Rational engineering of the fungal P450 monooxygenase CYP5136A3 to improve its oxidizing activity toward polycyclic aromatic hydrocarbons

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A promising polycyclic aromatic hydrocarbon-oxidizing P450 CYP5136A3 from Phanerochaete chrysosporium was rationally engineered to enhance its catalytic activity. The residues W129 and L324 found to be critical in substrate recognition were transformed by single (L324F) and double (W129/L324G, W129/L324F, W129A/L324G, W129F/L324G and W129F/L324F) mutations, and the engineered enzyme forms were expressed in Pichia pastoris. L324F and W129F/L324F mutations enhanced the oxidation activity toward pyrene and phenanthrene. L324F also altered the regio-selectivity favoring C position 4 over 9 for hydroxylation of phenanthrene. This is the first instance of engineering a eukaryotic P450 for enhanced oxidation of these fused-ring hydrocarbons.

Keywords: biocatalyst/cytochrome P450 monooxygenase/Phanerochaete chrysosporium/polycyclic aromatic hydrocarbon/protein engineering

Introduction

Fused-ring polycyclic aromatic hydrocarbons (PAHs) are environmentally ubiquitous and highly toxic pollutants known for mutagenicity and/or carcinogenicity in living organisms (Cerniglia and Heitkamp, 1989; Xue and Warshawsky, 2006; Haritash and Kaushik, 2009). These chemicals are normally resistant to microbial biodegradation and are highly persistent in the environment (Shuttleworth and Cerniglia, 1995; Haritash and Kaushik, 2009). Notably, the white-rot group of wood-decaying fungi possesses an extraordinary ability to completely degrade and/or mineralize (to CO₂) PAHs and have been shown to rely on P450-dependent and other mechanisms (reviewed in Syed and Yadav, 2012). However, progress in this field has been hampered due to lack of information on the specific P450 monooxynogenases catalyzing this rate-limiting initial step. Our recent ‘genome-to-function’ studies (Syed et al., 2010) on the model white-rot fungus Phanerochaete chrysosporium (henceforth abbreviated as Pc) led to a successful first identification of a set of six fungal PAH-oxidizing P450s (Pc-Pah1 to Pc-Pah6). Among these, Pc-Pah4 (CYP5136A3) showed oxidation of model PAH compounds phenanthrene and pyrene (Syed et al., 2010) as well as of the endocrine disrupting alkylphenols (Syed et al., 2011). Like a typical eukaryotic P450, this is a membrane P450 that requires electron delivery from NADPH via its cognate P450 reductase. Initial structure-activity analysis on this catalytically attractive Pc P450 showed that two amino acid residues (Trp129 and Leu324) are critical in substrate recognition and activity (Syed et al., 2011). Further improvement in the oxidizing ability of this potent P450 catalyst could enhance its competitiveness for application as a PAH bioremediation agent. In this context, there has been an increasing interest in engineering of the cytochrome P450 enzymes from mammalian and bacterial sources using both random and rational approaches (reviewed in Kumar, 2010; Jung et al., 2011; Urlacher and Girhard, 2012). However, P450 enzymes from these sources have shown inherent limitations in their potential for use as environmental bioremediation agents (Kumar et al., 2012 and references therein).

In light of the above background, the present study was aimed to engineer the fungal P450 enzyme CYP5136A3 to further improve its oxidation capability toward PAH compounds using a rational approach. We focused our protein engineering efforts using bioinformatically guided site-directed mutagenesis of the critical residues Trp129 and Leu324. The study led to two mutants of CYP5136A3 with significantly improved PAH oxidation activity.

Materials and methods

Strains and vectors

The microbial strains, plasmids and primers used in this study are listed in Supplementary Table SI. Media and growth conditions used for culturing Escherichia coli TOP10 and Pichia pastoris KM71H were as described in the Pichia expression manual (Invitrogen, USA).

Site-directed mutagenesis of CYP5136A3

A binary vector containing cDNAs of CYP5136A3 and its cognate reductase partner Pc-CPR (Supplementary Table SI), generated in our recent study (Syed et al., 2010), was used as a template to create mutations in CYP5136A3. The primers listed in Supplementary Table SI were used to create six mutations (one single mutation and five double mutations) in CYP5136A3 by using Strategene’s QuikChange Lightning

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Multi Site-directed Mutagenesis Kit (Cat. No. 210515) following the manufacturer’s instructions. Recombinant constructs were sequenced to select the ones showing the desired mutations for further studies.

**Heterologous expression of CYP5136A3 and its mutants**

The right construct for each mutation was transformed into *P. pastoris* KM71H using the Pichia Transformation Kit (Invitrogen). Recombinant clones were selected and confirmed for the co-presence of CYP5136A3 (mutated) and Pc-CPR. To check the CYP5136A3 gene copy number among the selected clones, real-time quantitative polymerase chain reaction (qPCR) was performed on the ABI Prism 9600 HT system (Applied Biosystems, USA). The following amplification conditions were used: initial denaturation at 90°C for 10 min followed by 40 cycles of amplification (denaturation at 95°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 30 s).

**Whole cell-based catalysis assay**

Whole cell-based estimations of P450 oxidation activity toward phenanthrene and pyrene and formation of their corresponding hydroxylated metabolites by the individual clones containing the mutated forms of CYP5136A3 were performed as described in our previous study (Syed *et al.*, 2011). Increase in the PAH oxidation activity (%) due to a mutation was then calculated using the wild-type (WT) value as 100%. All experiments were run in triplicate. For this, each mutant (single or double) was selected based on a screening of three recombinant clones for that mutation.

**Statistical analysis**

The substrate oxidation activities and the ratios of metabolites were analyzed for means and standard deviations, and compared for statistical differences by Student’s *t*-test using GraphPad QuickCalcs software package (GraphPad software, Inc. CA, USA).

**Computational methods for protein structure analysis**

A 3D model of CYP5136A3 was used from our recent study (Syed *et al.*, 2011). Briefly, it is a homology-based model built using the Phyre web-server (Kelley and Sternberg, 2009) with human CYP3A4 (PDB ID: 1tqn) as a template. Multiple sequence alignment (MSA) of the mammalian P450s known to oxidize PAHs with CYP5136A3 was performed using ClustalW2 (Larkin *et al.*, 2007). Following MSA analysis, WebLogo (Crooks *et al.*, 2004) was used to generate a sequence conservation profile in the substrate recognition sites (SRSs). In *silico* mutagenesis was conducted using Swiss-PdbViewer (Guex and Peitsch, 1997). Ligand docking simulations and binding energy estimates were obtained using AutoDock (Goodsell *et al.*, 1996).

**Results and discussion**

Mammalian PAH-oxidizing P450s have shown poor prospects for use as bioremediation agents due to their low stability and activity in bioremediation application-friendly microbial or plant hosts (Kumar *et al.*, 2012) for actual environmental applications, besides ecotoxicological concerns on the use of human-derived P450s. On the other hand, bacterial P450s (CYP101 and CYP102) that are known to have the capability to attack the PAH moiety have low or negligible native activity toward environmental PAHs such as phenanthrene and pyrene, among others (Harford-Cross *et al.*, 2000; Carmichael and Wong 2001). Hence, engineering of these P450 catalysts has received increasing attention in the recent past (Kumar, 2010; Jung *et al.*, 2011; Urlacher and Girhard, 2012), albeit with limited success in achieving the desired activity and compatibility for use as bioremediation agents toward PAHs. In the light of this background, engineering of a newly identified PAH-oxidizing fungal P450 with a potent native activity, such as CYP5136A3, offers promise for developing P450 biocatalysts for bioremediation of PAHs. Given that these fungal organisms are equipped with robust PAH biodegradation machinery, the engineered P450 could circumvent the rate-limiting nature of the initial oxidation step thereby increasing the turnover of the hydroxylated metabolites entering the downstream enzymes in the native PAH degradation pathways.

Whole cell-based P450 biocatalysts are more economical in terms of applications as compared with the reconstituted purified enzyme catalysts (P450 and reductase partner proteins), since the former will avoid external addition of the electron donor (NADPH) and other in *vitro* reaction components, and the likely uncoupling problems leading to lower catalytic efficiency in terms of enzyme activity and metabolite turn over (Rabe *et al.*, 2008). Motivated by these advantages of the whole cell-based systems as well as to circumvent the recognized practical problems in purifying these full-length membrane proteins without losing their native activity, we used *P. pastoris*-based whole cell system to generate and evaluate the engineered P450 forms. However, to use an integrative transformation-based system such as *P. pastoris* for comparing various P450 forms (WT and mutated), it is necessary to maintain the same copy number of the P450 insert to be expressed; changing the copy number could modulate the overall expression level and subsequent rate of substrate oxidation. Hence, in the present study, we selected *P. pastoris* recombinant clones containing the same copy number of CYP5136A3 as the WT. Considering that the vector-only (negative control) clone showed no PAH oxidation activity, the *P. pastoris* native P450s (or other enzymes) played no role in the observed oxidation of the test PAHs (phenanthrene and pyrene).

**Rational design of mutations to improve catalytic activity**

Two key residues for substrate recognition (Trp129 and Leu324) in CYP5136A3 identified in our preceding efforts (Syed *et al.*, 2011) are located at the SRSs SRS-1 and SRS-4, respectively. SRSs are short variable regions (labeled 1 through 6) within the active site cavity of any CYP that determine substrate specificity (for more details, refer to Graham and Peterson, 1999). MSA with the known PAH-oxidizing mammalian CYPs (Shimada, 2006) revealed the corresponding amino acids in other P450 enzymes. Figure 1 shows two segments (containing SRS-1 and SRS-4 regions, Fig. 1A and B) of the full protein length MSA on different human CYPs (SwissProt IDs: CP1A1_HUMAN, CP1A2_HUMAN, CP1B1_HUMAN, CP2A6_HUMAN, CP2E1_HUMAN and CP3A4_HUMAN) and one rat CYP (CP2B1_RAT, as no human ortholog is available). Following the MSA analysis, a sequence conservation profile was generated in these two segments (Fig. 1C and D for SRS-1 and SRS-4, respectively). Labeled with star are the positions of interest. As can be seen from Fig. 1C, position W129 is predominantly represented by Phe, except for CYP3A4, where it is Ile. Position L324 (Fig. 1D) is equally represented by Thr (52% out of the total) and Leu (48%).
occupied by either Gly or Phe. Our previous study showed that W129L (Leu is the closest amino acid to Ile) and L324G mutations have a detrimental effect on the catalytic activity of the enzyme (Syed et al., 2011). Hence, we rationalized that using alternative mutations (single or double) in these positions could yield the desired outcome. For instance, having Phe at these positions could benefit the enzyme in terms of PAH substrate recognition. This is because the two types of interactions, hydrophobic and π–π stacking, may increase binding affinity and direct orientation of an aromatic substrate with respect to heme (Lewis et al., 2004; Gonzalez et al., 2012). In order to further discern contributions of hydrophobic versus π–π stacking nature of the PAH binding, we probed Ala, Leu and Phe mutations (all hydrophobic, the first two being aliphatic and the last aromatic). Collectively, we considered probing the following mutations: a single mutant L324F and various double mutants (W129F/L324F, W129A/L324G, W129L/L324G, W129F/L324G and W129L/L324F). Before conducting the experiments, we compared these mutants on the computationally estimated binding energy ($E_{\text{bind}}$) produced occupied by either Gly or Phe. Our previous study showed that W129L (Leu is the closest amino acid to Ile) and L324G mutations have a detrimental effect on the catalytic activity of the enzyme (Syed et al., 2011). Hence, we rationalized that using alternative mutations (single or double) in these positions could yield the desired outcome. For instance, having Phe at these positions could benefit the enzyme in terms of PAH substrate recognition. This is because the two types of interactions, hydrophobic and π–π stacking, may increase binding affinity and direct orientation of an aromatic substrate with respect to heme (Lewis et al., 2004; Gonzalez et al., 2012). In order to further discern contributions of hydrophobic versus π–π stacking nature of the PAH binding, we probed Ala, Leu and Phe mutations (all hydrophobic, the first two being aliphatic and the last aromatic). Collectively, we considered probing the following mutations: a single mutant L324F and various double mutants (W129F/L324F, W129A/L324G, W129L/L324G, W129F/L324G and W129L/L324F). Before conducting the experiments, we compared these mutants on the computationally estimated binding energy ($E_{\text{bind}}$) produced

<table>
<thead>
<tr>
<th>CYP5136A3</th>
<th>$E_{\text{bind}}$, kcal/mol</th>
<th>Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>−6.68</td>
<td>61.74 ± 3.45</td>
</tr>
<tr>
<td>L324F</td>
<td>−6.96</td>
<td>75.73 ± 4.63</td>
</tr>
<tr>
<td>W129F/L324F</td>
<td>−6.75</td>
<td>79.59 ± 4.45</td>
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<tr>
<td>W129A/L324G</td>
<td>−5.92</td>
<td>32.87 ± 2.94</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>W129F/L324G</td>
<td>−6.22</td>
<td>15.38 ± 2.34</td>
</tr>
<tr>
<td>W129L/L324F</td>
<td>−5.76</td>
<td>0</td>
</tr>
<tr>
<td>Pyrene</td>
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<td></td>
</tr>
<tr>
<td>WT</td>
<td>−7.09</td>
<td>33.25 ± 3.20</td>
</tr>
<tr>
<td>L324F</td>
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<td>81.03 ± 4.87</td>
</tr>
<tr>
<td>W129F/L324F</td>
<td>−6.96</td>
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</tr>
<tr>
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<td>−6.45</td>
<td>1.07 ± 0.62</td>
</tr>
<tr>
<td>W129L/L324G</td>
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</tr>
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<td>W129L/L324G</td>
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</tr>
<tr>
<td>W129L/L324F</td>
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</table>
by AutoDock toward phenanthrene and pyrene (Table I, middle column). L324F was estimated to have the lowest binding energy to both phenanthrene and pyrene followed by WT and W129F/L324F, supporting our hypothesis about physico-chemical forces driving substrate recognition by the enzyme.

**Effect of mutations on substrate oxidation activity**

The mutant clones expressing the mutated CYP5136A3 forms were designated as L324F, W129F/L324G, W129L/L324G, W129F/L324F, W129A/L324G, and W129A/L324F. For each mutation, we selected a recombinant P. pastoris clone containing the same copy number of CYP5136A3 as the P. pastoris clone expressing the WT CYP5136A3. Similar Ct values for the two test clones (WT versus mutant) indicated the same copy number of the gene insert (Supplementary Fig. S1). For each mutation, triplicate clones were subjected to this screening. The oxidation activity results are presented in Fig. 2 and Table I (right column).

Among the double mutants, W129L/L324G and W129L/L324F showed complete loss of activity toward both PAHs (phenanthrene and pyrene). W129A/L324G and W129F/L324G retained partial oxidation activity toward phenanthrene (32 and 15%, respectively) but a complete loss of activity toward pyrene (Fig. 2A). In contrast, L324F and W129F/L324F mutants representing single and double mutations, respectively, showed greater oxidation of phenanthrene and pyrene compared with the WT (Fig. 2A). This showed that replacement of Leu324 with Phe caused enhancement of oxidation activity toward both PAHs. Using the WT activity value as the base value (denominator), the mutant showed a net increase of 23 and 144% for phenanthrene and pyrene, respectively (Fig. 2B). The corresponding activity increases for the double mutant W129F/L324F were 29% (phenanthrene) and 187% (pyrene) (Fig. 2B). All mutant clones showed the same biomass yield (Fig. 2C) as the WT and C clones indicating that the observed differences in PAH oxidation activity were due to the mutation(s) in the P450 enzyme. This result strongly supported our hypothesis that having Phe at these positions (Trp129 and Leu324) will benefit the enzyme in substrate recognition via two types of interactions, hydrophobic and π–π stacking, which increase binding affinity and direct orientation of a substrate with respect to heme. In this context, it is worth noting that the single mutation W129F was found to lower the enzymatic activity against PAHs in our previous mutagenesis study on CYP5136A3 (Syed et al., 2011). This indicates the difficulties in enzyme engineering due to complexity of structure–activity relationship for the residues at SRS, when the resulting enzymatic activity is determined not by a linear combination of single mutations improving activity individually but rather by a complex mixture of simultaneous physico-chemical changes introduced in multi-point mutations. At the same time, lower binding energy estimated for the mutants with enhanced enzymatic activity compared with other probed mutants may also indicate that improvements in catalytic activity were achieved due to a better substrate binding affinity, not by the increased electron transfer.

**Effect of mutations on CYP5136A3 regio-selectivity**

Comparison of the hydroxylated metabolite profiles for phenanthrene and pyrene between WT and mutant forms of CYP5136A3 is presented in Table II and Supplementary Fig. S2. All mutants (L324F, W129F/L324F, W129F/L324G and W129A/L324G) oxidized phenanthrene into three metabolites, 3-, 9- and 4-phenanthrols, and pyrene into two metabolites, 1- and 2-hydroxypyrene. The ratio of phenanthrene metabolites for the double mutants W129F/L324F, W129F/L324G and W129A/L324G was found to be the same as that
for WT suggesting that the respective mutations had no effect on regio-selectivity of oxidation of phenanthrene (Table II). However, L324F showed a lower amount of 9-phenanthrol and a higher amount of 4-phenanthrol compared with WT (Supplementary Fig. S2 and Table II). This suggested that replacing Leu324 with Phe changed the regio-selectivity of CYP5136A3 favoring the oxidation at position C4 in the phenanthrene moiety. The ratio of 1- and 2-hydroxyphenanthrene observed for L324F and W129F/L324F clones was the same as that for WT (Table II) suggesting that the mutations had no effect on regio-selectivity of oxidation of pyrene.

Conclusions

Rational engineering based on a combination of in silico and experimental approaches targeting critical amino acid residues in the SRS regions of the P450 protein enabled us to significantly improve the PAH oxidation activity of CYP5136A3 from the model white-rot fungus *P. chrysosporium*. An amino acid with a hydrophobic and aromatic side chain (phenylalanine) at position W129 and L324 turned out to be the most beneficial for the catalytic activity of CYP5136A3. To our knowledge, this study constitutes the first report on engineering a eukaryotic P450 for enhanced oxidation of PAHs (phenanthrene and pyrene). It is also the first demonstration of the use of *P. pastoris* whole cell-based oxidation assays for screening the engineered P450s with enhanced oxidation capability toward xenobiotic compounds. Furthermore, the study marks the beginning of protein engineering of P450 monoxygenases in this versatile bioremediation-relevant fungus.

Supplementary data

Supplementary data are available at *PEDI* online.

Funding

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