Protein engineering of oxidoreductases utilizing nicotinamide-based coenzymes, with applications in synthetic biology

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ABSTRACT

Two natural nicotinamide-based coenzymes (NAD and NADP) are indispensably required by the vast majority of oxidoreductases for catabolism and anabolism, respectively. Most NAD(P)-dependent oxidoreductases prefer one coenzyme as an electron acceptor or donor to the other depending on their different metabolic roles. This coenzyme preference associated with coenzyme imbalance presents some challenges for the construction of high-efficiency *in vivo* and *in vitro* synthetic biology pathways. Changing the coenzyme preference of NAD(P)-dependent oxidoreductases is an important area of protein engineering, which is closely related to product-oriented synthetic biology projects. This review focuses on the methodology of nicotinamide-based coenzyme engineering, with its application in improving product yields and decreasing production costs. Biomimetic nicotinamide-containing coenzymes have been proposed to replace natural coenzymes because they are more stable and less costly than natural coenzymes. Recent advances in the switching of coenzyme preference from natural to biomimetic coenzymes are also covered in this review. Engineering coenzyme preferences from natural to biomimetic coenzymes has become an important direction for coenzyme engineering, especially for *in vitro* synthetic pathways and *in vivo* bioorthogonal redox pathways.
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Peer review under responsibility of KeAi Communications Co., Ltd.
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1. Introduction

Protein engineering is the process of modifying the amino acid sequence of proteins toward desired properties, including improved substrate spectrum [1,2], product selectivity [3,4], enzyme activity [5], thermostability [6–8], and solvent tolerance [8]. Protein engineering has been a powerful tool in biotechnology to generate a vast number of enhanced or novel enzymes for industrial applications and has played a crucial role in advancing synthetic biology [9].

Synthetic biology is an emerging discipline that applies engineering principles for the design and assembly of biological components toward synthetic biological entities with an ultimate goal of cost-effective biomanufacturing [10]. The purpose of synthetic biology is to design and construct novel biological pathways, organisms and devices or to redesign the existing natural biological systems, in order to understand the complexity of biological systems and to improve various applications [11]. The most important application of synthetic biology may be the low-cost production of new drugs, chemicals, biomaterials, and bioenergy [12–18]. Synthetic biology can influence many other scientific and engineering fields as well as various aspects of daily life and society [17]. It can be classified into *in vivo* and *in vitro* synthetic biology [19]. *In vivo* synthetic biology mainly focuses on fundamental biological research facilitated by the use of synthetic DNA and genetic circuits on typical model microorganisms, such as *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* [14]. It is a current predominant research area because living organisms can self-duplicate without major concerns of the biocatalyst preparation, possibly due to a biotechnology paradigm based on thousands of years of fermentation. In contrast, *in vitro* synthetic biology, sometimes referred to as cell-free synthetic biology, is based on reconstituted enzyme mixtures or cell lysates in one pot for the ultimate purpose of biomanufacturing [20–24]. Strictly speaking, *in vitro* synthetic biology is slightly different from cell-free synthetic biology, where the former is based on the reconstitution of (purified) enzymes, coenzymes and/or other abiotic components (for example, using benzyl viologen as electron mediator for *in vitro* biohydrogen generation [25]), and the latter is mainly based on the cell lysates of one or multiple cell cultures. The *in vitro* synthetic biology platform has some distinctive advantages, such as high product yield, fast reaction rate, highly engineering flexibility, and high tolerance in toxic environments [19–21,26]. Recently, the first industrial biomanufacturing example of the cost-effective production of myoinositol from starch has been demonstrated in China [27].

Oxidoreductases are the largest group of enzymes in the Enzyme Commission nomenclature. These enzymes account for nearly 30% (1801/6300) of active enzyme classes according to Brenda database (http://brenda-enzymes.info/) [28]. Coenzymes are usually required in these oxidoreductase-catalyzed reactions to transport electron, hydride, hydrogen, oxygen, or other atoms or small molecules in different enzymatic pathways [29,30]. Typical coenzymes are nicotinamide adenine dinucleotide (NAD)/nicotinamide adenine dinucleotide phosphate (NADP), ubiquinone (CoQ), and flavin mononucleotide (FMN)/flavin adenine dinucleotide

(FAD). Nicotinamide-based coenzymes for the transport and storage of electrons in the form of hydride groups are the most important, because 80% of characterized oxidoreductases need NAD as a coenzyme, and 10% of them need NADP as a coenzyme [30]. NAD and NADP are two types of ubiquitous pyridine nucleotide coenzymes that differ only by the additional 2′-phosphate group esterified to the adenosine monophosphate moiety of NADP (Fig. 1a). Because the phosphate group of NADP is sufficiently distant spatially and covalently from the chemically active nicotinamide moiety (red rounded rectangle in Fig. 1a), nearly all oxidoreductases exhibit a strong preference for one to the other for implementing different metabolic roles [31].

Changing the coenzyme preference of oxidoreductases is an important area of protein engineering. It has also been recognized as an important tool for *in vitro* and *in vivo* synthetic biology projects. For *in vitro* synthetic biology and cascade biocatalysis projects, coenzyme preference is usually switched from NADP to NAD because the price of NADP is much higher than that of NAD (e.g., $200 per g for NADH (Sigma N8129), $6000 per g for NADPH (Sigma N5130), $140 per g for NAD$^+$ (Sigma N7004) and $1000 per g for NADP$^+$ (Sigma N5755)). Additionally, NAD is more stable than NADP [2,32,33]. Furthermore, more NADH-regeneration enzymes *in vitro* are available than NADPH-regeneration enzymes [29,34]. For *in vivo* synthetic biology projects, the switch of coenzyme preference can be conducted in both directions from NAD to NADP or from NADP to NAD to balance coenzyme availability and increase metabolic pathway efficiency [35–39]. Coenzyme engineering from natural to biomimetic nicotinamide-based coenzymes (Fig. 1b and c) may further decrease the production cost for *in vitro* synthetic biology because the cost and stability of biomimics are much better than those of natural coenzymes [40,41]. Engineered enzymes with specificities on biomimetic nicotinamide coenzymes can be used to develop bioorthogonal redox systems *in vivo* without interfering with native biochemical processes [42–44].

In this review, we focus on the methods of coenzyme engineering regarding switching the nicotinamide-based coenzyme preferences of oxidoreductases and the application of the mutant enzymes with different coenzyme preferences in product-oriented synthetic biology. The latest advances in the general design of coenzyme engineering and high-throughput screening methods for directed evolution are highlighted. Coenzyme preference changes from natural to biomimetic coenzymes could be extremely important, especially to *in vitro* synthetic biology such as biohydrogen and bioelectricity generation from oligosaccharides [25,45–51].

2. Coenzyme engineering methods of nicotinamide-based coenzymes

Coenzyme engineering that changes enzymatic coenzyme preferences has three major methods: rational design, semi-rational design and random mutagenesis (Fig. 2) [52,53]. Table 1 presents some representatives of product-oriented coenzyme engineering for *in vivo* and *in vitro* synthetic biology using these engineering methods. Rational design is a knowledge-based method
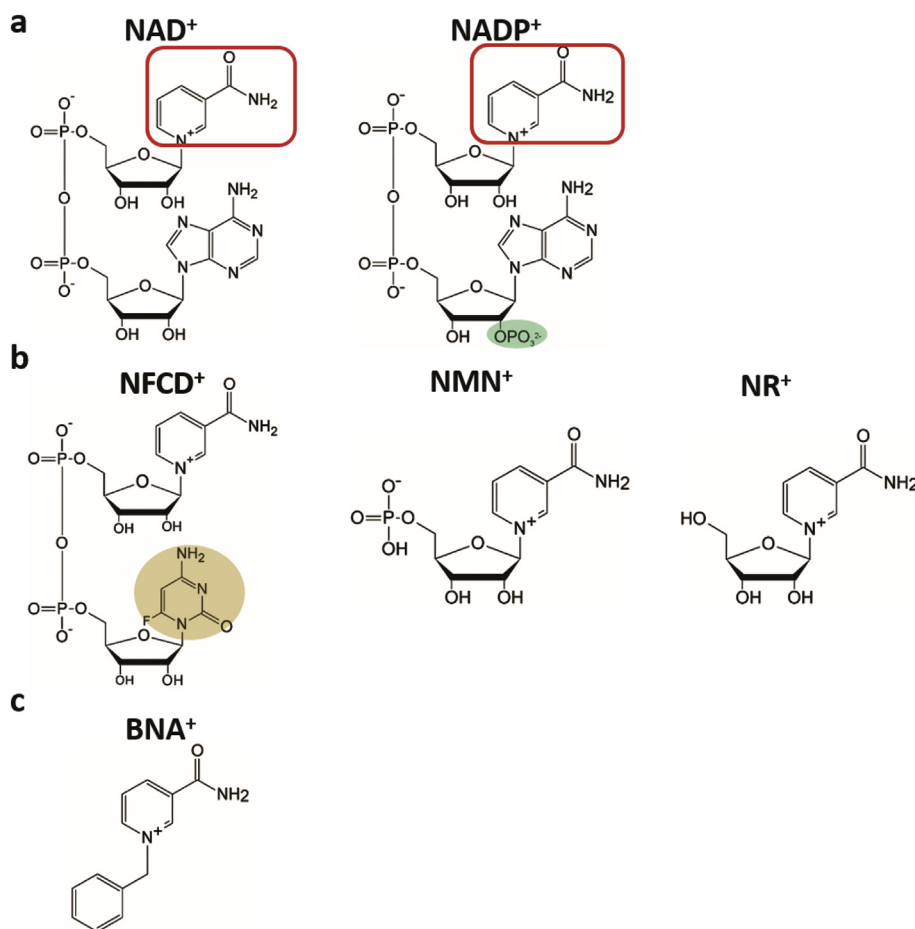
Fig. 1. Structures of nicotinamide-based coenzymes and biomimetic nicotinamide coenzymes. a) Two natural coenzymes, NAD$^+$ and NADP$^+$, in which the chemical groups in the open red rounded rectangles represent where the redox reaction occurs; these chemical groups are the same in all coenzymes. b) Biomimetic coenzymes derived from natural coenzymes, nicotinamide flucytosine dinucleotide (NFCD$^+$), nicotinamide mononucleotide (NMN$^+$), and nicotinamide mononucleoside (NR$^+$); the chemical group in the shaded area indicates the structural difference between NFCD$^+$ and NAD$^+$. c) Synthetic biomimetic coenzyme 1-benzyl nicotinamide (BNA$^+$).

that requires prior information on structure and/or function. Specific residues are used to replace the residues in specific sites of the targeted enzymes by site-directed mutagenesis, hoping to obtain mutants with the desired properties. Semi-rational design is also a knowledge-based approach that creates a mutant library by site-saturation mutagenesis (where all or a fraction of the 20 natural amino acids are tested) at specific residues. Random mutagenesis is a knowledge-free approach that generates a mutant library by error-prone PCR or gene shuffling for whole-gene randomization. The last two approaches always require an extra step for the screening or selection of the mutated enzymes possessing desired properties from the mutant library. Chica et al. proposed a flow-chart regarding the selection of the appropriate enzyme engineering approach based on the availability of experimental tools as well as the prior knowledge of structure and function [53]. Because most NAD(P)-based oxidoreductases share a highly conserved coenzyme-binding motif – the Rossmann fold, which is the first identified conserved protein domain based on sequence alignments and crystal structures [54,55], rational design and semi-rational design that creates 'small but smart' libraries based on this conserved motif are more widely used in coenzyme engineering projects than random mutagenesis that renders a large size of mutant library.

2.1. Rational design

Rational design is the oldest protein engineering tool to switch the coenzyme preference of oxidoreductases. It mutates specific amino acid residues to certain other residues using site-directed mutagenesis based on the structures of NAD(P)-enzyme complexes and related catalytic mechanisms. Generally, coenzyme engineering by rational design starts with the identification of residues near coenzyme-binding sites [56,57], residues binding with the 2′ phosphate group [58] or adenosine-binding pocket [59], or residues essential for catalytic activity [2,60−63]. Chen et al. performed an amino acid sequence alignment of the coenzyme-binding motifs of NADP- and NAD-preferred 6-phosphogluconate dehydrogenases (6PGDH) (Fig. 3a) [2]. The loop region amino acids (in red box of Fig. 3a) are responsible for the interaction between enzymes and 2′-phosphate of NADP. The alignment of the loop region indicates that NAD-preferred 6PGDH wild-type enzymes and mutants share a highly conservative, acidic aspartate residue at the N-terminal end of this loop region (site 32), while NADP-preferred 6PGDHs have three highly conserved amino acid residues at sites 32, 33, and 34 (Asn32, Arg33 and Ser/Thr34) (Fig. 3b). When these key amino acid residues responsible for the binding of the 2′-phosphate group of NADP$^+$ were modified by site-directed mutagenesis on a 6PGDH from *Thermotoga maritima*, the best mutant N32E/R33I/T34I exhibited a ratio of 96 for catalytic
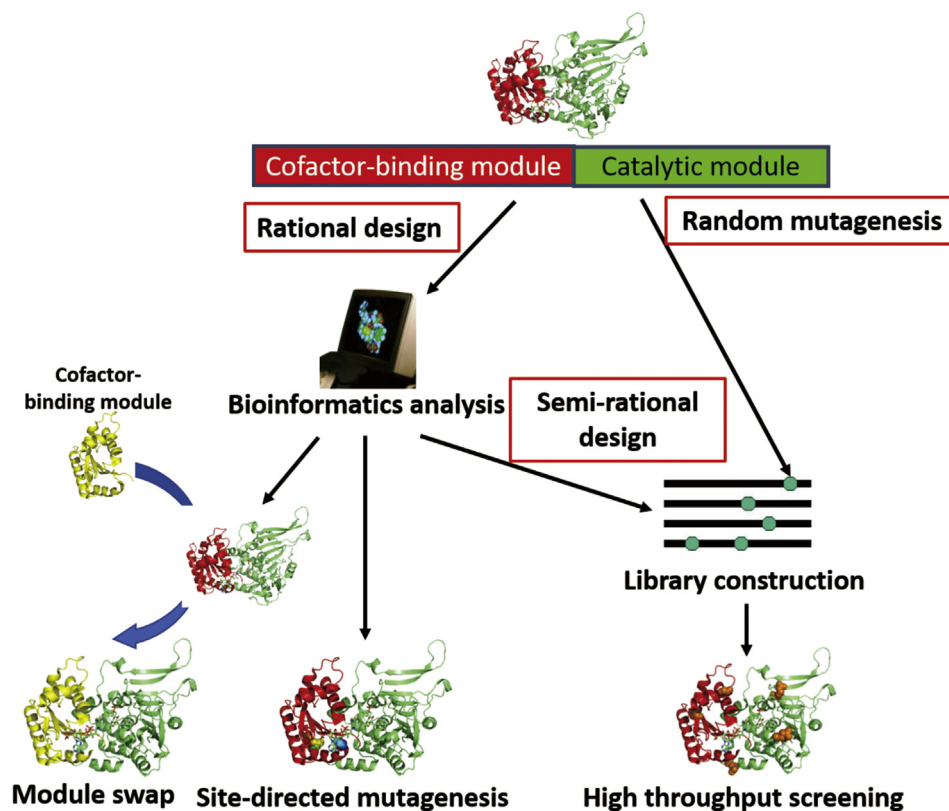
Fig. 2. Scheme of coenzyme engineering methods, including rational design, semi-rational design and random mutagenesis.

Table 1
List of product-oriented coenzyme engineering on natural nicotinamide coenzymes NAD(P).

Enzyme	Source	Specificity	Mutations	Product	Increasing effect	Reference
Glyceraldehyde 3-phosphate dehydrogenase	*Corynebacterium glutamicum*	NADH→NADPH	D35G/L36R/P192S	Lysine	~60% higher yield	[120]
NADH oxidase 2	*Streptococcus mutans*	NADH→NADPH	V193R/V194H	2-heptanone	NA	[121]
1,5-anhydro-D-fructose reductase	*Sinorhizobium Morelense*	NADPH→NADH	A13G	1,5-anhydro-D-mannitol	NA	[122]
Imine reductase	*Streptomyces* sp. GF3587	NADPH→NADH	K40A	2-methylpyrolidine	~64% higher conversion	[123]
Ketol-acid reductoisomerase	*Escherichia coli*	NADPH→NADH	A71S/R76D/S78D/ Q110V	2-methylpropan-1-ol (isobutanol)	3-fold higher titer	[39]
Xylose reductase	*Pichia stipitis*	NADPH→NADH	R276H	Ethanol	~20% higher yield	[91]
Xylose reductase	*Candida tenuis*	NADPH→NADH	K274R/N276D	Ethanol	~42% higher yield	[124]
6-phosphogluconate dehydrogenase	*Thermotoga maritima*	NADP$^+$→NAD$^+$	N32E/R33I/T34I	Electricity	~25% higher maximum power density and current density	[125]
6-phosphogluconate dehydrogenase	*Geobacillus stearothermophilus*	NADP$^+$→NAD$^+$	N33D/R34Y/K38L	Polyhydroxybutyrate	NA	[126]
Glucose 6-phosphate dehydrogenase	*Geobacillus stearothermophilus*	NADP$^+$→NAD$^+$	A47D	Polyhydroxybutyrate	NA	[126]
Glucose 6-phosphate dehydrogenase	*Thermotoga maritima*	NADP$^+$→NAD$^+$	S33E/R65M/T66S	Hydrogen	NA	Unpublished

NA: Not Available.

efficiency (k_{cat}/K_m) on NAD$^+$ to NADP$^+$, which is a ~64,000-fold reversal of the coenzyme selectivity from NADP$^+$ to NAD$^+$. Among these residues, arginine 33 plays a critical role in NADP$^+$ binding by contributing a positively charged planar residue that interacts primarily with the 2′-phosphate of NADP$^+$. Changing this arginine to aspartate or glutamate is often used to shift coenzyme preference from NADP to NAD [64–66]. Cui et al. (2015) developed a novel computational strategy to alter the coenzyme preference that enhances the hydrogen-bond interaction between an enzyme and a coenzyme. This novel computational strategy only required

the structure of the target enzyme without other homologous enzymes. By using this rational design method, *Gluconobacter oxydans* Gox2181, which belongs to the short-chain dehydrogenases/reductases superfamily (SDR superfamily), was engineered to show a much higher enzymatic activity with NADPH as its coenzyme through the two-site mutation of Q20R and D43S [67].

Module swapping is another rational design method to switch coenzyme preference by replacing the original coenzyme binding pocket with a new one from homologous enzymes [68]. For example, Yaoi et al. changed the coenzyme preference of an
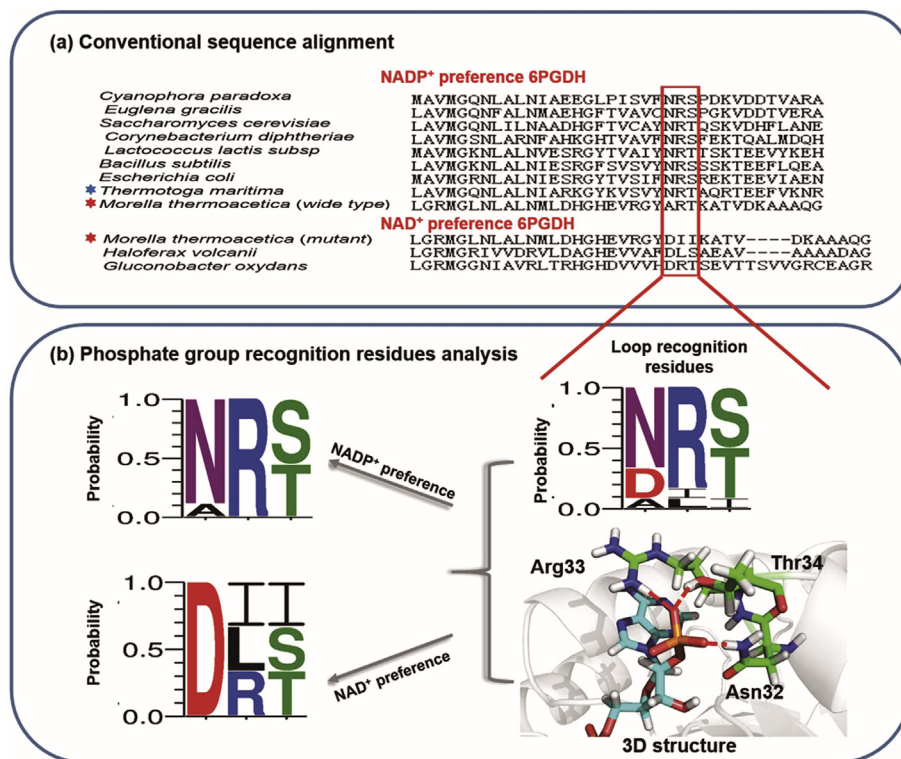
Fig. 3. a) Amino acid sequence alignment of the coenzyme-binding motif of various 6PGDH enzymes. The residues comprising the loop region and responsible for coenzyme recognition are boxed. Red stars represent *M. thermoacetica* wild-type NADP+-preferred 6PGDH and the NAD+-preferred 6PGDH mutant. The blue star indicates *T. maritima* 6PGDH studied in this research. (b) Sub-alignments of key amino acid residues playing an important role in 2′-phosphate interaction. The colors in the sequence logo refer to hydrophobic (black), positive charge (blue), negative charge (red) and polar (green) residues (This figure is a courtesy from Ref. [2]).

isocitrate dehydrogenase by replacing the NADP-binding pocket with a homogeneous NAD-binding pocket [69]. Similarly, the co-enzyme preferences of a β-isopropylmalate dehydrogenase [70] and a short-chain dehydrogenase [71] have been reversed using this strategy.

2.2. Semi-rational design

Semi-rational design is a powerful method to switch the coen-zyme preference by site-saturated mutagenesis of some critical amino acid residues deduced from bioinformatics analysis followed by the screening of mutant libraries. Coenzyme engineering of an *E. coli* ketol acid reductoisomerase (KARI) from NADP to NAD is a typical example of semi-rational design from Arnold's laboratory [39]. Five amino acids in the Rossmann fold of this KARI were determined based on previous work [72], sequence alignment and structure of the cofactor binding pocket. Five individual libraries of each amino acid were constructed by site-saturation mutagenesis and were screened for variants exhibiting a higher ratio of NADH to NADPH activities. A library was constructed by combining all beneficial mutations as well as the wild-type residues. The best variant, which had four mutation sites, exhibited much higher ac-tivity on NADH to NADPH, resulting in a 54,000-fold change in the ratio of the catalytic efficiency (k_{cat}/K_m) on NADH to NADPH compared with the wild-type enzyme [39]. Later, the same group proposed a general semi-rational approach to switch the coenzyme preference of KARI from NADPH to NADH by integrating previous results of an engineered NADH-dependent mutant of *E. coli* KARI, available KARI crystal structure information, and a comprehensive sequence alignment of KARI [61]. With this approach, the specific patterns of amino acid residue replacement in the β2αB loop

showed a positive effect on reversing the coenzyme specificity of KARI. The approach included the following steps: (1) identification of the loop, (2) determination of the β2αB-loop length and muta-tion based on the loop length by site-directed mutagenesis and site-saturated mutagenesis to achieve the coenzyme switch; and (3) improvement of the overall activity on NADH via random mutagenesis [61]. Recently, this group has developed a structure-guided, semi-rational strategy for reversing enzymatic nicotinamide-based coenzyme specificity of all NAD(P)-utilizing enzymes [31] based on the increased number of protein crystal structures with high resolution and homogeneous oxidoreductase sequences with different coenzyme preferences. This strategy is comprised of three steps: structural analysis of enzymes, design and screening of focused mutant libraries to reverse cofactor preference, and recovery of catalytic efficiency. Unlike the case of KARI engineering involving random mutagenesis of the entire gene [61], the recovery of catalytic efficiency in this strategy is based on the predicted positions in the amino acid sequence with dramati-cally increased probabilities of harboring compensatory mutations. This strategy has shown the efficacy of inverting the coenzyme preference of four structurally diverse NADP-dependent enzymes: glyoxylate reductase, cinnamyl alcohol dehydrogenase, xylose reductase, and iron-containing alcohol dehydrogenase. The analytical components of this approach have been fully automated and available in the form of a user-friendly online tool named Cofactor Specificity Reversal-Structural Analysis and Library Design (CSR-SALAD) [31].

2.3. Random mutagenesis

Random mutagenesis of the entire DNA sequence may be the

last solution to change the enzyme properties without relying on the crystal or modeling structure of the target protein [73,74]. This method is rarely used for shifting the coenzyme preference between NADP and NAD because coenzyme-binding domains are highly conserved at some specific residues close to coenzyme-binding sites. However, this method may be of particular importance for screening mutants that can work on biomimetic coenzymes, whose structures largely differ from NADP and NAD (Fig. 1). Random mutagenesis is also useful when compensatory mutations are remote from the cofactor-binding sites [31].

2.4. Directed evolution based on high-throughput screening (HTS)

A high-throughput screening method is urgently required to identify positive mutants from the library constructed by site-saturated mutagenesis or random mutagenesis. The use of 96-well microplate screening based on the absorbency of NAD(P)H at 340 nm or coenzyme-linked colorimetric assay is straightforward to measure the activities of dehydrogenases [33,61,75]. However, the microplate-based screening is labor-intensive, time-consuming and may require expensive automated machines [31,76]. It will be of great significance to develop a simple and effective HTS method to determine the coenzyme preference change of oxidoreductases. Recently, Zhang's group developed a Petri-dish double layer-based screening method to identify mutants of thermophilic 6-phosphogluconate dehydrogenase (6PGDH) from *Moorella thermoacetica* with reversed coenzyme preference from NADP$^+$ to NAD$^+$ [1]. The colonies of a 6PGDH mutant library were treated by heat to deactivate intracellular mesophilic dehydrogenases and reductive compounds (i.e., NADPH and NADH) and to disrupt the cell membrane. A second semi-solid layer was added by pouring the melted agarose solution containing tetranitroblue tetrazolium (TNBT), phenazine methosulfate (PMS), NAD$^+$, and 6-phosphogluconate. In the second layer, 6PGDH catalyzes the hydration of 6-phosphogluconate, coproducing NADH from NAD$^+$. In the presence of PMS and NADH, the colorless redox dye TNBT was reduced to black TNBT-formazan (Fig. 4A). As a result, 6PGDH mutants with improved activity on NAD$^+$ can be identified by naked eyes (Fig. 4B). Positive mutants were recovered by direct extraction of the plasmid from dead-cell colonies followed by plasmid transformation into *E. coli* TOP10 [1]. Using this method, our laboratory has also switched the coenzyme preference of *T. maritima* glucose 6-phosphate dehydrogenase (G6PDH) from NADP$^+$ to NAD$^+$ (submitted for publication).

3. Applications of coenzyme engineering in *in vivo* synthetic biology

In vivo synthetic biology and metabolic engineering are widely investigated for their potentials in the production of biofuels, amino acids, alcohols, natural products, and antibiotics [77,78]. Because NAD and NADP have different roles in catabolism and anabolism, respectively, their supply and consumption, as well as their balance, are essential for engineered organisms. Synthetic pathways that failed to match the coenzyme supply and consumption will possibly result in low product yields and slow volumetric productivity. For example, Liao's isobutanol synthesis pathway has a NADH-generation pathway to produce an isobutanol precursor followed by a NADPH-consumption step for the formation of isobutanol [79,80]. Fig. 5 illustrates a case in which one coenzyme is more prevalent in the cell organelle than the other. One enzyme in the pathway prefers the high-abundant coenzyme, while the other enzyme prefers the low-abundant coenzyme, coenzyme imbalance occurs, leading to the low-efficiency biosynthesis of the desired product. To balance different coenzymes,

several approaches can be taken: (1) Increase of oxygen supply can balance the energy flux, yet may still result in a lower-than-theoretical product yield. (2) Introduction of a transhydrogenase [81] that catalyzes the reversible transfer of a hydride ion between NADH and NADP$^+$. Nevertheless, the transhydrogenase may not always shift the hydride ion in the desired direction [82]. Additionally, introduction of new cellular components may increase the metabolic burden of the cells or may direct the energy flux toward undesired directions. (3) Replacement of native enzymes with those harboring a different coenzyme specificity [83,84]. However, finding a sequence with specific desired properties can be difficult, particularly when few members of a protein family have been characterized [62]. (4) The best strategy is changing the coenzyme specificity of the oxidoreductases in the pathway by protein engineering and then introducing the mutant enzyme into the cells for the replacement of the wild-type enzyme to solve the problem of coenzyme imbalance. Unlike *in vitro* synthetic biology in which the coenzyme engineering from NADP to NAD is preferred because of the cost issue, *in vivo* synthetic biology can change the coenzyme preference in both directions from NADP to NAD and from NAD to NADP [85]. In this section, we introduce some examples concerning the improvement of the productivity of microbial cell factories by changing the enzyme's coenzyme preference.

3.1. From NAD to NADP

Amino acids represent one of the largest classes of fermentative products, whose production closely correlates with the availability of NADPH. For example, the synthesis of one mole of lysine requires 4 mol of NADPH in *Corynebacterium glutamicum*. To produce more NADPH from glycolysis for the synthesis of lysine, Bommareddy et al. changed the coenzyme specificity of a native NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from *C. glutamicum* to NADP by rational protein design (D35G/L36T/T37K/P192S). The mutant GAPDH-containing *C. glutamicum* strain shows approximately 60% improvement in lysine production than the wild-type strain [86]. In another example, a recombinant *S. cerevisiae* strain containing *Pichia stipitis* xylose reductase (PsXR) and xylitol dehydrogenase (PsXDH) genes can convert xylose to ethanol, along with the unfavorable excretion of xylitol due to intercellular redox imbalance caused by the different coenzyme specificity between NADPH-preferring XR and NAD$^+$-dependent XDH. Watanabe et al. succeeded in generating several PsXDH mutants with a reversal of coenzyme specificity toward NADP$^+$ by multiple site-directed mutagenesis of the coenzyme-binding domain. A quadruple mutant (D207A/I208R/F209S/N211R) showed a more than 4500-fold increase of k_{cat}/K_m on NADP$^+$ than the wild-type enzyme, reaching a comparable value with the k_{cat}/K_m on NAD$^+$ of the wild-type enzyme [87]. These researchers further constructed a recombinant yeast coexpressing NADPH-preferring PsXR and NADP$^+$-dependent PsXDH, and the resultant recombinant yeast showed increased ethanol production and decreased xylitol excretion [35,88].

3.2. From NADP to NAD

Isobutanol can be produced from glucose by recombinant *E. coli* through the modified biosynthesis of branched-chain amino acids (BCAAs) pathway [61,62,79,80]. The pathway generates two pyruvates and two NADH per glucose molecule via glycolysis but consumes two equivalents of NADPH per isobutanol molecule synthesized, where NADPH is consumed by ketol-acid reductoisomerase (KARI) and alcohol dehydrogenase (ADH). The fermentation of this recombinant strain was operated aerobically or micro-aerobically to activate the pentose phosphate pathway (PPP)
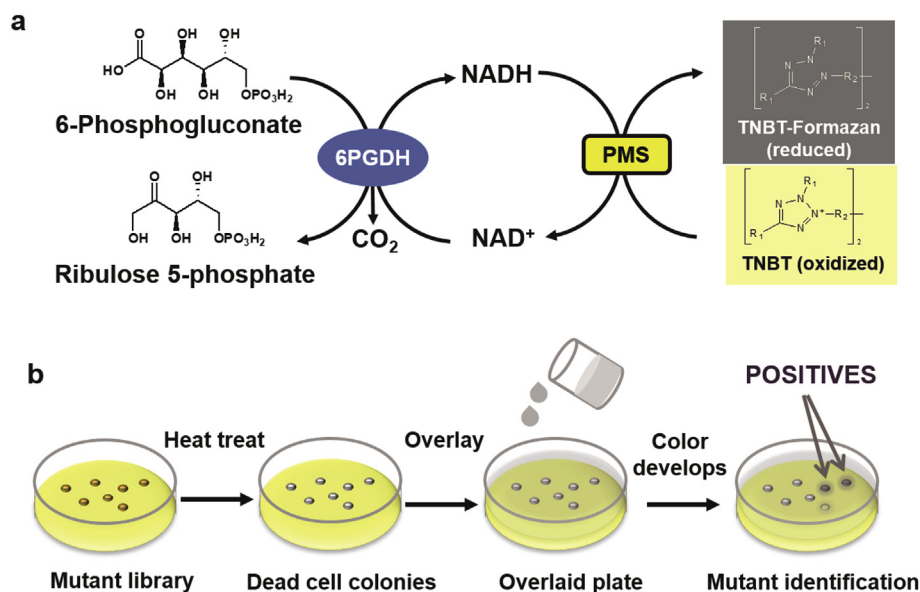
Fig. 4. Scheme of double layer-based screening. a) Mechanism of the colorimetric assay. 6PGDH catalyzes the oxidation of 6-phosphogluconate to ribulose 5-phosphate and CO_2, and the reduction of NAD^+ to NADH. In the presence of PMS, NADH transfers its hydride and reduces the colorless redox dye TNBT to black color TNBT formazan. b) The process of the double layer-based screening method. The mutant library was treated by heat and overlaid by a second agarose layer with reagents. The colonies with a darker color and halo were identified as positive mutants.
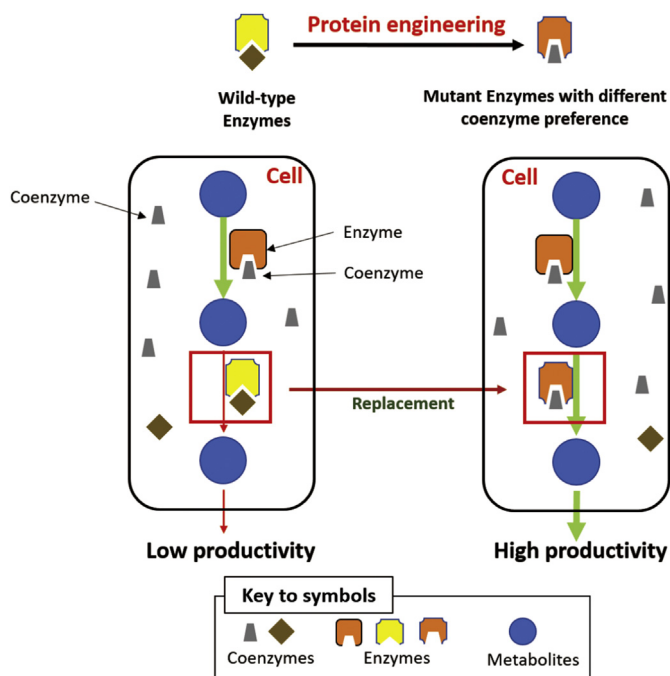

Fig. 5. Engineering the coenzyme preference of oxidoreductases in a metabolic pathway by protein engineering *in vitro* followed by the replacement of the wild-type enzyme with the mutant enzyme to solve the problem of coenzyme un-matching.

or the tricarboxylic acid (TCA) cycle for sufficient NADPH supply. Nevertheless, anaerobic conditions are preferred for large-scale biofuel production due to lower operating costs (e.g., cooling, mixing and aeration) as well as higher product yields [89]. Under anaerobic conditions, isobutanol production by engineered *E. coli* showed a limited supply of NADPH because of the shutdown of PPP or TCA cycle [37,39]. Bastian et al. investigated the construction of a NADH-dependent pathway using NADH-preferring engineered

E. coli KARI and ADH to produce high-yield isobutanol under anaerobic conditions. The introduction of this NADH-dependent pathway enabled anaerobic isobutanol production at a theoretical yield [39]. Similarly, the NADH-dependent pathway containing PsXDH and PsXR was also introduced into *S. cerevisiae* [90,91]. PsXR was engineered to use NADH by the mutation of R276H. The expression of the PsXR/R276H mutant and wild-type (WT) PsXDH in *S. cerevisiae* can lead to a 20% increase in ethanol production and a 52% decrease in xylitol excretion compared with the WT strain.

4. Applications of coenzyme engineering for *in vitro* synthetic biology

In vitro synthetic biology is an emerging biomanufacturing platform with advantages such as a high product yield, improved energy conversion efficiency, fast reaction rates, and broad reaction conditions [92]. This platform has shown great potential on the production of bioenergy (e.g., hydrogen and electricity), pharmaceuticals (e.g., heparin), and biochemicals (i.e., α-ketoglutarate, myo-inositol, isobutanol, fructose 1,6-biphosphate, polyhydroxybutyrate, and (R)-phenylethanol) [25,27,93–99]. The pathway design principle of the *in vitro* synthetic biology platform requires balances between the coenzyme supply and consumption as well as their types [100]. The product cost of this platform is crucial for manufacturing biocommodities [12,26], and can be reduced by the utilization of less costly coenzymes and the addition of coenzyme regeneration systems. NAD is preferable to NADP for *in vitro* synthetic biology because of its lower price [33,68], higher stability [101], and more regeneration methods [29,34]. In this section, we highlight several examples of *in vitro* synthetic (enzymatic) biosystems (ivSEB) involving coenzyme engineering from NADP to NAD. Cascade biocatalysis by engineered oxidoreductases with NADH or biomimetic cofactors along with coenzyme regeneration are not covered here and are referred elsewhere [41,102,103].

Biohydrogen is believed to be the best future transportation fuel. Hydrogen can be produced by ivSEBs from advanced water splitting energized by starch, sucrose and cellodextrins with a theoretical

yield of 12 mol H_2 per mol of hexose and water [25,47,48], breaking the Thauer limit of 4 mol of H_2 per mol of glucose [104,105]. In these ivSEBs, glucose 6-phosphate (G6P) is generated from ATP-free enzymatic phosphorylation of glucan (i.e., starch) and regenerated from the non-oxidative pentose phosphate pathway (PPP) and partial gluconeogenesis pathway. Two cascade dehydrogenases, G6PDH and 6PGDH, oxidize G6P to ribulose 5-phosphate (Ru5P) and simultaneously reduce two $NADP^+$ molecules to two NADPH molecules, which are converted into hydrogen with the help of a hydrogenase or even a biomimetic electron-transport chain containing an abiotic electron mediator [25]. Economic analysis suggests that the replacement of $NADP^+$ with NAD^+ shows great impact on the cost decrease of *in vitro* hydrogen production by changing the coenzyme preference of G6PDH and 6PGDH from $NADP^+$ to NAD^+ [106]. Chen et al. changed the coenzyme preference of hyperthermophilic *T. maritima* 6PGDH from $NADP^+$ to NAD^+ by rational design [2]. The best mutant shows ~64,000-fold reversal of the coenzyme preference from $NADP^+$ to NAD^+, resulting in 25% higher current density of the 6PGDH-diaphorase electricity production system [2]. Additionally, we further engineered *T. maritima* G6PDH to change its coenzyme preference from $NADP^+$ to NAD^+. The best mutant shows a more than 262-fold reversal of the coenzyme preference from $NADP^+$ to NAD^+ (submitted for publication). By coupling the G6PDH and 6PGDH mutants into the hydrogen production pathway, we achieved the highest *in vitro* hydrogen production rate of 530 mmol H_2/L/h at 80 °C from starch (submitted for publication). Polyhydroxybutyrate (PHB) is a type of biodegradable polyester. It can be produced by microbes in response to physiological stress [107] or engineered *E. coli* harboring *Streptomyces aureofaciens* PHB biosynthesis genes [108]. Recently, Opgenorth et al. designed an *in vitro* pentose-bifido-glycolysis (PBG) cycle to breakdown glucose for PHB synthesis. Through the PBG cycle, one mole of glucose can be converted into 2 mol of acetyl-CoA with 4 mol of NAD(P)H and 2 mol of CO_2. To prevent the accumulation of NADPH due to coenzyme imbalance, G6PDH and 6PGDH involved in the PBG cycle were engineered to change the coenzyme preference from $NADP^+$ to NAD^+. Engineered dehydrogenases were used to regulate the efficiency of the pathway by incorporation with NADH oxidase, $NADP^+$-dependent wide-type G6PDH and 6PGDH, exhibiting a more than two-fold improvement of the product yield [94]. Sieber and coworkers designed an ATP-free ivSEB to produce pyruvate from glucose with two NADH molecules per glucose molecule; pyruvate can then be converted to ethanol and isobutanol, consuming the 2 mol of NADH per 2 mol of ethanol and one mole of isobutanol molecule, respectively [109]. The NADH-generating enzymes are glucose dehydrogenase (GDH) and glyceraldehyde dehydrogenase (AIDH). However, AIDH has a very low activity on NAD^+ compared with $NADP^+$. To minimize the reaction complexity, the designed pathway was further consolidated to use the coenzyme NADH as the only electron carrier, and AIDH was engineered by directed evolution to have an 8-fold higher activity on NAD^+ [110].

5. Biomimetic coenzyme engineering

To further decease the coenzyme costs *in vitro*, the best solution is the replacement of natural coenzymes with low-cost biomimetic ones [40,68]. Biomimetic coenzymes, such as nicotinamide mononucleotide (NMN), nicotinamide mononucleoside (NR) (Fig. 1b) and 1-benzyl nicotinamide (BNA) (Fig. 1c), are not only less costly but also have better stability [41,68]. NMN and NR are precursors of NAD(P) and are much smaller in size than NAD(P) (Fig. 1b), and BNA is a typical biomimetic nicotinamide coenzyme. Few wild-type redox enzymes have been reported to have promiscuous activities on NMN, including liver alcohol dehydrogenase [111] and glutamic

dehydrogenase [112]. Scott and coworkers have engineered *Pyrococcus furiosus* alcohol dehydrogenase to act on NMN, but the enzyme activity remains very low [113]. Fish et al. found that the pyrophosphate and adenosine groups in NAD(P) are not essential for the hydride transfer for some flavin-containing oxidoreductases, and proposed the use of BNA chloride to replace NAD(P) [114]. Clark and Fish collaborated to show that an engineered flavin-containing P450 mutant with two amino acid changes can utilize BNA [115]. Additionally, another group showed that engineered P450 utilizes zinc dust rather than natural coenzymes as an electron source [116,117]. In 2011, Zhao and coworkers presented a bio-orthogonal system that catalyzed the oxidative decarboxylation of ʟ-malate with a dedicated biomimetic coenzyme, nicotinamide flucytosine dinucleotide (NFCD, Fig. 1b). The redox enzymes were engineered using site-saturation mutagenesis of the key amino acid sites [42], and the balance of this biomimetic coenzyme was achieved through a designed enzymatic pathway containing two engineered enzymes, both of which can use NFCD as coenzymes. This research opened the window to engineer bio-orthogonal redox systems for various applications in *in vivo* synthetic biology.

Despite several papers that pertain to the engineering of NAD/NADP preference of oxidoreductases exist [92,100,118] (Table 1), and some general rules have been proposed for coenzyme engineering [31,61,67], coenzyme engineering utilizing biomimetic coenzymes remains in its early stage due to the significant differences in structures and sizes among natural and biomimetic coenzymes (Fig. 1) [113]. This direction is becoming one of the top R&D priorities of *in vitro* synthetic biology [106].

6. Conclusions

Due to variations among different types of coenzymes, the imbalance of coenzyme supply and consumption, as well as coenzyme cost and stability issues, coenzyme engineering is one of the most important areas of protein engineering, with great application to *in vivo* and *in vitro* synthetic biology projects. With the increasing number of protein crystal structures with high-resolution and homogeneous oxidoreductase sequences and the development of novel high-throughput screening methods, the semi-rational design of switching coenzyme preferences between NAD and NADP is maturing. Coenzyme engineering utilizing biomimics is becoming more prevalent because such biomimics are more stable and less costly than natural ones [40,68]. It is increasingly acceptable that *in vitro* synthetic biology platforms could become a cornerstone of advanced biomanufacturing 4.0 for the cost-competitive biomanufacturing of low-value biocommodities and new foods [119].

Acknowledgements

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