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Abstract

Coenzymes are ubiquitous in Nature, assisting in enzyme-catalysed reactions. Several coenzymes, nicotinamides and flavins, have been known for close to a century, whereas variations of those organic molecules have more recently come to light. In general, the requirement of these coenzymes imposes certain constraints for *in vitro* enzyme use in biocatalytic processes. Alternative coenzymes have risen to circumvent the cost factor, tune reaction rates or obtain different chemical reactivity. This review will focus on these alternatives and their role and applications in biocatalysis.

Introduction on coenzymes

Coenzymes are organic molecules that assist certain enzymes in catalysis. Many coenzymes are vitamins or derivatives thereof, and often contain an adenosine monophosphate (AMP) moiety such as in β-nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), adenosine triphosphate (ATP) or coenzyme A. The common evolutionary origin of these cofactors made them indispensable for *in vivo* cellular metabolic processes. When applied to *in vitro* biocatalytic processes however, cost, instability or restricted reactivity may impede further development.

This review aims at describing new nicotinamide and flavin coenzyme derivatives that were discovered in Nature, as well as alternative synthetic coenzymes and their role and applications in biocatalysis. Nicotinamide and flavin coenzymes in oxidoreductases are first discussed, followed by *S*-adenosyl-L-methionine (SAM) in transferases. A previous review gives more details about the functional diversity of all the different coenzymes [1].

Alternative coenzymes for oxidoreductases

Oxidoreductases account for a quarter of all enzymes in the Enzyme nomenclature database (ExPaSy). Their substrate scope and large pool of diverse reactions lead to a wide range of applications and have brought oxidoreductases at the forefront in biotechnology and the pharmaceutical sector, where two-thirds of chiral products are obtained by enzymatic catalysis [2]. A remarkable proportion of oxidoreductases require β-nicotinamide adenine dinucleotides (NAD/NADP) or flavins (FAD/FMN) as coenzymes. NAD, a vitamin B3 derivative, is a ubiquitous redox cofactor in living cells central to many cellular processes that can act as an electron donor or acceptor through the release or acceptance of a hydride (Figure 1). Recently, a new nickel pincer cofactor was discovered in a lactate racemase enzyme. This (SCS)Ni(II) pincer complex (Figure 1) is derived from nicotinic acid and is involved in a hydride transfer for the racemisation of L-lactate [3].

NAD(P)-dependent enzymes represent half of the oxidoreductase activities registered in the Braunschweig Enzyme Database (BRENDA) [4]. The current price of these coenzymes can range from € 1400 (NAD) to € 70,000 (NADPH) per mole [5]. To reduce costs of biocatalysed redox reactions,

several well-established methods for NAD(P)H regeneration are available (see Table 1 for a comparison) [6, 7]. Nevertheless, significant efforts are undertaken to develop simpler, more efficient alternatives [5, 8]. Natural-based NADH analogues have been used to investigate the influence of substituents on the dihydropyridine ring, and synthetic nicotinamide coenzyme biomimetics (NCBs) were produced to investigate the hydride transfer mechanism, but more recently were attractive to provide inexpensive alternative coenzymes (Figure 1) [5, 9-11].

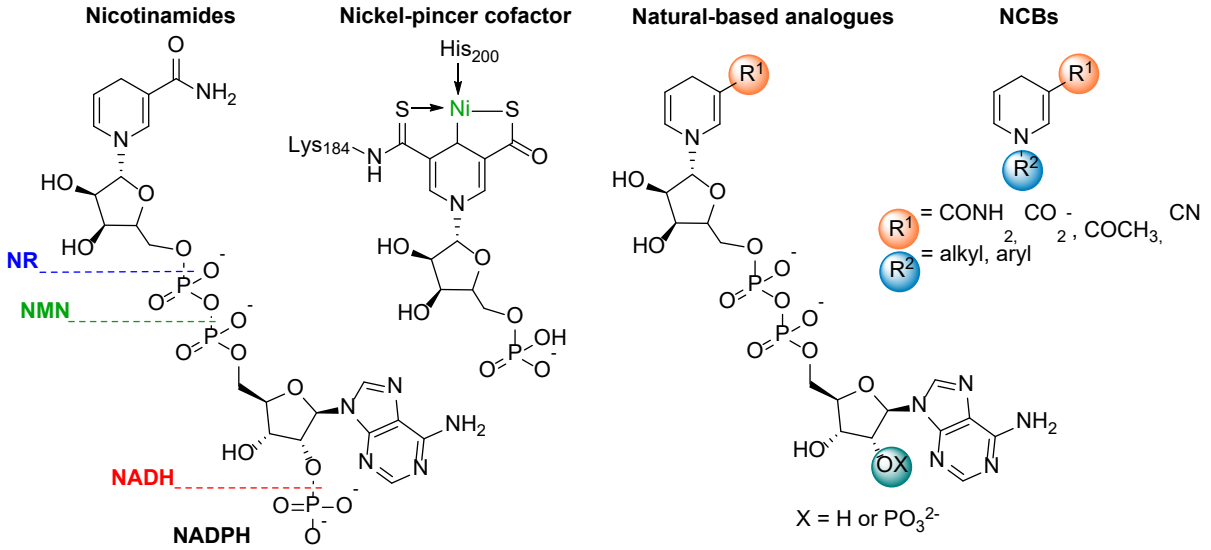

Figure 1. Schematic structures of nicotinamide coenzymes and derivatives (reduced forms; NR = nicotinamide ribose, NMN = nicotinamide mononucleotide).

Natural- and sugar-based (nicotinamide ribose NR, nicotinamide mononucleotide NMN) NAD analogues can be expensive alternatives to use in biocatalytic processes, whereas NCBs can be easily synthesised in good yields starting from cost-effective pyridine derivatives [5]. NCBs have been used in stoichiometric amounts since their *in situ* recycling is currently an open challenge. Nonetheless, when compared to the costs and disadvantages of enzymatic NAD(P)H recycling methods, which up to now are the only ones applied at industrial scale [7], stoichiometric amounts of biomimetics are shown to be viable (Table 1).

Table 1. Main nicotinamide coenzyme regeneration strategies and the use of stoichiometric amounts of NCBs.[a]

Approach	Pros	Cons	Selected reagents and coenzymes involved
Enzymatic *Coupled enzyme*	High TTN and selectivity	Enzyme instability and additional cost Interaction between species Product isolation	*NAD(P)H:* FDH[b] + formate; GDH[c] + glucose *NAD(P):* GLDH[d]
Enzymatic *Coupled substrate*	High TTN and selectivity	Product isolation Co-substrate in excess	*NAD(P)H:* isopropanol *NAD(P):* acetone
Chemical	Low cost	Low TTN Low selectivity	*NAD(P) and NAD(P)H:* NCBs; M[e]; $Na_2S_2O_4$
Electrochemical	Electrical energy as electron source	Low TTN and selectivity Electrode fouling Mediator required	*NAD(P)H:* modified electrode surface + M[e] or methylene blue *NAD*: modified electrode surface + ABTS^{2-}[f]
Photochemical	Solar energy as electron source	Mediator and photosensitizer required Low efficiency with visible light	*NADH:* carbon-doped TiO_2 + M[e] + 2-mercaptoethanol + H_2 *NAD(P)H:* oligothiophene + methyl viologen + EDTA + FDR[g]
Stoichiometric amount of NCB[h]	Low cost	Applicable only with NCB-accepting enzymes	*BNAH*

[a]Adapted from [6]; TTN = total turnover number; [b]Formate dehydrogenase; [c]Glucose dehydrogenase; [d]Glutamate dehydrogenase; [e]M=[Cp*Rh(bpy)(H_2O)]$^{2+}$; [f]2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate); [g]Ferrodoxin-NADP reductase; [h]Enzymatic and (photo)chemical recycling available however not currently efficient, see [11].

Flavin cofactors are omnipresent in Nature and are involved in a wide variety of chemical reactions [12, 13]. The most common flavin cofactors, FAD and FMN, are derived from vitamin B2 (riboflavin;

Figure 2), and occur as redox-active prosthetic groups in about 2% of all proteins [14]. In many flavoenzymes, including dehydrogenases, reductases and monooxygenases, the flavin cofactor exchanges electrons with NAD(P)(H). In most of these enzymes, the NAD(P)(H) co-substrate binds in an elongated conformation, and its nicotinamide moiety meets the isoalloxazine ring of the flavin for hydride transfer at the interface between the NAD- and FAD-binding domains [15-17].

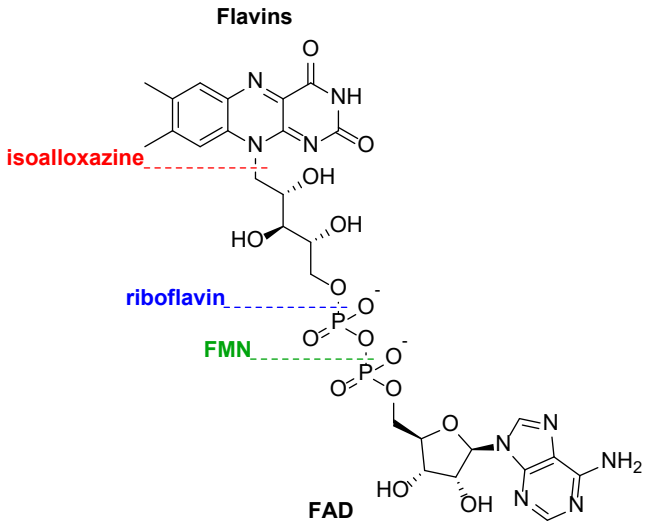

Figure 2. Schematic structural representation of flavin coenzymes.

Coenzymes for FMN-dependent ene-reductases and other reductases

Flavin-dependent ene-reductases (ERs) of the old yellow enzyme (OYE) family catalyse a wide range of asymmetric hydrogenation reactions [18]. NCBs are well accepted by OYEs, with variations. Since the first study published in 2013 [19], two main studies were carried out [20, 21]. Several NCBs (BNAH, BuNAH, BAPH, CO_2NAH, CNNAH, Figure 3) were screened against a panel of OYEs for the asymmetric reduction of α,β-unsaturated ketones and aldehydes (Figure 4a), leading to conversions and enantiomeric excess (*ee*) as good as those with the preferred natural coenzyme NADPH and even better conversion than with NADH [20]. It is worth noting that a substituted hydride donor such as the Hantzsch ester (HEH) does not seem to be accepted for steric reasons [19].

For three OYEs, PETNR, TOYE and XenA, the k_{cat} values were comparable and slightly higher for NCBs compared to NADPH, whereas the K_M values varied depending on the OYE and NCB: with PETNR and XenA, BNAH and BuNAH had a higher affinity than NADPH, but a lower affinity with TOYE. Interestingly, the rates of reduction of the flavin cofactor by these NCBs were orders of magnitude higher than with the natural coenzyme, research to explain this effect is ongoing [22]. For the nitrile substituted analogue (CNNAH), XenA was one of the few enzymes found (along with *Ts*OYE and *Rm*OYE) that gave high conversion [20]. According to the OYE classification proposed by Scholtissek *et al.*, XenA belongs to class III OYEs, which contains the only ERs showing activity with this mimic [23]. The BNAH-mediated reduction of ketoisophorone by *Ts*OYE was also coupled with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as a regeneration system for the mimic [20]. An iridium-based artificial metalloprotein was used to recycle MNAH, BNAH, BAPH, CO_2NAH and CNNAH for the *Ts*OYE-catalysed reduction of cyclic enones and a maleimide [24].

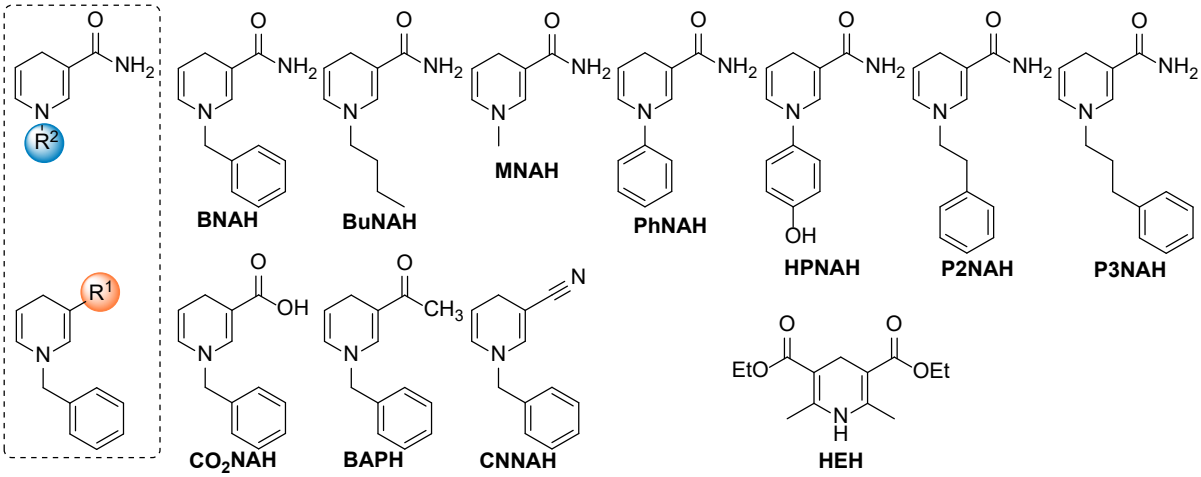

Figure 3. Structures of NCBs (reduced form) recently used for NAD-dependent oxidoreductase-catalysed reactions, and the Hantzsch ester HEH.

In another study, BNAH and its derivatives PhNAH and HPNAH (Figure 3) were tested on four OYEs, MR, NCR, OYE1 and OYE3, with enal substrates. All three synthetic coenzymes were accepted and HPNAH performed best with NCR [21]. Notably, these mimics showed efficient conversions with both isomers of citral, whereas this terpene is not efficiently reduced by NCR in presence of NADH [21].

Interestingly, a non-flavin reductase from *Nicotina tabacum* (*Nt*DBR) could catalyse the reduction of cinnamaldehyde with mimics BNAH, BAPH, CO_2NAH and BuNAH, albeit with low conversions [20]. An azoreductase AzoRo was used with BNAH, displaying a higher activity for the degradation of the dye methyl red at neutral pH [25].

Coenzymes for NAD(P)-dependent dehydrogenases, enzymatic NCB recycling and stability

To date, the regeneration of NCBs has been limited to chemical, chemoenzymatic and electrochemical methods, because of the lack of dehydrogenases able to accept those biomimetics [5, 11, 24]. Recently, a glucose dehydrogenase from *Sulfolobus sulfataricus* (*Ss*GDH) and its variants were tested with BNA, P2NA and P3NA (Figure 3 and 4b). The best kinetics results with the mimics were achieved with a double mutant that showed improved catalytic efficiencies for all the analogues, in particular P2NA [26]. Based on this, the reduction of 2-methylbut-2-enal by *Ts*OYE and P2NAH as coenzyme was coupled with the *Ss*GDH double mutant for the regeneration of P2NAH with a turnover frequency (TOF) of 99 h^{-1}. Control reactions revealed trace amounts of natural NADH present after purification of the mutant [26]. The same mimics were tested with other commercially available recycling enzymes and horse liver alcohol dehydrogenase (HLADH) but no activity was detected with purified enzyme [26].

Knaus *et al.* used a NADPH oxidase from *B. subtilis* with mimics BNAH, BAPH, CO_2NAH and BuNAH [20], while the group of Sieber used an NADH oxidase from *Lactobacillus pentosus* (*Lp*Nox) for the recycling of the oxidized form of BNAH and MNAH [27]. *Lp*Nox was active with P2NAH and P3NAH as well, with a catalytic efficiency of up to 0.49 $mM^{-1}s^{-1}$ with P3NAH [28].

NCBs are not necessarily more stable than their natural counterparts and are also sensitive to specific pH ranges, buffers and temperatures. Thus, efforts have been made recently to overcome the high instability of the mimic model BNAH. The derivative P2NAH was described to be as stable as NADH due to a potential stacking of the phenyl group against the dihydronicotinamide ring [28].

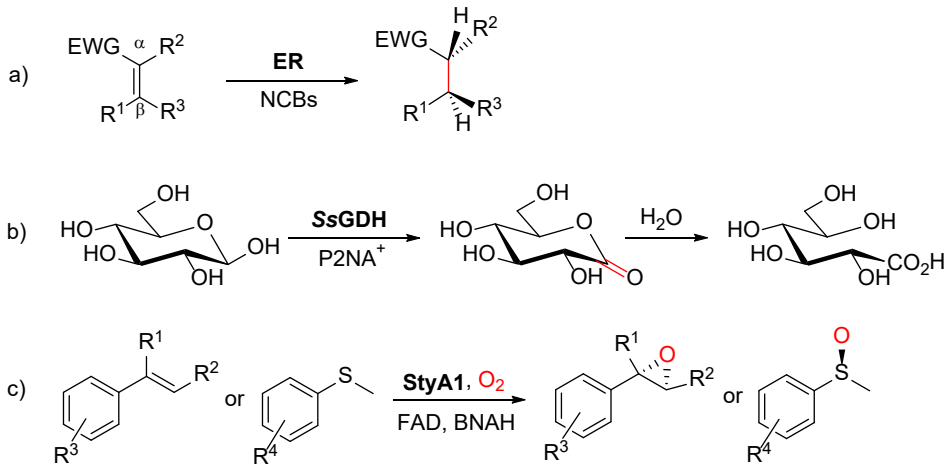

Figure 4. NAD-dependent oxidoreductase-catalysed reactions with NCBs. a) ER-catalysed asymmetric reduction of activated alkenes; b) GDH-catalysed oxidation of glucose and spontaneous hydrolysis of gluconolactone; c) Styrene monooxygenase-catalysed asymmetric epoxidation or sulfoxidation.

BNAH with two-component FAD-dependent monooxygenases

Two-component flavin-dependent monooxygenases use a reductase component (StyB) for generating reduced flavin [29]. The NCB model BNAH was used to directly reduce free FAD, which then could bind to styrene monooxygenase StyA1 from *Rhodococcus opacus* 1CP to generate the flavin hydroperoxide oxygenation species (Figure 4c). StyA1 was shown to catalyse the oxidation of styrene derivatives to their corresponding epoxides, retaining (*S*)-enantioselectivity. For styrene, a 433 h^{-1} TF was obtained compared to a 175 h^{-1} TF with the natural bi-enzymatic StyA1/StyB system [30]. Upscaling of the sulfoxidation of thioanisole in presence of BNAH gave 53% yield in two hours and 99% *ee* [30]. Therefore, the natural reductase partner StyB is not needed for the generation of $FADH_2$. The coenzyme mimic showed moderate to excellent electron transfer efficiency for both epoxidation and sulfoxidation reactions.

Artificial flavins for flavin-dependent enzymes

Kinetic studies on apoflavoproteins reconstituted with artificial flavins have provided valuable information on the accessibility of the flavin ring in these proteins [31]. Initially, artificial flavins were either used as chemically reactive or mechanistic probes. More recent work has shown that these compounds, especially when used in combination with protein engineering strategies, can also be useful for biocatalysis [13, 32].

Martinoli *et al.* showed that 1-deaza-FAD and a set of mono- or dichlorinated FADs (Figure 5a) can replace the natural FAD cofactor in the phenylacetone monooxygenase (PAMO)-mediated conversion of racemic bicyclo[3.2.0]hept-2-en-6-one [33]. In this Baeyer-Villiger oxidation reaction, which theoretically can yield four stereo- and regiodivergent products, the natural PAMO first preferentially converts the (1*R*,5*S*)-ketone to the “normal” lactone with an *ee* in favour of the (1*S*,5*R*)-enantiomer (Figure 6a). After depletion of the (1*R*,5*S*)-substrate, the (1*S*,5*R*)-ketone is also converted, yielding mainly the abnormal lactone. The PAMO variants reconstituted with artificial flavins gave virtually similar reaction patterns, although their reaction rates were somewhat slowed down. NADPH was also replaced with the acetylpyridine analogue APADPH (Figure 1 natural-based analogue, $R^1 = COCH_3$, X = PO_3^{2-}), which led to inefficient phenylacetone conversion.

Su *et al.* showed that exchange of the FMN cofactor of iodotyrosine deiodinase with a 5-deaza-FMN analogue (Figure 5a) suppresses the dehalogenase activity and leads to nitroreductase activity that supports full reduction of 2-nitrotyrosine to the amine product in the presence of sodium borohydride

[34].

Alternative artificial flavins in organic chemistry

Several studies reported the preparation of flavin adducts including flavin peptide polymer hybrids that might act as organocatalysts in biomimetic oxygenation reactions [35-37]. Bou-Nader *et al.* synthesised a flavin-methylene iminium compound that could act as a catalytically competent coenzyme intermediate of reconstituted RNA methyltransferase and thymidylate synthase [38].

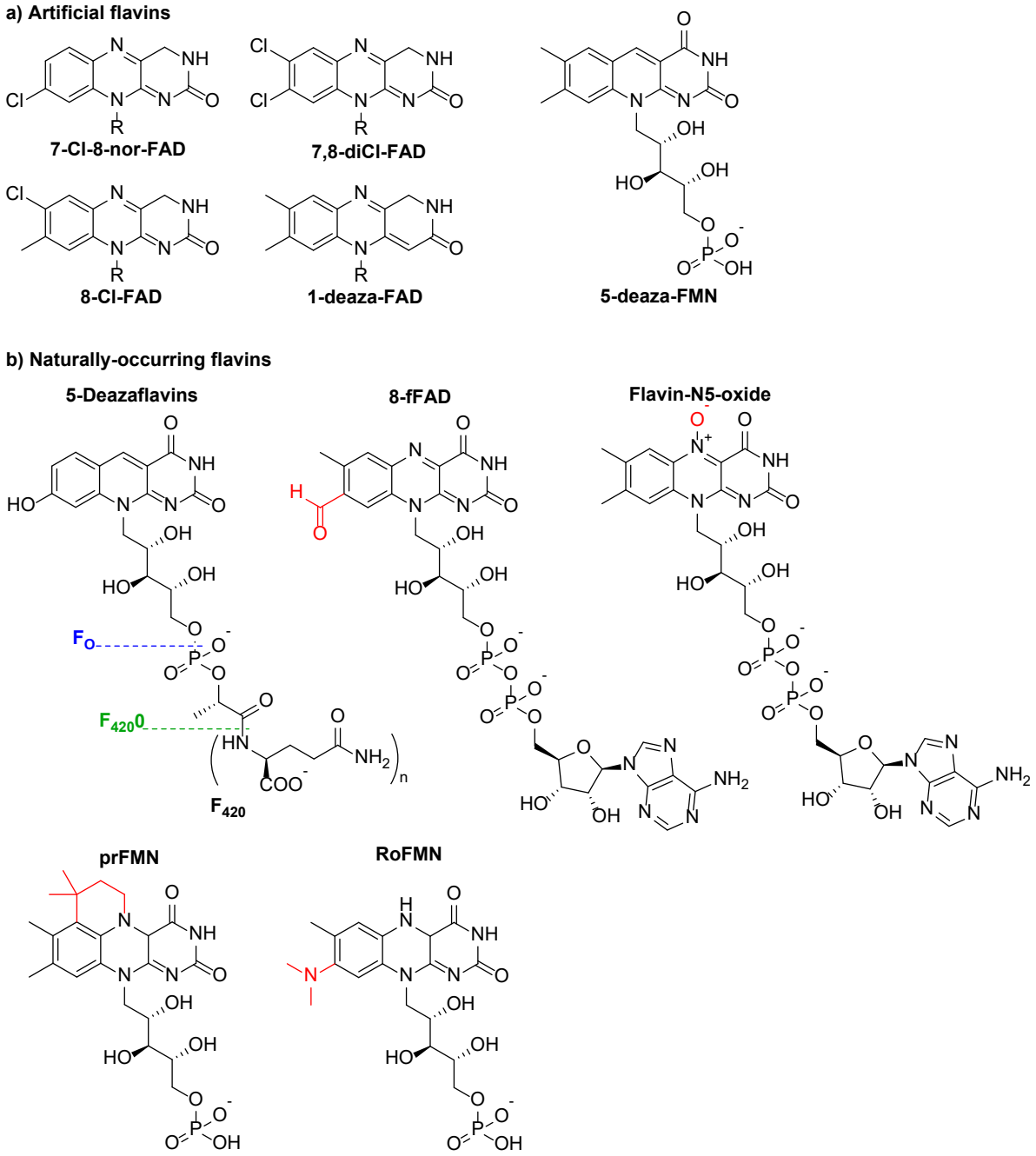

Figure 5. Schematic structural representation of flavin derivatives.

Naturally-occurring flavin derivatives

Recently, several new flavin-based cofactors were discovered (Figure 5b) [39]. 8-Formyl-FAD (8-fFADH) appeared to be the native cofactor in formate oxidase (FOX) [40]. A flavin-N5-oxide cofactor was encountered in EncM [41], while a prenylated form of FMN (prFMN) was found in (de)carboxylases (Figure 6b) [42].

Next to the FAD and FMN scaffolds, there is another natural flavin-like cofactor. This rare F_{420} deazaflavin cofactor, containing an oligoglutamate side chain of different length (Figure 5b), has recently been detected in a broad range of aerobic bacteria and therefore is more widespread than previously thought [43]. The redox potential of free F_{420} (-340 mV) is somewhat lower than that of free NAD(P)H (-320 mV) and much lower than that of free FMN/FAD (-220 mV). This makes F_{420} an obligate two-electron carrier that can perform a wide range of chemically demanding redox reactions [44, 45]. Kumar *et al.* isolated and characterized a thermostable F_{420}:NADPH oxidoreductase (FNO) from *Thermobifida fusca* [46]. Greening *et al.* showed that F_{420}-dependent reductases (FDRs) can reduce α,β-unsaturated alkenes [47]. Following this concept, Mathew *et al.* reported that FDRs from *Rhodococcus jostii* RHA1 can regioselectively reduce α,β-unsaturated ketones and aldehydes with high yields and excellent enantioselectivity (Figure 6c) [48]. They also found that the enantioselectivity of FDRs differs from that of FMN-dependent ene reductases [18].

The antibiotic roseoflavin (8-amino-8-demethyl-riboflavin) is another natural flavin that has been tested as an alternative coenzyme for flavoproteins [49]. Roseo-FMN (RoFMN, Figure 5b) bound with high affinity to apo azobenzene reductase, and the reconstituted enzyme showed about 30% activity of the native enzyme.

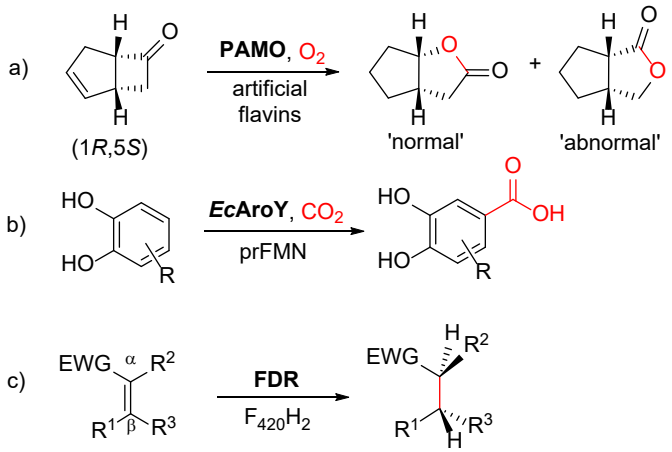

Figure 6. Flavin-dependent oxidoreductase-catalysed reactions with flavin derivatives. a) PAMO-catalysed oxidation of bicyclo[3.2.0]hept-2-en-6-one to the 'normal' or 'abnormal' lactone; b) Reaction catalysed by decarboxylase AroY from *Enterobacter cloacae* using the prenylated FMN; c) $F_{420}H_2$-dependent reductase-catalysed reduction of activated alkenes, giving opposite stereochemistry to that with OYEs.

Other alternative coenzymes

S-adenosyl-L-methionine (SAM) is a coenzyme required for the trans-methylation of biomolecules, playing a significant role in epigenetic regulation, cellular signalling and metabolite degradation [50]. SAM-dependent methyltransferases (MTases) are turning into versatile catalysts and advances of MTase in biocatalysis have been recently reviewed [51]. Several SAM analogues have been synthesised to transfer the activated groups on MTase substrates (Figure 7). As the *in situ* formation of the SAM coenzyme requires ATP, complex regeneration systems are being developed [52]. As well, an *S*-adenosyl-L-homocysteine hydrolase, providing the adenosine for SAM, requires NAD as a cofactor. Attempts to use NCBs resulted in finding a potential inhibitor [53].

MTases were also used for an enzymatic Friedel-Crafts alkylation reaction. Although the substrate specificity of the enzymes ranged from high to moderate, the cofactor scope is broad. Modified SAMs with alkyl groups other than methyl were used for biocatalytic Friedel-Crafts alkylation, achieving excellent conversions [54].

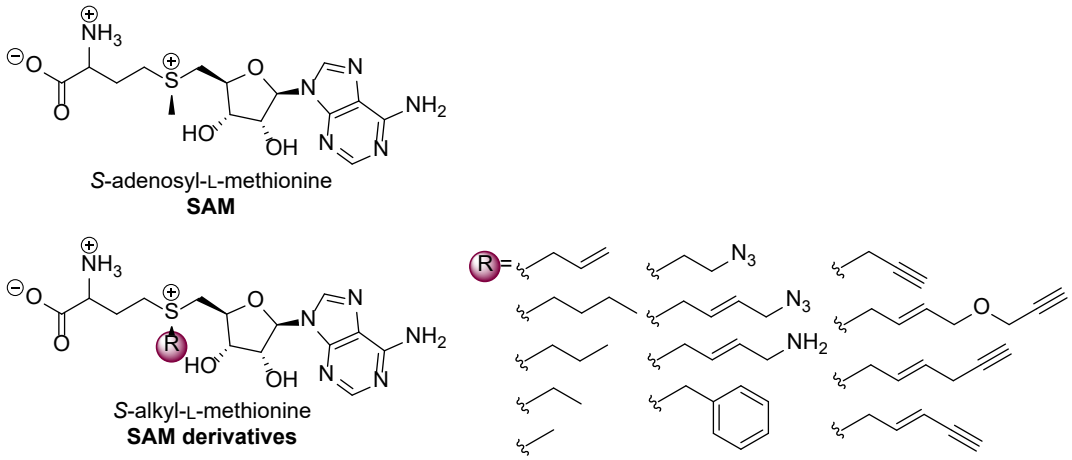

Figure 7. Schematic representation of SAM cofactor and derivatives reviewed in [51].

Conclusions and perspectives

The recent discovery of new coenzymes such as the nickel pincer complex and prenylated flavin shows that Nature is resourceful and stimulates research towards unravelling new reaction mechanisms. The use of alternative synthetic coenzymes for biocatalysis is promising: cost aside, the ability to change reaction rates or the type of reaction enables to catalyse highly selective reactions previously thought overly challenging. We hypothesise that NCBs are scarcely accepted by dehydrogenases due to their lack of an adenosine moiety required for enzyme recognition. This line of research would greatly benefit from a quality structure-activity relationship analysis, with the remaining challenge to alter cofactor specificity through protein engineering.

Declarations of interest

There are no conflicts of interest.

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