-fixing and diazotrophic bacteria [60]. Group I ETFs are usually physiologically electron acceptors of several dehydrogenases, and electrons flow to a membrane-bound ETF ubiquinone oxidoreductase. A well studied homologue of mammalian ETF is the ETF from the bacterium *Paracoccus denitrificans* [61-63].

In group II the α-ETF and β-ETF are homologous of FixB and FixA proteins, respectively [64]. Moreover, they receive electrons from the usual dehydrogenases, and also from ferredoxin and NADH [65]. It is postulated that the electron pathway to dinitrogen is via ETF:ferredoxin oxidoreductase, ferredoxin, nitrogenase reductase and nitrogenase [64]. The best studied ETF of this group is from the bacterium *Methylophilus methylotrophus* strain W3A1. Another well study ETF is the one from the anaerobe *Megasphaera elsdenii,* which is atypical, as it contains two FAD-binding sites and no AMP cofactor [58, 66].

2.2.2. Electron transfer flavoprotein ubiquinone oxidoreductase (ETF-QO)

ETF-QO is the link between several mitochondrial oxidative processes and the respiratory chain. Briefly, ETF-QO was first purified by Ruzicka and colleagues, who characterized it as a monomeric protein containing a [4Fe-4S] cluster and a FAD cofactor [67]. The ETF-QO gene has been mapped to chromosome 4q32-q35 [68], and its cDNA cloned and characterized [69].

The 66 kDa protein can be fully reduced by chemical reductants, such as dithionite, but its two electron reduction is only achieved in the presence of catalytic amounts of ETF [55]. The reduction potential of ETF-QO from pig liver mitochondria was determined using an EPR-spectroelectrochemical cell and dithionite as a reductant [23]. The reduction potential values were 28 mV, -6 mV and 47 mV, for first and second electron transfer to FAD and for the iron-sulfur cluster, respectively [23].

The crystal structure has revealed that the iron-sulfur cluster is closer to the protein surface while FAD molecule is closer to the ubiquinone, therefore it was postulated that the redox cluster is responsible for accepting electrons

from ETF and the flavin cofactor for reducing ubiquinone (Fig. 2.6) [22]. This proposed mechanism is supported by the recent work of Swanson on the determination of redox potential of *Rhodobacter sphaeroides* ETF-QO mutants [70]. The mutations had no impact on the reduction potential for the iron-sulfur cluster, but significantly decreased the first reduction potential of FAD. Lowering the midpoint potentials resulted in a decrease in the quinone reductase activity and negligible impact on disproportionation of ETF-semiquinone catalyzed by ETF-QO.

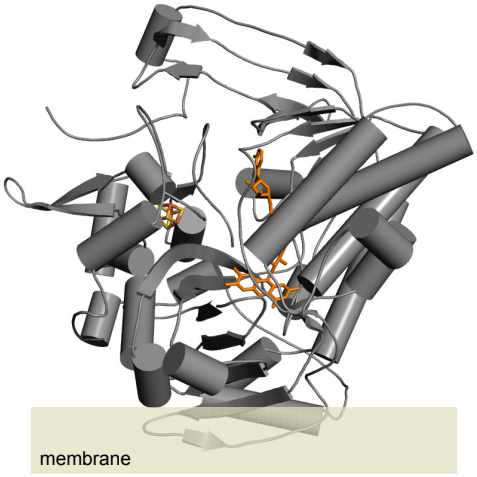

Figure 2.6: Representation of the structure of pig ETF-QO. Protein structure is represented as cartoon with helices represented as cylinders and beta-strands as arrows, cofactors are represented as sticks. The structure was obtained from the Protein Data Bank (PDB: 2gmj).

2.3. Fatty acid oxidation disorders

The disorders associated with the enzymes and transport proteins of the pathway described in the sections above are commonly denominated by fatty acid oxidation (FAO) disorders. The first report on these disorders dates to 1973, DiMauro and DiMauro, described CPT II deficiency [71]. Two years later, Karpati *et al.* described CAT deficiency [72], and in 1976 Gregersen *et al* reported MCAD deficiency [73] and Przyrembel *et al* reported multiple acyl-CoA dehydrogenase deficiency (MADD) [74]. Up until now defects in 18 enzymes of the pathway have been identified [4]. FAO disorders are inherited

autosomal recessive disorders with a wide spectrum of symptoms. In the beginning diagnosis was based only on metabolite profiles and no direct enzymatic studies or genetic analysis could confirm the exact enzyme alteration. More recently fatty acid oxidation defects are diagnosed via clinical, biochemical, pathological studies and mutational analysis of the genes encoding the suspected enzyme deficiencies [75].

Symptoms, which in some cases only manifest during episodes of metabolic crisis, are associated with energetic deficiency in tissues that depend on fatty acid oxidation. Other problems arise from the accumulation of intermediates of the pathway as these species may have toxic properties. In mild cases, disease symptoms can be triggered by prolonged fasting, exercise, infection, exposure to cold, or a fat-rich diet [4, 76].

Since the mid 1990s FAO disorders are part of the newborn screening programs in many countries, allowing an early diagnosis and treatment, decreasing morbidity and mortality. The newborn screening uses tandem mass spectrometry (MS/MS) to determine acylcarnitine profiles in blood spots allowing the identification of enzyme-specific anomalies in fatty acid oxidation [24].

In the following subsection a brief description of a selection of FAO disorders will be presented, namely MADD, MCADD, VLCADD and SCADD.

2.3.1. Multiple acyl-CoA dehydrogenase deficiency

Glutaric aciduria type 2 (GA2) (MIM 231680) was first described in 1976, and at that time the name was given because glutarate was described as the major urinary metabolite, in comparison to glutaric aciduria type 1 in which glutarate and its metabolites accumulate [74]. In 1980, Goodman and colleagues reported a new case where the amino and organic acids

abnormities were consistent with a defect in the flavoproteins which transfer electrons from the dehydrogenases to the respiratory chain, so the disorder should be renamed as "multiple acyl-CoA dehydrogenase deficiency" (MADD) [77]. Four years later a report suggested that the cause of MADD would be a defect in either ETF or ETF-QO [78], and this was confirmed by Frerman and Goodman through the immunological analysis of fibroblasts from five patients [79].

MADD is an autosomal recessively inherited disorder, and patients are affected in at least 12 mitochondrial flavoenzyme dehydrogenase activities, since their common flavin-dependent electron pathway to ubiquinone in the electron transport chain is partially or fully blocked. Over fifty-disease causing mutations are reported: 21 mutations on *ETFA*, 8 mutations on *ETFB* and 35 mutations on *ETFDH* genes, most of which are missense mutations (data extracted from The Human Gene Mutation Database accessed 30.08.2010) [80].

MADD clinical picture is rather heterogeneous, ranging from neonatal lethal forms (severe MADD or S:MADD) to later-onset forms with a milder clinical course and symptoms that may be ameliorated by high doses of riboflavin (mild MADD or M:MADD) [81-82]. Patients with the severe form can either present or not congenital anomalies. In the first case infants are often premature, and during the first 24-48 hrs of life present hypotonia, hepatomegaly, hypoglycemia, metabolic acidosis and sweaty feet odor. Usually kidneys are often palpably enlarged and cystic, and the patient can present facial dysmorphisms and rocker-bottom feet [83]. In the case of neonatal onset without congenital anomalies infants develop problems within the first few days of life, and the few patients who survive the first week die within a few months usually with severe cardiomyopathy. In the case of mild MADD the symptoms are extremely variable and characterized by recurrent episodes

of lethargy, vomiting, hypoglycemia, metabolic acidosis, and hepatomegaly often preceded by metabolic stress [83]. In some cases muscle involvement in the form of pain, weakness, and lipid storage myopathies has been reported.

The diagnosis of MADD is established by plasma acylcarnitines and urine organic acid and acylglycine profiles, but solely based on these data, the severity of the phenotype can not be established (Table 2.2). The diagnosis must be subsequently confirmed by enzymatic assays in fibroblasts cultures, and in some countries by molecular genetic analyses. A correlation between clinical phenotypes and both enzymatic defects and molecular genotypes as been suggested [83-84].

Table 2.2: Acylcarnitines that occur at elevated levels in MADD

	Acylcarnitine	MADD
Group I – almost always elevated levels in all MADD patients		
C8:0	octanoyl	Up
C10:0	decanoyl	Up
C12:0	dodecanoyl	Up
Group II – may have elevated levels (in severe forms/metabolic decompensation)		
C4	Butyryl	Up
C5	Isovaleryl	Up
C5-DC	Glutaryl	Up
C6:0	hexanoyl	Up
Group III – very rarely elevated levels (only in very severe forms/metabolic decompensation)		
C12:1	Dodecenoyl	Up
C14:0	tetradecanoyl	Up
C14:1	Tetradecenoyl	Up
C14:2	Tetradecadienoyl	Up
C16:0	Palmitoyl	Up
C18:0	Octadecanoyl	Up
C18:1	Linoleoyl	Up
C16:1	hexadecenoyl	Up

Treatment of MADD include avoidance of fasting, a diet low in fat and proteins and high in carbohydrates. In some cases additional supplements of

riboflavin and L-carnitine can be given. These treatments have been more successful when applied to patients with milder forms of the disease [85].

2.3.2. Medium-chain acyl-CoA deficiency

MCAD deficiency (MCADD) (MIM 201450) is in general associated to missense mutations on *ACADM* gene that compromise protein folding affecting the native conformation but not the thermal stability of the tetramer [86]. It is the most common defect of β-oxidation in humans, with a frequency in the population estimated at 1/15,000 [87]. The most prevalent mutation is a change of a lysine in position 304 for a glutamic acid (Lys304Glu), resulting in protein misfolding and aggregation [88-89].

The enzymatic deficiency results in a decreased ketone production and in an increase in medium chain fatty acids. Episodes of fasting or fever, commonly result in low appetite, hypoglycaemia and energy deficiency develop due to the rapid exhaustion of glycogen reserves. Usually patients are considered normal at birth, then around 3-24 months of age, acute decompensation may occur in response to prolonged or intercurrent fasting and common infections [24]. The disorder is characterized by a broad spectrum of symptoms that can range from hypoglycaemia to seizure, coma and sudden death.

Although, unexpected death during the first metabolic decompensation is common, the prognosis is excellent once the diagnosis is established, especially if detected by newborn screening before onset of symptoms [90-91].

2.3.3. Very-long chain acyl-CoA deficiency

VLCADD is considered as one of the most common β-oxidation

disorders, it results from mutations in the *ACADVL* gene and therefore patients have accumulation of C14 to C18 acylcarnitines. Patient with VLCADD can have a rather wide range of symptoms, so according to the phenotype the disorder can be classified in three different categories: a neonatal form, with cardiomyopathy, liver disease and myopathy; a childhood onset form, characterized by hypoketotic hypoglycemia; and a late-onset form with recurrent rhabdomyolysis and myoglobinuria [92].

Early diagnosis is highly important since the disease can be rapidly fatal due to cardiac or hepatic involvement. Clinical treatment include low-fat diet supplemented with medium-chain triglycerides and fasting precautions [93]. Until now around one hundred mutations have been described in the literature, and although a general correlation between genotype-phenotype has been reported [92], the molecular basis of VLCADD is very complex and genotypic analysis alone remains limited to the prediction of fatal cases [94-95].

2.3.4. Short–chain acyl-CoA deficiency

SCAD deficiency (SCADD) (MIM 201470) is less frequent, and results from multiple mutations or two common coding polymorphisms, which diminish or completely abolish SCAD enzymatic activity [96]. Impaired butyryl-CoA oxidation results in accumulation of butyrylcarnitine, butyrylglycine, ethylmalonic (EMA) acid, and methylsuccinic acid in blood, urine and cells [97]. The first case associated with SCAD was reported in 1984 by Turnbull and co-workers [98], but only in 1987 Amendt *et al* described a SCADD patient case, confirmed by enzymatic assays in skin fibroblasts [99]. The main features of the disorder are neuromuscular symptoms such as developmental delay, hypotonia, and seizures [86, 100].

More than one hundred patients have been reported, and although in the past the phenotypes were relatively severe now most patient are diagnosed through newborn screening and remain asymptomatic [33]. Moreover, the SCAD p.Gly209Ser variant, which has a mild effect on protein folding/stability [101], is found in the general population with a homozygous frequency of 7% but is over-represented in patients with ethylmalonic aciduria, a marker for SCAD deficiency (60%), indicating that other environmental and genetic factors contribute to disease development [94, 97, 102]. Therefore, there is still on going debate on whether the genetic variation accounts for the disorder or whether it is a simple result of polymorphic variation [94].

2.4. Riboflavin, a pharmacological chaperone

2.4.1. Riboflavin metabolism and deficiency

Riboflavin is widely used as therapy in different FAO disorders, therefore a brief description of its metabolism and of the known therapeutic effects will be presented. Riboflavin, or vitamin B2, was first described in the late 19th century by A. Wynter Blyth in a paper for the *Transactions of the Chemical Society*, where it was called 'lacto-chrome'. Approximately fifty years later, due to the need to clarify the nomenclature, the compound was renamed riboflavin. This designation derives from its ribityl side chain and yellow color (Fig. 2.7) [103]. Vitamin B2 is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Fig. 2.7), two very important protein cofactors present in a broad range of flavoenzymes, including oxidases, reductases and dehydrogenases [104].

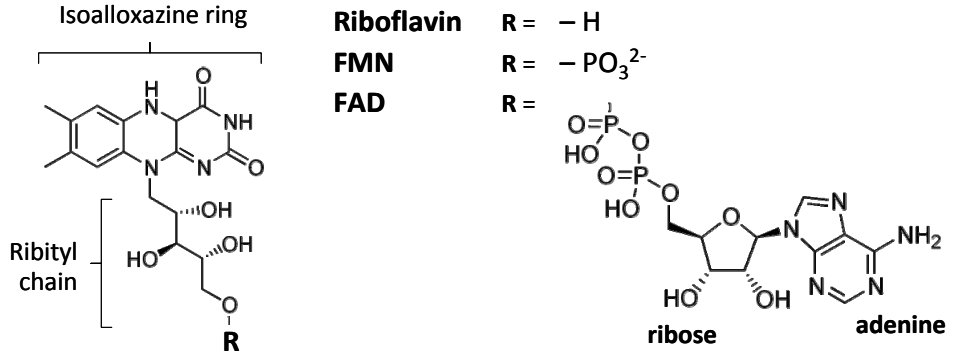

Figure 2.7: Chemical structures of riboflavin and its derivates FMN and FAD.

Vitamin B2 intake is made through regular diet, it is ingested in a protein bound form, or already as FAD and FMN in flavoproteins. The major sources of vitamin B2 are eggs, milk, some meat products, yeast and vegetables [105]. The normal intake for an adult is 1.6-2.6 mg/day, and deficiency symptoms are rarely observed with a normal diet [105]. The vitamin or its derivatives are released by stomach acid and via the action of gastric/intestinal proteases. FMN and FAD are sequentially hydrolysed to riboflavin by alkaline phosphatases and FMN/FAD pyrophosphatase [106]. The free riboflavin is transported into the enterocyte by an energy-dependent and sodium independent riboflavin carrier, probably the recently identified riboflavin transporter 2 (RFT2) [107]. After release to the hepatic portal vein, riboflavin is taken up by the hepatocyte riboflavin transporter which is regulated by intracellular Ca^{2+}/calmodulin [106]. Inside the cell, riboflavin is then phosphorylated by ATP and flavokinase to FMN, which is converted to FAD by FAD synthetase also in the presence of ATP (Fig. 2.8). The conversion of FMN and FAD can also occur inside the mitochondria, as this organelle also contains riboflavin kinase and FAD synthetase enzymes [106]. Free FAD can be incorporated in mitochondrial apoflavoproteins or, it can be carried by the Flx1p FAD carrier to the cytosol (Fig. 2.8) [108-109].

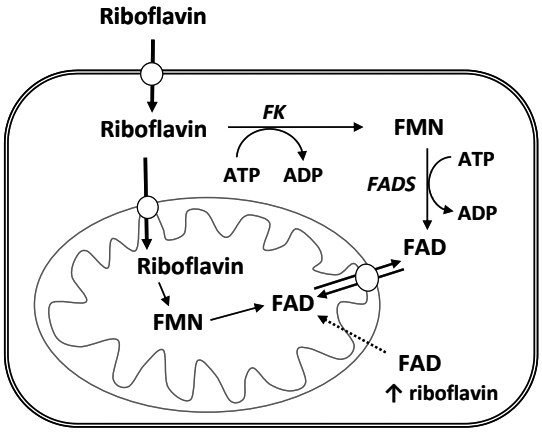

Figure 2.8: Riboflavin cellular processing pathways. Riboflavin is imported into the cell and later into the mitochondria via specific transporters (white circles in membranes). In the cytoplasm, Flavin kinase (FK) and FAD synthetase (FADS) consecutively convert riboflavin into FMN and FAD, at the expense of ATP. An identical mechanism is also thought to be present inside the mitochondria, although a mitochondrial FK remains to be identified. FAD can also be imported into the mitochondria, or diffuse passively when the riboflavin concentration is high.

Riboflavin is present, at least in small amounts, in all animal tissues. In humans the main storage site for riboflavin, mostly in the form of FAD, is the liver; it is also stored in spleen, kidney and cardiac muscle, so these organs and tissues are naturally protected against riboflavin deficiency. In the circulating plasma there is an equilibrium between the three species: riboflavin (50%), FAD (40%) and FMN (10%), where the total flavin concentration is 30 nM [106].

A diet low in riboflavin or an error in its metabolic pathway would result in low FAD and FMN contents in the cell, reducing the activity of a series of enzymes [110-113]. Different cellular systems can be affected directly or indirectly by lower amounts of riboflavin and several studies report the cellular consequences of reduced levels of riboflavin. In fact, the acyl-CoA dehydrogenases involved in the first dehydrogenation step of fatty acid oxidation (FAO) are among the first flavoenzymes to be affected during moderate riboflavin deficiency [114-117]. Reports using rodent models have shown that the major impact of moderate riboflavin deficiency is at the level of mitochondrial β-oxidation, due to the impairment of enzymatic activity of

the acyl-CoA dehydrogenases [114, 116]. Certain ACDHs have been shown to be specifically sensitive – namely short chain acyl-CoA dehydrogenase (SCAD) and isovaleryl-CoA dehydrogenase (IVD) [118-119]. Research work by the Tanaka laboratory in the 1990s has suggested that FAD regulates the ACDHs at different stages: fatty acid oxidation enzymes are regulated at the level of gene expression, and all ACDHs have an improvement of the ACDHs m-RNA translation, and an increase in stability of the mature protein [120].

Another type of mitochondrial disorders that seem to benefit from riboflavin supplementation are deficiencies involving enzymes from the respiratory chain, namely complex I (NADH:quinone oxidoreductase) and complex II (Succinate:quinone oxidoreductase). Bernsen and colleagues reported for the first time an improvement of enzymatic activity of complex I after riboflavin supplementation on a 6 year old boy with myopathy and pure motor neuropathy [121]. Later, additional cases were described and riboflavin supplementation was shown to improve the clinical condition of patients with myopathy and encephalomyopathy [122]. Riboflavin treatment also restored cognitive and motor development in a girl with mitochondrial myopathy associated with impaired NADH-dehydrogenase activity [123]. One report describes three patients which were treated with riboflavin for 4 to 6 years and whose clinical features did not improve, although disease progression was halted. In these cases, the activity of complex II increased 2-fold in fibroblast culture supplemented with 0.2 $mg.ml^{-1}$ riboflavin [124]. Another case of complex II deficiency, a 10 month old boy with severe neurological features showed improvements after riboflavin intake and by the age of 5 only a moderate psychomotor delay was observed [125]. Thus, riboflavin supplementation in mitochondrial disorders associated with complex II deficiencies thus appears to at least reduce disease progression.

Tests in HepG2 liver cells have revealed that a deficient riboflavin medium causes oxidative stress and cell damage, as decreased glutathione reductase activity, increased protein carbonylation (a process associated with oxidative damage), and increased DNA strand breaks are detected [126]. In addition, riboflavin deficiency impairs oxidative folding: in yeast it is known that Ero1, a protein involved in the control of disulfide formation during oxidative folding in the ER, is FAD dependent [127]; in human lymphoid cells cultured under flavin-deficient conditions, interleukin-2 accumulates intracellularly, probably due to an impairment of the human homologue of Ero1 [128]; and in flavin deficient human liver cells, it causes ER stress, activation of the unfolded protein response and decreased secretion of apoB which may interfere with lipid homeostasis *in vivo* [129]. Riboflavin deficiency can also affect heme biosynthesis via protoporphyrinogen oxidase, a FAD dependent enzyme of the pathway [106, 130]. It may also be involved, in some cases of anaemia [131] and other biological processes as reviewed in [132].

2.4.2. FAO disorders responsive to riboflavin

For most FAO disorders vitamin B2 supplementation is not suggested as first treatment approach, however some clinical reports have shown the beneficial effects of this vitamin. Duran and co-workers measured MCAD activities in lymphocytes of five MCAD deficient patients before and after a three weeks treatment with riboflavin (50-150 mg/day). All five MCAD deficient patients have at least double their MCAD activity. The smallest improvement registered was from 5 to 12% of control mean after treatment, however one of the patients had his MCAD activity increased to heterozygote levels. The clinical response and primary gene defects were not reported [133]. In another study involving eight patients with MCAD deficiency, no

effect on fibroblast MCAD enzyme activity was detected upon supplementation with 20 μM FAD [134]. Amendt and co-workers measured *in vitro* FAD response in fibroblasts from three patients with defects of long-chain fatty acid acyl-CoA dehydrogenation. Long-chain dehydrogenation activities in mitochondrial extracts were increased from 17-21% of controls to 27-36% of controls upon addition of 20μM FAD and the authors suggested merit of therapeutic riboflavin trials in patients [135].

Three papers have reported riboflavin responsiveness in patients with short-chain acyl-CoA dehydrogenase (SCAD) deficiency. In one paper the genetic defects were not addressed [136], but on the other two papers riboflavin responsiveness was associated to the p.Gly209Ser / c.625G>A SCAD variant [137-138]. Subclinical or biochemical riboflavin deficiency is quite common in the general population [139] and moderate riboflavin deficiency is known to selectively impair mitochondrial fatty acid oxidation by decreasing the activities of the flavin-dependant acyl-CoA dehydrogenases, in particular short-chain acyl-CoA dehydrogenase [115, 117]. Thus, mitochondrial flavin content and consequently riboflavin status could be a contributing factor to the development of SCAD deficiency. Recently van Maldegem and co-workers assessed the FAD status and evaluated the effect of riboflavin treatment in 16 patients with SCAD deficiency [138]. Blood FAD levels were normal in all patients before riboflavin supplementation therapy, but significantly lower in homozygous or heterozygous patients harboring the c.625G>A variation, as compared to heterozygous patients harboring two rare SCAD mutations. The common c.625G>A variation may be an example of a diet-gene interaction where a sizeable percentage of the population (7%) has a higher riboflavin requirement because of a polymorphic flavoenzyme as suggested by Ames *et al* [140]. However, even though van Meldegem and co-workers found the c.625G>A variant to be

associated with low FAD status, only a subgroup of patients (4/13) actually responded biochemically and clinically to the treatment with riboflavin. So the authors did not recommend riboflavin as a general treatment in SCAD deficiency [138].

In contrast to disorders of single fatty acid oxidation flavoenzymes like SCAD, MCAD and VLCAD, where riboflavin treatment has been tried only in single cases with appropriate deficiency states, riboflavin therapy is used as a general treatment in patients with multiple acyl-CoA dehydrogenase deficiency [141].

A certain group of MADD patients – the so-called riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency (RR-MADD) patients – is spectacularly responsive to riboflavin supplementation with clinical and biochemical abnormalities being normalized or near normalized after treatment.

The first patient, described in 1982 by Gregersen *et al.*, was a boy with episodic vomiting, lethargy and hypoglycemia during early childhood. He was diagnosed at 3 years of age during a Reye's syndrome-like crisis with metabolic acidosis, hepatomegaly and muscle hypotonia [142]. Since then, several further cases have been reported with heterogeneous clinical symptoms; mainly with encephalopathy or muscle weakness or a combination of these and often preceded by cyclical vomiting. In the late teenage years or adulthood these patients may develop a severe progressive proximal muscle weakness with lipid storage and secondary carnitine deficiency. In its most severe forms RR-MADD patients may end up being bound to the bed or a wheelchair and the acute metabolic crisis can be lethal [51, 143-144]. Since high levels of riboflavin can cure these symptoms and prevent lethal attacks, correct and timely diagnosis is important.

RR-MADD patients have metabolic profiles compatible with classical

MADD or ethylmalonic-adipic aciduria caused by genetic defects of ETF or ETF-QO, and also, in many cases, have decreased activities of single flavoenzymes, including acyl-CoA dehydrogenases and components of the respiratory chain, and decreased mitochondrial flavin content; all of which can be improved or corrected by high doses of riboflavin [51, 143-148]. These abnormalities resemble those found in rats fed with a riboflavin-deficient diet [117], and because they are not easily explained by ETF/ETF-QO mutations, it was for many years thought that RR-MADD patients had genetic defects of mitochondrial flavin metabolism or transport rather than defects of the *ETFA*, *ETFB* and *ETFDH* genes.

Today we know that mutations of ETF-QO are the most common cause of RR-MADD. This was established in 2007 when ETF-QO mutations were identified in all RR-MADD patients from 11 pedigrees [51]. At the same time Gempel *et al* reported ETF-QO mutations in a group of patients with the myopathic form of coenzyme Q10 deficiency, who had biochemical profiles consistent with RR-MADD [149]. Since then the molecular defect in RR-MADD has been confined to the *ETFDH* gene in a number of cases [144, 150-154] and to the *ETFA* gene in a single case [154] suggesting that RR-MADD may be caused by mutations in any of the three genes forming this flavin-dependent electron transfer pathway. At least one of two mutations, in all of these RR-MADD patients, are of the missense type and none of the 40 different missense mutations have been reported in classical MADD patients indicating that they are unique to RR-MADD.

Although RR-MADD in a large fraction of patients is clearly associated with ETF-QO mutations, the role of these mutant proteins on secondary mitochondrial dysfunction - with impaired function of multiple acyl-CoA dehydrogenases, respiratory chain complexes and coenzyme Q10 - remains controversial [94]. As low mitochondrial concentrations of flavins have been

observed in some patients with RR-MADD [145-147] it has been argued that the global changes of mitochondrial proteins could be due to genetic or environment related disturbances of flavin homeostasis. It could also be argued that the mitochondrial dysfunction is secondary to the ETF-QO mutations themselves.

New data show that MADD cells with null mutations of ETF/ETF-QO reduce oxidative phosphorylation and increase aerobic glycolysis, the consequences of which is a proliferative, apoptosis resistant phenotype, controlled by cell proliferative signaling pathways like the PPARG-ERK pathway [155]. Obviously, one could suggest that a similar metabolic switch accounts for the mitochondrial protein changes observed in pre-treated muscle samples from RR-MADD patients.

Although it might seem that MADD and RR-MADD share a similar metabolic adaptation to the genetic deficiencies of ETF/ETF-QO there are differences: the nonketotic hypoglycemic phenotype of classical MADD reflects the metabolic switch to glycolysis accompanied by decreased gluconeogenesis and decreased acetyl-CoA production as a consequence of genetic blockage of fatty acid oxidation and perhaps redirection of the pyruvate generated in glycolysis away from acetyl-CoA production and to lactate production. However, RR-MADD patients often show normal glucose levels and ketosis [51] reflecting that the acetyl-CoAs that are produced during β-oxidation of fatty acids are mostly used for ketone bodies. Moreover, dysfunction of respiratory chain components, including coenzyme Q10, seems to be variably deficient in different RR-MADD patients [146, 149-150]. Moreover the specific activities of FAO acyl-CoA dehydrogenases appear normal in MADD fibroblasts whereas they are decreased in muscle samples from RR-MADD patients, with SCAD and MCAD activities being more impaired than VLCAD activity, in most cases [156-158]. It remains to

be clarified if these observed differences are due to differences in the technical set-ups or whether they truly reflect different cell pathological responses to ETF-QO mutations. Oxidative stress and/or toxic metabolites have been suggested to be the link between ETF-QO mutations and general mitochondrial dysregulation [51, 94]. However, the exact trigger(s) of the metabolic switch and the implicated molecular pathways and mechanisms remain elusive.

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3 MUTATIONAL HOTSPOTS IN ELECTRON TRANSFER FLAVOPROTEIN UNDERLIE DEFECTIVE FOLDING AND FUNCTION IN MULTIPLE ACYL-COA DEHYDROGENASE DEFICIENCY

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3.1. Summary

We have carried out an extensive *in silico* analysis on 18 disease associated missense mutations found in electron transfer flavoprotein (ETF), and found that mutations fall essentially in two groups, one in which mutations affect protein folding and assembly, and another one in which mutations impair catalytic activity and disrupt interactions with partner dehydrogenases. We have further experimentally analyzed three of these mutations, ETFβ-p.Cys42Arg, ETFβ-p.Asp128Asn and ETFβ–p.Arg191Cys, which have been found in homozygous form in patients and which typify different scenarios in respect to the clinical phenotypes.

The ETFβ-p.Cys42Arg mutation, related to a severe form of multiple acyl-CoA dehydrogenase deficiency (MADD), affects directly the AMP binding site and intersubunit contacts and impairs correct protein folding. The two other variations, ETFβ-p.Asp128Asn and ETFβ–p.Arg191Cys, are both associated with mild MADD, but have a different impact on ETF. Although none affects the overall α/β fold topology as shown by far-UV CD, analysis of the purified proteins shows that both have substantially decreased enzymatic activity and conformational stability.

Altogether, this study combines *in silico* analysis of mutations with experimental data and has allowed establishing structural hotspots within the ETF fold that are useful to provide a rationale for the prediction of effects of mutations in ETF.

3.2. Introduction

Mutations in the *ETFA, ETFB* or *ETFDH* genes, which encode the two subunits of Electron Transfer Flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETF-QO), respectively, cause multiple acyl-CoA dehydrogenase deficiency (MADD), a congenital metabolic disease with a

broad clinical expression [1]. The systematic application of newborn screening programs has allowed for a wider coverage of MADD genotypes that range from missense or nonsense mutations to frameshifts.

One of the proteins affected in this disorder is ETF, a key enzyme in the mitochondrial fatty acid β-oxidation and amino acid degradation pathways, which transfers electrons from at least 12 dehydrogenases via ETF-QO to the respiratory chain. As in many other metabolic disorders, clear genotype-phenotype relationships remain elusive in MADD: in particular for milder disease forms, it is expected that the mutant proteins are partially functional and that cellular and environmental factors such as temperature and cofactors can modulate disease expression [2]. Progress in this issue has however also been constrained by the fact that only a very limited number of mutations has been characterized upon recombinant expression of the variant proteins. However, substantial steps forward can be achieved if the available data on clinical mutations are merged with a structural and functional analysis of the affected protein.

In this paper we have investigated for mutational hotspots on the ETF structure by analysing the protein for particularly susceptible regions in which MADD-related ETF variations detected in patients map. Complementary experimental validation for the putative mutational hotspots identified was subsequently obtained by carrying out a biochemical and biophysical analysis of purified ETF variants with representative disease-causing mutations. The data gathered by this strategy contribute to a better understanding of the molecular factors underlying functional deficiency and may, in the long term, provide a framework for the design of novel therapies.

3.3. Material and methods

Chemicals

All reagents were of the highest purity grade commercially available. Octanoyl-CoA, FAD, AMP and urea were purchased from Sigma. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from VWR International.

Structural Analysis

The crystallographic structures of human ETF (PDB code: 1efv, [3]) and ETF:MCAD complex (PDB code: 1T9G, [4]) were inspected using PyMOL (DeLano Scientific). Analysis of the molecular interactions, cofactor contacts, topological features and generation of models for the variants was carried out using the WhatIF web server [5] and the PDBsum database [6]. The PolyPhen (Polymorphism Phenotyping) web server was used to predict the effect of amino acid variations on protein structure and function [7]. The input for this server was the UniProt accession number (ETFα: P13804 and ETFβ:P38117), sequence position and the two amino acid variants characterizing the desired mutation.

ETF expression in *E. coli* JM109 cells

The *pK-lac-ETF-αβ3*(C/T) plasmid encoding both ETF subunits (here named pWt), described previously [8], was used as DNA template in the production of the two novel mutants, the ETFβ–p.Cys42Arg and ETFβ–p.Arg191Cys. The mutants were generated using the Quick change mutagenesis kit (Stratagene) as recommended by the manufacturer. Mutagenic primers were 5'-CCATGAACCCCTTC**C**GTGAGATCGCGGTGG-3' for the ETFβ–p.Cys42Arg variant, and 5'-GAGGCTCAACGAGCCC**T**GCTACGCCACGCTG-3' for the

ETFβ–p.Arg191Cys mutant (altered bases are underlined and in bold type). The second mutagenic primers required were the reverse complements of those shown. After PCR screening the sections containing the mutation were sequenced to exclude PCR errors and subcloned back into the original plasmid. The ETFβ–D128N mutant, also used in these studies, was already available [9].

E. coli JM109 cells transformed with the respective plasmid, pWt, pC42R, pD128N, pR191C, were grown in LB (Luria–Bertani) supplemented with 10 $\mu g.ml^{-1}$ kanamycin at 30ºC or 37ºC in a shaking incubator until OD_{532} of 0.5-0.8 was reached. The cells were than induced with 1 mM IPTG for 4 hours. Cells were harvested by centrifugation, re-suspended in 10 mM Hepes, 10% ethylene glycol and 0.5 mM phenylmethylsulphonylfluoride (Roth) in presence of DNase (Applichem) and disrupted in a French press. The soluble extract was obtained by centrifugation at 12,000 g for 30 minutes and used for the ETF activity assays [10] and for SDS-PAGE followed by western blotting analyses according to [11]. An ETF polyclonal antibody raised in rabbits against purified recombinant wild type ETF protein was used to identify ETF proteins and visualized with peroxidase conjugated secondary antibody reaction with 1-step TMB-Blotting (Pierce).

Co-expression of molecular chaperones with ETFβ–p.Cys42Arg

Different conditions were used to improve ETFβ–p.Cys42Arg mutant expression, always using LB as growth medium. First, *E. coli* cells transformed with pC42R plasmid were expressed at 30ºC and 18ºC. Induction was made with 1 mM IPTG.

Further, JM109 *E. coli* cells were co-transformed with the pC42R plasmid and a molecular chaperone plasmid, pOFX-BADSL2 and pOFX-BADKJE2, respectively for the expression of GroES/EL complex and dnaK, dnaJ and

GrpE [12]. Cells transformed with the ETFβ–p.Cys42Arg and with pOFX-BAD2 empty plasmid, control vector, or with the plasmid encoding the respective molecular chaperone system, were grown in LB supplemented with 10 $\mu g.ml^{-1}$ kanamycin and 10 $\mu g.ml^{-1}$ tetracycline at 30ºC and 18ºC until OD_{532} of 0.5-0.8 was reached. Cells were then induced with 0.2% arabinose and 30 minutes later with 1mM IPTG. Samples were withdrawn at 1, 3, 6 and 20 hours after the induction with IPTG. Cells were harvested by centrifugation and treated with Bug Buster solution (Novagen), soluble and insoluble fractions were analysed by SDS-PAGE and western blot analysis. Soluble fractions were also used for ETF activity assays [10].

Protein purification and biochemical assays

Soluble extract from 3L culture growth of wild type, ETFβ-p.Asp128Asn and ETFβ-p.Arg191Cys mutants, was applied on a Q-Sepharose fast flow column (GE Healthcare, 20 ml) previously equilibrated in 10 mM Hepes, 10% ethylene glycol and 0.5 mM phenylmethylsulphonylfluoride (buffer A). The column was washed with 5 volumes buffer A, and bound proteins were eluted by a linear gradient of 0 - 1M NaCl in buffer A. Pure ETF was eluted at a salt concentration around 250 mM, and purity was confirmed by SDS/PAGE. Protein concentration was determined using the Bradford assay. Flavin content was determined using the molar extinction coefficient ε^{436nm}=13400 $M^{-1}.cm^{-1}$ reported for FAD bound to ETF [13]. ETF enzyme activity was measured following 2,6-dichlorophenolindophenol (DCPIP) reduction at 600 nm in a coupled assay in which recombinant human MCAD and octanoyl-CoA were employed, as described in [10]. The unit of activity measurements is defined as nmol of DCPIP reduced per minute, in the conditions used in the assay. All specific activities reported are based on total flavin content. Pure ETF fractions with a 2.5 fold molar excess FAD were

fast-frozen using liquid nitrogen and stored at -80ºC.

Spectroscopic methods

Before each experiment FAD excess added to buffers as a preservative was removed by extensive washing using ultra filtration/dilution, and all experiments were performed with pure proteins containing full occupancy of FAD site.

UV/visible spectra were recorded at room temperature in a Shimadzu UVPC-1601 spectrometer with cell stirring. Fluorescence spectroscopy was performed using a Cary Eclipse instrument. For tryptophan emission excitation wavelength was set at 280 nm, and FAD emission was followed setting excitation wavelength at 436 nm; slits were 5 and 10 nm for excitation and emission, respectively. Typically protein concentration was 1 μM. CD spectra were recorded on a Jasco J-815 spectropolarimeter with a cell holder thermostatically controlled with a Peltier. A quartz polarized 1 mm path length quartz cuvette (Hellma) was used, and protein concentrations were typically 0.1 $mg.ml^{-1}$.

Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed on a BIAcore™ 2000 Instrument using NTA sensor chip (BIAcore, Inc.). The NTA sensor chip is designed to bind histidine tagged molecules after nickel activation of the surface. The surface was prepared as recommended by the manufacturer. The running buffer consisted of 10 mM Hepes, pH 7.4, 10% ethylene glycol, 50 mM NaCl, 0.005% Tween 20. Recombinant human MCAD with a histidine tag was immobilized in flow cell 2. The dehydrogenase was captured by manually injecting 25μl of a 200 nM solution at a flow rate of 5 $\mu l.min^{-1}$. Flow cell 1 was used to correct for refractive index

changes and nonspecific binding. ETF (ETF-WT, ETFβ–p.Asp128Asn or ETFβ–p.Arg191Cys) in 50 nM solutions was injected over flow cells 1 and 2 at a flow rate of 40 μl.min^{-1}, during 90 seconds. All SPR experiments were done at 25ºC.

Thermal stability

Thermal unfolding with a linear temperature increase was followed using circular dichroism (ellipticity variation at 222 nm) and fluorescence spectroscopy, tryptophan emission (λ_{ex} =280 nm; λ_{em} =340 nm), FAD emission (λ_{ex} =436nm; λ_{em} =530 nm) and FRET from tryptophan emission to FAD cofactor (λ_{ex} =280 nm; λ_{em} =530 nm). In all experiments, a heating rate of 1ºC.min^{-1} was used, and temperature was increased from 30 to 90ºC. Data were analysed according to a two-state model, and fits to the transition curves were made using OriginPro8.

Chemical stability

The denaturation curves were measured diluting ETF (ETF-WT, ETFβ–p.Asp128Asn or ETFβ–p.Arg191Cys) in different urea concentrations and the tryptophan emission spectrum was recorded after 15 minutes of incubation at 25°C. Transition curves were determined plotting the average emission wavelength against urea concentrations. Data were analysed according to a two-state model, and fits to the transition curves were made using OriginPro8.

3.4. Results and Discussion

Mutational hotspots in the ETF structure

A number of genetic defects in the *ETFA/ETFB* gene account for MADD. Most of the variations detected in patients are of the missense type, although frameshift and nonsense mutations are also described [14]. As the effect of modifying a single amino acid in ETF can be rather broad with respect to clinical expression, the possibility to predict whether a point mutation in a particular region of the protein would affect the folding, stability or activity, would be a potentially very useful parameter to establish genotype-phenotype relationships. In order to fill this gap we have set to define a structural mapping of mutations in ETF. For this purpose our strategy was twofold.

First, we have compiled a list of known disease-associated missense mutations described for human ETF, whose predicted effects were analysed using a combination of *in silico* tools for mutagenesis predictions and structural analysis (Table 3.1).

Secondly, we have analysed the ETF crystal structure (Fig. 3.1) in respect to specific structural factors that, if affected by mutations, are expected to perturb the structural integrity or the biological activity of the protein [3-4]. In this respect, we have analysed: i. positions that correspond to AMP-protein interactions; ii. residues that are involved in H-bond connections at the ETFα/ETFβ contact surface; iii. highly conserved segments throughout ETF ortologues; iv. amino acids involved in FAD binding or in the stabilization of the FAD moiety, and; v. positions involved in interactions with medium-chain acyl-CoA dehydrogenase (MCAD), both the anchor loop and the FAD domain. At this stage positions involved in the interaction with ETF-QO were not possible to analyze since there is no structural information available on the ETF:ETF-QO complex.

Table 3.1 : Mutations in ETF related to MADD.
The PSIC score is the position-specific independent count parameter computed by PolyPhen.

	PSIC score	Structural rationale	Ref
ETF α			
p.Leu95Val	0.085	Near inter subunit H-bonded region.	[15]
p.Gly116Arg	2.667	Changes at buried site: hydrophobicity, overpacking and charge.	[16-17]
p.Arg122Lys	1.507	Change at buried site hydrophobicity. Closest contact is ETFβ-Gln146. Affects subunits interface.	[18]
p.Phe144Ser	1.781	Changes at buried site: hydrophobicity and cavity creation.	[18]
p.Val157Gly	2.346	Closest contact is ETFβ-Ser223. Cavity creation. Increase in flexibility. Affects subunits interface.	[19]
p.Val165Ala	1.373	Near inter subunit H-bonded region.	[18]
p.Leu212Pro	2.798	Change at buried site hydrophobicity.	[18]
p.Arg249Cys	3.472	Disruption of FAD binding site. Stabilizes FAD semiquinone. Contacts with MCAD.	[18]
p.Gly255Val	2.988	Maps at FAD interacting region.	[20]
p.Thr266Met	2.834	Disruption of FAD binding site. Hydrophobicity change at buried site. Contacts with MCAD.	[9, 16-17]
p.Gly267Arg	2.982	Maps at FAD interacting region. Charged residue inserted in turn. Contacts with MCAD.	[20]
ETF β			
p.Ala17Pro	1.612	Maps at FAD and AMP interacting regions. Maps nearby ETFα-Ile284.	[21]
p.Cys42Arg	3.343	Disruption of AMP binding site. Changes at buried site: overpacking, hydrophobicity and charge.	[18, 22]
p.Asp128Asn	2.438	Charge change at buried site. Maps at AMP interacting regions. Maps nearby ETFα-Tyr149.	[9, 23]
p.Arg164Gln	2.492	Charge change at buried site. Near inter subunit H-bonded region. Maps nearby ETFα-Asn118.	[24]
p.Arg191Cys	3.515	Removal of charged residue. Contacts with MCAD.	[18]

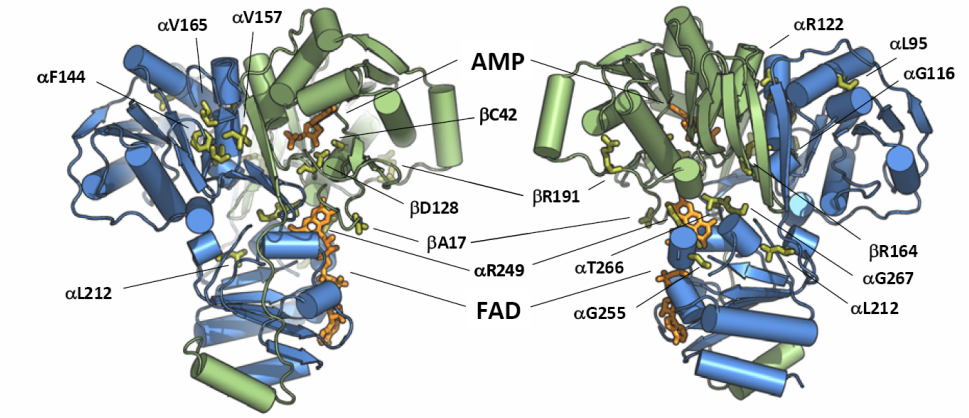

Figure 3.1 – Cartoon of human ETF structure (PDB: 1efv) highlighting the analysed mutations. Details on mutations are listed in Table 3.1, the two figures have been rotated 180° in respect to each other along the y-axis. Figure prepared using Pymol.

Merging the data obtained by these two complementary approaches shows that particular regions of the ETF structure are more susceptible to deleterious modifications than others and the clustering of the known mutations in those structure segments is rather suggestive of structural hotspots for MADD mutations (Fig 3.1 and 3.2).

This is clearly noted on ETFα, in which mutations map essentially in two regions: one corresponding to the region involved essentially in interactions with the ETFβ subunit (Glu91-Val165), and the other a conserved region rich in FAD and MCAD interacting residues and inter-subunit interactions (Ala244-Thr295).

Clearly mutations in the first region may result in folding defects due to poor dimer assembly or protein destabilisation, as changes in these positions modify the chemistry of buried sites and may result in cavity creation. The latter may arise if an amino acid with a bulkier side chain is replaced by another with a smaller volume (e.g. ETFα-p.Val157Gly) or if a charged residue replaces a hydrophobic one (e.g. ETFα-p.Gly116Arg) (Table 3.1).

On the other hand, mutations in the second region have an impact on

catalysis. This occurs either by disturbing directly the FAD protein interactions or the second coordination sphere ligands, which are important modulators of the redox properties of the cofactor. In fact, the chemical and catalytic properties of the flavin are tightly controlled by the polypeptide, not only at the level of the residues involved in direct close-range interactions (i.e. within the first coordination sphere), but also by those which are further away from the organic cofactor but that are nevertheless connected to it through hydrogen-bond networks or other weak interactions (i.e. second coordination sphere). Also, this segment comprises positions of the FAD domain that are known to be involved in complex formation with MCAD [25]. Therefore, mutations in this region are expected to affect mostly the biological activity of the protein, although concurrent destabilization may also occur.

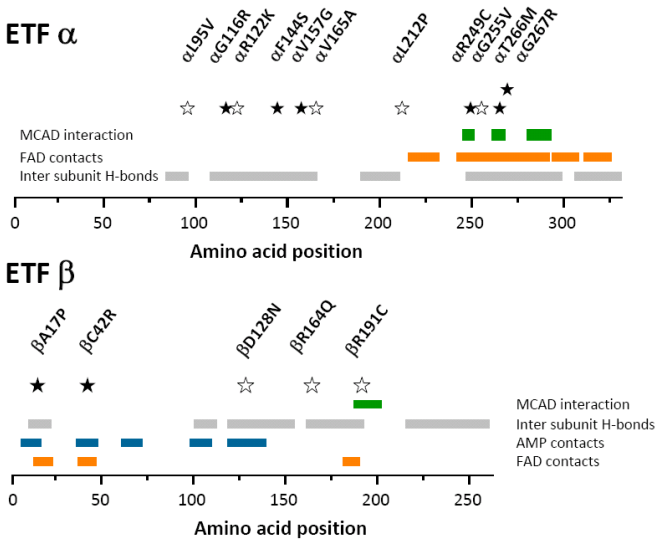

Figure 3.2 – Structural features mapped on ETF sequence. Analysis of the ETF structure (PDB: 1efv) allowed the identification of the regions involved in FAD and AMP binding, MCAD interactions, and H-bonding between the ETFα and ETFβ subunits, which are represented as boxed segments in respect to the primary sequence positions. Stars denote MADD mutations [14] and are filled according to the clinical phenotype: severe forms (filled stars) and mild (open stars).

In respect to the ETFβ subunit, the scenario is not so clear as a result of

the fact that only 5 missense mutations are known in this subunit. Nevertheless, some highly susceptible regions can be defined: those involving two N-terminal segments of ETFβ which harbour simultaneously FAD and AMP binding residues (ETFβ Arg5-Ile20 and ETFβ Val34-Ala45), a region rich in AMP-protein interactions (ETFβ Gly123-Thr134), and segments involved in interactions with partner proteins, namely the recognition peptide within the anchor domain that constitutes the primary interaction site of partner proteins with ETF (ETFβ Asp184-Lys200) [4], and also a short segment centred around ETFβ–Glu165, an essential residue that destabilises positions compatible with fast inter-protein electron transfer, thus ensuring high complex dissociation rates [25], a strict requirement for subsequent interaction of reduced ETF with ETF-QO (Fig. 3.1 and 3.2).

Analysis of prototypic mutations

In order to provide complementary experimental validation of the putative mutational hotspots identified in ETF, three mutations in ETFβ were selected for subsequent analysis: ETFβ–p.Cys42Arg, ETFβ–p.Asp128Asn and ETFβ–p.Arg191Cys. With the exception of ETFβ–p.Asp128Asn [9, 23], none of these mutations had been previously expressed and purified for a thorough *in vitro* functional and structural characterization. All three mutations were detected in patients homozygous for the respective mutations allowing to relate the results to the phenotypes. Also, this selection fills a gap in respect to the characterisation of mutations in the ETFβ subunit, in which a lower number of MADD related mutations have been described compared to ETFα.

Although an all-embracing division cannot be established, these mutations typify different functional and folding effects. To better predict the structural

effect of these individual mutations we have built molecular models of these variants by homology modelling with respect to the wild type protein (Fig. 3.3).

The ETFβ–p.Cys42Arg mutation, associated to a severe clinical expression, is expected to severely affect the interaction with the AMP cofactor, which plays a key role in the assembly of the ETF dimer [26-27].

The ETFβ–p.Asp128Asn modification, which occurs at an outer layer of the FAD interacting moieties, has been shown to affect the plasticity of the tertiary structure and decrease directly the specific activity [23], and its mutation likely affects a stabilizing H-bond to ETFβ–Lys11 (Fig. 3.3).

Finally, the ETFβ–p.Arg191Cys mutation is hypothesised to affect catalysis by impairing interaction with the partner dehydrogenase, as this residue is localized in the anchor domain. Also, analysis of the model of the mutant variation shows that removal of ETFβ–Arg191 may destabilize the fold by disrupting electrostatic interactions with the nearby ETFβ–Glu47, ETFβ–Arg51 and ETFβ–Glu54 (Fig. 3.3).

The patients with the latter two mutations display mild forms of the disease, indicating that the modified conformation is nevertheless able to fold and assemble as a dimer, retaining some catalytic proficiency.

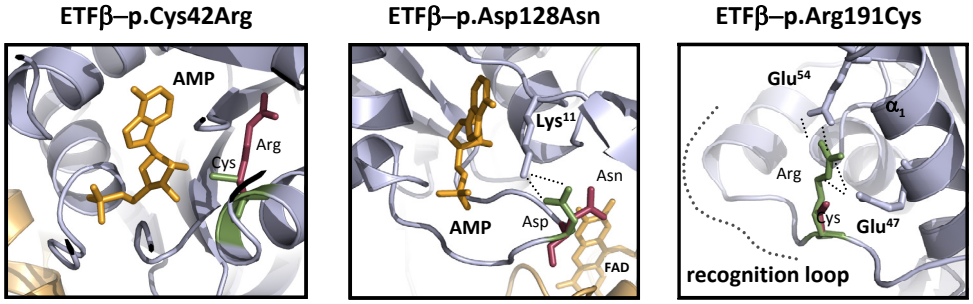

Figure 3.3– Structural details around the mutated positions The cartoons represent magnifications of the ETF structural regions in which the MADD variations are identified, overlaying the mutated and the original residues, as well nearby important. Straight dotted lines represent hydrogen bonds. Structures prepared using PyMOL.

Mutations affect folding efficiency

The effect of each mutation on the efficiency of protein folding was investigated by heterologous expression of the different variants and differential analysis of the soluble versus insoluble protein produced, in respect to that of wild type ETF and comparing cell cultures with standardized induction protocols displaying superimposable cell growth characteristics. Therefore, any difference strictly results from distinct expression levels of the heterologously expressed protein. Since it has been previously demonstrated that expression of ETFβ-p.Asp128Asn was improved upon cell growth at 30ºC [9], over expression of all the analysed ETF variants was carried out both at 30 and 37°C (Fig. 3.4). The results obtained show that while wild type ETF and the mild mutant variants (ETFβ–p.Asp128Asn and ETFβ–p.Arg191Cys) were well expressed as soluble proteins, the severe phenotype mutation (ETFβ–p.Cys42Arg) was not (Fig. 3.4).

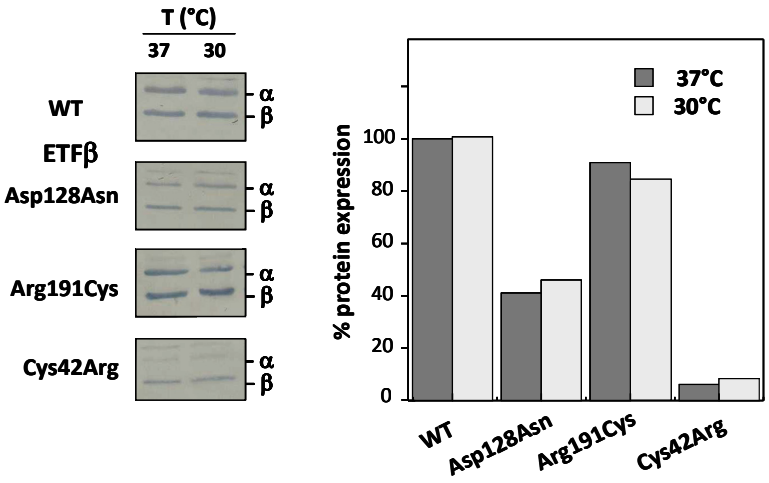

Figure 3.4 – Expression levels of ETF variants at 30 and 37°C. Expression of ETF variants at 30 and 37°C was quantified from Western Blot analysis (left) and expressed in a bar graph (right) in respect to that of the wild type protein. See text and materials and methods for details.

The expression level of ETFβ–p.Arg191Cys was 87% of that of the soluble protein detected in the wild type whereas that of ETFβ–p.Asp128Asn was decreased to 48%. This suggests that while the expression of the variant ETFβ–p.Arg191Cys is almost unaffected, the ETFβ–p.Asp128Asn modification has a destabilizing effect on the folding process, making it rather susceptible to misfold and aggregate, or be degraded. This observation during cell expression agrees with the data obtained *in vitro* using purified ETFβ–p.Asp128Asn for which a higher proteolytic susceptibility was determined [23].

On the other hand, expression of the ETFβ–p.Cys42Arg variant yielded only 10% of ETF protein in the soluble fraction, indicating that this mutation affecting the AMP binding severely impairs ETF folding and assembly. Further expression assays lowering the *E. coli* growth temperature to 18ºC in an attempt to further improve conditions for *in cell* folding did not change the scenario, and even at this low temperature almost all the ETFβ–p.Cys42Arg

protein expressed went to the insoluble fraction. This result agrees with western blot analysis of skin fibroblasts from a patient carrying this mutation that showed no detectable ETF protein [18, 22].

Molecular chaperones partly rescue ETFβ–p.Cys42Arg folding

An approach for the rescue of folding defects resulting from disease-causing mutations is that of stimulating the so called proteostasis network [28]. This involves recruiting the protein quality control machinery and/or the action of small molecule substrates and cofactors as effectors of the folding process. In order to investigate if molecular chaperones could rescue the defective folding resulting from the ETFβ–p.Cys42Arg mutation, we have carried out co-expression experiments with the chaperonins GroEL and GroES (homologs of the human mitochondrial Hsp60 and Hsp10 chaperone system), and the chaperone dnaK (homolog of mammalian Hsp70s) and its cofactors dnaJ and GrpE. This strategy has been shown to result in the effective rescue of proteins with folding defects in other cases [17, 29-31].

The results obtained show that the molecular chaperones could in fact partially rescue the protein to the soluble fraction (Fig. 3.5). However, enzymatic activity assays on the soluble fractions showed no detectable ETF activity, indicating that although molecular chaperones successfully rescue some soluble conformations these are still be incorrectly folded and/or with defective cofactor insertion: presumably these soluble forms are still substantially destabilised and poor incorporation of FAD and/or deficient interaction with the partner dehydrogenases impair activity.

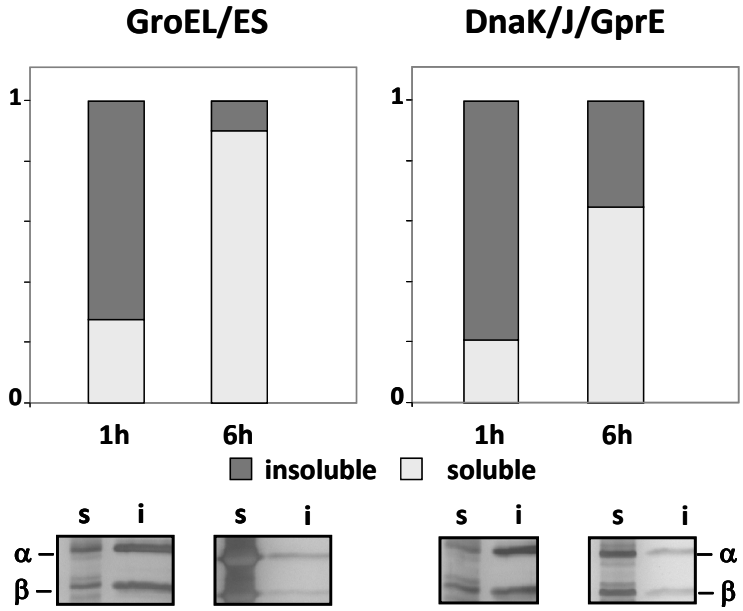

Figure 3.5 – Effect of molecular chaperones on the expression of soluble ETFβ-p.Cys42Arg. The amount of the ETFβ-p.Cys42Arg variant expressed in the soluble (s) and insoluble (i) form was quantified from western blots of cell extracts after inducing for 1 and 6h the co-expression of the molecular chaperones GroEL/ES (left) or DnaK/J/GrpE (right). The bar graph shows the relative fraction of soluble (light grey) and insoluble (dark grey) protein. See text and materials and methods for details.

Structural and conformational properties of the ETFβ–p.Asp128Asn and ETFβ–p.Arg191Cys variant proteins

The ETFβ–p.Arg191Cys variant was purified to homogeneity and spectroscopically characterised. The ETFβ–p.Asp128Asn was also purified and characterized as described in [23] and the spectroscopic data from this variant were redrawn from published data [23] in order to allow a direct comparison with the new variant described. The protein fold and secondary structure were analysed using far-UV circular dichroism (Fig. 3.6). The spectra obtained are typical of folded proteins with a α/β fold, and with the clear α-helix fingerprint with minima at 208 and 222nm.

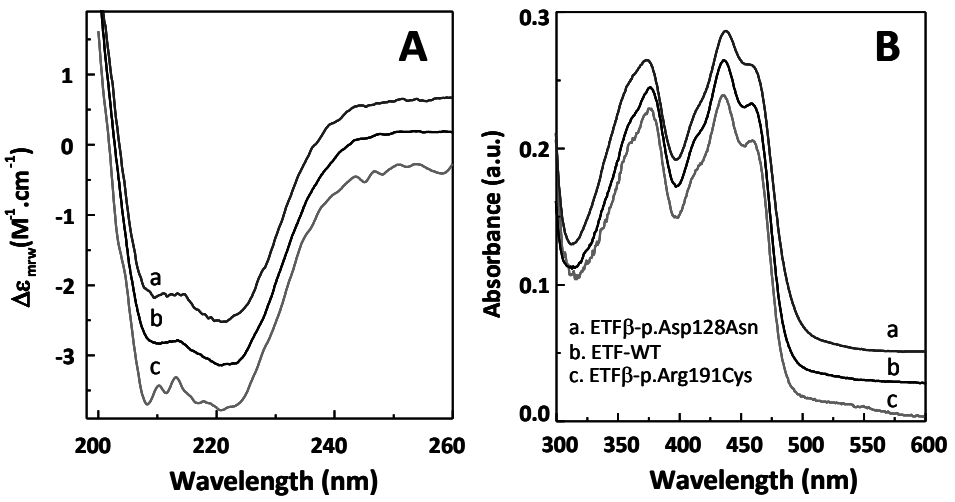

Figure 3.6 - Spectroscopic analysis (A) far-UV CD (B) Visible absorption. The different variants were analysed in respect to their far-UV CD (A) and visible absorption (B) spectra (the legend is common for both panels): a. ETFβ-p.Asp128Asn, b. ETFβ–WT, c. ETFβ–p.Arg191Cys. Spectra were slightly offset for clarity of comparison.

The mutant variants have superimposable spectra with respect to that of wild type ETF, indicating that the mutations do not disrupt the protein fold nor the secondary structure. Tryptophan emission spectra, which are indicative of tertiary structure interactions, were also recorded. ETF contains two tryptophan residues, and the fluorescence emission properties of this amino acid ranges typically from 315 to 350 nm and are related to solvent accessibility: a more solvent exposed tryptophan will emit at higher wavelengths. The results obtained show that both mutants have 10 nm red-shifted maxima of the emitting band, with maximum emission at 330 nm versus 320 nm of the wild type ETF. This suggests that the structure of the mutant variants is not as constrained as that of the wild type ETF and that they displays a more dynamic tertiary structure. The spectroscopic fingerprint of the catalytic FAD cofactor was also analysed using visible absorption spectroscopy, which is very sensitive to changes in the H-bound interaction

network around the cofactor. From the results obtained we could conclude that the environment of the functional cofactor does not change significantly in any of the mutants, as the two characteristic absorption bands with typical emission maxima at around 376 nm and 436 nm, are retained.

Overall, these results indicate that the ETFβ–p.Asp128Asn and ETFβ–p.Arg191Cys variants retain the fold and FAD interactions identical to those of the wild type protein but the differences in tryptophan fluorescence properties suggest decreased compactness and structural constraints in comparison to the wild type [23]. The latter aspect may have an impact on the enzymatic activity of the mutants as it likely contributes to a less efficient interaction with the partner acyl-CoA dehydrogenases and to increased protein instability.

Protein instability and MCAD interactions underpin functional deficiency

The specific activity of the ETFβ–p.Asp128Asn and ETFβ–p.Arg191Cys variants was only 30% of that of the wild type protein (400 U/mg *vs.* 1300 U/mg), which shows that, in addition to the lower folding efficiency, these mutant variants also display a decreased specific activity [23]. Since the mutant proteins retain structural features identical to those of the wild type protein under physiological conditions, we have investigated if differences in stability and interaction with the partner dehydrogenases could play a role. The interaction between the ETF variants and the medium chain acyl-CoA dehydrogenase (MCAD) was investigated in preliminary experiments by surface plasmon resonance, using MCAD immobilized in a NTA-sensor chip.

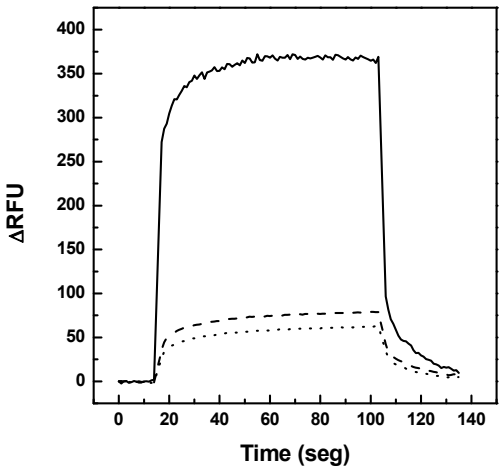

Figure 3.7 – Sensograms showing interaction of wild type ETF (—), ETFβ-Asp128Asn (– – –) and ETFβ-Arg191Cys (⋯) to MCAD. ETF variants in 50 nM solutions were injected over flow cells 1 and 2 at a flow rate of 40 μl.min^{-1}. The sensograms represented are the result of RFU intensity in flow cell 2 minus RFU intensity of flow cell 1.

The results obtained showed that the ETFβ–p.Asp128Asn and ETFβ–p.Arg191Cys variants interact with MCAD, as an increase in the resonance signal is observed; however, the magnitude of the interaction at equilibrium was only 20-30% of that observed for wild type ETF, which is suggestive of a weaker interaction (Fig. 3.7).

The effect of the β–p.Asp128Asn and ETFβ–p.Arg191Cys mutations on the conformational stability was also investigated spectroscopically using far-UV CD and fluorescence. Thermal denaturation of the proteins was monitored by following secondary and tertiary structure from far-UV CD and tryptophan and FAD fluorescence emissions (Fig. 3.8A). All the techniques used yielded similar transition curves for each of the proteins analysed, showing that all methods are monitoring the same unfolding event with no noticeable intermediates formed. The analysis showed the same order of relative stabilities of the proteins involved: ETF-WT > ETFβ–p.Arg191Cys > ETFβ–p.Asp128Asn. The thermal stability studies showed that wild type ETF unfolds at an apparent midpoint unfolding temperature (T_m) of 60 ºC, and that ETFβ–p.Arg191Cys is just slightly destabilised (ΔT_m=-3°C, T_m = 57

°C), whereas ETFβ–p.Asp128Asn exhibits a more significant destabilization (ΔT_m =-8°C, T_m = 52 °C) (Fig. 3.8A).

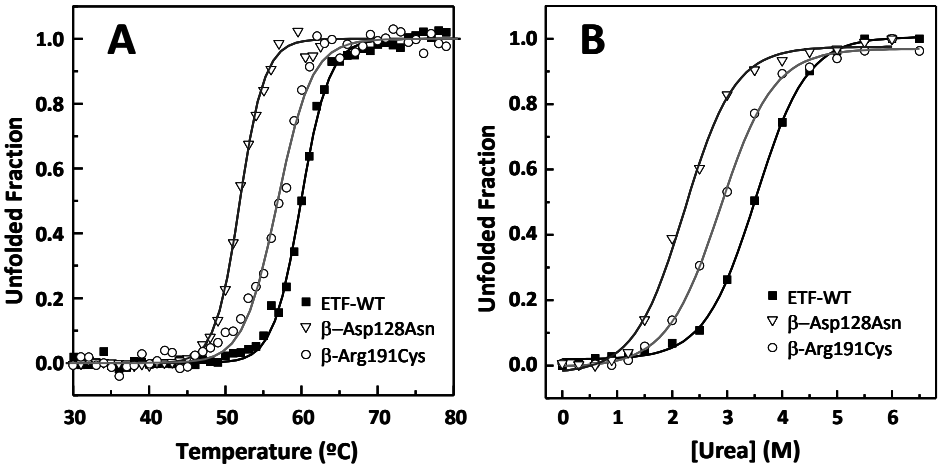

Figure 3.8 – Stability profiles of ETFβ-p.Asp128Asn and ETFβ–p.Arg191Cys variants
The thermal (A) and chemical (B) denaturation profiles of the ETFβ-p.Asp128Asn (inverted triangles) and ETFβ–p.Arg191Cys (open circles) were compared in respect to that of wild type ETF (closed squares). The solid curves represent two-state sigmoid curves from which the apparent midpoint denaturations were determined: melting temperatures (T_m) of 52, 57 and 60 °C and midpoint denaturant concentrations (C_m) of 2.2, 2.9 and 3.9 M urea for ETFβ–p.Asp128Asn, ETFβ–p.Arg191Cys, and ETF-WT, respectively. See text and materials and methods for details.

Chemical denaturation was followed using tryptophan emission, and the curves allowed the determination of apparent midpoint urea concentrations (C_m). The obtained values were 2.2, 2.9 and 3.9 M for ETFβ–p.Asp128Asn, ETFβ–p.Arg191Cys, and ETF-WT, respectively (Fig. 3.8B). Again, the order of stabilities was the same as in thermal denaturations indicating that the ETFβ–p.Asp128Asn variation has a stronger destabilizing effect than ETFβ–p.Arg191Cys. In fact it was already described that ETFβ–p.Asp128Asn exhibits some plasticity in tertiary structure and decreased specific activity as a function of time during incubation at 39°C, in an *in vitro* mimic of a fever episode (see next chapter) [23].

These differences among the studied variants can be rationalised from the molecular models made for the ETFβ–p.Asp128Asn and ETFβ–p.Arg191Cys variants (Fig. 3.3). The replacement of ETFβ–Asp128 propagates to the AMP coordination sphere by affecting an hydrogen bond between Asp128 and Lys11, therefore substantially destabilising the protein fold, as shown by thermal and chemical unfolding studies (Fig. 3.8). Likewise, the ETFβ–p.Arg191Cys variation also impacts on the conformational stability of the fold as removal of ETFβ–Arg191 disrupts salt bridges with Glu47 and Glu57 at the nearby α1 helix. This case illustrates how a mutation in a functional region can also have an impact on overall protein stability.

3.5. Conclusion

In this work we have analysed the effects of missense mutations in ETF which correspond to different clinical presentations in MADD patients, combining *in silico* studies with experimental biophysical and biochemical data. Overall, this study showed that ETF mutations map to structural hotspots with respect to subunit interaction, cofactor binding and interaction with partner proteins, and this provides a framework that allows to predict more accurately the probability for a given mutation to affect protein function. Variations leading to severe phenotypes do not map to a particular structural region and a direct correlation with the clinical display can not be generalised, a general problem that has hampered the prediction of mutation effects for many disease-relevant proteins. However, it appears that amino acid changes that lead to drastic alterations of the chemical properties of residues involved in catalytic regions and subunit interactions have a higher probability to result in severe effects. Among these is ETFβ-p.Cys42Arg which results in a protein that is unable to fold: such mutations will be clearly associated with severe

clinical phenotypes. Other mutations will affect folding, cofactor binding, and assembly to different degrees, and most of them will be associated with mild phenotypes. The degree of impairment in respect to the clinical display is rather difficult to predict, as different effects can be observed: cofactor binding, interaction with partner proteins and conformational stability. A key aspect arising from our study is the fact that conformational instability of dynamic substructures may be transferred to other parts of the protein, affecting the protein as a whole [32-34]. That is for example the case of ETFβ-p.Asp128Asn variant, in which the modification of a residue which has long range interactions with the cofactor, increases cofactor lability, decreases protein stability and interaction with the partner dehydrogenase. Likewise, the ETFβ–p.Arg191Cys variation directly affecting the recognition loop also decreases protein stability, as removal of the positively charged arginine will disrupt electrostatic interactions with the acidic residues within helix α_1 (Fig. 3.3). This effect then propagates to the whole structure, affecting its conformational stability. The observations made for ETF represent a scenario, which is also relevant for many other proteins, as it underpins the necessity to perform and carefully evaluate experimental expression of clinical protein variants in order to collect more data that will contribute to establish general principles that can be used to more accurately predict the functional outcome of genomic variations in metabolic disease.

3.6. Acknowledgments

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4 ROLE OF FLAVINYLATION IN A MILD VARIANT OF MULTIPLE ACYL-COA DEHYDROGENASE DEFICIENCY: A MOLECULAR RATIONALE FOR THE EFFECTS OF RIBOFLAVIN SUPPLEMENTATION

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4.1. Summary

Mutations in the genes encoding the α-subunit and β-subunit of the mitochondrial electron transfer flavoprotein (ETF) and the electron transfer flavoprotein:ubiquinone oxidoreductase (ETF-QO) cause multiple acyl-CoA dehydrogenase deficiency (MADD), a disorder of fatty acid and amino acid metabolism. Point mutations in ETF, which may compromise folding, and/or activity, are associated with both mild and severe forms of MADD.

Here we report the investigation on the conformational and stability properties of the disease-causing variant ETFβ-Asp128Asn, and our findings on the effect of flavinylation in modulating protein conformational stability and activity. A combination of biochemical and biophysical methods including circular dichroism, visible absorption, flavin, and tryptophan fluorescence emission allowed the analysis of structural changes and of the FAD moiety. The ETFβ-Asp128Asn variant retains the overall fold of the wild type, but under stress conditions its flavin becomes less tightly bound. Flavinylation is shown to improve the conformational stability and biological activity of the destabilized variant protein. Moreover, the presence of flavin prevented proteolytic digestion by avoiding protein destabilization. A patient homozygous for the ETFβ-Asp128Asn mutation developed severe disease symptoms in association with a viral infection and fever. In agreement, our results suggest that heat inactivation of the mutant may be more relevant at temperatures above 37 °C. To mimic a situation of fever *in vitro*, the flavinylation status was tested at 39 °C. FAD exerts the effect of a pharmacological chaperone, improving ETF conformation, and yielding a more stable and active enzyme. Our results provide a structural and functional framework that could help to elucidate the role that an increased cellular FAD content obtained from riboflavin supplementation may play in

the molecular pathogenesis of not only MADD, but genetic disorders of flavoproteins in general.

4.2. Introduction

Multiple acyl-CoA dehydrogenase deficiency (MADD, MIM 231680; also designated glutaric acidemia type II (GAII)) is caused by mutations in either of the genes encoding the two subunits of electron transfer flavoprotein (ETF) or the monomeric enzyme ETF:ubiquinone oxidoreductase (ETF-QO) [1-2]. The clinical features of patients suffering from MADD are rather heterogeneous. It ranges from lethal cases with neonatal anomalies to mildly affected individuals, presenting in childhood or adulthood with hypoglycemic, encephalopathy and/or myopathy [1, 3].

There is evidence that the severity of the clinical phenotype, to some extent, depends on the location and nature of mutations in the genes encoding ETF or ETF-QO, with null mutations severely affecting mRNA expression, processing and/or stability being associated with lethal disease and missense mutations leaving some residual ETF/ETF-QO enzyme activity being associated with milder clinical forms [3-6]. In patients with milder disease variants, symptoms are often intermittent and only become evident during periods of illness and catabolic stress, indicating that in this group of patients - in whom residual ETF/ETF-QO enzyme activity allows modulation of the enzymatic phenotype – the disease severity does not depend only on the nature of the gene defect but also on cellular factors that may modulate the enzymatic phenotype [3]. This potential for *in vitro* modulation of the enzymatic phenotype has been established for a large number of disease-causing mutations affecting flavin-containing mitochondrial acyl-CoA dehydrogenases [7]. In these cases, missense mutations have been shown to impair folding to the native structure and/or

destabilize the native folded structure, resulting in decreased enzyme levels, which are subject to modification by environmental conditions like temperature and availability of quality control chaperones and proteases [8-10].

The cofactor FAD is another cellular factor that may modulate the enzymatic phenotype of disease-causing mutations in mitochondrial flavoproteins. It has been observed for acyl-CoA dehydrogenases and for ETF that levels of available FAD have a strong impact on folding and maintenance of the native structure [11-14]. Moreover, supplementation of certain MADD patients [15-20], and some patients with isolated deficiencies of acyl-CoA dehydrogenases [21-23], with FAD or its precursor riboflavin results in an increase of the enzymatic activities and improvement of clinical symptoms (as reviewed in [24]), indicating that the enzymatic phenotype can be amenable to modulation by FAD *in vivo* also. Decreased dietary intake of riboflavin, and also certain physiological conditions like pregnancy (reviewed in [25]), fasting [26], exercise (reviewed in [27]), and infections (reviewed in [28]) may induce a depletion in FAD content, which will pose high demands on flavoproteins and perhaps especially on those with folding defects. This raises the interesting hypothesis that availability of FAD may be a predisposing factor in the cellular pathogenesis of genetic disorders of acyl-CoA dehydrogenations. However, the additional possibility of a ligand-induced folding effect exerted upon FAD binding to a mutant protein, which becomes destabilized upon an adverse physiological condition, has not yet been explored.

Here we address this hypothesis by studying *in vitro* the effect of flavinylation on the folding, conformational quality and proteolytic susceptibility of a mild variant of human ETF (ETFβ-Asp128Asn). Studies were also performed under physiological heat stress conditions, to mimic the

adverse physiological factors of a fever event.

4.3. Material and methods

Chemicals

All reagents were of the highest purity grade commercially available. Octanoyl-CoA, FAD, AMP and urea were purchased from Sigma. Isopropyl–β–D-thiogalactopyranoside (IPTG) was purchased from VWR International.

Gene expression and protein purification

Escherichia coli JM109 cells from Promega transformed with ETF plasmids for the wild type (WT) and ETFβ-Asp128Asn variants were grown as described previously [3]. Briefly, cells were grown in dYT medium (16 g Bacto Tryptone, 10 g Bacto Yeast extract and 5 g NaCl) supplemented with 10 $\mu g.ml^{-1}$ kanamycin at 37ºC or 30ºC, up to a O.D. at 532 nm between 0.5-0.8, and then induced with 0.1 mM IPTG, for 4h. Cells were harvested by centrifugation, re-suspended in 10 mM Hepes, 10% ethylene glycol and 0.5 mM phenylmethylsulphonylfluoride (Roth) (Buffer A) in presence of 0.1 $mg.ml^{-1}$ FAD, DNase (PVL) and disrupted in a French press. The soluble extract was applied to a 20 ml Q-Sepharose fast flow (Amersham Biosciences) equilibrated in buffer A. The column was washed with 5 column volumes of buffer A, and bound proteins were eluted by a linear gradient ranging from 0-1 M NaCl, in buffer A. ETF eluted as a pure protein at ~ 250 mM salt, as shown by SDS/PAGE. The proteins were fast-frozen using liquid nitrogen and stored at -80ºC. To ensure full occupancy of FAD sites, the protein was incubated with 2.5 fold molar excess FAD at 4 ºC overnight, and the free cofactor was removed with extensive washings by ultra filtration/dilution.

This procedure yielded FAD-to-protein ratios higher than 0.95 both for WT and ETFβ–Asp128Asn. Unless otherwise mentioned, all experiments were performed with the proteins containing full occupancy of FAD sites.

Structural analysis

The crystallographic structure of human ETF (PDB code: 1efv) was visualized using PyMOL (DeLano Scientific, USA). Analysis of the molecular interactions, cofactor contacts, topological features and generation of the model of the ETFβ-Asp128Asn was carried out using the WhatIF web server and the PDBsum database [29].

Biochemical methods and activity assays

Protein concentration was determined using the Bradford assay. Flavin content was determined using the molar extinction coefficient $\varepsilon^{436_{nm}}$=13400 $M^{-1}.cm^{-1}$ reported for FAD bound to ETF [30]. The enzymatic activity of the purified proteins was measured monitoring DCPIP reduction, in a coupled assay in which recombinant human MCAD (0.13 μM) and octanoyl-CoA (13 μM), were employed, as described in [17]. The unit of activity measurements is defined as nmol of DCPIP reduced per minute, in the conditions used in the assay. All specific activities reported are based on total flavin content.

Spectroscopic techniques

UV/visible spectra were recorded at room temperature in a Shimadzu UVPC-1601 spectrometer. Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter with Peltier temperature control. A quartz polarized 1 mm path length quartz cuvette (Hellma) was used, and protein concentrations ranged from 0.1-0.2 $mg.ml^{-1}$. Fluorescence spectroscopy was performed using a Cary Eclipse instrument. For tryptophan emission studies, excitation was set

at 280 nm, whereas for FAD emission, excitation was carried out at 436 nm. Unless otherwise noted, the slits for excitation and emission were set to 5 and 10 nm, respectively. Typically, ETF protein concentration in fluorescence studies was 1 μM.

Flavin depleted ETF

Apo ETF was prepared using KBr solutions as in [31]. Briefly, proteins were incubated in 3 M KBr, 10 mM Hepes pH 7.0 and 20% ethylene glycol solution for 1 hour. Released FAD was washed by repeated ultrafiltrations/dilutions in the presence of 1 mM dithiothreitol (DTT) and a 10 fold molar excess AMP. In the case of the Asp128Asn variant, we also used a milder procedure taking advantage of the spontaneous process of flavin release, which had already been described for ETF [32], to produce a FAD-depleted fraction: when the protein is stored at -20ºC during 60 days, a decrease in FAD content and in the specific activity was observed, without a significant alteration of the protein folding, as determined by far-UV CD. Unbound FAD was removed by repeated ultrafiltrations/dilutions, yielding a sample with FAD content as low as 10%.

Dissociation constant for FAD binding

Apo ETF (1 – 4 μM) in the presence of 1 mM dithiothreitol, 10 fold molar excess of AMP, 10 mM Hepes pH 7.0 and 20% ethylene glycol was placed in a quartz cuvette at 15 ºC. After each addition of known amounts of FAD the mixture was allowed to equilibrate for 2 minutes and the visible spectrum was measured. The spectra of free and bound FAD are clearly distinct, making possible to quantify the concentration of each species at a given point. The fraction of bound FAD (f_{bound}) was calculated using the proportionality $f_{bound} \propto A_1 - (\varepsilon_1/\varepsilon_2)\times A_2$, where A_i is absorbance at the

wavelength i, and ε_i is extinction coefficient of free FAD at the wavelength. In this case, the wavelengths 465 nm (ε_1= 10190 $M^{-1}cm^{-1}$) and 435 nm (ε_2=10150 $M^{-1}\,cm^{-1}$) were used to distinguish between bound and free FAD, respectively. The concentration of free FAD in the equilibrium was measured by the equation $[FAD]_{free} = [FAD]_{total} - [ETF]_{total} \times f_{bound}$. The dissociation constant (K_D) was obtained by fitting the data of two independent experiments, using the equation, $f_{bound} = [FAD]_{free}/(K_D + [FAD]_{free})$.

Kinetics of flavin release

Wild type ETF and the ETFβ–Asp128Asn (1 μM), both with full occupancy of FAD sites, were incubated at 39ºC for up to 60 minutes in 10 mM Hepes, 20% ethylene glycol, pH 7.0. The kinetics of flavin release were monitored from the increase of the 530 nm emission peak arising from free FAD upon excitation at 436 nm. After 1 hour of incubation the samples were boiled for 5 minutes, to release the remaining FAD and a spectrum was collected at 39ºC. The fraction of bound FAD was determined at each point in respect to the point of 100% release of FAD.

Chemical stability studies

ETFβ-Asp128Asn (16 μM) depleted of flavin by milder procedure (see above), was incubated overnight with 10 mM Hepes, 10% ethylene glycol pH 7.8 at 4ºC, in the absence and in the presence of a 2.5 –fold excess of FAD (40 μM). Samples were diluted in different urea solutions, in 2 mM Hepes pH 7.8, equilibrated at room temperature for 15 minutes, after which the Trp emission spectra were recorded. Accurate concentration of the urea stock solutions was confirmed by refractive index measurements [33], and the pH was verified before the experiment. Fluorescence emission data in the 300-400 nm interval was analyzed determining the average emission wavelength

(AEW), which takes into account both variations in the emission intensity and position [34]. The denaturation curves were determined plotting the AEW as a function of urea concentration. Data were fitted to a sigmoid curve allowing the determination of an apparent C_m (denaturant concentration at the curve midpoint). The biological activity of the samples incubated overnight was also determined using the assay described above [17].

Limited proteolysis with trypsin

Wild type and ETFβ-Asp128Asn with full occupancy of FAD sites were incubated overnight in 10 mM Hepes, 10% ethylene glycol pH 7.8 at 4ºC, in the presence of a 2.5–fold molar excess of FAD. Control samples without added FAD were also prepared. These fractions were used to test the susceptibility towards trypsin digestion. Samples were incubated with trypsin (bovine pancreas trypsin; PVL) at 35ºC in 0.1 M Tris/HCl pH 8.5, at a 10-fold excess over the protease. As a control, identical samples without trypsin were submitted to the same procedure. Aliquots with 0.05 nmol of protein were sampled at different time points up to 2 hours, and the reaction was stopped by adding SDS-PAGE loading buffer (2 % SDS and 5 % β-mercaptoethanol). As an internal standard for the quantity of loaded protein in the gel, bovine serum albumin (BSA) 5 μM final concentration was also added to loading buffer solution. The products of the proteolysis reaction were analyzed by 12% SDS/PAGE, which were stained with Coomassie. Protein was quantified densitometrically (Biorad Chemidoc XRS) and the percentage of undigested protein was calculated in respect to the total amount of protein at time zero. Reported values refer to the sum of intensities of α and β bands.

Thermal stress studies

ETFβ-Asp128Asn and wild type ETF (12 μM), in 10 mM Hepes, 20% ethylene glycol pH 7.0, were incubated (100 μL) different temperatures (33 - 44ºC) for one hour, and the residual activity was determined in relation to the activity at time zero (duplicate measurements were preformed for each temperature, and two independent protein batches were assayed). To study the effect of FAD on the loss of activity, ETFβ-Asp128Asn (16 μM) was incubated at 39ºC up to 90 minutes, in the presence of a 2.5 -fold excess of FAD (40 μM). Control samples without added FAD were also prepared and treated in the same conditions. At ~ 10 min intervals, 3.5 μL of the samples were collected to determine the residual activity as indicated above. For the measurements using fluorescence spectroscopy, spectra were collected at 2 minutes intervals, up to 1 hour. Tryptophan emission was recorded from 300-400 nm with excitation at 280 nm. Unless otherwise noted, ETF concentration used was 1 μM.

4.4. Results

Impact of the ETFβ-Asp128Asn mutation

ETF is a heterodimer composed of 30 kDa (ETFα) and 28 kDa (ETFβ) subunits, containing one structural AMP, and one catalytic FAD group [2]. The MADD ETFβ-Asp128Asn mutation [3, 35] which is here investigated occurs in a β-turn within a very conserved region of the β–subunit, that comprises the segment 126-AIDDD-130. These residues have contacts with the AMP and are involved in inter-subunit interactions with residues from the α–subunit, via hydrogen bonds and non-bonded contacts. In particular, Asp 128 is directly involved in a network of interactions: it is salt bridged to

the nearby Lys 11 (2.96 Å) from the β–subunit N-terminus; and it contacts with several residues from the α–subunit (Ile148, Tyr149, Asn152). Also, ETFβ–Asp130 is hydrogen bonded to ETFα–Gln265, a residue that contacts with the FAD ring. Therefore, although Asp128 is not directly involved in an interaction with the flavin, it is located in a relatively sensitive region of the protein, in respect to dimer contacts and flavin binding. Indeed, this is noted by the decreased catalytic activity of purified ETFβ-Asp128Asn variant (400 units.mg^{-1}), which is only ~30 % of that of the wild type protein. Also, the temperature deactivation profile of the ETFβ-Asp128Asn variant is much more pronounced than that of the wild type protein (Fig. 4.1). For the ETFβ-Asp128Asn variant, a rapid drop of the activity is observed if the protein is incubated for 60 min at temperatures above 37 ºC, whereas at 37 ºC and below, more than 85 % of the initial activity is retained.

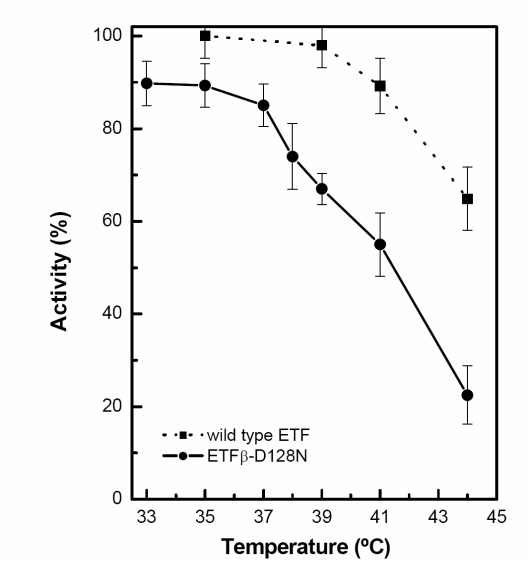

Figure 4.1: Thermal inactivation profile of ETFβ-Asp128Asn (—) and wild-type ETF (---). For each protein, independent samples were incubated for 60 min at different temperatures, and the remaining catalytic activity was determined (see "Experimental Procedures"). Two independent replicates were assayed, and each point represents the average of two experiments obtained with different protein batches (standard errors are plotted).

Folding and conformation of ETFβ-Asp128Asn

In order to define the structural characteristics of the ETFβ-Asp128Asn variant protein, we have investigated the folding and conformational properties of the purified protein. Circular dichroism (CD) analysis on the far-UV region showed that the spectrum of the mutant protein, is dominated by minima at 222 and 209 nm, which are typical of a well folded α/β protein (see Fig. 3.6). In fact, the two ETF subunits, although they share very little sequence identity, have the same structural topology, consisting of a three-layered αβα sandwich Rossman fold architecture. A comparison between the far-UV CD spectra of the mutant and wild type ETF shows that the mutation has no impact on the overall fold of the protein.

More subtle effects on the tertiary structure were monitored using fluorescence spectroscopy. The protein contains two tryptophan residues, one in each subunit (ETFα-Trp199 and ETFβ-Trp144), which are relatively accessible to the solvent, making them particularly sensitive conformational probes. The tryptophan emission maximum of ETFβ-Asp128Asn is 10 nm red-shifted in respect to that of wild type protein (Fig. 4.2). As a lower emission maximum denotes a more solvent-shielded tryptophan, this shift in the emission from ~320 to ~330 nm indicates that the aromatic moieties in ETFβ-Asp128Asn are more easily accessible to water molecules. This result suggests looser tertiary contacts in ETFβ-Asp128Asn, in comparison to wild type ETF.

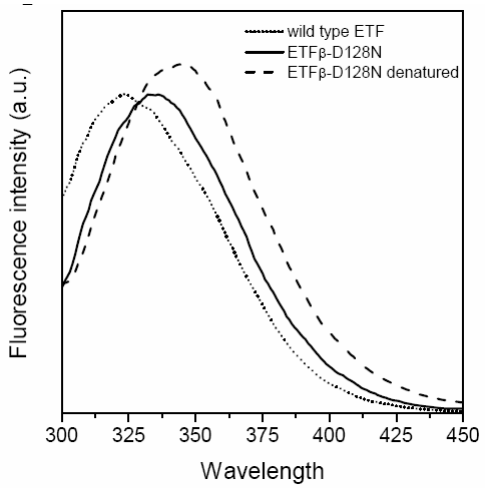

Figure 4.2: Fluorescence emission spectra of ETFβ- Asp128Asn (—) and wild type ETF (·····) and denatured ETFβ– Asp128Asn (– – –). Emission spectra were determined using λ_{ex} = 280 nm in 10 mM Hepes, pH 7.0, containing 20% ethylene glycol, at 39ºC; the concentration of proteins was 1 μM. The spectrum of denatured ETFβ– Asp128Asn was measured after boiling the protein for 5 min.

The FAD moiety in ETFβ-Asp128Asn was analyzed by visible absorption spectroscopy. The two bands typical of flavin, with maxima at 436 and 373 nm, were observed (see Fig. 3.6). The latter, is ~3 nm blue shifted in respect to the band observed in wild type ETF, which has a maximum at 376 nm. Additionally, the intensity of the FAD fluorescence is a reporter of the status of the flavin moiety, as the FAD emission is quenched when it is bound to the protein [36]. The FAD, which is in an extended conformation according to the crystal structure, has a similar emission spectrum in both proteins, denoting a characteristic weak band centered at ~500 nm.

Cofactor dissociation in ETF

To verify if the β-Asp128Asn mutation induces a perturbation of the flavin binding site, the dissociation constants for FAD binding to wild type ETF and ETFβ-Asp128Asn mutant were measured. When sub-stoichiometric quantities of FAD were added to wild type ETF apoprotein,

the characteristic ETF visible spectrum became immediately evident (Fig. 4.3A). In the case of ETFβ-Asp128Asn, an increased aggregation of the apoprotein was noted during the experiment, as evidenced from the increase of absorbance at lower wavelengths due to scattering (Fig. 4.3B). Nevertheless, the absorption spectrum characteristic of ETF was still observed.

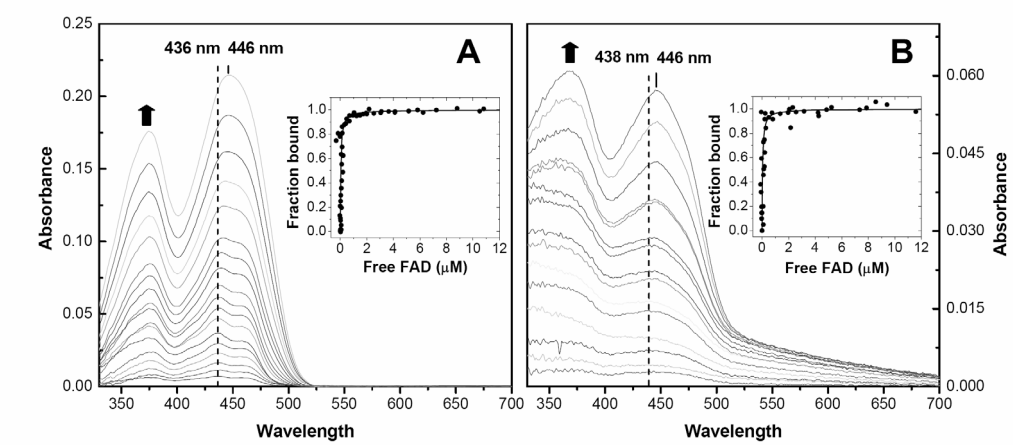

Figure 4.3: Binding of FAD to apoprotein wild-type ETF (*A*) and apoprotein ETFβ-Asp128Asn (*B*). Known amounts of FAD were added successively to the solution containing 4 μM apoprotein wild-type ETF or 1.4 μM apoprotein ETFβ-Asp128Asn in the presence of 10 mM Hepes, 20% pH 7.0, 20% ethylene glycol, containing 1 mM dithiothreitol and 10-fold molar excess AMP, at 15 °C. Spectra were measured after each addition of FAD, and the difference in respect to the apoprotein is shown (*arrows* point the spectral variation upon increasing concentrations of FAD). The binding curves (*inset*) were obtained by computing the fraction of bound FAD (see "Experimental Procedures"), and are the sum of two independent experiments. The *solid lines* in the *inset* correspond to a fit with a K_D= (0.04±0.01) μM for the wild-type ETF and K_D= (0.04±0.02) μM for ETFβ-Asp128Asn.

Since the spectra of free and bound FAD are distinct, it is possible to quantify the concentration of each species at a given point (as described in Experimental Procedures). By fitting the data with a one-site binding equation (Figure 4.3, A and B, *inset*) the dissociation constant of K_D = 0.04 ±0.01 μM and K_D = 0.04 ±0.02 μM was obtained for the wild type protein and ETFβ-Asp128Asn, respectively. These low values are close to the detection limit

that can be measured in these conditions, and should correspond to an upper limit.

Considering that the effect of the mutation on the specific activity is more pronounced above 37 °C (Fig. 4.1), the kinetics of flavin dissociation at 39°C was investigated for the ETFβ–Asp128Asn in comparison to wild type ETF (Fig. 4.4). Given that the intensity of the emission spectrum of free FAD is higher than that of bound FAD, the release of flavin was determined monitoring the increase in flavin fluorescence emission, similarly to a previously described methodology [31]. The point of 100% flavin release was measured after boiling the protein for 5 min. To rule out that the increase in intensity was due to conversion of FAD into FMN (10 fold more fluorescent), commercial FAD was treated in the same conditions, and no significant change in intensity was observed. Replicate assays were made, allowing to determine an apparent k_{off} of 0.38±0.06 h^{-1} for ETFβ-Asp128Asn and 0.13±0.02 h^{-1} for WT (n = 3). The value obtained for the wild type ETF is in excellent agreement with the reported value in the value in literature (0.12 h^{-1} [31]).

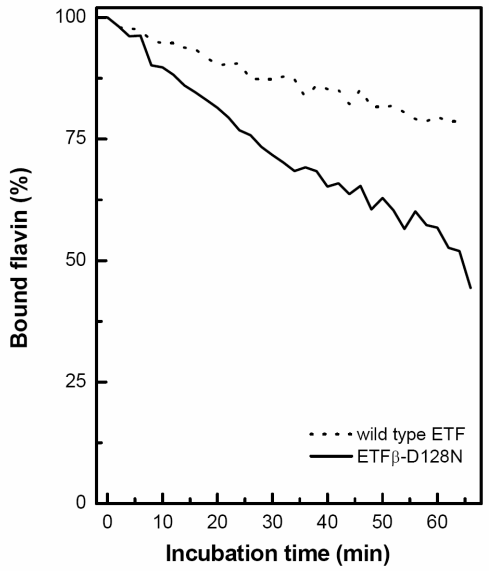

Figure 4.4: Kinetics of flavin release. ETFβ-Asp128Asn (—) and wild-type ETF (- - -) were incubated in 10 mM Hepes, 20% ethylene glycol pH 7 at 39 °C. The release of flavin was monitored as a function of time, following the emission of released FAD upon excitation at 436 nm.

After incubation at 39ºC, the original fluorescence spectrum could not be reverted by incubation at 4ºC for 24H. Moreover, the release of flavin correlates with a decrease in specific activity, which could not be rescued by incubation with excess FAD. The results showed that under these conditions, the ETFβ-Asp128Asn variant releases FAD irreversibly at a 3 fold higher rate than wild type ETF, denoting a weaker interaction of the cofactor with the protein at 39 °C or a less stable apoprotein.

Flavinylation improves the conformational stability of apo Asp128Asn variant

In order to test if flavin insertion or its absence has an effect on the conformational stability and overall folding of the protein, we analyzed the effect of flavinylation on the ETFβ-Asp128Asn variant. For this purpose, we determined urea denaturation curves, monitoring tertiary structure and packing changes in the protein from tryptophan-fluorescence emission. The effect of FAD was tested using as a starting point a fraction of apo ETFβ-Asp128Asn, which was depleted in flavin (<0.1 mol FAD/ mol protein) but that nevertheless retained, at least partially, the secondary structure and overall folding, as inferred from far-UV CD (see experimental procedures). In agreement with the lack of FAD, the enzymatic activity of this fraction was residual. The urea denaturation profile of this fraction yielded a cooperative transition, with an apparent midpoint denaturant transition (C_m) of 1.4 M (Fig. 4.5 A). However, incubation with FAD resulted in a dramatic increase of the C_m up to 2.3 M, and improved the cooperativity of the transition as reflected by the steeper slope (Fig. 4.5 A). These effects, which are indicative of a substantial increase in the conformational stability of ETF, correlate with an insertion of FAD in the protein, as denoted from the 14-fold increase in

the enzymatic activity upon incubation (from 12 to 166 units.mg^{-1}). Nevertheless this increase was less than the initial activity, when a ratio of 0.95 FAD:Protein was obtained, meaning that apoprotein could not be totally rescued. This suggests that a heterogeneous solution of apoprotein was obtained, in agreement with some degree of irreversible aggregation seen before for the apoprotein of ETFβ-Asp128Asn mutant in the reconstitution experiments. Inspection of the tryptophan emission spectrum at the different conditions, allowed us to evaluate the changes in the protein packing and tertiary contacts, as a result of FAD binding. The presence of flavin induced a more compact conformation as seen by the tryptophan emission shift from 337 nm in apoprotein to 333 nm in the reconstituted form, which denotes a more solvent-shielded Trp moiety [37] (Fig. 4.5 B).

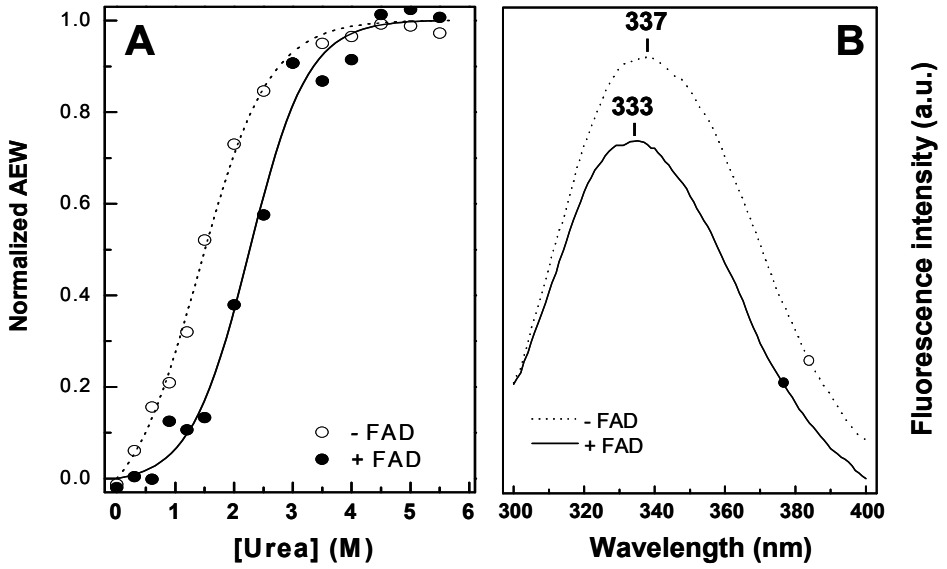

Figure 4.5: Urea-unfolding curves (*A*) and Trp fluorescence emission spectra (*B*) for ETFβ-Asp128Asn, in the presence (●) and in the absence (○) of FAD. Independent samples were incubated, with and without FAD, at different urea concentrations and the conformational changes were followed monitoring the average emission wavelength (AEW) in the 300–400 nm interval (see "Experimental Procedures"). The protein concentration used was 1 μM, and in the cases in which FAD was present, a 2.5-fold excess of cofactor over protein was added. The buffer used was 2 mM Hepes, pH 7.8, and the measurements were carried out at 25 °C. The curves correspond to a sigmoid fit to the experimental data.

Overall, ETF deflavinylation, which results in a structure with poorer tertiary interactions and decreased biological activity, in spite of the maintenance of an α/β fold, can be rescued to some extent by reincorporation of FAD in the protein.

Flavinylation modulates the proteolytic susceptibility of ETFβ-Asp128Asn

In order to test if the conformational stabilization afforded by flavinylation could contribute to minimize the propensity of the mutant ETFβ-Asp128Asn to be degraded, the resistance towards proteolysis was investigated. The assay used consisted of monitoring the extent of limited proteolysis by trypsin in the presence of FAD, and in its absence. The rationale for this approach is that a destabilized conformation will have a higher number of cleavage sites accessible to digestion, as a result of a higher flexibility of the polypeptide chain [38]. Therefore, this approach allows us to evaluate the propensity for *in vivo* degradation. Trypsin digestion was carried out at 35°C for up to 2 hours. Samples drawn every 30 minutes were analyzed by SDS-PAGE (Fig. 4.6). The protein samples without trypsin were heated for 2 hours. In the absence of FAD, the two subunits start to be substantially degraded after 60 minutes of incubation with trypsin (Fig. 4.6). After 120 minutes the protein amount decreases to 70% (Fig. 4.6). Interestingly, the presence of FAD could preserve the native conformation at 85% (Fig. 4.6). Control experiments in the absence of trypsin and on wild type ETF were also carried out (Fig. 4.6).

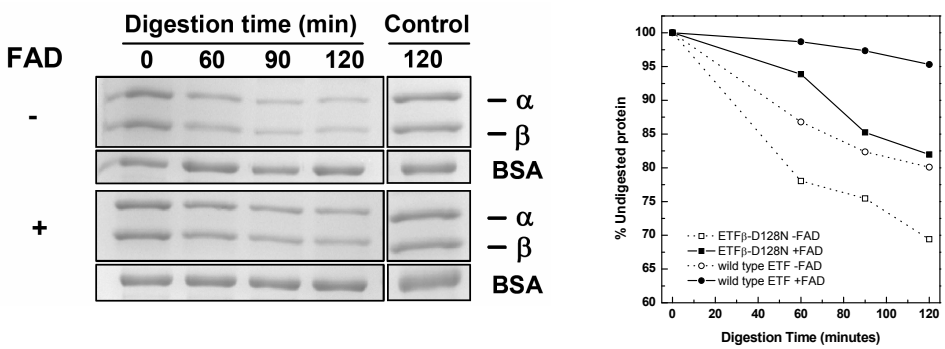

Figure 4.6: Effect of flavinylation on ETFβ-Asp128Asn - limited proteolysis. Proteolytic digestion during 2 h at 35°C was analyzed by SDS-PAGE. Protein was previously incubated overnight at 4 °C in 10 mM Hepes, 10% ethylene glycol in the absence and presence of 2.5 fold excess of FAD. The control represents protein treated in the same conditions and heated for 2 h in absence of trypsin. Bovine serum albumin was added to loading buffer as an internal standard and to correct for variations in the amount of loaded protein. The graph corresponds to the densiometric quantification of undigested protein. The sum of α and β band intensities are plotted as a percentage of undigested protein in respect to the total amount of protein at time zero.

These results suggest that flavinylation, and the presence of FAD, induce a protease-resistant conformation in ETF. Moreover, the presence of FAD induce changes in the conformation of the ETFβ-Asp128Asn variant, which restore its proteolytic resistance to levels of the wild type protein (Fig. 4.6).

External FAD preserves folding and enzymatic activity during thermal stress

We have designed an *in vitro* experiment aimed at testing a possible effect of dietary riboflavin supplementation for a mild mutation like ETFβ–Asp128Asn during thermal stress. To analyze the relevance of an increased FAD level during a fever episode, the effect of incubating ETFβ–Asp128Asn during 60 minutes at 39°C, in the absence and in the presence of a 2.5 fold excess of FAD, was investigated (Fig. 4.7). Activity measurements during the thermal perturbation showed that the presence of FAD prevents loss of activity that goes down to 50% of the initial value (225

units.mg^{-1}), in comparison to control samples (Fig. 4.7A). In contrast, in the control samples in the absence of FAD, the increased Trp emission is indicative of poorer and less compact tertiary structure (Fig. 4.7B). In conclusion, the presence of FAD during thermal stress of ETFβ–Asp128Asn prevents the loss of tertiary structure and protein compactness, as well as the loss of enzymatic activity.

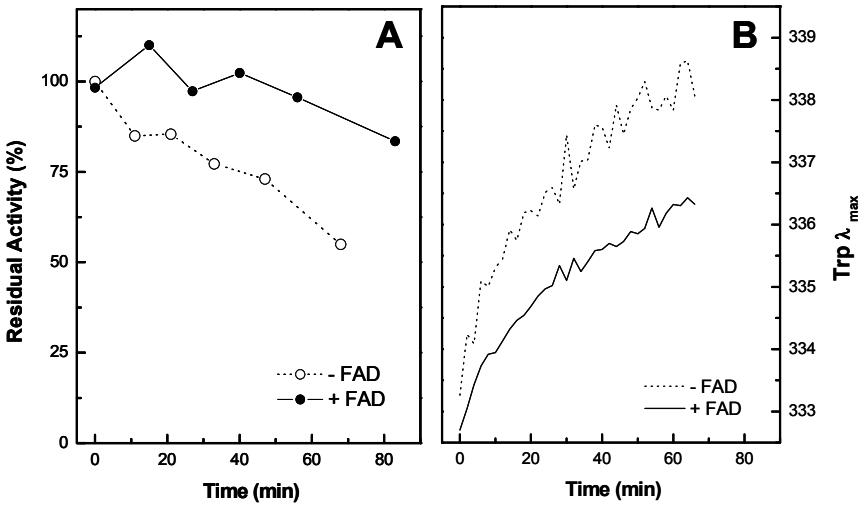

Figure 4.7: Effect of FAD during a thermal stress. Independent samples were incubated in 10mM Hepes, 20% ethylene glycol in absence (○,---) and presence of 2.5-fold excess of FAD (●,—) at 39 °C. *Panel A* represents loss of biological activity in relation to the initial value. *Panel B* shows decrease in tertiary contacts determined from the variation of the maximum wavelength on the tryptophan region (300–400 nm).

4.5. Discussion

In numerous diseases involving missense mutations in mitochondrial flavoproteins, symptoms are in some cases improved by dietary riboflavin supplementation [18-21, 24]. Perhaps because of the many reports on successful treatment of a variant group of MADD patients, the "riboflavin-responsive MADD patients" [18-20, 39-42], milder forms of MADD in general are treated with riboflavin, but without knowing the molecular mechanism of this treatment. MADD is therefore a good candidate to study

FAD sensitive conformational instability of flavoproteins due to disease-causing missense mutations. We chose the ETFβ-Asp128Asn protein for the following reasons: it was identified in homozygous form in a patient with MADD, who had no obvious symptoms until the age of two years when disease symptoms were precipitated in connection with virus infection and fever. The patient died after 2 days. A sibling from whom no genetic material was available, had died suddenly and unexpectedly at the age of 6 months [35]. Subsequent analysis in cultured fibroblasts from the patient showed residual activity and presence of reduced levels of both ETF subunits [3]. Upon expression of the mutant protein in an *E. coli* system, decreased levels of the mutant protein compared to wild type were observed. This could be partially rescued by growing the bacterial cells at lower temperature, but not by chaperone co-overexpression. Furthermore, the ETFβ-Asp128Asn mutant protein displayed reduced thermal stability [3]. Based on these observations, it was hypothesized that the mutant protein displays decreased conformational stability, which is sensitive to environmental conditions like temperature, and likely to the availability of FAD cofactor.

With our detailed analysis we have here shown that the ETFβ-Asp128Asn mutation decreases significantly the specific activity and introduces some plasticity on the tertiary structure, but does not negatively impact the overall protein fold. In agreement, the level and activity of this protein during heterologous expression was found to be improved when the cells were grown at low temperature [3], an indication that the mutation had a destabilizing effect on the protein. In more detail, the temperature inactivation profile of ETFβ-Asp128Asn demonstrates that the activity of the protein remains mainly unaltered by incubation at low temperatures, but starts to become more unstable at temperatures above 37 ºC. In agreement with this observation, we found that FAD binds very tightly in both ETFβ-

Asp128Asn and wild type protein at 15 ºC, but during heat stress (39 ºC) the flavin is irreversibly released three fold faster in the mutant, resulting in a concomitant loss in the specific activity. This *in vitro* result is in line with the *in vivo* situation where the symptoms emerged associated to an episode of stress. We then moved to analyze if flavinylation would have an effect on ETF stability, and found that apart from the expected increase in the enzymatic activity, saturation of the FAD binding site significantly improved the stability and conformation of the apoprotein, and rescued destabilized forms, as inferred from urea unfolding studies. The marked decrease in stability of the apoprotein and the fact that the irreversible deactivation at 39ºC is accompanied by flavin release suggest that if the cell is challenged with a stress condition or a decreased level of FAD, then the protein could become deflavinylated and as a consequence more destabilized. In addition, the presence of external FAD in the medium also increased the proteolytic stability of the protein, a finding which may be important as it again suggests that an excess of FAD in the patient cell may increase the lifetime and availability of an active form of the protein. Analysis of this effect in the context of a physiological heat stress condition, which mimicked a fever episode (incubation at 39°C for one hour), clearly showed that exogenous FAD prevented conformational destabilization and activity loss. Studies of tissue samples or cultured fibroblasts from patients with MADD have shown an activity increase upon supplementation with riboflavin or FAD [24]. This increase in activity may result from a direct saturation of the active site, or from an improvement of the *in vivo* maturation and assembly of the protein involved. It has been shown that FAD plays an important role during *in vitro* folding of mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD), nucleating the formation of a competent oligomeric conformation during hsp60 assisted folding [12]. Here we postulate another possibility, which may

occur concomitantly to the previous one. The hypothesis is based on the fact that externally added riboflavin leads to an increase in the FAD content, as shown in yeast mitochondria [43] and human mitochondria [41-42]. In this scenario, increased availability of the FAD ligand would promote its binding, which induces conformational changes which propagate to the overall structure. This may occur by a structure nucleation effect, by which certain motions in the molecule are restrained, leading to a decreased 'breathing' of the protein structure. This results in a better packed conformation, with improved local interactions, yielding a protein with a higher conformational stability. More importantly, in the context of the cell, this effect also leads to a reduction of the sites, which are available to proteolytic degradation by cellular proteases. A protein with decreased conformational packing and stability is a better substrate for the protein degradation machinery, as in this case, the structural destabilization makes digestion sites accessible which would be otherwise unavailable.

As the ETFβ-Asp128Asn mutation is not located in the FAD binding domain, our observations provide a structural and molecular rationale to understand the basis of one of the many possible effects of riboflavin supplementation in patients with milder forms of genetic deficiencies of acyl-CoA dehydrogenation, where the mutation sites are distributed all over the structure, suggesting that destabilization of local interaction may lead to long-distance conformational changes that may affect FAD binding. In these patients with mutations that do not totally impair protein folding, but result in a less stable or inactive conformation, the increase in FAD concentration resulting from riboflavin supplementation may enhance the conformational quality of the affected protein, increasing the cellular availability and life time of a biologically active molecule, by a mechanism identical to the one we report in this work. This effect could be particularly relevant under certain

pathophysiological conditions like fever but also fasting, infections, pregnancy and low dietary intake of riboflavin that may cause low cellular FAD content and thereby poses high demands on folding defective flavoproteins that are up-regulated under less favorable folding conditions. We have experimentally shown that flavinylation recovered the properties of the ETF mutant variant after a heat stress condition, which simulated a fever event, a condition which is known to aggravate the symptoms of patients suffering from mild fatty acid β-oxidation disorders [3]. These observations establish a proof of principle, which can be generalized and tested in many other genetic defects in mitochondrial flavoproteins.

4.6. Acknowledgments

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5 A POLYMORPHIC POSITION IN ELECTRON TRANSFER FLAVOPROTEIN MODULATES CONFORMATIONAL DYNAMICS AS EVIDENCED BY THERMAL STRESS

This chapter is under review as:

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"A polymorphic position in electron transfer flavoprotein modulates kinetic stability as evidenced by thermal stress"

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5.1. Summary

The electron transfer flavoprotein (ETF) is a dimeric flavoenzyme involved in mitochondrial beta-oxidation. Genetic defects in this pathway result in disease as a result of impaired folding and catalysis. ETF harbors a polymorphic variation at position 171 in its alpha-subunit, resulting in either a threonine (ETFα-Thr171) or an isoleucine (ETFα-Ile171). Interestingly, the ETFα-Thr171 variant is overrepresented in patients suffering from a mild form of very long chain acyl-CoA dehydrogenase deficiency, suggesting that this polymorphism may contribute to the phenotype, by an as yet unknown molecular mechanism.

Here we report an investigation on the effect of the variation of the ETFα-Thr/Ile171 polymorphism on the ETF folding, stability and dynamics under thermal stress. Our results show that the two variants have the same thermodynamic stability (T_m=58°C), although the ETFα-Thr171 variant has a decreased thermal inactivation midpoint (T_m^{inact}=49°C) in comparison to that of ETFα-Ile171 (T_m^{inact} =58°C). We found that upon thermal stress, the ETFα-Thr171 variant is prone to faster cofactor (FAD) loss and conformational destabilization. This variant also exhibits an increased conformational dynamics during thermal stress mimicking a fever episode *in vitro*: upon 2 hours at 39°C, ETFα-Thr171 looses half of its activity, whereas that of ETFα-Ile171 remains at ~85%. The GroEL chaperonin can rescue this effect, as it captures and refolds the dynamic populations of thermally destabilized ETFα-Thr171.

This polymorphic position thus affects the conformational landscape and protein function under thermal stress, a common metabolic trigger for the manifestation of the phenotype in mitochondrial beta oxidation defects.

5.2. Introduction

The mitochondrial oxidation of fatty acids and amino acid and choline catabolic reactions constitute important pathways through which energy is produced. Among others, it involves a series of acyl-CoA dehydrogenases (ACDHs) that selectively oxidize acyl-chains of different lengths, transferring this reducing power to the respiratory chain via electron transfer flavoprotein (ETF) and its partner ETF:ubiquinone oxidoreductase (ETF-QO), the terminal membrane-bound enzyme of this pathway [1-2]. Mutations in either ETF or ETF-QO account for a disease called multiple acyl-CoA dehydrogenase deficiency (MAAD) [3].

Besides rare disease-associated mutations, a few single nucleotide polymorphisms (SNPs) have also been reported. In particular, two polymorphisms have been described for human ETF, one in codon 171 of ETFα, and the other in codon 154 of ETFβ [4-5]. The polymorphism in ETFα replaces a threonine for an isoleucine, whereas at ETFβ a methionine is replaced by a threonine. The four possible combinations have been studied *in vitro* by heterologous prokaryotic expression [6]. It has been shown that the ETFα-Thr171 variant had a decreased enzymatic stability. The authors also assessed the frequency of the polymorphisms in a control group and patient groups associated with fatty acid oxidation disorders. Patients with the mild form of VLCAD deficiency [7] were found to have a significant overrepresentation of the α-Thr171 variant [6]. Although only a small number of patients was analysed, this suggested that the ETFα-Thr171 might modulate the phenotype by an as yet unknown mechanism [6].

Polymorphisms are nucleotide sequence variations occurring at a frequency greater than 1% within the general population. As suggested above these genetic variations may play a role as modulators of disease, especially in combination with other metabolic stressors such as increased body

temperature (fever), which has been documented to trigger and exacerbate the phenotype in patients with beta oxidation defects [8]. However, little is known about the molecular mechanisms through which this effect is exerted.

In this study we show that the polymorphic position ETFα-171 plays a role in modulating the conformational dynamics and biological activity under thermal stress conditions.

5.3. Materials and Methods

Chemicals

All reagents were of the highest purity grade commercially available. Octanoyl-CoA, and FAD were purchased from Sigma. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from VWR International.

Gene expression and protein purification

E. coli JM109 cells transformed with the ETF plasmids, ETFα-171Ile and ETFα-171Thr respectively [6], were grown in LB (Luria–Bertani) supplemented with 10 $\mu g.ml^{-1}$ kanamycin at 37ºC in a shaking incubator until OD_{532} of 0.5-0.8 was reached. The cells were then induced with 1 mM IPTG for 4 hours. Cells were harvested by centrifugation, re-suspended in 10 mM Hepes, 10% ethylene glycol and 0.5 mM phenylmethylsulphonylfluoride (Roth) (Buffer A) in presence of DNase (PVL) and disrupted in a French press. The soluble extract was applied to a Q-Sepharose fast flow (GE Healthcare, 20 ml) equilibrated in buffer A. The column was washed with 5 volumes of buffer A, and bound proteins were eluted by a linear gradient from 0-1 M NaCl, in buffer A. Pure ETF eluted at salt concentration around 250 mM salt, and purity was confirmed by SDS/PAGE. Protein concentration was determined using the Bradford assay. Flavin content was

determined using the molar extinction coefficient ε^{436nm}=13400 $M^{-1}.cm^{-1}$ reported for FAD bound to ETF [9]. Pure ETF fractions with a 2.5 fold molar excess FAD were fast-frozen using liquid nitrogen and stored at -80ºC.

GroEL expression and purification

The *E. coli* chaperonin, GroEL was isolated and purified as described earlier [10]. Briefly, the GroEL was purified using an Affi-Gel Blue treatment, after which the samples were precipitated in 45% (V/V) acetone at room temperature for 5 min. The precipitate was centrifuged at 10,000 *g* for 30 min and, after the removal of acetone, re-suspended in buffer. Residual protein aggregates and acetone were removed by a brief centrifugation followed by an extensive dialysis. Protein purity was confirmed by SDS-PAGE and by the second derivative analysis of the absorbance spectra.

Activity assays

ETF enzyme activity was measured following 2,6-dichlorophenol indophenol (DCPIP) reduction at 600 nm in a coupled assay in which recombinant human MCAD and octanoyl-CoA were employed, as described in [11]. The unit of activity measurements is defined as nmol of DCPIP reduced per minute, in the conditions used in the assay. All specific activities reported are based on total flavin content.

Spectroscopic methods

Before each experiment FAD excess added to buffers as a preservative was removed by extensive washing using ultra filtration/dilution, and all experiments were performed with pure proteins containing full occupancy of FAD site. UV/visible spectra were recorded at room temperature in a Shimadzu UVPC-1601 spectrometer with cell stirring. Fluorescence

spectroscopy was performed using a Cary Eclipse instrument. For aromatic emission excitation wavelength was set at 280 nm, and FAD emission was followed setting excitation wavelength at 436 nm; slits were 5 and 10 nm for excitation and emission, respectively. Typically protein concentration was 1 μM. CD spectra were recorded on a Jasco J-815 spectropolarimeter with a cell holder thermostatically controlled with a Peltier. A quartz polarized 1 mm or 10 mm path length quartz cuvettes (Hellma) was used, and protein concentrations were typically 0.1 or 1.0 $mg.ml^{-1}$, for far-UV and near-UV measurements, respectively.

Thermal stability

Thermal unfolding with a linear temperature increase was followed using circular dichroism (ellipticity variation at 222 nm and 288 nm) and fluorescence spectroscopy, tryptophan emission (λ_{ex} =280 nm; λ_{em} =340 nm), FAD emission (λ_{ex} =436nm; λ_{em} =530 nm) and FRET from tryptophan emission to FAD cofactor (λ_{ex} =280 nm; λ_{em}=530 nm). In all experiments, a heating rate of $1ºC.min^{-1}$ was used, and temperature was increased from 30 to 90ºC. For the thermal stability stepwise following activity, samples incubate for 10 minutes at different temperatures, from 30 to 75 ºC, and activity was measure for each time point. Data were analysed according to a two-state model, and fits to the transition curves were made using OriginPro8.

Kinetics of flavin release

ETF variants 1 μM were incubated at different temperatures (39, 45, 50 and 55 ºC) for up to 60 minutes in 10 mM Hepes, pH 7.8. The kinetics of flavin release were monitored from the increase of the 530 nm emission peak arising from free FAD upon excitation at 436 nm. After 1 hour of incubation the samples were boiled for 5 minutes, to release the remaining FAD and a

spectrum was collected at 25 ºC. The fraction of bound FAD was determined at each point in respect to the point of 100% release of FAD.

Aggregation

DLS measurements were performed at 90ºC scattering angle on a Malvern Instrument Zetasizer Nano-ZS. Measurements were carried out at 50ºC using a built-in temperature controller, and protein concentration of 1μM at pH 7.8. Protein samples were passed through a 0.22 μm filter before each experiment.

Thermal stress studies

ETF (8 μM) in 10 mM Hepes pH 7.8, was incubated at 39ºC up to 120 minutes and the residual activity was determined in relation to the activity at time zero (duplicate measurements were performed for each time point). The experiment was repeated in presence of 1.7 μM GroEL oligomer (10 mM Hepes, 1 mM ATP, 15 mM KCl, 5 mM $MgCl_2$, pH 7.8). A control with BSA instead of GroEL was made in order to distinguish unspecific effects.

5.4. Results

Effect of the ETFα-Thr/Ile171 variation on the conformational stability

ETF is composed of three structural domains: domain I and II are formed by the N- and C-terminal portions of the α subunit, respectively, while domain III is formed by the β subunit [12]. The polymorphic position ETFα−171 is located in domain I at the interface with domain II (Fig. 5.1). The Ile or Thr at this position have thus contacts with a set of side chains from the three nearby hydrophobic residues: Ile 269, Val 270 and Ala 271.

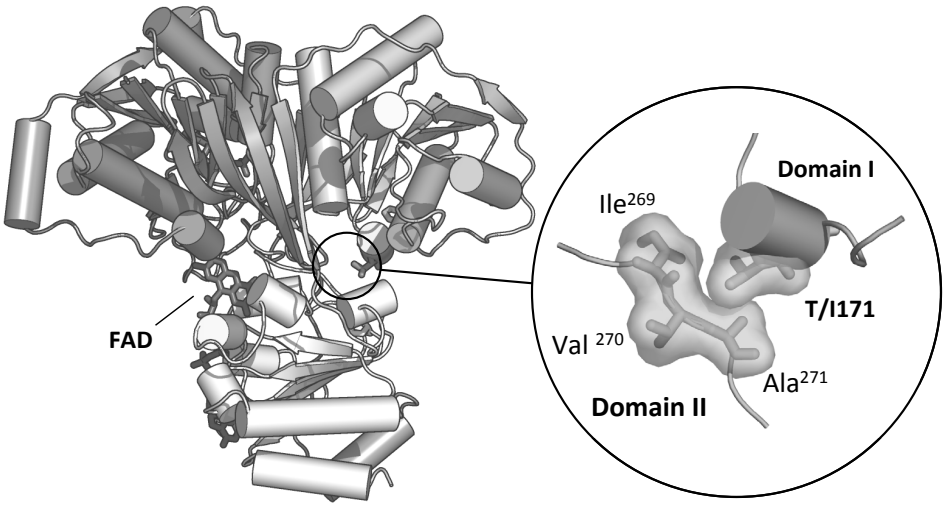

Figure 5.1: Structural model of human Electron Transfer Flavoprotein. This cartoon highlights the ETFα-171 position (shown by sticks).The three domains are represent in light gray (domain I), grey (domain II), and dark grey (domain III), the interactions of position 171 with the surrounding residues are highlighted. The cartoon was made using PyMOL, on the basis of PDB coordinates: 1efv.

The effect of the polymorphic variation at this interfacial position on protein stability was investigated from thermal melting experiments. Protein unfolding was monitored as a function of temperature using a combination of biophysical methods: far-UV CD for monitoring secondary structure changes, near-UV CD and aromatic fluorescence to monitor tertiary interactions and FAD fluorescence to report on cofactor contacts.

The data obtained showed that all these probes reported the same midpoint of thermal denaturation (T_m = 58°C) for the two variants (Fig. 5.2A). Also, there were no differences between the spectra of native preparations of the variants, in any of the spectroscopies used. This indicates that the alternation between an isoleucine and a threonine at the ETFα-171 position does not affect the fold nor the thermal stability of ETF.

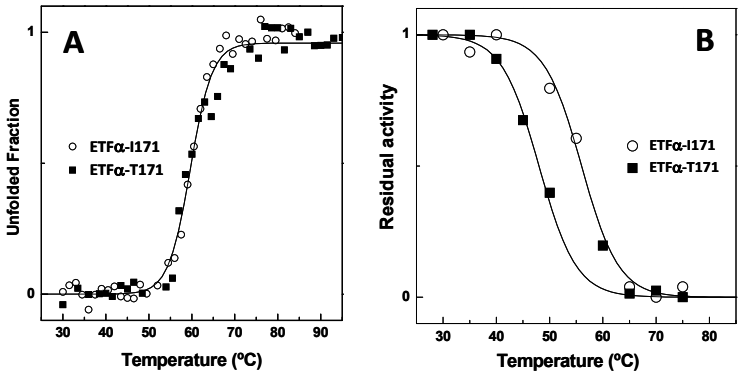

Figure 5.2: Thermal stability (A) and thermal inactivation (B) of ETF variants. A: The thermal unfolding curves for ETFα-Ile171 (○) and ETFα-Thr171 (■) were determined monitoring ETF unfolding using far-UV CD and aromatic fluorescence emission. Both techniques yielded the same results but for clarity purposes only the normalized variation of the CD signal at 222 nm is shown. Although ETF is a dimeric protein, its thermal unfolding can be described by a two-state process, as shown by the solid sigmoid curve (see materials and methods for details). The protein concentration used was 1.7 μM, in 2 mM Hepes, pH 7.8. **B:** The thermal inactivation of the ETF activity was monitored for the ETFα-Ile171 (○) and ETFα-Thr171 (■) variants up to 75 ºC. The plot represents the residual activity upon 10 minute incubation at the different temperatures tested (see materials and methods)**,** each point represents the average of two experiments obtained with different protein batches (standard errors less than 0.05).

Thermal inactivation profiles

A previous study had shown that ETFα-Thr171 is more susceptible to thermal inactivation than the ETFα-Ile171 variant [6]. This variant was found to be overrepresented among patients suffering from deficiency in the very long chain acyl-CoA dehydrogenase (VLCADD) and it was suggested to have a negative modulating effect in this disease [6]. However, the molecular origins of this different behaviour were not further addressed. Our data corroborate this difference between the two variants (Fig. 5.2B): upon incubation at different temperatures for 10 minutes, the ETFα-Thr171 activity decays at lower temperatures than that of ETFα-Ile171. This indicates that this difference is not preparation dependent. ETF is a protein known to undergo extensive conformational sampling during catalysis, some of which

are compatible with fast electron transfer [13-15]. Therefore, the observed discrepancy between the thermal denaturation and activity profile suggests that the ETFα-Ile/Thr171 polymorphism may influence the structural and most likely dynamic properties of the protein upon thermal perturbation, as suggested for other enzymes [16].

ETFα-Thr171 has a decreased kinetic stability upon thermal perturbation

In order to gain further insight into the structural changes occurring during thermal perturbation of the two ETF variants, the kinetics of thermal perturbation were determined under different conditions of thermal stress. Changes in protein folding were evaluated using fluorescence spectroscopy and FAD as a probe for structural changes. The dissociation rate of the cofactor was monitored at conditions of increasing thermal stress: 39, 45, 50 and 55°C. The results obtained showed that the two variants differ in kinetic stability, as the rates of FAD release during thermal perturbation by ETFα-Thr171 were always higher that those observed for ETFα-Ile171 and the difference between the variants increases with temperature (Fig. 5.3). Thus, the ETFα-Thr171 variant is more prone to undergo thermally driven conformational transitions that result in FAD release.

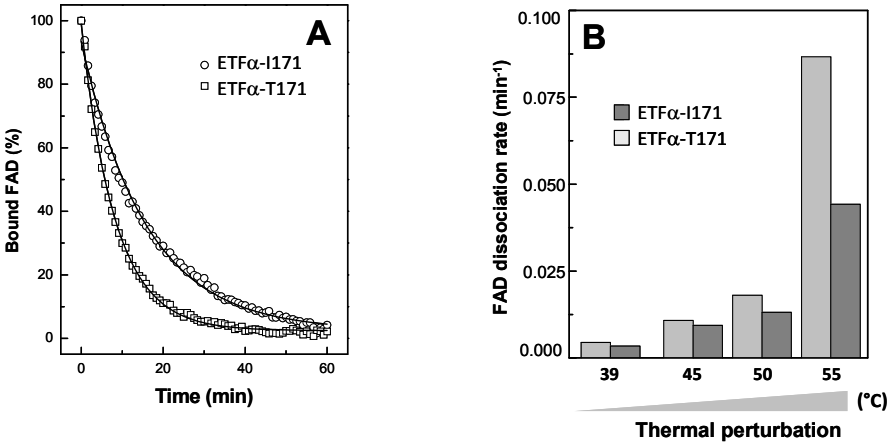

Figure 5.3: Kinetics of flavin release. A: ETFα-Ile171 (○) and ETFα-Thr171 (□) were incubated in 10 mM Hepes, pH 7.8 at 55 ºC and flavin release was followed monitoring increase in fluorescence emission at 530 nm upon excitation at 436 nm. **B:** Plot of the observed kinetic rates for flavin release at 39ºC, 45ºC, 50ºC and 55 ºC, according to a first order process (ETFα-Ile171 dark grey bars and ETFα-Thr171 light grey bars).

ETFα-Thr171 exhibits decreased conformational stability upon thermal stress

Thermal stress unmasked kinetic differences between the two polymorphic variants, which likely underlie distinct dynamic properties. We have further investigated this aspect by comparing the dynamic behavior of the two variants upon thermal stress at 50°C, a temperature below the midpoint of the thermal transition (T_m = 58°C). Incubation during 10 min under these conditions has a distinctly stronger effect on the enzymatic activity of the ETFα-Thr171 in respect to that observed for ETFα-Ile171 as it results in a residual activity of 38% versus 79%, respectively. However, this difference does not result from a macroscopic effect on the structural parameters, as both variants are equally affected in their secondary structure and FAD release. This suggests that a more dynamic behavior of the ETFα-Thr171 variant may allow a broader sampling of the conformational space thus resulting in subpopulations of inactive ETF. This possibility was investigated using dynamic light scattering (DLS) to monitor fluctuations in

the hydrodynamic radius of the two polymorphic variants, upon thermal stress in the conditions above. This experiment shows that at a very early stage (< 20 min) a substantial variation of particle size is observed exclusively on the ETFα-Thr171 variant (from ~3.2 to 34 nm). This suggests that a heterogeneous population is formed upon thermal stress.

Activity decay under physiological thermal stress

Fatty acid oxidation defect phenotypes are known to be triggered or exacerbated by stress factors such as fever. Following the finding that the polymorphic variation at ETFα-171 modulates the conformational dynamics under thermal stress, we have investigated if this effect would be notorious under conditions mimicking a fever episode. For this purpose, we have incubated the two variants at 39°C for up to 120 minutes, sampling periodically for activity measurements. In agreement with the previous results we have observed that the ETFα-Thr171 has an increased susceptibility towards thermal inactivation, in comparison to ETFα-Ile171 (Fig. 5.4).

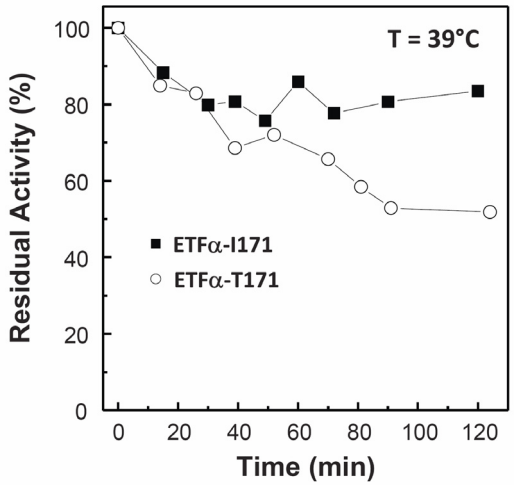

Figure 5.4: Thermal inactivation profile of ETFα-Ile171 (■) and ETFα-Thr171 (○). Each protein sample was incubated for 120 min at 39ºC, samples were taken at different time point and the remaining catalytic activity was determined (see Experimental Procedures). Two independent replicates were assayed, and each point represents the average of two experiments obtained with different protein batches (standard errors less than 5%).

Throughout the thermal perturbation assay, the activity of the ETFα-Ile171 variant was always higher, and the relative decrease in activity was of only 15%, whereas 50% of the ETFα-Thr171 was lost during this physiological thermal stress. This result suggests that the presence of the ETFα-Thr171 variant may decrease β-oxidation flux during fever episodes.

GroEL rescues the activity of ETFα-Thr171 during a fever-like thermal stress

As already stated above heat stress results in a deterioration of mitochondrial β-oxidation in cultured fibroblasts obtained from patients with fatty acid oxidation disorders [8]. Our findings on the decreased conformational stability of ETFα-Thr171 upon thermal stress contribute to a molecular understanding of this process at the protein level. Altogether these findings suggest that the homeostasis of proteins involved in this pathway is susceptible to thermal stress and we have investigated if the GroEL chaperonin could rescue destabilized conformations of ETF. GroEL binds to partially folded proteins in solution and then uses the energy of ATP hydrolysis to drive the protein to a folding-competent state.

In this experiment we have measured the residual activity of ETFα-Thr171 during incubation at 39°C, in the presence and in the absence of GroEL and excess ATP (Fig. 5.5). The results obtained showed that GroEL is rather efficient in rescuing the conformational destabilisation during thermal stress: after 120 min of incubation at 39°C the protein has still ~95% of its original activity when GroEL is present, whereas in its absence the activity has decayed to 50% (Fig. 5.5). A control experiment for unspecific interactions was carried out, in which BSA was added to the assay resulting in a decay comparable with the one observed in absence of GroEL, thus

showing the specificity of the effect.

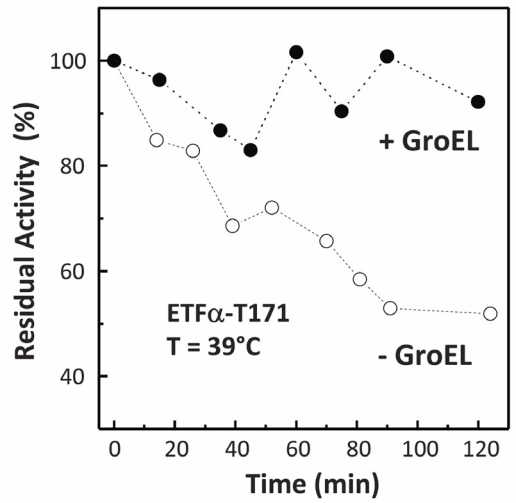

Figure 5.5: GroEL conformational rescue for ETFα-Thr171. Thermal inactivation profile of ETFα-Thr171 in presence (●) and absence (○) of GroEL. Protein was incubated for 120 min at 39 ºC, samples were taken at different time points and the remaining catalytic activity was determined (see Experimental Procedures). Two independent replicates were assayed, and each point represents the average of two experiments obtained with different protein batches (standard errors less than 5%).

The same experiment was also carried out using the ETFα-Ile171 variant, and the results obtained show that in this case the presence of GroEL has almost no effect, in agreement with the fact that the ETFα-Ile171 polymorphism is much less susceptible to destabilization upon thermal stress (Fig 5.6).

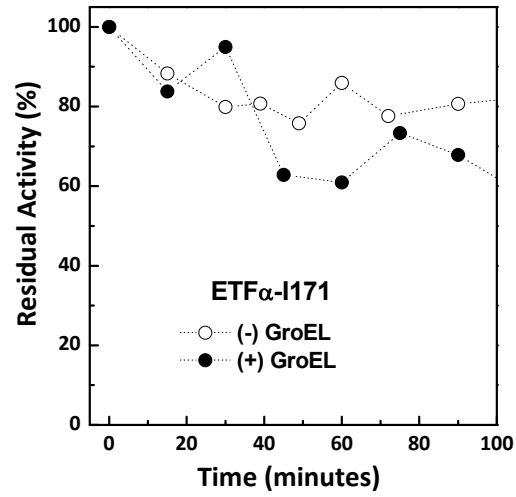

Figure 5.6: GroEL conformational rescue for ETFα-Ile171. Thermal inactivation profile of ETFα-Ile171 in presence (●) and absence (○) of GroEL. Protein was incubated for 90 min at 39 ºC, samples were taken at different time points and the remaining catalytic activity was determined (see Experimental Procedures). Two independent replicates were assayed, and each point represents the average of two experiments obtained with different protein batches (standard errors less than 5%).

Clearly, the distinct partitioning of the two variants into GroEL shows that, under thermal stress, the polymorphic position modulates the protein dynamics and its conformational landscape. In fact, such a preventive effect of GroEL during thermal stress is well known in several protein model systems, such as dihydrofolate reductase [17].

5.5. Discussion

Thermal stress is an important modulator of disease in mitochondrial beta oxidation disorders and point mutations that affect protein stability or activity are particularly susceptible to this effect [18-22]. In recent years, important progress has been made in the understanding of the molecular and structural basis of point mutations affecting different enzymes, including ETF and ETF-QO. However, the molecular effects of polymorphic protein variations in human disease remains poorly understood, and only now starts to be addressed. That is for example the case of the ApoE4 variant which plays a role in multiple disease [23], and of the polymorphic variation in the PrP protein that acts as a molecular switch in the control of interspecies disease transmission [24]. These examples clearly show that polymorphic variations may play a key role in the modulation of the biological function, by mechanisms which remain to be addressed at the protein-molecular level.

Focusing on the effects of the polymorphic variation Ile/Thr at ETFα-171, we have shown that it affects the protein conformation as evidenced by thermal stress, and that the ETFα-Thr171 variant is particularly susceptible to thermal destabilization as a result of an increased dynamics. ETF is reported to be a highly dynamic protein in the free form in solution and complex with it co-partners [25]. Domain II, which accommodates the FAD cofactor, was shown to rotate upon complex formation with partner ACDH's thus bringing the FAD bearing moieties in contact in order to allow electron transfer to

occur [26]. The dynamics of this domain induces a sampling of more flexible conformations that induce a disorder mechanism that leads to complex formation with the partner dehydrogenases [13]. Interestingly, the polymorphic position analysed is within domain I, in close contact to domain II. One may speculate, on the basis of the available crystal structures, that replacement of a isoleucine with a threonine at position 171 in ETFα- may influence ETF dynamics in the following way: an isoleucine would favor hydrophobic contacts with nearby aliphatic side chains from Domain II (Fig 5.1), whereas a threonine occupying this position would be less effective, and thus make the regions more flexible.

A very interesting correlation of our observations with clinical data arises from the fact that the ETFα-Thr171 is over represented among patients suffering from a mild form of VLCAD deficiency. This finding suggests that this polymorphism could eventually account for an increased susceptibility to inactivate ETF upon cell stress. This is a common circumstance in metabolic disease in which metabolic decompensation is intertwined with mitochondrial dysfunction resulting from metabolite accumulation, oxidative stress or fever.

Although we have shown that molecular chaperones can rescue these destabilised conformations and restore activity to higher levels, their overload as a result of massive proteostasis challenges may hinder complete recovery in patients. In agreement with this it has been recently reported that fever impairs beta oxidation in fibroblasts from patients suffering from different mitochondrial fatty acid beta oxidation defects. The analysis of additional polymorphic variations such as the one here analysed will contribute to a better understanding of the role of polymorphisms as modulators of human disease.

5.6. Acknowledgments

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6 GENERAL DISCUSSION

6.1. Functional and molecular consequences of ETF genetic variability

FAO disorders are included in the group of loss of function disorders, and as in other cases, the severity of the phenotype is dependent on the affected gene. This dissertation focuses on MADD, a FAO disorder associated to mutations in one of three genes: *ETFA*, *ETFB* or *ETFDH*. Since the first MADD report, in 1976, several studies involving patients' cells lead to the identification of over 55 mutations, that range from missense or nonsense mutations to frameshifts [1]. A correlation between *ETFA*/*ETFB*/*ETFDH* genotype and phenotype has been proposed; suggesting that homozygosity for two null mutations causes fetal development of congenital anomalies, type I patients [2]. However, even minimal amounts of residual ETF or ETF-QO activity seem to be sufficient to prevent congenital anomalies during embryonic development, type II patient [2]. Single point mutations that result at least in residual levels of active enzyme give rise to even milder phenotypes (type III patient). However, it has been suggested that milder forms can also be aggravated by environmental factors such as temperature [2]. Nevertheless, when considering milder phenotypes a directed correlation between genotype and the overall effect on protein structure and function that regulates the severity of the symptoms has not yet been fully described. The number of MADD clinical reports has been increasing over the last years in great extent, mostly due to the newborn screening programs; however, there are scarce reports focusing on understanding the molecular mechanism of MADD.

Salazar *et al* report an *in vitro* study on two ETF mutations associated with a severe MADD phenotype, ETFα-Thr266Met and ETFα-Gly116Arg. Over-expression of the ETFα-Gly116 mutant in *E. coli* was shown to be dependent

on the co-expression of GroEL and GroES chaperonins, and even so the mutant was not stable enough to further purification [3]. The ETFα-Thr266 mutant, the most frequent mutation found in patients, was purified and characterized. Although its overall structure is similar to wild type protein the flavin environment is altered by the mutation [3]. The activity of the ETFα-Thr266Met is strongly impaired, suggesting that this could be the cause of the severe phenotype. Another report has described the heterologous expression of ETFβ-Asp128Asn variant, containing a missense mutation identified in a patient with a mild phenotype [2]. Those studies have shown that the residual activity of the mutant enzyme could be rescued up to 59% of that of wild-type, when ETFβ-Asp128Asn transformed *E. coli* cells were grown at low temperature (30ºC) rather than at 37ºC. This was an important indication that environmental factors such as cellular temperature could play a role influencing disease progression.

In this perspective, our studies aimed at gaining a better understanding on the functional, cellular and molecular effects of the genetic variability in ETF. Also, we set to investigate how cellular factors such as increased temperature, modulate disease progression.

We have compiled an updated list of missense mutation described for ETF protein and analysed each mutation at the structural level aiming to find a correlation between genotype, phenotype and protein structural behaviour. The use of *in silico* tools for mutagenesis predictions and structural analysis permitted us to found that mutations fall essentially in two groups: one in which mutations affect protein folding and assembly; and another in which mutations impair catalytic activity and disrupt interactions with partner dehydrogenases. In the ETF α-subunit, for which 11 missense mutations are described, it was observed that there are two affected regions: one region that

involves essential interactions with the ETF β-subunit and another conserved region rich in FAD and MCAD interacting residues. In the case of the ETF β-subunit, we could suggest that there is a region rich in cofactor contacts, one that affects subunit interactions and another involved in the "recognition loop" implicated in interactions with ACDHs.

Moreover, we suggest that amino acid alterations that lead to drastic changes in the chemical properties of the residues involved in catalytic regions or regions rich in contacts between the subunits or with the cofactor have a higher probability to result in severe phenotypes. However, no direct correlation can be established between the genotype and the severity of the phenotype. Further, we have analysed three of these mutations, ETFβ-Cys42Arg, Asp128Asn and Arg191Cys, which typify different scenarios in respect to the clinical phenotypes.

The ETFβ-Cys42Arg mutation, associated to a severe form of MADD, directly affects the AMP binding site and intersubunit contacts, impairing correct protein folding. Upon co-expression with GroEL/GroES or DnaK/DnaJ/GrpE, ETFβ-Cys42Arg recombinant expression is increased; however, the protein has no activity probably due to lack of cofactor insertion.

The two other mutations, ETFβ- Asp128Asn and Arg191Cys, associated with milder forms of MADD, were purified after heterologous expression in a prokaryote system. Although none affected the overall α/β fold topology of native ETF, as shown by far-UV CD analysis of the purified proteins, both substantially decrease its enzymatic activity and conformational stability. The ETFβ-Asp128Asn mutation has a stronger impact on conformational stability (ΔT_m =-8ºC), as the mutation is near the subunit interface and the FAD binding site. Its decreased activity is likely a result of increased susceptibility

for FAD loss due to protein instability. The loss of activity in the ETFβ-Arg191Cys variant seems to result from impaired interaction with the electron-donor dehydrogenases since the mutation site is at the interface of the complex close to the "recognition loop", and no drastic decrease on stability was observed.

6.2. Molecular rationale for vitamin B2 effects

Further, we have used the mild variant ETFβ-Asp128Asn to study the molecular rationale of the impact of heat stress on FAO disorders, and moreover how disease symptoms could be rescued by vitamin B2 supplementation.

Why some FAO patients are responsive to riboflavin whereas others are not is one of the remaining challenges in this field [4-6]. An important approach to this topic is to study the flavinylation process *in vitro*, in order to better understand the structural and functional importance of the cofactor. Acyl-CoA dehydrogenases have occasionally been purified as apoenzymes, lacking FAD, or with substoichiometry FAD binding. Apoenzymes can usually be converted into holoenzymes by incubation with FAD. The FAD content in the cell is strongly dependent on dietary intake of flavins; low nutrient levels of riboflavin resulting in FAD shortage will likely affect the function of all these flavoenzymes.

Using *in vitro* translation/import into isolated mitochondria experiments, Nagao & Tanaka showed that the effect of low levels of riboflavin/FAD inside mitochondria resulted in a decreased proteolytic stability of the mature acyl-CoA dehydrogenase molecules. One may thus infer that this might be due to increased conformational flexibility of the oligomer lacking FAD, which results in higher susceptibility to endoproteases.

Later experiments by the Tanaka laboratory using MCAD as model showed that FAD also plays a very important role during the folding process, before the assembly of the subunits into the functional tetramer [7]. MCAD depends on the assistance of GroEL/GroES chaperonin system for its folding [8-9], and according to Saijo and Tanaka's experiments, FAD appears to be incorporated after MCAD monomers are released from the chaperonin complex or immediately before, thus assisting folding of the monomer to an oligomer assembly competent conformation. The disease-causing MCAD-Thr68Ala mutation directly affects a residue that is involved in FAD binding [10-11]. Although the major effect of this disease allele is on transcription levels, expression in a bacterial system and purification of the enzyme revealed that the amino acid replacement results in an enzyme that more readily looses FAD (~20% of wild type). In agreement, the enzymatic activity was decreased, as well as protein thermostability, thus also suggesting a structural role for the cofactor [11].

Sato *et al* also reported *in vitro* folding of ETF and, in connection with that, the roles of FAD and AMP on the folding and dimerization process [12]. Based on these experiments, a model in which AMP binds to a more loose conformation of the dimer was suggested, whereas FAD binds to a more compact form. Although this is suggestive of a sequential incorporation, *in vitro*, the two factors can be incorporated sequentially independently of each other.

In line with these studies, we have shown that the ETFβ-Asp128Asn clinical variant deflavinylates 3-fold faster than the wild-type protein during heat stress (mimicking a fever episode) with concurrently increased loss of activity. This is in agreement with the fact that a patient with this mutation has only developed disease symptoms in connection with a virus infection and fever [2]. Experiments in the presence of a 2.5 fold excess of FAD in

respect to ETF, corresponding to the increase observed in muscle mitochondria in riboflavin responsive patients [13], have shown that flavinylation improves the conformational and proteolytic stability of the protein, also increasing its biological activity [14]. Moreover flavinylation prevents activity loss and prevents loss of tertiary contacts during heat stress, up incubation at 39ºC. A noteworthy observation is the fact that ETFβ-Asp128Asn is not directly located in the FAD binding domain. Therefore, the observations made could be generalized to other mutations in different flavoproteins involved in fatty acid β-oxidation defects. Moreover, the use of this mild mutation, which was modulated by environmental factors, provides a concrete molecular rationale for the efficiency of riboflavin supplementation.

Presumably vitamin B2 supplementation increases the intramitochondrial flavin content, thereby compensating for the loss of flavin cofactors. Merging the different molecular, biochemical and cellular data described in this work, has allowed to outline a mechanism that accounts for the structural and functional rescue due to flavinylation (Fig. 6.1). After translation and import into the mitochondria the apoprotein form may become flavinylated *via* a chaperonin- independent (**A**) or chaperonin-dependent (**B**) pathway. In both cases, steps involving FAD insertion may eventually be mediated by FAD-chaperone proteins. The chaperonin-dependent pathway may involve folding of the apo monomer which then gets flavinylated upon release or immediately after release, like proposed for MCAD. Oligomerization into the functional forms (tetramers or dimers) is made starting from the holo-protein form. Upon an adverse cellular or patho-physiological condition such as a genetic mutation, stress (thermal, oxidative or other) or riboflavin and flavin depletion, cofactor lability may be enhanced thus resulting in an equilibrium of populations in which there is a significant amount of the enzyme in the

apo-form, as suggested by the ETF mild variant. The latter is known to be more conformationally destabilized and susceptible to degradation or misfolding, resulting in loss of function. In some cases, restoring the intra-mitochondrial flavin levels as a result of riboflavin supplementation, results in an increase of the activity of the affected proteins [13].

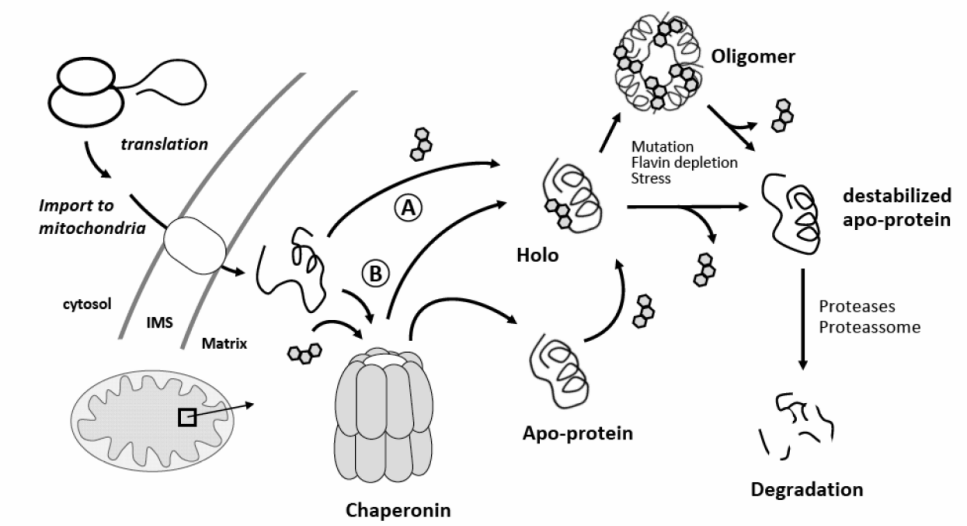

Figure 6.1: Cartoon representing different scenarios for pathways through which FAD may be inserted into proteins conferring structural and functional rescue. See text for details.

We have proposed a molecular mechanism that could provide a molecular rationale for the effect of riboflavin supplementation. However, even for the ETFβ-Asp128Asn, for which we proved that flavinylation has beneficial results, β-oxidation flux in patient fibroblasts cultured in riboflavin-supplemented media, using myristate or palmitate as substrates, was only 14% and 28% of controls, respectively [15]. This shows that *in vivo*, even though flavinylation can ameliorate the damaging effect of the ETFβ-Asp128Asn mutant, it cannot rescue protein activity to the level that is required to restore normal β-oxidation flux. This is analogous to other reports on riboflavin responsiveness in mild MADD, where biochemical and clinical abnormalities

are only partially restored [16-17].

Therefore, new strategies for improvement of the clinical treatments need to be prepared. Recent interest has been set in the use of fibrates, like bezafibrate, which act as an agonist of the peroxisomal proliferators-activated receptors (PPARs). The nuclear hormone receptor family of the peroxisomal proliferators–activated receptors (PPARs) is involved in the regulatory network that controls mitochondrial FAO, so drugs that act as PPAR activators could be relevant therapeutic targets to modulate FAO disorders [18]. A study on VLCAD patient cell showed that bezafibrate increases the level of VLCAD mRNA, protein, and residual enzyme activity [19]. In addition, it was reported that bezafibrate also stimulates the expression of several FAO genes in human fibroblasts, including carnitine palmitoyl-transferases, MCAD, LCHAD, ETF and ETF-QO [19]. As reviewed in [18], the beneficial effect of fibrates, in particular bezafibrate, has been proven in patient cells harbouring milder forms of CPT II or VLCAD deficiencies. Moreover, it is noteworthy that bezafibrate has been prescribed as a hypolipidaemic agent, and it is generally considered to have a good safe profile [20-21]. Hence, it seems clear that in parallel with a detailed study on the use of riboflavin in different FAO disorders, the use of bezafibrate could also be further tested.

6.3. Role of polymorphisms as modulators of human disease

Further, we investigated ETF polymorphism as a model to better understand how polymorphic variants can modulate disease development. Four polymorphic variants of ETF have been described (ETFα-171Ile/Thr and ETFβ-154Met/Thr) [22-23], and has been suggested that one of them (ETFα-Thr171) has decreased thermal stability in comparison to the others

[24]. It was also proposed that such polymorphisms could constitute susceptibility factor for the disease development in VLCAD patients, for which the less stable polymorphic variant was overrepresented.

In addition, Li and co-workers have recently shown that heat stress in cultured fibroblasts (41ºC), increases acetylcarnitine (C2) levels, while it significantly decreases the levels of other acylcarnitines in control and MCAD-deficient cells. However, CPT2, VLCAD or MTP patient cells have an enhanced accumulation of long chain acylcarnitines (C12, C14 and C16) [25-26]. Also, culture cells associated with MADD showed a reduction of short to medium-chain acylcarnitines but a significant increase in long-chain acylcarnitines [26]. These results suggest that long-chain associated FAO disorders are more susceptible to heat stress.

Aiming to dissect the molecular effects of the polymorphic variation in the folding, stability and dynamics of ETF we have performed a detailed *in vitro* investigation of the polymorphism in position 171 of the α-subunit. We have shown that the ETFα-Thr171 is prone to faster FAD release, and that it exhibits increased conformational dynamics during thermal stress.

Through heat stress, such as during fever, cells up regulate several molecular chaperones, which are able to rescue thermally destabilized proteins. We have shown, *in vitro*, that in fact, the GroEL chaperonin can rescue ETFα-Thr171, as it captures and refolds the dynamic populations of the protein, as suggested by the recovery of its biological activity. In the cell, this effect could eventually be less significant since several other proteins will be affected, and an overload of the cell quality control machinery would probably decrease its efficiency. These results are especially important to outline how polymorphic variants, which are apparently innocuous, may significantly influence disease progression and severity via indirect effects.

As a final remark, it would be interesting, in particular in patients with

long-chain fatty acid impaired oxidation, to systematically investigate expression levels and activity of the FAO enzymes, especially ETF in order to better judge on global and indirect effects on the pathway. This knowledge could contribute to the design of better therapeutic alternatives.

6.4. References

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