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Review article

Multiple functionalities of reduced flavin in the non-redox reaction catalyzed by UDP-galactopyranose mutase

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ABSTRACT

Flavin cofactors are widely used by enzymes to catalyze a broad range of chemical reactions. Traditionally, flavins in enzymes are regarded as redox centers, which enable enzymes to catalyze the oxidation or reduction of substrates. However, a new class of flavoenzyme has emerged over the past quarter century in which the flavin functions as a catalytic center in a non-redox reaction. Here we introduce the unifying concept of flavin hot spots to understand and categorize the mechanisms and reactivities of both traditional and noncanonical flavoenzymes. The major hot spots of reactivity include the N5, C4a, and C4O atoms of the isoalloxazine, and the 2′ hydroxyl of the ribityl chain. The role of hot spots in traditional flavoenzymes, such as monooxygenases, is briefly reviewed. A more detailed description of flavin hot spots in noncanonical flavoenzymes is provided, with a focus on UDP-galactopyranose mutase, where the N5 functions as a nucleophile that attacks the anomeric carbon atom of the substrate. Recent results from mechanistic enzymology, kinetic crystallography, and computational chemistry provide a complete picture of the chemical mechanism of UDP-galactopyranose mutase.

Contents

1. Introduction

Enzymes exhibit extraordinary catalytic power and diversity, which remains unmatched by man-made catalysts. All enzymes leverage amino acid functional groups to catalyze chemical reactions [1]. Three-dimensional constellations of amino acids optimized by evolution are capable of catalyzing diverse reactions, including phosphoryl transfer, peptide bond hydrolysis, and isomerization reactions among many others [2–4]. Cofactors expand the chemical repertoire of enzymes, allowing more complex chemical reactions to occur. For example, heme-dependent enzymes can cleave C-H bonds via the formation of Fe-oxo species [5]. Pyridoxal phosphate-dependent enzymes form a covalent aldimine

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intermediate that facilitates α-C-H bond cleavage, which is important in racemization and elimination reactions [6]. Numerous oxidoreductases use nicotinamide adenine dinucleotide cofactors in hydride transfer reactions. Photosynthetic reaction centers perhaps represent the pinnacle of cofactor-assisted catalysis [7].

Flavin cofactors are also widely used by enzymes to catalyze a broad range of chemical reactions. Flavins are derived from riboflavin (vitamin B2) and are processed into flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) in the cell [8]. Typically, flavoenzymes catalyze oxidation-reduction reactions, where the flavin cycles between the oxidized and reduced states, such as in hydride transfer reactions. In other redox reactions, flavoenzymes form a transient semiquinone, such as in electron transfer reactions [8–12].

The isoalloxazine ring of the flavin contains several reactive centers or "hot spots" that play important roles in catalysis. In reactions catalyzed by flavin monooxygenases, the activation of oxygen involves a single electron transfer from the reduced flavin and recombination of the flavin semiquinone with the superoxide ion forming a covalent intermediate at the C4a-atom, the C4a-peroxyflavin (Scheme 1A) [13]. Formation of this intermediate is essential for O-O bond cleavage and substrate oxygenation [14,15]. Similarly, C4a-thiol covalent intermediates are formed in glutathione reductase reactions and are involved in the mechanism of light activation by light-, oxygen-, or voltage-sensitive (LOV) domains (Scheme 1B) [16].

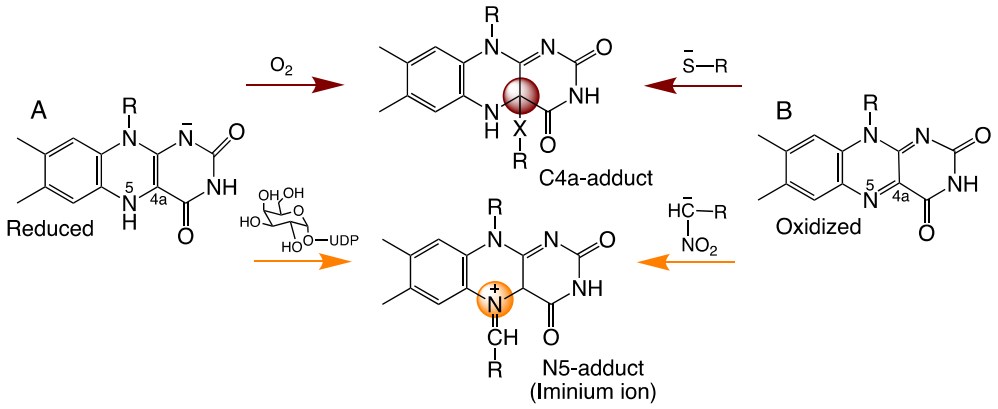
In addition to covalent intermediates at the C4a-position of the isoalloxazine ring, covalent intermediates involving the flavin N5 have also been characterized. Reactivity of the N5-atom was shown using model compounds and oxidized flavoenzymes reacting with cabanions (Scheme 1B) [17–19]. Our group and others demonstrated the role of a covalent flavin N5 intermediate (Scheme 1A) in the isomerization of UDP-galactopyranose (UDP-Gal*p*) to UDP-galactofuranose (UDP-Gal*f*) by UDP-galactopyranose mutase (UGM). The role of the reduced flavin in the non-redox reaction catalyzed by UGM is the focus of this review. We will discuss recent biochemical, structural, and computational data that suggest that the flavin cofactor in UGM plays multiple roles in catalysis. We will focus on the mechanism of activation of the flavin-N5 for covalent catalysis, the role of the oxygen atom at the C4 positon (C4O) in proton transfer and activation of flavin-sugar intermediates, and of the 2′-OH of the ribityl chain in stabilization of conformational changes that modulate bending of the reduced flavin, which is essential for the proton transfer function of the C4O.

2. Formation of flavin N5-adducts

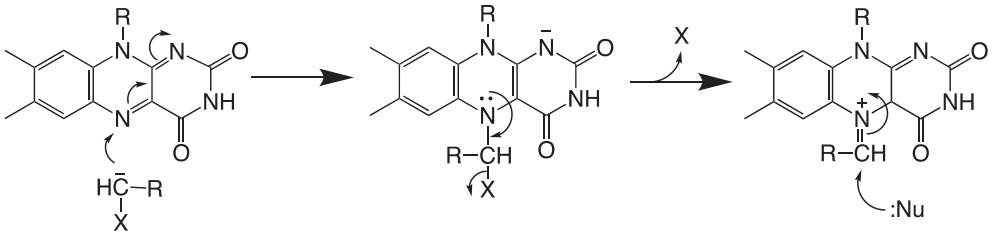
The reactivity of the N5-atom of the isoalloxazine ring was originally investigated in model compounds where the spectral and fluorescent properties of N5-alkylated flavins were reported [17,18]. The presence of N5-flavin adducts in enzymes was demonstrated in the reaction of D-amino acid oxidase with a nitroethane anion (Scheme 2) in the presence of cyanide and lactate oxidase with glycolate [19]. It was later demonstrated that an N5-adduct played a role in the catalytic cycle of nitroalkane oxidase [20]. This adduct was later trapped with cyanide and the structure elucidated by x-ray crystallography [21]. N5-adducts have also been proposed in the reaction of monoamine oxidase with several substrates and inhibitors [22]. The β-Cl-elimination activity of D-amino acid oxidase has been proposed to occur via formation of a transient N5-covalent intermediate [23]. In these examples, after the initial attack of the substrate/analogs, formation of a N5-iminium ion or decay of the flavin N5-adduct is coupled to an elimination step (e.g., nitrite, chloride) (Scheme 2). In the N5-iminium ion, a carbon atom is activated for nucleophilic attack by a hydroxide ion (or other nucleophile) as part of the catalytic cycle (Scheme 2). Formation of flavin N5-iminium ions in the methyl transfer steps of the reaction catalyzed by flavin-dependent enzymes in thymidylate biosynthesis and tRNA modifications have also been documented [24,25]. Formation of prenylated FMN in decarboxylation reactions by ferulic acid decarboxylase, has been proposed to form via N5-iminum ion intermediates [26]. More recently, formation of N5-oxoammonium ions, which are important for hydroxylation in enterocin biosynthesis and in dibenzothiophene degradation, have been proposed [27,28]. These reactions result in reduction of the flavin and oxidation of the substrate/analog. Thus, the flavin must cycle back to the oxidized state for sustained catalysis.

Formation of N5-adducts in non-redox reactions involving the flavin in the oxidized or reduced forms have also recently been demonstrated. In the reaction of alkyl-dihydroxyacetone phosphate synthase (ADPS), the substrate attacks the N5-atom of the oxidized flavin, and formation of the N5-iminum ion facilitates elimination of the fatty acid. Formation of the N5-iminium ion activates the C1-atom, which is attacked by the fatty alcohol (e.g., Nu: in Scheme 2) [29,30]. In the ADPS reaction, the substrate functions as a nucleophile and attacks the N5-atom of the flavin, which functions as an electrophile.

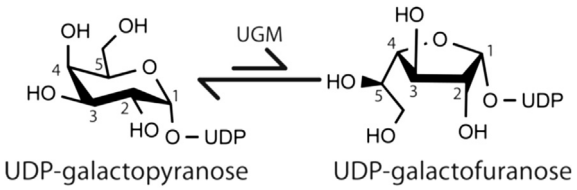
In the reaction catalyzed by UGM (Scheme 3), the flavin is required to be in the reduced state and functions as a nucleophile. In this reaction, the substrate is not activated for N5-adduct



Scheme 1. Structure of the isoalloxazine ring highlighting hot spots for covalent catalysis. (A) Hot spots of the 2-electron reduced flavin. Molecular oxygen is activated and form a covalent intermediate at the C4a position in monooxygenases. The N5-atom functions as a nucleophile attacking the C1-atom of UDP-Galp in UGM. (B) Hot spots of the oxidized flavin. The N5-atom can function as an electrophile that is attacked by carbanions forming N5-iminium ions. Similarly, covalent intermediates at the C4a-position occur by attack of thiols.



Scheme 2. Nucleophilic attack of carbanions results in the formation of an N5-alkyl intermediate. Formation of the N5-iminium ion is coupled to an elimination step.



Scheme 3. The reaction catalyzed by UGM.

formation; instead, priming of the flavin-N5 is required. In the sections that follow, we describe the mechanism that primes the flavin-N5 for nucleophilic attack and the role of the flavin-C4O in proton transfer, which is required to activate reaction intermediates.

3. Priming the flavin-N5 for reactivity in non-redox reactions

Work on UGMs from bacterial sources by the Liu and Blanchard groups established that the reduced flavin is required for catalysis and that the glycosidic bond was broken during turnover [31,32]. Later, the Kiessling group demonstrated that a flavin-N5-covalent intermediate was formed during turnover [33]. The structures of bacterial UGMs (bUGMs) in various redox states revealed the basic UGM fold and insight into substrate recognition [34–36]. However, they were less informative in explaining the various steps involved in the activation of the flavin for catalysis in the UGM reaction [35]. Crystal structures of eukaryotic UGMs (eUGMs) eventually provided insight into the mechanism of flavin activation, as described next.

We have been working with eUGM, which share low sequence identity to bUGMs (~15%) and are, on average, ~100- amino acid longer than bUGMs [37–40]. The presence of a covalent flavin-N5-adduct was demonstrated in eUGMs [41] and the structures of the free enzyme and in complex with UDP-Gal*p* in the oxidized and reduced state have been elucidated [42–45]. These structures revealed conformation changes in the active site flaps, which open in the absence and close in the presence of substrate. Other conformational changes that are coupled to the redox state of the flavin were also shown and included bending of the isoalloxazine ring and movement of several amino acids in the active site that are predicted to place the sugar in proper orientation for flavin attack [43,46]. In addition, the conformational change of a loop (the histidine loop), moves a conserved His residue over 7 Å so that it can hydrogen bond with the ribityl 2′-OH (Fig. 1A). Conformational change of the histidine loop also places the carbonyl oxygen of a conserved Gly residue in hydrogen bonding distance with the N5-atom of the reduced flavin [42,43,46]. These interactions were described to be important to properly orient the reacting molecular orbital centered around the N5-atom and hindering the flavin from reacting with molecular oxygen. The importance of the histidine loop was validated through biochemical studies of a variant of UGM from *Aspergillus fumigatus* (AfUGM) in which the conserved His63 was mutated to Ala (AfUGMH63A). The mutant enzyme was shown to be inactive, and the reduced AfUGMH63A was 100 times more reactive with molecular oxygen than the wild-type enzyme [47].

The flavoenzyme type II isopentenyl diphosphate isomerase (IDI-2) also requires reduced flavin to catalyze a redox neutral reaction [48]. Analysis of the oxidized and reduced structures of *Sulfolobus shibatae* IDI-2 revealed the presence of redox-mediated conformational changes in a flexible loop [49,50]. In the structure of the reduced enzyme, backbone carbonyl oxygen atoms of the flexible loop form hydrogen bonds with the 2′OH and N5-atom (Fig. 1B). These interactions are reminiscent of those observed between the histidine loop and the reduced FAD in UGM. It is reasonable to assume that these interactions in IDI-2 modulate the

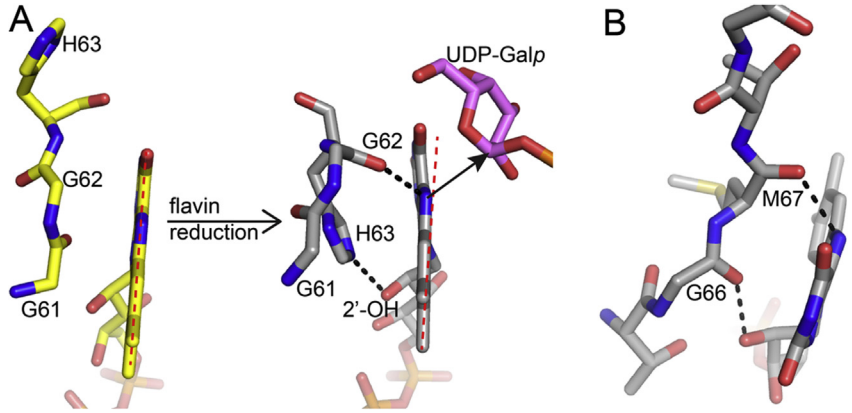


Fig. 1. Hydrogen bonds that prime the flavin N5 for reactivity. (A) Redox-mediated conformational changes of the histidine loop in AfUGM. Upon reduction of the FAD, the histidine loop rearranges to bring conserved Gly62 and His63 within hydrogen bonding distance of the FAD. (B) Structure of reduced IDI-2 (PDB 2ZRV) showing the hydrogen bonding of an active site loop with the FAD. These hydrogen bonds are reminiscent of those in reduced UGM.

reaction of the N5-atom in the acid/base steps; however; this remains to be demonstrated.

4. Activation of intermediates in catalysis: role of the flavin-C4O in proton transfer

The structure of AfUGMH63A complexed with the substrate UDP-Gal*p* showed the presence of a C1-galactose-N5-FAD adduct (PDB 5HHF)(Fig. 2) [47]. This remains the only crystal structure containing a covalent intermediate of UGM. Besides the new bond formed between the sugar and the FAD-N5 and the absence of the bond between the sugar and the UDP (Fig. 2A), differences from the E-S complex structure (PDB code 3UTH) were observed. These include the lack of a hydrogen bond between His63 and the FAD 2′-OH, shifting of the Gal*p* O5 atom by 1.2 Å (Fig. 2B), and the displacement of the carbonyl of Gly62, which now faces away from the N5-atom (Fig. 2C). The fact that the mutant enzyme is unable to turnover suggests that the interactions involving Gly62 and His63 in the wild-type enzyme are essential for catalysis.

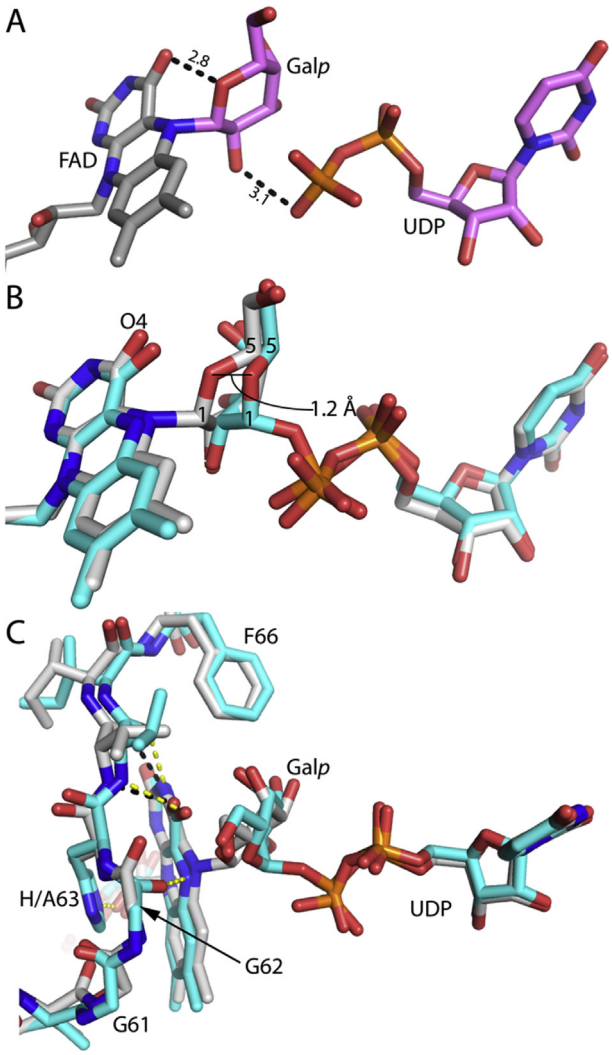


Fig. 2. Structure of a covalent intermediate bound to AfUGMH63A (PDB 5HHF). (A) Structure of the FAD-Gal*p* intermediate with severed UDP. Reduced FAD is colored gray. Severed UDP-Gal*p* is colored pink. (B) Comparison of the covalent intermediate in AfUGMH63A (white) and the noncovalent E-S complex (cyan, PDB code 3UTH). Formation of the N5-C1 bond draws the Gal*p* O5 closer to the flavin O4. (C) Comparison of the loops of AfUGMH63A (white) and the E-S complex (cyan). Black and yellow dashes indicate hydrogen bonds in AfUGMH63A and the E-S complex, respectively. Note that Gly62 fails to hydrogen bond to the FAD N5 atom in the covalent complex, suggesting that this interaction in the wild-type enzyme is essential for turnover.

The structure of the UGM adduct in combination with quantum mechanics/molecular mechanics (QM/MM) molecular dynamics (MD) studies performed by Pierdominici-Sottile et al., provided a clear view of the important steps for catalysis in UGM and evidence for new functionalities for the flavin cofactor [47,51]. The simulations indicate that after formation of the N5-galactose adduct, the next step is deprotonation of the N5-atom by the C4O (Fig. 3). The distance between the N5-H and the C4O in the reduced FAD is 2.4 Å (from ~2.7 Å in the oxidized form) due to bending of the flavin. MD simulations indicate that the bending is further increased in the transition state, decreasing the distance between these two atoms to 1.5 Å. This process is stabilized by interactions of the positively charged His63 with the electron rich flavin (Fig. 3).

The next step in the reaction is opening of the sugar ring. This step is coupled to the formation of the N5-iminum ion and is facilitated by protonation of Gal*p* O5 atom. Bending of the flavin, which brings the FAD C4OH and the Gal*p* O5 together for proton transfer, is also required in this step (Fig. 3). Without His63, the distance between the FAD C4OH and the Gal*p* O5 is not decreased by bending of the flavin ring preventing the proton transfer step. Therefore, the reaction will stall at the FAD-Gal*p* step, accounting for why this intermediate is observed in the AfUGMH63A structure [47]. Further support of this step comes from the observation that the Gal*p* O5 atom is shifted 1.2 Å towards the FAD C4O in the FAD-Gal*p* adduct structure (Fig. 2B). Formation of the FAD-Gal*p*-iminium ion activates the Gal*p* C1 for attack by the C4-OH to generate the furanose form of the sugar. Deprotonation of the sugar C4-OH prior to this step is facilitated by the FAD C4O atom. The proton now at the FAD C4O position is then transferred back to the FAD N5 atom (Fig. 3). The final step is the attack of the Gal*f* C1 by UDP to form UDP-Gal*f*, which yields the reduced flavin.

5. Summary and outlook

The chemical properties of the flavin cofactor in redox reactions are well-characterized. In some of these reactions, it has been shown that covalent flavin reactions occur, such as in the formation of C4a-(hydro)peroxyflavins where the semiquinone reacts with the superoxide ion. Formation of the N5-iminium ion in the reaction of DAAO and NAO with nitroalkanes occurs via attack of a carbanion intermediate. In these examples, the substrate is activated in the active site and reacts with the N5- or-C4-atoms, which can be considered as “hot spots” of the isoalloxazine ring. In the non-redox reaction catalyzed by UGM, the substrate is not activated for reaction with the flavin. Instead, redox-mediated conformational changes place the substrate in proper position and prime the flavin-N5 for nucleophilic attack. Interestingly, a similar conformation is observed in the “activation loop” in ID-II. Thus, conformational changes that prime the N5-atom are observed in non-redox reactions where the reduced flavin plays a role as a nucleophile or an acid/base.

In addition to modulating the flavin reactivity, the protein scaffold (e.g., histidine loop in UGM) plays an essential role in the bending of the flavin ring. Bending of the flavin in the UGM reaction is essential for the proton transfer steps, which are required for advancement of the catalytic cycle (e.g., ring opening) and reformation of the reduced flavin. These proton transfer steps involve the FAD C4O atom (Fig. 3). Studies on UGM show that the FAD C4O-atom plays a role in acid/base reactions and should also be considered as a reactive center or hot spot. The role of the flavin in the acid/base reaction is also observed in IDI-2, however, the N5-atom is predicted to be the main player in that system [52].

Interaction of the imidazole group of H63 in AfUGM with the 2′-

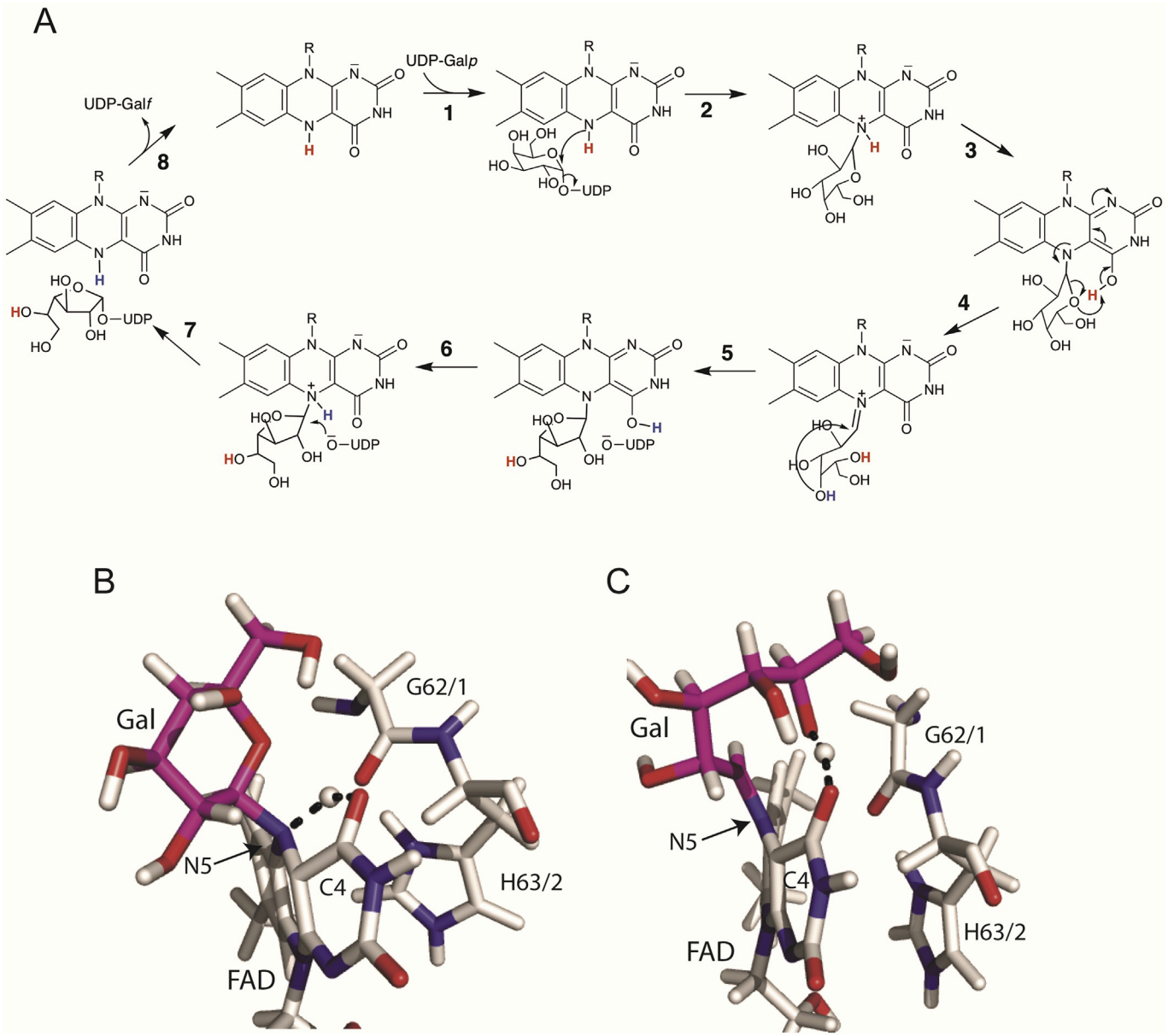


Fig. 3. (A) Diagram showing the major steps in the chemical mechanism. UGM with reduced flavin binds to UDP-Gal*p* (1) and a covalent flavin-galactose adduct is formed via the direct attack of the FAD-N5 to the Galp-C1 atom (2). This step leads to cleavage of the glycosidic bond. Bending of the flavin permits the transfer of the proton from the FAD N5 (shown in red) to the C4O (3). This proton is next transferred to the Gal*p* C5O-atom, facilitating the opening of the sugar ring and formation of the flavin iminium ion (4). The FAD C4O is predicted to accept the proton from the Gal*p* C4-OH (shown in blue) during ring contraction (5). The next step is the transfer of the proton from the FAD C4O to the FAD N5 (6). The final product is formed by direct attack of UDP to the FAD-Gal*f* adduct (7) and release of UDP-Gal*f* from the active site completes the catalytic cycle (8). (B) Structure of the transition state for the transfer of the proton from the FAD N5 to the C4O (Step 3). (C) Structure of the transition state for the protonation of the Galp5O by the FAD4O (Step 4). The coordinates were provided by Prof. Pierdominici [51]. The first number for the amino acids are those for AfUGM and the second for TcUGM.

OH of the ribityl chain is important in establishing the various interactions of the His loop with the flavin ring. Other evidence of the 2′-OH of the ribityl chain playing a role in catalysis has been observed in NAO where it interacts with and facilitates elimination of the nitrate group [21]. Hydrogen bonding between the ribityl 2′-OH atom and the carbonyl oxygen of the substrate in medium chain acyl-CoA dehydrogenase have been shown to play an important role in the activation of C-H bond during hydride transfer [53]. In the bifunctional enzyme proline utilization A, reduction of the FAD induces dramatic conformational changes of the ribityl chain, which alter the hydrogen bonding pattern of the 2′-OH [54–57]. The new interactions are thought to be important for stabilizing the reduced FAD and broadcasting the flavin redox state to the rest of the enzyme. Thus, the 2′-OH of the ribityl chain can be considered as a flavin hot spot.

In summary, the flavin cofactor in UGM plays a role in the formation of N5-adducts and in intra-flavin and flavin-substrate proton transfer steps (between the N5 and C4O atoms and with the Gal*p* O5). Priming of the N5-atom and bending of the flavin during proton transfer requires redox-mediated conformations changes,

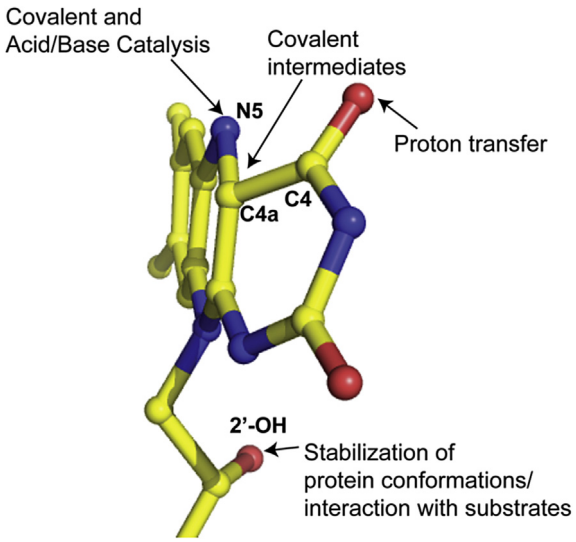


Fig. 4. The major reactive centers of flavin cofactors.

where the 2′-OH of the ribityl chain plays a key stabilizing role. Thus, the 2′-OH of the ribityl chain and the C4O can be added to the list of reactive groups or hot spots of flavin cofactors (Fig. 4). It is likely that these functional groups are involved in proton transfer in other flavoenzyme reactions. A combination of biochemical, structural, and computational approaches, as implemented in UGMs, will be required to reveal these important steps in other mechanisms.

Acknowledgments

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