J. Microbiol. Biotechnol. (2006), **16**(6), 974–978

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Hydroxylation of Indole by PikC Cytochrome P450 from *Streptomyces venezuelae* and Engineering Its Catalytic Activity by Site-Directed Mutagenesis

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Received: November 17, 2005
Accepted: January 6, 2006

Abstract The cytochrome P450 monooxygenase from the pikromycin biosynthetic gene cluster in *Streptomyces venezuelae*, known as PikC, was observed to hydroxylate the unnatural substrate indole to indigo. Furthermore, the site-directed mutagenesis of PikC monooxygenase led to the mutant enzyme F171Q, in which Phe171 was altered to Gln, with enhanced activity for the hydroxylation of indole. From enzyme kinetic studies, F171Q showed an approximately five-fold higher catalytic efficiency compared with the wild-type PikC. Therefore, these results demonstrate the promising application of P450s originating from *Streptomyces*, normally involved in polyketide biosynthesis, to generate a diverse array of other industrially useful compounds.

Key words: PikC cytochrome P450 monooxygenase, *Streptomyces venezuelae*, indole-hydroxylating, indigo, site-directed mutagenesis

P450 enzymes are well known for their potential usefulness in the catalysis of diverse oxygenation reactions, and are found throughout nature in bacteria, yeasts, mammalian tissues, plants, and insects. The cytochrome P450 monooxygenases from the genus *Streptomyces* catalyze the monooxygenation of a diverse range of polyketides [12, 19], which are crucial for structural diversity and biological activity [19]. The PikC cytochrome P450 monooygenase, which is involved in the pikromycin and methymycin biosynthetic pathway from *Streptomyces venezuelae*, can accept 12- and 14-membered ring macrolides as substrates and act at different positions [1, 9, 22, 23]. Furthermore, this monooxygenase catalyzes the C-12 hydroxylation of the 14-membered ring macrolide narbomycin to pikromycin and functionalizes the C-10 position of the 12-membered ring macrolide YC-17 to methymycin or the C-12 position to neomethymycin [1, 9, 22, 23]. Functionalization at both positions of YC-17 yields novamethymycin [25]. This unique substrate flexibility of PikC has recently been exploited to generate a range of novel macrolide polyketides with a structural homology to the native substrates of PikC [8, 11, 24]. Accordingly, to prove the substrate flexibility of PikC towards an unnatural substrate with a completely different structure to the natural substrates of PikC, this study selected indole as a target for hydroxylation. Indole is a product of tryptophan catabolism in tryptophanase-synthesizing bacteria, such as *Escherichia coli* [4], and several microbial oxygenases have already been identified to accept indole as a substrate for 3-hydroxyindole, which is rapidly oxidized in air and dimerized to form indigo, the oldest known dyestuff [2, 6, 10, 17]. Furthermore, the present study also subjected PikC to site-directed mutagenesis to generate mutants with enhanced activity towards indole as a structurally unrelated substrate.

The *pikC* gene was amplified by a polymerase chain reaction (PCR) from the genomic DNA of *S. venezuelae* ATCC15439 using the following oligonucleotide primers: forward, carrying an NdeI site (underlined) 5'-GGAGTTC-CATATGCGCCGTACCCAG-3', and reverse, carrying a HindIII site (underlined) 5'-CGTAAGCTTGACGTGCGGG-TTCAACC-3'. The PCR fragments were isolated, confirmed by DNA sequencing, and placed into the expression vector pET-24a(+) containing 6× His-tag (Novagen, U.S.A.) as a NdeI-HindIII fragment. The restriction enzymes were purchased from New England Biolabs (U.S.A.). The PCR and ligation were performed with *Pfu* polymerase and T4 DNA ligase purchased from Roche (Germany). The DNA

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manipulation in *E. coli* and transformation of *E. coli* were performed using standard procedures [20]. *E. coli* DH5α and *E. coli* BL21(DE3) were used as the host for the DNA manipulation and protein expression, respectively.

The transformed *E. coli* cells were grown overnight at 37°C in a Luria-Bertani (LB) medium containing 50 μg/ml of kanamycin. One ml of the overnight culture was then inoculated into 100 ml of Terrific Broth [18] containing kanamycin and incubated at 37°C until the OD_{600}=0.6. After the addition of 0.75 mM IPTG, the cells were allowed to grow overnight at 26°C, harvested by centrifugation at 4°C, and then suspended in a lysis buffer [23]. The lysate was prepared on ice by sonication using a Vibra-Cell VCX750 (Sonics & Materials, Inc., U.S.A.). The cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant was then mixed with Ni-NTA agarose beads (Qiagen, U.S.A.) for 5 h at 4°C and collected by centrifugation at 3,000 rpm for 10 min at 4°C. Thereafter, the beads were washed three times with a washing buffer [23] and eluted using an elution buffer [23]. Finally, the enzyme fractions were dialyzed against a 100 mM potassium phosphate buffer (pH 7.2) using a Slide-A-Lyzer Dialysis Cassette (Pierce, U.S.A.) and concentrated using a Centricon Plus-20 (Amicon, U.S.A.). The protein content was estimated using a Bradford assay [3] with bovine serum albumin as the standard.

The *in vitro* conversion of indole to indigo was accomplished by combining 1 μM purified enzyme, 2 mM indole (Aldrich, U.S.A.), 10 μM spinach ferredoxin (Sigma, U.S.A.), 0.2 units spinach ferredoxin-$NADP^+$ reductase (Sigma, U.S.A.), and 1 mM NADPH (Jülich Fine Chemicals, Germany) in a total volume of 1 ml of a 100 mM potassium phosphate buffer (pH 7.2) for 20 min at 37°C. The *in vitro* reaction was then stopped by the addition of 6 N KOH (50 μl) and a light blue pigment formed. The pigments in these samples were extracted two times with 1 ml of ethyl acetate and the partitioned solvent phase evaporated to dryness using a Centra-Vac (Vision Scientific, Korea).

The residue was dissolved in a small volume of methanol and analyzed by TLC, HPLC (Thermo Hypersil-Keystone LC, U.S.A.), and HPLC-ESI MS/MS (Finnigan LCQ-Advantage, U.S.A.). The extracts from the enzyme reaction were initially analyzed by TLC using silica gel plates (Merck, Germany) developed with chloroform/acetone (30:1,v/v). The R_f value of the blue component was exactly the same as that of a commercial indigo standard (Aldrich, U.S.A.), indicating that indigo was produced by the enzyme reaction (data not presented). The HPLC analysis was carried out with a reversed-phase C_{18} column (4.6 mm× 250 mm Watchers 120 ODS-BP 5 μm, DAISO, Japan) using the same conditions as reported previously [14]. The hydroxylation of indole to indigo using the purified enzymes was assayed in comparison with the HPLC profile of authentic indigo. A UV peak corresponding to indigo from the extract of the *in vitro* reaction was detected at the same retention time as authentic indigo (Figs. 1A and 1B). The HPLC analysis of the *in vitro* reaction samples spiked with authentic indigo also yielded evidence of indigo production (Fig. 1C). The molecular ion of indigo equivalent to *m/z* 263 was monitored by HPLC-MS (Fig. 1D), and further fragmentation of the *m/z* 263 ion by MS/MS yielded *m/z* 235 $[M+H-CO]^+$, 219 $[M+H-CO-NH_2]^+$, 206 $[M+H-CO-CHO]^+$, 180 $[M+H-CO-CO-C_2H_2]^+$, 132 $[M/2+H]^+$, and 77 $[M/2+H-CO-HCN]^+$ (Fig. 1E). This MS/MS spectrum was in good agreement with those obtained by others [5, 6], and the same daughter ions were observed when authentic indigo was fragmented using the same conditions (data not

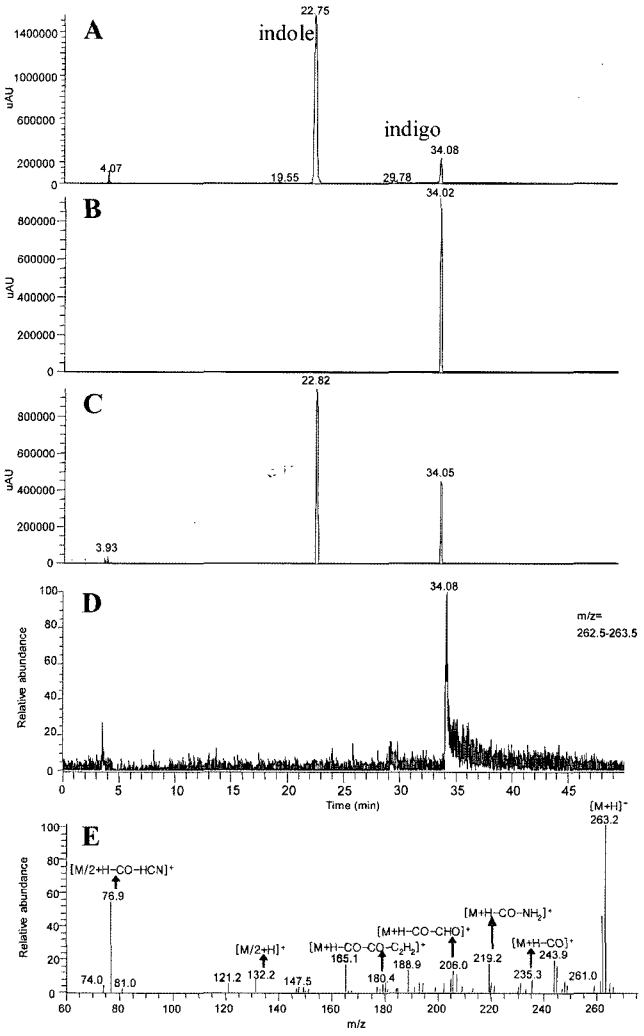

Fig. 1. HPLC and HPLC-ESI MS/MS analyses of indigo formation.

A. HPLC analysis of extract from *in vitro* reaction with purified wild-type PikC. **B**. HPLC analysis of authentic indigo. **C**. HPLC analysis of *in vitro* reaction sample (**A**) spiked with authentic indigo. **D**. LC/MS selected for *m/z* 263 from *in vitro* reaction sample (**A**), corresponding to indigo. **E**. MS/MS trace corresponding to indigo formed from *in vitro* reaction sample (**A**).

wild-type BM-3	^{70}NLSQ**A**LKFV^{78}	^{184}AMNK**L**QRAN^{192}
mutated BM-3	^{70}NLSQ**G**LKFV^{78}	^{184}AMNK**Q**QRAN^{192}
wild-type PikC	^{63}AVLA**D**PRFS^{71}	^{167}DRAA**F**RVWT^{175}
mutated PikC	^{63}AVLA**G**PRFS^{71}	^{167}DRAA**Q**RVWT^{175}

Fig. 2. Amino acid sequence alignments of cytochrome P450 BM-3 and cytochrome P450 PikC.
Ala74 and Leu188 in the wild-type BM-3 were altered to Gly74 and Gln188, respectively, to yield the mutated BM-3 with catalytic activity toward indole [13]. In the present study, Asp67 and Phe171 in the wild-type PikC were replaced with Gly67 and Gln171, respectively. The altered amino acids are in bold and underlined.

presented). Therefore, these results indicate that the wild-type PikC was able to catalyze the hydroxylation of the unnatural substrate indole to indigo. However, the catalytic efficiency of the wild-type PikC was relatively low and required further improvement. Consequently, PikC was subjected to site-directed mutagenesis to generate mutants with enhanced catalytic activity towards indole.

The self-sufficient cytochrome P450 BM-3 enzyme from *Bacillus megaterium* [15, 16] is one of the most extensively studied microbial P450 monooxygenases. Although the wild-type P450 BM-3 is incapable of hydroxylating indole, several engineered BM-3 mutants have been reported to catalyze the hydroxylation of indole to indigo [13] by altering Ala74 and Leu188 to Gly74 and Gln188, respectively (Fig. 2). The mutagenesis sites in PikC, including Asp67, Phe171, and Asp67/Phe171, were selected according to an amino acid sequence alignment between the wild-type PikC and the mutated BM-3 enzymes (Fig. 2). As such, the Asp67 and Phe171 of PikC were replaced with Gly67 and Gln171, respectively. The site-directed mutagenesis was performed by a PCR using a QuikChange kit (Stratagene, U.S.A), and the mutagenesis carried out using the following mutagenic oligonucleotide primers for the respective sites: for D67G, 5'-GCGGTCCTCGCCGGTCCCCGGTTCAG-CAA-3' and 5'-TTGCTGAACCGGGGACCGGCGAGG-ACCGC-3'; for F171Q, 5'-ACCGCGCCGCCCAACGC-GTCTGGACCGA-3' and 5'-TCGGTCCAGACGCGTTG-GGCGGCGCGGT-3'. The PCR reaction was started at 95°C for 30 s and ran through 15 cycles using the following conditions: 95°C for 30 s, 50°C for 30 s, and 68°C for 10 min. The template DNA was then digested with 20 units of DpnI (New England Biolabs, U.S.A.) for 1 h at 37°C. Recombinant colonies were selected from the transformed *E. coli* DH5α plated on LB agar plates and the nucleotides sequenced to confirm the mutations. The mutant PikC enzymes were purified and subjected to an *in vitro* enzymatic reaction using the same procedures as in the case of the wild-type PikC. According to an HPLC analysis of the indigo formation, F171Q produced a notably higher amount of indigo than the wild-type PikC based on the peak intensity. However, the amount of indigo produced by the D67G and D67G/F171Q mutants was slightly lower or similar to the level produced using the wild-type PikC.

Table 1. Kinetic parameters of wild-type PikC and mutant PikC enzyme for indole hydroxylation

Mutants	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
WT	2.0	10.1	199.3
F171Q	2.8	2.8	992.8

The kinetic parameters for the indole hydroxylation by the wild-type PikC and F171Q were determined. Assuming a 2:1 stoichiometry NADPH consumption to indigo formation [13], the initial velocities were measured spectrophotometrically at various indole concentrations (0.1–1.6 mM) by monitoring the rate of absorbance decay at 340 nm in a VERSAmax microplate reader (Molecular Devices, U.S.A.). The same *in vitro* reaction mixture as described above was combined with a 0.2 ml total volume of a potassium phosphate buffer (pH 7.2) in a 96-well plate (Nunc, Denmark) and placed in a microplate reader adjusted to 30°C. The reaction was initiated by the addition of 1 mM NADPH and the rate of NADPH consumption monitored over 60 s. The data from the first 20 s of the reaction were used to fit the Michaelis-Menten equation for the enzyme kinetic parameters. The catalytic efficiency value (k_{cat}/K_m) of F171Q for the indole hydroxylation was about five times that of the wild-type PikC (Table 1).

Although a lack of information on the crystal structure of PikC has limited the analysis of the possible effects of amino acid substitutions on substrate binding and catalytic activity, the above results suggest that the Phe171 position may be involved in the substrate binding in PikC. The ClustalW [21] amino acid sequence alignments of PikC with several other cytochrome P450 monooxygenases in actinomycetes showed that Phe171 in PikC is a conserved residue maintained as Phe or Leu in most P450 monooxygenases (Table 2). The site-directed mutagenesis of both Phe171 in the wild-type PikC (in this study) and Leu188 in the wild-type BM-3 [13] into Gln provided not only a higher catalytic efficiency but also a new functionality towards the unnatural substrate indole. Accordingly, these results suggest that engineering the conserved sites in the P450s of actinomycetes may generate mutant enzymes with potential catalytic properties that are unlikely to be generated by natural selection.

In summary, it was demonstrated that PikC can catalyze the oxidation of indole, which has a widely divergent structure from the natural substrates of PikC. As a result of subsequent studies on site-directed mutagenesis, the catalytic activity of PikC towards indole was considerably enhanced.

The formation of indirubin, which is a promising anticancer agent [7] and often seen in the microbial preparation

Table 2. Comparison of amino acid sequences selected for site-directed mutagenesis in PikC with other actinomycete P450 sequences.

Strain	P450[a]	Sequence	Accession number[b]
S. venezuelae	PikC	^{167}DRAA**F**RVWT^{175}	AAC68886
Sac[c]*. erythraea*	EryF	^{163}YRGE**F**GRWS^{171}	AAA26496
Sac. erythraea	EryK	^{144}DHEQ**F**GDWS^{152}	AAC45584
S. antibioticus	OleP	^{167}DRDL**F**RTFS^{175}	AAA92553
S. lavendulae	Orf3	^{170}DRKR**F**CVWS^{178}	AAD28449
S. fradiae	Orf1	^{171}DRDL**F**REWV^{179}	AAA21341
S. ansochromogenes	SanL	^{171}QRRD**F**HEWA^{179}	AAL85695
S. griseolus	SuaC	^{169}DHEF**F**QDAS^{177}	AAA26823
S. hygroscopicus	GdmP	^{159}DFAE**F**QQAS^{167}	AAO06929
S. hygroscopicus	RapJ	^{150}EGPV**L**EGQM^{158}	CAA60469
S. nanchangensis	NanP	^{178}DYEF**L**FERT^{186}	AAP42875
S. nanchangensis	MeiE	^{170}GNAI**L**TVAR^{178}	AAM97314
S. nodosus	AmphL	^{162}EQGQ**L**LAVL^{170}	AAK73504
S. noursei	NysL	^{162}EQGK**L**IAAL^{170}	AAF71769
S. cinnamonensis	MonD	^{185}DHDF**L**FDRS^{193}	AAO65808
S. caeruleus	NovI	^{168}DRKY**L**L- - -^{176}	AAF67502
S. avermitilis	AveE	^{165}AEAA**L**R- - -^{173}	BAC68651
S. natalensis	PimD	^{164}RRAE**L**TTLL^{172}	CAC20932

[a,b]Protein names and accession numbers as described in the NCBI database.
[c]*Saccharopolyspora.*

of indigo as an isomer of indigo, was not found with either the wild-type or mutated P450 PikC. However, the hydroxylation of indole is known to produce indirubin in much smaller amounts than indigo [13], possibly beyond the detection limits of the present study. Thus, further engineering studies on mutated PikC may lead to indirubin production as well as a higher production of indole.

Cytochrome P450 monooxygenases with catalytic activities towards alternative substrates may be very useful as industrial biocatalysts, yet attempts to utilize the broad substrate specificity of P450s from the genus *Streptomyces* towards a diverse array of commercially valuable chemical compounds that are structurally divergent from their natural substrates have rarely been reported. Therefore, the identification of new monooxygenases from *Streptomyces* is needed, along with extensive efforts to improve their activities and substrate flexibility through protein engineering, such as directed evolution, for the biotechnological application of these valuable monooxygenases.

Acknowledgments

This work was supported by grant No. R01-2002-000-00050-0 from the Basic Research Program of the Korea Science & Engineering Foundation and an Ewha Womans University Research Grant of 2004 (to Y.J.Y.). J.S.A. is grateful to the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant MG05-0303-4-0), Republic of Korea, and KRIBB Research Initiative Program.

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