Emerging Roles for Riboflavin in Functional Rescue of Mitochondrial β–Oxidation Flavoenzymes

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Abstract: Riboflavin, commonly known as vitamin B2, is the precursor of flavin cofactors. It is present in our typical diet, and inside the cells it is metabolized to FMN and FAD. As a result of their rather unique and flexible chemical properties these flavins are among the most important redox cofactors present in a large series of different enzymes. A problem in riboflavin metabolism or a low intake of this vitamin will have consequences on the level of FAD and FMN in the cell, resulting in disorders associated with riboflavin deficiency. In a few number of cases, riboflavin deficiency is associated with impaired oxidative folding, cell damage and impaired heme biosynthesis. More relevant are several studies referring reduced activity of enzymes such as dehydrogenases involved in oxidative reactions, respiratory complexes and enzymes from the fatty acid β-oxidation pathway. The role of this vitamin in mitochondrial metabolism, and in particular in fatty acid oxidation, will be discussed in this review. The basic aspects concerning riboflavin and flavin metabolism and deficiency will be addressed, as well as an overview of the role of the different flavoenzymes and flavin chemistry in fatty acid β-oxidation, merging clinical, cellular and biochemical perspectives. A number of recent studies shedding new light on the cellular processes and biological effects of riboflavin supplementation in metabolic disease will also be overviewed. Overall, a deeper understanding of these emerging roles of riboflavin intake is essential to design better therapies.

Keywords: Flavin, Riboflavin-responsive, Mitochondrial metabolism, Disease, Protein folding, conformational destabilisation, biophysics.

RIBOFLAVIN METABOLISM AND DEFICIENCY

Riboflavin, or vitamin B2, was first described in the end of the XIX 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, and as a necessity to clarify the nomenclature, the compound was renamed riboflavin. This designation derives from its ribityl side chain and yellow color [1]. Vitamin B2 is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), two very important protein cofactors involved in a broad range of flavoenzymes, including oxidases, reductases and dehydrogenases [2] (Fig. 1).

Vitamin B2 intake is made through regular diet, and is ingested in a protein bound form, or already as FAD and FMN in flavoproteins (Fig. 2). The major sources of vitamin B2 are eggs, milk, some meat products, yeast and vegetables [3]. The normal intake for an adult is 1.6-2.6 mg/day, and deficiency symptoms are rarely observed with a normal diet [3]. The vitamin or its derivatives are released by stomach acid and via gastric/intestinal proteases. FMN and FAD are sequentially hydrolysed to riboflavin by alkaline phosphatases and FMN/FAD pyrophosphatase [4]. The free riboflavin is transported into the enteroctye by an energy-dependent and sodium independent riboflavin carrier, probably the recently identified riboflavin transporter 2 (RFT2) [5]. After release to the hepatic portal vein, riboflavin is taken up by the hepatocyte riboflavin transporter that is regulated by intracellular Ca²⁺/calmodulin [4]. Inside the cell, riboflavin is then phosphorylated by ATP and flavokinase to FMN, which is converted to FAD by FAD synthetase and is present in the presence of ATP. The conversion of FMN and FAD can also occur inside the mitochondria, as this organelle also contains riboflavin kinase and FAD synthetase enzymes [4]. Free FAD can be incorporated in mitochondrial apoflavoproteins or, it can be carried by the Flx1p FAD carrier to the cytosol [6, 7].

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%), in a concentration of 30 nM total flavin [4] (Fig. 2).

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 [8-11]. Different cellular systems can be affected directly or indirectly by a lower amount of riboflavin and there are several studies reporting the cellular consequences of reduced riboflavin. In fact, the acyl-CoA dehydrogenases working in the first dehydrogenation step of fatty acid oxidation (FAO) are among the first flavoenzymes to be affected during moderate riboflavin deficiency [12-15]. 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 (ACDHs) [12, 14]. It appeared that certain ACDHs are specifically sensitive – namely short chain acyl-CoA dehydrogenase (SCAD) and isovaleryl-CoA dehydrogenase (IVD), one of the ACDHs involved in amino acid metabolism, [16, 17]. Investigations in the Tanaka laboratory in the 1990s suggested that FAD regulates the ACDHs on 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 [18].
Tests in HepG2 liver cells revealed that a deficient riboflavin medium causes oxidative stress and cell damage [19]. In this study the authors pursued the idea that a decrease in riboflavin content would decrease glutathione reductase activity, as FAD serves as a cofactor for the enzyme. The importance of this enzyme is related with the production of reduced glutathione, a scavenger of free radicals and reactive oxygen species. As major conclusions the authors described a decrease of glutathione reductase activity, an increase in protein carbonylation (a process associated with oxidative damage), and an increase of DNA strand breaks [19]. 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 [20]; in human lymphoid cells cultured under flavin-deficient conditions, interleukin-2 accumulates intracellularly, probably due to an impairment of the human homologue of Ero1 [21]; 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 [22]. Riboflavin deficiency can also affect heme biosynthesis via protoporphyrinogen oxidase, a FAD dependent enzyme of the pathway [4, 23]. It may also be involved, in some cases of anaemia [24] and other biological processes as reviewed in [25].

**FLAVOPROTEIN CHEMISTRY**

Flavins are redox active protein cofactors that participate in many biological processes as they are able to carry out two-electron or one-electron transfer reactions and to be involved in diverse catalytic reactions (Fig. 3). These include two-electron dehydrogenation of several substrates, aromatic hydroxylations, one or two electron redox reactions, activation of molecular oxygen and emission of biological light [1, 26, 27]. Recently they were even described as participating in signal transduction in programmed cell death [28] and regulation of biological circadian clocks [29]. Flavins are composed of an isoalloxazine ring system, the catalytic moiety, a ribityl side chain, and either a 5'-terminal phosphate ester in the case of FMN, or a pyrophosphate linkage of FAD with an aminomorphophosphate (AMP) moiety, in the case of FAD. The isoalloxazine ring system has an amphipathic structure: it can interact both with hydrophobic regions within a protein through its xylene moiety, or it can form hydrogen bonds through the pyrimidine ring [26]. Flavins can be analyzed using diverse biophysical methods, such as visible absorption, visible circular dichroism, resonance raman and fluorescence emission, which take advantage of a set of well defined spectroscopic signatures which are characteristic of different redox states and protein environments [27]. Also, a substantial number of chemically modified flavin variants are available allowing studies of the chemical environment by exchanging the natural flavin with a modified one. One example is the use of 8-chloroflavins, as the substitution in position 8 is readily eliminated by sulphur oxidation, resulting in a 8-SR-flavin with different spectral characteristics constitutes a valuable tool to analyse enzymatic reactions and to study flavin chemistry within the flavin-protein complex. The redox properties of flavins that account for their functional properties are determined by the ring system and are modulated by the protein environment. Free flavins have a redox potential of about -210 mV (versus the normal hydrogen electrode) but those of protein-bound flavins vary rather widely, spanning up to 600 mV. This results from non-covalent interactions such as π-stacking, hydrophobic effects, hydrogen bonding, electrostatic interactions, flavin binding and protonation state [1, 34]. The semiquinone is thermodynamically stabilized in many flavoproteins, and this affords a separation of the redox potentials of the two one-electron transfer reactions (Figs. 3B and 3C). The same is achieved by kinetic stabilization of the radical, i.e. as a result a one-electron reduction step of the flavoprotein, or if the acceptor available for a fully reduced flavin is another flavin radical, which can only accommodate one-electron. If the redox potentials for the first and second steps are equivalent (i.e. for E(ox/sq)=E(sq/red)), around 30% of the flavin will be stabilized in the semiquinone form during the two-electron transfer reaction. Fig. (3B) shows a theoretical curve for a two-electron reduction of FAD in which E(ox/sq) and E(sq/red) are equal to 0 mV. However, protein stabilisation of the semiquinone results in an effective separation of the redox potentials of the two steps, and in this case FAD may in fact be populated to high levels (Fig. 3C, for example theoretical curves with E1 =0 mV and E2 = -150 mV). These aspects are mechanistically important for the diversity of reactions catalyzed by flavoenzymes and underlie the functional variety afforded by these cofactors. In fact, the increasing redox potentials of these flavoenzymes assures the functional coupling: acyl-CoA dehydrogenases (-114 mV), ETF (25/30mV) and ETF-QO (28/-6 / 47 mV) [35-37].

**FLAVOENZYMES INVOLVED IN FATTY ACID OXIDATION**

This review is especially focused on the consequences of riboflavin availability in the mitochondria and how it affects fatty acid metabolism. 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. 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 beta oxidation. 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), acylcarnitine translocase (CAT) and carnitine palmitoyl transferase II (CPT II). 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. The subsequent steps are catalyzed by enoyl-CoA hydratase, 3-L-hydroxacyl-CoA dehydrogenase and β-ketoacyl-CoA thiolase and lead to the formation of an acetyl-CoA and a fatty acyl-CoA two carbon
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shorter [38]. The disorders associated with these enzymes and transport proteins are commonly denominated by fatty acid oxidation (FAO) deficiencies. The first report on these disorders dates from 1973 by DiMauro and DiMauro, and described a CPT II deficiency [39]. Two years later Karpati et al. described CAT deficiency [40], and in 1976 Gregersen et al. reported MCAD deficiency [41] and Przyrembel et al. reported multiple acyl-CoA dehydrogenation deficiency (MADD) [42]. Until now defects in at least 18 enzymes of the pathway have been identified [38]. FAO disorders are inherited autosomal recessively 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 [43]. 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 [38, 44].

Since the mid-90s the 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 diagnostic acylcarnitine profiles in blood spots permitting to identify enzyme-specific anomalies in fatty acid oxidation [45].

At this point it is important to draw the attention of the reader to the fact that all acyl-CoA dehydrogenases catalyzing the first step in fatty acid β-oxidation as well as the two enzymes, electron transferring flavoprotein (ETF) and electron transfer flavoprotein ubiquinone oxidoreductase (ETF:QO), which transfer the electrons gained in the dehydrogenation reactions to ubiquinone in the mitochondrial inner membrane, are flavoenzymes with FAD as an essential co-factor (Table 1, Fig. 4). 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)). The ACDHs of fatty acid β-oxidation form a gene family with four additional acyl-CoA dehydrogenases involved in amino acid metabolism: glutaryl-CoA dehydrogenase (GCD), isovaleryl-
CoA dehydrogenase (IVD), short-branched chain acyl-CoA dehydrogenase (SBCAD) and isobutyryl-CoA dehydrogenase (IBD). All these ACDHs carry one non-covalently bound FAD molecule per subunit and the binding sites reside within each monomer, i.e., FAD contacts only one subunit at a time. ETF is a heterodimer with one FAD and a catalytically inactive AMP molecule, whereas the monomeric, membrane-embedded ETF:QO contains one FAD molecule and an iron-sulfur center (Fig. 4). The availability of FAD is thus essential for the functioning of the first step in fatty acid oxidation and exploitation of the gained electrons for ATP synthesis.

**FATTY ACID OXIDATION DISORDERS RESPONSIVE TO RIBOFLAVIN**

As an essential source of flavin cofactors riboflavin therapy – in theory – could be beneficial in most fatty acid oxidation disorders caused by missense mutations in single flavoenzymes like the acyl-CoA dehydrogenases, ETF and ETF:QO, which result in mild misfolding or destabilization of protein structure that directly or indirectly affect FAD binding. However, despite extensively studied during the last decades, only few papers have reported on riboflavin responsiveness in disorders of single fatty acid acyl-CoA dehydrogenases and in most cases, the primary gene defect, and consequently the molecular mechanisms of riboflavin responsiveness, are unknown. One exception to this is the enigmatic Riboflavin-Responsive Multiple Acyl-CoA Dehydrogenation Deficiency (RR-MADD) patients, who show a spectacular response to riboflavin treatment. By the recently identification of *ETFDH* as the major disease-associated gene in RR-MADD, defect flavinylation – and not defect flavin metabolism as previously suggested - could be the primary molecular mechanisms of riboflavin responsiveness in RR-MADD. We will discuss RR-MADD in later sections. This section will deal with knowledge on riboflavin responsiveness in disorders of single fatty acid acyl-CoA dehydrogenases.

To our knowledge no large clinical trials on riboflavin responsiveness in fatty acid oxidation disorders have been conducted, so the knowledge discussed in this section is derived from single case reports.

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common fatty acid oxidation disorder. Duran and coworkers measured MCAD activities in lymphocytes of five MCAD patients before and after three weeks treatment with riboflavin (50-150 mg/day). All five MCAD deficient patients at least doubled their MCAD activity from 5 to 12% of control mean after treatment. In one patient MCAD activity was increased to the heterozygote level. The clinical response and the primary gene defects were not reported [46]. In another study of eight patients with MCAD deficiency patients at least doubled their MCAD activity from 5 to 12% of control mean after treatment. In one patient MCAD activity was increased to the heterozygote level. The clinical response and the primary gene defects were not reported [46]. In another study of eight patients with MCAD deficiency no effect on fibroblast MCAD enzyme activities could be detected upon supplementation with 20 μM FAD [47]. Amendt and coworkers 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 [48].

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**Table 1. Properties of Selected Mitochondrial Flavoenzymes**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Structure</th>
<th>Subunit MW (kDa)</th>
<th>Preferred Substrate(s)</th>
<th>OMIM</th>
<th>Gene symbol</th>
<th>Mutation†</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>very-long-chain acyl-CoA dehydrogenase (VLCAD)</td>
<td>3B96</td>
<td>67 x2</td>
<td>C16</td>
<td>609575</td>
<td>ACADVL</td>
<td>65</td>
<td>[97]</td>
</tr>
<tr>
<td>medium-chain acyl-CoA dehydrogenase (MCAD)</td>
<td>1EGE</td>
<td>45 (x4)</td>
<td>C8</td>
<td>607008</td>
<td>ACDAM</td>
<td>51</td>
<td>[35, 98]</td>
</tr>
<tr>
<td>short-chain acyl-CoA dehydrogenase (SCAD)</td>
<td>2VIG</td>
<td>41 (x4)</td>
<td>C4</td>
<td>606885</td>
<td>ACADAS</td>
<td>14</td>
<td>n.a.</td>
</tr>
<tr>
<td>glutaryl-CoA dehydrogenase (GCDH)</td>
<td>ISIQ</td>
<td>43 (x4)</td>
<td>Glutaryl-CoA</td>
<td>608801</td>
<td>GCDH</td>
<td>95</td>
<td>[99, 100]</td>
</tr>
<tr>
<td>isovaleryl-CoA dehydrogenase (IVD)</td>
<td>1IVH</td>
<td>43 (x4)</td>
<td>3-methylbutyl-CoA</td>
<td>607036</td>
<td>IVD</td>
<td>22</td>
<td>[101]</td>
</tr>
<tr>
<td>short-branched chain acyl-CoA dehydrogenase (SBCAD)</td>
<td>2JIF</td>
<td>44 (x4)</td>
<td>(s)-2-methylbutyl-CoA</td>
<td>600301</td>
<td>ACADSB</td>
<td>7</td>
<td>n.a.</td>
</tr>
<tr>
<td>isobutyryl-CoA dehydrogenase (IBD)</td>
<td>1RX0</td>
<td>44 (x4)</td>
<td>Isobutyryl-CoA</td>
<td>604773</td>
<td>ACAD8</td>
<td>18</td>
<td>[102]</td>
</tr>
<tr>
<td>electron transfer flavoprotein (ETF)</td>
<td>1EFV</td>
<td>30</td>
<td>Reduced acyl-CoA dehydrogenases</td>
<td>608053</td>
<td>ETFA</td>
<td>13</td>
<td>[36, 103]</td>
</tr>
<tr>
<td>electron transfer flavoprotein ubiquinone oxidoreductase (ETF-QO)</td>
<td>2GMJ</td>
<td>64</td>
<td>ETF</td>
<td>231675</td>
<td>ETFDH</td>
<td>21</td>
<td>[37, 104]</td>
</tr>
</tbody>
</table>

†Number of missense mutation, data extracted from The Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/index.php (accessed 14.05.10). Accession numbers: Protein Data Bank.
Three papers have reported on riboflavin responsiveness in patients with short-chain acyl-CoA dehydrogenase (SCAD) deficiency. In one paper the genetic defect was not investigated [49]. In two other papers riboflavin responsiveness is associated with the p.Gly209Ser / c.625G>A SCAD variant [50, 51]. The SCAD p.Gly209Ser variant has a mild effect on protein folding/stability [52] and is found in the general population with a homozygous frequency of 7% but is overrepresented in patients with ethylmalonic aciduria, a marker for SCAD deficiency (60%), indicating that other environmental and genetic factors contribute to disease development [53-55]. Subclinical or biochemical riboflavin deficiency is quite common in the general population [25] and moderate riboflavin deficiency is known to selectively impair mitochondrial fatty acid oxidation by decreasing the activities of the flavin-dependant acyl-CoA dehydrogenases and in particular short-chain acyl-CoA dehydrogenase [13, 15]. Thus, mitochondrial flavin content and consequently riboflavin status could be a contributing factor in the development of SCAD deficiency. Recently van Maldegem and coworkers assessed the FAD status and evaluated the effects of riboflavin treatment in 16 patients with SCAD deficiency [51]. Blood FAD levels were normal in all patients before riboflavin supplementation therapy, but significantly lower in patients harboring the c.625G>A variation, as compared to 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. [56]. However, even though van Meldegem and coworkers 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 treatment with riboflavin. So the authors did not recommend riboflavin as a general treatment in SCAD deficiency [51].

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 dehydro-
RIBOFLAVIN-RESPONSIVE MULTIPLE ACYL-CoA DEHYDROGENATION DEFICIENCY

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 [65]. 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 attacks can be lethal [66-68]. 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 [11, 66-70]. These abnormalities resemble those found in rats fed with a riboflavin-deficient diet [15], 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 ETF, ETFB and ETFDH genes encoding the ETF/ETF-QO pathway.

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 [67]. 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 [71]. Since then the molecular defect in RR-MADD has been confined to the ETFDH gene in a number of cases [68, 72-76] and to the ETFA gene in a single case [76] 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 closely associated with ETF-QO mutations, the role of these mutant proteins in production of the secondary mitochondrial dysfunction - with impaired function of multiple acyl-CoA dehydrogenases, respiratory chain complexes and coenzyme Q10 - remains controversial [55]. As low mitochondrial concentrations of flavins have been observed in some patients with RR-MADD [11, 69, 70] 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 PPAR-ERK pathway [77]. 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 similar metabolic adaption to 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 [67] 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 [69, 71, 72], and 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 in most cases being more impaired than VLCAD [78-80]. It remains to be clarified if these observed differences are due to differences in the technical setups 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 [55, 67]. However, the exact trigger(s) of the metabolic switch and the implicated molecular pathways and mechanisms remain elusive.

Apart from clinical reports, in vitro studies of the pathogenic nature of missense mutations associated with the RR-MADD phenotype have not yet been published so the molecular rationale for the spectacular riboflavin-responsiveness remains elusive. Intercurrent illness, often associated with fever and suboptimal caloric intake, is known to aggravate the symptoms in RR-MADD. This means that flavin-dependent fatty acid oxidation enzymes are upregulated during impaired cellular folding conditions with high temperature and perhaps decreased flavin content. It is likely that the RR-MADD missense mutations confer a riboflavin-responsive phenotype by a mechanism similar to the one observed with the ETFβ-p. Asp128Asn mutation, where the flavin becomes less tightly bound during heat stress causing loss of stability and activity [81] (further discussed below). Like in this case most RR-MADD missense mutations are not located in the FAD binding domain but are distributed all over the protein structure [55], suggesting that destabilization of local interactions may lead to long-distance conformational changes that may affect FAD binding indirectly. In contrast to MADD fibroblasts, which only show a partial correction of fatty acid β-oxidation flux when cultured at high concentrations of riboflavin [47, 82-85], RR-MADD patient fibroblasts show normal or near normal β-oxidation flux at high riboflavin concentrations [11, 67]. Thus, even though MADD patients with missense mutations affecting protein folding/stability probably may benefit from riboflavin supplementation the beneficial effect depends on the nature and location of the amino acid substitution, with amino acid substitutions associated with RR-MADD likely being more benign. Future studies of the pathogenic nature of RR-MADD missense mutations are clearly needed to elucidate the molecular mechanisms of the amazing phenotypic response to riboflavin. Such studies may also add to customize riboflavin therapies to suit the genotype and individual needs instead of treating the phenotype.

EVIDENCE OF ALTERED FLAVIN METABOLISM IN RR-MADD

Primary genetic defects in ETFDH do not easily explain the decreased activities of mitochondrial flavoenzymes, including acyl-CoA dehydrogenases and respiratory chain enzymes, observed in pre-treated mitochondrial protein samples from RR-MADD patients. Therefore, these changes have been suggested to result from altered mitochondrial riboflavin metabolism, but so far only few studies have attempted to investigate the molecular causes and consequences of altered flavin metabolism in RR-MADD and no study has tried to unravel the connection(s) – if any - between ETFDH gene defects and disturbances of mitochondrial flavin homeostasis.

In 1999, Vergani and co-workers described two patients with RR-MADD and lipid storage myopathies in which they have also analysed enzymes involved in the regulation of the mitochondrial flavin pool [69]. They observed a decrease in the amount of mitochondrial FAD and FMN, and have correlated this with reduced activity of several flavoproteins, namely SCAD, MCAD and complex II. The activity of mitochondrial FAD pyrophosphatase, which converts FAD into riboflavin, was significantly increased in one patient but normal in the other patient.

Another important study was a detailed investigation using a proteomic approach recently carried out by Gianazza and colleagues. This elegant report aimed at establishing a correlation between flavin metabolism and mitochondrial flavoenzyme dysfunction [70]. The study focused on muscle mitochondria from a patient with profound muscle weakness associated with ethylmalonic-adipic aciduria, which upon riboflavin medication underwent a substantial improvement, as assessed by biochemical parameters. In this patient the ratio between acyl/free carnitine and the amount of intracellular lipids, two features associated with MADD, were evaluated. The activities of the fatty acid β-oxidation acyl-CoA dehydrogenases and those of respiratory chain complexes were measured, and found to be decreased in respect to controls. These data led the authors to evaluate the FAD and FMN concentrations in whole muscle, and the results evidenced a lower amount of available FAD. At this point, to better characterize the extent of dysfunction of mitochondrial pathways, the mitochondrial subproteome of muscular tissue from the patient was analysed using a combination of 2D PAGE and MS methods. A reference map of normal human muscle mitochondria was constructed to serve as control. The authors noticed that several proteins were down regulated such as the 75kDa Fe-S subunit of NADH:quinone oxidoreductase, ETF:QO, MCAD, the β subunit of the tri functional enzyme, 3-hydroxy-isobutyryl-CoA hydrolase, the E2 component of the branched-chain α-ketoacid dehydrogenase complex, the E2 component of pyruvate dehydrogenase complex, among others. All these proteins reverted to normal levels after riboflavin treatment. Many of the down regulated enzymes were flavoproteins suggesting a dysfunction of the flavin regulator pathway, in agreement with the reduced amount of FAD in muscle.

A very interesting and novel observation resulting from this study was the fact that RR-MADD mitochondria were in fact depleted in not only flavin-dependant acyl-CoA dehydrogenases and respiratory chain components but also other flavoproteins, some of which were completely absent. The affected flavoenzymes belong to flavin-dependent mitochondrial pathways and to a subset of mitochondrial calcium-binding proteins. These results prompted the question that...
riboflavin cofactors maybe important for transcriptional or translational regulatory factors similar to what was observed in yeast [86], or that they are important players during the folding process or that they exert a key function as conformational flavoprotein stabilizers, as in the cases of MCAD and ETF [81, 87, 88]. This work therefore established the proof of principle in respect to the application of proteomics to the analysis of fatty acid β-oxidation disorders, which can be extended to the study of different tissues from patients with diverse clinical phenotypes. This would contribute to identify defective cellular processes, including affected flavoenzymes that would help to better rationalize the molecular basis of therapeutic riboflavin supplementation.

Genetic testing of the ETFA, ETFB or ETFDH genes were not reported in the three RR-MADD patients described above to have altered mitochondrial flavin metabolism. Therefore, we do not know if disturbances of mitochondrial flavin metabolism are primary disease causes in these patients or rather associated to primary gene defects in the ETFDH gene as reported in another RR-MADD patient recently (63, 65). In fact, despite suggested for many years, genetic defects of riboflavin metabolism as primary causes of RR-MADD were only very recently indicated, when a an unknown genetic defect of riboflavin metabolism was suggested to cause maternal riboflavin deficiency during pregnancy and transient significant RR-MADD disease in the newborn child [10].

FLAVINYLA TION: STRUCTURAL AND FUNCTIONAL PROTEIN RESCUE

Another important approach is to study the flavinylation process in vitro in order to better understand the structural and functional importance of the cofactor; merging the different molecular, biochemical and cellular data allows to outline the mechanisms that account for the structural and functional rescue due to flavinylation (Fig. 5). Acyl-CoA dehydrogenases have occasionally been purified as apoenzymes, lacking FAD or with only partial loading of the FAD binding site. A poenzymes can usually be converted into holoenzyme by incubation with FAD. Also, it has been reported that the ACDHs may differ in their affinity for FAD, although some of the evidence is contradicting. As the FAD is non-covalently bound, FAD is distributed between the ACDHs, other flavoproteins and possible FAD binding proteins. The FAD content is therefore dependent on dietary availability and environmental conditions. Low nutrient levels of riboflavin resulting in FAD shortage will affect the function of these enzymes. In vitro translation/import into isolated mitochondria experiments, Nagao & Tanaka showed that the effect of low levels of riboflavin/FAD manifested inside mitochondria leading to decreased proteolytic stability of the mature acyl-CoA dehydrogenase molecules. One might thus infer that this is due to increased conformational breathing of the oligomer lacking the FAD molecules which results in higher susceptibility to endoproteases. However, later experiments by the Tanaka laboratory using MCAD as an example showed that FAD plays also a very important role during the folding process, before assembly of the subunits to the native enzyme occurs [87]. When FAD was depleted, acquisition of the native structure was strongly hampered and the unfolded/partially folded polypeptide chain remained in complex with the chaperonin. Using a bacterial expression system in which the levels of the GroEL/GroES chaperonin system, a homolog of mitochondrial Hsp60/Hsp10, can be manipulated, we have shown that MCAD depends on the assistance by this specific molecular chaperone for its folding [89, 90]. According to Saijo and Tanaka’s experiments, FAD appears to be incorporated after monomers of MCAD are released from the chaperonin complex or immediately before, thus finishing folding of the monomer to an oligomer assembly competent conformation. Moreover FAD plays a critical role for medium-chain acyl-CoA dehydrogenase folding [91], presumably by exerting nucleation effects during monomer folding. This study used recombinant expression in bacteria of artificial mutant variants targeting amino acids, Thr136 and Glu137, which play a role in the binding of the FAD molecule. The disease-causing MCAD pThr68Ala mutation directly affects a residue that is directly involved in FAD binding [92, 93]. Although the major effect of this disease allele is on transcript 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 suggesting also a structural role for the cofactor. Interestingly, mutations distant from the active site and FAD binding region may also have an effect on the environment of the co-factor. This has been exemplified by biochemical and biophysical analyses of the MCAD p.Lys304Glu variant, which is prevalent in MCAD deficiency [94]. 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 [95]. 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. This may suggest a sequential incorporation, however, in vitro the two factors can be incorporated sequentially independently of the orders.

More recently we have shown that an ETFβ p.Asp128Asn variant, identified in a patient with M:MADD disease [82, 85], deflavinylates 3-fold faster than the wild-type protein during a fever-mimicking heat stress, with concomitantly increased loss of activity. In the case of this patient, disease symptoms developed in connection with a virus infection and fever. The study demonstrated that although the mutant protein had only 30% of wild type activity it presented a native like fold, but with looser tertiary contacts as suggested by a modified fluorescence emission spectrum. Experiments in the presence of a 2.5 fold excess of external FAD, corresponding to the increase observed in muscle mitochondria in riboflavin responsive patients [70], and at 39°C, simulating a fever event, showed that flavinylation improved the conformational and proteolytic stability of the protein, also increasing its biological activity [81]. Presumably riboflavin supplementation increases the intramitochondrial flavin content, thereby compensating for the loss of flavin cofactors. Beta-oxidation flux in patient fibroblasts homozygous for the ETFβ p.Asp128Asn mutant, using myristate or palmitate as substrates, was 14% and 28% respectively of that of controls when cultured in riboflavin-
supplemented media [82]. This shows, that even though flavinylation can ameliorate the damaging effect of the ETFβ p.Asp128Asn mutant, it cannot rescue protein activity to a level that is required to restore normal β-oxidation flux. This is analogous to other reports on riboflavin responsiveness in M:MADD, where biochemical and clinical abnormalities are only partially restored [47, 83]. A noteworthy observation is the fact ETFβ Asp128 is not directly located in the FAD binding domain, therefore the observations made could be extrapolated to several mutations of different flavoproteins of fatty acid the β-oxidation. Moreover the use of this mild mutation, which was modulated by environmental factors, provides a concrete molecular rationale for effectiveness of riboflavin supplementation.

OUTLOOK

The molecular consequences of riboflavin supplementation in the functional rescue of defective fatty acid β-oxidation flavoenzymes is becoming increasingly clear with the combination of diverse studies made during the last decades, which have ranged from in vitro analysis on 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 besides being an essential chemical component of the active site, FAD appears to be directly and actively involved in monomer folding, possibly by stabilizing intermediary conformational states which are part of a preferred folding pathway, leading to a faster/more efficient acquisition of the native state. Also, flavin-protein interactions are known to modulate the conformational stability of the recipient protein, as well as the protease sensitivity of the folded protein. Thus, FAD availability and the protein-flavin affinity which may be altered in mutant variants, can impact on the enzyme lifetime and degradation turnover. In fact, a diversity of missense mutations are expected to affect protein folding and stability [76, 85, 96] and it has been suggested [59], but only recently experimentally proven, that riboflavin therapy may overcome a genetic defect that directly or indirectly affects FAD coenzyme binding [81]. In relation to patients with mutations causing defects that are sensitive to co-factor availability, optimizing the supply of FAD by a riboflavin rich diet [51] or supply of other factors like chemical chaperones or conformation stabilizers have the potential to be used. Research on this subject is mainly based on work performed in the last decade of the last century. A renewed interest is presently observed and it will be necessary to systematically apply current methodology to elucidate the basic principles and disentangle the different possible effects.

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REFERENCES


Emerging Roles for Riboflavin in Functional Rescue

Furman, F. E.; Goodman, S. I. Defects of electron transfer flavoprotein and electron transfer flavoprotein ubiquinone oxidoreductase: glutaric acidemia type II. In The metabolic and molecular


