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An Overview of P450 Enzymes: Opportunities and Challenges in Industrial Applications

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Abstract

Cytochrome P450 enzymes (P450s) containing a heme-iron center, are biocatalysts from all kingdoms, involved in a large variety of reactions. Their potential in catalyzing a broad range of substrates makes perfect candidates for biotechnology applications and the production of high-value compounds. Biocatalytic reactions performed by P450s have a great interest in the pharmaceutical industry, fine chemicals, cosmetics, and for bioremediation procedures. However, the complex nature of this protein is still a major hurdle in the prospect of using their promising ability for expanding the number of industrial applications. Multiple approaches of protein engineering are currently conducted to improve activity, stability and/or substrate specificity for a given reaction. Furthermore, in combination with the appropriate biocatalyst, a suitable bioengineering process is a key step in the implementation of P450s at the industrial scale.

Keywords: Cytochrome P450 enzyme; Limitations P450-based reactions; Biotechnology applications

Introduction

Cytochrome P450 monooxygenases (termed CYPs or P450s) are highly promising heme proteins ubiquitously found across all kingdoms, catalyzing a large variety of reactions under mild conditions [1]. Originally discovered in rat liver microsomes, the P450 name is derived from their unusual property to display a maximum peak of absorption at 450 nm of the reduced carbon monoxide gas-bound complex [1,2]. This method also enables the rapid evaluation of protein content [2,3].

P450s have the great ability to catalyze oxido-reduction reactions as e.g hydroxylation, oxidation, sulfoxidation, decarboxylation or dealkylation of a broad range of substrates including alkanes, fatty acids, steroids, terpenes, antibiotics or xenobiotics [4]. A vast majority of P450s are monooxygenases and the transfer of an oxygen atom in a substrate, is the result of the reductive scission of a dioxygen bond and the release of one molecule of water [5]. A catalytic wheel was proposed in early 70's and described using $P450_{cam}$, an enzyme isolated from *Pseudomonas putida* and responsible for the hydroxylation of camphor [6–9]. $P450_{cam}$ was also the first crystal structure solved and was used as reference until the discovery of $P450_{BM3}$, from *Bacillus megaterium*, the most catalytically efficient P450 known to date [10–13]. $P450_{BM3}$ has been extensively studied and engineered to perform a large variety of reactions and still serves as a model for the P450 mechanism [14].

The P450-based reactions occur in the heme domain of CYPs where the substrate binds. However, catalysis also requires the transfer of two electrons from the cofactor nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) (for most of the P450s) usually provided by the reductase domain [5]. Ten different classes of P450 have been described based on the structure of the enzyme and on the redox partners associated e.g. heme domain fused to the reductase domain ($P450_{BM3}$), membrane bound P450s (mammalian CYPs), or three component soluble system (most of the bacterial P450s) [4,15].

Due to their versatility in catalyzing chemically challenging regio- and stereoselective reactions, P450s are promising candidates for biotechnology use [16]. However their complex nature hampered their potential for establishing cost-competitive industrial processes. The combination as a suitable biocatalyst, host and appropriate process engineering for a given reaction is a crucial study to increase the number of implementation of cytochrome P450 at the industrial scale [17].

We will discuss the common limitations associated to P450-based reactions, at the enzymatic level and during whole-cell biotransformations, the alternatives to address challenges and current industrial biotechnological opportunities and applications of CYPs.

Addressing P450 Challenges

Substrate specificity, activity, and stability of P450s are key barriers in industrial applications

Protein engineering is the most common approach to expand diversity, tackle low enzyme activity, stability, substrate specificity, and reduce side product formation [18]. Possible strategies for generating improved biocatalysts include gene shuffling, rational protein design or directed evolution [19,20].

The increased frequency of available P450 sequences from databases, provides functional and structural information, which in turn greatly facilitates rational protein design strategies [21–24]. As an example, mutant libraries of CYP106A2 isolated from *Bacillus megaterium*, a difficult protein to crystallize, were based on rational design and docking studies using a homology model and have provided structural and functional information. This led to the generation of improved biocatalysts able to hydroxylate steroids in a more effective manner [25]. Similarly a homology model of the heme domain of CYP153A from *Marinobacter aquaeolei* ($CYP153A_{M.aq}$) was created and allowed to facilitate targeted mutagenesis within the binding pocket and at the substrate entrance tunnel. Based on visual assessment,

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docking studies and high conservation of CYPs sequences alignment analysis, an engineered biocatalyst demonstrated enhanced activity for the terminal hydroxylation of short to long chain fatty acids [26]. The crystal structure of $CYP153A_{M.aq}$ was ultimately solved and revealed a crucial anchoring residue in the active site. Additional mutations demonstrated extended flexibility in the substrate recognition region which enhanced product formation [27].

Directed evolution, generating diversity via random mutagenesis, has the great advantage of identifying improved enzymes in the absence of structural, sequence or functional information [28]. However, the establishment of a high-throughput screening assay or selection method is indispensable to isolate enhanced variants from large mutant libraries [29]. Pioneering work has been establish in Frances Arnold's lab, including the engineering of $P450_{BM3}$ for diverse types of reactions and substrates have been accomplished [30,31]. The promiscuity of the heme protein enabled Arnold and coworkers to tailor $P450_{BM3}$ to catalyze the cyclopropanation reaction [32,33].

Besides activity and substrate specificity, P450 instability is a major hurdle for industrial processes. Efforts have been made to design and identify proteins with increased thermostability and high resistance to co-solvents. For instance, $P450_{BM3}$ was tailored to display high temperature resistance, and stability in the presence of ethanol, acetone, dimethylformamide, dimethyl sulfoxide and acetonitrile, which are commonly utilized to dissolve poorly soluble substrates [34,35]. Due to their complex nature, immobilization of P450s to optimize their stability has proven difficult. Nevertheless, $P450_{BM3}$ immobilized in a sol-gel matrix displayed increased stability and activity towards three tested substrates including β-ionone, octane, and naphthalene [36,37].

Manipulating redox partners and uncoupling events for optimizing P450s activity

Most P450s receive two electrons essential for the oxygen activation, usually from the electron donor NAD(P)H. Both inefficient electron transfer between the reductase domain and the heme domain and side reactions known as uncoupling events, can result in diminished catalytic rates. The latter encourage formation of reactive oxygen species, leading to heme loss [16]. Protein engineering of P450 has shown to increase coupling efficiency in combination with improved turnover rate but this postulate is not a general case [16]. $P450_{BM3}$ is a self-sufficient natural protein in which the heme domain is fused to the reductase domain. This results in improved electron transfer rates and high catalytic turnover numbers [38]. Therefore, based on such model, a large number of artificial fusion constructs have been created and the suitable selection of redox protein associated to the heme have generated catalytically enhanced enzymes [39]. Furthermore, the linker region between the heme domain and the reductase domain has been shown to be a decisive element to facilitate electron transfer along with increasing enzyme activity and stability. Generated chimera proteins have resulted in greater activity, simply by modifying the linker with an appropriate amino acid length [40–42].

Bioprocess development with P450s

In addition to the challenges identified at the enzyme level, efficient whole-cell P450-based reaction development is a key step for potential industrial applications. For an economically feasible process, various parameters must be carefully considered and evaluated including the choice of the host, of the biocatalyst but also the operating mode (growing cells *vs.* resting cells) [17]. The potential substrate and/or product inhibition or toxicity to the cells and/or to the enzyme is another factor to examine, as well as substrate transport limitation across the cell membrane and overoxidation and/or degradation of the product [43–46].

In the case of a whole-cell biotransformation, the cofactor availability has already been shown as a limiting parameter, resulting in lower P450 activity [45]. Several options can be considered to address this limitation as enzyme engineering. $P450_{cam}$ was tailored into a peroxidase to utilize H_2O_2 in the absence of cofactor for the hydroxylation of naphthalene [47]. The addition of a glucose dehydrogenase for cofactor recycling is a second possibility, which has been demonstrated in *Bacillus amyloliquefaciens* for the conversion of 1-hexene by $P450_{BM3}$ [48]. Another strategy involves switching the cofactor from NADPH to the more stable NADH, thereby reducing process costs [49, 50]. If necessary, a larger choice of NAD^+-dependent dehydrogenases can also be co-expressed for cofactor regeneration. The chemical synthesis of NADPH or NADH cofactor analogues has also recently demonstrated successful catalysis reactions [51,52]. The utilization of electrochemical way by immobilizing the P450 on electrode surfaces for the electron transfer has also been investigated [53,54]. Another novel approach involved the light-driven catalysis and the creation of an artificial hybrid $P450_{BM3}$ heme domain and a photosensitizer Ru(II), demonstrating the hydroxylation of dodecanoic acid using light only [55].

Overview of Industrial Applications of P450s

Light of P450s in pharmaceutical industry

P450s have a great interest for the production of drug metabolites, and a vast number of publications demonstrated the successful use of CYPs in the industrial level [16]. As an example, hydrocortisone synthesis is a well-established process via the conversion of the compound Reichstein S steroid by P450 isolated from *Curvularia sp*., and pravastatin has recently been produced from compactin in a single step fermentation process using an engineered bacterial P450 [56–58]. In addition, CYP1051A from *Streptomyces griseolus* enables the conversion of vitamin D3 into 1α25-dihydroxyvitamine D3 [59]. Moreover, the company Novartis engineered strains expressing recombinant human P450 for the production of drug metabolites. CYP107 from *Saccharopolyspora erythraea* and plant P450s are involved in the synthetic steps required for the production of derivatives of erythromycin and Taxol, respectively [60,61]. Production of the anti-cancer drug perilly alcohol from limonene, has been demonstrated in *P. putida* expressing a P450 from the bacterial CYP153 family [44]. Recently, Sanofi has started producing 25 g L^{-1} of antimalarial artemisinin from an engineered strain. One biosynthetic route includes the integration of CYP71AV1 isolated from *Artemisia annua*, executing successive oxidations of the artemisinic acid precursor [62, 63].

Deployment of P450s in bioremediation process

P450s can play an essential role in bioremediation processes as environmental contaminants have been shown to be degraded by mammalian and fungal cytochrome P450s. For instance, CYP5145A3 from the white rot fungus *Phanerochaete chrysosporium* and the rat CYP1A1 showed great activity towards two different polychlorinated dibenzo-*p*-dioxins [64]. A mammalian cytochrome–expressed in *Arabidopsis thaliana,* was able to neutralize explosive particulates which remained in soils after a decade of military activity [65]. Furthermore, several P450s were described as able to metabolize polycyclic aromatic hydrocarbons (PAHs) [66]. Besides detoxification of pollutants, engineered plants were also developed to contain mammalian CYPs, conferring a resistance to herbicides [67].

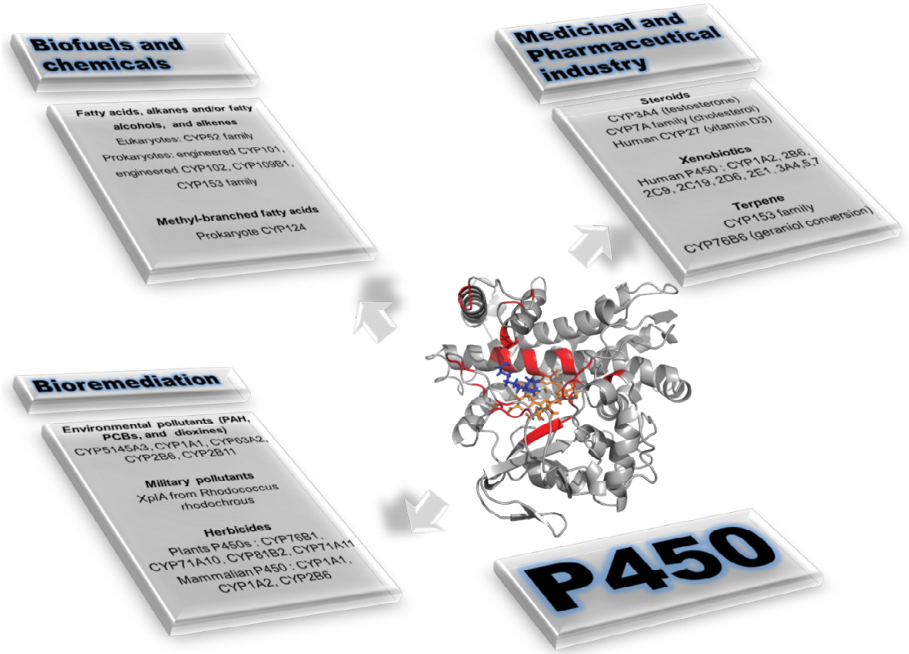

Figure 1: Overview of P450s involved in metabolic steps and applications in the bioindustry. The P450 family is represented by a model structure of the heme of CYP153A from *Marinobacter aquaeolei*; the fatty acid substrate in blue is in close proximity to the iron heme, colored in orange [88].

Market for renewables

Using the power of cytochrome P450, common dyes including indigo and indirubin were synthesized in cell cultures, which illustrates their potential application within the horticulture industry for the generation of flowers with new colors [68–70].

In the perspective of bioconversion of alkanes for fuels and renewable chemicals, engineered $P450_{BM3}$ and CYP101 from *P. putida* have been utilized to produce alcohols from small chain-length alkanes, such as ethane to ethanol or propane to propanol [71–73]. Furthermore, a new type of CYP decarboxylase from the CYP152 family, isolated from *Jeotgalicoccus* sp., has been shown to generate olefins from fatty acids biosynthesis intermediates [74]. Terminally hydroxylated fatty acids and α, ω-dicarboxylic acids can be used as anticancer agents, but also as precursors for polymers and in the cosmetics area for flavours and fragrances [75–78]. The challenging activation and oxidation of C-H bond can be achieved by the versatile P450 instead of chemically-based processes requiring toxic metals and costly thermodynamic conditions, yielding low regioselectivity [79]. The cultivation of the yeast *Candida* and *Yarrowi*a sp. on alkanes and fatty acids has demonstrated the production of terminal hydroxy fatty acids and the corresponding dicarboxylic acid due to the expression of CYP52 family [80,81]. The engineered *Candida tropicalis* was able to produce 174 g L^{-1} of terminal hydroxy fatty acids and 6 g L^{-1} of diacids from 200 g L^{-1} methyl tetradecanoate after six days of biotransformation [82]. As alternative to the yeast platform, bacterial CYP153 family was shown to catalyze a large variety of substrates as fatty acids and alkanes but also terpenes and primary alcohols [43,83–87].

Conclusion

Cytochrome P450s are remarkable biocatalysts of great interest for the industry due to their versatility for a wide substrate range and in performing a large number of oxidative reactions (Figure 1). The identification of limitations associated to P450-based catalysis and whole-cell process are the first step towards an industrial application of such powerful proteins. Extensive research and multiple strategies including protein engineering are currently being conducted to circumvent the challenges as low enzyme activity, robustness and/or substrate specificity, required for cost-competitive implementation. With the incessant development of technologies and synthetic biology tools, there is a no doubt that an increased repertoire of industrial applications of P450s will be established in the near future.

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