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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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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 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 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

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