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Design, construction, and application of noncanonical redox cofactor infrastructures

William B Black[1], Sean Perea[1] and Han Li[1,2,3]

Controlling the flow of carbon and reducing power in biological systems is a central theme in metabolic engineering. Often, trade-offs in pushing carbon flux through targeted pathways while operating in conditions agreeable to the host are required due to the central pools of the shared native redox cofactors NAD(P)/H. Noncanonical redox cofactors (NRCs) have emerged as promising tools to transform how engineers develop biotransformation systems. These new-to-Nature redox cofactors have been demonstrated to function orthogonally to the endogenous cofactors, support pathway thermodynamics optimization, and achieve product scopes previously difficult to reach due to endogenous pathway crosstalk. This review will discuss the development of NRC infrastructures, comprising NRC pools, cofactor reduction sources, and cofactor oxidation sinks, the (pool–source–sink) infrastructure.

Addresses
[1] Department of Chemical and Biomolecular Engineering, University of California, Irvine, Irvine, CA 92697-3900, United States of America
[2] Department of Biomedical Engineering, University of California, Irvine, Irvine, CA 92697-3900, United States of America
[3] Department of Biological Chemistry, University of California, Irvine, Irvine, CA 92697-3900, United States of America

Corresponding author: Li, Han (han.li@uci.edu)

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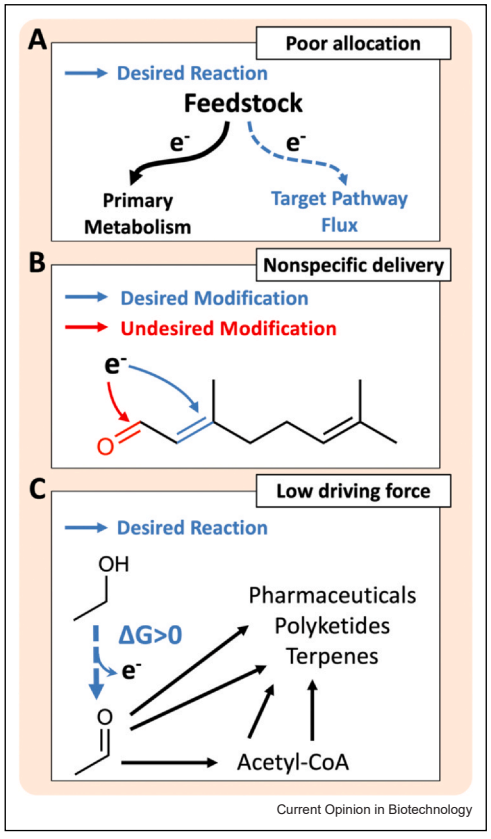
Introduction

Metabolic engineers work to wrangle the metabolism of microorganisms to direct the flows of carbon and reducing power toward their desired products. The universal redox cofactors nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (NAD(P)/H) are central in metabolic engineering. Engineering efforts surrounding these native cofactors regularly encompass maintaining redox balances, genome mining, protein engineering for alternate cofactor-preferring enzymes, and concentrating reducing power to the desired pathway through manipulating the host's endogenous metabolic background.

However, there are limitations of relying solely on natural cofactors (Figure 1). First, poor allocation of reducing power can make directing flux to a desired biosynthetic pathway difficult (Figure 1a). For example, despite efforts to increase the global reduced nicotinamide adenine dinucleotide phosphate (NADPH) pool in the cells, P450 enzymes in the secondary metabolism still receive a low proportion of NADPH since the primary metabolism runs at a higher rate [1]. Second, nonspecific reducing power delivery can derail the desired biosynthetic route (Figure 1b). For example, the vast number of promiscuous alcohol dehydrogenases native to the microbial host will dissipate the aldehyde substrates or products uncontrollably since these alcohol dehydrogenases all receive electrons from NAD(P)H, resulting in the rampant reduction of aldehydes to their respective alcohols [2,3]. Third, lack of driving force renders the desired biosynthetic reactions thermodynamically inaccessible. The relatively mild reduction potential (E° = –320 mV) of NAD(P)H is critical for their role as versatile electron carriers to reversibly react with a maximal number of other biological molecules. However, this mild reduction potential also means that NAD(P)/H can struggle to drive difficult oxidation/reduction reactions. For instance, the $NAD(P)^+$-mediated oxidation of alcohols to aldehydes is an attractive starting point for biosynthetic pathways. However, this thermodynamically uphill reaction ($\Delta G > 0$) regularly struggles to achieve sufficient flux to drive carbon assimilation because the reduction potential of $NAD(P)^+$ is too low relative to the reduction potential of alcohol oxidation, resulting in the reaction equilibrium lying heavily toward the substrates, alcohols, and $NAD(P)^+$ (Figure 1c) [4–6].

Over the last decade, a renewed thrust of utilizing noncanonical redox cofactors (NRCs) in metabolic engineering has begun to overcome these limitations both

Figure 1



Limitations of relying on natural redox cofactors. **(a)** Carbon feedstocks and central pathway intermediates are often readily consumed by endogenous pathways. This often results in limited flux into the desired heterologous pathway as key intermediate metabolites are diverted away. **(b)** Nonspecific reducing power from the shared pool of NAD(P)H provides reducing power to the entire biological milieu. Target products or intermediates can suffer from undesired modifications, as reducing power is not insulated to the enzymes that perform the desired reactions. **(c)** Biological systems operate within the thermodynamic constraints of natural redox cofactors, restricting user control and ability to power reactions in thermodynamically uphill directions. As an example, the ability to upgrade inexpensive feedstocks, such as bioethanol, into key metabolic intermediates, such as acetyl-CoA, is limited by the thermodynamically challenging, NAD(P)H-dependent oxidation of ethanol to acetaldehyde.

in vitro and *in vivo*. Recent advances have demonstrated the ability for these NRCs to function orthogonally to the endogenous metabolic background [2,7,8], to optimize driving forces and shift reaction equilibrium [4,5,9], and serve as low-cost alternatives to NAD(P)/H in cell-free biotransformation processes [10–13].

As NRC-utilizing enzymes continue to accumulate, an orthogonal redox infrastructure that can sustain new-to-nature metabolic capabilities starts to emerge. This review will map recent developments to this infrastructure containing three parts: a pool of NRCs for catalysis, electron sources to reduce them, and electron sinks to oxidize them (Figure 2). Finally, we will discuss the unique applications accessible with a fully developed NRC pool–source–sink infrastructure.

Pool–source–sink infrastructure of redox cofactors

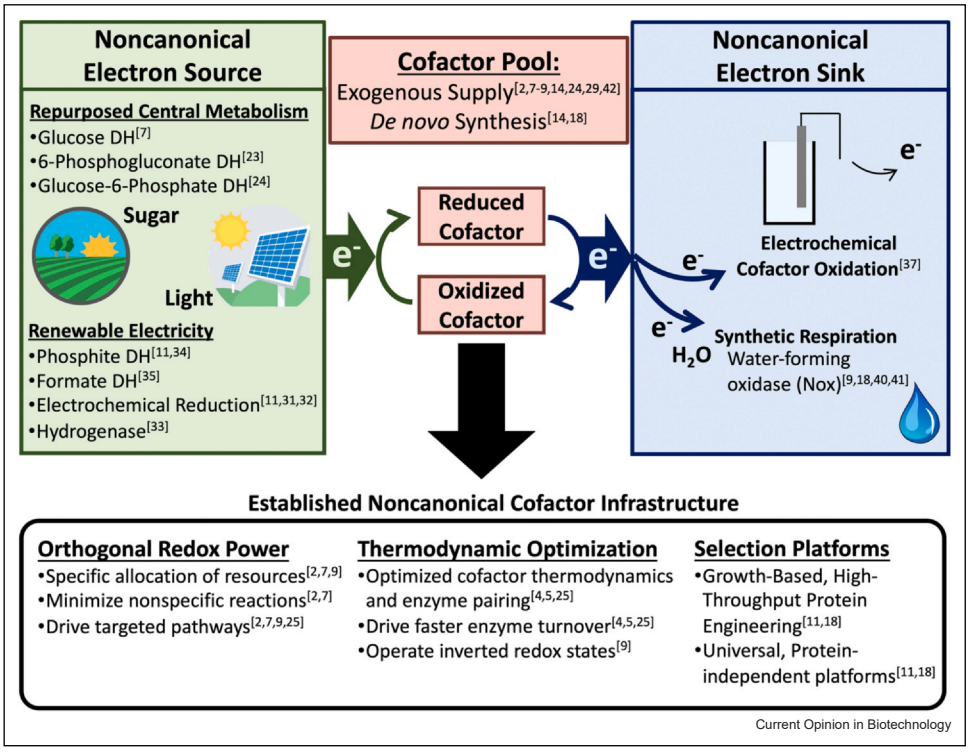
Using NAD(H) as an example, we can illustrate the necessary infrastructure for a redox cofactor to function: first, cells must maintain an intracellular concentration of NAD(H) (a pool of cofactor) that matches their enzymes' cofactor affinity; second, central metabolism, such as glycolysis, reduces nicotinamide adenine dinucleotide (NAD^+) to reduced nicotinamide adenine dinucleotide (NADH) (an electron source); third, respiration or fermentation oxidizes NADH to NAD^+ (an electron sink). When any of the three components is disrupted, all NAD(H)-dependent metabolism comes to a halt. Cells also have exquisite mechanisms to regulate the pool, source, and sink, which vary NAD(H) level and NADH/NAD^+ ratio to suit their fluctuating metabolic needs. To successfully deploy NRCs as a versatile and robust tool in biomanufacturing, construction of a similar infrastructure is paramount.

Development of noncanonical redox cofactor pools

NRCs can largely be broken down into three classes based on their degree of structural deviation from NAD^+ (Figure 3a). Establishing a pool of a particular NRC largely depends on the NRC's structure.

First, dinucleotide-based biomimetic cofactors, such as nicotinamide cytosine dinucleotide (NCD^+) and carba-nicotinamide adenine dinucleotide phosphate ($cNADP^+$), offer high structural similarity to NAD^+. This can enable facile engineering of enzymes to accept them, if not already accepted by wild-type enzymes (Figure 3, Table 1) [4,5]. These cofactors are typically produced using enzymatic or chemo-enzymatic reactions [14,15]. Of particular interest is the 'head swapping' reaction catalyzed by the pig brain NADase [16], which replaces the redox-active nicotinamide moiety. This results in NRCs such as thionicotinamide adenine dinucleotide ($SNAD^+$) and 3-acetylpyridine dinucleotide ($ApAD^+$) with a higher reduction potential than $NAD(P)^+$ [13] (Figure 3a and b), ideal for driving oxidative reactions. Nicotinic acid adenine dinucleotide, another 'head swapping' NRC with a lower reduction potential (Figure 3b), can be biosynthesized as an intermediate in NAD^+ biosynthesis [17]. Its lower reduction potential makes it desirable to drive reduction reactions. These altered redox potential cofactors can be strategically paired with thermodynamically limiting reaction steps to minimize pathway bottlenecks [4,5].

Figure 2



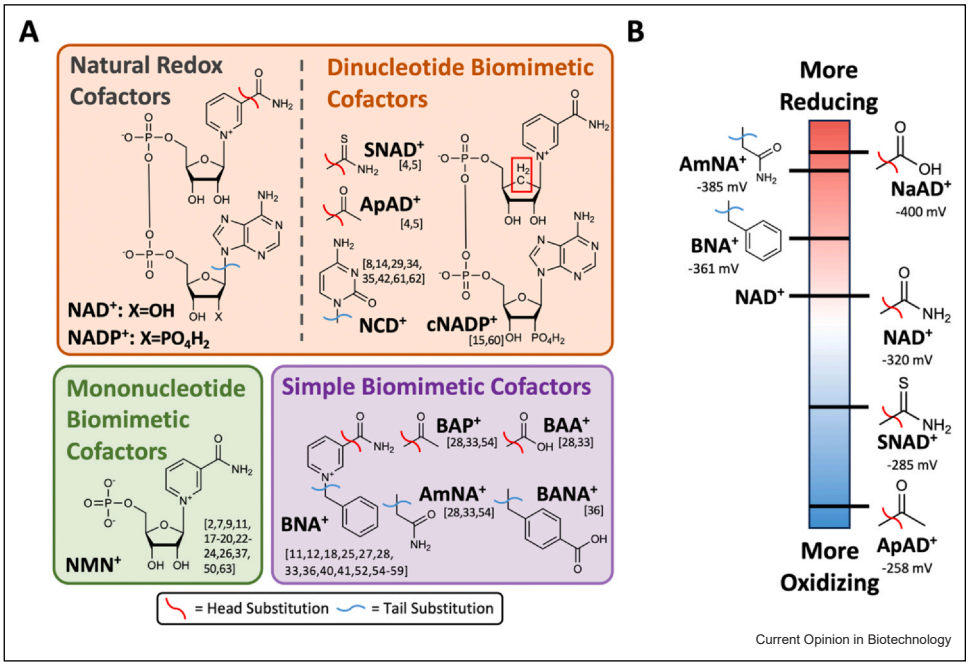
Cofactor systems with pool, source, and sink infrastructure. Noncanonical cofactor systems have been developed to mimic native redox cofactor systems, where a fundamental infrastructure of a cofactor pool, electron sources for noncanonical cofactor reduction, and redox sinks to purge excess reduced cofactors to their oxidized form. Noncanonical cofactor pools can be developed through exogenous supply or engineered *de novo* synthesis pathways. Source platforms have been developed for noncanonical cofactor reduction through the key enzymes in the central metabolism or through electrochemical means. Noncanonical cofactor sinks have been developed to offer fine control of noncanonical redox ratios through water-forming oxidases and electrochemical oxidation. Once a complete pool–source–sink infrastructure is developed around a cofactor, systems can be constructed to realize the new-to-nature capabilities of these cofactors. DH = dehydrogenase. Numbers represent references utilizing these enzymes or pool-building concepts.

Second, mononucleotide-based biomimetic cofactors, such as nicotinamide mononucleotide (NMN^+), exhibit substantial structural deviation from $NAD(P)^+$ (Figure 3a). This can greatly minimize crosstalk with the endogenous enzymes [2,7]. NMN^+ has also been efficiently synthesized and accumulated at high concentrations in engineered *Escherichia coli* and yeast by introducing exogenous synthesis pathways and disruption of genes encoding NMN^+-degrading enzymes [17–20]. In the future, the 'head swapping' chemistry mentioned above can potentially be extended to the mononucleotide-based scaffold; previous efforts have produced different mononucleotide NRCs through the cleavage of the pyrophosphate bond of their respective 'head-swapped' dinucleotide forms [21]. This would result in NRCs that are both orthogonal to NAD^+ and exhibit a broader range of reduction potentials than NAD^+. Wild-type enzymes typically have nominal activity toward NMN(H) [7,11,22–24]. To overcome this enzyme engineering barrier, our work has demonstrated universal, high-throughput selection platforms to facilitate the engineering of NMN(H)-dependent enzymes [11,18]. These directed evolution efforts revealed general design principles whereby enzymes can be reprogrammed to utilize mononucleotide cofactor [11,18].

Third, simple biomimetic cofactors, such as 1-benzylnicotinamide (BNA^+), 1-methylnicotinamide (MNA^+), 1-(2-carbamoylmethyl)-nicotinamide ($AmNA^+$), and 3-carbamoyl-1-(4-carboxybenzyl) pyridin-1-ium ($BANA^+$), offer substantially simpler structures. This enables their facile, inexpensive chemical synthesis [10,25] (Figure 3a). However, their substantial structural deviation from NAD^+ regularly requires extensive protein engineering efforts to achieve activity with enzymes [12,26,27]. These NRCs represent 'tail-swapped' derivatives of NAD^+, which interestingly also afford different reduction potentials (Figure 3b).

In vitro biotransformation systems offer unique, direct access to reaction systems, where users can build a cofactor pool by simply adding NRCs. In these systems,

Figure 3



Classes and composition of noncanonical cofactors. **(a)** Redox cofactors are broken down into different classes, primarily based on structure. Different 'head' groups and/or 'tail' substitutions modify cofactor recognition and function. Numbers represent references utilizing these cofactors. Red lines indicate a 'head' group substitution. Blue lines indicate a 'tail' group substitution. **(b)** Right side, 'head' group substitution at the nicotinamide moiety of NAD^+ modifies the cofactor's redox potential. Left side, 'tail' group modifications additionally modify the redox potential of the biomimetic cofactor. Red lines indicate a 'head' group substitution. Blue lines indicate a 'tail' group substitution. NAD^+: nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; $SNAD^+$: thionicotinamide adenine dinucleotide; $ApAD^+$: 3-acetylpyridine dinucleotide; NCD^+: nicotinamide cytosine dinucleotide; $cNADP^+$: carba-nicotinamide adenine dinucleotide phosphate; NMN^+: nicotinamide mononucleotide; BNA^+: 1-benzylnicotinamide; BAP: 1-benzyl-3-acetylpyridine; BAA^+: 1-benzylnicotinic acid; $AmNA^+$: 1-(2-carbamoylmethyl)-nicotinamide.

cost is a primary driving factor in cofactor selection, where inexpensive synthetic cofactors can significantly reduce cost of cofactor supply [10]. Furthermore, since the majority of engineered enzymes to date exhibit a relatively high K_M for NRCs [7,23,27,28] (Table 1), the ability to supply high concentrations of cofactors *in vitro* without a formidable cost can circumvent this limitation.

In vivo applications require more intricate steps to establish an NRC pool. The direct supplementation of NRCs in the cell culture medium is one option [7,8,11,22,29], which depends on the cell's ability to uptake the desired NRC without degrading it. A more ideal option is to biosynthesize NRCs intracellularly [14,17,18], especially for dinucleotide-based cofactors that are not readily transported across the cell membranes. In one example, Zhao and coworkers engineered the *E. coli* nicotinic acid mononucleotide adenylyltransferase to favor cytidine triphosphate and NMN^+, which provided a synthesis route for NCD^+ in *E. coli*. This supported the NCD^+-dependent production of lactate from malate, without the need for exogenous cofactor supply [14]. Similar efforts have also been shown for NMN^+ in *E. coli*, where this NRC accumulates to a level sufficient to support central carbon metabolism [7,18].

Engineering electron sources for noncanonical redox cofactor reduction

In their simplest form, electrons can be sourced from electricity. The direct reduction of redox cofactors on electrodes may result in the production of inactive side products. Mediators and catalysts, either immobilized on the electrode surface or supplied in the solution, have been applied to overcome side product formation [30–32].

Within a similar vein, enzymes have been developed which use electrochemically producible substrates, such as hydrogen, phosphite, and formate, to reduce NRCs. Reeve and coworkers investigated soluble hydrogenases capable of utilizing H_2 to reduce a variety of synthetic biomimetic cofactors, such as BNA^+, 1-benzyl-3-acetylpyridine (BAP^+), and $AmNA^+$ [33]. We and others have engineered phosphite dehydrogenase to cycle NMN^+, BNA^+, and NCD^+

Table 1

Apparent kinetic parameters of engineered enzymes with NRCs.

Enzyme category	Enzyme	Mutant	Cofactor	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	Entry
Source enzyme	*Pseudomonas* sp. 101 Formate dehydrogenase [35]	V198I/C256I/P260S/ E261P/S381N/S383F	NAD^+	7.97	0.07	0.00878	1
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.12382	0.18	1.45	
Source enzyme	*Pseudomonas putida* Formaldehyde dehydrogenase [61]	A192R/L218V/L236V/ R267V	NAD^+	4	0.5	0.125	2
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	1.5	14.6	9.73	
		A192R/L223V/L236V	NAD^+	2.5	0.8	0.32	3
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.2	11.3	56.5	
Source enzyme	*Pseudomonas stutzeri* 12X-TS Phosphite dehydrogenase [11]	LY-7	NAD^+	0.75	0.71	0.95	4
			$NADP^+$	0.11	0.56	5.09	
			NMN^+	1.4	0.16	0.114	
		LY-13	NAD^+	0.18	2.06	11.44	5
			$NADP^+$	0.05	0.96	19.2	
			NMN^+	0.62	0.27	0.435	
Source enzyme	*Ralstonia* sp. 4506 Phosphite dehydrogenase [34]	I151R/P176R/M207A	NAD^+	4.7	0.21	0.045	6
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.0991	0.20	2.04	
		I151R/P176E/M207A	NAD^+	11.7	0.40	0.034	7
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.2815	0.35	1.24	
Source enzyme	*Thermobifida fusca* F420:NADPH oxidoreductase [28]	G29Y	NAD^+	n.r.	n.r.	n.r.	8
			$NADP^+$	0.0082	0.17	20.73	
			$AmNA^+$	23	3.2	0.139	
			$EtOHNA^+$	23	6.2	0.269	
			BNA^+	9.5	8.7	0.915	
		P89H	NAD^+	n.r.	n.r.	n.r.	9
			$NADP^+$	0.014	20	1428	
			$AmNA^+$	14	2.5	0.179	
			$EtOHNA^+$	20	2.9	0.145	
			$ProOHNA^+$	5.7	0.27	0.047	
		G29L	NAD^+	n.r.	n.r.	n.r.	10
			$NADP^+$	1.2	0.63	0.525	
			$ProNA^+$	18	0.36	0.02	
		G29W	NAD^+	n.r.	n.r.	n.r.	11
			$NADP^+$	0.697	2.6	3.73	
			BNA^+	4.3	25	5.81	
			BAP^+	7.4	252	34.1	
Source enzyme	*Saccharolobus solfataricus* Glucose dehydrogenase [12]	I192T/V306I	NAD^+	0.17	0.95	5.65	12
			$NADP^+$	n.r.	n.r.	n.r.	
			BNA+	5.49	0.00900	0.00164	
			$P2NA^+$	8.16	0.04217	0.00517	
			$P3NA^+$	4.1	0.0070	0.00170	
Source enzyme	*Saccharolobus solfataricus* Glucose dehydrogenase [27]	3M	NAD^+	n.r.	n.r.	n.r.	13
			$NADP^+$	n.r.	n.r.	n.r.	
			$P4NA^+$	1.7	70.3	41.35	
			$P5NA^+$	2.1	122	58.1	
		5M	NAD^+	n.r.	n.r.	n.r.	14
			$NADP^+$	n.r.	n.r.	n.r.	
			$P2NA^+$	7.3	144	19.7	
			$P4NA^+$	3.2	159	49.7	
			$P5NA^+$	4.1	199	48.5	
Source enzyme	*Saccharolobus solfataricus* Glucose dehydrogenase [36]	E44D/E114L	NAD^+	n.r.	n.r.	n.r.	15
			$NADP^+$	n.r.	n.r.	n.r.	
			$BANA^+$	9.08	4.809	0.529	
Source enzyme	*Saccharolobus solfataricus* Glucose dehydrogenase [60]	T189M	NAD^+	n.r.	n.r.	n.r.	16
			$NADP^+$	1.5	57	38	
			$cNADP^+$	0.1	5.7	57	
		V63M	NAD^+	n.r.	n.r.	n.r.	17
			$NADP^+$	0.03	5.9	197	
			$cNADP^+$	0.01	5.5	550	
Source enzyme	*Bacillus subtilis* Glucose dehydrogenase [7]	GDH Triple	NAD^+	3.7	0.41	0.11	18
			$NADP^+$	0.61	4.4	7.5	

Table 1 (*continued*)

Enzyme category	Enzyme	Mutant	Cofactor	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	Entry
			NMN^+	6.4	3.1	0.51	
		GDH Ortho	NAD^+	6.5	0.025	0.0038	19
			$NADP^+$	2.0	0.022	0.011	
			NMN^+	5.9	1.2	0.21	
Source enzyme	*Zymomonas mobilis* Glucose-6-phosphate dehydrogenase [24]	R4	NAD^+	0.37	3.7	10	20
			$NADP^+$	0.06	6.3	105	
			NMN^+	0.18	8.6	47.8	
Source enzyme	*Thermotoga maritima* 6-phosphogluconate dehydrogenase [23]	Mut6-1	NAD^+	n.r.	n.r.	n.r.	21
			$NADP^+$	0.19	28.9	148.6	
			NMN^+	13.5	27.4	2.04	
Source enzyme	*Streptococcus mutans* Glyceraldehyde-3-phosphate dehydrogenase [22]	GapN Penta	NAD^+	n.d.	n.d.	0.043	22
			$NADP^+$	2.6	0.26	0.1	
			NMN^+	12	0.82	0.068	
		GapN Ortho	NAD^+	2	0.021	0.011	23
			$NADP^+$	8.3	0.016	0.002	
			NMN^+	7.7	0.081	0.01	
Sink enzyme	*Escherichia coli* Glutathione reductase [11]	Gor Ortho	NADH	n.a.	n.a.	0.06	24
			NADPH	n.a.	n.a.	0.10	
			NMNH	n.a.	n.a.	0.012	
Sink enzyme	*Lactobacillus pentosus* NADH oxidase [18]	LP-7	NADH	1.7	47	27.65	25
			NADPH	0.89	27	30.34	
			NMNH	n.d.	n.d.	0.01	
		LP-3-EP	NADH	n.d.	n.d.	0.049	26
			NADPH	n.d.	n.d.	0.051	
			NMNH	n.d.	n.d.	0.023	
Sink enzyme	*Lactobacillus lactis* NADH oxidase [9]	Nox Ortho	NADH	n.r.	n.r.	20	27
			NADPH	n.r.	n.r.	20	
			NMNH	n.r.	n.r.	55	
Pathway enzyme	*Ascaris suum* Malic enzyme [42]	L310R/Q401C	NAD^+	10.4	3.8	0.36	28
			$NADP^+$	n.r.	n.r.	n.r.	
			$NCFD^+$	1.70	162.4	96.7	
			NCD^+	1.02	158.2	154.6	
Pathway enzyme	*Lactobacillus helveticus* Lactate dehydrogenase [62]	V152R/I177K/N213E	NAD^+	1.60	0.08	0.049	29
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	1.38	2.95	2.1	
		V152R/I177K/N213I	NAD^+	1.05	0.08	0.074	30
			$NADP^+$	n.r.	n.r.	n.r.	
			NCD^+	0.6600	2.02	3.1	
Pathway enzyme	*Comamonas testosteroni* 3α-Hydroxysteroid dehydrogenase [63]	A70K	NAD^+	n/a	n/a	2.90E-04	31
			$NADP^+$	n.r.	n.r.	n.r.	
			NMN^+	n/a	n/a	1.27E-05	
Pathway enzyme	*Pyrococcus furiosus* Alcohol dehydrogenase [37]	K249G/H255R	NAD^+	0.46	3	6.5	32
			$NADP^+$	n.r.	n.r.	n.r.	
			NMN^+	2.6	0.027	0.0104	
Pathway enzyme	*Serratia sp.* AS13 Butanediol dehydrogenase [9]	*Ser* S-Bdh Ortho	NAD^+	1.6	0.019	0.012	33
			$NADP^+$	1.4	0.002	0.0014	
			NMN^+	4.3	0.38	0.086	

NAD^+: nicotinamide adenine dinucleotide; $NADP^+$: nicotinamide adenine dinucleotide phosphate; NCD^+: nicotinamide cytosine dinucleotide; NMN^+: nicotinamide mononucleotide; $AmNA^+$: 1-(2-carbamoylmethyl)-nicotinamide; $EtOHNA^+$: 1-hydroxyethylnicotinamide; BNA^+: 1-benzylnicotinamide; $ProOHNA^+$: 1-hydroxypropylnicotinamide; $ProNA^+$: 1-propylnicotinamide; BAP^+: 1-benzyl-3-acetylpyridine; $P2NA^+$: 3-carbamoyl-1-phenethylpyridin-1-ium; $P3NA^+$: 3-carbamoyl-1-(3-phenylpropyl)pyridin-1-ium; $P4NA^+$: 3-carbamoyl-1-(4-phenylbutyl) pyridinium; $P5NA^+$: 3-carbamoyl-1-(5-phenylpentyl) pyridinium; $BANA^+$: 3-carbamoyl-1-(4-carboxybenzyl)pyridin-1-ium; $cNADP^+$: carba-nicotinamide adenine dinucleotide phosphate; $NCFD^+$: nicotinamide flucytosine dinucleotide. NADH: reduced nicotinamide adenine dinucleotide; NADPH: reduced nicotinamide adenine dinucleotide phosphate; NMNH: reduced nicotinamide mononucleotide. LY-7: A155N-E175W-A176G-L208V; LY-13: A155N-E175A-A176F; 3M: E44D/I192T/V306I; 5M: E44D/D176G/I192T/A253T/V306I; GDH Triple: Y39Q/A93K/I195R; GDH Ortho: S17E/Y39Q/A93K/I195R; GapN Ortho: P179K/F153S/S330R/I234E/G214E; GapN Penta: P179K/F153S/S330R/I234E/G210Q; Gor Ortho: I178T/R198M/R204L; LP-7: I158S/D177W/V240L; LP-3-EP: I158S/D177W/G178E/V240L/P362H/V395L; Nox Ortho: I159T/D178N/A179F/I243E; *Ser* S-Bdh Ortho: L39Q/A92K/M194T. n.r.: not reported, metrics are not reported by the authors of the cited work. n.d.: not determined, authors of the cited work state the metric was not determined. n/a: not available, authors of the cited work state the metric was not available.

[8,11,34] (Table 1, Entries 4, 5, 6, 7). Zhao and coworkers have engineered formate dehydrogenase as an electron source for NCD^+ [35] (Table 1, Entry 1).

Conversely, the cell's central metabolism can be repurposed to serve as the source of noncanonical reducing power, enabling the NRC systems to directly channel

resources from feedstock digestion, mimicking that of a natural redox cofactor system. Recently, an extensive effort has undergone to repurpose glycolysis for the reduction of NMN^+ [7,18,22–24]. Li and coworkers have repurposed the Embden–Meyerhof–Parnas (EMP) [22] and Entner–Doudoroff [7,18] pathways to specifically reduce NMN^+ through nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GapN) (Table 1, Entries 22 and 23) and glucose dehydrogenase (GDH) (Table 1, Entry 19), respectively. Additionally, Zhang and coworkers have repurposed both cofactor-reducing enzymes in the pentose-phosphate pathway, glucose-6-phosphate dehydrogenase [24] (Table 1, Entry 20) and 6-phosphogluconate dehydrogenase [23] (Table 1, Entry 21), to reduce NMN^+, completing an entire suite of upper glycolysis NMN^+-reducing candidates. Other NRCs have also been reduced using glucose via GDH engineering [7,12,27,36].

For *in vitro* applications where the reduced NRCs will be used by pathways that are mediated by purified enzymes, cofactor recycling systems need to have high productivity and high total turnover number (TTN) (e.g. GDH Triple (Table 1, Entry 18) and PTDH LY-13 (Table 1, Entry 5)) [7,11]. On the other hand, if the reduced NRCs will be tasked to specifically direct reducing power to a target reaction within a complex system such as *in vivo* or in crude cell extract, then the cofactor recycling also needs to discriminate against NAD^+ (e.g. GDH Ortho) [7], NCD-dependent PTDH [34], and NCD-dependent FDH [35] (Table 1, Entries 19, 7, and 1) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) (e.g. GDH Ortho [7], Entry 19), allowing the electron sources to be specifically designated to NRCs.

Engineering electron sinks for noncanonical redox cofactor oxidation

Cells utilize electron sinks to purge excess reduced cofactors. These electron sinks, typically fermentation in anaerobic conditions and respiration in aerobic conditions, are critical to cell survival and maintaining effective control over their redox state. Engineering these electron sinks for NRCs is critical because they enable the user to dictate the specific redox state of the NRC pools (Figure 2). Banta and coworkers demonstrated the use of an electrode to serve as a redox cofactor sink, where excess reduced nicotinamide mononucleotide (NMNH) was oxidized at the electrode surface to drive enzymatic oxidation of D-arabinose in an enzymatic fuel cell [37]. Within this system, NMN(H) outperformed NAD(H) as superior mass transfer rates of the smaller cofactor supported improved diffusion to and from the electrode.

Dedicated electron sinks are also being developed for rapid oxidation of NRCs via enzymatic reactions. Water-forming NADH oxidase (Nox), the workhorse cofactor recycling enzyme in industry [38,39], has been shown to have promiscuous activity toward reduced NRCs [40,41]. To amplify this activity, King and coworkers utilized an NMN-dependent, ultra-high-throughput growth selection platform to engineer the Nox from *Lactobacillus pentosus*, which was capable of the rapid oxidation of NMNH, BNAH, and MNAH [18] (Table 1, Entries 25 and 26). Aspacio and coworkers continued to build on this concept, engineering the Nox from *Lactococcus lactis* to specifically oxidize NMNH, deemed *Ll* Nox Ortho [9] (Table 1, Entry 27). They subsequently utilized this NMNH-specific oxidase as an orthogonal electron sink, maintaining a highly oxidized NMN^+/NMNH pool without the interference from NAD(H) or NADP(H).

Applications of the noncanonical redox cofactor infrastructure

Construction of a complete pool–source–sink infrastructure enables intricate control of NRC systems. Recently, researchers have begun to apply these systems to realize the unique capabilities afforded by these new-to-nature reagents.

With engineered orthogonal enzymes, NRCs promise to deliver reducing or oxidizing power precisely to the target reactions without cross-talking with natural metabolism in the hosts. Zhao and coworkers have developed orthogonal metabolic circuits around NCD^+, where an engineered phosphite dehydrogenase (Table 1, Entry 7) serves as the orthogonal electron source, selectively supplying reducing energy to an engineered malic enzyme (Table 1, Entry 28), allowing for the NCD-mediated control over the flux of electrons between pyruvate and malate [8,42]. Subsequent work utilized an *in vivo* NCD synthesis platform to produce an intracellular NCD^+ pool, enabling the delivery of orthogonal reducing power in a fully self-sufficient NRC system in *E. coli* [14]. Richardson and coworkers utilized their orthogonal NMNH redox system to supply specific, directed reducing power to reduce citral to citronellal, both monoterpene aldehydes, without suffering the rampant nonspecific consumption of the aldehydes to alcohols that occurs in an equivalent system using native cofactors, achieving high aldehyde purities of 97–100% and 83% in crude lysate- and whole-cell-based systems, respectively [2]. The ability to preserve aldehydes [3,43–45] and other short-lived intermediates [46,47] without relying on exhaustive knowledge of the numerous, broad-substrate range, endogenous degradation enzymes will remove the bottleneck to access many value-added chemicals in metabolic engineering and synthetic biology.

NRCs have also been used to shift the equilibrium of redox reactions by providing thermodynamic driving forces. Both *in vitro* and *in vivo*, Aspacio and coworkers

utilized a dedicated electron source GDH Ortho (Table 1, Entry 19) and electron sink Nox Ortho (Table 1, Entry 27) to maintain NMN(H) at an inverted redox state compared with NAD(H) or NADP(H), respectively [9]. This system is used to drive stereo-upgrading of meso-butanediol to (2S,3S)-butanediol or (2R,3R)-butanediol with high purity. This process requires two consecutive steps of first an oxidation and next a reduction to both go to completion. NMN(H)'s insulated reduction potential with NAD(H) and NADP(H) minimized cross-talking and self-canceling of opposing driving forces. Furthermore, many NRCs with lower reduction potentials have been established (Figure 3), which may serve as more potent reductants that are much needed in the recent push to utilize CO_2 in biomanufacturing. CO_2 is inert and requires strong reducing power to convert to formate, formaldehyde, and methanol, which are more reactive to feed into biosynthetic pathways [48,49]. Paul and coworkers have showcased simple biomimetic cofactors with lower redox potentials (more reducing than NADH), which can accelerate enzymatic reduction reactions [25]. Taken together, NRC's capability to provide strong, insulated redox driving force unlocks the ability to perform challenging, thermodynamically uphill reactions that would otherwise not be feasible when working within the confines of natural biology.

Ultimately, broad utilization of NRC systems relies on a diverse toolkit of enzymes that receive these cofactors efficiently. In addition to the dedicated electron sources and sinks, a number of enzymes for pathway engineering have been demonstrated to support NRC biocatalysis, such as nitroreductases [50,51], alcohol dehydrogenases [4,5,52,53], monooxygenases [7,54,55], azoreductases [56], enoate reductases [25,57–59], P450's [7,55], and a number of other enzyme chemistries. Additional protein engineering strategies capable of elucidating rapid, on-demand NRC activity will transform the field. To this end, efforts that tie NRC cycling to cell survival open opportunities to evolve NRC-utilizing enzymes. Recently, Li and coworkers have built a pair of universal, growth-based selection platforms capable of engineering NMN(H)-oxidizing and -reducing enzymes, respectively [11,18]. This is achieved by metabolically engineering two auxotroph *E. coli* strains: one whose glycolysis requires the oxidized NMN to function, and the other requires the reduced NMN (NMNH) to combat oxidative stress. Excitingly, universal design principles have emerged from deeply navigating the protein sequence space, which was not possible without the high-throughput methods. These growth selection platforms complement the high-throughput screening architecture developed around droplet sorting [60] and colorimetric-based colony screening [23,24] to afford the full range of optimization and engineering capability.

Conclusion

As the fields of metabolic engineering and synthetic microbiology continue to push the limits of feasibility with the development of novel biocatalytic systems to produce higher-value, more complicated products, tools to help control the fate of substrate carbon and energy are becoming increasingly paramount. Noncanonical cofactor biomimetics are a powerful tool to provide increased insulation and regulation of flux through a given pathway by supplying high, nonnatural driving forces in a manner inaccessible to the natural pathways. The field has just begun to realize the potential of these broadly applicable cofactors. As additional pool–source–sink infrastructures are developed, a suite of noncanonical cofactor-driving forces will be available to reach biocatalytic pathways previously thought to be thermodynamically impossible.

CRediT authorship contribution statement

William B. Black: Writing and editing. **Sean Perea:** Writing and editing. **Han Li:** Writing and editing.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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