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Construction and functional analysis of a whole-cell biocatalyst based on CYP108N7

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ABSTRACT

Cytochrome P450 enzymes are versatile biocatalysts with great potential in biotechnology. A new bacterial P450 was identified from the genome of *Rhodococcus wratislaviensis* NBRC 100605 and annotated as CYP108N7. The enzyme accepted the ferredoxin and ferredoxin reductase from *spinach* as surrogate redox partners for improved electron transfer efficiency. It was heterologous expressed in *Escherichia coli* together with the redox partners and a glucose dehydrogenase which supplied the reduced cofactor NADPH. The resulting whole-cell biocatalyst catalyzed a variety of reactions including sulfoxidation, epoxidation, hydroxylation, demethylation and dehalogenation. Remarkable stereoselectivity was observed in asymmetric sulfoxidation reaction, which could deliver chiral sulfoxides with > 99% ee from thioanisole and derivatives.

1. Introduction

Cytochrome P450 enzymes (P450s, CYPs) form a huge superfamily of heme-containing external monooxygenases that are ubiquitously distributed in all domains of life [1]. They catalyze a number of chemically diverse reactions like hydroxylations, epoxidations, dealkylations, which makes them very attractive and versatile biocatalysts in the synthesis of fine chemicals and pharmaceuticals [2–4].

The eukaryotic P450s, mainly mammalian, have been extensively studied and applied, especially in drug metabolism and pharmaceutical industry [5]. They are membrane bound within cells, and often tolerate low substrate concentration with physiological relevance and low catalytic activities. In contrast, bacterial P450s are soluble and can be heterologously overexpressed, bringing a higher catalytic potential in biotransformation [4,6]. Members of self-sufficient P450s, such as CYP116B2 (P450 RhF) and CYP102A1 (P450 BM3), are categorized into the class VII or VIII electron transport systems [7] with fused reductase domains. They have been the most attractive P450 biocatalysts for synthetic application and protein engineering due to the system simplicity, high catalytic activity and catalytic diversity [3,8]. Mutants of CYP102A1, in particular, have shown human P450-like activities and altered specificities, such as catalyzing non-natural carbine- and nitrene-transfer reactions [9,10].

The class I electron transport system [7], on the other hand, consists of most bacterial P450s, but is more challenge for heterologous expression due to the requirement for separate redox partners, such as ferredoxin and ferredoxin reductase. CYP101A1 (P450cam) and CYP108A1 (P450terp) are representative class I bacterial P450s with resolved crystal structures and characterized substrate specify. CYP101A1 catalyzed the stereoselective oxidation of camphor to 5-*exo*-hydroxycamphor. The systematic research on CYP101A1 and its redox partners, putidaredoxin reductase (PdR) and putidaredoxin (Pdx) has provided a basis for the understanding of P450 reaction mechanisms [11,12]. Nowadays, Pdx and PdR have been widely adapted for the electron delivery for other P450s. The CYP108 family comprises three members with established function, namely CYP108A1, CYP108D1 and CYP108J1. CYP108A1 was originally isolated for α-terpineol oxidation. Its active site was found to be more sterically restricted compared to CYP101A1 and CYP102A1, resulting higher stereo-selectivity in the oxidation of substituted thioanisoles and styrenes [13]. CYP108D1 and CYP108J1 were reported in the oxidative degradation of polycyclic aromatic hydrocarbons [14,15].

The search and characterization of new P450s is an important strategy to meet the constant need of novel biocatalysts with varied substrate scope and selectivity, especially when the increasing sequences in the database reveal the presence of an unexpectedly vast number of P450 genes [16]. Here, we report the functional characterization of a new bacterial P450 that belongs to the CYP108N subfamily. The constructed whole-cell system was optimized *via* regulating electron transfer system on different levels using surrogate redox partners,

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and displayed excellent stereoselectivity in the oxidation of substituted thioanisoles.

2. Materials and methods

2.1. Chemicals and nucleotide sequences

Substrates and product standards of **11b–14b** were obtained from Tokyo Chemical Industry (Shanghai, China) or Alfa Aesar (Shanghai, China) and used without further purification. Racemic standard of products (**1b–10b**) were prepared from the corresponding substrates using H_2O_2 as the oxidant [17]. δ-Aminolevulinic acid hydrochloride (5-ALA) was a gift of Professor Jibin Sun (Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences) [18]. Restriction enzymes, High-Fidelity DNA polymerase and T4 ligase were purchased from New England Biolabs (Beijing, China).

The amino acid sequence and the native DNA sequence of CYP108N7 were obtained from NCBI database (accession number: WP_037231399.1). Then the DNA sequence of CYP108N7 was optimized for heterologous expression using DNAWorks (https://hpcwebapps.cit.nih.gov/dnaworks) and synthesized by Sangon (Shanghai, China). The GenBank accession numbers of the DNA sequences of glucose dehydrogenase (GDH), ferredoxin (Fdx), ferredoxin reductase (FNR), flavodoxin (Fld), flavodoxin reductase (Fpr), Pdx and PdR are D90044.1, M35660.1, X07981.1, AAG55007.1, CQR83320.1, AAA25759.1 and AOE86720.1, respectively. The DNA sequence encoding GDH was synthesized by Invitrogen Biotechnologies (Shanghai, China). The DNA fragments encoding the redox partners were each cloned from plasmids pET-Fdx [19], pMBP-Fdr (for FNR) [20], pET-Fpr, pET-Fld, pCW-Pdx [21] and pET-PdR [21].

2.2. Plasmids construction and gene manipulation

The DNA fragment encoding CYP108N7 was digested with *Nde* I and *Hin*d III, and ligated into pET28a (+) which was digested with the same restriction enzymes. The constructed plasmid was designated as pET28-108N7. To coexpress CYP108N7 with redox partners and GDH, the target gene was each amplified using primers that contained the desired restriction sites (Table S1). After digestion with the corresponding restriction enzymes, the fragments were ligated into the pETDuet-1 and pRSFDute-1 vectors (Novogen) with varied combinations (Table S1). In all instances, restriction enzymes of *Nco* I and *Hin*d III were used to clone the digested DNA fragment into the first multiple cloning site (MCS) and restriction enzymes of *Nde* I and *Xho* I were used for the second MCS of the two vectors. The resulting plasmids were designated based on the nomenclatures of the vector and the two targeted genes (Table S1). All the constructed plasmids were verified by DNA sequencing at Invitrogen Biotechnologies (Shanghai, China).

2.3. Expression and purification of recombinant CYP108N7

The *E.coli* BL21 (DE3) strain transformed with the pET28-108N7 vector was inoculated into Terrific Broth (TB) medium containing kanamycin (50 mg/l), and incubated at 37 °C. After the OD_{600} of the culture reached 0.8, IPTG (0.5 mM), 5-ALA (0.5 mM), thiamine (1.0 mM), and trace elements [22] were added, and the culture was grown at 22 °C with shaking at 180 rpm for 22 h. Bacterial soluble fractions containing CYP108N7 were prepared by high pressure homogenizer (ATS-AH100B, ATS Engineering Inc., Canada) followed by centrifugation at 4 °C. CYP108N7 proteins were purified using a Ni^{2+}-NTA column (Qiagen, Valencia, CA) and eluted with Tris-HCl buffer (100 mM, pH 7.4) containing 500 mM NaCl and 250 mM imidazole, and dialyzed against Tris-HCl buffer (10 mM, pH 7.4). Protein analysis was done with SDS-PAGE and a Nano-drop 2000 spectrophotometer (Nano Drop Technologies, USA).

2.4. Spectroscopic characterization

P450 concentrations were estimated using the reduced-CO versus reduced difference spectrum as described by Omura and Sato [23]. UV–vis spectra were collected on a spectrophotometer (Shimadu UV-1800, Kyoto, Japan) in Tris-HCl buffer (100 mM, pH 7.4) at room temperature. Sodium dithionite was added to reduce the purified ferric CYP108N7 enzymes. With the sample cuvette saturated with bubbles of CO, spectra from 400 to 500 nm were recorded until the 450 nm peak reached a maximum. P450 content is determined as follows: P450 (μmol/l) = $(A_{450} - A_{490})_{\text{baseline}}/0.091$.

2.5. Preparation of the whole-cell catalyst

The expression plasmids were transformed into *E.coli* BL21 (DE3), and single colonies were inoculated in TB medium supplemented with kanamycin (50 mg/l) and carbenicillin (100 mg/l). After overnight incubation at 37 °C at 180 rpm, 2 ml of the starter culture was transferred into 200 ml TB medium supplemented with kanamycin (50 mg/l) and carbenicillin (100 mg/l), and incubated at 37 °C. When the OD_{600} of the culture reached 0.8, IPTG (0.5 mM), 5-ALA (0.5 mM), thiamine (1.0 mM), and trace elements were added, and the incubation was continued for 16 h at 22 °C with shaking at 180 rpm. Cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C, washed twice, and resuspended in potassium phosphate buffer (100 mM, pH 7.4).

2.6. General method for whole-cell biocatalysis and product analysis

The biotransformation was carried out in a 100 ml screwed flask in 10 ml potassium phosphate buffer (100 mM, pH 7.4) containing 0.1 g dry cell weight (DCW) of the resting cells, 1% (w/v) glucose, 32 mg/l polymyxin B and 5 mM substrate, and incubated at 30 °C for 12 h. Control reactions were set up under the same conditions except using the resting cells carrying the empty plasmids. To determine the yield of product, samples were taken at intervals and extracted twice with an equal volume of ethyl acetate.

Analysis was performed at 30 °C on a Shimadzu Prominence LC-20AD system connected to a PDA-detector using a Daicel Chiralcel AS-H (for **11b**; 2-propanol/hexane 2:98; 0.5 ml/min), or OB-H column (for all the others; 2-propanol/hexane 15:85; 0.6 ml/min) to determine the enantiopurity, and a ZORBAX Rx-SIL column (2-propanol/hexane 30:70; 0.5 ml/min) to determine the product yield. ^{1}H NMR spectra were recorded on a Brucker-600 (600/150 MHz) spectrometer in $CDCl_3$. All signals are expressed as ppm down field from tetramethylsilane. Optical rotations were measured with a Perkin Elmer 341 polarimeter. The absolute configurations were determined by comparing the specific rotation values (**1b–8b**,**10b**), or the elution orders on chiral HPLC (**10b** [24], **11b–12b** [25]) with published data.

2.7. Determination of the coupling efficiency

The coupling efficiency is defined as the ratio between the product formation rate and the NAD(P)H oxidation rate [26]. In specific, NADPH was used for the redox partners of FNR/Fdx and Fpr/Fld, and NADH was used for PdR/Pdx. The bioconversions were performed at 20 °C in 1.0 ml potassium phosphate buffer (100 mM, pH 7.4) containing 0.5 mM thioanisole (**1a**) and 2.0 μM cell-free CYP108N7. The reaction was initiated by the addition of 1 mM NAD(P)H, and the consumption rate of the cofactor was monitored at 340 nm over 30 min and calculated based on the absorbance coefficient (ε_{340} = 6.22 mM^{-1} cm^{-1}). The background consumption was measured in the absence of substrate. The product formation rate was calculated based on HPLC analysis results after the termination of the reaction.