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

Chun You a,*, 1, Rui Huang b,1, Xinlei Wei a, Zhiguang Zhu a, Yi-Heng Percival Zhang a, b, **

* Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 West 7th Avenue, Tianjin Airport Economic Area, Tianjin, 300308, People’s Republic of China
** Biological Systems Engineering Department, Virginia Tech, 304 Seitz Hall, Blacksburg, VA 24061, USA

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 vivo synthetic pathways and in vivo bioorthogonal redox pathways.

© 2017 The Authors. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Contents

1. Introduction .................................................................................................................. 209
2. Coenzyme engineering methods of nicotinamide-based coenzymes .................................................. 209
   2.1. Rational design ........................................................................................................ 210
   2.2. Semi-rational design ................................................................................................ 212
   2.3. Random mutagenesis .............................................................................................. 212
   2.4. Directed evolution based on high-throughput screening (HTS) ................................ 213
3. Applications of coenzyme engineering in in vivo synthetic biology ................................................. 213
   3.1. From NAD to NADP ............................................................................................... 213
   3.2. From NADP to NAD ............................................................................................. 213

* Corresponding author. Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 West 7th Avenue, Tianjin Airport Economic Area, Tianjin, 300308, People’s Republic of China. Tel: +86 22 24828795.
** Corresponding author. Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 West 7th Avenue, Tianjin Airport Economic Area, Tianjin, 300308, People’s Republic of China. Tel: +86 22 24828789.
E-mail addresses: you_c@tib.cas.cn (C. You), zhang_yh@tib.cas.cn (Y.-H.P. Zhang).
Peer review under responsibility of KeAi Communications Co., Ltd.
1 CY and HR contributed equally on writing this review.

https://doi.org/10.1016/j.synbio.2017.09.002
2045-805X © 2017 The Authors. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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-replicate 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 myo-inositol 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 vitro 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, semirational 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...
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 flowchart 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
efficiency ($k_{\text{cat}}/K_m$) 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. 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.

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. For example, Yaoi et al. changed the coenzyme preference of an
isocitrate dehydrogenase by replacing the NADP-binding pocket with a homogeneous NAD-binding pocket \[69\]. Similarly, the coenzyme preferences of a \(\beta\)-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 coenzyme 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 activity on NADP to NADPH activities. A library was constructed by combining all beneficial mutations as well as the wild-type residues.

The approach included the following steps: (1) identification of the loop, (2) determination of the \(\beta2\alphaB\)-loop length and mutation 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 \[61\]. 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 \(\beta2\alphaB\) 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 \(\beta2\alphaB\)-loop length and mutation 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 dramatically increased probabilities of harboring compensatory mutations. This strategy has shown the efficacy of inverting the coenzyme preference of four structurally diverse NAD(P)-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 preferences 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 thermaacetica 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 tetrabromo blue tetrazolium (TNBT), phenazine methosulfate (PMS), NADH, 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, Bommarcoeddy 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 xyitol dehydrogenase (PsXDH) genes can convert xylose to ethanol, along with the unfavorable excretion of xyitol due to intercellular redox imbalance caused by the different coenzyme specificity between NADP-harboring 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 kcat/Km on NADP⁺ than the wild-type enzyme, reaching a comparable value with the kcat/Km on NAD⁺ of the wild-type enzyme [87]. These researchers further constructed a recombinant yeast coexpressing NADP⁺-preferring PsXR and NAD⁺-dependent PsXDH, and the resultant recombinant yeast showed increased ethanol production and decreased xyitol 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 (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 synthetized, 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)
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-prefering 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, polyhydroxybutyrates, 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 celloextrins with a theoretical...
yield of 12 mol H₂ per mol of hexose and water [25,47,48], breaking the Thauer limit of 4 mol of H₂ per mol of glucose [104,105]. In these ivSEBs, glucose 6-phosphate (G6P) is generated from ATP-free enzymatic phosphorylation of glucon (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⁺ 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⁺ into 2 mol of acetyl-CoA with 4 mol of NAD(P)H and 2 mol of CO₂. To further decrease the coenzyme costs 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 6PGD-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 H₂/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 CO₂. 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 decrease 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 l-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 bioorthogonal 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

This study was mainly supported by the Key Research Program of the Chinese Academy of Sciences (Grant No. ZDRW-ZS-2016-3), 1000-youth program of China to CY and the National Natural Science Foundation of China (Grant No. 31600636). Funds were partially provided by the DOE EERE award (DE-EE0006968) to YPZ.

References

[1] Huang R, Chen H, Zhong C, Kim JE, Zhang Y-HP. High-throughput screening of coenzyme preference change of thermophilic 6-phosphogluconate dehydrogenase from NADP⁺ to NAD⁺. Sci Rep 2016;6:32644.
[2] Chen H, Zhu Z, Huang R, Zhang Y-HP. Coenzyme engineering of a hyperthermophilic 6-phosphogluconate dehydrogenase from NADP⁺ to NAD⁺, its application to biobatteries. Sci Rep 2016;6:36311.
[3] McIntosh JA, Coelho PS, Farwell CC, Wang ZJ, Lewis JC, Brown TR, et al.
Enantioselective intramolecular C-H amination catalyzed by engineered cytochrome P450 enzymes in vitro and in vivo. Angew Chem Int Ed Engl 2010;52:3909–3927.

[4] Agudo R, Rolban GD, Reetz MT. Achieving regio- and enantioselectivity of P450-catalyzed oxidative CH activation of small functionalized molecules by structure-guided directed evolution. ChemBiochem 2012;13:1465–73.

[5] van Leeuwen JG, Wijma HJ, Floor RJ, van der Laan JM, Janssen DB. Directed evolution strategies for enantioselectively haloalkane dehalogenases: from chemical waste to enantiopure building blocks. ChemBiochem 2012;13:37–48.

[6] You C, Huang Q, Xue H, Xi Y, Lu H. Potential hydrophobic interaction between two cysteines in interior hydrophobic region improves thermostability of a family 11 xylanase from Neocallimastix patriciarum. Biotechnol Bioeng 2010;105:861–70.

[7] Blum JK, Ricketts MD, Bommarius AS. Improved thermostability of an enzyme based on combining B-FIT analysis and structure-guided consensus method. J Biotechnol 2012;160:214–21.

[8] Reetz MT, Soni P, Fernandez L, Gumulya Y, Carballo JC. Increasing the stability of an enzyme toward hostile organic solvents by directed evolution based on iterative saturation mutagenesis using the B-FIT method. Chem Commun (Camb) 2010;46:8657–8.

[9] Foo JL, Ching CB, Chang MW, Leong SS. The important role of protein engineering in synthetic biology. Biotechnol J 2012;30:541–9.

[10] Wellhausen R, Oye KA. Intellectual property and the commons in synthetic biology: strategies to facilitate an emerging technology. In: 2007 atlanta conference on science, technology, and innovation policy. 2007. p. 1–2.

[11] Cachon AE, Othome FE, Ressor SJ, Lindsey K. Synthetic biology. New Phyto 2012;196:671–7.

[12] Zhang Y-HP, Myung S, You C, Zhu Z, Rollin J. Toward low-cost bio- manufacturing through in vitro synthetic biology: bottom-up design. J Mater Chem 2011;21:18877–86.

[13] Chang MC, Keasling JD. Production of isoprenoid pharmaceuticals by engineered microbes. Nat Chem Biol 2006;2:674–81.

[14] Stephanopoulos G. Synthetic biology and metabolic engineering. ACS Synth Biol 2012;1:154–25.

[15] Cheng AA, Lu TK. Synthetic biology: an emerging engineering discipline. Annu Rev Biomed Eng 2012;14:155–78.

[16] Haseloff J, Ajouj J. Synthetic biology: history, challenges and prospects. J R Soc Interface 2009;6(Suppl 4):S389–91.

[17] Andrianantoandro E, Basu S, Karig DK, Weiss R. Synthetic biology: new engineering rules for an emerging discipline. Mol Syst Biol 2006;2.

[18] Heinemann M, Panke S. Synthetic biology—putting engineering into biology. Bioinformatics 2006;22:2790–9.

[19] Forster AC, Church GM. Synthetic biology projects in vitro. Genome Res 2007;17:1–6.

[20] Dudley OM, Karim AS, Jewett MC. Cell-free metabolic engineering: bio-manufacturing beyond the cell. Biotechnol J 2015;10:69–82.

[21] Hodgman CE, Jewett MC. Cell-free synthetic biology: thinking outside the cell. Metab Eng 2012;14:261–9.

[22] Bopp S. Next-generation bioproduction systems: cell-free conversion concepts for industrial biotechnology. Eng Life Sci 2013;13:19–25.

[23] Morgado G, Gerngross DT, Roberts TM, Panke S. Synthetic biology for cell-free engineering rules for an emerging discipline. Mol Syst Biol 2006;2.

[24] Fessner WD. Systems Biocatalysis: development and engineering of cell-free biological hydrogen production at theoretical yield from sucrose. Metab Eng 2014;24:70–7.

[25] Ye X, Wang Y, Hopkins RC, Adams MW, Evans BR, Mielenz JR, et al. Spontaneous high-yield production of hydrogen from cellulose materials under catalyzed cell-free synthetic biology. Biotechnol Biofuels 2012;5:456.

[26] Zhu Z, Zhang Y-HP. Use of nonimmobilized enzymes and mediators achieved high power densities in closed bioreactors. Energy Sci Eng 2015;4:390–7.

[27] Zhu Z, Zhang Y-HP. In vitro metabolic engineering of hydrogen production at theoretical yield from sucrose. Metab Eng 2014;24:70–7.

[28] Zhu Z, Wang Y, Miinter SD, Zhang Y-HP. Maltodextrin-powered enzymatic fuel cell through a non-natural enzymatic pathway. J Power Sources 2011;196:7505–9.

[29] Li Y. Beyond protein engineering: its applications in synthetic biology. Enzyme Eng 2012;11:1000e103.

[30] Chun RA, Doucet N, Pelletier JN. Semi-rational approaches to engineering enzyme activity: combining the effects of directed evolution and rational design. Curr Opin Biotechnol 2005;16:378–84.

[31] Lesk AM. NAD-binding domains of dehydrogenases. Curr Opin Struct Biol 2002;12:52–68.

[32] Ji D, Wang L, Hou S, Liu W, Wang J, Wang Q, et al. Creation of bioorthore reduced systems dependent on altered coenzyme preference improves ethanol xylitol dehydrogenase. Bioci Biochem Biotechnol 2011;75:1994–2000.

[33] Haitagawa H, Ishishina S, Yoshida S. Ethanol production from xylose by a recombinant Candida shehatae expressing xylose reductase and xylitol dehydrogenase. Biosci Biotechnol Biochem 2011;75:1994–2000.

[34] Bastian S, Liu X, Meyerowitz JT, Snow CD, Chen MM. Arnold FH. Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in Escherichia coli. Metab Eng 2011;13:345–52.

[35] Matsuhashi A, Watanabe S, Kodaki T, Makino K, Inoue M, Murakami K, et al. Expression of protein engineering rules for an emerging discipline. Mol Syst Biol 2006:2.

[36] Matsushika A, Watanabe S, Kodaki T, Makino K, Inoue H, Murakami K, et al. Expression of protein engineering rules for an emerging discipline. Mol Syst Biol 2006:2.

[37] Paul CE, Arends IWCE, Holmann F. Is simpler better? Synthetic nicotinamide cofactor analogues for redox chemistry. ACS Catal 2014;4:788–97.

[38] Paul CE, Holmann F. A survey of synthetic nicotinamide cofactors in enzymatic processes. Appl Microbiol Biotechnol 2016;100:4773–8.

[39] Ji D, Ji D, Wang L, Hou S, Liu W, Wang J, Wang Q, et al. Creation of bioorthore reduced systems dependent on altered coenzyme preference improves ethanol xylitol dehydrogenase. Bioci Biotechnol Biochem 2011;75:1994–2000.

[40] Bastian S, Liu X, Meyerowitz JT, Snow CD, Chen MM. Arnold FH. Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in Escherichia coli. Metab Eng 2011;13:345–52.
Zhang X, Jantama K, Moore JC, Shanmugam KT, Ingram LO. Production of L

Liu W, Hong J, Bevan DR, Zhang Y-HP. Fast identification of isocitrate dehydrogenase from S. lactis, a role for arginine residues in binding substrate and coenzyme. Biochem J 1999;338:55–60.

Tatew E, Hanau S, Wells JM, Le Page RWF, Adams MJ, Arkison S, et al. Phosphogluconate dehydrogenase from S. lactis, a role for arginine residues in binding substrate and coenzyme. Biochem J 2006;281:38683–10.

Cui D, Zhang L, Jiang S, Yao Z, Gao B, Lin J, et al. A computational strategy for altering an enzyme in its cofactor preference to NAD(H) and/or NADP(H). FEMS J 2015;282:2339–51.

Rollin JA, Tam TK, Zhang Y-HP. New biotechnology paradigm: cell-free biosystems for biomanufacturing. Green Chem 2013;15:1708–19.

Yao T, Miyazaki K, Oshima T, Komukai Y, Go M. Conversion of the coenzyme specificity of isocitrate dehydrogenase by module replacement. J Biochem 1999;116:1044–9.

Miller SP, Lunzer M, Dean AM. Direct demonstration of an adaptive constraint. Science 2006;314:658–61.

Takase R, Mikami B, Kawai S, Murata K, Hashimoto W. Structure-based random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. Methods Mol Biol 2004;270:319–34.

Kim JE, Huang R, Chen H, You C, Zhang Y-HP. Facile construction of random gene mutation libraries for directed evolution without the use of restriction enzyme in Escherichia coli. Biotechnol J 2016;11:1422–90.

Chusacultanachai S, Yuthavong Y. Random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. Methods Mol Biol 2004;260:319–34.

Mayer KM, Arnold FH. A colorimetric assay to quantify dehydrogenase activity in cell-free reactions. J Biochem Biophys Res Commun 1970;42:970–5.

Takase R, Mikami B, Kawai S, Murata K, Hashimoto W. Structure-based random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. Methods Mol Biol 2004;270:319–34.

Mayer KM, Arnold FH. A colorimetric assay to quantify dehydrogenase activity in cell-free reactions. J Biochem Biophys Res Commun 1970;42:970–5.

Takase R, Mikami B, Kawai S, Murata K, Hashimoto W. Structure-based random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. Methods Mol Biol 2004;270:319–34.

Mayer KM, Arnold FH. A colorimetric assay to quantify dehydrogenase activity in cell-free reactions. J Biochem Biophys Res Commun 1970;42:970–5.

Takase R, Mikami B, Kawai S, Murata K, Hashimoto W. Structure-based random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. Methods Mol Biol 2004;270:319–34.

Mayer KM, Arnold FH. A colorimetric assay to quantify dehydrogenase activity in cell-free reactions. J Biochem Biophys Res Commun 1970;42:970–5.

Takase R, Mikami B, Kawai S, Murata K, Hashimoto W. Structure-based random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. Methods Mol Biol 2004;270:319–34.

Mayer KM, Arnold FH. A colorimetric assay to quantify dehydrogenase activity in cell-free reactions. J Biochem Biophys Res Commun 1970;42:970–5.
[118] Zhang Y-HP, Huang W-D. Constructing the electricity-carbohydrate-hydrogen cycle for a sustainability revolution. Trends Biotechnol 2012;30:301–6.

[119] Zhang Y-HP, Sun J, Ma Y. Biomanufacturing: history and perspective. J Ind Microbiol Biotechnol 2017;44:773–84.

[120] Bommareddy RR, Chen Z, Rappert S, Zeng AP. A de novo NADPH generation pathway for improving lysine production of Corynebacterium glutamicum by rational design of the coenzyme specificity of glyceraldehyde 3-phosphate dehydrogenase. Metab Eng 2014;25:30–7.

[121] Petschacher B, Stauning N, Müller M, Schürmann M, Mink D, De Wildeman S, et al. Cofactor specificity engineering of Streptococcus mutans NADH oxidase 2 for NAD(P)+ regeneration in biocatalytic oxidations. Comput Struct Biotechnol J 2014;9:1–11.

[122] Dambe TR, Kühn AM, Brossette T, Giffhorn F, Scheidig AJ. Crystal structure of NADP (H)-dependent 1,5-anhydro-d-fructose reductase from Sinorhizobium morelense at 2.2 Å resolution: construction of a NADH-accepting mutant and its application in rare sugar synthesis. Biochemistry 2006;45:10030–42.

[123] Gand M, Thole C, Müller H, Brundiek H, Bashiri G, Höhne M. A NADH-accepting imine reductase variant: immobilization and cofactor regeneration by oxidative deamination. J Biotechnol 2016;230:11–8.

[124] Petschacher B, Nidetzky B. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of Saccharomyces cerevisiae. Microb Cell Fact 2008;7:9.

[125] Chen H, Zhu Z, Huang R, Zhang Y-HP. Coenzyme engineering of a hyperthermophilic 6-phosphogluconate dehydrogenase from NADP+ to NAD+ with its application to biobatteries. Sci Rep 2016;6:36311.

[126] Opgenorth PH, Korman TP, Bowie JU. A synthetic biochemistry module for production of bio-based chemicals from glucose. Nat Chem Biol 2016;12:393–5.