



Engineering natural and noncanonical nicotinamide cofactor-dependent enzymes: design principles and technology development

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Nicotinamide cofactors enable oxidoreductases to catalyze a myriad of important reactions in biomanufacturing. Decades of research has focused on optimizing enzymes which utilize natural nicotinamide cofactors, namely nicotinamide adenine dinucleotide (phosphate) (NAD(P)⁺). Recent findings reignite the interest in engineering enzymes to utilize noncanonical cofactors, the mimetics of NAD⁺ (mNADs), which exhibit superior industrial properties *in vitro* and enable specific electron delivery *in vivo*. We compare recent advances in engineering natural versus noncanonical cofactor-utilizing enzymes, discuss design principles discovered, and survey emerging high-throughput platforms beyond the traditional 96-well plate-based methods. Obtaining mNAD-dependent enzymes remains challenging with a limited toolkit. To this end, we highlight design principles and technologies which can potentially be translated from engineering natural to noncanonical cofactor-dependent enzymes.

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Introduction

Nicotinamide cofactor-utilizing enzymes are versatile catalysts for both *in vitro* chemical synthesis and *in vivo* metabolic engineering. Although more than 15,000 sequences have been confirmed or predicted to encode NAD(P)⁺ or NAD(P)H utilizing enzymes [1], natural enzymes frequently do not meet the catalytic needs of compatibility with the metabolism of a chassis host *in vivo* and viability at large scales *in vitro*, and often require

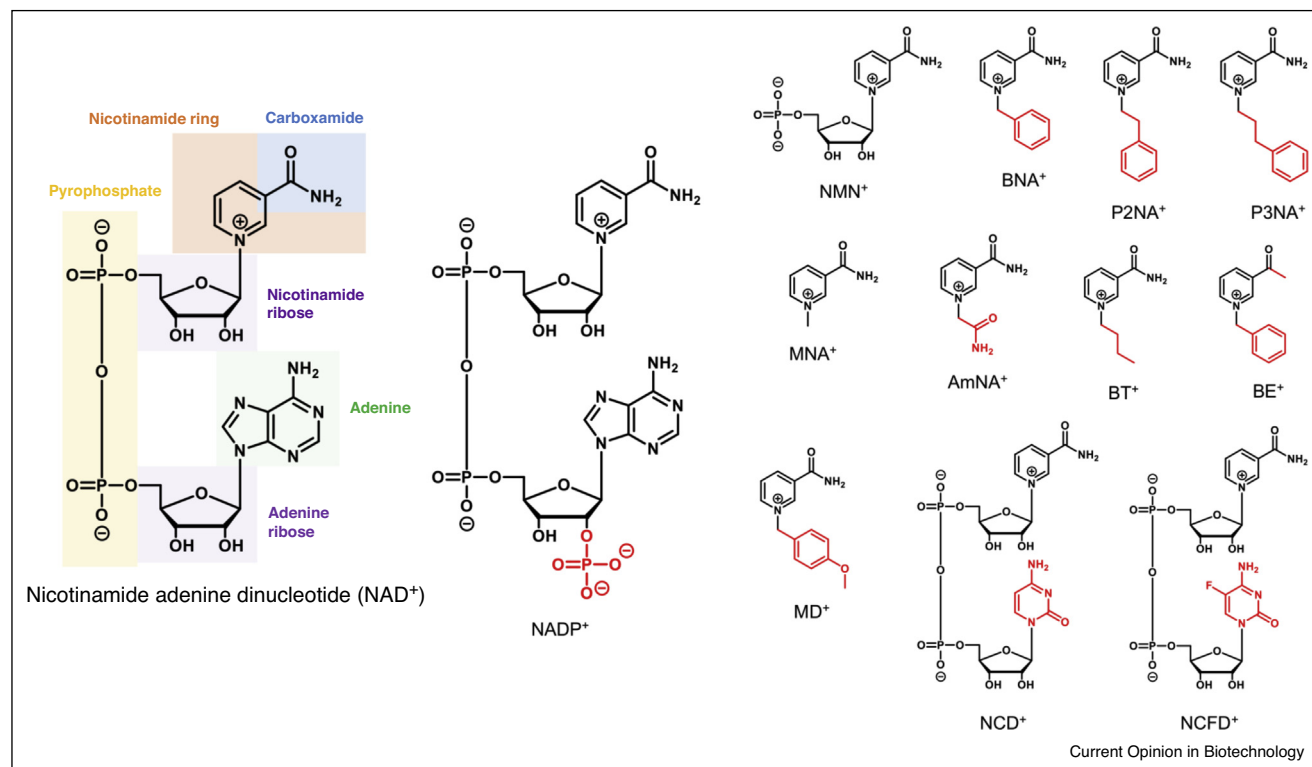
engineering of the enzyme's natural cofactor specificity, substrate scope, and robustness.

Recent studies highlight the value of engineering these enzymes to use noncanonical nicotinamide cofactors, which are mimetics of NAD⁺ (mNADs). The nicotinamide ring is the only fragment required for a small molecule to function as a redox cofactor [2–4]. Molecules with alternative functional groups replacing the NAD(P)/H carboxamide [4], the adenine base [5,6,7], the nicotinamide ribose [8], and mimics truncated at different atoms have been explored as artificial redox cofactors [9,10] (Figure 1). These mimics have industrial value for lowering feedstock costs as mNADs are often simpler to synthesize [11] and have greater stability than native cofactors [12], permit access to new chemistries with altered redox potential [13], reduce oxygenase decoupling [14,15], and importantly enable specific delivery of electrons in both whole cells and crude cell lysates [9,11,16]. Metabolic pathways engineered to specifically utilize mNADs are orthogonal from the host's metabolism, as they do not cross-talk with native pathways which only use natural cofactors. This allows precise control of chemical reactions in the cells without interference and has been demonstrated *in vivo* for the production of malate from the carboxylation of pyruvate via nicotinamide cytosine dinucleotide (NCD⁺) [6,16], and selective generation of the pharmaceutical intermediate levodione by nicotinamide mononucleotide (NMN⁺) mediated reduction [9].

Design principles in engineering natural cofactor-dependent enzymes

Many general design principles are derived from decades of research in engineering NAD(P)/H-dependent enzymes. For example, altering specificity between natural cofactors commonly relies on the mutagenesis of binding pocket amino acids interacting with the signature 2'-phosphate or 2'-hydroxyl groups that differentiate NADP(H) and NAD(H), respectively. Fundamental semi-rational design rules have been captured by an easy-to-use web tool Cofactor Specificity Reversal-Structural Analysis and Library Design (CSR-SALAD) [17]. This computational method incorporates structural activity and genetic information to automatically design focused libraries [18]; however, it has met with limited success for enzymes that utilize cofactors in complex reaction mechanisms [17,19]. Flexible loop grafting has emerged as another design principle in engineering the

Figure 1



Chemical structures of natural nicotinamide redox cofactors and mNADs. The natural nicotinamide redox cofactor NAD⁺ is composed of the catalytic nicotinamide ring attached to a ribose, pyrophosphate, a second ribose, and adenine base. NADP⁺ differs in that a phosphate group replaces a hydroxyl on the 2'-carbon of the adenine ribose. mNADs maintain the central nicotinamide ring, but are truncated or incorporate alternative functional groups compared to NAD⁺. The cofactors are illustrated in their oxidized form, complete cofactor names are listed under Tables 1 and 2.

cofactor preference of TIM barrel oxidoreductases. Swapping of cofactor binding loops between homologous ene reductases (ERs) with NADPH and NADH preference [20] shows promise as a means of generating flexibility in cofactor preferences. Additional studies on aldo-keto reductase (AKR) inverted cofactor preference from NAD⁺ to NADP⁺ by inserting either additional residues [21[•]] or a calcium controllable repeats-in-toxin (RTX) domain [22] into substrate binding loops.

Beyond cofactor specificity, complex traits such as stability and conformational dynamics are a challenging task for rational design. Recent reports revealed the effects of modulating the microenvironment surrounding oxidoreductases, which can potentially be a universal design principle in engineering both NAD(P)/H and mNAD-dependent enzymes. For example, fusing a variant of superfolder green fluorescent protein (sfGFP) with extreme surface charges enhanced the activity of AKR, possibly by influencing the apparent ionic strength of the active site [23[•]]. Furthermore, increased cofactor availability has been explored with DNA-enzyme nanostructures [24] acting as local reservoirs of cofactors, fusing of

redox cycling partners by co-expression [25–29], and directly tethering NAD(H) to proteins with polyethylene glycol chains [30].

Design principles in engineering noncanonical cofactor-dependent enzymes

We summarize efforts in enhancing mNAD catalysis and evaluate the extent of success through the following metrics [31] (Tables 1 and 2):

- (1) Coenzyme Specificity Ratio (CSR), a measure of preference for the mNAD over natural cofactors. While most wild type enzymes use mNADs very poorly, as reflected by near zero CSR, many flavoenzymes including enoate reductases, nitroreductases, and para-hydroxybenzoate hydroxylase exhibit promising activities [9[•],14,32] (Table 1). In particular, the xenobiotic reductase from *Pseudomonas putida* (*P. putida* XenA) utilizes a range of mNADs more efficiently than natural cofactors [32] (Table 1). High CSR is desirable for creating orthogonal redox circuitry [6,9[•],16]; however, while most studies only

consider NAD^+ , it is important to measure CSR for both NAD^+ and NADP^+ [9•] when determining orthogonality *in vivo*.

$$CSR = \frac{\left(\frac{k_{cat}}{K_m}\right)_{mNAD}}{\left(\frac{k_{cat}}{K_m}\right)_{NAD(P)}} \quad (1)$$

- (2) Relative Catalytic Efficiency (RCE) is the ratio of the mutant's catalytic efficiency with mNAD compared to wild type with native cofactor. Since wild type enzymes have been optimized by Nature with its native cofactor, RCE essentially indicates how effective the engineering approaches are compared to natural evolution. RCEs for mNADs are extremely low for most engineered enzymes, indicating that catalytic activities are a small fraction of the wild type with native cofactor. We note that P450-BM3 R966D-W1046S reported by Lo *et al.* [33] had an exceptional RCE of ~96 for 1-benzyl-1,4-dihydronicotinamide (BNAH) and ~60 for N-4-methoxybenzyl-1,4-dihydronicotinamide (MDH) (Table 2). In comparison, RCEs of >1 are frequently achieved in switching NAD^+ and NADP^+ specificity [31].

$$RCE = \frac{\left(\frac{k_{cat}}{K_m}\right)_{mNAD}^{mut}}{\left(\frac{k_{cat}}{K_m}\right)_{NAD(P)}^{WT}} \quad (2)$$

- (3) Relative Specificity (RS), the CSR of a variant compared to that of the wild type, which is often referred to as the fold of cofactor specificity switch toward noncanonical cofactors. This parameter is useful for comparing the effectiveness of different engineering approaches in general, independently of the specific enzymes targeted (Table 2).

$$RS = \frac{\left(\frac{k_{cat}}{K_m}\right)_{mNAD}^{mut}}{\left(\frac{k_{cat}}{K_m}\right)_{NAD(P)}^{WT}} \quad (3)$$

Because the number of successful cases is still relatively small, core design principles for switching cofactor specificity toward noncanonical cofactors have yet to clearly emerge. The field still largely relies on semi-rational and random engineering which often yields beneficial mutations with unknown mechanisms. Gaining fundamental understanding on enzyme-mNAD interaction through structural and kinetic studies is crucial to deriving design principles to streamline engineering. Nevertheless, the following trends are notable:

First, relaxation of cofactor specificity is linked to enhanced activity with mNADs. *Bacillus stearothermophilus* lactate dehydrogenase F16Q-C81S-N85R with specificity switched from NAD^+ to NADP^+ was found to

reduce NMN^+ with trace activity [34]. The K249G-H255R variant of *Pyrococcus furiosus* alcohol dehydrogenase designed to increase the volume of the active site for NADP^+ binding unexpectedly gained the ability to utilize NMN^+ (Table 2), and showed a 40% increase in maximum current density when used in a biofuel cell, postulated to be due to improved mass transfer of NMN^+ compared to NAD^+ [35]. The P450-BM3 mutant R966D-W1046S (Table 2) capable of using both NADPH and NADH was also able to utilize BNAH for the reduction of cytochrome *c* with a catalytic efficiency of $41.3 \text{ min}^{-1} \mu\text{M}^{-1}$, while the wild type had no detectable activity [33,36]. A similar variant P450-BM3 W1046S also gained activity for utilizing both natural cofactors and reduced NMN^+ (NMNH) [9•].

Second, size reduction of the cofactor binding pocket to improve packing often affords increased activity toward mNADs. For example, the phosphite dehydrogenase from *Ralstonia* sp. 4506 harboring I151R-P176R-M207A mutations had significantly enhanced activity toward NCD^+ . Crystallography suggested activity was achieved through compression of the binding pocket around the smaller cytosine [7•]. Interestingly, natural flavoenzymes that efficiently utilize mNADs also employ this strategy. The bulky Trp302 residue in *P. putida* XenA active site adopts a different conformation when smaller mNADs are bound to pack more tightly against the cofactors [32].

Third, design to install polar interactions, which in principle contribute more strongly to binding affinity than hydrophobic packing, is effective for achieving stringent binding of mNADs. We recently engineered a highly orthogonal *Bacillus subtilis* glucose dehydrogenase S17E-Y34Q-A93K-I195R to use NMN^+ [9•] which showed the highest RS (Eq. (3)) reported to date of 1.1×10^7 for NADP^+ and 2.1×10^7 for NAD^+ . We first utilized Rosetta modeling to identify the positively charged I195R mutation which is predicted to form a salt bridge with the highly negative NMN^+ phosphate. Next, we achieved exclusive specificity for NMN^+ by introducing S17E which is modeled to repel the phosphate in the adenosine monophosphate (AMP) moiety that is only present in the natural cofactors but not in NMN^+ . Because of the high conservation of residues lining the cofactor binding pocket, we hypothesize that these mutations should be readily transferable and support NMN^+ binding in homologs.

Technology development for engineering natural cofactor-dependent enzymes

Limited throughput has driven the use of semi-rational strategies to minimize the number of variants screened and to maximize the likelihood of isolating promising candidates. Many of these focused libraries have been screened based on readouts that can be determined by a microplate reader [37,38] or visualized on an agar plate

Table 1

Performance of wild type enzymes with noncanonical cofactors.

Enzyme	Uniprot	Native cofactor	Noncanonical cofactor ^a	CSR ^b	Source
D-Lactate dehydrogenase (<i>L. helveticus</i>)	P30901	NAD ⁺	NCD ⁺	4.8×10^{-2}	Liu <i>et al.</i> , 2020 [5]
Formate dehydrogenase (<i>Pseudomonas</i> sp. 101)	P33160	NAD ⁺	NCD ⁺	4.7×10^{-2}	Guo <i>et al.</i> , 2020 [6]
Glucose dehydrogenase (<i>B. subtilis</i>)	A0A1B2ATD9	NAD ⁺	NMN ⁺	2.6×10^{-6}	Black <i>et al.</i> , 2019 [9*]
6-Phosphogluconate dehydrogenase (<i>T. maritima</i>)	A0A2N5RL69	NADP ⁺	NMN ⁺	1.7×10^{-6}	
Phosphite dehydrogenase (<i>Ralstonia</i> sp. 4506)	A0A2N5RL69	NADP ⁺	NMN ⁺	3.1×10^{-6}	Huang <i>et al.</i> , 2019 [10**]
3-Hydroxy benzoate 6-hydroxylase (<i>R. jostii</i>)	G4XDR8	NAD ⁺	NCD ⁺	5.8×10^{-2}	Liu <i>et al.</i> , 2019 [7*]
para-Hydroxybenzoate hydroxylase (<i>P. fluorescens</i>)	Q0SFK6	NADH	BNAH	7.5×10^{-4}	Guarneri <i>et al.</i> , 2019 [14]
			AmNAH	4.9×10^{-4}	
			BNAH	4.8×10^{-3}	
			AmNAH	3.2×10^{-3}	
Salicylate hydroxylase (<i>P. putida</i>)	P20586	NADH	AmNAH	2.1	Nowak <i>et al.</i> , 2017 [8*]
Glucose dehydrogenase (<i>S. solfataricus</i>)	—	NADPH	AmNAH	2.4×10^{-3}	
		NADH	BNAH	8.3×10^{-3}	
		NADPH	BNAH	8.8×10^{-1}	
NADH oxidase (<i>L. pentosus</i>)	O93715	NAD ⁺	BNA ⁺	1.0×10^{-2}	Nowak <i>et al.</i> , 2015, 2017 [12,15]
Xenobiotic reductase (<i>P. putida</i>)	F6IXY6	NADH	P2NA ⁺	1.5×10^{-2}	
			P3NA ⁺	5.6×10^{-3}	
			MNAH	—	
			BNAH	—	
Pentaerythritol tetranitrate reductase (<i>E. cloacae</i>)	P71278	NADPH	P2NAH	—	Knaus <i>et al.</i> , 2016 [32]
			P3NAH	—	
			BNAH	3.1×10^2	
			BTH	1.8×10^3	
Thermophilic old yellow enzyme (<i>T. pseudethanolicus</i>)	B0KAH1	NADH	BEH	2.7×10^2	Paul <i>et al.</i> , 2015 [54]
			BNAH	2.7	
			BTH	1.5×10	
			BEH	2.7×10^2	
Styrene monooxygenase (<i>R. opacus</i>)	C7ACG0	NADH	BNAH	1.4	Campbell <i>et al.</i> , 2012 [35]
Alcohol dehydrogenase (<i>P. furiosus</i>)	—	NAD ⁺	BTH	5.2	
Malic enzyme (<i>E. coli</i>)	P26616	NAD ⁺	BEH	1.0×10^{-1}	
			BNAH	8.2×10^{-1}	
Styrene monooxygenase (<i>R. opacus</i>)	C7ACG0	NADH	BTH	1.2	Paul <i>et al.</i> , 2015 [54]
			BEH	3.3×10^{-1}	
			BNAH	2.2×10^{-2}	
			BTH	3.2×10^{-2}	
Alcohol dehydrogenase (<i>P. furiosus</i>)	—	NAD ⁺	BEH	8.7×10^{-3}	Campbell <i>et al.</i> , 2012 [35]
Malic enzyme (<i>E. coli</i>)	P26616	NAD ⁺	BNAH	—	
Alcohol dehydrogenase (<i>P. furiosus</i>)	—	NAD ⁺	NFCD ⁺	9.3×10^{-3}	Ji <i>et al.</i> , 2011 [53*]
			NCD ⁺	1.3×10^{-2}	

^a Full names of the noncanonical cofactors: AmNA⁺, 1-(2-carbamoylmethyl)-1,4-dihydronicotinamide; BNA⁺, 1-benzyl-1,4-dihydronicotinamide; BT⁺, 1-butyl-1,4-dihydronicotinamide; BE⁺, 1-(1-benzyl-1,4-dihydro-3-yl) ethanone; MD⁺, N-4-methoxybenzyl-1,4-dihydronicotinamide; MNA⁺, 1-methyl-1,4-dihydropyridine-3-carboxamide; NCD⁺, Nicotinamide cytosine dinucleotide; NCFD⁺, Nicotinamide flucytosine dinucleotide; NMN⁺, Nicotinamide mononucleotide; P2NA⁺, 1-phenethyl-1,4-dihydropyridine-3-carboxamide; P3NA⁺, 1-(3-phenylpropyl)-1,4-dihydropyridine-3-carboxamide. Reduced cofactor ends with 'H'.

^b CSR, Cofactor Specificity Ratio (Eq. (1)).

[39,40]. Application of a 4-nitrophenylacetonitrile microplate assay provided a colorimetric screen to isolate cytochrome P450-BM3 variants for hydroquinone production with 70-fold improvement over wild type activity [37] (Figure 2). In another example, an agar screen leveraged the solubility difference of the substrate and product to evolve the substrate scope of a cyclohexanone

monooxygenase (CHMO) for pilot-scale applications [40,41]. For enzymes that do not produce color or absorbance change during catalysis, a mass spectrometry-based screening platform was developed (Figure 2) to use 'click' chemistry to enhance throughput [42]. This mass spectrometry-based platform may be readily applicable to engineering mNAD-dependent enzymes. Despite

Table 2

Performance of engineered enzymes with noncanonical cofactors.

Enzyme	Uniprot	Strategy	Native cofactor	Noncanonical Cofactor ^a	Mutations	CSR ^b	RCE ^c	RS ^d	Source
D-Lactate dehydrogenase (<i>L. helveticus</i>)	P30901	Bibliography, Saturation, Structure	NAD ⁺	NCD ⁺	V152R-N213E	4.3×10	2.1×10^{-1}	9.0×10^2	Liu <i>et al.</i> , 2020 [5]
Formate dehydrogenase (<i>Pseudomonas</i> sp. 101)	P33160	Bibliography, Saturation, Structure	NAD ⁺	NCD ⁺	V152R-I177K-N213I V198I-C256I-P260S-E261P-S381N-S383F	4.2×10 1.7×10^2	3.1×10^{-1} 3.7×10^{-2}	8.8×10^2 3.5×10^3	Guo <i>et al.</i> , 2020 [6]
Glucose dehydrogenase (<i>B. subtilis</i>)	A0A1B2ATD9	Computational, Structure	NADP ⁺	NMN ⁺	Y34Q-A93K-I195R	6.8×10^{-2}	1.8×10^{-3}	4.1×10^4	Black <i>et al.</i> , 2019 [9]
			NAD ⁺	NMN ⁺	S17E-Y34Q-A93K-I195R	1.9×10	7.5×10^{-4}	1.1×10^7	
			NAD ⁺	NMN ⁺	Y34Q-A93K-I195R	4.6	2.8×10^{-3}	1.8×10^6	
6-Phosphogluconate dehydrogenase (<i>T. maritima</i>)	A0A2N5RL69	Computational, Random, Saturation, Structure	NADP ⁺	NMN ⁺	S17E-Y34Q-A93K-I195R	5.5×10	1.2×10^{-3}	2.1×10^7	Huang <i>et al.</i> , 2019 [10]
Glucose-6-phosphate dehydrogenase (<i>T. maritima</i>)	A0A2N5RP10	Computational, Random, Saturation, Structure	NADP ⁺	NMN ⁺	Mut 5-1 ^e	1.4×10^{-2}	1.2×10^{-4}	4.6×10^3	
			NADP ⁺	NMN ⁺	Mut 6-1 ^f	1.4×10^{-2}	1.5×10^{-4}	4.4×10^3	
Phosphite dehydrogenase (<i>Ralstonia</i> sp. 4506)	G4XDR8	Bibliography, Computational, Saturation, Structure	NAD ⁺	NCD ⁺	I151R-P176R	2.0×10	1.3×10^{-1}	3.4×10^2	Liu <i>et al.</i> , 2019 [7]
Glucose dehydrogenase (<i>S. solfataricus</i>)				BNA ⁺	I151R-P176R-M207A	4.5×10	9.4×10^{-3}	7.8×10^2	
					I192T-V306G	5.5×10	6.5×10^{-2}	5.5×10^2	
	O93715	Bibliography, Saturation, Structure	NAD ⁺	P2NA ⁺	I192T-V306I	2.9×10^{-1}	1.0×10^{-1}	2.9×10	Nowak <i>et al.</i> , 2017 [8]
					I192T-V306G	3.1	3.6×10^{-2}	2.0×10^2	
					I192T-V306I	9.2×10^{-1}	3.2×10^{-1}	6.1×10	
				P3NA ⁺	I192T-V306G	3.8×10	2.6×10^{-1}	6.8×10^2	Wang <i>et al.</i> , 2017 [16]
					I192T-V306I	3.0×10^{-1}	1.1×10^{-1}	5.3×10	
					I151R	4.3×10	1.4×10^{-2}	5.4×10^2	
Phosphite dehydrogenase (<i>Ralstonia</i> sp. 4506)	G4XDR8	Bibliography	NAD ⁺	NCD ⁺	I151R	4.3×10	1.4×10^{-2}	5.4×10^2	Lo <i>et al.</i> , 2017 [33]
P450-BM3 (<i>B. megaterium</i>)	P14779	Bibliography, Structure	NADH	BNAH	R966D-W1046S	1.0×10^{-1}	9.6×10	–	
Alcohol dehydrogenase (<i>P. furiosus</i>)	–	MSA ^g , Structure	NAD ⁺	MDH	R966D-W1046S	6.4×10^{-2}	6.0×10	–	
Malic enzyme (<i>E. coli</i>)	P26616	Computational, MSA, Saturation, Structure	NAD ⁺	NMN ⁺	K249G-H255R	1.6×10^{-3}	4.4×10^{-4}	1.8×10^2	Campbell <i>et al.</i> , 2012 [35]
				NFCD ⁺	L310R	9.8×10	4.1×10^{-1}	1.1×10^4	Ji <i>et al.</i> , 2011 [53]
					L310R-Q401C	2.7×10^2	4.5×10^{-1}	2.9×10^4	
				NCD ⁺	L310R	1.9×10^2	7.9×10^{-1}	1.4×10^4	
D-Lactate dehydrogenase (<i>L. helveticus</i>)	P30901	MSA, Structure	NAD ⁺	NCFD ⁺	L310R-Q401C	4.3×10^2	7.2×10^{-1}	3.3×10^4	Flores <i>et al.</i> , 2005 [34]
Malate dehydrogenase (<i>E. coli</i>)	P61889	MSA, Structure	NAD ⁺	NCFD ⁺	V152R	–	–	–	
Lactate dehydrogenase (<i>B. stearothermophilus</i>)	P00344	MSA, Saturation, Structure	NAD ⁺	NMN ⁺	L6R	–	–	–	

^a Full names of the noncanonical cofactors: AmNA⁺, 1-(2-carbamoylmethyl)-1,4-dihydronicotinamide; BNA⁺, 1-benzyl-1,4-dihydronicotinamide; BT⁺, 1-butyl-1,4-dihydronicotinamide; BE⁺, 1-(1-benzyl-1,4-dihydro-3-yl) ethanone; MD⁺, N-4-methoxybenzyl-1,4-dihydronicotinamide; MNA⁺, 1-methyl-1,4-dihydropyridine-3-carboxamide; NCD⁺, Nicotinamide cytosine dinucleotide; NCFD⁺, Nicotinamide flucytosine dinucleotide; NMN⁺, Nicotinamide mononucleotide; P2NA⁺, 1-phenethyl-1,4-dihydropyridine-3-carboxamide; P3NA⁺, 1-(3-phenylpropyl)-1,4-dihydropyridine-3-carboxamide. Reduced cofactor ends with 'H'.

^b CSR, Cofactor Specificity Ratio (Eq. (1)).

^c RCE, Relative Catalytic Efficiency (Eq. (2)).

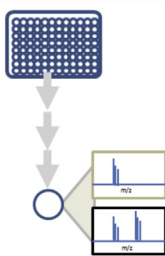
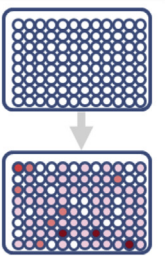
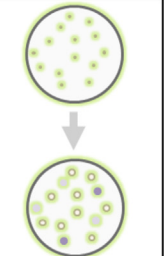
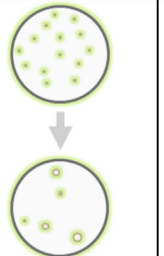
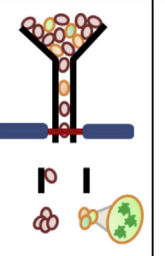
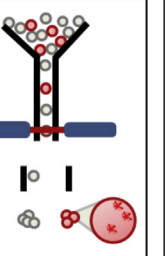
^d RS, Relative Specificity (Eq. (3)).

^e Mut 5-1 contains A11G-K27R-R33I-T34I-F60Y-D82L-T83L-Q86L-K118N-I120F-D294V-F326S-Y383C-N387S-A447V.

^f Mut 6-1 contains A11G-K27R-R33I-T34I-F60Y-D82L-T83L-Q86L-K118N-I120F-D251E-D294V-F326S-F329Y-Y383C-N387S-V390G-A447V.

^g MSA, Multiple Sequence Alignment.

Figure 2

	PECAN (Mass Spectrometry)	NpCN Assay (Microplate Plate)	NESPA Assay (Agar Plate Screen)	Redox Balance Growth Assay (Plate Selection)	Redox Sensor SoxR (FACS)	HRP Assay (FADS)
Enzyme	P450 _{BM3}	P450 _{BM3}	<i>Tm</i> 6PGDH	<i>Ld</i> Idh	<i>Lb</i> adh	<i>Bo</i> CHAO
Throughput	~10 ³	10 ² -10 ⁴	10 ⁴ -10 ⁵	>10 ⁶	>10 ⁶	>10 ⁶
Readout	m/Z	UV-vis	Digital Imaging	Growth	Fluorescence (GFP)	Fluorescence (Amplex Red)
Screening Process						
Benefits	Improves Mass Spectrometry Throughput	Simple and common	Sensitive to non-canonical cofactors	Low-cost, broad application	Rapid, broad application	Rapid
Limitations	Multi-step processing and substrate analog required	Requires colorimetric substrate analog	Multi-step Processing	Substrate permeability and toxicity	Substrate permeability and toxicity, high background	Requires H ₂ O ₂ production
Reference	de Rond et al. (2019)	Weingartner et al. (2018)	Huang et al. (2019)	Zhang et al. (2018)	Spielmann et al. (2020)	Debon et al. (2019)

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Representative screening methods used to facilitate the directed evolution of oxidoreductases. PECAN (probing enzymes with click-assisted NIMS), NpCN (4-nitrophenylacetone), NESPA (NAD(P)-eliminated solid-phase assay), soxR (Redox-sensitive transcriptional activator), FACS (Fluorescence Activated Cell Sorting), HRP (Horse radish peroxidase), and FADS (Fluorescence Activated Droplet Sorting). Targeted Oxidoreductases: P450_{BM3} (NADPH-dependent Cytochrome P450 BM3), *Tm* 6PGDH (NADP⁺-dependent *Thermotoga maritima* 6-phosphogluconate dehydrogenase), *Ld* Idh (NADH-dependent *Lactobacillus delbrueckii* d-lactate dehydrogenase), *Lb* adh (NADPH-dependent *Lactobacillus brevis* alcohol dehydrogenase), *Bo* CHAO (FADH₂-dependent *Brevibacterium oxydans* cyclohexamine oxidase).

success, throughput remains limiting (10³–10⁵); furthermore, reduced library sizes may miss potential cooperative effects critical for dramatic improvements [9*].

Recent campaigns apply ‘ultra-throughput’ (>10⁶) methods using reactions that can be detected by fluorescence sorting [43,44,45*]. For example, *Brevibacterium oxydans* cyclohexylamine oxidase (*Bo* CHAO) variants were compartmentalized in droplets and screened for their activity towards a non-natural substrate using fluorescent activated droplet sorting (FADS) (Figure 2), which yielded a mutant with 960-fold increased catalytic efficiency [45*]. However, this method is only applicable to enzymes that produce H₂O₂ which is detected by a fluorescent dye, Amplex-Ultra Red. To overcome this limitation, an *Escherichia coli* strain harboring SoxR-regulated GFP cassette to report the intracellular NADPH/NADP⁺ ratio was developed to screen NADPH-dependent enzymes via fluorescent activated cell sorting (FACS) [43,44]. This system enabled screening of a random library and isolated

a *Lactobacillus brevis* alcohol dehydrogenase variant with improved activity for the reduction of 2,5-hexanedione to (2R,5R)-hexanediol [44] (Figure 2). Advanced sorting techniques offer rapid screening of the library being explored, but are often hindered by narrow dynamic ranges and high background signal. Selections, as opposed to screens, do not rely on special instrumentation and automatically eliminate undesirable candidates.

In vivo selection platforms modulate cell growth by disrupting intracellular cofactor cycling within engineered *E. coli* strains. These platforms were pioneered in early work aiming to accumulate NADH in anaerobic condition by disrupting the host’s native fermentative pathways, for example in strain JCL166 (*ΔadhE ΔldhA Δfrd*). In this strain, anaerobic growth is only restored when an NADH-recycling enzyme is present. This system has identified endogenous *E. coli* enzymes which form a 2,3-butanediol production pathway [46]. The same principle of cofactor recycling is the foundation for a variety of ultra-high

throughput ($>10^6$) growth-based selections of nicotinamide-dependent oxidoreductases in directed evolution [47^{••},48,49^{••},50,51]. A recent growth-based selection strain has an engineered NADPH-dependent glycolysis, and therefore required a NADPH-consuming 'fermentative' reaction to grow anaerobically. This platform enabled the selection of $\sim 6.2 \times 10^7$ variants in one round, and produced a *Lactobacillus delbrueckii* D-lactate dehydrogenase with a 470-fold increase in activity with NADPH [49^{••}] (Figure 2).

This selection strategy has since been expanded to include both NADPH and NADH-dependent selections in aerobic conditions, to be compatible with engineering oxygenases such as p-hydroxybenzoate hydroxylase [47^{••}] and cyclohexanone monooxygenase [48]. These results highlight the usefulness of *in vivo* growth platforms for oxidoreductase selections.

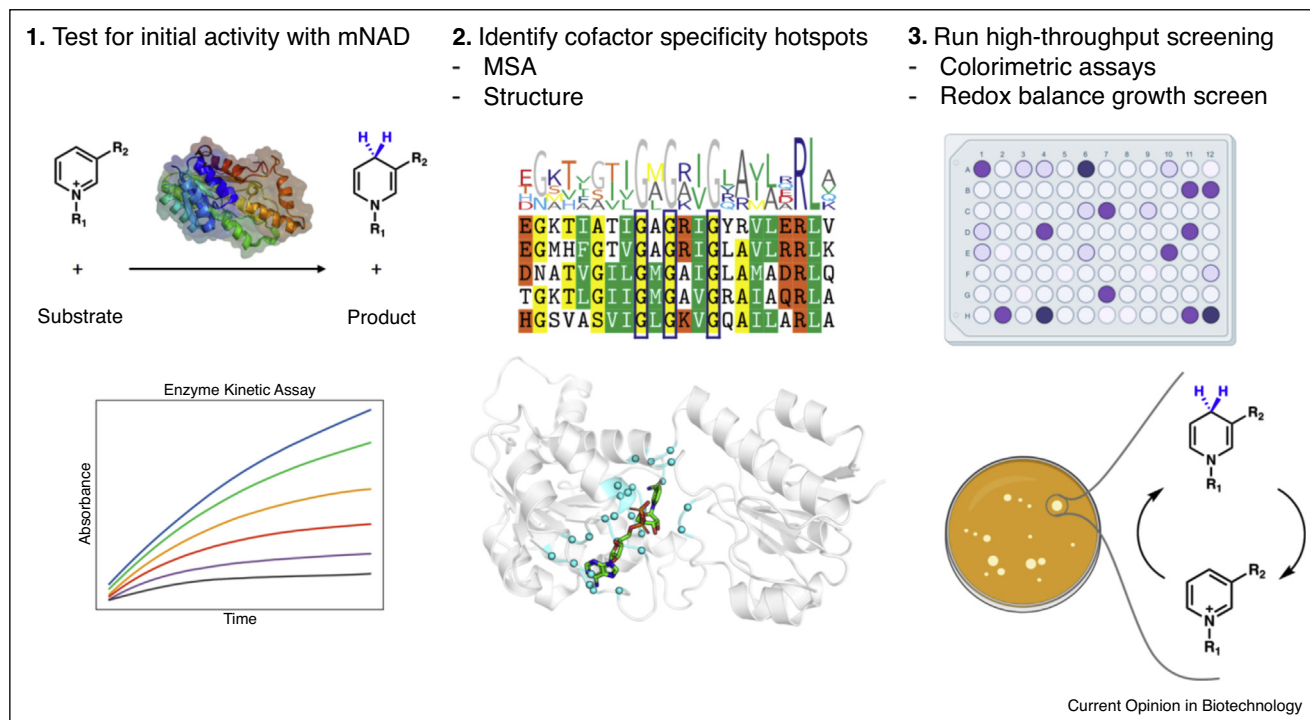
Growth selection has not been applied in engineering noncanonical cofactor-dependent enzymes. However, our recent work where *E. coli* growth was obligately linked to the cycling of the noncanonical cofactor NMN⁺ presents a platform for future studies. This was achieved by disrupting standard glycolysis networks and directing glucose entry into the life-essential carbon metabolism

through our NMN⁺-specific glucose dehydrogenase (GDH) [9[•]]. Cell growth was only restored when the NMN⁺-cycling partner of GDH was present to complete the NMN⁺ based redox cycle and prevent cofactor depletion. The specific function of the partner is not linked to cell survival and we anticipate that the complementary partner can be exchanged.

Technology development for engineering noncanonical cofactor-dependent enzymes

In general, efforts in engineering mNAD-dependent enzymes follow three steps (Figure 3): First, wild type enzymes from different organisms are screened with the mNAD of interest to identify a starting template. Second, sequence alignment or computational models predict positions surrounding the cofactor binding pocket. Third, identified positions are targeted by mutagenesis, often in combinatorial fashion. To achieve high diversity, site-saturation mutagenesis is typically performed with degenerate primers, and variants are screened with 96-well plate-based absorbance assays detecting reduced cofactor [5,8[•]] or colorimetric assays detecting reactions of reduced cofactor with nitroblue tetrazolium and phenazine methosulfate producing the purple dye formazan [7[•],52]. Future tool developments will include growth-

Figure 3



Outline of protocol to engineer enzymes for mNAD activity. An initial screen is performed with the mNAD of interest and wild type enzyme to determine the baseline performance. Positions surrounding the cofactor binding pocket and those that contribute to cofactor specificity found through sequence alignment are chosen for mutagenesis. Variants are screened through colorimetric assay measuring activity through color development reflecting production of reduced cofactor. Future tool developments to improve throughput will include growth-based selection assays where the ability of the cell to regenerate mNAD is linked to survival.

based selection where the ability of the cell to cycle the target mNAD is linked to life-essential functions such as carbon metabolism. Variants with more active mNAD cycling will readily outcompete those with lower fitness resulting in facile, high-throughput selection of mNAD-dependent enzymes through readout of cell growth.

We highlight two recent reports departing from the standard saturation mutagenesis and 96-well plate based screening approach. Huang *et al.* [10**] developed the NAD(P)-eliminated solid phase assay (NESPA) (Figure 2), a colorimetric screen performed with colonies grown on agar plate advancing throughput to over 10⁵ samples per round while managing low background noise. A heat treatment step is performed to permeabilize cells, followed by washing to remove endogenous NAD(P)⁺. Rounds of saturation mutagenesis at the cofactor binding site, followed by error prone PCR to raise diversity in more distal regions, resulted in a *Thermotoga maritima* 6-phosphogluconate dehydrogenase variant with 50-fold enhanced NMN⁺-dependent activity. However, heat treatment limits the assay to screening thermostable enzymes, and manual washing steps may lead to high variance. In our recent study [9*], *in silico* screening was performed in lieu of experimental screening. Bioinformatic analysis was used to identify positions with high plasticity to tolerate mutations. Then, by simulating the effects of mutations on the mNAD binding pose using Rosetta, we greatly narrowed down candidates that warranted experimental testing and eliminated the need to broadly sample with site-saturation mutagenesis. The best mutant *B. subtilis* glucose dehydrogenase S17E-Y34Q-A93K-I195R was obtained from just experimentally testing <20 candidates.

Conclusion

When engineering enzymes to utilize noncanonical cofactors, even the most developed variants often show low relative catalytic efficiencies with mNADs. The sampling cap from utilizing 96-well plate-based screens greatly restricts our ability to identify rare, highly functional variants. Future directions to expand the mNAD evolution toolbox will involve adapting principles and methods currently used for natural cofactors.

In addition, computational methods will be highly instrumental in engineering mNAD-dependent enzymes. Without crystal structures of the target enzymes with noncanonical cofactors bound, molecular modeling tools are essential for visualizing enzyme-cofactor interaction. Furthermore, homology modeling tools and sequence alignment facilitate the translation of successful mutations between different enzymes. For example, *E. coli* malic enzyme L310R gained the ability to utilize nicotinamide flucytosine dinucleotide (NCFD⁺) and NCD⁺ [53*]. High sequence conservation at L310 inspired the rational design of *E. coli* malate dehydrogenase L6R for

NCFD⁺ binding [53*], *Lactobacillus helveticus* D-lactate dehydrogenase V152R [5,53*], and *Ralstonia* sp. 4506 phosphite dehydrogenase I151R for NCD⁺ binding [16].

Conflict of interest statement

The authors declare no conflict of interest.

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