Review Article

RIBOFLAVIN METABOLISM: CURRENT STATUS AND FUTURE POSSIBILITIES: A REVIEW

DEY, M. AND BANDYOPADHYAY, D.[?]

Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009.
E. mail: debasish63@gmail.com, Cell: 09836035535

Abstract: The role of riboflavin resides in its being the precursor of flavin mono nucleotide (FMN) and flavin-adenine-dinucleotide (FAD), two coenzymes that are required for a wide variety of important oxidation-reduction reaction. The flavoproteins are involved at least peripherally in every metabolic pathway. For normal cellular functions, the concentration of FMN & FAD within the cell should be maintained at an optimum level. This depends upon the riboflavin metabolizing enzymes which converts riboflavin to FMN and FAD. There are several important works explaining the nature and mechanism of action of the enzymes involved in riboflavin metabolism. The flavoproteins are important component of the enzyme complexes involved in mitochondrial electron transport chain. The level of reduced glutathione in our body depends upon the riboflavin status. This indicates that for combating oxidative stress related disorders, the level of flavoproteins should be maintained at an optimum level. Besides, clinically riboflavin has several applications. Research supports the use of riboflavin and its metabolites (FMN and FAD) in amelioration of a wide number of clinical conditions.

Key words: Riboflavin, FMN, FAD.

INTRODUCTION

Riboflavin (vitamin B_2) is easily absorbed micronutrients with a key role in maintaining health in human and animals. As one of the family of B vitamins, riboflavin contributes to cellular growth, enzyme function, and energy production. It is the central component of the coenzyme FAD and FMN. They play a key role in energy metabolism and also in the metabolism of fat, ketone bodies, carbohydrate and protein. Knowledge of the nutritional and biochemical aspects of riboflavin has accumulated steadily since its discovery and structural elucidation in early 1930s. Warburg termed it the "yellow enzyme" [1] and in 1935, Kuhn and Karrer were both able to synthesize the vitamin and were awarded the Nobel Prize in chemistry for their work with

vitamins [2,3]. The nutritional significance of riboflavin is dictated by the fact that mammals do not synthesize it and, therefore must rely on ingestion of foods that contain amounts sufficient to meet metabolic needs. Normal colonic bacteria synthesize riboflavin, contributing to a soluble pool of the vitamin that can be utilized in addition to dietary intake [4]. In the body, the active forms of riboflavin are synthesized both in cytosol and in the mitochondria, forming FMN and FAD through phosphorylation. Riboflavin is phosphorylated to form FMN by flavokinase and ATP. The conversion of FMN to FAD is catalyzed by FAD pyrophosphorylase and ATP. Riboflavin is transported into the plasma as both free riboflavin and FMN, both of which are bound in appreciable amounts to plasma proteins. Transport of free riboflavin in the plasma takes place both as

albumin bound form or by binding with certain immunoglobulins [5]. Riboflavin uptake appears to occur by a manner similar to its enteric absorption, by a Na ion-dependent high affinity carrier mediated process probably also involving the Ca^+/calmodulin pathway and protein kinase A [6-9]. After it is taken up by the cell, free riboflavin is converted into its co-enzyme forms. The flavins act as electron carriers in a number of oxidation-reduction (redox) reactions involved in energy production and in numerous metabolic pathways. They are also necessary for the activation of other vitamins and enzyme systems. Folate and pyridoxine are vitamins that rely on riboflavin for activation. Clinically, riboflavin has several applications due to its ubiquitous nature in metabolism. Research supports the use of riboflavin in anemia, cataracts, hyperhomocysteinemia, migraine prophylaxis, and alcoholism.

Riboflavin, FMN and FAD- why are they important?: Riboflavin (7,8-dimethyl-10-ribityl-isoallozaxine) consists of a conjugated isoalloxazine ring (flavin) and a five-carbon carbohydrate, ribitol. The 5'-hydroxymethyl terminus of the ribityl side chain of the vitamin is phosphorylated to become the simpler coenzyme. The main antioxidant activity of riboflavin is due to its involvement in the conversion of glutathione. Riboflavin and the glutathione reductase enzyme are both used to convert oxidized glutathione to the reduced form. When riboflavin levels are low, the activity of the glutathione reductase in the body actually increases in an attempt to help convert sufficient glutathione to the reduced form [10]. Riboflavin also affects levels of other antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase.

Flavin mononucleotide (FMN) or riboflavin-52 - phosphate, is a biomolecule produced from riboflavin (vitamin B_2) by the enzyme flavokinase and functions as prosthetic group of various oxidoreductases including NADH dehydrogenase as well as cofactor in biological blue-light sensing photo receptors [11]. During catalytic cycle, the reversible interconversion of oxidized (FMN), semiquinone (FMNH•) and reduced (FMNH$_2$) forms occurs in the various oxidoreductases. FMN is a stronger oxidizing agent than NAD and is particularly useful because it can take part in both one- and two-electron transfers. It is the principal form in which riboflavin is found in cells and tissues. It requires more energy to produce, but is more soluble than riboflavin.

Flavin adenine dinucleotide (FAD) is a redox cofactor involved in several important reactions in metabolism. FAD can exist in two different redox states, which it converts between by accepting or donating electrons. The molecule consists of a riboflavin moiety bound to the phosphate group of an ADP molecule. The flavin group is bound to ribitol, a sugar alcohol, by a carbon-nitrogen bond, not a glycosidic bond.

The biosynthesis of flavocoenzymes in our body is tightly regulated and dependent on riboflavin status. The tissue content of FMN at any point of time is dependent on the activity of FMN-phosphatase and modulation of the activity of this phosphatase may exert an important controlling influence on the entire flavin metabolizing pathway. A great number of mammalian enzymes require FMN or flavin adenine dinucleotide (FAD) as co-enzyme for their activities and both the flavin co-enzymes are associated with many of the components of the electron transport chain of mitochondria associated with oxidative phosphorylation. Flavin coenzymes are critical for the metabolism of carbohydrates, fats, and proteins into energy. Therefore, maintenance of an optimum tissue concentration of both FMN and FAD is very important for normal cellular function. The tissue level of FAD or FMN at any point of time depends on the degree of activities of FAD-pyrophosphatase (which hydrolyses FAD to FMN) and FMN-phosphatase (which hydrolyzes FMN to riboflavin). Impairment of flavin metabolism causes severe depletion of cellular FMN-FAD concentration which in turn leads to serious health effects which include disorders like mitochondrial myopathies [12,13], inefficient energy metabolism and activities of enzymes that are responsible for synthesis of other vital coenzymes such as NAD.

Flavin dependent enzymes and their significance: In the electron transport chain, FMN is one of the components of complex I while FAD is involved in the activity of complex II. FAD acts as an electron carrier and takes part in both the Kreb's Cycle and oxidative phosphorylation. It accepts electrons and is transformed into FADH$_2$. FADH$_2$ then transfers its electrons to complex II of the electron transport chain. For each pair of electrons from FADH$_2$ passed along the electron transport chain, a number of ATP molecules are formed. FAD also affects enzymes that are responsible for the synthesis of other vital coenzymes such as NAD. Severe deficiencies in riboflavin can lower levels of

Table 1: Enzymes requiring FMN and FAD in our body

Flavoprotein	Flavin	Metabolic Functions
Mitochondrial electron transfer flavoprotein (ETF)	FAD	e$^-$ acceptor for acyl-CoA, branched chain acyl CoA.
Ubiquinone reductase	FAD	1-e$^-$ transfer from ETF and co enzyme Q of respiratory chain.
Succinate dehydrogenase	FAD	Transfer reducing equivalents from succinate to ubiquinone yielding fumerate.
Acyl-CoA dehydrogenase	FAD	2-e$^-$ transfer from substrate to flavin, in oxidation of the N-methyl groups of choline and sarcosine.
D-amino acid oxidase	FAD	Dehydrogenation of D-amino acid substrates to imino acids, which are hydrolysed to μ-keto acids.
Xanthine oxidase	FAD	Oxidation of hypoxanthine and xanthine to uric acid .
L-gulonolactone oxidase	FAD	Oxidation of L-gulonolactone to ascorbic acid.
Microsomal flavoprotein monooxygenase	FAD	Oxidation of N, S, Se, and I centers of various substrates in drug metabolism.
NADH cytochrome P-450 reductase	FMN	1 e$^-$ transfer to cytochrome p-450.
NADP dehydrogenase	FMN	2-e$^-$ transfer from NADP to FAD,then to ubiquinone.
L-amino acid oxidase	FMN	Dehydrogenation of L-amino acid substrates to imino acids, which are hydrolysed to μ-keto acids.

coenzymes, leading to inefficient energy metabolism and consequent energy depletion.

Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are essential cofactors for a variety of enzymes that participate in metabolic processes in all organisms [14]. FMN and FAD function as cofactors for a wide variety of oxidative enzyme systems. In plants, these cofactors are required for photosynthesis, mitochondrial electron transport, photoreception, DNA repair and metabolism of other cofactors [15-18].

List of some of the enzymes requiring FMN and FAD: Roles of flavoproteins in intermediary metabolism:

1. Oxidative decarboxylation of pyruvate and α-ketogluterate.
2. Succinate dehydrogenase removes electrons from succinate to form fumerate.
3. The electrons are passed into the electron transport chain via coenzyme Q.
4. Fatty acyl CoA dehydrogenase requires FAD in fatty acid oxidation.
5. Xanthine oxidase, another FAD-dependent enzyme, catalyzes the oxidation of hypoxanthine and xanthine to uric acid. Uric acid is one of the most effective water-soluble antioxidants in the blood. Riboflavin deficiency can result in decreased xanthine oxidase activity, reducing blood uric acid levels [19].
6. Aldehyde oxidase uses FAD to oxidize aldehydes. For example: 1. Pyridoxal (vitamin B_6) to pyridoxic acid (excreted), 2. Retinal (vitamin A) to retinoic acid.
7. Pyridoxine phosphate oxidase which converts pyridoxamine phosphate and pyridoxine phosphate to pyridoxal phosphate (primary coenzyme form of vitamin B6) is dependent on FMN.
8. Synthesis of an active form of folate, N5 methyl tetrahydrofolate, requires FADH$_2$.
9. Enzymes for choline catabolism require FAD. a. Choline dehydrogenase, b. Dimethylglycine dehydrogenase.
10. Metabolism of some amines requires FAD-dependent monoamine oxidase, e.g. Dopamine , Tyramine, Histamine
11. Glutathione reductase is an FAD-dependent enzyme that participates in the redox cycle of glutathione. The glutathione redox cycle plays a major role in protecting organisms from reactive oxygen species, such as hydroperoxides.

The unique ability of the flavo-coenzymes to catalyse a diverse array of cellular processes is a reflection of their structural and chemical versatility [20]. The Protein Data Base Bank (PDB) contains about 200 entries for FAD and FMN dependent proteins [21]. Flavo-enzymes can catalyse a wide range of biochemical reactions, as well as carry out dehydrogenation of variety of metabolites, including lipid, protein and carbohydrate metabolism biosynthetic pathways [22], in one- and two-electron transfer from and to redox centres [23], in light emission, and in the activation of oxygen for oxidation and hydroxylation [24], ion pumping, antibiotic synthesis and the metabolism of biogenic amines [25,26]. Flavins also participate in the photo-repair of DNA damage and in the regulation of caspase-independent apoptosis [27,28]. In addition, they are important components of the blue-light sensing photoreceptors (cryptochromes) involved in the

regulation of biological clocks and development and in the generation of light in bacterial bioluminescence [11,29].The number of biological processes that are known to require FMN and FAD are increasing which is evident from the recent discoveries of flavo-enzymes that take part in protein folding [30] and chromatin remodelling [31].

Flavin metabolizing enzymes: The first step is the adenosine-triphosphate (ATP) dependent phosphorylation of the vitamin to FMN by the enzyme flavokinase. FMN is subsequently converted to FAD in a second ATP- dependent reaction catalyzed by FAD synthetase. FAD pyrophosphatase catalyzes the conversion of FAD to FMN. FMN phosphatase finally converts FMN to riboflavin.

Flavokinase: Flavokinase (ATP: riboflavin 5'-phosphotransferase, EC 2.7.1.26) catalyses the ATP dependent phosphorylation of riboflavin to form FMN- the first step in the biosynthesis of flavo-coenzymes. Modulation of its activity exerts an important controlling influence on the entire flavin metabolizing pathway [32,33]. This enzyme is present in most mammalian tissues, the largest amount being present in liver (34). The enzyme has been shown to be located in the cytosolic fraction [35]. This cytosolic enzyme was first purified to apparent homogeneity from rat liver by affinity chromatography [36], but the purified enzyme was reported to be very unstable. McCormick [37] studied the properties of this enzyme with partially purified preparation and later confirmed some of the properties with the purified enzyme especially with respect to flavin and phosphate donor specificity, cation requirement and specificity and pH dependency. Later, Bandyopadhyay et al. [38,39] studied the interaction of divalent cations both with the partially purified and the purified preparations of flavokinase from rat liver in considerable details.

FAD synthetase: FAD synthetase (EC 2.7.7.2) is the enzyme that catalyses the conversion of flavin mono nucleotide (FMN) to flavin adenine di nucleotide (FAD).Two isoforms having FAD synthetase activity in human have been identified by bioinformatics analysis. The first isoform (isoform1) differs from the second isoform (isoform 2) by an extra sequence of 97 residues at the N- terminus which is not present in isoform 2 . At least two more isoforms could be predicted in humans by genetic

analyses but they remain uncharacterised [40,41]. The enzyme requires Mg^{2+} as cofactor.

FAD pyrophosphatase: FAD pyrophosphatase (EC 3.6.1.18) is an enzyme that catalyses the chemical reaction FAD + $H_2O \longrightarrow$ AMP + FMN. Thus, the substrate of this enzyme is FAD, whereas its two products are AMP and FMN. This enzyme belongs to the family of hydrolases. The systematic name of this enzyme class is FAD nucleotidohydrolase.

FMN phosphatase: The enzyme FMN phosphatase is the most poorly studied among all the riboflavin metabolizing enzymes. This enzyme catalyses the conversion of FMN back to riboflavin with the liberation of inorganic phosphate.

Research works on riboflavin metabolism: The most eminent contributions throwing light on the reaction sequence and the enzymes of riboflavin metabolism was made by Donald B. McCormick. He purified the enzyme flavokinase and characterized it. A very important work revealing the possible mechanism of the enzyme action was done by Bandyopadhyay et al. [38,39]. It was found that the activity of the enzyme flavokinase was inhibited by cadmium indicating the involvement of sulfhydryl group in the reaction catalysis. FAD synthetase was also purified and characterized from rat liver by Oka and McCormick [42]. Besides, the effect of riboflavin status on the activity of flavin metabolising enzymes were also studied [43]. The enzyme was extracted from several lower animal species. The existence of FMN phosphatase was pointed out in several works of McCormick et al. Recently the enzyme was purified and characterized from goat heart and goat liver. The activity of mammalian FMN phosphatase was inhibited by divalent cations indicating the involvement of sulfhydryl groups in their active sites Thyroxine and triiodothyronine stimulate FMN and FAD synthesis in mammalian system [44]. This seems to involve a hormone mediated increase in the activated form of the enzyme flavokinase. In hypothyroidism, the flavin adenine dinucleotide level of the liver decreases to levels observed in riboflavin deficiency. Thyroxine therapy resulted in normal levels of this enzyme while the subjects were on a controlled dietary regimen. This demonstrates that thyroid hormone regulates the enzymatic conversion of riboflavin to its active coenzyme forms in the human adult.

Future possibilities: Targeting riboflavin metabolism can open a wide area of therapeutic possibilities. Bernsen, et al. [45] evaluated the effects of riboflavin treatment in five patients with mitochondrial myopathies. Mitochondrial myopathies are disorders often characterized by defects in the electron transport chain. Riboflavin supplementation is hypothesized to improve the efficiency of the Complex I protein by increasing the concentrations of available FMN molecules in the cell [46]. Ogle et al. [47] reported the effects of riboflavin in a case involving a female patient with a myopathy caused by complex I deficiency. Studies in animals suggests that riboflavin deficiency may impair iron absorption, increase intestinal loss of iron, and/or impair iron utilization for the synthesis of hemoglobin (Hb) [48]. In humans, it has been found that improving riboflavin nutritional status increases circulating Hb levels [49]. The flavoprotein, methylene tetra-hydrofolate reductase (MTHFR), plays a very important role in folate-mediated homocysteine metabolism. Deficiency of folate and elevated homocysteine concentrations may increase cancer risk. Intake of riboflavin is a determinant of homocysteine concentration. This indicates that riboflavin status can influence MTHFR activity and the metabolism of folate, thus potentially affecting cancer risk [50]. Besides riboflavin can also act as a photosensitizer, and this property may have value in photodynamic therapy of cancer [51]. Anti-cancer agents poses various side effects that may force patients to limit the dose or to discontinue the treatment. Supplementation of riboflavin along with niacin and coenzyme Q10 prevented the oxidative stress associated with such treatments [52].

Studies indicates that impaired mitochondrial oxygen metabolism in the brain may play a role in the pathology of migraine headaches. As riboflavin is the precursor of two flavocoenzymes (FAD and FMN) required by the flavoproteins of the mitochondrial electron transport chain, supplemental riboflavin has been investigated as a treatment for migraine (53). Riboflavin (as FAD) is required for the key folate-metabolizing enzyme, MTHFR. A reduced riboflavin status may interfere with the metabolism of folate, such individuals exhibit a higher risk of cardiovascular disease (CVD). Emerging evidence from intervention trials supports an important role for riboflavin against hypertension in individuals with the MTHFR 677TT genotype.

CONCLUSIONS

Studies suggest that oxidative stress play a major role in the pathogenesis of type-2 diabetes mellitus. Abnormally high levels of free radicals and decline of antioxidant defense mechanisms can cause damage of cellular organelles and enzymes. Riboflavin supplementation showed increased glucose uptake in skeletal muscle and white adipose tissue. There is also recovery from liver and tissue injury and also cellular DNA damage [54]. Riboflavin metabolism is thus a wide area which can be targeted for disease prevention as well as disease management. Keeping pace with the antioxidant status as well as generating ATP by ETC in the mitochondria are the key in maintaining health and fighting off disease. Riboflavin plays a pivotal role in both these processes suggesting its immense future implications.

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