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The Diverse Roles of Flavin Coenzymes—Nature's Most Versatile Thespians

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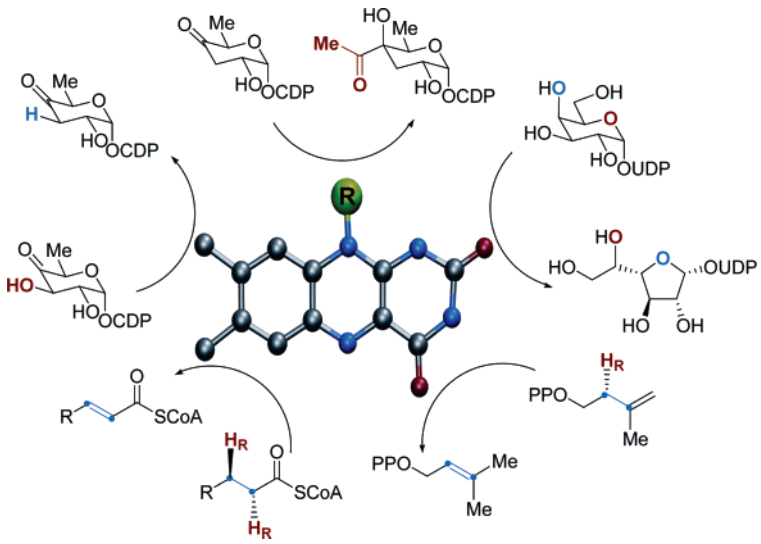

Flavin coenzymes play a variety of roles in biological systems. This Perspective highlights the chemical versatility of flavins by reviewing research on five flavoenzymes that have been studied in our laboratory. Each of the enzymes discussed in this review [the acyl-CoA dehydrogenases (ACDs), CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase (E_3), CDP-4-aceto-3,6-dideoxygalactose synthase (YerE), UDP-galactopyranose mutase (UGM), and type II isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-2)] utilizes flavin in a distinct role. In particular, the catalytic mechanisms of two of these enzymes, UGM and IDI-2, may involve novel flavin chemistry.

Introduction

Flavin mononucleotide (FMN, **1**) and flavin adenine dinucleotide (FAD, **2**), the biologically active forms of riboflavin (vitamin B_2, **3**), play a lead role in a diverse array of biological processes, which is a reflection of their structural and chemical versatility (Scheme 1).[1−3] Flavoenzymes, which contain FMN and/or FAD as prosthetic groups, catalyze many of the one- and two-electron oxidation/reduction reactions critical to the four major energy metabolism systems (photosynthesis, aerobic respiration, denitrification, and sulfur respiration).[4] Many flavoenzymes are capable of catalyzing oxygenation reactions, such as Baeyer−Villiger oxidations and aromatic hydroxylations, which are crucial for soil detoxification processes.[5] Flavin-dependent halogenases are required for the biosynthesis of a myriad of halogenated natural products, many of which have therapeutic potential.[6] Flavins are also involved in the photorepair of DNA damage and in the regulation of caspase-

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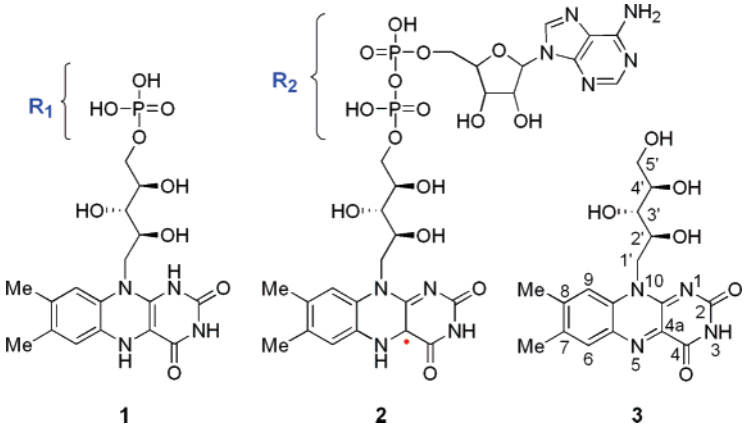
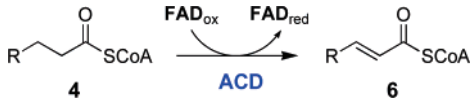
SCHEME 1. Structures and Redox States of the Natural Flavins[a]



[a] FMN (**1**) and FAD (**2**) differ from riboflavin (**3**) in their ribityl side chains, which contain a phosphate residue ($\mathbf{R_1}$) or an ADP moiety ($\mathbf{R_2}$) in ester linkage with its terminal hydroxyl group, respectively. The isoalloxazine ring of flavins can exist in any one of three oxidation states. FMN is shown in the two-electron-reduced hydroquinone state, FAD as a one-electron-reduced neutral semiquinone, and riboflavin in its oxidized form.

SCHEME 2. Reaction Catalyzed by Acyl-CoA Dehydrogenases (ACDs)



independent apoptosis.[7,8] 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.[9−11]

Because of the chemical versatility of flavin coenzymes and the diverse roles they play in biology, studies of several physiologically significant and/or mechanistically intriguing flavin-dependent enzymes have been carried out in our laboratory in an effort to learn more about this important class of biocatalysts. This Perspective reviews research on five of these flavoenzymes [acyl-CoA dehydrogenase (ACD), CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase (E_3), CDP-4-aceto-3,6-dideoxygalactose synthase (YerE), UDP-galactopyranose mutase (UGM), and type II isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-2)], each of which utilizes flavin in a distinct role.

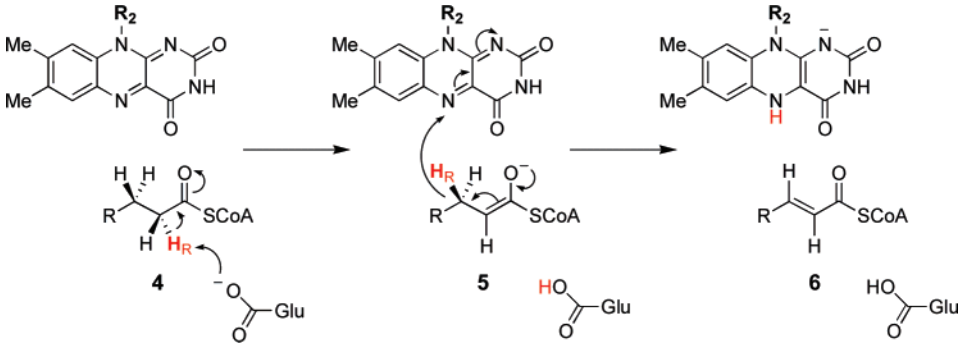
Acyl-CoA Dehydrogenases: Flavin Playing a Redox Role

Acyl-CoA dehydrogenases (ACDs) constitute a homologous family of FAD-dependent enzymes that catalyze the conversion of acyl-CoA thioesters (**4**) to the corresponding *trans*-α,*β*-enoyl-CoA (**6**) (Scheme 2).[12−15] Members of this family share significant structural and biochemical similarities, but differ widely in their substrate specificities.[16−18] ACDs are involved in the *β*-oxidation of short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD), and very long chain (VLCAD) fatty acids and in the catabolism of amino acids, such as leucine (isovaleryl-CoA dehydrogenase, IVD), isoleucine, (short/branched-chain acyl-CoA dehydrogenase, SBCAD), valine (isobutyryl-CoA dehydrogenase, IBD), and lysine (glutaryl-CoA dehydrogenase, GCAD).[18−22] Mutations in seven of these ACDs have been linked to disease states in humans, including sudden infant death syndrome.[23−26] The majority of ACDs are soluble, homotetrameric enzymes with a subunit molecular weight of ∼43 kDa and are present in the inner mitochondrial matrix.[27] The lone exception is VLCAD, which exists as a homodimer with a subunit molecular weight of ∼67 kDa and is associated with the inner mitochondrial membrane.[28]

Each of the ACDs has been found to share the same complicated catalytic mechanism (Scheme 3).[29,30] First, ACD-FAD_{ox} forms a Michaelis complex with its acyl-CoA substrate through a two-step binding mechanism.[31,32] Next, a conserved glutamate residue abstracts the pro-*R* α-proton of the substrate, generating an enolate intermediate (**5**).[33−35] Dehydrogenation of the acyl-CoA substrate is then achieved by the transfer of the pro-*R* *β*-hydrogen of the nascent enolate intermediate to the flavin coenzyme as a hydride equivalent.[36−38] This results in the formation of a characteristic, bluish-green ($\lambda_{max} \approx 570$ nm) charge-transfer complex between ACD-FAD_{red} and the enoyl-CoA product.[39] In the absence of an external electron acceptor, the *trans*-α,*β*-enoyl-CoA product is slowly released from the enzyme, though this equilibrium strongly favors the charge-transfer complex.[40−42] The tight binding interaction between ACD-FAD_{red} and product is thought to help drive dehydrogenation of the acyl-CoA thioester substrates, which are fairly weak thermodynamic reductants.[40,43,44] The physiological electron acceptor for all ACDs is the electron-transferring flavoprotein (ETF), which channels the reducing equivalents from the reduced flavin coenzyme of ACD into the mitochondrial respiratory chain. ETFs are typically heterodimeric proteins and contain one equivalent of FAD and a non-redox-active AMP molecule.[45,46] The electrons are transferred sequentially in two one-electron steps to ETF.[47,48] Though the charge-transfer complex is a poorer thermodynamic reductant than free ACD-FAD_{red}, binding of the enoyl-CoA product greatly enhances the rate of the electron-transfer reaction to ETF.[43,44,49] This rate enhancement may be linked to the decrease in pK_a of the reduced flavin that is observed upon thioester binding.[50−52] ETF then transfers the electrons derived from the acyl-CoA thioester to ETF-ubiquinone oxidoreductase—an intrinsic inner mitochondrial membrane protein that contains a [4Fe-4S] cluster and binds one equivalent of FAD.[53] Recently, the crystal structure of ETF-ubiquinone oxidoreductase was solved with and without bound ubiquinone, illuminating the likely electron-transfer pathway from ETF to ubiquinone.[54]

The pK_a of the conserved glutamic acid residue in MCAD-FAD_{ox} is ∼6 and that of an α-proton of a typical acyl-CoA

SCHEME 3. Chemical Mechanism of Acyl-CoA Dehydrogenases (ACDs)[a]



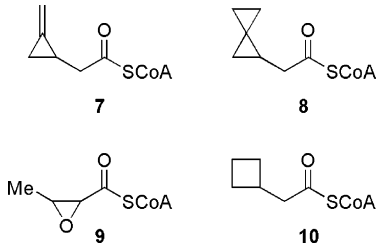
[a] In this reaction, FAD acts as a hydride acceptor.

thioester is ~21.[55−58] Thus, the pK_a of the active-site base must be raised and/or the pK_a of the α-proton of the substrate must be substantially lowered for catalysis to be efficient. Indeed, upon thioester binding, the pK_a of the glutamic acid residue is found to increase to a value of ~9.[32,59] This elevation is primarily attributed to the desolvation of the active site that accompanies substrate binding. Binding of the substrate analogue 3-thiaoctanoyl-CoA, which can be deprotonated but not dehydrogenated, to MCAD results in the lowering of the pK_a of its α-proton from ~16 in free solution to ~5.[60,61] The crystal structure of this complex was solved, and it was found that the backbone amide of the conserved glutamate residue and the 2′-hydroxyl of the ribityl moiety of FAD form hydrogen bonds with the carbonyl oxygen of the thioester.[62] The pK_a of the α-proton of 3-thiaoctanoyl-CoA observed upon binding to MCAD reconstituted with 2′-deoxy-FAD was ~11, illustrating the importance of hydrogen-bond formation in substrate activation.[56] The isoalloxazine ring of flavin itself contributes to a lowering of the thioester substrate pK_a. A series of flavin analogues substituted at the 7- or 8-position were used to reconstitute MCAD, and their influence on the activation of 3-thiaoctanoyl-CoA was monitored.[63] As the electron-withdrawing properties of the flavin substituent increased, the pK_a of the substrate α-proton decreased, from a value of 7.4 with 8-amino-FAD to 4.0 with 8-cyano-FAD. This is consistent with an earlier observation that the electron density at N5 of FAD, which is modulated by hydrogen-bonding interactions with a nearby threonine residue, is important for the activation of the α-proton of 3-thiaoctanoyl-CoA.[30]

In addition to the large pK_a difference that must be overcome for efficient catalysis, the disparity between the redox potentials of the ACD-FAD$_{ox}$ complexes and their acyl-CoA substrates represents another energetic hurdle. The midpoint potentials of the *trans*-α,*β*-enoyl-CoA/acyl-CoA redox couples have been found to be virtually independent of chain length over a range of C4 to C16, varying from −38 to −45 mV (relative to the SHE), respectively.[43] In the absence of substrate, the midpoint potential of the MCAD-FAD$_{ox}$/MCAD-FAD$_{red}$ couple is −136 mV. However, the binding of an acyl-CoA thioester to MCAD results in a dramatic shift of the redox potential of the enzyme to −26 mV, allowing the reaction to proceed in the thermodynamically favorable direction.[43] A similar redox potential shift upon substrate/product binding was also observed for SCAD.[64]

In the chemical mechanism of ACDs (Scheme 3), the FAD coenzyme functions as a hydride acceptor. Kinetic isotope effect (KIE) measurements indicate that cleavage of the C_β−H bond is rate-limiting.[15] When MCAD was reconstituted with reduced 5-deazaFAD, which is incapable of single-electron chemistry, a hydride equivalent was transferred from C5 of the coenzyme to crotonyl-CoA, generating butyryl-CoA.[14] In addition, the crystal structure of MCAD in complex with octanoyl-CoA/octenoyl-CoA revealed that the *β*-carbon of the substrate/product was ideally positioned for direct hydride transfer with N5 of the flavin coenzyme.[33]

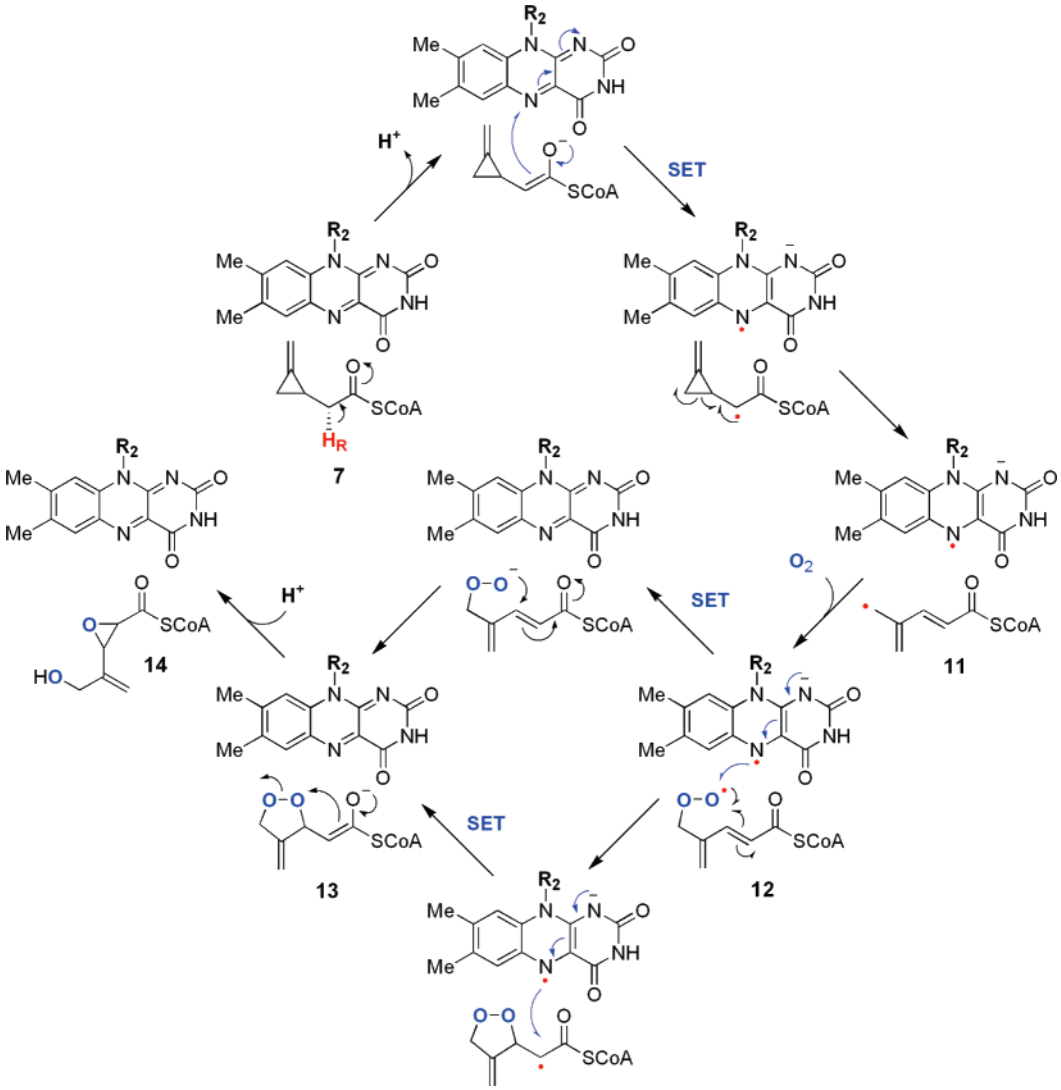
SCHEME 4. Mechanism-Based Inactivators of ACDs: (Methylenecyclopropyl)acetyl-CoA (7), spiropentylacetyl-CoA (8), (*E*)-2,3-epoxybutyryl-CoA (9), and Cylcobutylacetyl-CoA (10)



Although the oxidation of acyl-CoAs is thought to proceed via hydride transfer to ACD-FAD$_{ox}$, studies using mechanism-based inactivators (Scheme 4) have shown that ACDs are also capable of mediating one-electron oxidations. When MCAD is incubated with (methylenecyclopropyl)acetyl-CoA (MCPA-CoA, **7**), a metabolite of hypoglycin A and the causative agent of Jamaican vomiting sickness, time-dependent loss of enzyme activity is observed, concomitant with the bleaching of the flavin chromophore (λ_{max} = 446 nm).[65,66] The inactivation process is initiated by abstraction of the α-proton of MCPA-CoA. Subsequent fragmentation of the cyclopropyl ring of the inactivator leads to the covalent modification of the flavin coenzyme. The rate of bleaching of the flavin chromophore is slightly slower than the rate of inactivation, and levels off while a small loss of enzyme activity continues.[67] This suggests the existence of a minor inactivation pathway, which was found to result from modification of the flavin at a site other than the isoalloxazine ring.[68] Both enantiomers of MCPA-CoA are potent inactivators of MCAD with nearly identical partition ratios.[67,69,70] Since cleavage of the C_β−H bond of the acyl-CoA substrate is pro-*R* specific, the lack of stereospecificity in the inactivation process suggests the ring-opening reaction is not enzyme-controlled. Rapid ring fragmentation is consistent with the rearrangement of an α-cyclopropyl radical to a ring-opened alkyl radical, which is known to be an extremely facile process.[71,72]

Further support of a radical-mediated inactivation mechanism comes from the identification of the major turnover product of

SCHEME 5. Chemical Mechanism of Aerobic MCPA-CoA (7) Turnover by MCAD[a]



[a] In this reaction, FAD plays a redox role in catalyzing the dioxygenation reaction (SET: single-electron transfer).

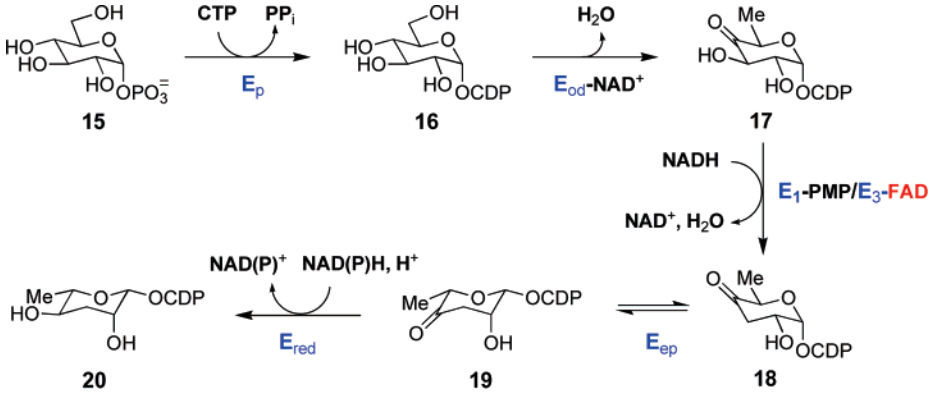
MCPA-CoA with MCAD obtained under aerobic conditions (**14**), which was found to be a CoA thioester containing a disubstituted terminal olefin, a hydroxymethyl group, and an epoxide.[68,73] The structure of this turnover product is consistent with the generation of an allylic ring-opened radical (**11**) that is intercepted by O_2 to form a transient peroxy radical (**12**). Subsequent reduction by FAD_{sq} results in the formation of **13**, which undergoes an intramolecular epoxidation reaction (Scheme 5).

Studies using spiropentylacetyl-CoA (SPA-CoA, **8**), a close analogue of MCPA-CoA, yielded similar results.[74] Chemical model studies, in conjunction with high-level ab initio calculations, on the ring-opening reactions of 4-butylspiropentylcarbinyl radicals identified a single ring-opened primary cyclopropylcarbinyl radical as the species which forms an adduct with FAD and identified N5 as the likely site of covalent modification.[75] Interestingly, both stereoisomers of SPA-CoA inactivate MCAD, whereas the (*R*)-epimer is an inactivator and the (*S*)-epimer is a competitive inhibitor of SCAD.[74] Similar ACD class-dependent inactivation properties were observed with (*E*)-2,3-epoxybutyryl-CoA (**9**), which is an inactivator of SCAD and an inhibitor of MCAD, and cylcobutylacetyl-CoA (CBA-CoA, **10**), which is an inactivator of SCAD but a substrate for MCAD.[76,77] The different reactivities of these compounds likely reflect differences in the steric and electronic environments of the active sites of the various ACD classes. These differences may allow the rational design of class-specific inhibitors for the manipulation of fatty acid metabolism.

CDP-6-Deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase Reductase: Flavin Acting as a Redox Mediator

3,6-Dideoxysugars are found predominantly in the lipopolysaccharide of Gram-negative bacteria.[78] These sugars are well-known antigenic determinants and play important roles in the pathogenicity and virulence of bacteria.[79] Ascarylose is a 3,6-dideoxyhexose found in the *O*-antigen of *Yersinia pseudotuberculosis* V.[80] The biosynthetic pathway of CDP-L-ascarylose (**20**) is shown in Scheme 6. The first step, catalyzed by α-D-glucose-1-phosphate cytidylyltransferase (E_p), involves formation of CDP-D-glucose (**16**) from α-D-glucose-1-phosphate (**15**) and cytidine triphosphate (CTP).[81] Next, CDP-D-glucose 4,6-

SCHEME 6. Biosynthetic Pathway of CDP-L-Ascarylose (20)



dehydratase (E_{od}) catalyzes C6 deoxygenation via a NAD^+-dependent oxidation−reduction reaction.[82] This transformation is followed by deoxygenation of **17** at C3 catalyzed by the combined actions of two enzymes, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase (E_1) and its reductase (E_3). The configuration at C5 of the product of this reaction, **18**, is then inverted by CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose 5-epimerase (E_{ep}).[83] Finally, the 4-keto group of **19** is stereospecifically reduced by CDP-3,6-dideoxy-D-*glycero*-L-*glycero*-4-hexulose 4-reductase (E_{red}).[83]

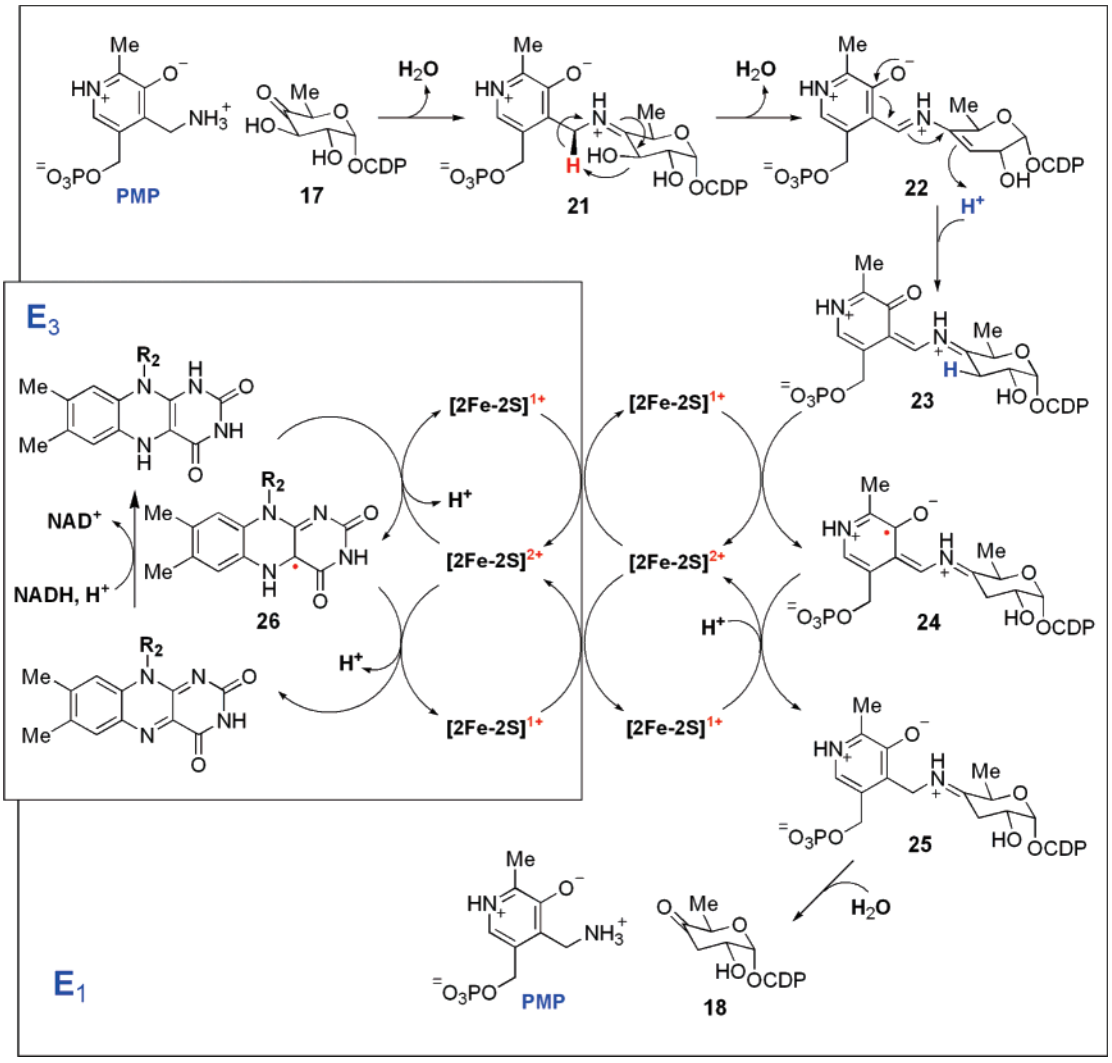
The conversion of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**17**) to CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**18**) by E_1/E_3 occurs in two stages (Scheme 7). The first stage, which is catalyzed by E_1, consists of the pyridoxamine phosphate (PMP)-dependent dehydration of **17** to a conjugated $\Delta^{3,4}$-glucoseen intermediate (**22**). E_3 then catalyzes the second stage of the reaction, wherein reducing equivalents from NADH are transferred to the $\Delta^{3,4}$-glucoseen intermediate, ultimately leading to the formation of the product, **18**, and regeneration of the PMP coenzyme.[84,85]

E_1 is a homodimeric enzyme with a subunit molecular weight of ∼49 kDa.[84] Each subunit binds one equivalent of PMP, as determined by radiometric and fluorometric quantitations.[86,87] The highly conserved lysine residue that is typically present in coenzyme B_6-dependent enzymes is replaced with a histidine residue (His220) in E_1.[88−93] Thus, the binding mode of PMP to E_1 is unusual in that the coenzyme is associated with the active site through strictly noncovalent interactions. In addition to PMP, analytical assays of E_1 revealed the presence of stoichiometric amounts of iron and sulfur.[94] Subsequent electron paramagnetic resonance (EPR) spectroscopic analysis confirmed the presence of a [2Fe-2S] cluster.[94,95] This iron−sulfur cluster, which is essential for catalysis, is bound via an unusual $C-X_n-C-X-C-X_7-C$ coordination motif.[96] Substrate **17** is capable of binding to E_1 only in the presence of the PMP coenzyme. Upon binding, **17** forms an imine (**21**) between its C4 keto group and the amino group of PMP. Subsequent abstraction of the pro-*S* 4′-hydrogen of PMP by an active-site base triggers elimination of the C3 hydroxyl group, leading to the formation of the conjugated $\Delta^{3,4}$-glucoseen intermediate (**22**).[84,87,97] Since the replacement of the C3 hydroxyl group of **17** by a hydrogen atom in the deoxygenated sugar product (**18**) proceeds with net retention of stereochemistry, the dehydration reaction is likely a suprafacial process that occurs on the solvent-accessible *si* face of the PMP−substrate adduct (**21**).[98] The E_1-catalyzed dehydration of **21** to the $\Delta^{3,4}$-glucoseen intermediate (**22**) is a reversible process (as evidenced by the incorporation of an ^{18}O-label at C3 of recovered **17** when the reaction is carried out in $H_2{}^{18}O$), which is driven to completion by the reduction of **22** to **25**. A number of reductants (such as dithionite, diaphorase, and methane monooxygenase reductase) are capable of reducing **22**, albeit with much lower efficiency than E_3-NADH.[86,97,99]

E_3, which is a monomer with a molecular weight of ∼36 kDa, binds one equivalent of FAD and contains a plant-type ferredoxin [2Fe-2S] cluster, as determined by both UV−visible and EPR spectroscopic analysis and iron and sulfur quantitation.[100] On the basis of sequence homology, E_3 is a member of the flavodoxin-$NADP^+$ reductase (FNR) family of proteins.[101] NADH is capable of binding to both oxidized and two-electron-reduced forms of E_3, with dissociation constants (K_d's) of 52.5 ± 2 and 12.1 ± 1 μM, respectively. E_3 catalyzes the stereospecific transfer of a hydride derived from the C4 pro-*R* hydrogen of NADH to FAD at a rate of 107.5 ± 3 s^{-1}.[102] This reaction is analogous to the aforementioned hydride transfer catalyzed by ACDs. The FAD coenzyme of E_3 is capable of transferring reducing equivalents from NADH to many external electron acceptors, including O_2, 2,6-dichlorophenolindophenol (DCPIP), and exogenous FAD and FMN, with varying degrees of efficacy.[103] This process is independent of the presence of the [2Fe-2S] cluster.[102] However, both iron−sulfur centers in E_1 and E_3 are required for the FAD-mediated transfer of reducing equivalents from NADH to the $\Delta^{3,4}$-glucoseen intermediate bound at the active site of E_1.[95,100,102,104] The presence of the two [2Fe-2S] clusters, which are obligatory one-electron-transfer cofactors, indicates that FAD acts as a two-electron/one-electron switch in mediating the transfer of electrons from NADH through the chain of redox-active cofactors.[102,104] The midpoint potentials of FAD and the [2Fe-2S] clusters in E_3 and E_1 were determined to be −212, −257, and −209 mV, respectively.[104] The presence of the [2Fe-2S] cluster in E_3 modulates the redox properties of the flavin coenzyme, as its removal causes a shift in the midpoint potential of FAD from −212 to −227 mV. The thermodynamic barrier to electron transfer from FAD to the [2Fe-2S] cluster within E_3 might serve a regulatory role in suppressing the E_1/E_3 reaction unless an excess of NADH is present in the cell. The E_1/E_3 system may also be regulated by pH since redox titrations and stopped-flow spectroscopic studies showed that this thermodynamic barrier to electron transfer is inverted when the pH is increased from 7 to 10.[102,104]

The transfer of reducing equivalents from E_3 to the $\Delta^{3,4}$-glucoseen intermediate (**22**), which is bound at the active site of E_1, requires formation of an intermolecular complex between E_1 and E_3. This was confirmed both *in vitro*, using size-exclusion

SCHEME 7. Chemical Mechanism of E_1/E_3[a]



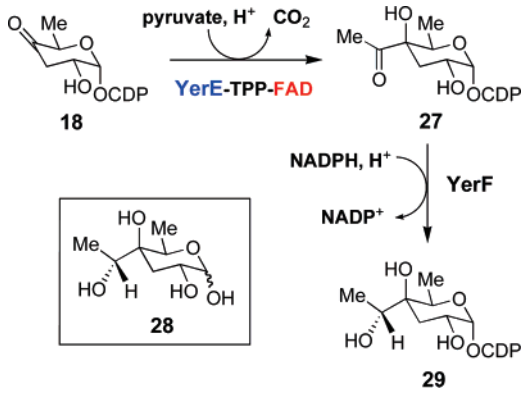
[a] In this reaction, FAD plays the role of a two-electron/one-electron switch in mediating electron transfer.

chromatography and circular dichroism (CD) spectroscopy, and *in vivo*, using the yeast two-hybrid system.[105] The apparent dissociation constant of the E_1-E_3 complex was estimated to be 288 ± 22 nM, with a stoichiometry of 1.7 between E_3 and E_1.

Two organic radical intermediates have been detected in the catalytic cycle of E_1/E_3 (Scheme 7) by EPR spectroscopy.[95,99] The first radical was unambiguously assigned as a flavin semiquinone (**26**), formed as the reduced flavin transfers a single electron to the [2Fe-2S] center of E_3. The second radical, which was observed only in the presence of substrate, was a featureless EPR singlet centered at $g = 2.003$. The detailed kinetic mechanism of the early stages of the E_1/E_3 reaction was determined using stopped-flow spectroscopy.[95,102] By fitting the absorbance data obtained at different wavelengths using a diode-array spectrophotometer to a series of sequential exponential equations, it was determined that at least six intermediates were formed during the catalytic cycle.[95] The rate of formation and decay of Intermediate V was nearly identical to that of the second organic radical, as determined by rapid freeze-quench EPR spectroscopy. The UV−visible difference spectrum of Intermediate V has a λ_{max} at 456 nm with a shoulder at 425 nm, which bears resemblance to substrate−PMP quinonoid species typically observed in PLP-dependent β-elimination reactions.[95]

To determine the structure of the radical Intermediate V, several isotopically labeled PMP analogues were synthesized and used to reconstitute E_1 for EPR analysis.[106] The EPR spectrum obtained with [4′,5′-$^2H_4$]PMP resulted in a ~3 G narrowing of the EPR line shape. The presence of ^2H hyperfine couplings in samples of E_1 reconstituted with [4′,5′-$^2H_4$]PMP and [2′-$^2H_3$]PMP were directly detected using Mims electron nuclear double-resonance (ENDOR) spectroscopy. These results clearly indicate the presence of electron spin-density in the pyridine ring of the PMP coenzyme and support the assignment of Intermediate V as an unprecedented PMP−glucoseen radical (**24** and its resonance forms). Intermediate **24** is then reduced by a second electron from the [2Fe-2S] cluster of E_1 to give **25**, which is hydrolyzed to yield the 3,6-dideoxysugar product (**18**) and regenerate PMP. As the E_1/E_3 system illustrates, flavin coenzymes play crucial physiological roles as mediators between two-electron- and one-electron-transfer reactions.

SCHEME 8. Biosynthetic Pathway of CDP-Yersiniose A (29)[a]



[a] Steps Leading to the Formation of **18** are the Same as in Scheme 6.

CDP-4-Aceto-3,6-dideoxygalactose Synthase (CADS): Flavin Playing a Structural Role

Branched-chain sugars are an important class of carbohydrates.[107] Most naturally occurring branched-chain sugars contain a methyl branch, though examples of sugars with longer side chains are known.[78,82,108] Yersiniose A (see **28** in Scheme 8) is a prototypical hydroxyethyl-branched 3,6-dideoxysugar found in the *O*-antigen of *Yersinia pseudotuberculosis* VI.[109,110] Early feeding experiments have shown that the two-carbon chain present in these branched-chain sugars derives from pyruvate, which is consistent with the branch attachment being a thiamine pyrophosphate (TPP)-dependent process.[111−113] Curiously, well-known inhibitors of TPP-dependent enzymes failed to affect the production of these branched-chain sugars, and radioactive products were not detected when cell-free extracts of the producing strains were incubated with 1-([1-^{14}C]-hydroxyethyl)-TPP.[111−113]

In previous experiments, such as the aforementioned studies on ascarylose biosynthesis, CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**18**) was established as a common intermediate in the biosynthesis of 3,6-dideoxyhexoses.[114,115] Since yersiniose A (**28**) is a C4-branched 3,6-dideoxyhexose, and the two strains producing ascarylose and yersiniose A are closely related, it seemed likely that the sequence and organization of the yersiniose (*yer*) biosynthetic gene cluster would show a high degree of homology to the *asc* cluster. Therefore, the *ascC* gene encoding E_1 was used as a probe to screen a genomic library of *Y. pseudotuberculosis* VI in an effort to identify the *yer* cluster.[116] The *yer* cluster was successfully characterized and found to contain eight ORFs, two of which (*yerE* and *yerF*) are essential for incorporation of the hydroxyethyl branch of yersiniose A.[116]

YerE shares 32% sequence identity with the large subunit of acetolactate synthase, a FAD-containing TPP-dependent enzyme. YerF shows moderate homology to NAD(P) binding enzymes, such as UDP-D-galactose-4-epimerase and CDP-D-tyvelose-2-epimerase. Thus, the structure of the *yer* cluster is consistent with the CDP−yersiniose A (**29**) biosynthetic pathway shown in Scheme 8.

Studies on acetolactate synthases have shown that FAD plays no obvious role in the catalysis, whereas TPP is critical for the formation of an acetyl carbanion from pyruvate and facilitation of the subsequent condensation reaction.[117] To verify the catalytic role of YerE, recombinant protein was overexpressed in *Escherichia coli* and the purified enzyme was found to contain bound FAD, giving a typical flavin spectrum with two absorption maxima at 370 and 450 nm. YerE exists as a homodimer, with a molecular weight of 58.5 kDa per monomer. In the presence of TPP and Mg^{2+}, YerE was shown to convert pyruvate and **18** (prepared enzymatically using purified E_{od}, E_1, and E_3) to **27**, whose structure was verified by ^{1}H and ^{13}C NMR.[116] At the optimal pH of 8.0, this transformation was found to proceed with a k_{cat} of 72.3 ± 2.8 min^{-1} and K_m of 7.9 ± 1.8 and 3.11 ± 0.24 mM for pyruvate and **18**, respectively.[118]

When YerE is prereduced with dithionite and the reactions are performed under anaerobic conditions, there is no discernible loss of catalytic efficiency.[116] This result strongly suggests that FAD does not play a redox role in the YerE-catalyzed transformation. Instead, the reaction likely proceeds according to a *de facto* "umpolung" (charge-reversal) reaction typical of other TPP-dependent enzymes. In this mechanism (Scheme 9), the coupling of pyruvate and TPP generates a nucleophilic carbanionic adduct (**30**), which subsequently attacks the 4-keto group of **18**. Collapse of the resulting tetrahedral adduct (**31**) leads to the formation of **27** and regeneration of the coenzyme. Given the similarity of the YerE-catalyzed reaction to the reaction catalyzed by acetolactate synthase, we propose herein to designate this enzyme CDP-4-aceto-3,6-dideoxygalactose synthase (CADS).

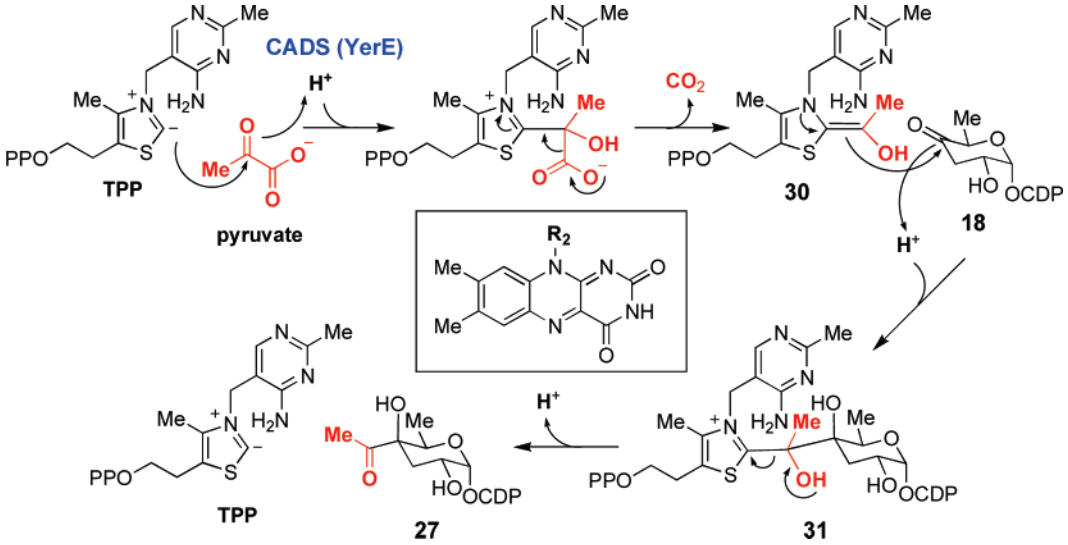
Thus, CADS represents an unusual class of flavoproteins in which the flavin coenzyme appears to play a structural role in maintaining the integrity of the enzyme active site. This class, which includes the acetolactate synthases and glyoxylate carboligases, is thought to be an evolutionary descendent of pyruvate oxidase (or a related enzyme), and the flavin requirement exists only as a vestigial remnant.[119] Interestingly, a similar structural role has been proposed for the FAD coenzyme of certain hydroxynitrile lyases, which are distantly related to the glucose−methanocholine−oxidoreductase family of FAD-dependent enzymes.[3] In the case of hydroxynitrile lyases, however, it has recently been proposed that, in addition to its role in maintaining active site integrity, the oxidized flavin coenzyme helps maintain a positive electrostatic potential in the active site, which stabilizes the cyanide anion that forms during turnover.[120] Thus, while the FAD is not involved in redox chemistry, it may electrostatically stabilize the reaction intermediates.

UDP-Galactopyranose Mutase: Flavin Acting as a Covalent Catalyst

D-Galactose is a sugar found ubiquitously in nature in its thermodynamically stable pyranose form (Gal*p*).[121,122] The furanose form of D-galactose (Gal*f*) is decidedly less common, being limited primarily to the cell walls and/or cell membranes of microorganisms, including several human pathogens such as *Mycobacterium tuberculosis*.[123−126] UDP-Gal*p* (**32**) is converted to UDP-Gal*f* (**33**) through the action of the FAD-dependent enzyme UDP-galactopyranose mutase (UGM) (Scheme 10), which has an equilibrium constant of 0.057 in the forward direction.[127−129] Since Gal*f* is not a constituent of human glycoconjugates, UGM is an attractive drug target.[130]

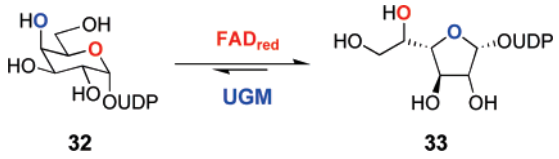
UGM is a homodimeric enzyme with a subunit molecular weight of ∼43 kDa.[128,131] Each subunit binds one equivalent of FAD. The role of flavin in the unusual ring contraction reaction catalyzed by UGM is not immediately apparent since there is no net redox change of the substrate or product. The

SCHEME 9. Chemical Mechanism of CDP-4-Aceto-3,6-Dideoxygalactose Synthase (YerE)[a]



[a] In this reaction, FAD does not participate directly in catalysis but instead may play a structural role in maintaining active site integrity.

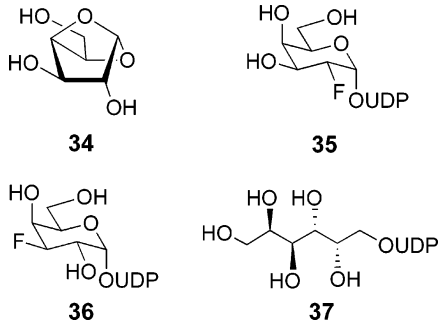
SCHEME 10. Reaction Catalyzed by UDP-Galactopyranose Mutase (UGM)



reduced form of the flavin coenzyme is required for catalytic activity.[131,132] UGM reconstituted with 1-deazaFAD (k_{cat} = 22 s^{-1} and K_m = 39 μM) has comparable catalytic activity to UGM reconstituted with regular FAD (k_{cat} = 25 s^{-1} and K_m = 30 μM), whereas enzyme reconstituted with 5-deazaFAD is catalytically inactive.[133] Since 5-deazaFAD is limited to two-electron-transfer processes, while FAD and 1-deazaFAD can undergo both one- and two-electron processes, these results are consistent with a radical mechanism for UGM catalysis. Further evidence in support of a radical mechanism comes from a thermodynamic analysis of UGM which showed that the FAD_{sq} is stabilized in the presence of substrate and the reduced flavin is present in its anionic form, which would facilitate electron transfer to the substrate in a crypto-redox process.[134] Alternatively, the fact that UGM reconstituted with 5-deazaFAD is inactive may reflect the importance of having a nitrogen atom, with its lone pair of electrons, at position 5 of the flavin ring. Indeed, an N5-alkylated flavin was trapped in UGM reaction mixtures treated with sodium cyanoborohydride, suggesting that a FAD−substrate adduct may be an intermediate in the catalytic cycle of UGM (see **39** in Scheme 12).[135]

The crystal structure of UGM from several bacterial sources has been solved with FAD bound at a putative active site lined with conserved residues.[131,136] Site-directed mutagenesis confirmed the importance of three conserved tyrosine residues and a conserved tryptophan residue in catalysis, which are thought to interact with the substrate through hydrogen-bonding and stacking interactions with the UDP moiety, respectively.[131] The flavin coenzyme is situated between one of the conservedtyrosines and a conserved histidine residue.[136] In contrast to ACDs, in which the N5 atom of FAD_{ox} accepts a hydrogen bond from a threonine residue (thereby increasing its electrophilicity), the

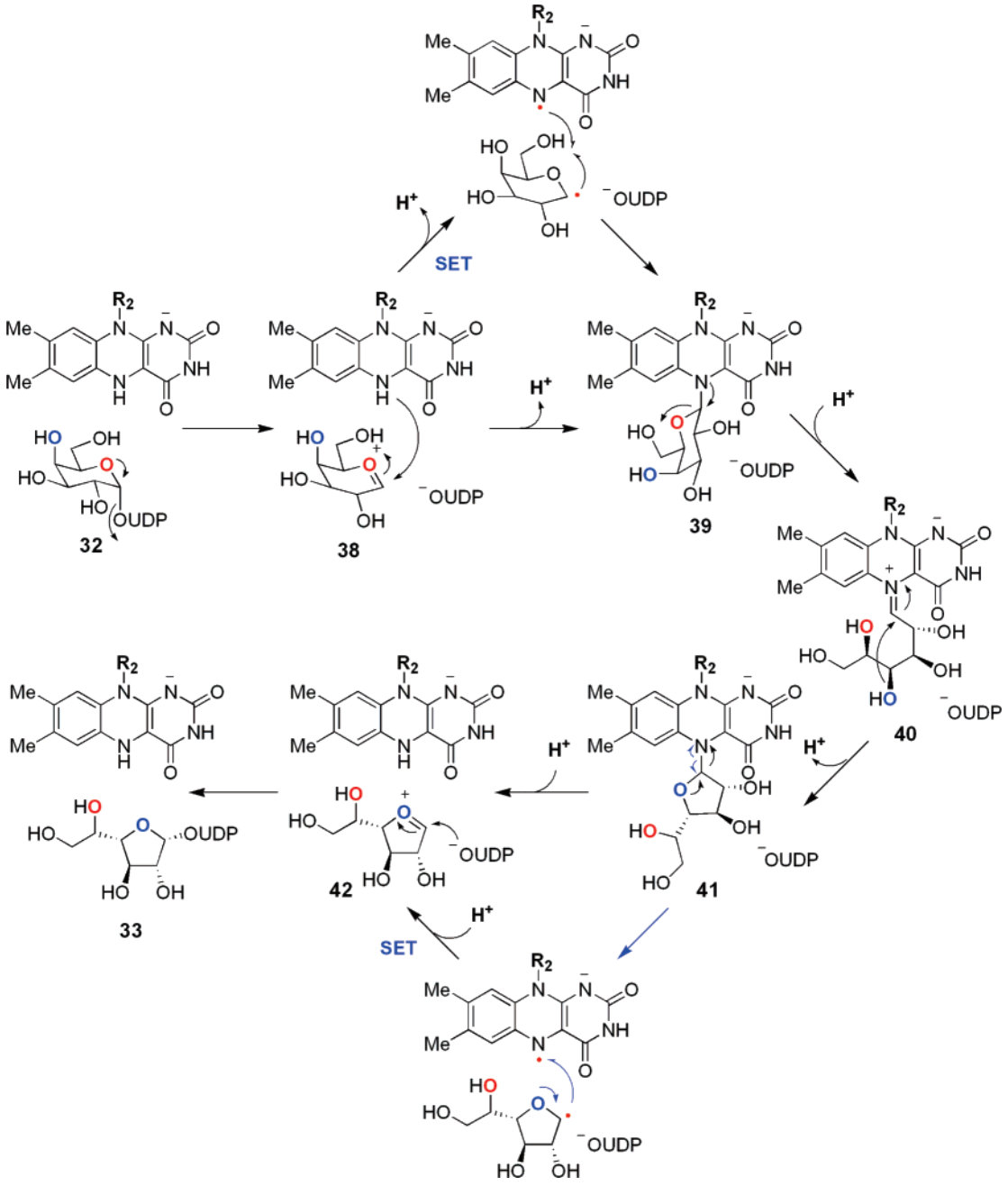
SCHEME 11. Mechanistic Probes Used to Study UGM Catalysis: 1,4-Anhydrogalactopyranose (34), UDP-[2-F]Gal*p* (35), UDP-[3-F]Gal*p* (36), and UDP-galactitol (37)



N5 of FAD_{red} in UGM donates a hydrogen bond to a main chain carbonyl, which may help to increase the ability of the coenzyme to act as a nucleophile/reductant.[136] Molecular modeling, either alone or in conjunction with saturation transfer difference−nuclear magnetic resonance (STD−NMR) spectroscopy, has been used to predict the binding mode of UDP-Gal*p* (**32**) to UGM from the available crystallographic data.[136,137]

On the basis of positional isotope exchange (PIX) experiments, it was determined that the anomeric C−O bond of the substrate is cleaved and re-formed during the course of the reaction.[138] This important finding led to a mechanistic proposal involving a 1,4-anhydrogalactopyranose intermediate (**34**, Scheme 11). However, when authentic 1,4-anhydrogalactopyranose was synthesized and incubated with UGM and UDP, UDP-Gal*p* (**32**) and UDP-Gal*f* (**33**) were not formed as products.[139] Mechanisms involving oxidation of sugar hydroxyl groups at C2 or C3 were effectively ruled out in experiments with 2- and 3-fluorinated UDP-Gal*p*, which are both substrates for UGM, exhibiting k_{cat} and K_m values of 0.033 s^{-1} and 65 μM for UDP-[2-F]Gal*p* (**35**) and 5.7 s^{-1} and 861 μM for UDP-[3-F]Gal*p* (**36**).[129] The particularly slow rate observed with UDP-[2-F]Gal*p* (**35**) may be due to the inductive effects of the fluorine substituent at C2 and is consistent with the formation of oxocarbenium intermediates (such as **38** in Scheme 12) or transition states during turnover. Additional support for an oxocarbenium ion intermediate comes from recent studies using the linear substrate

SCHEME 12. Two Possible Mechanisms of UGM Catalysis[a]



[a] In these mechanisms, FAD plays a role in facilitating the ring contraction reaction through the formation of an N5 adduct. In forming the flavin−substrate adduct, FAD could be acting as a nucleophile (inner path) or as a SET cofactor (outer path).

analogue, UDP-galactitol (UDP-GalOH, **37**).[140] UDP-GalOH, whose UDP moiety can be displaced via S_N2 but not S_N1 mechanisms, was found to be an inhibitor, but not a substrate, of UGM, with a binding affinity similar to that of UDP-Gal*p* (**32**).

On the basis of the above observations, a mechanism for UGM catalysis can be envisioned where a Gal*p* oxocarbenium ion intermediate (**38**) is intercepted by N5 of FAD_{red}, either by direct nucleophilic attack or single-electron transfer (SET) followed by radical−radical recombination (Scheme 12, inner and outer paths, respectively). Formation of the FAD−substrate adduct (**39**) can then facilitate ring opening through the generation of an iminium ion (**40**). Recyclization using the C4 hydroxyl of the galactose moiety leads to the formation of a FAD−product adduct (**41**). Collapse of this adduct leads to the formation of a Gal*f* oxocarbenium ion (**42**), which can recombine with UDP to generate the final product (**33**). However, more research is needed to distinguish between the various mechanistic proposals for UGM catalysis. This knowledge will be essential for the rational design of novel therapeutic agents targeting Gal*f* formation in human pathogens.

Type II Isopentenyl Diphosphate:Dimethylallyl Diphosphate Isomerase: Flavin Playing a Novel Role?

Isoprenoids are a large and structurally diverse class of metabolites that are essential to life.[141−143] Included in this class

SCHEME 13. Chemical Mechanism of IDI-1[a]

[a] This mechanism involves acid/base chemistry and proceeds with formation of a 3°-carbocation intermediate (**44**).

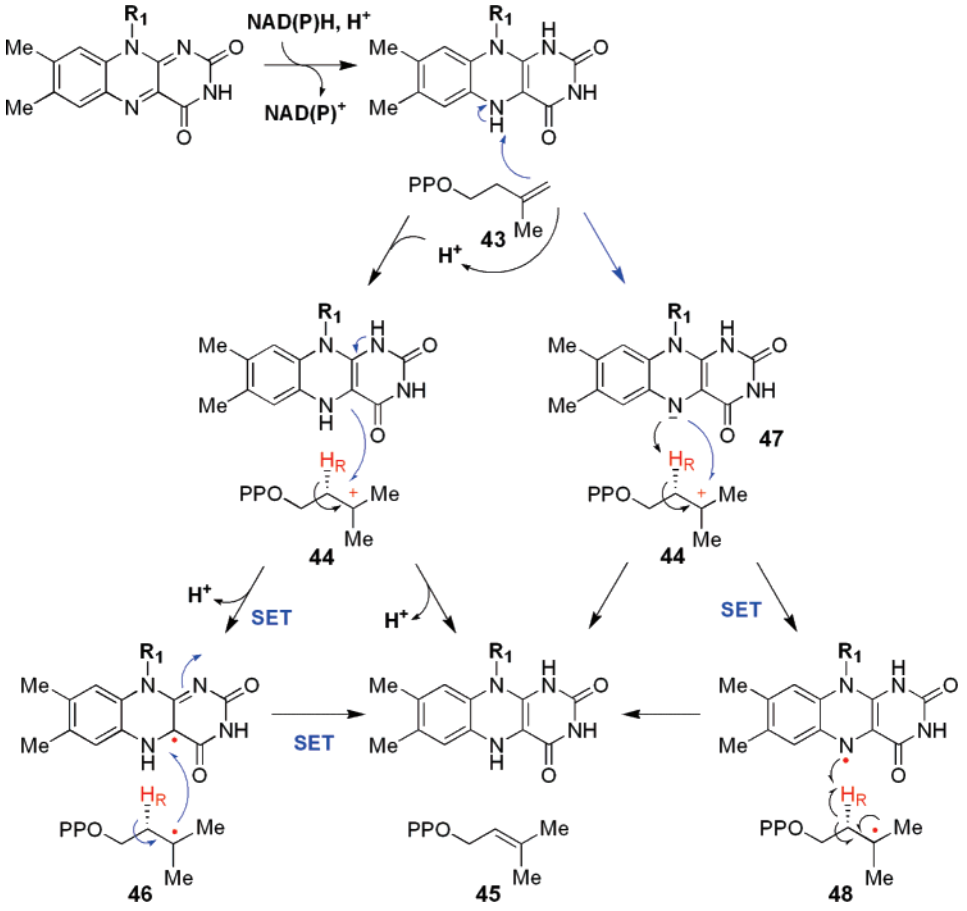
are the sterols, dolichols, carotenoids, ubiquinones, and the lipophilic side chains of prenylated proteins. These compounds are utilized in a variety of ways by living organisms, functioning as hormones, vitamins, visual pigments, pheromones, toxins, and components of cell membranes. All isoprenoids derive from the same five-carbon building blocks, isopentenyl diphosphate (IPP, **43**) and dimethylallyl diphosphate (DMAPP, **45**). Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI) catalyzes the reversible interconversion of IPP and DMAPP. There are two distinct types of IDI. Type I IDI (IDI-1) has been extensively studied and requires two divalent metal ions (Mg^{2+} and Zn^{2+}) for activity.[144] The Mg^{2+} ion promotes substrate binding by coordinating the diphosphate moiety of IPP/DMAPP, while the Zn^{2+} ion binds to a hexacoordinate site in the enzyme that contains an essential glutamate residue. It is believed that this glutamate, in conjunction with a nearby tyrosine residue, acts as a catalytic acid, protonating the double bond of IPP to generate a 3°-carbocation intermediate (**44**). Subsequent removal of the pro-*R* C2 proton of **44** by a conserved cysteine residue completes the isomerization reaction (Scheme 13). In contrast to IDI-1, type II IDI (IDI-2) requires a reduced flavin coenzyme in addition to divalent metal ions.[145,146] Humans rely exclusively on IDI-1 for the synthesis of isoprenoids, while several pathogenic bacteria, including *Staphylococcus aureus*, only possess IDI-2. Because of the distinct evolutionary origins and unique cofactor requirements of the two types of IDIs, IDI-2 represents a promising new drug target.

IDI-2 is a member of the (*S*)-α-hydroxyacid dehydrogenase superfamily, and several crystal structures of IDI-2 enzymes are now available.[147−149] However, mechanistic studies on this enzyme remain sparse, and the role of the flavin coenzyme in catalysis is unknown. During preliminary mechanistic studies conducted in our laboratory, we have shown that the IDI-2−FMN_{ox} complex is reduced with stoichiometric amounts of NAD(P)H via stereospecific transfer of the pro-*S* hydride, and that the resultant IDI-2−FMN_{red} is capable of performing multiple turnovers.[150] Initially, this led to the proposal of a mechanism where, following FMN_{ox} reduction and substrate binding, IPP is protonated by an active-site acid to give a 3°-carbocation (**44**), similar to the reaction catalyzed by IDI-1 (Scheme 14, outer left path). FMN_{red} could then transfer one electron to the intermediate, yielding a neutral FMN_{sq}/substrate radical pair (**46**).[3] Electron transfer back to FMN_{sq} and removal of the pro-*R* proton from C2 by an active-site base could then complete the isomerization and regenerate FMN_{red} for another round of catalysis.[151] From the available crystal structures, which were solved with FMN_{ox} in the absence of substrate, it is not clear what the putative acid/base residues could be, although de Ruyck et al. have suggested that a conserved glutamate residue (E155 in *S. aureus*) could be the base that removes the pro-*R* proton from C2.[149]

In support of a mechanism involving SET, we have found that IDI-2 reconstituted with 1-deazaFMN is active, whereas enzyme reconstituted with 5-deazaFMN has no activity. The inability of 5-deazaFMN to support IDI-2 catalysis was previously demonstrated by Hemmi et al.[145] Using UV/visible difference spectroscopy, we have shown that FMN_{ox} and 5-deazaFMN_{ox} have similar binding affinities for the active site, while 1-deazaFMN_{ox} exhibits slightly weaker binding. These results suggest that the flavin coenzyme does not simply play a structural role in catalysis, as has been previously suggested.[149] Instead, the flavin (and more specifically, N5 of FMN) most likely plays a direct role in chemistry or intermediate stabilization. Interestingly, a neutral flavin semiquinone has been observed in anaerobic stopped-flow assays and IDI-2 thermodynamically stabilizes FMN_{sq} during photoreduction experiments and redox titrations in the presence of substrate.[150,152] Although these results appear to support a mechanism involving SET chemistry mediated by FMN, we have yet to establish the catalytic competence of the neutral FMN_{sq}. Furthermore, the anionic reduced FMN, which is present in the resting state of the enzyme, is rapidly converted to a neutral FMN_{red} upon substrate binding.[152] Protonation of the flavin N1−C2═O locus could be carried out by Lys186 or His149 (*S. aureus* numbering), both of which are conserved and are within hydrogen-bonding distance to either N1 or O2.[147] If IDI-2 employs a mechanism involving electron transfer, the accumulation of a neutral reduced FMN, which is a poorer thermodynamic reductant than the more electron dense anionic FMN_{red}, may indicate that the SET reaction occurs in the inverted Marcus region. As an alternative to electron transfer, it is possible that FMN_{red} serves to electrostatically stabilize the developing substrate carbocation (Scheme 14, inner left path). This would be similar to the putative catalytic role performed by the FAD_{ox} coenzyme of hydroxynitrile lyase.[120]

The behavior of the flavin coenzyme during IDI-2 catalysis bears a striking resemblance to that observed in the reaction catalyzed by chorismate synthase (CS). CS catalyzes the conversion of 5-enolpyruvyl-3-shikimate phosphate (EPSP, **49**) to chorismate (**50**) in the final step of the shikimate biosynthetic pathway. This reaction involves the *anti*-1,4-elimination of the 3-phosphate group and the unactivated C6 pro-*R* proton from EPSP and requires reduced FMN for activity (Scheme 15).[3,153,154] Like the reaction catalyzed by IDI-2, there is no net redox change in the CS-catalyzed reaction. The enzyme binds anionic FMN_{red}, but upon substrate binding, a large conformational change leads to the rapid accumulation of neutral reduced FMN.[155−158] Protonation at the N1−C2═O locus of the coenzyme is likely carried out by a conserved histidine residue.[159,160] Transient kinetic experiments and KIE measurements suggest that the *anti*-1,4-elimination is stepwise in nature, with phosphate elimination preceding C6 deprotonation.[158,161−164] Studies using fluorinated EPSP substrate analogues support the formation of an electron-deficient cationic intermediate (**51**) during turnover.[163] A neutral semiquinone species (**52**) has been observed under certain conditions in various CS reaction mixtures, suggesting that FMN_{red} may transiently reduce this cationic species to an allylic radical intermediate (**53**).[156,165,166] However, the FMN_{sq} has never been observed to form in a catalytically competent manner and has not been detected in single turnover reactions with EPSP.[161] Recently, the crystal structure of CS in complex with FMN_{ox} and EPSP has been solved.[159] EPSP was found to stack on the flavin coenzyme, with N5 of the isoalloxazine ring in close proximity to the pro-*R* hydrogen atom at C6 of EPSP. The lack of any other base in

SCHEME 14. Four Possible Mechanisms of IDI-2 Catalysis[a]



[a] In these mechanisms, FMN plays a role in the isomerization reaction by participating in SET (outer left path), modulating the electrostatic potential of the active site (inner left path), acting as an acid/base catalyst (inner right path), or by sequentially protonating, reducing, and abstracting a hydrogen atom equivalent from the substrate (outer right path).

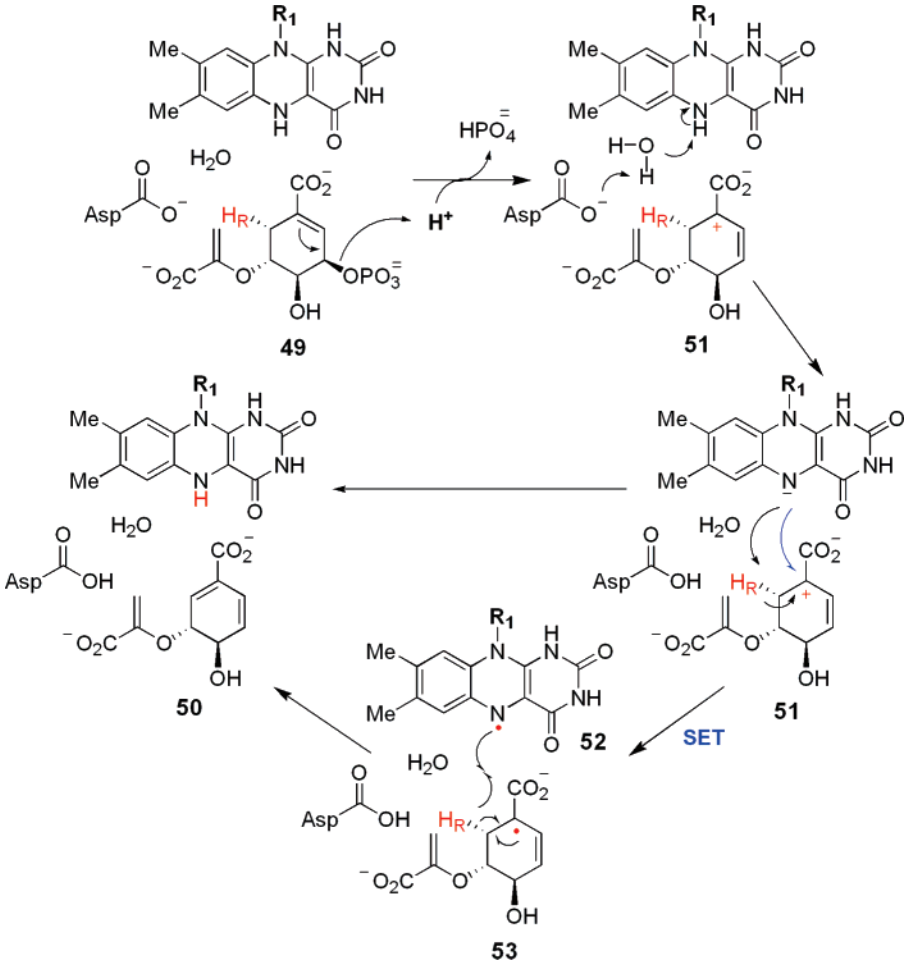
the vicinity of C6 suggests that N5 most likely removes this proton, either by hydrogen atom abstraction from the allylic radical intermediate or by direct deprotonation of the cationic intermediate with the aid of a strictly conserved aspartate residue via an intervening water molecule (Scheme 15, outer and inner paths, respectively).[159,160,167] The essential role for N5 in CS catalysis is also supported by the inactivity of the enzyme reconstituted with reduced 5-deazaFMN.[168]

Given the similarities between the IDI-2 and CS reactions, it is likely the flavin coenzymes act in similar capacities. Therefore, it is possible that the flavin N5 in IDI-2 is directly involved in protonating IPP (**43**), generating the 3°-carbocation intermediate (**44**) and an N5 anionic species (**47**). The N5 anion could then deprotonate C2 directly or the anionic flavin could transfer a single electron to the carbocation **44**, forming a radical pair (**48**) that is converted to products by hydrogen atom transfer from C2 (Scheme 14, inner and outer right paths, respectively). The radical pair (**48**) could also be generated directly from **43** by hydrogen atom transfer from N5 to IPP (**43**) in a concerted proton-coupled electron-transfer (PCET) mechanism. Interestingly, Eguchi and co-workers have recently shown that an epoxide analogue of IPP inactivates IDI-2 from *Methanocaldococcus jannaschii* by forming a covalent flavin N5 adduct.[169] This result clearly shows that the substrate binds in close proximity to the flavin ring and suggests that electron density is capable of accumulating at N5. Though the involvement of a formal reduced N5 anion in acid/base chemistry has never before been demonstrated in flavin-dependent enzyme catalysis, such a species could both electrostatically stabilize a developing substrate carbocation and act as a general base. While the N5 proton of reduced flavin is not typically very acidic, specific active-site geometries and flavin interactions could depress this pK_a to an accessible value, especially if a positively charged intermediate forms in close proximity during the course of the reaction.[170,171] Consistent with this hypothesis, an absolutely conserved threonine residue (T67 in *S. aureus*) was found to form a hydrogen bond with the N5 atom of FMN.[148] The involvement of N5 in acid/base chemistry may also be facilitated by the protonation of the N1−C2=O locus of the anionic reduced flavin that occurs upon substrate binding, as this should help to lower the pK_a of N5. Further mechanistic studies will be needed to verify the role flavin plays in IDI-2 catalysis, but it is clear that this enzyme may employ novel flavin chemistry, with the coenzyme acting as a general acid/base catalyst.

Conclusion

From electron transport and DNA repair to the biosynthesis of primary and secondary metabolites, flavin-dependent enzymes have worked their way into many facets of cellular metabolism. This is undoubtedly a function of their versatile and adaptable chemistry, which has been exploited by many different enzymes

SCHEME 15. Two Possible Mechanisms of CS Catalysis[a]



[a] FMN plays a role in this elimination reaction by acting as an acid/base catalyst (inner path) or by performing a SET and then abstracting a hydrogen atom equivalent from the substrate (outer path).

to carry out a diverse array of reactions. Flavin coenzymes are employed by nature for numerous energetically difficult reactions, such as the oxidation of aliphatic substrates catalyzed by ACDs and the activation of molecular oxygen catalyzed by monooxygenases. Because of their ability to accept and donate either one or two electron equivalents, flavoenzymes are essential mediators between obligate two-electron and one-electron carriers. This is exemplified by E_3 during the biosynthesis of 3,6-dideoxysugars, where two electrons from NADH are relayed through two separate [2Fe-2S] clusters by a FAD coenzyme to the active site of E_1. Similar functions are carried out by flavoenzymes involved in cellular respiration and photosynthesis. In addition to their well-documented roles in redox chemistry, some flavoenzymes, such as CADS, appear to serve only structural roles in enzyme catalysis. Other enzymes, such as UGM, IDI-2, and CS, catalyze rearrangement, isomerization, and elimination reactions, respectively, with no net change in the redox states of the substrates/products and coenzymes. While the mechanisms for these enzymes potentially involve the transient one-electron oxidation of the reduced flavin coenzyme, they could also utilize flavin in polar nucleophilic chemistry (UGM) or perhaps even for acid/base chemistry (CS and IDI-2). The examples highlighted in this Perspective clearly illustrate the diverse chemical and biological roles performed by flavin coenzymes—truly making them one of Nature's most versatile thespians.

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