Sweating the assets of flavin cofactors: new insight of chemical versatility from knowledge of structure and mechanism

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Flavins are arguably one of the most versatile cofactors by virtue of the reactivity of the isoalloxazine ring system. A varied catalogue of reactions for the diverse family of flavoenzymes has been reported, leading to unifying concepts in (long-range) electron transfer, oxygen activation, photochemistry and substrate redox reactions. Recent examples of unprecedented flavin chemistry have been reported that uncover hidden depths of the flavoenzyme chemical repertoire. These include ring expansion of flavin through prenylation and formation of the superoxidized flavin-N5 oxide species. These and other new flavin based species are reviewed here and suggest further exciting discoveries await the flavoenzymology field.

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This rich chemical repertoire might suggest the flavin potential is exhaustively documented leading one to ask how can the chemical versatility of flavin cofactors be expanded further to enrich the diversity of reaction types beyond that already established?

Over the years, mechanistic understanding of flavoprotein chemistry has emerged from integrated studies of structure, spectroscopy, kinetics, model chemistry and computation. Unifying concepts have emerged, but controversies remain where the balance of available data do not provide unequivocal evidence in support of a generally accepted reaction mechanism. Notable have been the current and historical debates concerning mechanisms of amine oxidation [6], or C–H bond breakage [7], based on reasoning from structural, computational and kinetic/spectroscopic investigations. These are rehearsed in some detail elsewhere, and are therefore not the focus of this article. Likewise, the versatility of flavins in ‘non-chemical’ transformations that is in photochemistry/light sensing [8] or long-range electron transfer (in selected cases involving complex bifurcation mechanisms [9,10]), has also been reviewed elsewhere. The purpose here is not to recapitulate this familiar territory. Instead, our aim is to establish to what extent fundamentally new aspects of flavin enzymology have arisen from integrated structural, computational and other experimental investigations reported in recent years (review period 2012–2016). Our focus in particular is on (i) new flavoprotein chemistry, (ii) deeper mechanistic insight of established flavin mechanisms, and (iii) exploitation of the above in the new world of synthetic and industrial biology.

Introduction, flavin cofactors: old dogs and new tricks
The chemical versatility of flavins has long been recognized, with these cofactors traditionally shown to be involved in a wide range of substrate reductions and oxidations [1,2]. This reactivity is attributed to the canonical flavin isoalloxazine ring, focused on the N5 and C4a atoms, the ‘business end’ of the flavin cofactor. By virtue of their various oxidation states (i.e. quinone, semiquinone and dihydroquinone), flavins sit at the crossroads of 1 and 2-electron chemistry. This versatility defines many established reaction classes, encompassing the oxidation of alcohols [3] and amines [4], long-range electron transfer chemistry, and multiple reactions involving the transfer of molecular oxygen [5] (e.g. Baeyer–Villiger oxidations, epoxidation, and oxidase reactions).

New flavin cofactors empower new chemistry
The recent discovery that the reversible decarboxylase UbiD enzymes contain a highly modified FMN cofactor essential for activity confirms that an even wider scope for flavin chemistry is possible [11**]. These enzymes make use of a prenylated FMN cofactor, synthesized by the associated UbiX enzyme (Figure 1a). The latter covalently links the prenyl group of dimethylallyl-monophosphate to both the isoalloxazine N5 and C6 generating a fourth, non-aromatic ring [12**]. The UbiX mechanism proceeds via an unusual sp3 N5-prenyl intermediate that could be visualized using kinetic crystallography. The UbiX prenylated FMN product is converted to the corresponding oxidized form in UbiD. This oxidative maturation leads to formation of
a N5-prenyl C1’ iminium group, generating an azomethine ylide-like cofactor.

The prenylation of FMN by UbiX followed by the oxidative maturation in UbiD bears some chemical resemblance to the FMN dependent UDP-galactopyranose mutase [13], where recent efforts have also allowed visualization of a key \( sp^3 \) N5-galactose adduct (Figure 1b [14]). In the latter enzyme, a N5-sugar iminium bond is created coupled to ring opening in the absence of any formal oxidation. The mutase reaction comes from the ability of distinct sugar alcohol groups to attack the N5-sugar iminium, a step that is somewhat similar to the inactivation of the UbiD cofactor by hydrolysis. In contrast to the proposed non-redox dependent formation of the N5-iminium species in UDP-galactopyranose, UbiD cofactor maturation via the N5-iminium is dependent on oxygen, leading to oxidation of the prenyl moiety via an unknown pathway.

The UbiD prFMN is proposed to support reversible decarboxylation via a 1,3 dipolar cyclo-addition reaction with the unsaturated substrate (a dipolarophile, Figure 2a). In fact, the prenylation and subsequent oxidation generating the prFMN azomethine ylide allows for formation of a covalent substrate–prFMN adduct. The latter is proposed to readily decarboxylate with development of a (transient) negative charge on the isoalloxazine ring system before protonation and product formation (via a retro 1,3 cyclo-addition step). Although present characterization of UbiD enzymes is limited to those family members specific for cinnamic acid-type substrates, other members work on benzoic acid derivatives. It has been proposed a similar mechanism could prevail in the latter [15].

As the oxidized prFMN cofactor is unstable when isolated from UbiD, it would appear UbiX has evolved to use reduced FMNH\(_2\) in order to generate the more stable reduced prFMN form, allowing for cofactor transfer.
Proposed mechanism for (de)carboxylation of cinnamic acid by the prenylated FMN containing UbiD [11**]. Following pyrrolidine ring formation via 1,3 dipolar cycloaddition, decarboxylation is proposed to occur. A covalent adduct could be observed in crystal structures of the Aspergillus niger Fdc enzyme following incubation with a hydroxylated substrate analogue that support the proposal of a substrate adduct with the prFMN via the prenyl C1’ atom (see inset; PDB 4ZA9 [11**]). It is proposed a conserved acidic residue serves as proton donor to catalyze the formation of a 2nd pyrrolidine ring adduct, leading ultimately to the decarboxylation product (in this case styrene) via retro 1,3 cycloaddition.

between both enzymes. UbiX is the second enzyme shown to use flavin as a substrate, in addition to BluB, an enzyme that ‘cannibalizes’ FMN to generate dimethylbenzidine group required for B12 biosynthesis [16]. However, while both enzymes use FMNH₂ as substrate, exploiting its inherent reactivity, the DMAP-dependent UbiX reaction leads to ring expansion while the oxygen dependent reaction of BluB ultimately leads to isoalloxazine fragmentation.

New chemistry with canonical flavin cofactors
In the review period, the English idiom ‘you cannot teach old dogs new tricks’ was shown to be untrue for flavin chemistry. Mechanistic understanding of flavin chemistry has been established for decades. That said, the review period witnessed chemistries previously undocumented for conventional flavin cofactors. Two distinct enzymes have been shown to use flavin for methylene transfer, coupling the transfer of the C1-unit from methylene-THF to the substrate via a transient N5-iminium adduct species similar to those adducts outlined above. While one enzyme is involved in methylation of tRNA (TrmFO [17]), the other is a distinct member of the thymidylate synthases (ThyX [18*]; Figure 3a).

The diversity of catalytically relevant N5 adducts is further expanded by the recent report of a N5-oxide species responsible for the 4-electron oxidation of the
EncM polyketide substrate (Figure 3b; [19**]). Unlike the conventional C4a-peroxide oxidative species, the N5-oxide is remarkably stable in absence of reducing agents. The unexpected oxidative chemistry catalyzed by EncM and the methylene transfer chemistry proposed to occur in TrmFO and ThyX serve to illustrate that the flavin N5 position is an equal asset (compared to the C4a locus) in terms of enzyme catalysis. In EncM, the superoxidized N5-oxide species is proposed to be formed by hydrogen transfer from the reduced flavin to molecular oxygen, followed by radical coupling of the anionic semiquinone N5 with the protonated superoxide [20]. Elimination of water from the resultant N5-peroxide is postulated to lead to the N5-oxide species.

**New flavin chemistry on the horizon**

The N5=C iminium is proposed to act as an electrophile in TrmFO/ThyX, UDP-galactopyranose mutase and during the hydrolytic inactivation of holo-UbiD. A recurrent feature in the proposed mechanisms for the N5-adduct forming enzymes described above is the zwitterionic nature of the N5-adducts formed, with the N5 cation counteracted by a negative charge on the N1–O2 isosalloxazine moiety. This conveys dipolar character to the various N5-adducts formed. The active UbiD species makes use of the azomethine ylide character to support a cyclo-addition reaction at both the C4a and N5=C carbon atoms. It is possible other flavoenzymes exist that exploit the dipolar nature of zwitterionic N5=C adducts for nucleophilic attack.

These enzymes offer exciting new avenues for further mechanistic investigation and exploitation in industrial biocatalysis and metabolic engineering. The fact that UbiD is distantly related to other flavoproteins suggests prFMN binding evolved from a flavin binding scaffold. Therefore, the possibility exists that other distinct prFMN dependent enzymes are yet to be discovered. Indeed, while UbiD makes use of the oxidized prFMN form, the reduced prFMN (and possibly the radical prFMN as observed in UbiX) offer the possibility to further widen the scope for (prenylated)-flavin based chemistry. The group of Fraaije et al. have reported on the recent development of artificial flavoenzymes containing N5-alkylated flavins capable of H2O2-driven enantioselective sulfoxidations, exploiting the remarkably stable 4a-peroxy form of N5-alkylated flavins as powerful oxidizing catalysts [21]. It is possible as yet undiscovered prFMN dependent enzymes could use similar strategies.

**New insight into established C4a focused mechanisms — reactions with dioxygen**

**Oxygen activation**

While mechanisms of dioxygen activation by flavin cofactors emerged decades ago (and have been reviewed extensively [5,22,23]), new insight has emerged from a
combination of density functional theory calculations, time-resolved spectroscopy and site-directed mutagenesis with selected enzymes such as pyranose 2-oxidase (P2O). Conventional pathways for the reactions of monooxygenases and oxidases with molecular oxygen involve electron transfer to oxygen, and in the case of monooxygenases formation of a flavin C4a-(hydro) peroxylavin (Figure 4). These established mechanisms have now been revised with enzymes such as P2O where formation and decay of the C4a-(hydro) peroxylavin intermediate can be visualized directly by time resolved spectroscopy [24\*]. Recent studies have established the P2O reaction with oxygen as one involving single electron transfer coupled with proton transfer from a conserved histidine residue. The proton transfer has been shown to enhance the electron accepting ability of the oxygen. Calculations have shown that the resulting *OOH–flavin semiquinone diradical pair is then optimally positioned to allow formation of the C4a-(hydro) peroxylavin, before uni-molecular decay of the intermediate to form hydrogen peroxide.

Aspects of the P2O mechanism also map to formation of the C4a-(hydro) peroxylavin intermediate formed in monooxygenases, specifically the oxygenase component (C2) of β-hydroxyphenylacetate 3-hydroxylase (PH3H). Here density functional calculations and transient kinetics demonstrate that an active site His is required to protonate dioxygen in a proton coupled electron transfer reaction to form the triplet diradical *OOH–flavin semiquinone complex [25\*]. Intersystem crossing then occurs to form the open-shell singlet diradical complex, before forming the closed-shell singlet C4a-hydroperoxylavin intermediate. The notable key aspect of this mechanism is that formation of the C4a-hydroperoxylavin intermediate is essentially barrierless, in part due to the optimal positioning of the proximal oxygen of *OOH for nucleophilic attack of the flavin C4a atom. Both the P2O and PH3H studies emphasize the importance to catalysis of proton coupled electron transfer (PCET) achieved by the positioning of the active site histidine residue in the vicinity of the flavin. Both studies extend our appreciation of ‘established’ mechanisms by highlighting novel features that will no doubt reoccur in other flavin-dependent oxidases/monooxygenases, and ultimately in the design of new catalysts that seek to exploit the ability of flavin to activate molecular oxygen.

A further refinement of our understanding of flavin-dependent monooxygenases has been observed in SidA, a N-hydroxylating monooxygenase involved in siderophore biosynthesis [26]. As with P2O and PH3H, SidA must

**Figure 4**

Flavin reaction mechanisms with molecular oxygen can be divided into the conventional (a) and (b) pathways for monooxygenase and oxidase reactions respectively [9]. Path c depicts the essentially barrierless formation of the C4a-hydroperoxylavin following the coupled proton and electron transfer to form the semiquinone and HOO\* radical pair [24\*–25\*]. The crystal structure of pyranose 2-oxidase (in complex with acetate) reveals a histidine is ideally positioned to act as proton donor (see inset; PDB 1TTO).
stabilize the C4a-hydroperoxyflavin intermediate. In this case, kinetic isotope effects and density functional theory have suggested that proton transfer from the 2'-OH of the nicotinamide ribose of NADP⁺ to the C4a-peroxyflavin facilitates formation of the C4a-hydroperoxyflavin. This is yet another mechanistic variation that satisfies the need to stabilize a key intermediate (the C4a-hydroperoxyflavin) in monooxygenase/oxidase reactions.

Mechanistic switching between oxidase and monooxygenase chemistry is a major challenge, especially for structure–mechanism based redesign of flavoprotein catalysis for applications in industrial biocatalysis and/or metabolic engineering. The review period saw an elegant demonstration of such switching, namely conversion of a Baeyer–Villiger monooxygenase (BVMO) into a NADPH oxidase [27*]. Most BVMOs can catalyze hydrogen peroxide formation in the absence of a suitable substrate but this so-called uncoupling reaction is typically very slow (<0.1 s⁻¹). Using phenylacetone monooxygenase (PAMO) Mattevi and Fraaije screened several variants of the enzyme for oxidase activity. Surprisingly, they found that a single mutation (Cys45 to Asp54) was sufficient to provide ca 250-fold stimulation of NADPH oxidase activity. Analysis of the crystal structure of wild-type and the C45D variant in the oxidized and flavin-reduced forms revealed only a small change in position of Asp66 in the oxidized form of the C45D (compared with the three other structures), with the Asp residue rotating closer to the flavin — a conformation also seen in a complex of wild-type PAMO and APADP⁺, which also shows enhanced uncoupled oxidase activity [28]. The authors suggest that, through the repositioning of Asp66, the C45D variant displays enhanced NADPH-dependent oxidase activity because of a favored rate of decay of the C4a-peroxyflavin intermediate. This was attributed to (a) steric hindrance to C4a and (b) the ability to rapidly protonate the proximal oxygen of the intermediate that enables formation of hydrogen peroxide. Whatever the mechanistic interpretation, the study demonstrates that subtle restructuring of the flavin environment can have major catalytic outcomes, in this case a switching from BVMO to NADPH-dependent oxidase chemistry.

Macheroux and coworkers also reported the engineering of oxidase activity by mutagenesis in the review period. They identified a gatekeeper residue in the bar berine bridge enzyme (BBE) and BBE-like enzyme Ph1 p4. When targeted in Ph1 p4 (I153V) oxidase activity was increased 60,000-fold; an inverse exchange in the related position of BBE (V169I) reduced oxidase activity by ca 500-fold [29]. Through biochemical and structural analysis the authors concluded that these enzymes have a cavity close to the flavin ring that accommodates dioxygen. They suggested that dioxygen reactivity is determined by steric control of access to this cavity, and that this might be a general feature for controlling oxidase activity beyond their model BBE and BBE-like enzymes.

Catalytic cycles and new chemistries

Insight into structural change accompanying the catalytic cycle of BVMOs has emerged from studies with *Rhodocyclus* cyclohexanone monooxygenase (CHMO). The crystal structure of CHMO was reported several years ago and this highlighted multiple conformations of the bound nicotinamide coenzyme required to reduce the flavin and stabilize formation of the Criegee intermediate formed in the reaction cycle [30]. More recent studies have reported the structure of a CHMO complex with NADP⁺ and 2-cyclohexenone and reveal major rotation of the NADP⁺ cofactor away from the flavin cofactor so that it is no longer stacked with the tricyclic isaloxazine ring [31]. In this new conformation, cyclohexanone is now located over the flavin in a position that enables formation of the Criegee intermediate. In combination with new structures (so-called ‘tight’ and ‘loose’ CHMO complexes [32]) reported for CHMO — product (ε-caprolactone) complexes, these ligand complexes emphasize the dynamic nature of the CHMO active site and the conformational transitions that are required to facilitate the chemical changes throughout the complex reaction cycles of BVMOs.

In the nitro-FMN reductase superfamily, flavin dependent dehalogenases can stabilize the flavin semiquinone following substrate binding [33]. A single mutation was recently shown to switch a dehalogenase to a nitro-reductase, by altering the N5 hydrogen pattern and thereby the inherent ability to promote one versus two-electron chemistry [34]. This suggests catalytic function is primarily controlled by the protein through hydrogen bonding at the N5 position in the nitro-FMN reductase superfamily.

Despite these recent advances, there is still more to be learned — it seems the idiomatic old dog has further tricks to reveal. There have been tantalizing reports of new chemistries catalyzed by oxidases and monooxygenases. These include simple oxidations of thiols by oxidases to form thiocarbonyls [35], thereby extending the chemistry of oxidases beyond oxidation of C–O, C–C and C–N bonds. Furthermore, complex cascade chemistry has been postulated to occur in a flavin dependent decarboxylase–dehydrogenase–monooxygenase during assembly of the warhead α,β-epoxycetone proteaseome inhibitors [36] and for the multifunctional monooxygenase XanO4 that catalyzes sequential insertion of two oxygen atoms and a cryptic demethylation in the biosynthesis of xantholipids [37]. These intriguing reactions extend further the already large family of biocatalysts represented by the flavin-dependent monooxygenases [22]. These are interesting developments and mechanistic understanding will no doubt accrue from future
integration of structural, computational and spectroscopic studies of the reaction cycles for these newly discovered family members.

**Perspectives for the future**

The flavin field continues to surprise despite it being an established field of investigation. New mechanisms have emerged from structural, computational and solution studies. In parallel new modes of covalent linkage to flavins [38,39], the use of flavins as redox shuttles in microbe mineral electron transfer [40], and interfacing flavoproteins with man-made electron donors [41] have been uncovered. All this combines to open up new possibilities for the application of flavoproteins in biocatalysis, metabolic engineering and synthetic biology where fundamental knowledge of structure and mechanism can underpin exciting new applications.

**Conflict of interest statement**

Nothing declared.

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


The first identification of flavoenzymes that can make use of the flavin-N5-oxide as an intermediate.

Use of density functional calculations and transient kinetics is used to demonstrate nearly barriless formation of C4a-hydroperoxyflavin in a flavin dependent monooxygenase.


An elegant demonstration of how targeted enzyme engineering can achieve a monooxygenase-to-oxidase switch.


Recent determination of the Na+-pumping NADH:quinone oxidoreductase from Vibrio cholerae identified a new member of the family of covalent flavoproteins. Here Thr255 forms a covalent phosphoester link with the phosphate group of FMN, although the functional consequences of forming such a link are not known.

