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Review

Use of directed evolution of mammalian cytochromes P450 for investigating the molecular basis of enzyme function and generating novel biocatalysts

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Abstract

Directed evolution has been successfully applied to the design of industrial biocatalysts for enhanced catalytic efficiency and stability, and for examining the molecular basis of enzyme function. Xenobiotic-metabolizing mammalian cytochromes P450 with their catalytic versatility and broad substrate specificity offer the possibility of widespread applications in industrial synthesis, medicine, and bioremediation. However, the requirement for NADPH-cytochrome P450 reductase, often cytochrome b_5, and an expensive cofactor, NADPH, complicates the design of mammalian P450 enzymes as biocatalysts. Recently, Guengerich and colleagues have successfully performed directed evolution of P450s 1A2 and 2A6 initially by using colony-based colorimetric and genotoxicity screening assays, respectively, followed by in vitro fluorescence-based activity screening assays. More recently, our laboratory has developed a fluorescence-based in vitro activity screening assay system for enhanced catalytic activity of P450s 2B1 and 3A4. The studies indicate an important role of amino acid residues outside of the active site, which would be difficult to target by other methods. The approach can now be expanded to design these as well as new P450s using more targeted substrates of environmental, industrial, and medical importance.

Keywords: Cytochrome P450; P450; Directed evolution; Random mutagenesis; Biocatalysts

Natural evolution is a spontaneous process that occurs during reproduction and survival of the whole organism, whereas directed evolution has a defined goal, and the key processes such as mutation, recombination, and screening or selection are controlled by the experimenter [1]. Directed evolution allows us to explore enzyme functions that were not required in the natural environment and for which the molecular basis is poorly understood [2]. This approach contrasts with the more conventional one, in which proteins are tamed 'rationally' using site-directed mutagenesis. The main requirements for successful directed evolution are: (1) the desired function must be physically and biologically feasible; (2) the libraries of mutants should be simple enough to screen for desired function and complex enough to contain rare, beneficial mutations; (3) there must be a rapid and cost-effective screen or selection that reflects the desired function. Directed evolution has the ability to tailor individual proteins (for enhanced catalytic efficiency, stability, and novel activities) as well as whole biosynthetic and biodegradation pathways for biotechnological applications [3,4].

In recent years, there has been increasing realization of the power of biocatalysts for the industrial synthesis of bulk chemicals, pharmaceuticals, agrochemicals, and food ingredients, especially when high stereoselectivity is required [3,5]. In addition, there is a greater demand of biocatalysts for detoxification of environmental contaminants and in cancer gene therapy [6–8]. Hydroxylation has been one of the most difficult enzymatic reactions to harness because of low stability and turnover and expensive cofactor

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requirements. However, exciting recent results with the bacterial enzymes P450 BM3 and P450cam have illustrated the potential of directed evolution for engineering new and efficient hydroxylation pathways and for enabling the utilization of artificial oxygen donors, such as H_2O_2, instead of molecular oxygen and NAD(P)H [9–12]. Xenobiotic-metabolizing mammalian cytochromes P450, with their generally broader substrate specificity than bacterial P450s [13–15], offer the possibility of even greater applications in industrial synthesis, medicine, and bioremediation.

In this review, first we will briefly describe directed evolution of bacterial P450s followed by the significance of designing xenobiotic-metabolizing mammalian P450s by directed evolution. Since it is not possible to provide a complete account of all the methods used for directed evolution, we will mainly introduce the methods and strategies used successfully in our laboratory. Alternative methods applied to biocatalyst evolution in other laboratories along with some computational approaches will be discussed briefly. The main focus of this review will be on directed evolution of mammalian P450s, which was successfully pursued in Dr. Fred Guengerich's and our laboratory.

Directed evolution of bacterial cytochromes P450

Unlike most other P450s, in P450 BM3 an FMN/FAD-containing reductase domain is directly linked to the C-terminus of the heme domain, yielding a self-sufficient fatty acid hydroxylase, which makes it a simple system for directed evolution [16]. Through directed evolution, a P450 BM3 mutant (139-3) was created that displayed up to a 2-fold increase in hydroxylation of fatty acids and up to 100-fold higher activities with unnatural alkane substrates compared with other monooxygenases known to metabolize the same compounds [9,10]. Interestingly, this evolved enzyme has 11 simultaneous mutations, only two of which are in the active site. A recent crystal structure of P450 BM3 mutant (139-3), however, showed no significant difference in the overall architecture compared with wild-type, and only the two active site mutations were found to be responsible for improved alkane hydroxylase activity [17]. More recently, directed evolution of P450 BM3 generated a series of variants active towards smaller alkanes. Mutants 53-5H and 35-E11, having at least 15 amino acid substitutions, supported thousands of turnovers of propane, and catalyzed the selective conversion of ethane to ethanol without over-oxidation [18].

In addition to increased activity and substrate diversity, the following studies have strengthened the potential of directed evolution for engineering P450 BM3 for industrial applications: (1) utilization of H_2O_2 to catalyze hydroxylation and epoxidation of fatty acids [11,12]; (2) enhanced thermal stability and T_{50} from 43 to 61 °C [19]; (3) improved catalytic activity by 10-fold in 2% THF and 6-fold in 25% DMSO [20]; (4) development of the gram scale production and purification as well as whole cell (WC) high-throughput screening assays for improved activity and enhanced catalytic tolerance to organic solvents [21]. However, limited substrate diversity is the major disadvantage in designing bacterial P450 enzymes for a variety of industrial purposes. In contrast, because of broad substrate specificity mammalian P450s may be more suitable for design as biocatalysts for industrial, environmental, and medical purposes [13–15].

Xenobiotic metabolizing mammalian cytochromes P450: significance

Mammalian cytochromes P450s comprise a superfamily of monooxygenases that are of considerable interest because they are the major catalysts involved in the oxidation of steroids, drugs, carcinogens, pesticides, and other xenobiotics [13–15]. Among the human enzymes, P450s 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 3A4, and 3A5 are involved in the oxidation of >90% of environmental toxicants, drugs, and carcinogens. Human 3A4 alone accounts for oxidation of more than 50% of drugs and plays a significant role in metabolizing carcinogens [22]. 3A4 demonstrates homotropic cooperativity with a number of substrates as well as heterotropic cooperativity, which is characterized by increased oxidation of one substrate in the presence of an effector [23]. The homotropic and heterotropic cooperativity of 3A4 is critical for drug-drug and drug-food interactions. P450s from the 2B subfamily are involved in the metabolism of anti-cancer prodrugs such as cyclophosphamide (CPA) and ifosfamide (IFA), and environmental contaminants such as polychlorinated biphenyls (PCBs) [24–26]. P450s 1A2 and 2A6, which have been studied extensively by Guengerich and co-workers [27,28] and are a major subject of this review, are the major enzymes involved in the bioactivation of heterocyclic aromatic amine and indole oxidations, respectively. P450s from the 2C subfamily, which are also being considered for directed evolution, play a major role in drug metabolism (accounting for >30% of available drugs) [29].

Because of their pharmacological and environmental significance, P450 3A4 and P450s from the 2B subfamily have been the subject of a large number of structure–function studies involving X-ray crystallography, homology modeling, and site-directed mutagenesis of substrate recognition site (SRS) or active site residues [30–38]. The above mentioned subject and a number of other studies have revealed that substrate binding and oxidation are influenced not only by SRS or active site residues, but also by residues outside of the active site [30,32,33,35–42]. Non-SRS residues may influence reductase binding, substrate access or product exit, and conformational changes that occur in the catalytic cycle. Non-SRS residues may also be involved in global changes that affect residues in the substrate binding region. For example, comparison of the P450 2B4 X-ray crystal structures in the absence and in the presence of inhibitor, site-directed mutagenesis of 2B1 in B′ helix, F–G loop, and I helix regions, and molecular modeling studies suggests very dynamic substrate access channels [32,33,39–42], In addition, X-ray crystal structures of