Review

Cofactor F$_{420}$-Dependent Enzymes: An Under-Explored Resource for Asymmetric Redox Biocatalysis

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Abstract: Asymmetric reduction of enoates, imines and ketones are among the most important reactions in biocatalysis. These reactions are routinely conducted using enzymes that use nicotinamide cofactors as reductants. The deazaflavin cofactor F$_{420}$ also has electrochemical properties that make it suitable as an alternative to nicotinamide cofactors for use in asymmetric reduction reactions. However, cofactor F$_{420}$-dependent enzymes remain under-explored as a resource for biocatalysis. In this review, we consider the cofactor F$_{420}$-dependent enzyme families with greatest potential for the discovery of new biocatalysts: the flavin/deazaflavin-dependent oxidoreductases (FDORs) and the luciferase-like hydride transferases (LLHTs). We discuss characterized F$_{420}$-dependent reductions that have potential for adaptation for biocatalysis, and we consider the enzymes best suited for use in the reduction of oxidized cofactor F$_{420}$ to allow cofactor recycling in situ. We also discuss recent advances in the production of cofactor F$_{420}$ and its functional analog F$_{O-5'}$-5'-phosphate, which remains an impediment to the adoption of this family of enzymes for industrial biocatalytic processes. Finally, we discuss the prospects for the use of this cofactor and dependent enzymes as a resource for industrial biocatalysis.

Keywords: cofactor F$_{420}$; deazaflavin; oxidoreductase; hydride transfer; hydrogenation; asymmetric synthesis; cofactor biosynthesis

1. Introduction

Enzymes that catalyze the asymmetric reduction of activated double bonds are among the most important in biocatalysis, allowing access to chiral amines from imines (C=N), sec-alcohols from ketones (C=O), and enantiopure products derived from enoates (C=C). To date the reduction of imines, ketones and enoates has been achieved largely using enzymes that draw their reducing potential from the nicotinamide cofactors NADH and NADPH; e.g., imine reductases, ketoreductases and Old Yellow Enzymes [1-4]. However, there has been recent interest in an alternative reductive cofactor, cofactor F$_{420}$ (8-hydroxy-5-deazaflavin) [5,6].

Cofactor F$_{420}$ is a deazaflavin that is structurally similar to flavins (Fig. 1), with a notable difference at position 5 of the isoalloxazine ring, which is a nitrogen in flavins and a carbon in deazaflavins. Additionally, while C-7 and C-8 are methylated in riboflavin, they are not in cofactor F$_{420}$: C-7 is hydroxylated and C-8 is unsubstituted. These structural differences cause significant differences in the electrochemical properties of cofactor F$_{420}$ and flavins: a $\Delta E_{1/2}$ of 360-340 mV the redox mid-point potential of cofactor F$_{420}$ is not only lower than that of the flavins (−205 mV to −220 mV), but it is also lower than that of the nicotinamides (−320 mV) [7]. Additionally, as a consequence of the substitution of N-5 for a
carbon, cofactor F₄₂₀ cannot form a semiquinone (Fig. 1), which means that unlike other flavins cofactor F₄₂₀ can only perform two-electron reductions.

**Figure 1.** The structures of NAD(P) (top), cofactor F₄₂₀ and its synthetic analog F₀P (center) and common flavins (riboflavin, FMN and FAD; bottom). The oxidized and reduced forms are shown, as is the flavin semiquinone. Dashed lines indicate the differences in the structures of F₀P and cofactor F₄₂₀, and riboflavin, FMN and FAD.
Cofactor $F_{420}$ was originally described in methanogenic archaea, where it plays a pivotal role in methanogenesis [8,9]. Cofactor $F_{420}$ has since been described in a range of soil bacteria supporting a range of metabolic activities, including catabolism of recalcitrant molecules (such as picric acid) and the production of secondary metabolites, such as antibiotics [10]. A comprehensive review of the biochemistry and physiological roles of cofactor $F_{420}$ was recently published by Greening and coworkers [10]. In this review we will consider the potential of $F_{420}$-dependent enzymes in industrial biocatalysis, focusing on the enzyme families relevant to biocatalytic applications and the reactions that they catalyze. We will also discuss cofactor recycling strategies and cofactor production, with a focus on the prospects for achieving low-cost production at scale in the latter case.

2. Families of $F_{420}$-dependent enzymes relevant to biocatalysis

With respect to their prospective biocatalytic applications, the two most important families of $F_{420}$-dependent enzymes are the Flavin/Deazaflavin Oxidoreductase (FDOR) and Luciferase-Like Hydride Transferase (LLHT) families, albeit $F_{420}$-dependent enzyme from other families have also been shown to have catalytic activities of interest (e.g. TomJ, the imine reducing flavin-dependent monoxygenase) [11]. The FDOR and LLHT families are large and contain highly diverse flavin/deazaflavin-dependent enzymes; in both families there are enzymes with preferences for flavins, such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), as well as those that use cofactor $F_{420}$ [12,13]. Moreover, there are $F_{420}$-dependent FDORs that have been shown to be able to promiscuously bind FMN and use it in oxidation reactions [14]. In this section, we will discuss the FDOR and LLHT families and the classes of reaction that they catalyze.

2.1 The FDOR superfamily

The FDOR superfamily (PFAM Clan CL0336) can be broadly divided into two groups: the FDOR-A$s$ (which includes a sub-group called the FDOR-AAs) and the FDOR-B$s$. The FDOR-A$s$ are restricted to Actinobacteria and Chloroflexi and to date no FDOR-A has been described that use cofactors other than $F_{420}$ [7,12]. The FDOR-B$s$ are found in a broader range of bacterial genera than the FDOR-A enzymes, and in addition to $F_{420}$-dependent enzymes this group also includes heme oxygenases, biliverdin reductases, flavin-sequestering proteins, pyridoxine 5’ oxidases and a number of proteins of unknown function [12,15-17]. Both groups of FDOR are highly diverse, with many homologs often found within a single bacterial genome (e.g., Mycobacterium smegmatis has 28 FDORs)[18]. Addition, the majority of the enzymes of this family are yet to be characterized with respect to either their biochemical or physiological function, and therefore the FDORs represent a currently under-explored source of enzymes for biocatalysis.

The FDOR enzymes share a characteristic split β-barrel fold that forms part of the cofactor-binding pocket. The majority of the protein sequences of enzymes currently identified as belonging to this family are small single-domain proteins. The topologies of the two FDOR subgroups are broadly similar (Fig. 2), with the split-barrel core composed of 7–8 strands and with 4–5 helices interspersed. All FDOR-B$s$ studied so far have been demonstrated to be dimeric, with stands β2, β3, β5 and β6 making up the core of the dimer interface (Fig. 2). In structures of full-length FDOR-A$s$ solved to date, the N-terminal helix (if present) lies on the opposite face of the beta sheet to that in FDOR-B$s$. Thus, the N-terminus occupies part of the dimer interface region and prevents interaction between the sheets of adjacent monomers. In contrast to the FDOR-B$s$, the oligomerization state of the FDOR-A$s$ is more varied. While a number of FDOR-A$s$ have been determined to be monomeric [18], the deazaflavin-dependent nitroreductase (DDN) from M. tuberculosis forms soluble aggregates through the amphipathic N-terminal helix [19]. DDN and the FDOR-AA subgroup have been shown to be membrane-associated [20-22], and FDOR-AAs have been associated with fatty acid metabolism [12]. No structures of FDOR-AAs have been solved to date.
2.2 The LLHT family:

The LLHT family form part of the Luciferase-Like Monooxygenase family (PFAM PF00296). They adopt an (α/β)-TIM-barrel fold with three insertion regions, IS1–4 (Fig. 3). IS1 contains a short loop and forms part of the substrate cleft. IS2 contains two antiparallel β-strands, and IS3 contains a helical bundle at the C-terminus of the β-barrel and contains the remainder of the substrate-binding pocket (Fig. 3). All structures solved to date from the LLHT family contain a non-prolyl cis peptide in β3 [23-26]. Recent phylogenetic reconstructions have shown that the F420-dependent LLHTs form two clades: the F420-dependent reductases and the F420-dependent dehydrogenases [27]. The F420-reductases contain methylenetetrahydromethanopterin reductases (MERs), which catalyze the reversible, ring-opening cleavage of a carbon-nitrogen bond during the biosynthesis of folate in some archaea [28-30]. The F420-dependent dehydrogenases can be further divided into three subgroups. The first contains F420-dependent secondary alcohol dehydrogenases (ADFs) and the hydroxymycolic acid reductase from M. tuberculosis [31]. The second contains the F420-dependent glucose-6-phosphate dehydrogenases (FGDs) from Mycobacteria and Rhodococcus, while the third appear to be more general sugar-phosphate dehydrogenases [27]. In contrast to the heterodimeric structure of bacterial luciferase the F420-
dependent dehydrogenases form homodimers with the dimer interface burying a relatively large portion of the surface area of the monomers (= 2000 Å², roughly 15% of the total surface area) [23,25,26]. A number of enzymes involved in the F₄20-dependent degradation of nitroaromatic explosives such as picrate and 2,4-dinitroanisole appear to belong to the Luciferase-Like Monooxygenase family as well [32,33].

**Figure 3.** Structure of representative LLHT (PDB: 1RHC). A) A 3D representation of the biologically relevant dimer (panel A). Monomer of an LLHT with insertion sequences IS1–4 highlighted, along with the helical bundle composed of α7–9 (panel B). Topology diagram showing (α/β)₈ fold with insertion sequences highlighted: IS1, red; IS2, orange; IS3, light green, IS4, pink. The helical bundle of α7–9 is highlighted in purple (panel C).

2.3 Cofactor F₄20-dependent reactions with relevance to biocatalysis

From the perspective of biocatalysis, cofactor F₄20-dependent enzymes catalyze a number of key reductions including enoate reduction, imine reduction, ketoreduction and nitroreduction (Table 1; Fig 4).
Figure 4. Representative cofactor F$_{420}$-dependent reductions with potential for adaptation to biocatalytic applications. Included are: nitroreduction, enoate reduction, ketoreduction and imine reduction (from top to bottom). For clarity, only the dehydropiperidine ring of the thiopptide is shown.

For enoate reductions, a small number of FDORs have been studied, albeit the substrate range for most of these enzymes is yet to be fully elucidated. The ability of the mycobacterial FDORs to reduce activated C=C double bonds was first identified when these enzymes were shown to be responsible for the reduction of aflatoxins, these enzymes were then shown to also reduce enoates in coumarins, furanocoumarins and quinones [6,12,16,18,34-39]. Recent studies have shown that these enzymes are promiscuous and can use cyclohexen-1-one, malachite green and a wide range of other activated ene compounds as substrates [36]. However, there have been few FDOR studies to date that have examined their kinetic properties and stereospecificity. In one of these studies, FDORs from *Mycobacterium huiaci*um (FDR-Mha) and *Rhodococcus jostii* RHA1 (FDR-Rh1 and FDR-Rh2) were shown to reduce a range of structurally diverse enoates with conversions ranging from 12 to >99 % and e.e. values of up to >99 % [40]. Interestingly, it has been proposed that both the hydride and proton transfer from F$_{420}$H$_2$ in these reactions was directed to the Re-face of the activated double bond (Fig. 5), which is promotes the opposite enantioselectivity compared to that of the FMN-dependent Old Yellow Enzyme family of...
enoate reductases [40], suggesting that the F420-dependent FDORs may provide a stereocomplementary enoate reductase toolbox. However, other studies suggest that protonation of the substrate is mediated by solvent or an enzyme side-chain (as it is in Old Yellow Enzyme) [38]. Further structure/function studies are needed to fully understand the mechanistic diversity of this family of enzymes.

Figure 5. Enoate reduction by a flavin-dependent enzyme (Old Yellow Enzyme) and the proposed cofactor F420-dependent mechanism. Notably the mechanism of reduction yields trans-hydrogenation products for Old Yellow Enzyme and cis-hydrogenation products for the F420-dependent enzymes.

The LLHT family contains several enzymes with alcohol oxidase or ketoreductase activity (Table 1; Fig. 4). The F420-dependent glucose-6-phosphate dehydrogenases of several species have been investigated [25,26,41]; although an extensive survey of their substrate ranges has yet to be conducted it has been demonstrated that glucose is a substrate for the Rhodococcus jostii RHA1 enzymes [26]. An F420-dependent alcohol dehydrogenase (ADH) from Methanogenium liminatans has been shown to catalyze the oxidation of the short chain aliphatic alcohols 2-propanol, 2-butanol and 2-pentanol (85, 49 and 23.1 s⁻¹ kcat, 2.2, 1.2 and 7.2 mM KM respectively) [42], but it was unable to oxidize primary alcohols, polyols or secondary alcohols with more than five carbons. It is unclear whether these alcohol oxidations are reversible, but in the oxidative direction these reactions provide enzymes that can be used to recycle reduced cofactor F420 (see section 4). Alcohol oxidation can also be used to produce ketones as intermediates in biocatalytic cascades that can then be used in subsequent reactions, such those catalyzed by transaminases or amine dehydrogenases in chiral amine synthesis [2,43-45] or by ketoreductases or alcohol dehydrogenases in chiral sec-alcohol synthesis (i.e., deracemization or stereoinversion of sec-alcohols). This approach can be achieved in a one pot cascade if different cofactors are used for the oxidation and reduction (Fig. 6) [46].
At least one \( \text{F}420 \)-dependent ketoreductase has been described. The mycobacterial \( \text{F}420 \)-dependent phthiadiolone ketoreductase catalyzes a key reduction in the production of phthiocerol dimycocerosate, a diacylated polyketide found in the mycobacterial cell wall [47]. Although the physiological role of this enzyme has been elucidated, biochemical studies of the catalytic properties and substrate range will be required to assess this enzyme’s potential for use as a biocatalyst.

Figure 6. Proposed scheme for one-pot, enzyme cascades for deracemization/steroiinversion of sec-alcohols (top) and chiral amine synthesis (bottom) using cofactor \( \text{F}420 \)-dependent alcohol oxidation.

\( \text{F}420 \)-dependent enzymes have also been shown to reduce imines (Table 1; Fig. 4). An FDOR for \textit{Streptomyces tateyamensis} (Tpn1) is responsible for the reduction of dehydropiperidine in the piperidine-containing series \( \alpha \) group of thiopeptide antibiotics produced in this bacterium (Fig. 4). Tpn1 was identified as the \( \text{F}420 \)-dependent dehydropiperidine reductase responsible for the reduction of dehydropiperidine ring in thiostrepton A to produce the piperidine ring in the core macrocycle of thiostrepton A [48]. Tpn1 activity was affected by substrate inhibition at concentrations higher than 2 \( \mu \text{M} \) of thiostrepton A, preventing measurement of the \( K_M \), but its \( k_{cat}/K_M \) was measured at \( 2.80 \times 10^4 \text{ M}^{-1} \text{S}^{-1} \) [48]. The substrates for phthiadiolone ketoreductase and Tpn1 are large secondary metabolites and, as yet, it is unclear if it will accept smaller substrates or substrates with larger/smaller heterocycles (e.g., dehydropyrroles).

Another \( \text{F}420 \)-dependent imine reductase (TomJ) has been described from \textit{Streptomyces achromogenes} that reduces the imine in 4-ethylidene-3,4-dehydropyrrole-2-carboxylic acid during production of the secondary metabolite tomaymycin, which has been shown to have potentially interesting pharmaceutical properties [11]. Additionally, the reduction of a prochiral dihydropyrrole to a pyrrole is a reaction with a number of biocatalytic applications [5].

Nitroreductases have potential application in the reduction of a prochiral nitro group to form a chiral amine [49]. The LLHT family \( \text{F}420 \)-dependent nitroreductase Npd from \textit{Rhodococcus} catalyzes the two-electron reduction of two nitro groups in picric acid during catabolism of the explosive TNT (Table 1; Fig. 4)[50]. While this stops short of reducing the nitro group to an amine, this catalytic activity may contribute to a reductive cascade that achieves this conversion.

The final class of reaction for consideration in this review is the unusual, reversible ring-opening/closing reaction catalyzed by the MERs (Fig. 4; Table 1). This reaction is required for folate biosynthesis in some archaea [24,28-30]; however, ring-closing reactions of this type could be used for producing N-containing heterocycles, which are intermediates in the synthesis of numerous pharmaceuticals [51,52]. The promiscuity of the MERs has not yet been investigated, and so the potential to re-engineer these enzymes is not fully understood.
Table 1. Characterized F420-dependent enzymes with activities that could be adapted for biocatalytic applications.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Family</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Enoate reduction</td>
<td></td>
<td></td>
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<tr>
<td>Aflatoxins</td>
<td>FDOR</td>
<td>[18,34,35]</td>
</tr>
<tr>
<td>Coumarins</td>
<td>FDOR</td>
<td>[34-36]</td>
</tr>
<tr>
<td>Quinones</td>
<td>FDOR</td>
<td>[37]</td>
</tr>
<tr>
<td>Biliverdin reduction</td>
<td>FDOR</td>
<td>[12,16]</td>
</tr>
<tr>
<td>Nitroimidazoles</td>
<td>FDOR</td>
<td>[38]</td>
</tr>
<tr>
<td>Cyclohexenones</td>
<td>FDOR</td>
<td>[6,36,39]</td>
</tr>
<tr>
<td>Citral/Neral/Geranial</td>
<td>FDOR</td>
<td>[6]</td>
</tr>
<tr>
<td>Carvone</td>
<td>FDOR</td>
<td>[6]</td>
</tr>
<tr>
<td>Ketoisophorone</td>
<td>FDOR</td>
<td>[6]</td>
</tr>
<tr>
<td>Alcohol oxidation / ketoreduction</td>
<td></td>
<td></td>
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<tr>
<td>Glucose-6-phosphate</td>
<td>LLHT</td>
<td>[26,53]</td>
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<tr>
<td>Phthiodiolone dimycocerosate</td>
<td>LLHT</td>
<td>[47]</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>LLHT</td>
<td>[54]</td>
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<tr>
<td>Imine reductions</td>
<td></td>
<td></td>
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<tr>
<td>Dehydropiperidine (in thiopeptins)</td>
<td>FDOR</td>
<td>[48]</td>
</tr>
<tr>
<td>4-ethylidene-3,4-dihydropyrrole-2-carboxylic acid</td>
<td>Flavin-dependent monooxygenase</td>
<td>[11]</td>
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<tr>
<td>Nitroreductions</td>
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<tr>
<td>Picrate</td>
<td>LLHT</td>
<td>[55,56]</td>
</tr>
<tr>
<td>2,4-DNP</td>
<td>LLHT</td>
<td>[55,56]</td>
</tr>
<tr>
<td>Ring opening/closing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-N bond cleavage/formation in methylenetetrahydromethanopterin</td>
<td>LLHT</td>
<td>[24,28-30]</td>
</tr>
</tbody>
</table>

4. Cofactor recycling for cofactor F420

Cofactor recycling is essential for the practical application of the F420-dependent enzymatic processes in biocatalysis. There are various strategies for cofactor regeneration for NADH and NADPH, including enzymatic, chemical, electrochemical and photochemical methods [57]. In this section we will discuss potential enzymes for the regeneration of cofactor F420. As most of the industrially relevant F420-dependent reactions are asymmetric reductions, F420-dependent oxidases are required for cofactor regeneration. Figure 7 shows the characterized enzymes that catalyze F420-dependent oxidations that could be applied in cofactor F420 reduction.

Emulating methods developed for nicotinamide cofactors, both formate dehydrogenase (FDH) and glucose 6-phosphate dehydrogenase (G6PD) enzymes are attractive enzymatic routes for cofactor reduction both in vitro [58-61] and in vivo [62,63]. Fortunately, F420-dependent G6PDs and FDHs have been identified and characterized. The F420-dependent G6PD from Mycobacteria (FGD) is one potential cofactor F420-recycling enzyme; FGD is the only enzyme in these bacteria known reduce oxidized cofactor F420. The intracellular concentration of G6P in Mycobacteria is up to 100-fold higher than it is in...
E. coli, which provides a ready source of reducing power for F_{420}-dependent reduction reactions [64]. FGD from Rhodococcus jostii and Mycobacterium smegmatis have been studied and expressed in E. coli, both the enzymes were stable in in vitro assays [41,53,65]. Both FGDs have been expressed in engineered E. coli producing cofactor F_{420} together with FDORs [39,66]. FGDs have been shown to efficiently regenerate reduced cofactor F_{420} both in vivo and in vitro. However, the cost of the glucose-6-phosphate and the need to separate reaction products from the accumulated FGD byproduct (6-phosphoglucono-D-lactone) may prove to be impediments for the adoption of FGD as a recycling system for cofactor F_{420} in in vitro biotransformations.

Formate is an excellent reductant for cofactor recycling, with FDH-dependent cofactor reduction yielding carbon dioxide, a volatile byproduct that can be easily removed from the reaction mixture, thereby simplifying the downstream processing of the product of interest. Additionally, formate is a low-cost reagent, leading to favorable process economics. Most methanogens have the capability to use formate as sole electron donor using F_{420}-dependent formate dehydrogenase [67,68]. The soluble F_{420}-dependent FDH from Methanobacterium formicium has been expressed in E. coli [69], purified and studied in vitro with the reduction of 41.2 µmol of F_{420} min^{-1} mg^{-1} of FDH, with non-covalently bound FAD required for optimal activity [70,71]. Methanobacterium ruminantium FDH reduces cofactor F_{420} at a much slower rate than that of M. formicium: 0.11 µmol of F_{420} min^{-1} mg^{-1} of FDH [72]. As yet, the use of F_{420}-dependnt FDHs for in vitro cofactor recycling has been sparsely studied; however, as these enzymes are soluble and can be heterologously expressed, they represent a promising system for use in cofactor F_{420}-dependent biocatalytic processes.

Another potential recycling system for cofactor F_{420} is the F_{420}:NADPH oxidoreductase (Fno), which couples the reduction of cofactor F_{420} with oxidation of NADPH. Methanogenic archaea use this enzyme to transfer reducing equivalents from hydrogenases to produce NADPH via F_{420}, while in bacteria it functions in the opposite direction: to provide cell with reduced F_{420} via NADPH [73]. Fno is also required for production of reduced F_{420} for tetracycline production in Streptomyces [74]. The Fno enzymes from the thermophilic bacteria Thermobifida fusca and the thermophilic archaeon Archeoglobus fulgidus have been expressed in E. coli [75,76]. These enzymes are thermostable, with their highest activity observed at 65 °C. As the redox midpoint potentials of NADP and cofactor F_{420} are very similar, it is perhaps unsurprising that pH has a significant influence on the equilibrium of the reaction, with the reduction of NADP^+ favored at high pH (8–10) and the reduction of F_{420} favored at low pH (4–6) [75,76]. The Fno Streptomyces griseus has also been purified and characterized, and also displayed pH-dependent reaction directionality [73]. Fno may be an excellent enzyme for in vivo reduction of cofactor F_{420}, where NADPH would be provided from central metabolism. However, for use as a cofactor F_{420} recycling enzyme in vitro, Fno would need to be coupled with an NADPH regenerating enzyme, such as an NADPH-dependent formate dehydrogenase [77]. This added complexity and cost may limit the use of Fno-dependent cofactor F_{420} recycling in vivo.
5. Cofactor production

The lack of a scalable production system for cofactor $F_{420}$ has been noted as a major impediment to the adoption of $F_{420}$-dependent enzymes by industry [5]. Cofactor $F_{420}$ is available as a research reagent (http://www.gecco-biotech.com/), but its production at scale is not yet economic. In fact, most research labs with an interest in cofactor $F_{420}$-dependent enzymes synthesize and purify the cofactor themselves using slow-growing $F_{420}$ producing microorganisms, most commonly methanogens and actinobacteria (Table 2). Economic production of cofactor $F_{420}$ at large scale is not feasible using natural producers as they are ill-suited to industrial fermentation and generally lack the genetic tools required to improve cofactor $F_{420}$ yield.

Recently, there have been significant advances towards the scalable production of cofactor for $F_{420}$-dependent enzymes. $M.\ smegmatis$ has been engineered to overexpressing the biosynthetic genes for cofactor $F_{420}$ production, leading to a substantial improvement in yields (Table 2) [78]. However, $M.\ smegmatis$ is not ideally suited as a fermentation organism as it is slow growing, forms clumps during cultivation and is not recognized as GRAS. More recently, the biosynthetic pathway for cofactor $F_{420}$ has been successfully transplanted to $E.\ coli$ [66], allowing heterologous production of the cofactor at levels similar to those of the natural $F_{420}$ producers (Table 2) [66], accumulated to 0.38 µmol of $F_{420}$ per gram of dry cells.

Table 2. Published production systems for cofactor $F_{420}$.

<table>
<thead>
<tr>
<th>Source</th>
<th>$F_{420}$ yield (µmol / g Cell weight)</th>
<th>Growth conditions</th>
<th>Ref</th>
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<tr>
<th>Organism</th>
<th>Concentration (µg)</th>
<th>Growth Conditions</th>
</tr>
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<tbody>
<tr>
<td>Methanobacterium thermoautotrophicum</td>
<td>0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Grown at 60 °C using complex media in fermenter, under pressurized hydrogen gas</td>
</tr>
<tr>
<td>Methanobacterium formicum</td>
<td>0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Grown at 37 °C using complex media in fermenters</td>
</tr>
<tr>
<td>Methanospirillum hungatii</td>
<td>0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Grown at 37 °C using complex media in fermenters</td>
</tr>
<tr>
<td>Methanobacterium strain M.o.H</td>
<td>0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Grown at 40 °C using complex media in fermenters</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum</td>
<td>1.7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Grown using complex media in fermenters, under pressurized hydrogen gas</td>
</tr>
<tr>
<td>Streptomyces flocculus</td>
<td>0.62&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Grown using complex media in fermenters</td>
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<tr>
<td>Streptomyces coelicolor</td>
<td>0.04&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Grown using complex media in fermenters</td>
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<td>Streptomyces griseus</td>
<td>0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Growth conditions not mentioned in the publication</td>
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<tr>
<td>Rhodococcus rhodochrous</td>
<td>0.11&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Grown using complex media in fermenters</td>
</tr>
<tr>
<td>Mycobacterium smegmatis</td>
<td>0.30&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Grown using complex media in fermenters</td>
</tr>
<tr>
<td>Mycobacterium smegmatis</td>
<td>3.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Overexpression of F&lt;sub&gt;420&lt;/sub&gt; pathway genes, cultivation in complex media at 37 °C in shake flasks</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Overexpressing F&lt;sub&gt;420&lt;/sub&gt; pathway genes, grown in minimal media at 30 °C in shake flasks</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mol weight of F<sub>420</sub> with 1 glutamate tail is 773.6 Da, which was used to convert values published as µg of F<sub>420</sub>, noting that micro-organisms produce mixture of F<sub>420</sub> with different number of glutamates (1–9) attached.

<sup>b</sup>Concentration estimated through absorbance at 420 nm and using extinction coefficient of 41.4 mM<sup>−1</sup>cm<sup>−1</sup>[81]

<sup>c</sup>F<sub>420</sub> concentration per g of wet cell weight

<sup>d</sup>Concentration of F<sub>420</sub> not mentioned in the publication, but F<sub>420</sub> yield was stated to be 10 times higher than wild-type M. smegmatis.

<sup>e</sup>Concentration estimated through absorbance at 400 nm and using extinction coefficient of 25.7 mM<sup>−1</sup>cm<sup>−1</sup>[79]

There is scope to further improve the production of F<sub>420</sub> in E. coli. Cofactor F<sub>420</sub> does not appear to be toxic to E. coli [66], which suggests that there is little interaction between F<sub>420</sub> and the enzymes E. coli (although this is yet to be confirmed experimentally). The thermodynamics of cofactor F<sub>420</sub> production are favorable (Appendix A), suggesting that there are no major thermodynamic impediments to improving yield. Interestingly, the first dedicated step of cofactor F<sub>420</sub> production (catalyzed by CofC/FbiD) is not energetically favorable and may consequently be sensitive to intracellular metabolite concentrations. In addition to the engineering considerations that this may impose, it may also be responsible for the biochemical diversity of this step in different microorganisms. In different microbes the CofC/FbiD-dependent step uses 2-phospholactate [78], 3-phosphoglycerate [82] or phosphoenolpyruvate [66] as a substrate, which may reflect the relative abundance of those metabolites in various bacteria and archaea and the thermodynamic constraints on this step.

Another recent advance is the production of a synthetic analog of cofactor F<sub>420</sub>, called F<sub>O</sub>-5′-phosphate (FoP). FoP was derived from F<sub>O</sub>, the metabolic precursor of cofactor F<sub>420</sub>, which is phosphorylated using an engineered riboflavin kinase [83]. FoP has also been shown to function as an active cofactor for cofactor F<sub>420</sub>-dependent enzymes activities, albeit there is a penalty in the rates of
these reactions [83]. Drenth and coworkers prepared F₀ by chemical synthesis, using a method developed by Hossain et al. [84]; however, it is likely that the engineered kinase for the phosphorylation of F₀ could be introduced into an organism that over-produces F₀ allowing for the production of F₀P by fermentation. This semisynthetic pathway would have the advantage that it needs only two biosynthetic steps, instead of the four steps needed for cofactor F₄₂₀ production, and demands less metabolic input from the native host metabolism (e.g., no glutamate is required) [83]. The production of F₀P also opens the possibility of making deazaflavin analogs of FMN and FAD, which would be electrochemically more like F₄₂₀ than flavins, but may still bind FMN and FAD-dependent enzymes and potentially allow us to access new chemistry with already well-characterized enzymes.

5. Prospects

Reduced cofactor F₄₂₀ is electrochemically well suited for biocatalytic applications, and the small number of F₄₂₀-dependent enzymes characterized to date show promise as potential biocatalysts (as discussed above). However, before these enzymes can be widely and effectively used as biocatalysts, further research will be needed to better characterize them as the biochemistry of cofactor F₄₂₀-dependent enzymes remains under-explored. The LLHT and FDOR families are a rich source of highly diverse enzymes with considerable potential for biocatalysis, albeit much of the research to date has focused on the physiological roles of these enzymes, rather than their in vitro enzymology. Although some of these enzymes have been shown to have small molecule substrates, those involved with secondary metabolite biosynthesis tend to act on high molecular weight substrates and it is not yet clear whether they will accept lower molecular weight molecules.

To be cost competitive, cofactor F₄₂₀ will need to have effective recycling systems. Enzymes for cofactor recycling have already been identified, although there have been few studies investigating their performance in this role. Moreover, alternative cofactor recycling strategies, such electrochemical or photochemical recycling, have not yet been investigated for cofactor F₄₂₀. The production of cofactor F₄₂₀ at scale and at low cost remains a roadblock for the use of these enzymes by industry. However, considerable progress has been made on this front in the last few years and it is likely that low cost cofactor F₄₂₀, or F₄₂₀ surrogates, will soon be available. Additionally, the availability of F₄₂₀-producing bacteria with tools for facile genetic manipulation, along with a growing number of empirically determined protein structure, opens up the prospect of improving this class of enzymes using in vitro evolution and rational design. It is notable that there is still some uncertainty concerning the mechanistic detail of F₄₂₀-dependent reactions, which will need to be addressed through detailed structure/function analysis to enable rational design of these enzymes.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A: Thermodynamics of F₄₂₀ biosynthesis

The thermodynamic properties of each of the steps in cofactor F₄₂₀ biosynthesis were estimated to evaluate the feasibility of increasing the production of the cofactor in an engineered microorganism. The standard transformed Gibbs free energy (ΔrGt) of each step were calculated under the physiological conditions (25°C, pH 7, and ionic concentration of 0.25 M) as described elsewhere [85,86]. The overall Gibbs free energy (ΔGt) was then calculated by summing up all individual ΔGt (Table 3). The Gibbs free energy of metabolite formation (ΔGF) for each metabolite in the pathway was obtained (Supplementary Information) from comprehensive lists of metabolites whose ΔG were estimated using
a group contribution method [87,88]. The ΔG for each metabolite was then converted into its transformed type (ΔGt) method of Alberty [86]. Data were collected from relevant biochemical databases and literature for any metabolite with missing ΔG [89-91]. Owing to possessing different protonation states, inconsistencies in ΔG of certain metabolites such as the glutamates in F420-n among databases and literature are inevitable. Thus, ΔGt for reactions containing metabolites with varying ΔG were calculated considering the differences in their ΔG leading to the generation of a total of four sets of ΔGt. Finally, the mean and standard deviations were calculated for these sets to yield the variation in each reaction as well as in the overall pathway (Table 3).

The data shown in Table 3 confirms that the overall cofactor F420 biosynthesis pathway is thermodynamically feasible under the given conditions. However, certain steps in this pathway impose a thermodynamic barrier with respect to the physiological conditions examined. For example, CofC production (i.e., F420) molecule seems to be the most favorable step among other F420 biosynthesis of F420 appears to be the most thermodynamically favorable step in the whole pathway driving the energy-dependent synthesis of EPPG, one of the precursors for making F420. CofG/H combined seems to be the most thermodynamically favorable step in the whole pathway possibly due to the high levels of inconsistencies of data available for ΔG of certain metabolites such as the glutamates in F420 biosynthesis. Interestingly, formation of F420-2 molecule seems to be the most favorable step among other F420 molecules downstream of the pathway. It should be noted that the thermodynamic calculations were only performed up to three steps of F420 molecule production (i.e., F420-3) largely because of the high levels of inconsistencies of data available for ΔG of higher F420 molecules.

### Table 3. Standard transformed Gibbs free energy of reaction (ΔGt), for the F420 biosynthesis pathway shown in Figure X, calculated based on Gibbs free energy of metabolite formation (ΔGf) calculated at 25°C, pH of 7, and ionic concentration of 0.25 M.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
<th>ΔGf (kJ)</th>
<th>ΔGt (kJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CofC / FbiD</td>
<td>PEP + GTP → EPPG + PPI</td>
<td>+71.27±(67)</td>
<td>+25.13±(44)</td>
</tr>
<tr>
<td>CofG / FbiC</td>
<td>5ARP + Tyr + SAMe → 5ARP4HB + ImAcet + Met + 5AD</td>
<td>-1192.39±(36)</td>
<td>-1080.88±(80)</td>
</tr>
<tr>
<td>CofH / FbiC</td>
<td>5ARP4HB + SAMe → Fd + NH3 + Met + 5AD</td>
<td>+71.90±(36)</td>
<td>+29.56±(33)</td>
</tr>
<tr>
<td>CofD / FbiA</td>
<td>Fd + EPPG → d420-0 + GMP</td>
<td>-31.3±(128)</td>
<td>-24.5±(115)</td>
</tr>
<tr>
<td>CofX / FbiB</td>
<td>d420-0 + FMNH2 → F420-0 + FMN</td>
<td>-74.59±(87)</td>
<td>-67.8±(83)</td>
</tr>
<tr>
<td>CofE / FbiB</td>
<td>F420-0 + GTP + Glu → F420-1 + GDP + Pi</td>
<td>-7.5±(24)</td>
<td>-4.3±(23)</td>
</tr>
<tr>
<td>CofE / FbiB</td>
<td>F420-1 + GTP + Glu → F420-2 + GDP + Pi</td>
<td>-39.4±(35)</td>
<td>-32.8±(34)</td>
</tr>
<tr>
<td>CofE / FbiB</td>
<td>PEP + 5ARP + Tyr + (2) SAMe + FMNH2 + (3) Glu + (4) GTP</td>
<td>-21.99±(38)</td>
<td>-19.49±(37)</td>
</tr>
</tbody>
</table>

Overall → F420-3 + (2) Met + (2) 5AD + ImAcet + NH3 + FMN + (3) GDP + (3) Pi + GMP + PPI | -1224.05±(82) | -1160.5±(79) |

*For simplicity, protons were omitted in these equations and subsequent calculations as the ΔG of a proton under the set conditions is ~0.08 kJ. However, all ΔGt calculations are based on a balanced equation. The mean values of four sets and their standard deviations in parenthesis shown for each reaction.*

Abbreviations:

5AD: 5'-Deoxyadenosine; 5ARP: 5-amino-6-(d-ribitylamino)uracil; 5ARP4HB: 5-amino-5-(4-hydroxybenzyl)-6-(d-ribitylimino)-5,6-dihydrouracil; d420-0: Dehydro coenzyme ferredoxin Fd-0 (oxidized); EPPG: Enolpyruvyl-diphospho-5'-guanosine; Fd: 7,8-Dimethyl-8-hydroxy-5-deazariboflavin; Fd-0: Coenzyme ferredoxin Fd-0 (oxidized); Fd-1: Coenzyme ferredoxin Fd-1 (oxidized); Fd-2: Coenzyme ferredoxin Fd-2 (oxidized); Fd-3: Coenzyme ferredoxin Fd-3 (oxidized); FMN: Flavin mononucleotide (oxidized); FMNH: Flavin mononucleotide (reduced); GDP: Guanosine diphosphate; GMP: Guanosine monophosphate; Glu: l-Glutamate; GTP: Guanosine triphosphate; H+: Proton; ImiAce: 2-iminoacetate or Dehydroglycine; Met: l-Methionine; NH3: Ammonium; PEP: Phosphoenolpyruvate; Pi: Phosphate; PPI: Diphosphate; SAMe: S-Adenosyl-l-methionine; Tyr: l-Tyrosine
References


