Expanding the repertoire of flavoenzyme-based biocatalysis

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The flavin tricyclic conjugated isoalloxazine ring system can undoubtedly be recognized as one of the most versatile cofactor types. Flavin-dependent enzymes display a remarkable wide range of chemical reactions which involve both one- and two-electron transfers and even non-redox reactions. Flavoenzymes and their catalogue of reaction types have been exhaustively documented over the past 100 years. Yet, recent discoveries have unveiled new hidden troves of flavoenzymology. The N5-oxide flavin cofactor was shown to be involved in a new mechanism of oxidizing a substrate. The prenylated flavin cofactor was found to be key in catalyzing carboxylation and decarboxylation reactions. Another atypical flavin cofactor is also discussed: the deazaflavin cofactor F$_{420}$. Enzymes utilizing this cofactor show great potential as they display activities that are distinct from flavoproteins that use regular flavins. Furthermore, recent findings on the role of the frequently observed covalent flavin–protein linkages reveal how such covalent interactions allow enzymes to be equipped with special catalytic properties. In addition to providing a way to tune the redox potential through covalent tethering the flavin to a protein, the observed bicovalent linkage allows flavoproteins to evolve towards oxidases that deal with rather bulky substrates by virtue of their exceptionally open active sites. The emerging biocatalytic properties of newly discovered flavoenzymes will open up new possibilities for applications in various fields, including biocatalysis, pharmaceuticals, and biotechnology.

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1.1. Introduction

Since the discovery of flavins about a century ago, flavin-dependent enzymes are recognized as ubiquitous and versatile catalysts, participating in a wide range of substrate reductions and oxidations. These reactions include essential steps in numerous biosynthetic pathways involved in both primary and secondary metabolism in all domains of life. Such a unique and versatile reactivity of flavoenzymes is largely attributed to various thermodynamically and kinetically accessible flavin redox states, i.e., quinone, semiquinone, and dihydroquinone, which position flavin at the crossroad of one- and two-electron transfer chemistry. Of special note is the versatile reactivity of the N5-C4a locus on the canonical flavin isoalloxazine ring system (Walsh 1980; Walsh and Wencewicz 2013). The enormously wide range of flavoenzymes-catalyzed transformations are well established, encompassing amongst others the oxidation of alcohols, aldehydes (Romero and Gadda 2014; Pickl et al. 2015), and amines (Scrutton 2004; Pollegioni et al. 2008), the hydroxylation of (cyclo)alkanes (Franceschini et al. 2012) and aromatic compounds (Sazinsky et al. 2006), the epoxidation of alkenes (Heine et al. 2016), the Baeyer–Villiger oxidations (Bučko et al. 2016), and other oxygenation reactions (Brondani et al. 2016). Astonishingly, in the last few years two new flavin cofactors were discovered, highlighting that there remain unexplored treasure troves for flavoenzymology within this seemingly already mature field (Leys and Scrutton 2016). Most notable is the discovery of the highly modified prenylated-FMN, where the isoalloxazine is expanded with a fourth ring (Figure 1.1). It was shown that such flavin cofactor is key on catalyzing (reversible) decarboxylations in UbiD/Fdc1 enzyme system. Another major find was the discovery of a new FAD derivative: the FAD-N5-oxide. This unanticipated oxygenated flavin assists in the enzymatic oxygenation of the precursor of enterocin, a secondary metabolite, and triggers a rather complex cyclisation reaction (Figure 1.1). The discovery of the abovementioned novel flavins and their respective enzymes has largely expanded the chemical versatility of flavin cofactors, unearthing a broader catalytic diversity for flavoenzymes.

In line with the occurrence of these newly identified flavin species, nature has also evolved other strategies of flavin modifications to enhance or tune the chemical reactivity of flavin-dependent biocatalysts. Recent studies have demonstrated that the naturally occurring covalent flavin–protein linkage can be regarded as a way to equip flavoenzymes with new catalytic properties (Figure 1.1). The understanding on the role and mechanism of covalent flavin attachment has increased within these years, opening up the possibility to enhance the oxidative power of (bi)covalent flavin-dependent oxidases in a tailored manner for synthetic applications. Another example of drastic modifications of the flavin cofactor in nature is the occurrence of the 5-deazaflavin F$_{420}$ (Figure 1.1). Genome sequence analyses have revealed that this obligate hydride-transfer cofactor is commonly used in various microorganisms. The growing number of discovered F$_{420}$-dependent enzymes may develop as a valuable toolbox for organic chemists to perform selective reductions that require a unique low redox potential. In this chapter, we provide an overview of the biocatalytic potential of newly discovered flavoenzymes and how they may broaden the diversity of flavoenzyme-based biocatalysis.
Figure 1.1: Overview of naturally occurring flavin modifications. The flavin can covalently attach to the polypeptide chain (in green) to form mono- or bicovalent flavin–protein linkages (in red). The common covalent flavinylation sites are indicated with red arrows. Another newly found flavin carries an N5-oxide (FAD-N5-oxide) and is responsible for the 4-electron oxidation catalyzed by EncM. Alternatively, the flavin can be prenylated at the N5 and C6 (in purple), forming the so-called prenylated-FMN, which is used by (de)carboxylases. In 5-deazaflavins, the N5 is substituted by a carbon, resulting in distinct physico-chemical properties. \( R = \text{H (FMN), AMP (FAD)}; R_1 = \text{ribityl–ADP}; R_2 = \text{ribitylphosphate}; R_3 = \text{ribityl–lactyl–oligo(γ-L-glutamyl)} \).

1.2. Modulating biocatalytic properties of flavoenzymes by covalent flavin attachment

Although the vast majority of flavoproteins contain a tightly but non-covalently bound flavin, it was estimated that the prevalence of covalent attachment \textit{in vivo} is relatively high, accounting for 10% of all flavoproteins (Heuts et al. 2009). Several types of covalent flavin–protein linkages have been discovered so far, including \( 8\alpha-N^3\)-histidyl–FAD/FMN, \( 8\alpha-N^1\)-histidyl–FAD/FMN, \( 8\alpha-S\)-cysteinyl–FAD/FMN, \( 8\alpha-O\)-tyrosyl–FAD, \( 8\alpha-O\)-aspartyl–FAD, \( 6-S\)-cysteinyl–FMN, \( 8\alpha-N^1\)-histidyl–6-S-cysteinyl–FAD/FMN, and phosphoester-threonyl–FMN (Figure 1.2) (Heuts et al. 2009; Mewies et al. 1998; Starbird et al. 2015). Early experimental evidence for the existence of covalent flavoproteins dates back to the 50s with the work on succinate dehydrogenase (Kearney and Singer 1955), which was later revealed to feature an \( 8\alpha-N^3\)-histidyl–FAD (Walker et al. 1972). To date, the histidyl–FAD represents the most abundant covalent flavin linkage type, found in a large number of enzymes. Such covalent tethering is especially common in flavoenzymes that belong to the vanillyl alcohol oxidase (VAO) family of flavo-
Flavoenzyme-based biocatalysis

Figure 1.2: Chemical structure of riboflavin and amino acid sidechains involved in covalent flavin–protein linkages. Potential positions of the covalent tethering are depicted in red. R = ribityl–ADP.

proteins (Leferink et al. 2008). Less widespread are the cysteinyl–bound flavins cofactors. Well-known examples of cysteinyl flavoproteins are the extensively studied monoamine oxidase (Edmondson et al. 2004), monomeric sarcosine oxidase (Hassan-Abdallah et al. 2005), and trimethylamine dehydrogenase (Scrutton et al. 1994). The tyrosyl–FAD, aspartyl–FAD, and threonyl–FMN bonds have been observed in some isolated cases: p-cresol methyl hydroxylase (PCMH) (Mathews et al. 1991), chloramphenicol halogenase (Podzelinska et al. 2010), and the NprB/C subunits of a Na+–translocating NADH:quinone reductase (Hayashi et al. 2001; Steuber et al. 2014), respectively.

1.2.1. Importance of flavin covalent formation

With a number of new flavoprotein structures solved and information from site-directed mutagenesis studies, the role of flavinylation has become more clear in recent years. Several lines of evidence have shown that covalent incorporating of the flavin into the polypeptide chain can significantly raise the redox potential of the respective flavoprotein. Such effect can also be seen when comparing redox potentials determined for non-covalent, mono-covalent, and bi-covalent flavoproteins (Heuts et al. 2009). An increased redox potential renders flavoenzymes more efficient in oxidizing substrates by virtue of raising the thermodynamic driving force of the chemical reactions catalyzed. For instance, VAO harbors an 8α-N3-histidyl–FAD linkage and displays an unusually high midpoint potential of +55 mV (Fraaije et al. 1999). Upon removal of the histidine involved in the covalent attachment, the enzyme midpoint potential drastically dropped to −55 mV, associated with a tightly bound but noncovalent FAD. This decrease in potential resulted in an ~10-fold lower in substrate turnover, although the enzyme active site geometry and its mechanism remained unchanged (Fraaije et al. 1999).
The resultant higher redox potential in turn narrows down the range of available electron acceptors, leaving in most cases molecular oxygen as the solely suitable candidate. As a consequence, most covalent flavoproteins were found to be oxidases, whereas the noncovalent counterparts are often dehydrogenases/reductases (Heuts et al. 2009).

Enhancing structural integrity and retention of the flavin cofactor
Another crucial role of covalent flavinylation seems to be assisting in protein folding. A large body of work has demonstrated that, in several flavoenzymes, disruption of the covalent flavin–protein bond resulted in formation of misfolded apoproteins. For instance, expression of alditol oxidase from *Streptomyces coelicolor* in which the FAD-linking histidine (His46) was replaced by an alanine resulted in formation of inclusion bodies (Heuts et al. 2007). Intriguingly, covalent flavinylation was suggested to be a strategy to outcompete unproductive binding of other competitive ligands. This was particularly compelling in the case of bacterial putrescine oxidase (PuO) of which the apo form has equal affinities to both FAD and ADP, the latter affording a non-functional holoenzyme (van Hellemont et al. 2008). Replacing Ala394 in PuO—corresponding to the FAD-linking cysteine in human monoamine oxidase B, a close homolog of PuO—by a cysteine indeed afforded the formation of a covalent FAD–protein linkage. This process is totally self-catalytic and does not induce any significant conformational alterations; albeit the extent of FAD covalent incorporation critically depends on a proper flavin microenvironment (Kopacz et al. 2011).

Bicovalent flavoproteins have evolved to accept bulky compounds as substrates
The recently discovered bicovalent flavoproteins appear to be an attractive group of enzymes for biocatalytic applications as they catalyze the oxidation of a wide range of bulky substrates such as oligosaccharides, alkaloids, and antibiotics (Huang et al. 2005; Winkler et al. 2008; Alexeev et al. 2007). This feature may be attributed to the fact that the two covalent bonds (8α-N-histidyl & 6-S-cysteinyl) allow tight anchoring of the flavin in a catalytically competent position in a relatively open active site. The first and prototypic structure described for flavoproteins with a bicovalent FAD linkage is glucooligosaccharide oxidase (GOOX) from the fungus *Acremonium strictum* (Huang et al. 2005). GOOX harbors a strikingly open carbohydrate-binding groove, allowing bulky oligosaccharide substrates to enter. Such open active site geometry was also observed in other bicovalent flavoprotein structures reported later (Alexeev et al. 2007; Ferrari et al. 2016). In GOOX, the flavin is tethered to the polypeptide chain via the C6 atom and the 8α methyl group of the isoalloxazine chromophore with Cys130 and His70, respectively (Figures 1.1 and 1.2). Flavinylation was shown to occur independently at these two residues, and both covalent linkages are crucial for proper cofactor binding, substrate binding as well as maintaining the structural stability (Huang et al. 2008). GOOX represents attractive for analytical biochemistry applications in the food industry as it is one of the few enzymes with high selectivity for oligosaccharides. GOOX oxidizes the reducing ends of di- and oligosaccharides such as maltose, cellobiose, and lactose. Maltooligosaccharides up to seven units are also readily accepted by GOOX (Fan et al. 2000;
Figure 1.3: Mechanism for covalent flavin attachment at the 8α position in the isoalloxazine ring via an quinoneimine methide intermediate. It is proposed that specific residues act as bases (blue) whereas L (red) represents the linking residue that is covalently attached to the flavin (His, Tyr, Cys, or Asp). R = ribityl–ADP.

Lee et al. 2005). The best GOOX substrates as determined by steady-state kinetics are cellotriose ($k_{\text{cat}} = 13 \text{ s}^{-1}, K_m = 26 \mu\text{M}$) and celllobiose ($k_{\text{cat}} = 5.0 \text{ s}^{-1}, K_m = 48 \mu\text{M}$). Recent studies have also shown that by structure-inspired mutagenesis, the oligosaccharide preference of GOOX and sequence-related oxidases can be tuned towards specific mono- or oligosaccharides (Ferrari et al. 2015). Other flavoproteinoxidases acting on oligosaccharides are lactose oxidase, celllobiose dehydrogenase, chitooligosaccharide oxidase, and xylooligosaccharide oxidase (Ferrari et al. 2016; van Hellemond et al. 2006; Lin et al. 1991; Heuts et al. 2007).

1.2.2. Mechanisms of covalent flavin attachment

Numerous structural and biochemical data in the last two decades has unveiled the role and mechanisms of covalent flavinylation. Until lately, most evidence indicated that formation of the covalent bond between the polypeptide chain and flavin is a fully autocatalytic, post-translational process (Hassan-Abdallah et al. 2005; Jin et al. 2008). The proposed mechanism for covalent flavinylation at the 8α carbon in the isoalloxazine ring involves an attack of the imino quinone methide by nucleophilic amino acid residues (Figure 1.3) (Walsh 1980). Intriguingly, more recent data suggest a role for proteins that assist in flavin covalent bond formation. Studies on the eukaryotic succinate dehydrogenase has identified a small protein (~10 kDa), SdhAF2, acting as an additional assembly factor crucial for formation of the covalent flavin–protein bond (Hao et al. 2009). The SdhAF2 bacterial counterpart, SdhE, has also been characterized in detail (McNeil et al. 2012; McNeil and Fineran 2013; McNeil et al. 2014). These ancillary proteins are believed to either stabilize a conformation of the flavoproteins ready for the flavinylation process, or harboring catalytic residues directly
involved in covalent flavinylation. A similar mechanism has been proposed for
the formation of the 8α-O-tyrosyl-FAD linkage in PCMH (Kim et al. 1995; Cun-
nane et al. 2005) and the Oγ-threonyl-phosphoester-FMN bond in both the Na+-
translocating NADH:quinone reductase and urocanate reductase (Bertsova et al.
2013).

Altogether, in recent years, various aspects concerning the role and mechanism
of covalent flavinylation have been unraveled (Heuts et al. 2009; Mewies et al.
1998; Starbird et al. 2015). The recent discoveries of novel flavin linkages via two
amino acid residues and threonyl-FMN suggest that nature has evolved a wide
variety of cofactor–protein crosslinks of which some may await discovery. With
new insights into the mechanism of covalent attachment, structure-based redesign
of covalent linkage has become increasingly more reachable, enabling elegant fine-
tuning flavoenzymes into tailored powerful oxidative biocatalysts (Kopacz et al.

1.3. The newly found FAD-N5-oxide cofactor in EncM
and DszA

EncM is a remarkable internal monooxygenase that catalyzes a 4-electron ox-
idation reaction involved in a rare Favorskii-type carbon-carbon rearrange-
ment. This reaction is essential for generating the structural tricyclic scaffold
of enterocin produced by Streptomyces maritimus (Figure 1.4). Thorough bio-
chemical and structural studies of EncM’s mechanism led to the discovery of an
unprecedented flavin-N5-oxide species (FlN$_{5}$[O]) that is responsible for the EncM-
catalyzed oxygenation reaction. The N5-oxide species is proposed to take part
in the dual oxidation—a hydroxylation and a dehydrogenation—at the C4 of the
octaketide enterocin precursor followed by a Favorskii-type rearrangement, af-
fording the desmethyl-5-deoxyenterotocin intermediate (Teufel et al. 2013; Teufel
et al. 2016). In EncM, the proposed FlN$_{5}$[O] formation is the result of the reaction
between the fully reduced flavin (Fl$_{\text{red}}$) and molecular oxygen. This reaction ini-
tially generates the anionic red semiquinone (SQ) and a protonated superoxide,
which allows a radical coupling at N5, yielding a transient flavin-N5-peroxide
species. The resultant N5-peroxide undergoes water elimination affording the
EncM–FlN$_{5}$[O] (Teufel et al. 2015). EncM exploits its substrate as an electron
donor and is therefore independent of external reductants, e.g., NADP(H) as for
most canonical flavin oxygenases. As a result, EncM can be classified as member
of the unusual “internal monooxygenases.” Interestingly, NAD(P)H, however,
can participate in an auxiliary route to efficiently restore the N5-oxide species
in the case when the labile flavin-N5-peroxide intermediate eliminates H$_2$O$_2$
instead of water, thus generating Fl$_{\text{ox}}$ instead of FlN$_{5}$[O] (and thereby rendering
the enzyme inactive).

The reactivity of EncM with oxygen is modulated by a confined substrate
binding tunnel which is complementary to the elongated natural polyketide sub-
strate (Teufel et al. 2013; Teufel et al. 2015). Upon product release, oxygen can
enter the active site and react with Fl$_{\text{red}}$ to regenerate the FlN$_{5}$[O]. A distinctive
characteristic of the FlN$_{5}$[O] species is its remarkable stability within the EncM
Flavoenzyme-based biocatalysis

Figure 1.4: Proposed mechanism for the EncM-catalyzed dual oxidation of the polyketide enterocin precursor and formation of the FAD-N5-oxide species. The flavin cofactor is covalently attached to His78 of EncM via its C8α atom. For clarity, the EncM-mediated Favorovskii-type rearrangement and subsequent steps in the biosynthetic pathway of enterocin are not shown.

active site in the absence of reducing agents. This is in contrast to the short-lived C4a-peroxyflavin intermediate in the more common flavoprotein monooxygenases.

In addition to EncM, another flavoenzyme, DszA (Adak and Begley 2016) was shown to utilize a FMN-N5-oxide (in the oxygenating a sulfur-containing aromatic compound). As DszA is using a FMN instead of a FAD cofactor and it is sequence-unrelated to EncM, flavin-N5-oxides may be more commonly used than literature suggests. Other Fl\(_{N5}[O]\)-utilizing enzymes may have been overlooked in the past due to its close spectral feature with that of Fl\(_{ox}\). Considering the chemical versatility of this stable, superoxidized flavin cofactor, it is expected that additional Fl\(_{N5}[O]\)-utilizing enzymes will be discovered in the near future (Teufel et al. 2016).

1.4. Flavoenzyme (de)carboxylases utilize a prenylated-FMN—a novel cofactor with a fourth ring added by flavin prenyltranferases

Decarboxylases play an important role in catalysis affording alcohols, amines, terminal olefins, carboxylic acids, and other important chemicals under mild reaction conditions (Kourist et al. 2014). For instance, styrene is enzymatically obtained from the α,β-unsaturated acid decarboxylation catalyzed by a pair of enzymes in yeast: Fdc1 (ferrulic acid decarboxylase) and Pad1 (phenylacrylic acid decarboxylase) (Figure 1.5a) (Mukai et al. 2010; Lin et al. 2015). Their bacterial counterparts UbiD and UbiX (homologs of Fdc1 and
Pad1, respectively) were shown to be involved in the non-oxidative decarboxylation of 3-polyisoprenyl-4-hydroxybenzoate, an intermediate in the prokaryotic ubiquinone biosynthetic pathway (Aussel et al. 2014; Gulmezian et al. 2007; Liu and Liu 2006). It has been recently discovered that the decarboxylase activity of Fdc1/UbiD requires an unexpected prenylated-FMN cofactor (prFMN), which is generated via the functionally associated enzymes Pad1/UbiX (Clarke and Allan 2015; Payne et al. 2015; White et al. 2015). The latter enzymes modify FMN by covalently linking the isoprenyl group of dimethylallyl monophosphate (DMAP) to both the N5 and C6 of the isovaloxazine ring via an unusual $sp^3$ N5-prenyl intermediate, resulting in the formation of a fourth non-aromatic ring (Figure 1.5a). The obtained prFMNH$_2$ species then undergoes an oxidative maturation in UbiD to generate the active form—an azomethine ylide-like flavin—containing an N5-prenyl C1’ iminium group. Detailed mechanism of the oxygen-dependent maturation of prFMNH$_2$ moiety leading to the formation of the N5-imidinium species remains to be elucidated. When supplied with DMAP, UbiX was shown to be able to prenylate oxidized FMN into prFMNH$_2$, enabling Fdc1 to decarboxylate cinnamic acid in vitro in the absence of UbiX (White et al. 2015). Interestingly, DMAP is a non-canonical prenyl donor, as the common source of prenyl groups is dimethylallyl diphosphate. The metabolic origin of DMAP and its role in other physiological reactions remains largely obscure; one plausible source could be the interconversion from the isomer isopentenyl monophosphate generated in certain archaea (Clarke and Allan 2015; Vinokur et al. 2015).

The UbiD-mediated decarboxylation reaction was proposed to occur via a 1,3-dipolar cycloaddition in which the matured prFMN species (an azomethine ylide) and the $\alpha,\beta$-unsaturated carboxylic acid (a dipolarophile) react, generating a transient covalent substrate–prFMN pyrrolidine adduct (Figure 1.5b). The latter was proposed to undergo a Grob-type fragmentation concomitantly with the breakage of the C$\beta$–prFMN C4a bond, leaving the two species connected by a single bond (between the substrate C$\alpha$ and the prFMN-C1’) (Payne et al. 2015). Protonation of the substrate C$\alpha$ by Glu282 (Lan and Chen 2016) was proposed to take place, coupled with the formation of a second pyrrolidine species before product release via a retro 1,3-dipolar cycloaddition.

UbiX is the first known enzyme shown to convert FMN leading to the isovaloxazine ring expansion (Leys and Scrutton 2016). On the other hand, BluB—or flavin deconstructase, a known enzyme using FMN as substrate—catalyzes the fragmentation of FMN into 5,6-dimethylbenzimidazole required for vitamin B$_{12}$ biosynthesis (Yu et al. 2012). The prenylation followed by oxidative maturation of the isovaloxazine ring catalyzed by UbiX/Pad1 proved to be an extreme case for the versatility in flavin chemistry (Walsh and Wencwicz 2013). The azomethine ylide dipole property of the prFMN species enables members of the Fdc1/UbiD enzyme family to decarboxylate or carboxylate a wide range of (aromatic) substrates that could be attractive for organic chemists. Obviously, prFMN may well be utilized by other enzymes that await forthcoming explorations.
1.5. Deazaflavins—resurrection of an old, long-neglected cofactor

$F_{420}$ is a 7,8-didemethyl-8-hydroxy-5-deazaisoalloxazine chromophore first identified in *Methanobacterium bryantii* (Figure 1.6, Cheeseman et al. 1972). Its name was coined after the specific visible absorption maximum at 420 nm. Two biologically relevant cofactor variants are 7,8-didemethyl-8-hydroxy-5-deazrriboflavins ($F_0$ or FO, equivalent to riboflavin), and its lactyl oligoglutamate phosphodiester derivative $F_{420}$. The seemingly little modifications on the isoalloxazine ring when compared with flavins (i.e., a substitution of N5 by a carbon, a lack of the 7- and 8-methyl groups, and the addition of an 8-hydroxy group) render deazaflavin’s physicochemical properties remarkably different from that of common flavins. Whereas flavins can mediate one or two electron redox chemistry and react with molecular oxygen, deazaflavins participate exclusively in hydride transfer and react very slowly with O$_2$. Therefore, deazaflavins appear to be more analogous to nicotinamide cofactors and can be regarded as “a nicotinamide in a flavin’s clothing” (Walsh 1980). Moreover, deazaflavins display a much lower redox potential (−340 mV) than that of flavins (−220 mV) and even nicotinamides (−320 mV), thus represent one of the lowest-potential redox cofactors in biological systems (de Poorter et al. 2005; Jacobson and Walsh 1984).

Originally, $F_{420}$ was thought to be narrowly distributed in archaea and a few select actinomycetes. Nevertheless, comparative genomic analysis and numerous biochemical data in recent years strongly suggest that the cofactor is much more widespread than previously believed (Ahmed et al. 2015; Greening et al. 2016). The atypical flavin cofactor is predicted to be present in 11% of all sequenced bacteria and archaea (Selengut and Haft 2010). Despite the early characterization of $F_{420}$ in the 70s of the past century in Wolfe’s laboratory (Cheeseman et al. 1972), $F_{420}$-dependent enzymes have been mostly studied by a few dedicated research groups working on archaea, perhaps partly due to the cofactor’s commercial unavailability. However, the last decade has witnessed an immense surge in $F_{420}$’s interest following the discovery of the novel antitubercular prodrugs that require an *in vivo* reductive activation by a dedicated $F_{420}H_2$-dependent reductase in *Mycobacterium tuberculosis* (Mtb) (Taylor et al. 2013). These bicyclic nitroimidazoles, delamanid (OPC-67683) (Matsumoto et al. 2006) and pretomanid (PA-824) (Stover et al. 2000), are highly promising antitubercular agents as they not only act on actively replicating bacteria but also kill non-replicating bacilli. These dormant microbes are responsible for a prolonged treatment period and causative agents of latent tuberculosis infection which is recalcitrant to conventional chemotherapeutics (Nathan 2008). Importantly, they exhibit no cross-resistance with drugs currently used due to their novel mechanism of action. Delamanid was approved for multidrug-resistant tuberculosis in 2013, whereas pretomanid has entered phase III clinical trial. The reductive activation of these nitroimidazole prodrugs was mainly investigated with pretomanid (Figure 1.6). A hydride from $F_{420}H_2$ (regenerated by an $F_{420}$-dependent glucose-6-phosphate dehydrogenase—FGD) is transferred to the nitroimidazole mediated by Rv3547, a deazaflavin-dependent nitroreductase (Ddn). This yields an unstable intermediate which quickly decomposes into three major metabolites, of which des-nitro...
Figure 1.5: a) Schematic view of the proposed UbiX/Pad1 reaction generating the prenylated-FMN species and the decarboxylation catalyzed by UbiD/UbiX or Fdc1/Pad1. Atoms derived from dimethylallyl monophosphate (DMAP) are depicted in red. $R_1 = \text{ribitylphosphate}, R_2 = \text{polyprenyl}$. b) Proposed mechanism for the (de)carboxylation of cinnamic acid catalyzed by prFMN-containing UbiD. The reaction entails a 1,3-dipolar cycloaddition leading to the pyrrolidine ring formation followed by a decarboxylation.
PA-824 is the predominant one (Stover et al. 2000; Manjunatha et al. 2006; Singh et al. 2008). During the decomposition, nitrous acid is eliminated and rapidly disproportionates, giving rise to nitric oxide and other reactive nitrogen species. The level of NO release was shown to highly correlate with anaerobic bactericidal activity, presumably via reacting with cytochromes and cytochrome c oxidase thus leading to respiratory poisoning (Singh et al. 2008). Besides, transcriptomic analysis suggests that the drugs may also disrupt cell wall biosynthesis, possibly by inhibiting an F_{420}\textsuperscript{b}-dependent hydroxymycolic acid dehydrogenase (Matsumoto et al. 2006; Manjunatha et al. 2009; Purwantini and Mukhopadhyay 2013). Interestingly, there may exist other F_{420}\textsuperscript{b}-dependent reductases that activate this class of prodrugs. For example, the compound CGI-17341 (the original lead bicyclic nitroimidazole, which was discontinued due to side effects) appeared to be reduced by other Ddn homolog(s) rather than Rv3547 (Manjunatha et al. 2006). This opens up the possibility for developing next-generation nitroimida-zoles whose activation depend on various F_{420}\textsuperscript{b}H\textsubscript{2}-dependent reductases and thus minimize the cross-resistance propensity (Greening et al. 2016).

With a unique low redox potential of the reduced deazaflavin cofactor, F_{420}\textsuperscript{b}H\textsubscript{2}-dependent reductases are capable of catalyzing the hydrogenation of a wide range of organic compounds which are otherwise recalcitrant to reductive activation.
such as enones (Taylor et al. 2010; Lapalikar et al. 2012b; Lapalikar et al. 2012a) and imines (Coats et al. 1989; Li et al. 2009a; Li et al. 2009b) in various heterocycles (Schrittwieser et al. 2015). One intimidating obstacle for large-scale applications of \( \text{F}_{420}\text{H}_2\)-reductases may lie heavily in the accessibility of this (to date) commercially unavailable cofactor and its regeneration machinery thereof. However, established \( \text{F}_{420}\) isolation methods from the soil bacterium *Mycobacterium smegmatis* (Isabelle et al. 2002) or an \( \text{F}_{420}\)-overproducing variant (Bashiri et al. 2010), together with a robust FGD system (Nguyen et al. 2016) have become recently available. With these tools in hand, \( \text{F}_{420}\text{H}_2\)-enzymes may develop into industrially applied biocatalysts (Greening et al. 2016; Taylor et al. 2013; Ney et al. 2016).

1.6. Concluding remarks and future perspectives

Flavoproteins are arguably the most well-studied redox enzymes ever since the identification of the flavin cofactor over 100 years ago. Yet, novel discoveries and flavoenzyme-based applications have continued to emerge. The astonishing versatility of flavin chemistry is exemplified by the characterization of the previously unknown species prenylated-FMN and flavin-N5-oxide. The physicochemical properties of these species together with the corresponding in vivo reactions catalyzed, remain to be fully tapped and underpinned. In parallel, flavoenzyme engineering by cofactor redesign to enhance the oxidative power by virtue of elevating the redox potential has proven to be feasible in the last few years. Modulating the reactivity and structural stability of flavin-dependent oxidases can be accomplished by introducing the (bi)-covalent attachment to the polypeptide chains. This strategy has been largely facilitated by the increasing availability of structural and biochemical data that deciphered the precise modes of covalent flavinylation. The newly discovered bicovalent flavoenzymes are particularly attractive for biotechnological purposes as they accept a wide range of surprisingly bulky substrates, such as oligosaccharides, alkaloids, and antibiotics. Alternatively, discovery of novel biocatalysts can be achieved by resurrecting old, yet long-neglected cofactors, of which the deazaflavin cofactor \( \text{F}_{420}\) is an example. With an interesting redox profile, \( \text{F}_{420}\text{H}_2\)-dependent reductases are believed to be potentially useful for asymmetric synthesis, as they complement those employing the nicotinamide cofactors. Taken together, these findings suggest that the well-established flavoenzymology field can still await major surprises, and feed new exciting applications in pharmaceuticals, biocatalysis, and bioremediation.

1.7. Aim and outline of the thesis

This thesis describes studies that aimed at discovery, structural analyses, and biocatalytic evaluations of several newly found (deaza)flavoenzymes. The enzymes were identified by genome mining approaches and the obtained results nicely illustrate the intimate blending between enzymology and structural biology.

In the first part of this thesis (chapters 2 and 3), two robust flavin-containing oxidases are described. Chapter 2 reports on eugenol oxidase (EUGO) from *Rhodococcus jostii* RHA1, belonging to the vanillyl alcohol oxidase (VAO) flavo-
protein superfamily. In fact, EUGO is highly similar to the prototypical member of this enzyme family, VAO, and also shares the way by which the FAD cofactor is bound: covalently tethered to a histidine. Substrate profiling of EUGO revealed that, besides the oxidation of eugenol and vanillyl alcohol, EUGO can efficiently perform the dehydrogenation of various α,β-unsaturated ketones and the selective oxidation of a racemic secondary alcohol. Crystal structures of the enzyme in complexes with isoeugenol, coniferyl alcohol, vanillin, and benzoate have been determined at 1.7–2.6 Å resolution. Chapter 3 describes the expression and structure analysis of another flavoprotein oxidase, an alcohol oxidase (also known as methanol oxidase) from the white-rot basidiomycete Phanerochaete chrysosporium (PcAOX). PcAOX belongs to the glucose–methanol–choline (GMC) oxidoreductase superfamily and contains a noncovalently but tightly bound FAD cofactor. The work has resulted in: 1) the development of the first efficient AOX expression system using *Escherichia coli* as a host (rather than yeast), and 2) the second AOX crystal structure. The AOX crystal structure has revealed the molecular basis for the substrate acceptance profile of this newly discovered flavoprotein alcohol oxidase.

The second part of the thesis (chapters 4 and 5) demonstrates that novel bacterial deazaflavin-dependent enzymes can be identified by genome mining, confirming the presence of this rare cofactor in these bacteria. Chapter 4 describes the expression, characterization, and structure of a robust F₄₂₀-dependent glucose-6-phosphate dehydrogenase (Rh-FGD1) from *R. jostti* RHA1. Rh-FGD1 represents an attractive alternative for the poorly expressed FGD from *Mycobacterium tuberculosis* that is currently used by others for producing reduced F₄₂₀. In chapter 5, a thermostable F₄₂₀:NADPH oxidoreductase (Tfu-FNO) from the mesophilic bacterium Thermobifida fusca is reported. Based on the three-dimensional structure of the enzyme in complex with NADP⁺, residues in direct contact with the 2′-phosphate group this cofactor were selected for targeted mutagenesis. This confirmed the role of some of these residues in recognizing the nicotinamide coenzymes and several mutants possessing an improved performance with NAD(H), a cheaper cosubstrate than NADP(H). This newly found enzyme may develop into a handy tool to (re)generate F₄₂₀H₂ for biocatalytic purposes.

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