Chapter 1

General Properties of Flavins

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Abstract

The general properties of flavins in free solution and when bound to flavoproteins as cofactors are analysed. The extremely high chemical versatility of flavins is reflected in the remarkable versatility of flavoproteins, when considered as a whole. However, each flavoprotein is also characterized by a strict specificity, thus implying that one of the most important roles of the protein component is to limit the wide range of possible flavin–protein interactions to those beneficial to the reaction to be catalysed.Many attempts have been made to achieve a rational classification of flavoproteins, depending on their different properties. However, when general classifications are based on the different possible catalytic mechanisms, in most cases there is uncertainty, owing to the wide range of reaction mechanisms potentially available to the flavin cofactor. Despite the significant accumulation of information in recent years on catalysis, biomimetics and structural studies, the factors that determine the specificity of flavoproteins are still poorly understood.

1.1. Introduction

The yellow-coloured compounds with the basic structure of 7,8-dimethyl-10-alkylisoalloxazine are generally termed as flavins. Flavins are ubiquitous in nature, and they take part in many biochemical reactions as coenzymes and photoreceptors. Riboflavin, the precursor of all the biologically important flavins, was first reported as lactochrome, a bright yellow pigment isolated from

cow milk in 1879.[1] Later, in the late 1920s and early 1930s, yellow pigments with bright greenish fluorescence were isolated from different sources, and they were named as lactoflavin, ovoflavin, *etc.*, indicating the source from which they had been isolated. Concomitantly, it was recognized that the yellow pigment was a constituent of the vitamin B complex. Two important groups determined the structure and proved it by chemical synthesis.[2,3] The name riboflavin (RF) was given to the compound; it derives from the ribityl side chain and from the yellow-conjugated ring system (see Figure 1).

There is a broad distribution of flavins in tissues, but little is present as free RF. The majority is found in flavocoenzymes, mainly as flavin adenine dinucleotide (FAD), and in lesser amounts as flavin mononucleotide (FMN), the common name of riboflavin-5′-phosphate, despite the known fact that RF is

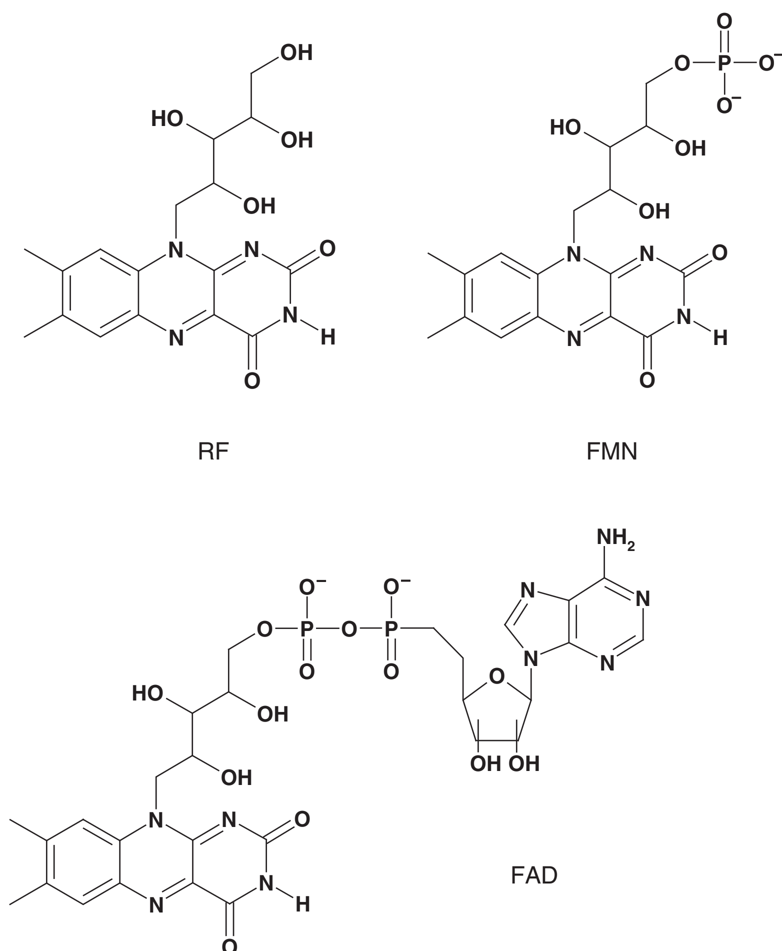


Figure 1. Chemical structures of riboflavin (RF), flavin momonucleotide (FMN) and flavin adenine dinucleotide (FAD)

not a real nucleoside because the linkage between the ribityl chain and the N_{10} of the flavin is not glycosidic; therefore, FMN and FAD are not real nucleotides. The structures are shown in Figure 1.

Since the pioneer study of Theorell,[4] who demonstrated in 1935 that the biochemical basis for the necessity of RF as a vitamin is its role as precursor of the FMN cofactor in enzyme catalysis (coenzyme), and those by Krebs[5] and Warburg[6] who showed its role as precursor of FAD cofactors, hundreds of flavoprotein enzymes have been known, and new ones are reported every year. Most of them contain non-covalent-bound FAD or FMN, and are specific for binding either of the two flavin forms as nature initially provided them with. Nowadays, there are many flavoproteins crystal structures known, which reveals that the majority of the flavin–protein interactions are with the N-10 ribityl side chain of FMN or FAD.[7] A recent study on the sequence-structure relationship in 32 families of FAD-containing proteins, showed that in every case the pyrophosphate moiety binds to the most strongly conserved sequence motif, suggesting that pyrophosphate binding is a significant component of molecular recognition.[8]

1.2. Properties of Flavins

The redox potential for the two electron reduction of the flavin is about −200 mV. However, this value can greatly vary in flavoproteins, due to the crucial role of the protein environment in the properties of flavins, spanning a range from approximately −400 mV to +60 mV. In general, the proximity of a positive charge is believed to increase the redox potential and a negative charge or a hydrophobic environment are expected to lower it.[9] A few flavoenzymes have a covalent-bound FAD molecule, and site-directed mutagenesis studies suggest that the covalent interaction could increase the oxidative power of the flavin.[10]

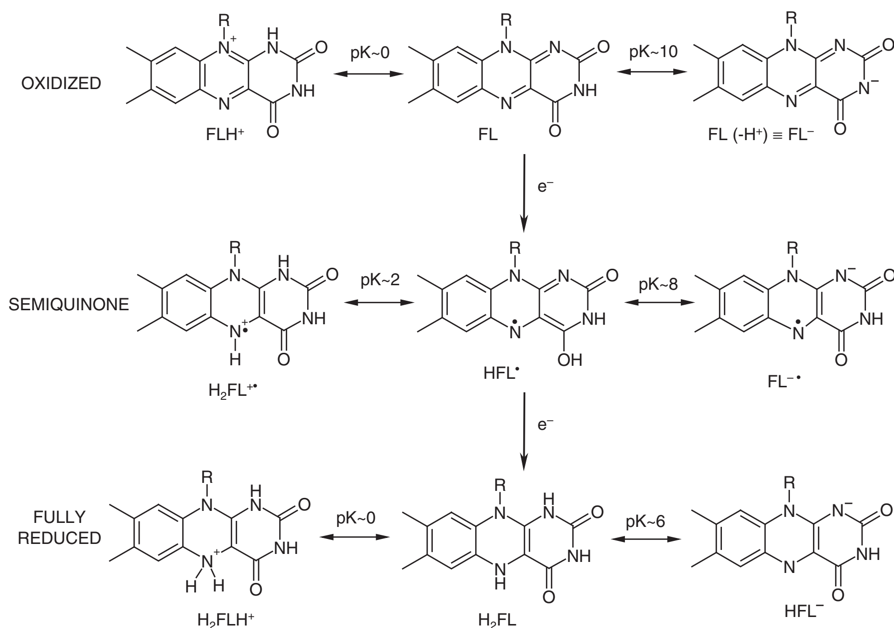
Since the discovery and characterization of RF and its derivatives FMN and FAD, they have been recognized by their ability of participate in both one- and two-electron transfer processes. This means that flavin molecules can exist in three different redox states: oxidized, one-electron reduced (semiquinone) and two-electron reduced states. Therefore, they can participate in redox reactions as either one- and two-electron mediator making the flavoenzymes very versatile in terms of substrate and type of catalysed reactions. This is a major reason for the ubiquity of flavin-dependent enzymes in biological systems. Flavins have the potential for transfer of single electrons, of hydrogen atoms and of hydride ions. Therefore, they can participate in redox reactions as either one- and two-electron mediator making the flavoenzymes very versatile in terms of substrate and type of reactions, which is a major reason for the ubiquity of flavin-dependent enzymes in biological systems. In addition, the oxidized flavin molecule is susceptible to nucleophilic attack, especially at N-5 and C-4α.[11]

In free solution (when not bound to an enzyme), the equilibrium of the different flavin species is pH dependent, as shown in Scheme 1 (proposed by Heelis, 1982).[12]

This Scheme presents the different redox states: oxidized, one-electron reduced (semiquinone) and two-electron reduced (fully reduced) and also the different protonation states for each of them. From the nine forms in Scheme 1, at least six are physiologically possible on the basis of their pK_a values.

At pH 7, only about a 5% radical is stabilized in an equimolar mixture of oxidized and reduced flavin. The semiquinone can exist in a neutral or anionic form, with a pK_a of $\sim$8.5.

On binding to a specific protein, this equilibrium can change dramatically: some enzymes show no stabilization of semiquinone, while others give almost 100% stabilization. In some cases, if the protein can stabilize the neutral radical species over the whole range of pH values at which the enzyme is stable, the pK_a is shifted up significantly from 8.5. In other cases if the semiquinone anion is stabilized, the pK_a is decreased significantly. There are some enzymes, of which glucose oxidase was the first example,[13] which show such a pK_a that the identification of both forms is possible. In addition to these redox/ionic forms (each of them with different canonical forms), there are other electronic states, known as charge-transfer states. They are electronic states that do not belong to any of the three redox states, but are those in which partial charge is transferred to or from one of the three redox states. All these redox, ionic and charge-transfer states are the origin of the different colours of flavins and



Scheme 1. Redox and acid-base equilibria of flavins

flavoproteins.[14] The large spectral differences between the various flavin redox–ionic–electronic states make it possible to monitor the events occurring in catalysis using the flavin itself as a reporter.

1.3. Classification of Flavoproteins

Flavins show an extremely high chemical versatility, which is reflected in the remarkable versatility of flavoenzymes; however, each of the enzymes is also characterized by a strict specificity. Many attempts have been made to achieve a rational classification of the many different types of flavoproteins, depending on their different properties.

In 1970, Hemmerich *et al.*[15] proposed four classes of flavoproteins according to their behaviour at half reduction in the absence of substrates.

(A) Flavoproteins exhibiting stoichiometric amounts of blue semiquinone
(B) Flavoproteins exhibiting stoichiometric amounts of red semiquinone
(C) Flavoproteins exhibiting no paramagnetic intermediates
(D) Flavoproteins exhibiting non-stoichiometric amounts of semiquinone.

The red radicals of class B have been identified as flavosemiquinone anions (Fl^{-}), and the blue radicals of class A as neutral semiquinones ($FlH^{\bullet}$). According to this classification, glucose oxidase belongs to class A and B, depending on pH. The authors proposed that in class A and B flavopropteins N-5 is protected by forming a strong hydrogen bond in the radical state, so that no "in plane" transfer of electrons can occur. These radicals are stable as long as the hydrogen bond at N-5 is not removed. They identified class D as metalloflavoproteins, where the radical yields at half reduction never exceed 50% of total flavin. Class C includes those flavoproteins that exhibit a cyst(e)ine residue participating in the catalysis. Both internal redox systems (flavin and disulfide) are in covalent contact at 2e-reduction of the enzyme. The point of attachment of the sulfur component to the flavin has been proposed to be C-4α.

Despite the fact that several hundred flavoenzymes have been described, Massey and Hemmerich[16] proposed that a large number of flavoproteins may be classified into two classes, based on distinct spectroscopic properties: regiospecific reactivities of the flavin skeleton and hydrogen bonding patterns with the apoprotein. The results reported by Shinkai *et al.* in 1985[17] support this proposal.

The first group (dehydrogenases/oxidases) is characterized by a "red" semiquinone radical, a bent structure of the reduced form, and hydrogen bonding to the N-1 nitrogen atom, activating the N-5 position.

The second group (electron transferases) is characterized by a blue semiquinone radical, a planar structure of the reduced form, and hydrogen bonding to N-5, activating of the C-4α position.

With the aim of addressing some characteristics of the two classes of flavoenzymes described above, Wouters *et al.*[18] determined the electronic properties of the three different protonated forms HFlox, H_2Flox^{+}(N-5) and

H_2Flox^+(N-1) using lumiflavin as model compound. Lumiflavin is a photoproduct of RF, in which the ribityl side chain is replaced by a methyl group. In order to describe the electrophilic/nucleophilic properties, they investigated the geometry, charge distribution, and HOMO–LUMO topologies of the three forms of lumiflavin adopting the RHF/3-21G level. The electronic spectra were evaluated at the CNDO/CI level with a particular attention to the blue or red shifts respect to the oxidized form. The authors concluded that a larger electron delocalization, demonstrated by a smaller bond length alternation upon protonation, as well as a larger basicity associated with a more negative charge can explain the favourable protonation of N-1 with respect to N-5, and the unusual electrophilic affinity of N-5 when lumiflavin is protonated at N-1. The calculations correctly place the $\pi-\pi^*$ transition as the lowest energy transition for neutral oxidized lumiflavin and predict a blue shift of the low-lying electronic transition upon monoprotonation (formation of the stable N-1 protonated form). They found in their calculation on radical intermediates of lumiflavin, a theoretical rationalization for the experimental classification of flavoenzymes in two distinct classes. Hydrogen bonding at N-5 and the fact that the semiquinone radical is red appear consistent with protonation at N-1 and larger wavelength absorption. Hydrogen bonding at N-1 and the fact that the semiquinone radical is blue are consistent with protonation of N-5 and a smaller wavelength absorption.

Among the known redox coenzymes, flavocoenzymes are unique in that they can participate in both one-electron and two-electron processes. The other redox cofactors usually catalyse exclusively either one- or two-electron processes. Active redox metalloenzymes catalyse only one-electron process, and nicotinamide nucleotides, with wide distribution in biological systems, are involved in only two electron redox reactions, because the radical forms of the pyridine ring are not sufficiently stable as to be involved in enzymatic reactions. For these reasons, flavoenzymes mediate two-electron and one-electron processes, as is the case of the well-known mitochondrial and chloroplast electron transport chains.

The reactions catalysed by a flavoenzyme always involve two separate half-reactions: reductive and oxidative half-reactions, both of which are necessary for the turn over of the enzyme. The former is the process in which a substrate or an electron donor is oxidized with the concomitant flavin reduction. In the latter process, the reduced flavin is oxidized by another substrate or an electron donor. Hemmerich *et al.*[19] proposed in 1977 a scheme consisting of five broad classes of flavoenzymes based on the nature of the substrate involved in the two separate half-reactions.

(i) Transhydrogenase, where two-electron equivalents are transferred, along with the appropriate hydrogen ions, from one organic substrate to another.

(ii) Dehydrogenase–oxidase, where two-electron equivalents are transferred to the flavin from an organic substrate, where molecular oxygen is the oxidizing substrate, being reduced to H_2O_2.

(iii) Dehydrogenase–monooxygenase, where the flavin is reduced generally by a reduced pyridine nucleotide, and where on oxidation with O_2 in the presence of a cosubstrate one atom of oxygen is inserted into the cosubstrate, while the other is reduced to H_2O.
(iv) Dehydrogenase–electron transferase, where the flavin is reduced by two-electron transfer from a reduced substrate and then reoxidized in sequential single electron transfers to acceptors, such as cytochromes and iron–sulphur proteins. This class might be further subdivided to distinguish those enzymes which function in the reverse sense, *i.e.*, those which receive electrons one at a time and then transmit them in a two-electron step in the reduction of a pyridine nucleotide.
(v) Electron transferase, where the flavin is reduced and reoxidized in one-electron steps.
(vi) This scheme was adopted by the International Union of Biochemistry.[20]

In the last decade, several interesting studies about flavins properties have been published. Recently, Miura has proposed an alternative classification of flavoenzymes based on the number of electrons involved in the catalytic cycle.[14] Because these half-reactions can be either one-electron or two-electron processes, there are four possible combinations of the two half-reactions; therefore, the author proposed that flavoenzymes can be classified according to the combination of one- and two-electron processes for reductive and oxidative half-reactions. Although four categories are theoretically possible; 1/1, 1/2, 2/1 and 2/2, the author points that flavoenzymes of the 1/2 category are not known at present. The author proposed that specificity of each flavoenzyme is understood in terms of the regulatory mechanism of the broad reactive potential of flavin. The regulatory mechanisms include hydrogen-bonding networks, electrostatic effects, charge transfer interactions, positioning between substrate and flavin, and modulation of resonance hybridization.[14]

The reaction between the fully reduced flavin H_2Fl and oxygen is essentially irreversible in free solution, according to the redox potentials, but it is also slow because of the spin inversion associated with the reaction of the singlet-reduced flavin and the triplet molecular oxygen. In flavoproteins, the reaction with oxygen may be orders of magnitude faster or slower, depending on the specific flavoprotein. They have been classified in four well-defined groups, according to the rate of their reactions with oxygen and the nature of the products formed, such as electron transferases, dehydrogenases or transhydrogenases, oxidases and monooxygenases. In 1994, Massey published an interesting review about the reactions of flavin and flavoproteins with oxygen.[21] The author discussed the common characteristics of the flavoproteins that are classified according their reactivity with oxygen, and proposed that they have many characteristic properties of a particular group, not shared with other groups of flavoproteins. Considering that the ratios of the rates of reaction with oxygen are at least 10^6 among flavoproteins, and that enormous differences in the decay rates of the hydroperoxides are found, the author also pointed out that there are important questions that remain to be answered What are the flavin–protein interactions

that control the rate of reaction with oxygen? What factors determine whether the C-4α hydroperoxide should be formed? What are the factors that determine the stability of this hydroperoxide for different flavoenzymes?

In 1997, Wouters *et al.*[18] published a study of the electronic properties of lumiflavin as model compound. The authors found in their calculation a theoretical rationalization for the behaviour of flavoproteins and its classification in two groups, as described above.[18] They also gave an explanation, in terms of energy level differences, for the red shift observed for the first absorption band of the isoalloxazine ring upon complex formation with appropriate ligands. The favourable stacking observed in the crystal structures of lumiflavin–hydroquinone complexes was also rationalized in terms of the complementarity of the molecular electrostatic potential generated by the two molecules, and/or by the superposition of the HOMO of hydroquinone and the LUMO of the oxidized flavin.[18]

In 2000, Massey[7] published an interesting review, where the catalytic versatility of flavoproteins is explained by the catalytic mechanisms of selected flavoenzymes. The author studied the catalytic mechanisms of the different enzymes by replacing the native flavin with artificial flavins, such as 8-chloroflavins, 8-mercaptoflavins, and the photoreactive 6-azido-FAD. He also replaced the substrate with derivatives such as β-chloro amino acids or β-chloro α-hydroxyacids, and used the information available from crystal structures. He proposed possible reaction mechanisms for each of the groups of flavoenzymes: that catalyse the oxidation of α-hidroxyacids and α-amino acids, disulphide reductases, monooxygenases, and reductases, dehydrogenases and electron transferases (including the old yellow enzyme).

Flavoproteins are now known to have a variety of folding topologies. In 2000, Fraaije and Mattevi[9] examined the three-dimensional structures of flavoenzymes that were available in the protein data bank (PDB),[22] searching for recurrent features in their catalytic apparatus. They focused their analysis to the dehydrogenases, a group of enzymes that catalyse a reaction involving the rupture of a kinetically stable C–H bond, coupled with the transfer of two electrons to the flavin. The authors found that these enzymes share a few invariant features in the hydrogen-bond interactions between the protein and the flavin and that the positioning of the reactive part of the substrate with respect to the flavin is generally conserved. The authors recognized that the mechanistic problems cannot be solved solely on the basis of structural data; however, they pointed out that all the different mechanistic proposals (hydride transfer, radical mechanism and carbanion mechanisms) require, at least to some extent, juxtaposition between the flavin N-5-C4α locus and the reactive C–H group of the substrate. Although many of the reviewed enzymes are proposed to function via hydride transfer, there is no general consensus about the exact mechanisms for some of them. Nonetheless, the stereochemical principles underlying the mutual interactions between the substrate C–H group and the flavin are surprisingly well conserved, and they represent a validation test for the plausibility of any proposed mechanism, which must be compatible with the observed stereochemistry of substrate binding. The authors proposed

that in the future the stereochemical requirements of each of the proposed mechanisms would be defined, to evaluate their compatibility with the three-dimensional structures.[9]

Dym and Eisenberg published in 2001[8] an interesting review, where they analyzed structure–sequences relationships in 32 families of FAD-binding proteins. Their work illustrates how similar cofactors are utilized by nature in a wide variety of proteins families. The availability of the three-dimensional structures of a large collection of FAD-proteins (150 flavoprotein X-ray structures solved in the presence of FAD were retrieved from PDB) allow to identify four different FAD-family folds, each with distinctive conserved sequence motifs. Furthermore, the FAD pocket shape can be distinguished from one fold to another.

In contrast to the diversity of the FAD fold and its sequence motif, the pyrophosphate moiety makes hydrogen bonds with residues from the most conserved sequence motifs of each fold family. The authors proposed that the pyrophosphate moiety is crucial for molecular recognition, whereas the isoalloxazine ring is involved in catalytic function and the adenine ring stabilizes cofactor binding, both rings interacting with protein residues in partially conserved sequence motifs. As a result, the isoalloxazine and adenine rings interact with different residues in the different proteins belonging to the same FAD-family fold. This is consistent with the variability in bond type (covalent or non-covalent) and cofactor conformation (elongated or bent) observed in the FAD-family folds. However, in each FAD-family fold there is a clear correlation between the fold, the shape and position of the pocket and the cofactor conformation. This is in agreement with the finding that the cofactor exhibits directionality, which is highly conserved within a family fold.[8]

The same year, Fitzpatrick[23] reported a review about recent progress on the elucidation of the mechanisms of oxidation of organic substrates of flavoenzymes that catalyse dehydrogenation of bonds between carbon and either nitrogen or oxygen. The work is focused on the oxidation of alcohols, amino and hydroxy acids, amines and nitroalkanes. The author combined information obtained from site-directed mutations, effect of inhibitors and substrate analogues, kinetic isotope effects and structural information, with an initial goal to develop unified mechanisms for each of the different families of flavoprotein oxidases. In most, if not all the cases, multiple mechanisms still remain viable despite intensive study. This uncertainty can be attributed to the wide range of reaction mechanisms potentially available to the flavin cofactor. The author concluded that radical and nucleophilic mechanisms must be considered in addition to the more accepted hydride transfer mechanism. The possibility that the reaction observed with substrate analogues may diverge from the normal catalytic mechanisms must also be considered.[23]

Our understanding of flavin chemistry has increased in the last years by the wealth of information obtained by catalysis and biomimetics as well as from structural studies of flavoproteins. However, as discussed in this work, versatility and specificity are essential concepts to describe flavoproteins/flavoenzymes.

The wide range of possible reactions give such a high versatility to these proteins as to make very complex any attempt to describe their behaviour by general patterns. However, each flavoprotein has a strict specificity, thus implying that one of the most critical roles of the protein component is to limit the whole range of possible flavin–protein interactions to those beneficial to the reaction to be catalysed. Despite the significant progress that has been made in recent years, the factors that determine the specificity of flavoproteins are still poorly understood, and many research groups are contributing to this area.

In this context, advances in the research on the photochemical and photobiological properties of flavins and flavoproteins are necessary for a complete understanding of these important compounds.

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