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In vitro characterization of CYP102G4 from *Streptomyces cattleya*: A self-sufficient P450 naturally producing indigo

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ABSTRACT

Self-sufficient CYP102As possess outstanding hydroxylating activity to fatty acids such as myristic acid. Other CYP102 subfamily members share substrate specificity of CYP102As, but, occasionally, unusual characteristics of its own subfamily have been found. In this study, only one self-sufficient cytochrome P450 from *Streptomyces cattleya* was renamed from CYP102A_scat to CYP102G4, purified and characterized. UV–Vis spectrometry pattern, FAD/FMN analysis, and protein sequence comparison among CYP102s have shown that CYP102 from *Streptomyces cattleya* belongs to CYP102G subfamily. It showed hydroxylation activity toward fatty acids generating ω-1, ω-2, and ω-3-hydroxyfatty acids, which is similar to the general substrate specificity of CYP102 family. Unexpectedly, however, expression of CYP102G4 showed indigo production in LB medium batch flask culture, and high catalytic activity (k_{cat}/K_m) for indole was measured as 6.14 ± 0.10 min^{-1} mM^{-1}. Besides indole, CYP102G4 was able to hydroxylate aromatic compounds such as flavone, benzophenone, and chloroindoles. Homology model has shown such ability to accept aromatic compounds is due to its bigger active site cavity. Unlike other CYP102s, CYP102G4 did not have biased cofactor dependency, which was possibly determined by difference in NAD(P)H binding residues (Ala984, Val990, and Tyr1064) compared to CYP102A1 (Arg966, Lys972 and Trp1046). Overall, a self-sufficient CYP within CYP102G subfamily was characterized using purified enzymes, which appears to possess unique properties such as an only prokaryotic CYP naturally producing indigo.

1. Introduction

Cytochrome P450 (CYP) enzyme is a heme-containing monooxygenase, which introduces an oxygen atom from atmospheric oxygen molecule into C—H bonds of its substrate [1,2]. Due to its hydrophobic substrate pocket, CYPs are able to hydroxylate wide range of hydrophobic compounds [3,4]. Therefore, applications of CYP family are very diversified in detoxification [5,6], lipid degradation [7], secondary metabolite production [8,9], and drug discovery [10,11]. Unusual ability of regio/stereospecific hydroxylation in mild condition, which is difficult to achieve through chemical catalysts, makes this enzyme to be more important and unparalleled among biocatalysts [12]. Since one CYP can do various different oxidation reactions (> 20 different reactions), which is very unique property, CYPs are indispensable to biosynthesis of natural products in secondary metabolism, but frequently pointed out as rate-limiting steps in the biosynthesis due to their low activities [13].

A single catalytic cycle of CYP requires two equivalent electrons, cleaving O—O bonds [1]. Electrons are delivered from NAD(P)H by electron-donating proteins through a relay of flavin and/or Fe—S cluster proteins, providing stepwise supply of electrons according to the difference in individual redox potentials [14,15]. Issues in electron transfer from the partner reductase to CYP have been often identified as a bottleneck within the catalytic cycle [2]. To enhance the coupling efficiency between its electron-donor and CYP, finding most compatible reductases and constructing artificial fusion proteins with the CYP, so called self-sufficient (SSF) CYPs, are very common strategies to improve its catalytic activity and performance [16–18].

CYP102 family members are one of few CYP groups with natural SSF CYPs found in prokaryotes and exhibit the activity of oxidation of fatty acids at ω-positions [19,20]. Although the reason for existing throughout bacterial species are uncertain, it is inferred to have a role in degrading xenobiotics including unsaturated fatty acids [21,22]. Among CYP102 family members, CYP102A1, commonly known as BM3, has been widely studied because of its high activity and stable expression in *E. coli* [2]. CYP102A1 has not only been demonstrated as an important model for studying mechanisms of CYP systems, but also as a starting template for the evolution of the biocatalysts to hydroxylate non-native substrates such as sterols [23], flavonoids [24], and terpenes [25]. Many reports on characteristics of other CYP102s have shown that they share a lot of common properties with CYP102A1 in substrate specificity and structures [20,21,26–31]. Occasionally, distinctive

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characteristics from CYP102A1 have been discovered within individual CYP102s, and it will provide new insights on the mechanisms of CYP102 family or new possibilities for industrial application. For example, CYP102A5 showed extremely higher rate of electron transfer [29], CYP102A7 showed hydroxylation activity for monoterpenes [30], CYP102D1 showed substrate specificity toward isoflavonoids [31], and CYP102s from *Ktedonobacter racemifer* DSM44963 showed extraordinary regiospecificity toward long chain fatty acid [20].

Streptomyces cattleya NRRL 8057 is a gram-positive bacterium which produces thienamycin and has a rare ability to produce fluoro-metabolites [32]. Like other *Streptomyces* species, about 38 CYPs have been identified in the full genome of *S. cattleya* [33]. Previously, we have showed that CYP102A_scat had an interesting activity for production of indigo, which was the first report among wild-type prokaryotic CYPs, and maximized the production yield through optimizing media [34]. In this study, we re-classified CYP102A_scat, the only one self-sufficient cytochrome P450 in *S. cattleya*, to CYP102G4 and focused on its *in vitro* characterization using purified enzymes with additional substrates. CYP102G4 was compared with CYP102A1 in substrate specificity, components of electron transfer system, and critical residues holding the structures. We further compared its homology model with CYP102A1 and understand what makes such unique changes in the activity of CYP102G4.

2. Materials and methods

2.1. Chemicals

LB broth and yeast extract were purchased from BD bioscience. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), isopropyl-β-thiogalactopyranoside (IPTG), δ-aminolevulinic acid (ALA), and various substrates (fatty acid, indole, flavonone, benzophenone, coumarin) were purchased from Sigma-Aldrich Korea (Suwon, South Korea). Chloroindoles were purchased from Santa Cruz Biotechnology. Oligomers and sequencing were purchased from Cosmogenetech (Seoul, South Korea). Enzymes involved in restriction reaction, ligation, and PCR were purchased from Thermo Scientific, Promega, and Novagen, respectively.

2.2. Phylogenetic sequence analysis and alignment

In this study, the protein sequence of WP_014145542.1 (NCBI protein_ID) and corresponding nucleotide sequence were used. Catalytic domain of the protein sequence was aligned with CYP102 family using Clustal Omega [35]. The library of protein sequences of CYP102*s* was obtained from the bacterial CYP library of Dr. David Nelson (http://drnelson.uthsc.edu/cytochromeP450.html) [36]. Based on the identity among CYP102 family, the self-sufficient CYP was categorized as subfamily G. A molecular phylogenetic sequence analysis was conducted through MEGA6 using the maximum likelihood method [37]. Additional alignment analysis was performed with BioEdit software [38].

2.3. Purification of CYP102G4 and CYP102A1

Plasmid prepared in the previous study was used for expression and purification of CYP102G4 [34]. To be more specific, the self-sufficient CYP gene was amplified using oligonucleotide forward primer 5′-ATATGAATTCATGAGTCCTACGCCGCACAGC-3′ and backward primer 5′-ATATAAGCTTTCACCCGGCGGCGTACACG-3′ from genomic DNA of genomic DNA of *S. cattleya* NRRL8057. The PCR product was cloned into *Eco*RI and *Hind*III sites of pET28a(+), resulting pET28a::*cyp102G4* with a 6xHig-tag at N-terminus. pET28a::*cyp102A1* was prepared as described previously [39].

Each of the constructed vectors was transformed into competent *E. coli* BL21(DE3) and selected on a LB agar plate supplemented with kanamycin (30 μg/ml). One colony was inoculated into LB broth supplemented with 0.2% glucose and kanamycin (30 μg/ml) and grown overnight at 37 °C. Addition of glucose significantly reduced leaky expression. The seed culture was inoculated into 50 ml TB-medium containing kanamycin (30 μg/ml) and grown at 37 °C until the cell density reached OD_{600} = 0.750–0.850. Expression of CYP enzyme was induced with IPTG and ALA (5-aminolevulinic acid) to a final concentration of 0.02 mM and 0.5 mM, respectively, and incubated on an orbital shaker (200 rpm) at 30 °C for 8 h. When LB medium was used, produced indigo hindered efficiency of further cell disruption and binding of 6xHis-tagged protein to NTA beads. The cells were harvested by centrifugation at 4000 xg for 20 min, and washed with PBS (pH 7.5) buffer solution. The cell pellets were resuspended in 5 ml of buffer solution, composed of 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM phenylmethylsulfonyl, and 1 mM DTT, and disrupted by ultrasonication in ice-cold water for 8 min (5 s on, 8 s off). The soluble fraction was collected by centrifugation at 11,000 xg for 20 min at 4 °C. The protein profile was analyzed with 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The CYP enzymes were purified using Ni-NTA his-tag purification kit (QIAGEN Korea Ltd., Seoul, Korea). The soluble fraction was loaded into a column filled with Ni-NTA resin, prewashed with 40 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 10% glycerol. The Ni-NTA bound enzymes were washed twice with the same buffer. The 6xHis-tagged enzymes were eluted with the same buffer containing 80 mM histidine. The eluents were dialyzed with 50 mM Tris-HCl buffer (pH 8.0) with 10% glycerol to remove histidine and salt. When imidazole was used in washing or elution buffer, the dialysis solution turned yellow and catalytic activity of CYP102s were lost, which is suspected to be caused by loss of either FMN or FAD. Purified enzymes were analyzed with 8% SDS-PAGE and used in kinetics parameter measurements. The purified enzyme was stored at 4 °C along the experiments and used within three days.

To confirm condition and calculate concentration of enzyme state, various forms of the purified enzymes were observed between 350 nm and 700 nm. The spectrum of the purified proteins diluted in 50 mM Tris-HCl buffer (pH 8.0) (oxidized form), the diluted sample with an addition of a few amount of sodium dithionite (reduced form), and the reduced sample with CO bubbling for 30 s (CO-bound form) were analyzed using 50 mM Tris-HCl buffer (pH 8.0) as a blank. The concentration of purified CYP102G4 was estimated by using CO-binding spectral assay. The spectral profile of the reduced form was compared with that of the CO-bound form. The concentration was measured from the difference in absorbance between 450 nm and 490 nm using an extinction coefficient of active CYP ($\varepsilon_{450-490}$ = 91 mM^{-1} cm^{-1}) [40].

2.4. Kinetic parameter measurement of indole

In order to compare kinetic parameters with indigo-producing CYPs from previous studies, k_{cat}, K_m and k_{cat}/K_m values were measured by monitoring production of indigo. Activity assay for indole hydroxylation from the previous studies was used with slight modification [41,42]. The 800 μl of reaction mixture contained 1 μM CYP102G4, 50 mM Tris-HCl buffer (pH 8.0), 200 μM NAD(P)H and varying concentration of indole (10 μl of DMSO stock) ranging from 1 mM ~ 100 mM. After pre-incubation at 30 °C for 3 min, corresponding cofactor was added to initiate the reaction. NADH and NADPH oxidation rates were measured by monitoring the decrease in absorbance at 340 nm using a UV–vis spectrophotometer at 30 °C (ε_{340} = 6.220 mM^{-1} cm^{-1}). The reaction was quenched by addition of 15 μl KOH 4 M after 30 min. Concentration of indigo was measured at 670 nm after 5 min. The empirical data was fitted to the *Michaelis-Menten* equation for determination of kinetic parameters.

2.5. NADPH consumption rate analysis, product identification, and K_d measurement

The reaction condition was identical to the description above, except that various substrate concentrations were fixed to a final concentration of 100 μM. NADPH was used to follow reaction profile rather than NADH because NADPH showed slightly higher consumption rate.