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Protein design for pathway engineering

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ABSTRACT

Design and construction of biochemical pathways has increased the complexity of biosynthetically-produced compounds when compared to single enzyme biocatalysis. However, the coordination of multiple enzymes can introduce a complicated set of obstacles to overcome in order to achieve a high titer and yield of the desired compound. Metabolic engineering has made great strides in developing tools to optimize the flux through a target pathway, but the inherent characteristics of a particular enzyme within the pathway can still limit the productivity. Thus, judicious protein design is critical for metabolic and pathway engineering. This review will describe various strategies and examples of applying protein design to pathway engineering to optimize the flux through the pathway. The proteins can be engineered for altered substrate specificity/selectivity, increased catalytic activity, reduced mass transfer limitations through specific protein localization, and reduced substrate/product inhibition. Protein engineering can also be expanded to design biosensors to enable high through-put screening and to customize cell signaling networks. These strategies have successfully engineered pathways for significantly increased productivity of the desired product or in the production of novel compounds.

1. Introduction

Engineering highly efficient enzymatic pathways for industrial-scale production of fuels and chemicals remains an overwhelming challenge in metabolic engineering and synthetic biology (Keasling, 2010; Khosla and Keasling, 2003). The poor performance of pathways may result from unbalanced protein expression and activity levels, low availability of precursors and cofactors, toxic intermediates and end-products, and overall metabolic burden (Du et al., 2011). Several transcriptional engineering strategies have been developed to address these inefficiencies, such as varying plasmid copy number (Ajikumar et al., 2010; Jones et al., 2000), promoter engineering (Alper et al., 2005; Du et al., 2012), intergenic region engineering (Pfleger et al., 2006; Smolke et al., 2000), ribosome binding site (RBS) engineering (Salis et al., 2009), and codon optimization (Redding-Johanson et al., 2011). However, these strategies cannot overcome the limitations associated with enzymes themselves. Innate enzyme characteristics can produce bottlenecks, generate unwanted by-products, and limit high titers. To overcome these deficiencies, shrewd protein design can be indispensable when engineering an optimal pathway. In designing efficient proteins, one may choose to engineer activity, substrate specificity/selectivity, solubility, and stability. Additionally, substrate/product inhibition and protein localization can be considered in the design process to optimize the pathway. Protein function can also be designed as a major messenger of cellular signals, in detection of cell–cell communication and environmental inputs. Thus, protein engineering is a powerful tool in developing biosensors for high-throughput methods in metabolic engineering and designing customized cellular signaling networks.

This review will first briefly describe experimental and computational tools for protein engineering and design. Next, a few examples will be highlighted to illustrate how these tools can be used to improve the efficiency of pathways for the production of fuels and chemicals. Though there are innumerable examples of protein engineering to improve the performance of enzyme biocatalysts (Bornscheuer et al., 2012; Cobb et al., 2012; Rubin-Pitel and Zhao, 2006; Wang et al., 2012), this review will focus on engineering enzymes within a pathway, wherein the engineered enzyme is coupled with the entire pathway for an increased flux, titer, and productivity of the final product. Protein design for biosensor development and signaling pathway engineering will also be discussed, as systems can be engineered to yield novel output responses or react to novel inputs.

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2. Tools for protein engineering

2.1. Directed evolution

Directed evolution has become one of the most powerful tools in protein engineering. The process mimics Darwinian evolution in a test tube and involves iterative rounds of creating genetic diversity followed by selection or screening (Cobb et al., 2012; Rubin-Pitel et al., 2006; Wang et al., 2012) (Fig.1A). The most common methods to generate genetic diversity include error-prone PCR, DNA shuffling, chemical mutagenesis, and use of a mutator strain. To identify improved mutants from this genetic diversity, a myriad of screening/selection methods have been developed such as colorimetric assays, colony size-based growth assays, and fluorescence activated cell sorting (FACS). A major advantage of directed evolution is that no prior knowledge of the enzyme structure or mechanism is required to improve enzymatic properties. Another advantage is the ability to mutate the entire enzyme, thus identifying residues distant to the active site that could affect activity through allosteric interactions. However, a major disadvantage of random mutagenesis-based directed evolution is the large library size; this limits the exploration of the full sequence diversity, even with the most powerful screening or selection method. Additionally, it can be difficult and time consuming to develop a high-throughput screening/selection method for a target enzyme property (Fig.1A).

2.2. Rational design

Rational design is a knowledge-driven process which uses *a priori* information about the enzyme such as its structure or sequence. This knowledge is used to make specific, targeted amino acid mutations which are predicted to affect enzymatic properties vital for the desired reaction (Fig.1B). This strategy can be valued more than directed evolution because it limits the onerous task of screening the large libraries of random mutagenesis-based directed evolution. In a sequence-based approach, researchers pursue systematic comparisons of homologous protein sequences to identify possible residues that could alter protein activity. When the three-dimensional crystal structure of the target enzyme or a homologous enzyme is available, a more direct structure–function relationship study of residues within the active site can be investigated. Through this visualization, the active site structure can be redesigned, allowing for modified chemistry to occur. Though there are many options for modifications, one example is to mutate large residues to smaller, hydrophobic residues, thus enlarging the active site which allows a larger substrate to bind. Various computational tools have been developed to compare the homologous sequences and structural databases to create a mutability map for a target protein (Damborsky and Brezovsky, 2009; Pavelka et al., 2009; Pleiss, 2011) (Fig.1B).

Rational design is not only used to modify existing enzymes, it can also create new ones. Statistical methods linking structure and function relationships are becoming more successful for de novo protein design. A detailed understanding of the desired catalytic mechanism and its associated transition states and reaction intermediates is typically required for this method. An idealized active site is created by positioning protein functional groups to provide the lowest free energy barrier transition state between the substrates and the product (Rothlisberger et al., 2008; Siegel et al., 2010). Siegel and coworkers developed a de novo enzyme which could catalyze the Diels–Alder reaction. In this design, the most dominant interaction of the transition state is interaction of the

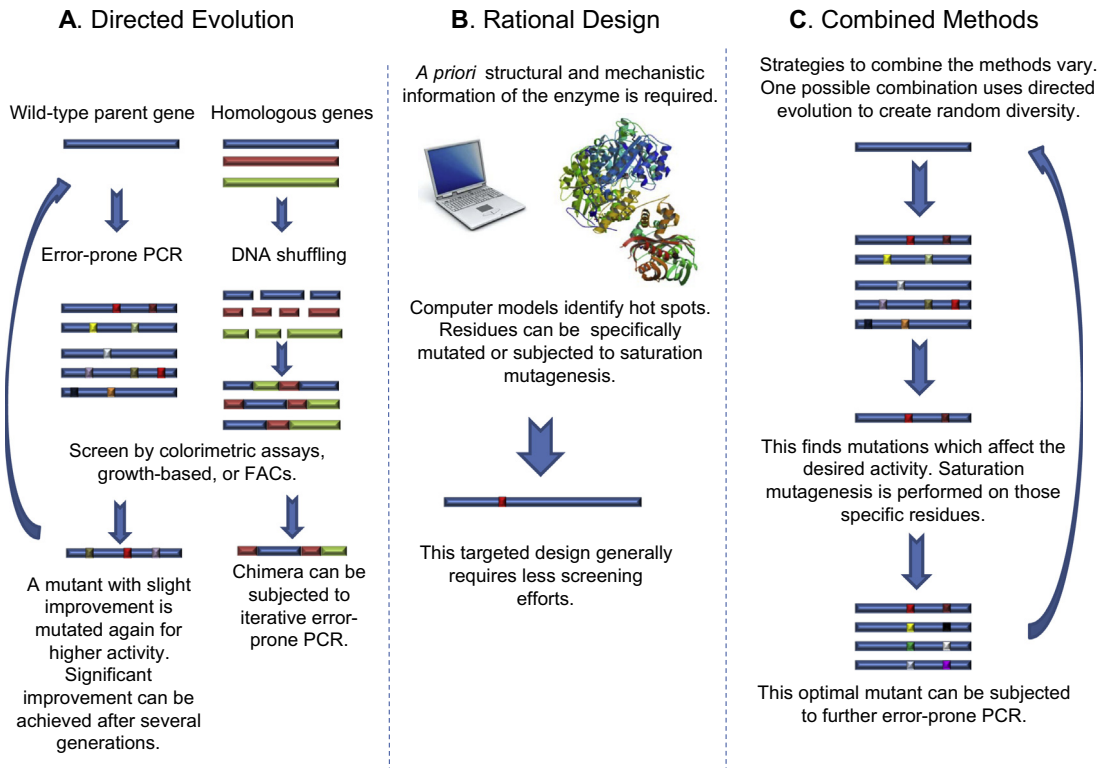

Fig.1. A schematic of the main protein engineering strategies consisting of directed evolution, rational design, and a combined approach. (A) Directed evolution involves the iterative rounds of genetic diversity, being screened/selected for higher activity. The genetic diversity can be introduced through either error-prone PCR or DNA shuffling. (B) Rational design identifies residues which are expected to increase the desired activity through *a priori* sequence or structure knowledge. (C) Though the methodology to combine these strategies can vary, one example of the conjoined method is using directed evolution to identify hotspots, and then rational design to target residues proximal to those hot-spots.