Journal of Inherited Metabolic Disease

Riboflavin Transport and Metabolism in Humans

--Manuscript Draft--

Manuscript Number:	BOLI-D-16-00043R2
Full Title:	Riboflavin Transport and Metabolism in Humans
Article Type:	Invited Articles
Keywords:	Riboflavin homeostasis; Flavin cofactors; Riboflavin transporters; Riboflavin kinase, RFK; FAD synthase, FADS or FMNAT; FLAD1 gene; FAD delivery; Riboflavin Responsive Multiple Acyl-Coa Dehydrogenase Deficiency, RR-MADD
Manuscript Classifications:	10: Inborn errors of metabolism – general, adult; 40: Dietetics and nutrition; 140: Disorders of fatty acid oxidation and ketone body metabolism; 280: Disorders of vitamins, cofactors and trace elements
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Funding Information:	MIUR (Ministry of Instruction, University and Research - Italy) (PON01_00937) — Dr Maria Barile
Abstract:	Recent studies elucidated how riboflavin transporters and FAD forming enzyme work in humans and create a coordinated flavin network ensuring the maintenance of cellular flavoproteome. Alteration of this network may be causative of severe metabolic disorders such as MADD (Multiple Acyl-CoA Dehydrogenase Deficiency) orBrown-Vialetto-van Laere syndrome. A crucial step in the maintenance of FAD homeostasis is riboflavin uptake by plasma and mitochondrial membranes. Therefore, studies on recently identified human plasma membrane riboflavin transporters are presented, together with those in which still unidentified mitochondrial riboflavin transporter(s) have been described. A main goal of future research is to fill the gaps still existing as for some transcriptional, functional and structural details of human FAD synthases (FADS) encoded by FLAD1 gene, a novel "redox sensing" enzyme. In the frame of the hypothesis that FADS, acting as a "FAD chaperone", could play a crucial role in the biogenesis of mitochondrial flavo-proteome, several basic functional aspects of flavin cofactor delivery to cognate apo-flavoenzyme are also briefly dealt with. The establishment of model organisms performing altered FAD homeostasis will improve the molecular description of human pathologies. The molecular and functional studies of transporters and enzymes herereported, provide guidelines for improving therapies which may have beneficial effects on the altered metabolism.

Dear Editor,

thank you very much for your final decision concerning our manuscript entitled "Riboflavin Transport and Metabolism in Humans", an invited submission for the **SSIEM2015 conference issue,** to be published in the JIMD**.**

As suggested by Reviewer 1 we have included, as in press, a-just accepted for publication in HJHG-manuscript, concerning the involvement of FLAD1 variants in severe mitochondrial respiratory chain derangements and RR-MADD. Moreover, we have substituted Fig. 2 and the corresponding legend since we noticed that RFK abbreviation was not correct.

Other minor points:

- Brown-Vialetto-van Laere syndrome – in the summary - and gene names are now correctly written. Sorry for this!
- The quite confusing denomination of the bifunctional enzyme (FAD synthetase) with respect to the single animal module FAD synthase (corresponding to the more clear and correct nomenclature FMN:ATP adenylyl transferase or FMNAT) is now clarified at lines 29-30 at page 6. We decided to leave, also in this review, the" classical" acronyms FADS, that was used since the beginning, for readers' sake.
- We beg your pardon if the sequence of sentences (page 9 line 1-4 in the previous version now at line 17-19 page7) was not as clear as we expected. We have omitted the speculation and added a reference (Yazdanpanah et al 2009) probing the lethality of RFK mutation in mice.
- LSD1 EC has been filled in the hyphens.

We thank you for the precious contribute in ameliorating our manuscript and we hope that this revised version of the manuscript is now suitable for publication in IJHD.

Since we found in the Instruction to Authors that a figure could be indicated for the cover of the issue I suggest Fig. 2 of the manuscript as a potential candidate for this, if you agree.

Looking forward to hearing from you, I remain

Yourssincerely

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Riboflavin Transport and Metabolism in Humans

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KEYWORDS

1. Riboflavin homeostasis
2. Flavin cofactors
3. Riboflavin transporters
4. Riboflavin kinase, RFK
5. FAD synthase, FADS or FMNAT
6. *FLAD1* gene
7. FAD delivery
8. Riboflavin Responsive Multiple Acyl-Coa Dehydrogenase Deficiency, RR-MADD

Recent studies elucidated how riboflavin transporters and FAD forming enzyme work in humans and create a coordinated flavin network ensuring the maintenance of cellular flavoproteome. Alteration of this network may be causative of severe metabolic disorders such as MADD (Multiple Acyl-CoA Dehydrogenase Deficiency) orBrown-Vialetto-van Laere syndrome. A crucial step in the maintenance of FAD homeostasis is riboflavin uptake by plasma and mitochondrial membranes. Therefore, studies on recently identified human plasma membrane riboflavin transporters are presented, together with those in which still unidentified mitochondrial riboflavin transporter(s) have been described. A main goal of future research is to fill the gaps still existing as for some transcriptional, functional and structural details of human FAD synthases (FADS) encoded by *FLAD1* gene, a novel "redox sensing" enzyme. In the frame of the hypothesis that FADS, acting as a *"FAD chaperone"*, could play a crucial role in the biogenesis of mitochondrial flavo-proteome, several basic functional aspects of flavin cofactor delivery to cognate apo-flavoenzyme are also briefly dealt with. The establishment of model organisms performing altered FAD homeostasis will improve the molecular description of human pathologies. The molecular and functional studies of transporters and enzymes herereported, provide guidelines for improving therapies which may have beneficial effects on the altered metabolism.

Introduction

This review briefly deals with molecular aspects linked to cellular FAD homeostasis and its derangements; it presents recent research concerning the mechanisms of riboflavinabsorption, cellular trafficking and metabolism. A section is devoted to the most recent achievements on the mechanism of FAD assembly to nascent mitochondrial and nuclear apo-flavoproteins, enlarging the idea that beside mere enzymatic cofactors, vitamin derived cofactors may function in protein stabilisation as well as in regulation of apo-protein synthesis. The comprehension of how the cell regulates flavin homeostasis as well as the precise understanding of the physiological role exerted by FAD biosynthetic pathways in different sub-cellular compartments require further investigation. Answering such questions appears to be of special interest in the light of the recent notion that impairment in flavoenzymes activity and flavin supply/metabolism could have a role in the pathogenesis of Rf-responsivemyopathies. Human metabolic diseases affecting mainly nervous and muscular systems are caused by flavoprotein derangements or inadequate availability of flavin cofactors caused by genetic mutations. Among these, there are the Rf-Responsive Multiple Acyl-CoA Dehydrogenase Deficiency (RR-MADD) and the Brown-Vialetto-Van Laere syndrome (BVVLS). Moreover, a role of FAD and other vitamin-derived cofactors in regulation of epigenetic

events and in cancer is emerging [(Olsen et al 2007; Giancaspero et al 2015) and see (Barile et al 2013) and Refs therein].

Riboflavin-derived cofactors are essential for cellular function

Riboflavin or vitamin B2, the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), is a key molecule in the aerobic cell. The vitamin (7,8-dimethyl-10-ribitylisoalloxazine) is made up of a substituted isoalloxazine ring, whose N-10 atom is bound to a ribityl residue. In FMN the 5'end of the ribityl moiety is esterified by a single phosphoryl group; adenylation of FMN gives rise to FAD (Fig. 1). FMN and, more frequently FAD, are destined to tightly-and sometimes covalently-bind to one out of the hundreds of the so called apo-flavoenzymes(McCormick 1989; Joosten and van Berkel 2007; Heikal 2010; Macheroux et al 2011; Barile et al 2013). During the catalytic cycle, enzyme-bound flavin cofactors can undergo one-electron and two-electron transfer processes, giving rise to the semi-reduced (only stabilized by the action of the protein environment) or fully reduced forms, which can be differentiated from one another and from the oxidized flavins on the basis of their optical properties (Fig. 1). Functionally active holoenzymes have a typical flavin UV/Vis spectrum. They are essential for energy generation and are also involved in a wide variety of reactions which, in eukaryotes, are preferentially localised in mitochondria (Merrill et al 1981; McCormick 1989; Massey 1995; Massey 2000; Depeint et al 2006). Thanks to their natural auto-fluorescence properties, which are sensitive to protein binding and local environment, flavin cofactors may contribute to a varying extent to cellular fluorescence, thus allowing non-invasive imaging of activities of living cells and tissues, with an increasing interest in using them as intrinsic biomarker for cellular bioenergetics (Reinert et al 2007; Tohmi et al 2009; Heikal 2010).

As summarised in Fig. 2, the oxidative metabolism of carbohydrates, fatty acids, certain amino acids, choline, betaine, and a number of other bioenergetically relevant metabolites in mammals, depends on the functionality of mitochondrial flavoproteins. In particular, subunits of the respiratory chain complexes I and II, as well as the electron transfer flavoprotein (ETF) and its ubiquinone oxidoreductase (ETFQO), localised in the inner mitochondrial membrane, drive electrons from a number of reduced flavoproteins to ubiquinone and then to complex III of respiratory chain.

Riboflavin availability could also be correlated to the biosynthesis of heme(Powers 2003) and inorganic enzymatic cofactors: those derived from iron, such as FeS clusters (Wollers et al 2010), deserve special attention, given their long-established simultaneous co-presence in many flavoenzymes or multi-enzymatic complexes, among which complex II (Maio et al 2015).

In the intermembrane space of mitochondria, the flavoprotein apoptosis inducing factor (AIF) initiatescaspase-independent programmed cell death (Sevrioukova 2011) and a FAD-dependent pathway controls oxidative protein folding (Bragoszewski et al 2015). A better defined FAD-dependent pathway controls protein folding in the endoplasmic reticulum (Hudson et al 2015), where FAD trafficking was described (Tu et al 2000). Flavin cofactors have also unique roles in redox balance since flavoproteins, distributed in mitochondrion, peroxisome, plasma membrane, and nucleus are involved in both generating and scavenging of oxygen reactive species (ROS) and reactive nitrogen species (RNS) (Fransen et al 2012). In the nucleus, FAD dependent oxidases play some roles in epigenetic events, controllingthe expression of genes involved in energy metabolism (Hino et al 2012; Giancaspero et al 2013). The wide spectrum of existence, function and localisation of all these FAD dependent enzymes, raises the problem of their biogenesis route and dependence on FAD availability in each organelle. Given this multiplicity of cellular functions, it is not surprising that inadequate riboflavin availability, as well as derangements in FAD homeostasis, may be causative of cell degeneration and cancer (Powers 2003; Nakano et al 2011; Powers et al 2011), as well as severe metabolic defects some of which treatable with high doses of the vitamin (Gregersen 1985; Gianazza et al 2006; Barile et al 2013).

Nutritional status, absorption and transport of riboflavin

Riboflavin can be synthesized by bacteria, fungi and plants starting from GTP and ribulose 5-Pi (Bacher et al 2000). Yeast can also take riboflavin from the outside (Perl et al 1976; Sibirnyi et al 1977), being the product of *MCH5* gene (Mch5p) the first plasma membrane transporter cloned in eukaryotes (Reihl and Stolz 2005). Conversely, higher organisms have lost the ability to synthesize the vitamin and have to obtain it, as other water soluble class B vitamins, from food or to a lesser extent from intestinal microflora's production. Best nutritional sources of riboflavin are seafood, poultry, lean meat, milk and dairy products, eggs, cereals and vegetables. To a lesser extent riboflavin derives from intestinal microflora. The average requirement for riboflavin is 1.3 mg/day and 1.1 mg/day for adult men and women, respectively, with some variations depending on age and status (e.g. pregnancy and lactation), as assessed by the Commission of the European Communities (1993). Other aspects of riboflavin nutrition were exhaustively dealt with in previous reviews (McCormick 1989; Foraker et al 2003; Powers 2003; Said 2011).

Riboflavin absorption in mammals takes place mainly in small intestine and partly in large intestine. As summarized in Fig. 3, following dietary protein denaturation, vitamin is released from the non-covalently bound cofactors by pyrophosphatases and phosphatases located on the brush border membrane of the intestinal epithelium. These intestinal hydrolases are still unidentified in

humans. A question remains also about the destiny of flavin cofactors that are covalently bound to dietary proteins. Free riboflavin is transported in enterocytes via carrier-mediated processes (Fig. 3 and see below). Once in the intestinal cell, it undergoes ATP-dependent phosphorylation to form FMN, most of which is further converted into FAD. Non-specific or still not well characterized hydrolases act on intracellular flavin cofactors to make again free riboflavin available for being transported through the basolateral membrane of the enterocytes into the plasma. There is little or no storage of riboflavin in the body; any surplus intake which overcomes the capacity of renal re-absorbtion, is eliminated in the urine in the form of riboflavin as such or of its catabolites 7-alpha-hydroxy riboflavin,10-hydroxyethylflavin, and lumiflavin(Chastain and McCormick 1987). This is why, as for other class B vitamins, riboflavin has a relatively low toxicity even at pharmacological doses (Gregersen 1985; Olsen et al 2015).

The physiological mechanisms regulating uptake of riboflavin by the different mammalian organs and tissues are only partially understood. As reviewed in (Said 2011; Barile et al 2013; Yonezawa and Inui 2013), functional studies on riboflavin transport in different mammalian and human tissues demonstrated that riboflavin transport is carrier-mediated, inhibited by structural analogues and metabolic inhibitors. Regulatory features are tissue specific, sometimes involving intracellular signal transduction pathways (Said and Ma 1994). Plasma membrane riboflavin transporters remained unidentified in mammals, for many years. A scarce homology of the mammalian riboflavin transporters with *MCH5*(Reihl and Stolz 2005) or with the bacterial riboflavin transporters - *RibU*, *impX*or *RibM*(Vitreschak et al 2002; Burgess et al 2006; Vogl et al 2007; Hemberger et al 2011), is one of the reasons of this gap. Recently, three human riboflavin transporters have been cloned and characterized, belonging to the SLC52 family of solute carriers, whose more recent nomenclature is: *hRFVT1*, *hRFVT2*, and *hRFVT3*, corresponding to*SLC52A1*, *SLC52A2* and *SLC52A3*, respectively (Yonezawa and Inui 2013). This novel nomenclature and classification substituted the previous acronyms *hRFT1*, *hRFT3*, and *hRFT2*(Yonezawa et al 2008) and see table 1. *hRFVT1*, *hRFVT2*, and *hRFVT3* genes are located at 17p13.2, 8q24.3 and20p13 loci, respectively. *hRFVT1*and *hRFVT2* proteins exhibit 86% amino acid identity with each other, and only 42 or 43 % identity, respectively, with *hRFVT3* (Fig. 4 A).

The three proteins have different sub-cellular and tissue-specific expression profile, as well as functional and kinetical properties (see Table 1) (Fujimura et al 2010; Barile et al 2013; Yonezawa and Inui 2013; Subramanian et al 2015; Subramanian et al 2015; Subramanian et al 2015; Wu et al 2016). They were predicted to have 10 (hRFVT1 and 2) or 11 (hRFVT3) membrane-spanning domains (Moriyama 2011; Yonezawa and Inui 2013). A novel homology model of hRFVT3 is presented here (Fig. 4B). Following absorption, most endogenous blood

flavinsincluding taken up riboflavin, are localized in circulating cells, particularly in erythrocytes, which contain only trace amounts of riboflavin, and median concentrations of 44 and 469 nmol/L, for FMN and FAD, respectively (Said and Mohammadkhani 1993). Circulating plasma riboflavin is bound both to albumin (*kd*= 3.8 to 10.4 mM) and, more tightly, to a sub-fraction of immunoglobulins (Innis et al 1985) with a median plasma concentrations of 10.5, 6.6 and 74 nmol/L for Rf, FMN and FAD (Hustad et al 2002). Therefore, under normal condition, binding to albumin may not be relevant. Several methods have been used to measure either urinary riboflavin excretion (Chastain and McCormick 1987) or riboflavin levels in erythrocytes/serum (Hustad et al 1999; Petteys and Frank 2011), whose values are, however, subjected to small circadian variations (Zempleni et al 1996) which impair the actual evaluation of riboflavin status. Thus, the most reliable method for flavin content determination is based on the estimation of enzymatic tissue saturation with cofactors, represented by the calculation of the Erythrocyte Glutathione Reductase Activation Coefficient (EGRAC) (Weber et al 1973). The normal EGRAC for riboflavin corresponds to a value lower than 1.2; a value between 1.2 and 1.4 indicates inadequate nutritional status; values above 1.4 indicate severe riboflavin deficiency. The EGRAC is unreliable in case of glucose 6-phosphate dehydrogenase deficiency, beta-thalassemia, or respiratory infections (Hustad et al 2002). In these cases measurements of pyridoxamine phosphate oxidase activity should be used (Mushtaq et al 2009). A link between riboflavin and vitamin B_6 has long been known, with FMN being required for pyridoxal-5'phosphate cofactor synthesis (McCormick 1989; Powers 2003). Riboflavin status correlates with folate and vitamin B_{12} metabolism. (Moat et al 2003; Depeint et al 2006; McNulty and Scott 2008) (see also Fig. 2). Deficiency of hRFVT2 and 3 have been reported to be a cause of BVVLS. hRFVT1 could not compensate for the defects probably due to different tissue expression (Yonezawa and Inui 2013; Jaeger and Bosch 2016).

Flavin cofactors synthesis and assembly to apo-enzymes

Once in the cell, riboflavin is quickly transformed into its catalytically active cofactors, before the assembly with specific apoproteins occurs. Two enzymes are required for flavin cofactor synthesis: riboflavin kinase (RFK, ATP: riboflavin 5'phosphotransferase, EC 2.7.1.26), which transfers a phosphoryl group from ATP to riboflavin to form FMN; FAD synthase, or better ATP:FMN adenylyltransferase (FADS or FMNAT, EC 2.7.7.2), that adenylates FMN to FAD (Fig. 1).In bacteria both RFK and FADS activities are fused in a bifunctional enzyme, which is still named FAD synthetase (see (Yatsyshyn et al 2009) and (Frago et al 2008; Herguedas et al 2010)). Beside the bifunctional enzymes, some monofunctional RFKs have recently been reported in prokaryots(Herguedas et al 2015). In archaea FAD biosynthetic pathway is performed by aunique

monofunctional RFK (namely *RibK*) and by a unique monofunctional FADS(Mashhadi et al 2008; Mashhadi et al 2010).

In lower eukaryotes and in animals, two physically distinct polypeptides with either RFK or FADS activities have been purified and characterised, as extensively reviewed in (McCormick 1989; McCormick 2000; Barile et al 2013).Their genes were firstly identified in *Saccharomyces cerevisiae* genome and named *FMN1* and *FAD1* respectively (Wu et al 1995; Santos et al 2000).

The human orthologue of *FMN1* is the *RFK* gene (EC 2.7.1.26) localised on chromosome 9q21.13: it codes for a 17.6 kDa polypeptide consisting of a single domain, whose crystal structures (PDB codes: 1NB0, 1NB9, 1P4M, 1Q9S) revealed a novel kinase fold that contains a six-stranded anti parallel β-barrel core and a unique ATP and flavin-binding site (namely a nucleotide binding motif) (Karthikeyan et al 2003). Upon binding of riboflavin, the enzyme undergoeslarge conformational changes which allows binding of ATP and catalysis (Karthikeyan et al 2003; Karthikeyan et al 2003). Structural observations correlate well with kinetics of RFK, largely regulated by the relative concentration of substrates/products (Yamada et al 1990). These data together with observations made in RFK-KD (knock down) cells (Hino et al 2012) allow to propose that RFK is the limiting step of the intracellular conversion of riboflavin into FAD, which in turn is regulated by the rate of riboflavin transport across the plasma membrane via RFVTs. Therefore, RFK deficiency, even if not jet described in humans so far, might be lethal as reported in mice (Yazdanpanah et al 2009).

This flavin-based kinetic control of flavin cofactor forming enzyme could be exerted together with other regulatory mechanism, such as those triggered by thyroid hormones (Lee and McCormick 1985).

*FAD1*localized on chromosome IV of *S. cerevisiae*, encodes for a monofunctional protein (Fad1p) essential for yeast life (Wu et al 1995). The *S. cerevisiae* Fad1p crystal structure was solved at 1.90Å resolution in a complex with the FAD product in the active site (Leulliot et al 2010). The extensive interaction observed between the enzyme and the product FAD, never observed in bacterial FADS crystal structures (Wang et al 2005), is in good agreement with the kinetic analysis and the molecular characterisation performed in our laboratories, where the human orthologue of *FAD1*, i.e. the *FLAD1*gene was identified and cloned (Brizio et al 2006) and the first human FADS was over-produced and purified to homogeneity (Galluccio et al 2007; Torchetti et al 2011). hFADS structure has yet to be resolved by X-ray crystallography. Studies performed with a recombinant form of hFADSshowed that it is a FAD-binding protein, and corroborate the proposal of a possible regulatory role of eukaryotic FAD synthesizing enzyme on cellular FAD homeostasis and flavoprotein biogenesis. Our structural observations are in line with the definition of hFADS as

a “jealous protein” for which a profound destructuration is necessary to remove the cofactor (Torchetti et al 2011; Giancaspero et al 2015). This protein feature is strictly connected to the problem of FAD delivery to nascent apo-flavoproteins (see below).

FLAD1 gene is localised on chromosome 1 at 1q21.3; it is organized in 7 exons and codes for different putative variants (Supplementary Fig. 1 with recent nomenclature and AC numbers), generated by alternative splicing. Only two of them have been characterised in detail up to now at the protein level. The isoform 2 is referred as the cytosolic form of the enzyme, while isoform 1 is the mitochondrial one (Torchetti et al 2010). Indeed, the existence of FADS in mitochondria is matter of debate since many years, as well as the direction of FAD movement across the mitochondrial translocator named Flx1p in *S. cerevisiae* and MFT in humans. Besides mediating FAD (or FMN) transport, Flx1p may have a role as "nutrient sensor" maintaining the normal status of mitochondrial FAD-binding enzymes such as lipoamide dehydrogenase and succinate dehydrogenase (Tzagoloff et al 1996; Bafunno et al 2004; Spaan et al 2005; Giancaspero et al 2008; Giancaspero et al 2014). This correlates well with the recent finding of a mitochondrial FAD transporter deficiency in a patient (Schiff et al 2016).

Studies on subcellular localisation of FADSs are still in progress and represent the ongoing research efforts in our laboratory, especially related to neuronal biochemistry (Lin et al 2009). More recently, FADS was found also in the nucleus (Giancaspero et al 2013); even if the nuclear isoform has not been characterised yet, nuclear FADS could concur, together with cytosolic and mitochondrial FADS, to the creation of a "flavin network", a scenario which is strictly in line with the recent literature demonstrating a fundamental role for cellular FAD biosynthesis in allowing for lysine demethylase1 (LSD1 or lysine-H3 histone demethylase 1, EC 1.14.11.B1) biogenesis and redox epigenetics (Hino et al 2012; Giancaspero et al 2013; Giancaspero et al 2014). Moreover, very recently, using RNAseq analysis combined with protein mass spectrometry, novel FADS isoforms have been described,both at the protein and at the mRNA levels in the frame of the identification and description of flavin cofactor homeostasis derangements found in different 9 patients suffering fromMultiple Acyl-CoA Dehydrogenase (MADD) and Combined Respiratory Chain Deficiency(Olsen et al 2016).

Even if tissue distribution, subcellular localization,as well as kinetical and molecular features or novel FADs isoforms remain to be fully characterized ((Torchetti et al 2010; Barile et al 2013) the emerging concept concerning FAD forming enzymes is that a dynamic control, exerted by alternative splicing, could regulate the expression/localization of specific FADS isoforms). It could be also hypothesized that one of hFADS isoforms may migrate to the nucleus in response to

chemical-physical changes induced by post-translational modifications that may be caused by different metabolic/redox states of the cell.

An interesting feature of both the FADSs characterised so far in humans (as in most higher eukaryotes), is that they differ from their yeast counterpart in being organized in two domains (Fig. 5). Besides the PAPS reductase domain, which is *per se* able and sufficient to catalyse FAD synthesis starting from FMN and ATP (Miccolis et al 2012), an additional N-terminal located domain is present in the hFADSs characterised up to now. It resembles a molybdo-pterin-binding (MbPt) domain.This domain is lacking in the novel hFADS isoforms, which only encode the FAD synthase domain and which are still able to catalyze the synthesis of FAD (Olsen et al 2016).

The function of MbPt domain in hFADS remained completely unknown since last year, when the first 231 amino acids of the N-terminusof hFADS2 were aligned with the sequences of two representative members of the COG1058 family(Cialabrini et al 2013; Giancaspero et al 2015). A Co^{++} dependent hydrolytic activity was revealed, as dependent on the redox state of one of the two redox sensitive cysteines of hFADS2 (Giancaspero et al 2015). Modelling of PAPS (3'phosphoadenosine 5'phosphosulfate) reductase domain and of the MbPt (molybdo-pterin-binding) domain hFADS have been obtained and reported in Fig. 5.

Since the FAD-forming enzyme in humans as well as in yeast is unrelated to the bacterial enzyme, and the latter is strictly required for bacterial viability, FADS is particularly interesting as a potential target for the development of novel antimicrobial drugs (Gerdes et al 2002; Serrano et al 2013).

From our recent studies concerning the molecular organization of human FADSs, the knowledge about FAD cleavage was enlarged (see above and Fig. 5). Differently from the well described FAD synthesis in humans, very little isknown about FAD cleavage and flavoprotein turnover,requiring the sequential action of two enzymatic activities: namely FAD pyrophosphatase (EC 3.6.1.-) and FMN phosphohydrolase (EC 3.1.3.2). Functional studies reported the existence of FAD hydrolysing enzyme in lysosomes and peroxisomes in mammals. Our group indicated the existence of FAD hydrolysing enzymes in the inter-membrane space of rat liver mitochondria (Barile et al 1997) and in the nucleus (Giancaspero et al 2013): kinetic studies suggest that they could be due to NUDIX (NUcleosideDIphosphate linked to another moiety, X) hydrolases. Muscular FAD pyrophosphatase was found to be altered in case-reports of patients affected by a rare human neuromuscular disorder called RR-MADD (see below) (Vergani et al 1999). The existence of 24 genes and 5 pseudogenes encoding NUDIX hydrolases have been described in humans, but only three of them have been described at the protein level (Safrany et al 1998; Kasprzak et al 2001). The members of this super-family show diverse substrate specificity as well

as different subcellular localizations(cytoplasm, nucleus, mitochondria, peroxisomes, plasma membrane) (McLennan 2006). A NUDIX hydrolase that efficiently hydrolyses FAD has been characterized in mammals, namely NUDT12(Abdelraheim et al 2003), but its localization appears to be only peroxisomal. Therefore, the problem of cleavage of FAD during flavoprotein degradation and recycling is far from being elucidated. In particular, the ability of the nuclear FAD hydrolysing enzyme to discriminate between the redox states of pyridine nucleotides might suggest a novel role for nuclear NAD(H) redox status in regulating nuclear FAD homeostasis. This feature was studied in some details using*S. cerevisiae*as a model (Giancaspero et al 2013; Giancaspero et al 2014).

Thus the question arises whether and how (but also where and when) the FAD forming enzyme have to choose between FAD delivery to cognate apo-flavoenzymes and FAD degradation. This is a fine problem of molecular recognition (Fig. 5). Our working hypothesis, that deserves further research, is that other ancillary proteins and probably the redox status of some out of the ten cysteine residues of the protein are responsible for the choice (Miccolis et al 2014).

A better knowledge of the structural relationships between the two domains of the protein (Miccolis et al 2012; Giancaspero et al 2015), as well as of the structural changes associated to the redox switch cysteine-related architecture (Miccolis et al 2014), might enlighten a number of relevant cellular processes as outlined in Fig. 2.

The problem of FADS delivery to nascent client apo-enzymes

As above mentioned, the purified recombinant hFADS2 behaves as a FAD binding protein, showing a typical flavoprotein absorbance spectrum, with a main peak at 274 nm and two minor peaks at 350 and 450 nm. In its PAPS reductase domain, the enzyme binds one mole of the FAD product very tightly, although non-covalently, with a FAD/protein ratio equal to 0.86 ± 0.2 mol FAD per monomer (Torchetti et al 2011; Miccolis et al 2012). In a number of "client" flavoproteins, like EFF/ETFQO (Olsen et al 2007; Cornelius et al 2012), acyl-CoA dehydrogenases (Saijo and Tanaka 1995), DMGDH (dimethylglycine dehydrogenase) (Decker and Brandsch 1997; Brizio et al 2004; Brizio et al 2008)apo-holo transition has an effect on folding/stability and, thus, a FAD-chaperon theory was developed (Olsen et al 2007).

Conversely, in the recombinant hFADS2, bound FAD does not affect chemical and thermal stability of its secondary structure (Torchetti et al 2011), a priori excluding chaperoning protein structuration, at least *in vitro*. Conversely, complete release of FAD from the recombinant protein required extensive structural changes (denaturation) *in vitro*, which is surprising, since *in vivo* newly synthesized FAD is expected to be rapidly delivered to "client" apo-flavoproteins (Fig. 5). Thus, a big question arises about the chemico-structural requirements for FAD release.

The observation that the relatively low k_{cat} values of the recombinant hFADS2 (Torchetti et al 2011) significantly increased under reducing condition, as well as the finding that the tightly bound FAD can be removed from the protein upon reduction (Pedrolli et al 2011) (M. Barile, unpublished results), suggested that the redox state of the hFADS could be crucial for the cofactor release. The concept of the protein as a redox sensor was strengthened by demonstration of the existence of certain redox sensitive disulphide bridges (Miccolis et al 2014). As demonstrated in other cases for assembling and inserting in some human proteins of inorganic (Bonomi et al 2008; Ye and Rouault 2010; Leitch et al 2012; Rouault 2015) and organic cofactors (Padovani and Banerjee 2009), we propose that hFADS is a part of a "flavinylation machinery" previously named mtFSF(Brizio et al 2000; Hao et al 2009). Thus, FAD transfer event seems not to occur in solution, but in the course of molecular recognition i.e. protein-protein interaction between FAD forming and client proteins. Also the entire molecule of the FAD-bound FADS has a chaperoning effect during client flavoenzyme biogenesis (Brizio et al 2000; Kim and Winge 2013; Maio et al 2015). The machinery should require the presence of an apo-protein accepting the cofactor and some accessory proteins, like mitochondrial chaperon Hsp60 and - presumably - additional still not characterised ancillary redox chaperones (Hsp10). A direct protein-protein interaction as well as a direct transfer of FAD from the donor hFADS to two "clients" rat DMGDH and human LSD1 was recently demonstrated (Giancaspero et al 2015).

A derangement of this "flavinylation machinery" is expected to be causative of misfolding of those mitochondrial polypeptides schematised in Fig. 2, presumably triggering mitochondrial unfolding response (Bender et al 2011; Cornelius et al 2012).This phenomenon should be relevant in particular for ETF/ETFQO complex, thus underlying the profound and coordinated metabolic and proteomic derangement(Vergani et al 1999; Gianazza et al 2006; Rocha et al 2011) connected to the oxidative stress (Olsen et al 2015) observed in RR-MADD patients.

To validate the hypothesis that defects in flavin cofactor supply/delivery may cause flavoproteome-dependent derangements in mitochondrial bioenergetics and protein homeostasis, two novel model organisms i.e. the yeast *S. cerevisiae* (Bafunno et al 2004; Giancaspero et al 2008) and the nematode *Caenorhabditis elegans* (Liuzzi et al 2012) have been established in which as a consequence of changing flavin cofactor metabolismalteration of complex II biogenesis, ATP production and ROS homeostasis and proteomic derangement were observed(Giancaspero et al 2014).

As outlined above, recent findings definitively identified the mitochondrial FAD transporter (*SLC25A32)*(Schiff et al 2016), as well as *FLAD1* variants as cause for riboflavin treatable MADD and more severe combined Respiratory Chain Deficiencies (Olsen et al 2016).

Other models have been established in the frame of the relationships between RVTs and BVVLS(Bosch et al 2011; Haack et al 2012; Biswas et al 2013). An exhaustive description of other riboflavin responsive human pathologies have been reviewed elsewhere (Gregersen et al 2008; Barile et al 2013).

Conclusions

Studies performed in the last decade have demonstrated the existence of a coordinated flavin network which is involved in the maintenance of cellular flavoproteome. Riboflavin transporters and FAD synthase isoenzymes have been highlighted as major players of the network, as demonstrated by the occurrence of severe metabolic disorders caused by defects of these molecular components. Even though several functional and molecular aspects of the network have been recently described, the structure and the regulation of the major players remain to be elucidated. Defining these still unknown issues has a strategical importance due their link with human health from the knowledge of the molecular basis of inherited pathologies to the development of novel antimicrobial drugs.

Acknowledgments and funding

The authors thank: the student M.T. Damiano, (UniversitàdegliStudi di Bari "Aldo Moro") for the aid in figure drawings and Mr V. Giannoccaro (UniversitàdegliStudi di Bari "Aldo Moro") for the technical assistance.

This work wassupported by PON-ricerca e competitività 2007-2013, MIUR (Ministry of Instruction, University and Research - Italy) project 01_00937: "Modelli sperimentali biotecnologici integrati per la produzione ed il monitoraggio di biomolecole di interesse per la salute dell'uomo" to M.B.

Conflict of interest and ethics declarations

Authors have nothing to declare, based on the "Competing Interests Questions".

Authors confirms that the content of the article has not been influenced by the sponsors".

Ethics approval was not required for all research studies.

Author contribution

Maria Barile, Teresa Anna Giancaspero, Piero Leone, Michele Galluccio, Cesare Indiveri contributed in preparing materials, writing and critically revising the manuscript and updating bibliography; Maria Barile coordinated all the work.

Table 1. Riboflavin transporters in *Homo sapiens*

Transporter	Tissue	Mechanism	References
hRFVT1 (hRFT1/SLC52A1)	Placenta, intestine, kidney	Na^+, potential and pH-independent	(Yonezawa et al 2008; Subramanian et al 2011; Yonezawa and Inui 2013)
hRFVT2 (hRFT3/SLC52A2)	Ubiquitously, highest expression: brain, salivary glands	Na^+ and pH-independent	(Yao et al 2010; Subramanian et al 2011; Yonezawa and Inui 2013)
hRFVT3 (hRFT2/SLC52A3)	Intestine, prostate, testis, stomach, pancreas	Na^+-independent and pH-dependent	(Yamamoto et al 2009; Fujimura et al 2010; Eli et al 2012; Ghosal and Said 2012; Yonezawa and Inui 2013)

Legend to Figures

Fig. 1. Structure of flavins. The structures of Riboflavin, FMN and FAD are reported with the indication of enzymes involved in conversion of riboflavin to FAD. While the oxidized flavins are yellow and the fully reduced molecules are colourless, the half-reduced forms can be red or blue depending on pH. Thanks to their natural auto-fluorescence properties, flavin cofactors, may contribute to a varying extent to cellular fluorescence, thus allowing non-invasive imaging of activities of living cells and tissues. Since intracellular flavin fluorescence properties are sensitive to protein binding and local environment there is an increasing interest in using them as intrinsic biomarker for cellular bioenergetics and neuronal activities (Gibson et al 1962; Murataliev 1999). The inset shows the absorbance spectrum of oxidized/reduced (blue curve) FAD with the titration curved of the three compounds.

Fig. 2. Flavoproteins involved in cell metabolism and relationships with oxidative pathways. Cytosolic, mitochondrial and nuclear flavoprotein metabolism are depicted. A complex network of transporters and enzymes accomplish flavin homeostasis and its relationships with other cell oxidative and regulatory pathways in cytosol, mitochondria and nucleus. Flavoproteins are depicted in yellow. Abbreviated names of transporters and enzymes are indicated in the figure as listed below:

RFK: Riboflavin kinase (EC 2.7.1.26); FADS2: FAD synthase isoform 2 (EC 2.7.7.2); RTs: plasma membrane riboflavin transporters; FADS1: FAD synthase isoform 1 (EC 2.7.7.2); mRT: mitochondrial riboflavin transporter; MFT: mitochondrial folate transporter; CACT: mitochondrial carnitine acylcarnitine translocase; OCTN2: organic cation transporter novel 2; ChT: choline transporter; LSD1: lysine specific demethylase 1 (EC 1.-.-.-); AIF: apoptosis inducing factor; CPT1: carnitine palmitoyltransferase 1; CPT2: carnitine palmitoyltransferase 2; I: respiratory chain complex I (NADH-ubiquinone oxidoreductase); II: respiratory chain complex II (succinate dehydrogenase); III: respiratory chain complex III (ubiquinol-cytochrome c reductase); IV: respiratory chain complex IV (cytochrome c oxidase); V: respiratory chain complex V (ATP

synthase); 2-OGDH: 2-oxoglutarate dehydrogenase complex; ACADs: acyl-CoA dehydrogenase various isoforms; BCKAD: branched-chain α-keto acid dehydrogenase complex; SaDH: sarcosine dehydrogenase; DMGDH: dimethylglycine dehydrogenase; SHMT: serine hydroxymethyl transferase; BHMT: betaine hydroxymethyl transferase; ETF: electron transfer flavoprotein; ETF-QO: electron transfer flavoprotein-ubiquinone oxidoreductase; CDH; Choline dehydrogenase; GCDH: glutaryl-CoA dehydrogenase; BCAD: acyl-CoA dehydrogenase branched chain specific (2-methyl-butyryl-CoA dehydrogenase); SAM: S-Adenosyl methionine; MTRR: Methionine synthase reductase; MTHFR: Methylenetetrahydrofolate reductase; FH4: tetrahydrofolate.

Fig. 3. Riboflavin, FMN and FAD distribution among polarized epithelia and blood. FAD and FMN from food proteins are converted to riboflavin by non-specific hydrolases on the brush-border membrane of intestine. Riboflavin vitamin is absorbed by action of RFVT3 at the apical membrane and is released in blood by RFVT1 and 2, where riboflavin associates with albumin or globulins, or is converted into a coenzyme form in erythrocytes or leukocytes, which contain several flavoproteins. RFVTs allow riboflavin uptake in tissue cells, such as hepatocytes, where the vitamin is converted into enzymatically active cofactors (not shown). Riboflavin and its catabolites are released in urine by renal epithelium. In this district RFVTs are also localized.

Fig. 4. Alignment of the hRFVT transporters isoforms and structural model of hRFVT3. (A) Alignment of the three isoforms of RFVTs was performed by ClustalW and manually adjusted. (B) The homology structural model of hRFVT3 was built, for the first time, using as template the glucose transporter from *Staphylococcus epidermidis* using the Phyre2 online software. The protein shows the 11 transmembrane α-helical segments nearly parallel to the membrane axis. Cys residues are highlighted in red. These residues are distributed along the structure. Some of these residues are close enough to be oxidized to disulphides, thus suggesting the possible occurrence of a redox control of the protein function/structure.

Fig. 5. Homology structural model of the MPTb and PAPS domains of hFADS2. The homology models were built using Modeller 9.15 software (Webb and Sali 2014). The N-terminal (aa 102 to

aa 266) portion of hFADS was first aligned with the template CinA (PDB ID:3KBQ) of *T. acidophilum* by ClustalW and manually adjusted; then the MPTb domain homology model was obtained. The C-terminal portion (aa to 232 to 490) of hFADS2 was aligned with the template FMN adenylyltransferase (PDB ID:3G5A) of *C. glabrata*(Huerta et al 2009) as for the MPTb; then the PAPS domain homology model was obtained. Ribbon representation of the MPTb and PAPS redecutase domains domain of the hFADS2: the Cys residues are highlighted in red ball representation and numbered. Predicted mechanism of FAD delivery to client apo-flavoproteins is shown below the structural models. Modeling of the human PAPS reductase domain will also allow high-throughput screening for the identification of specific inhibitors/modulators.

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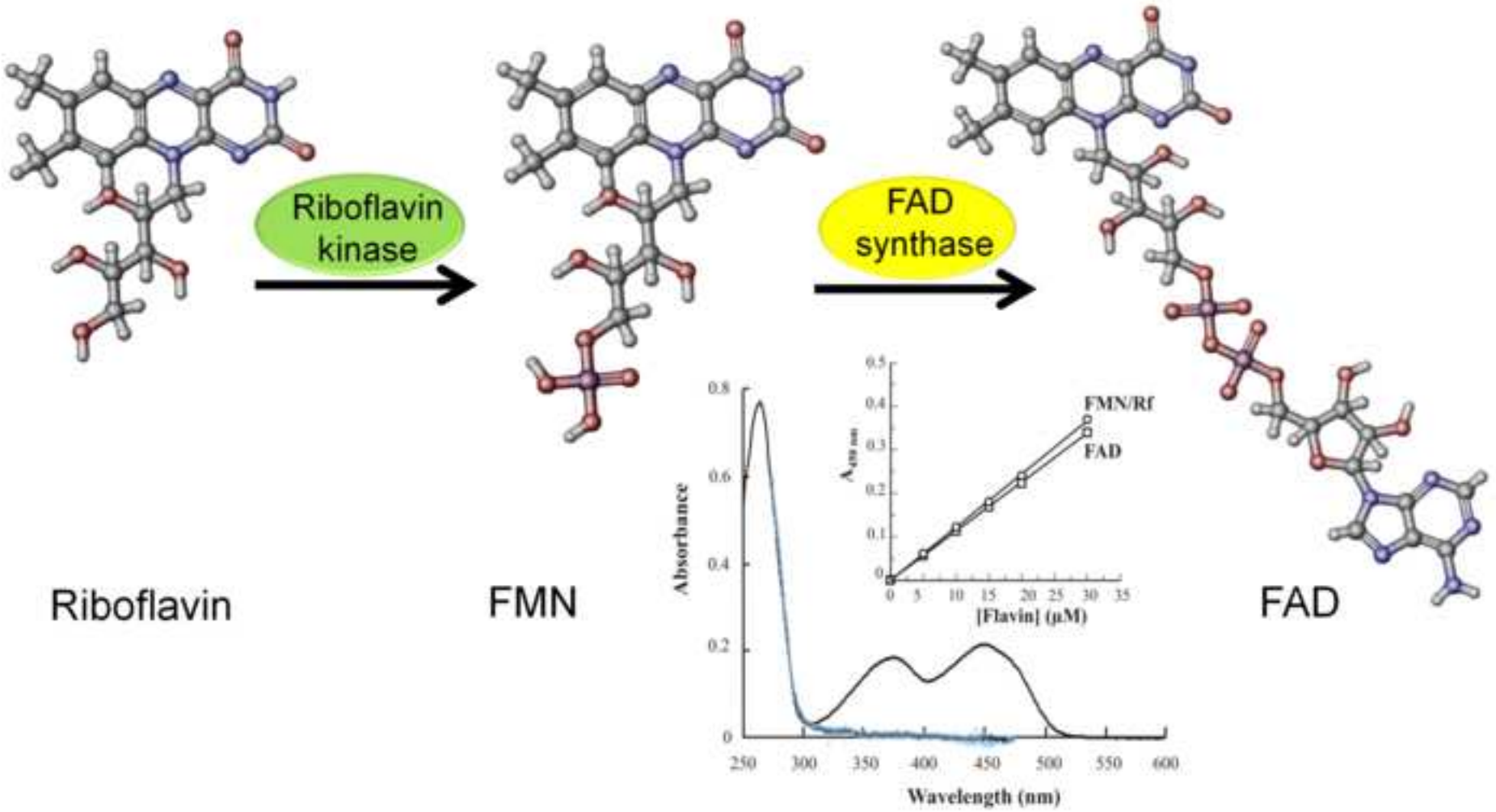

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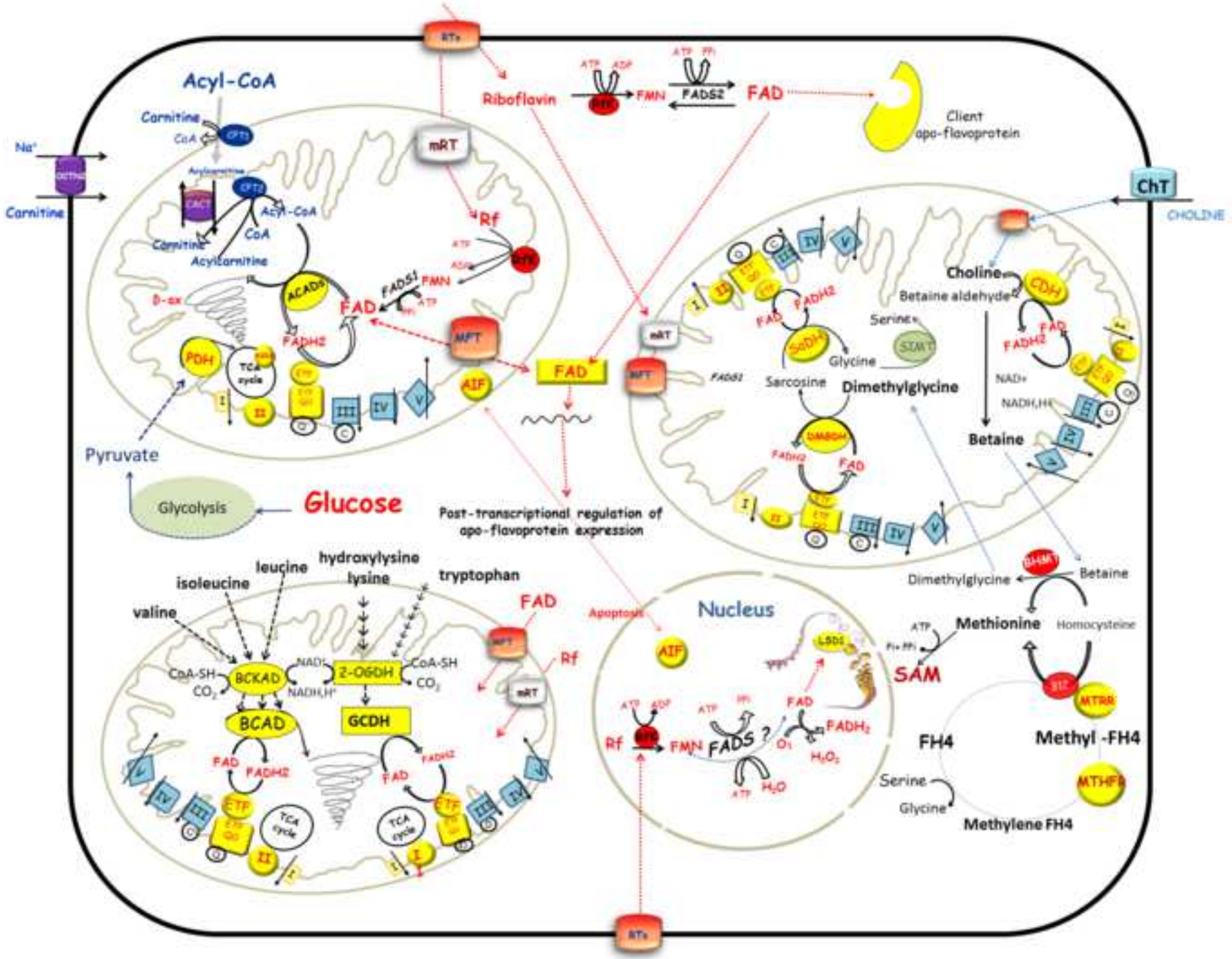

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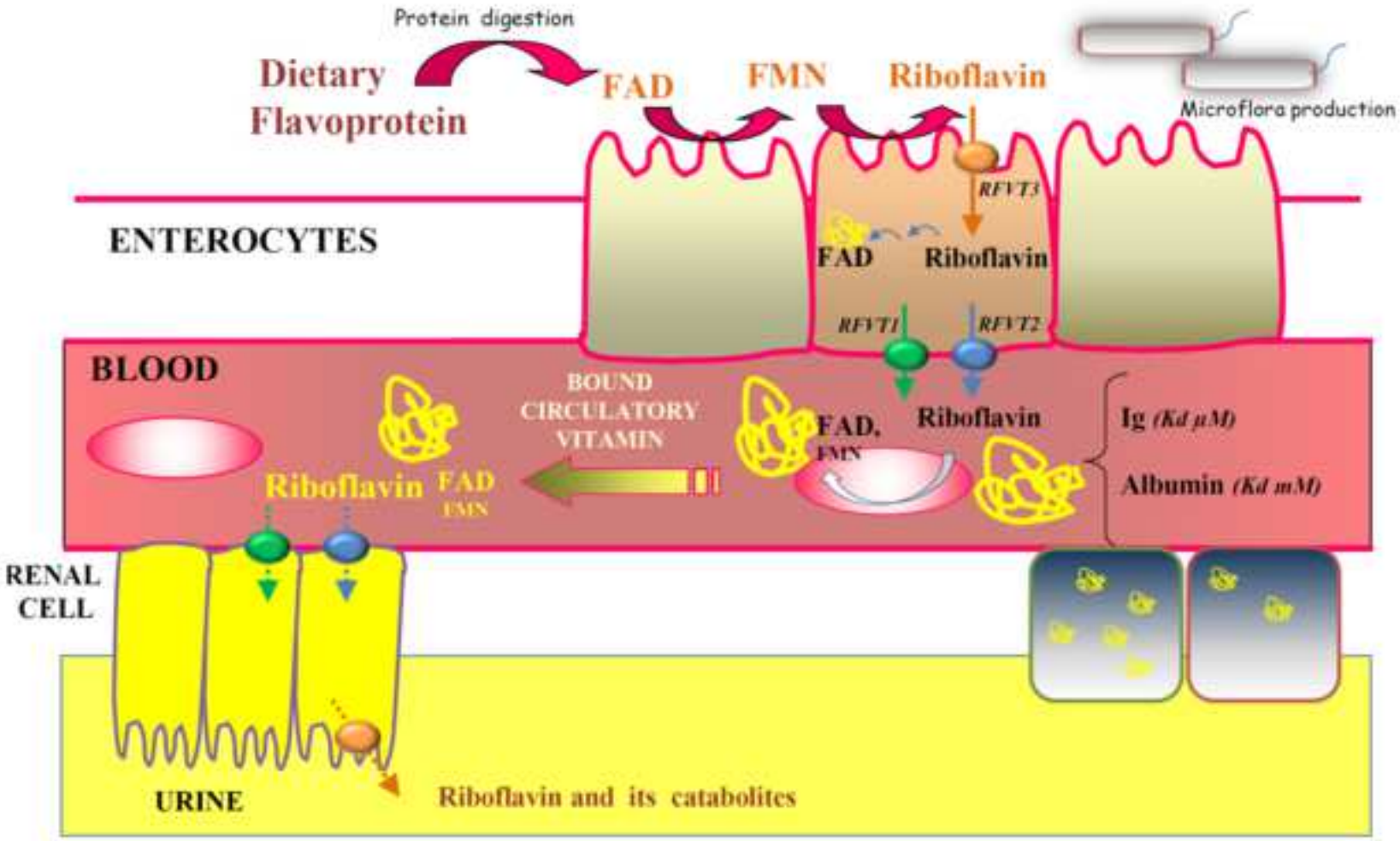

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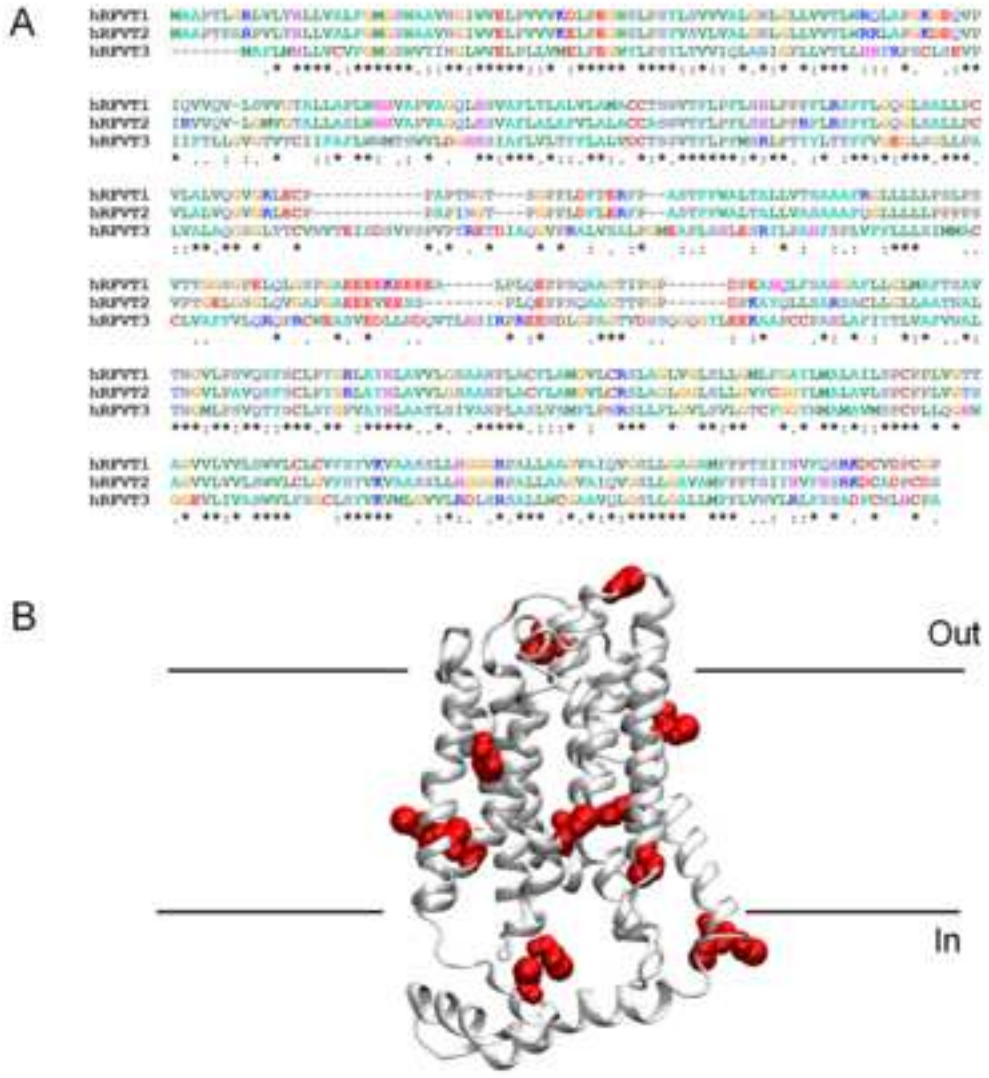

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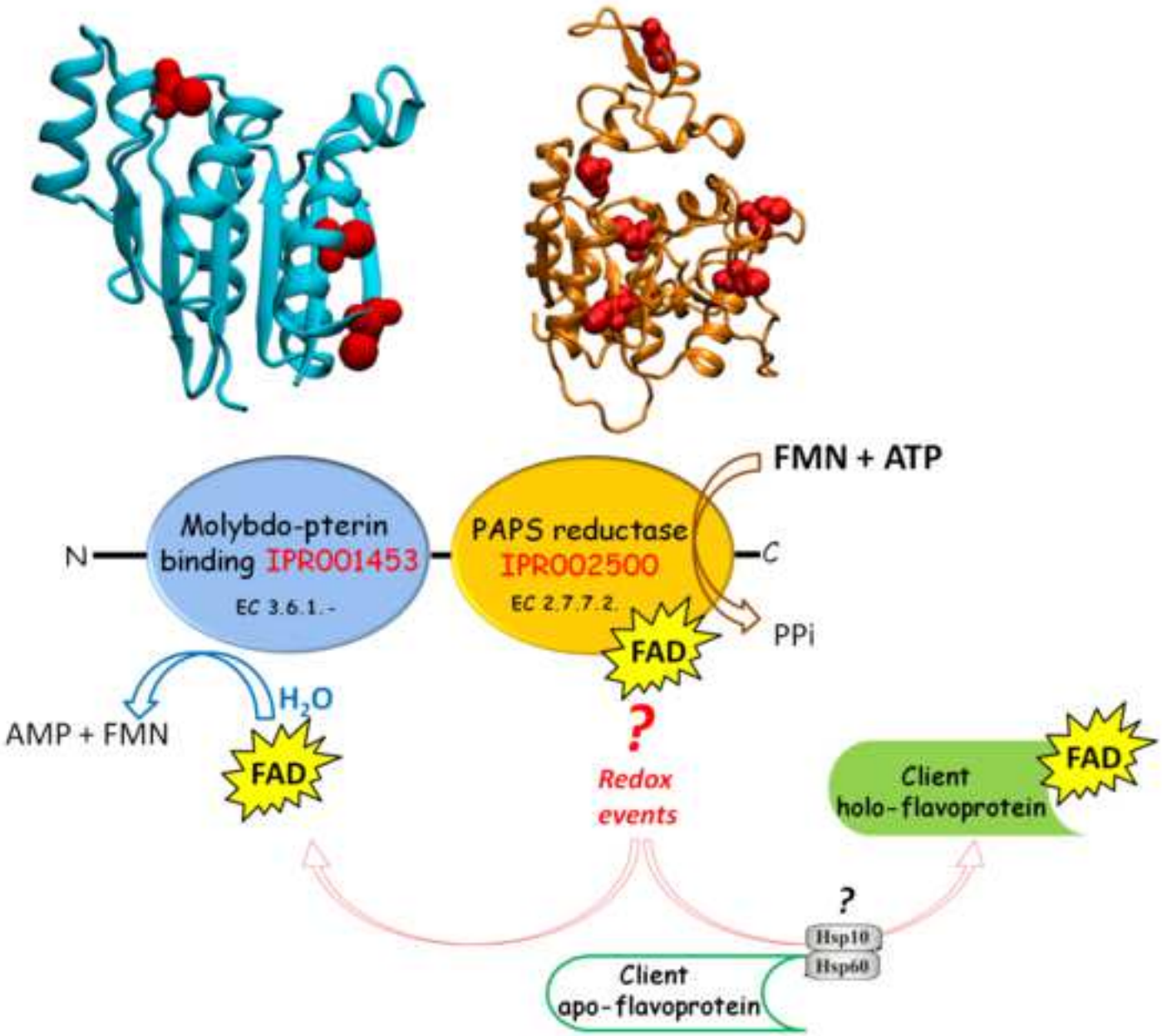

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