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Exploring deazaflavoenzymes as biocatalysts

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Introduction

Hemant Kumar and Marco W. Fraaije

1.1. Flavins and deazaflavins

The role of vitamins in human health was first realized over a century ago. It was found that the deficiency of these essential small molecules, which were required in trace amounts, caused many diseases. The term vitamin, composed of the terms 'vita' (in Latin, *vita* = life) and 'amine', was coined in 1912 by Casimir Funk (Funk 1911). As it was later realized that vitamins are not always amines, the 'e' was omitted. Since then, almost one century long research on vitamins has significantly contributed towards our understanding of human health and disease.

Like other vitamins, the role of riboflavin (vitamin B2) has been very well studied over the last few decades. Riboflavin consists of a tricyclic isoalloxazine ring with a ribityl tail attached at position N10 (Figure 1A). The isoalloxazine moiety provides riboflavin an intense yellow color (in Latin, *flavus* = yellow). In fact, it is also used as colorant in food products. Riboflavin serves as a precursor of the flavin cofactors that are generated and used in cells for specific enzymes, the so-called flavoproteins. There are two ubiquitous riboflavin-based flavin cofactors: (1) flavin mononucleotide (FMN), which is the 5'−OH phosphorylated product of riboflavin, and (2) flavin adenine dinucleotide (FAD), which is the result of the condensation of FMN with adenosine monophosphate (AMP). FAD and FMN generally form a non-dissociable (covalently as well as non-covalently attached) part of a flavoprotein hence serving as prosthetic groups. Flavin cofactors equip enzymes with redox functionalities making them versatile biocatalysts. Flavoenzymes are found in many different enzyme classes, including e.g. dehydrogenases, oxidases, monooxygenases, reductases, and halogenases. Due to their catalytic versatility the range of processes in which flavoproteins play a crucial role is astounding and includes photoreception, light production, electron transfer pathways and degradation of xenobiotics.

Except for the flavin cofactors mentioned above, there is another natural cofactor that shows quite some resemblance: cofactor F_{420} (Figure 1B). F_{420} is a so-called deazaflavin cofactor found in certain groups of microorganisms. It was first discovered and isolated in 1972 by Cheesman et al from a methanogen (Cheesman et al. 1972) in which it plays a pivotal role in methane metabolism. For a long time, it was considered a very rare cofactor that only occurs in specific archaea. Later, it was also found to exist in various actinobacteria (Daniels et al. 1985) and recent genome sequence analyses have revealed that it also plays a role in other bacteria (Selengut and Haft 2010), such as cyanobacteria and some members of betaproteobacteria (Li et al. 2014; Ney et al. 2017). Its widespread occurrence in bacteria and archaea suggests that it fulfils important roles in these microbes.

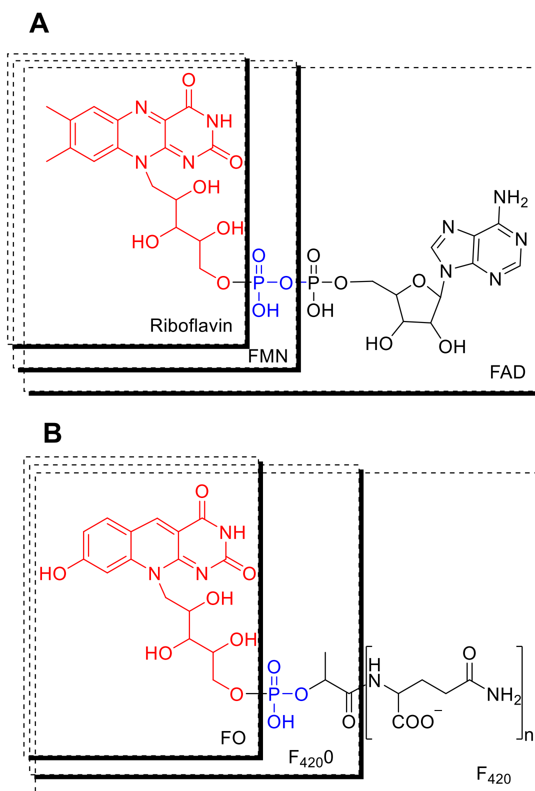

Figure 1. Natural flavins (A) and 5-deazaflavins (B). FO stands for 7,8-didemethyl-8-hydroxy-5-deazariboflavin. $F_{420}0$ has an additional phospho-L-lactate group attached to FO. Cofactor F_{420} carries a poly-γ-glutamate moiety (2-9 glutamates) attached to the lactyl group.

1.2. Structure and properties of F_{420}

The redox-active moiety of cofactor F_{420} is the tricyclic ring which is structurally quite similar to the regular flavins. Yet, when compared with the isoalloxazine part of riboflavin, F_{420} has a carbon at position 5 instead of nitrogen and is regarded as a deazaflavin (Figure 1). This absence of the N5 attributes to its obligate two electron chemistry (hydride transfer) while, in contrast, riboflavin-based cofactors are also capable to support single electron transfers and oxygen reactivity. Besides that, there is a hydroxyl group at position 8 instead of methyl group and the no methyl group at position 7. The riboflavin analog of F_{420} is 7,8-didemethyl-8-hydroxy-5-deazariboflavin and is called FO. Similar to riboflavin for the

biosynthesis of the FMN and FAD flavin cofactors, FO is the precursor for the full F_{420} cofactor. The absorption spectrum of FO has a blue shift of about 50 nm when compared with the absorption spectrum of riboflavin which is typical for a 5-deazaflavin. The 8-hydroxy group of FO/F_{420} allows extensive (*p*-quinoid) conjugation as a result of relatively facile deprotonation of the phenolic moiety. This conjugation is interrupted in the reduced form of the deazaflavin cofactor (Figure 2). As a result of the differences in the isoalloxazine moiety, the redox potential of F_{420} (-360 mV) is much lower when compared with FAD or FMN (-240 mV). In fact, its redox properties are more similar to the nicotinamide cofactors by only catalyzing hydride transfer and displaying a low redox potential, which is even lower then that of $NAD(P)^+$ (-320 mV). F_{420} can be regarded as a nicotinamide cofactor in disguise.

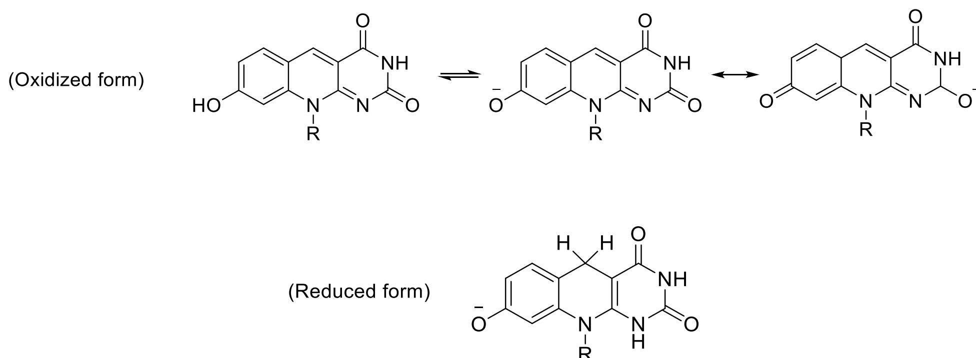

Figure 2. Protonation states of 5-deazaflavin (oxidized and reduced).

Riboflavin is converted into a common flavin cofactor, FMN, by phosphorylation. In a next step, FMN is converted into FAD by the addition of an AMP moiety. The biosynthesis of F_{420} is fundamentally different. While the deazariboflavin cofactor FO, shows still some similarity with riboflavin, the first step in converting this precursor of F_{420} includes, except phosphorylation, also the incorporation of a lactyl moiety (vide infra). The next step in maturing the F_{420} cofactor involves the attachment of a poly-γ-glutamate tail, which is catalyzed in a step-wise manner. Clearly, there is a dedicated biosynthetic route towards F_{420} which is totally different from the route towards FMN/FAD and which involves unique enzymes to build this atypical redox cofactor. Only in the synthesis of the FO precursor some enzymes are shared with the riboflavin synthesis route. Intriguingly, the length of the poly-glutamate moiety is organism dependent and is essential to prevent diffusion of the cofactor out of the cell while it does not play a role in catalysis. In most of the crystal structures, the

poly-glutamate chain has relatively little interactions with the protein and is often only partly bound to a patch on the surface of the respective deazaflavoprotein.

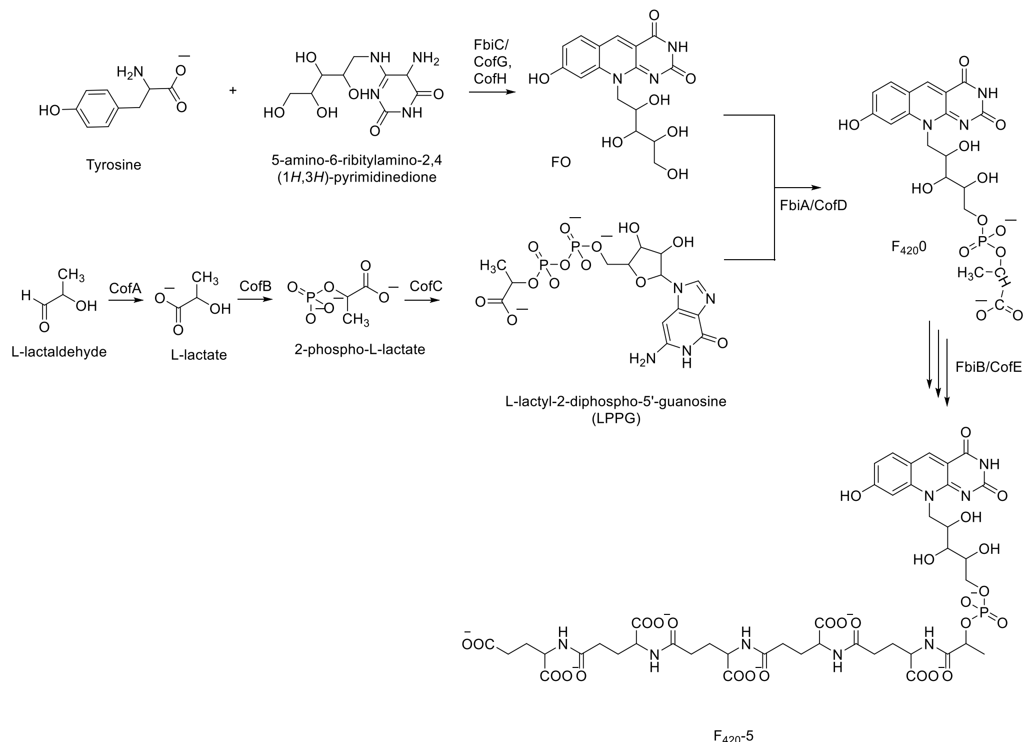

Figure 3. Proposed biosynthetic pathway for F_{420}.

1.3. Biosynthesis of F_{420}

1.3.1. Biosynthesis of the deazariboflavin core, FO

Analogous to riboflavin and its derivatives FMN and FAD, F_{420} is synthesized starting from a deazariboflavin precursor, the chromophore FO. FO already contains the catalytic moiety of the F_{420} cofactor and some F_{420} enzymes can even use this minimal deazaflavin as a co-factor for catalysis (Hossain et al. 2015). FO also serves as light antenna molecule for DNA photolyases which repair thymine-thymine dimers. FO, in these enzyme complexes, trans-fers energy to FAD (Glas et al. 2009). Intriguingly, the FO-containing DNA photolyases are from eukaryotic origin and therefore it is still unclear how these enzymes sequester FO as no deazaflavin biosynthetic genes have been reported for eukaryotes.

The biosynthetic pathway for riboflavin and the deazariboflavin FO diverges from a common intermediate; 5-amino-6-(ribitylamino)-2,4(1H,3H)-pyrimidinedione (ribityldiaminouracil) (Figure 3) which is obtained from GTP after a multistep reaction. FO is synthesized by condensation of ribityldiaminouracil with tyrosine. A radical S-adenosyl-L-methionine (SAM) dependent enzyme, FO synthase, catalyzes this reaction. In archaea and cyanobacteria, FO synthase comprises two proteins encoded by two adjacent or non-adjacent genes (*cof*G and *cof*H in *Methanocaldococcus jannaschii*)(Graham et al. 2003). Both enzymes contain one radical SAM site each and are capable of generating radicals independently. However, for FO production, the first reaction is catalyzed by CofH and it forms an intermediate product. This intermediate serves as substrate for CofG and eventually deazaflavin chromophore formation takes place (Decamps et al. 2012). In bacteria, the same reaction is catalyzed by a bifunctional enzyme, FbiC, which is encoded by one *fbi*C gene. FbiC has N- and C-terminal domains each containing one radical SAM site (Choi et al. 2002; Graham et al. 2003). Detailed mechanistic studies has been carried out on these enzymes (Philmus et al. 2015). The *fbi*C gene, when cloned and expressed in *E. coli* cells, resulted in *in vivo* production of FO (unpublished data). Since FO can diffuse across the cell membrane, FO production was detected in the growth medium.

1.3.2. Phospho-L-lactylization of FO

Addition of the phospho-L-lactate group to the ribityl tail of FO leads to the formation of a polar molecule, F_{420}-0. Unlike FO, F_{420}-0 cannot easily diffuse across the cell membrane due to the charged group. The lactate group added to the FO has been argued to originate from lactaldehyde (Grochowski et al. 2006). An NAD^+ dependent lactate dehydrogenase (CofA) catalyzes this reaction. As shown in figure 3, phosphorylation of lactate to 2'-phospho-l-lactate is believed to be catalyzed by CofB. The reaction mechanism for this reaction is still not clear. The pyrophosphate linkage from GTP is used in this process. Two enzymes have been identified that catalyze the steps of forming an activated phospho-L-lactate intermediate, L-lactyl-2-diphospho-5'-guanosine (LPPG) (Grochowski et al. 2008) and to add this to the ribose moiety of FO (Figure 3). In *Methanocaldococcus jannaschii,* CofC and CofD are involved in such phosphorylation event. CofD (FbiA in actinobacteria) is a 2-phospho-l-lactate transferase and structural data show that substrate dependent conformational changes initiate the condensation process. Upon action of CofD, the F_{420} precursor F_{420}-0 is formed (Forouhar et al. 2008). It has not been reported whether this deazaflavin has any relevance as a cofactor. It is seen as precursor of the mature F_{420} cofactor.

1.3.3. Addition of poly-γ-glutamyl tail

The last steps of maturation of the F_{420} cofactor entail the addition of an unusual poly-γ-glutamyl tail. For this, dedicated enzymes, γ-glutamyl ligases, have been identified. The coupling of each L-glutamate moiety goes at the expense of a GTP molecule. For the archaeon *M. jannaschii*, CofE has been found to be responsible for this processive decoration of F_{420}-0. In that case, as in other archaea, on average only 2 glutamates are coupled to form F_{420}-2. In actinobacteria, the poly-γ-glutamyl tail is typically longer, in the range of 4 to 9 glutamates long. The respective enzyme from *Mycobacterium tuberculosis*, FbiB, has been studied in detail recently. The longer peptide formed by FbiB, when compared with CofE, may be explained by an additional C-terminal domain in the bacterial enzyme. However, the exact mechanism of formation of the poly-γ-glutamyl tail is still unclear. Although a crystal structure of FbiB has been elucidated, even the details on how L-glutamates are incorporated in the growing peptide (by insertion or extension) remain to be established.

1.4. Physiological role of F_{420}

Cofactor F_{420} has an important role in metabolism of many microorganisms. The first comprehensive studies on F_{420}-dependent enzymes focused on methanogenic archaea and revealed a role of the deazaflavin cofactor in multiple pivotal enzymes. In fact, methanogens contain such high amounts of F_{420} that they can be detected by its cofactor specific fluorescence (Doddema and Vogels 1978). Since the discovery of the F_{420} cofactor in 1972, a relatively small number of F_{420}-dependent enzymes have been reported, mainly from methanogenic archaea and *Streptomyces* species (Greening et al. 2016). In methanogens, CO_2, H_2 and acetate are mainly fixed into methane. In the process of the reduction of CO_2 to CH_4, F_{420}-dependent hydrogenases/dehydrogenases come into play. The required electrons mainly come from H_2 by action of F_{420}-dependent hydrogenases (Muth et al. 1987; Michel I et al. 1995; Mills et al. 2013), and in some cases from formate dehydrogenases (Tzeng et al. 1975) and secondary alcohol dehydrogenases (Berk and Thauer 1997).

Only recent genome sequence analyses revealed that the F_{420} cofactor is also produced by many bacteria (Selengut and Haft 2010). Except for conservation of the F_{420} biosynthesis genes, predicted proteomes of many bacteria appear rather rich in F_{420}-dependent proteins. For example, the proteome of *Rhodococcus jostii* RHA1 is predicted to include >100 deazaflavoproteins, most of them with unknown function. The deazaflavin biosynthetic genes are well conserved in actinobacteria and it has been shown that these bacteria contain relatively high levels of the cofactor. *M. smegmatis* is typically used for isolating F_{420}

because its high content of the cofactor and ease of cultivation (Isabelle et al. 2002; Bashiri et al. 2010). The role of F_{420} in actinobacteria has been most intensely studied for the pathogen *M. tuberculosis*. It appears that F_{420} is essential to resist oxidative stress for which *M. tuberculosis* cells sustain a high level of glucose-6-phosphate (Hasan et al. 2010) which is used by a F_{420}-dependent glucose-6-phosphate dehydrogenases to generate $F_{420}H_2$. Several quinone reducing $F_{420}H_2$-dependent reductases appear essential for this. The same reductases were found to be essential for activating antitubercular prodrugs. Also a $F_{420}H_2$-dependent biliverdin reductase, generating bilirubin, adds to the capacity of *M. tuberculosis* to withstand stress conditions (Ahmed et al. 2016). Except for combating (oxidative) stress, the deazaflavin cofactor is also used by other enzymes in *M. tuberculosis*: for example, for biosynthesis of a special kind of mycobacterial lipid, phthiocerol dimycocerosates, a $F_{420}H_2$-dependent phthiodiolone ketoreductase is produced, while a F_{420}-dependent hydroxymycolic acid dehydrogenase is essential for the biosynthesis of ketomycolic acids (Purwantini and Mukhopadhyay 2013). The genome of *M. tuberculosis* is predicted to contain more deazaflavoenzymes, awaiting identification and future biochemical studies to reveal their role in metabolism.

1.5. Biocatalytically relevant F_{420}-dependent enzymes

F_{420}-dependent enzymes represent a diverse group of unexplored biocatalysts which play an important role in archaeal and bacterial metabolism(Greening et al. 2016). In archaea, F_{420}-dependent enzymes serve a function in central metabolic pathways. It is estimated, based on genome sequence analysis, that around 1 out of 10 bacteria contain the required genes for F_{420} biosynthesis (Selengut and Haft 2010). Based on homology searches using the sequences of known deazaflavoproteins, F_{420} producing bacteria are predicted to contain many (uncharacterized) F_{420}-dependent enzymes. Future studies will reveal the full biocatalytic potential of these redox enzymes. Being an obligate hydride transferring cofactor, F_{420}-dependent enzymes are expected to correspond to dehydrogenases and reductases. Yet, it may also be that new activities will be revealed by studying novel F_{420}-dependent enzymes. For example, some F_{420}-dependent enzymes may have evolved ways to utilize the unique photoreactive properties of the deazaflavin cofactor, similar to FO in DNA photolyases.

Based on the current biochemical knowledge, it is clear that there are several distinct structural families that contain F_{420}-dependent enzymes. Especially the elucidation of crystal structures of a number of these F_{420}-dependent enzymes has helped to realize the distinct

features of various deazaflavoprotein classes. Concerning their catalytic properties, they can be divided into two types: 1) those which use oxidized form of the F_{420} (dehydrogenases), and 2) those that use the reduced form of the cofactor (reductases). This is analogous to the superfamily of nicotinamide cofactor dependent enzymes. Below, some F_{420}-dependent enzymes are highlighted in the context of their potential use as biocatalysts. In Table 1, an overview of some F_{420}-dependent enzymes is provided.

1.6. $F_{420}H_2$ regenerating enzymes

Since F_{420} is not commercially available, it has to be purified from a suitable host. Previous work has shown that the levels of the deazaflavin cofactor varies considerably among F_{420} producers. Due to the intracellular level of F_{420} and the ease by which the organism can be grown, F_{420} is mostly isolated from *M. smegmatis*. Still, the yield of the cofactor is very low, upto 1.4 µmol/L (Isabelle et al. 2002). Clearly, when considering F_{420}-dependent enzymes for biocatalysis, efficient cofactor recycling systems will be essential. Alternatively, one could opt for whole cell conversion using a host that expresses the deazaflavin cofactor. However, this does not seem to be a real option because the most common hosts for recombinant protein expression (e.g. *E. coli*, yeast, filamentous fungi) do not harbor the F_{420} biosynthetic pathway. Therefore, it is essential to have efficient F_{420} regenerating enzymes available, analogous to the developed systems for NAD(P)H dependent biocatalysts. Both in archaea as well as in prokaryotes, such enzymes are available with a similar physiological function. As F_{420}-dependent enzymes show greatest potential in performing selective reductions, it will be most valuable to develop a toolbox of enzymes for the regeneration of $F_{420}H_2$ at the expense of a sacrificial cosubstrate. For generating the reduced deazaflavin coenzyme, F_{420}-dependent glucose-6-phosphate dehydrogenases (FGD) and F_{420}-dependent alcohol dehydrogenases (ADF) are promising candidates. Alternatively, one could also consider the so-called F_{420}:NADPH oxidoreductase (FNO). Though this enzyme assists methanogens in transferring the surplus of electrons from reduced F_{420} to $NADP^+$, generating NADPH, FNO can also be used in the reverse mode. More details on these $F_{420}H_2$ regenerating enzymes are provided below.

1.6.1. F_{420}-dependent dehydrogenases

F_{420}-dependent D-glucose-6-phosphate dehydrogenases (FGD) catalyze the oxidation of the substrate to 6-phospho-D-glucono-1,5-lactone which is spontaneously hydrolyzed to 6-phosphogluconate. FGDs from several actinomycetes have been characterized. In case of *M. tuberculosis,* the physiological role of FGD is to provide the reduced form of F_{420} for

$F_{420}H_2$ dependent reductases. Interestingly, *M. tuberculosis* has both a $NADP^+$-dependent D-glucose-6-phosphate dehydrogenase as well as a F_{420}-dependent glucose-6-phosphate dehydrogenase, both tapping from the same pole of glucose-6-phophate. It has been shown that cells that accumulate glucose-6-phosphate as response to oxidative stress. *M. tuberculosis* knockout mutants lacking FGD (*Δfgd*) were significantly more sensitive to oxidative stress. A similar observation was made for the FO synthase knockout mutant (*ΔfbiC*). This shows that the FGD plays a crucial role in *M. tuberculosis*, to sustain a sufficient level of $F_{420}H_2$ in the cytosol, to serve the $F_{420}H_2$-dependent enzymes. Work in this thesis (Chapter 4) investigates new subclass of F_{420}-dependent glucose-6-phosphate dehydrogenases from *Nocardiacae* and *Cryptosporangium* sp*.* These enzymes, unlike previously described enzymes, also accept other sugar-6-phosphates as substrate.

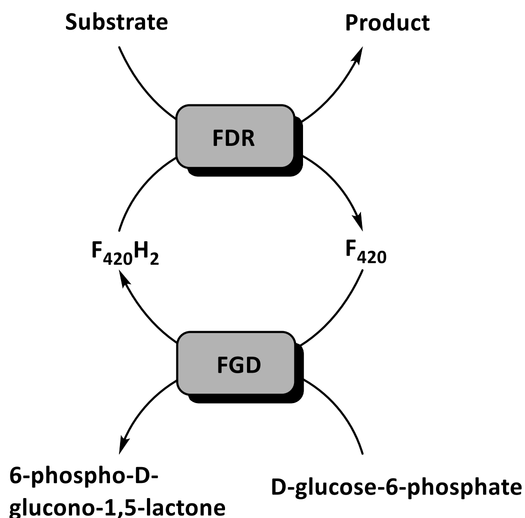

Figure 4. F_{420}-dependent glucose-6-phosphate dehydrogenases as $F_{420}H_2$ cofactor recycling system.

A disadvantage of the F_{420}-dependent glucose-6-phosphate dehydrogenase is the required cosubstrate, glucose-6-phophate. As this is a rather expensive compound, FGD can only be considered when synthesizing high value compounds. As an alternative, one may consider F_{420}-dependent alcohol dehydrogenases which belong to the same structural family as the FGDs (TIM barrel containing luciferase family). So far only two ADFs have been reported in literature. These enzymes accept a range of small (and cheap) alcohols and therefore are

interesting candidates for use in biocatalysis. With the available crystal structure of one of these ADFs, it may also be possible to improve them for biocatalytic purposes through enzyme engineering.

1.6.2. F_{420}:NADPH oxidoreductase (FNO)

Another interesting candidate enzyme for the generation of $F_{420}H_2$ is the F_{420}:NADPH oxidoreductase (FNO). FNO is thought to connect the anabolic NADPH pathway to the catabolic F_{420} pathway as it catalyzes the reversible reduction of $NADP^+$ using $F_{420}H_2$. Its physiological function within the cell is to shuttle the reducing equivalents between nicotinamide and deazaflavin molecules. In methanogens, this enzyme acts as a $F_{420}H_2$-dependent $NADP^+$ reductase, whereas in prokaryotes, it seems to act as a NADPH-dependent F_{420} reductase. In the latter catalytic mode, one can envisage its use for the generation of $F_{420}H_2$ at the expense of NADPH. Many systems have been developed for generating NADPH using cheap starting compounds, such as glucose in combination of a NADP-dependent glucose dehydrogenase. Though such $F_{420}H_2$ regeneration system would rely on several enzymes, the well-developed nicotinamide recycling systems make this approach appealing when a robust FNO is available. In addition to that, FNO may serve as 'bridge' recycling enzyme in cascade reactions involving both F_{420};NADPH or $F_{420}H_2$:$NADP^+$ dependent enzymes.

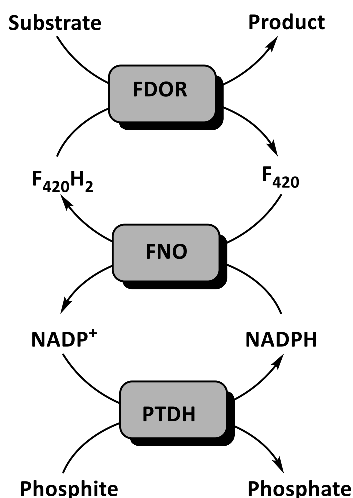

Figure 5. F_{420}:NADPH oxidoreductase as $F_{420}H_2$ recycling system. Phosphite dehydrogenase (PTDH) can be used for NADPH recycling.

F_{420} Oxidoreductase	Reaction catalyzed	Reference
Rossman fold		
F_{420}:NADPH oxidoreductase (FNO)/ $F_{420}H_2$ dependent $NADP^+$ reductase	NADPH + F_{420} ⟷ $NADP^+$ + $F_{420}H_2$	This thesis
TIM barrel fold		
F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD)	D-glucose-6-phosphate + F_{420} → D-6-phosphoglucono-1-lactone + $F_{420}H_2$	(Nguyen et al. 2016)
F_{420}-dependent sugar-6-phosphate dehydrogenase (FSD)		This thesis
Alcohol dehydrogenase (ADF)	Isopropanol + F_{420} → Acetone + $F_{420}H_2$	(Aufhammer et al. 2004)
Methylene hydropterin reductase (Mer)		
F_{420} dependent hydroxymycolic acid dehydrogenase	Hydroxy-mycolic acid + F_{420} → Keto-mycolic acid + $F_{420}H_2$	(Purwantini and Mukhopadhyay 2013)
Split β-barrel like fold		
Deazaflavin dependent nitroreductase (Ddn)	PA824 (Prodrug) + $F_{420}H_2$ → PA824 (Active) + F_{420}	(Cellitti et al. 2012)
Biliverdin reductase	Biliverdin + $F_{420}H_2$ → Bilirubin + F_{420}	(Ahmed et al. 2016)
Aflatoxin degrading FDRs	Reduction of α,β unsaturated bonds	(Gurumurthy et al. 2013)
F_{420} dependent oxidoredutases (FDORs)		
F_{420} dependent *ene*-reductase	Reduction of α,β unsaturated bonds	This thesis

Table 1. Some F_{420}-dependent enzymes.

1.7. F_{420}-dependent reductases

Due to the relatively low redox potential, F_{420}-dependent enzymes are predicted to be effective in reductions. In line with this, many of the described deazaflavoenzymes function as reductases. The generation of the required reduced form of the deazaflavin coenzyme is accomplished by the enzymes mentioned above. Intriguingly, the F_{420}-dependent reductases described in literature have hardly been explored for biocatalytic purposes. Most of the known examples have been studied in the context of elucidating a metabolic pathway or understanding the mode of action of prodrugs. The latter mainly refers to the finding that is *M. tuberculosis* a deazaflavin-dependent nitroreductase (Ddn) (Cellitti et al. 2012) has been shown to be responsible for the activation of the prodrug PA-824 (Pretonamid) into an active toxic form (Manjunatha et al. 2006; 2008). This promising prodrug PA-824 and similar nitroimidazoles are currently in clinical trials. The activated form of PA-824 leads to the release NO after reduction by Ddn. This NO, in turn, kills *M. tuberculosis*, including the non-replicating form of the organism (Singh et al. 2008). This is important because other drugs can only target the active form of *M. tuberculosis* and TB, most of the cases is dormant for years.

Apart from *M. tuberculosis*, other members of actinobacteria such as *R. jostii* RHA1*, M. smegmatis,* and *T. fusca* also have a large number of F_{420}-dependent enzymes most of which are yet to be characterized. F_{420}-dependent reductases are part of the split β-barrel enzyme superfamily (FDORs) (Ahmed et al. 2015). These enzymes are distantly related to FMN-dependent pyridoxamine 5'-phosphate oxidases (PNPOx).

Some members of F_{420}-dependent reductases (FDR) catalyze the reduction of α,β-unsaturated esters of recalcitrant aflatoxin compounds (Taylor et al. 2010). Some enzyme from *M. tuberculosis* help in persistence by reducing quinones, xenobiotics and bactericidal agents (Gurumurthy et al. 2013; Jirapanjawat et al. 2016). Reductases from *M. smegmatis* has been also shown to reduce diverse compounds through common mechanism (Greening et al. 2017). However, these enzymes have not been explored as biocatalysts for enantio- and/or regioselective reductions. Our work on F_{420}-dependent reductases (chapter 5) shows that these enzymes display a broad substrate scope and can catalyze *ene*-reduction reactions in an enantio- and regioselective manner. Crystal structures of several of these small F_{420}-dependent reductases have been solved which will accelerate the improvement of these enzymes as efficient biocatalysts through enzyme engineering.

1.8. Aim and outline of the thesis

The main goal of the work described in this thesis was to identify, characterize and engineer bacterial F_{420}-dependent enzymes for their potential use as biocatalysts. Both genome mining and proteomic techniques were explored in order to identify such deazaflavoenzymes. Since there are relatively few deazaflavoenzymes characterized till date, we did not restrict ourselves to one particular class of enzyme. Several novel F_{420}-dependent enzymes have been discovered and studied in detail (chapters 2-5, see below). Except for deazaflavoenzymes, also work has been performed on identifying flavin-containing monooxygenases that can convert furanoid compounds (chapter 6).

Chapter 2 involves a proteomic approach aimed at identifying F_{420}-binding proteins. By generating F_{420}-bound column material, an affinity chromatography method was developed and validated. By using recombinantly expressed F_{420}-dependent glucose-6-phosphate dehydrogenase it could be shown that this method indeed is able to isolate F_{420}-binding proteins. The method was used to identify the novel F_{420}-binding proteins by analyzing cell free extract of *M. smegmatis* cells. Upon SDS-PAGE and MS analyses, several putative F_{420}-binding proteins were identified. While, based on their protein sequence, a large portion of the isolated proteins are predicted to be F_{420}- dependent, some of the identified proteins have not been studied before. Future studies will reveal for what purpose these proteins utilize the deazaflavin cofactor.

In chapter 3, a newly identified F_{420}:NADPH oxidoreductase (Tfu-FNO) from the mesothermophile *Thermobifida fusca* is described. Except for establishing an expression and purification protocol for this deazaflavoenzyme, also a detailed characterization was performed. This resulted in elucidation of its crystal structure, in complex with $NADP^+$. Tfu-FNO is a valuable biocatalyst for regenerating reduced F_{420} at the expense of NADPH, or vice versa. Since wild-type Tfu-FNO is specific for $NADP^+$/NADPH and has very poor activity towards NAD^+/NADH, mutant enzymes were prepared in order change the nicotinamide coenzyme specificity.

Chapter 4 describes a thorough sequence analysis of the family of sequence-related $F_{420}H_2$-dependent alcohol dehydrogenases. This revealed that this specific family of deazaflavoenzymes has evolved from an FMN-dependent ancestral enzyme. A predicted ancestral sequence of a F_{420}-dependent alcohol dehydrogenases was used to resurrect the corresponding protein. By using a synthetic gene, this ancestral protein was expressed and purified. Biochemical characterization revealed that it acts as a glucose-6-phosphate dehydrogenase and that it exhibits a relatively high thermostability. As part of this study, also a new

subgroup of dehydrogenases was identified. Expression and characterization of a representative revealed that it has a somewhat more relaxed substrate acceptance profile when compared with the closely related F_{420}-dependent glucose-6-phosphate dehydrogenases. These data provide new insights in how F_{420}-dependent dehydrogenases have evolved over time.

The work described in chapter 5 concerns an explorative study of $F_{420}H_2$-dependent reductases. While such reductases had been described in literature as enzyme capable to reduce quinoid substrates, here it was shown for the first time that they can be exploited as biocatalysts for regio- and enantioselective reductions of α,β-unsaturated ketones and aldehydes. The observation that the enantioselectivity is opposite to the flavin-dependent reductases that are typically used for such reactions, confirm that the $F_{420}H_2$-dependent reductases are structurally and mechanistically different from the most widely applied reductases. The observed excellent enantioselectivities indicate that this is an interesting group of reductases in the context of biocatalysis. Future enzyme engineering efforts aimed at improving the rate of catalysis may turn them into potent biocatalysts.

In chapter 6, FAD-containing Baeyer-Villiger monoxygenases (BVMO) were tested for their ability to convert furanoid aldehydes. Interestingly, most of the tested BVMOs were found to be active on these compounds. Product analysis revealed that the acid form (instead of formate ester) was formed as product using furfural and furfural derivatives (HMF, DFF and FFA) as substrates. A mutant of phenylacetone monooxygenase (PAMO) showed a relatively high activity towards furanoid aldehydes. This study shows that BVMO are interesting biocatalysts for the conversion of furanoid compounds and could perhaps serve a role in the recent interest in producing 2,5-furandicarboxylic acid (FDCA) starting from 5-(hydroxylmethyl)furfural (HMF) Although at slower rate, these enzymes could oxidize both the aldehyde groups of DFF to a bioplastic precursor, FDCA.

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