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Hydroxylation of non-substituted polycyclic aromatic hydrocarbons by cytochrome P450 BM3 engineered by directed evolution

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Abstract

Cytochromes P450 are monoxygenases involved in the metabolism of xenobiotics, including recalcitrant pollutants persistent in the environment and dangerous to human health such as polycyclic aromatic hydrocarbons (PAH). In mammals, cytochromes P450 oxidise PAHs leading to active metabolites that act as carcinogens, while in bacteria, they are involved in the first rate-limiting step oxidation for their metabolism. Here we apply random mutagenesis to generate libraries of mutants of P450 BM3 active towards PAHs. After three rounds of error-prone PCR, three mutants were identified for improved activity toward chrysene and new activity toward pyrene in comparison to the wild type enzyme. The mutants show higher affinity and coupling efficiency for chrysene with faster rates of product formation compared to the wild type. Furthermore, the mutants are able to hydroxylate chrysene in different positions, producing four metabolites, 1-, 3-, 4-, and 6-hydroxychrysene, and to hydroxylate pyrene to 1-hydroxypyrene.

The different product profiles obtained for the different P450 BM3 mutants indicate that substrate orientation in the catalytic pocket of the protein can be modified by protein engineering. The mutants can be used for metabolic engineering for safe and cost-effective sustainable production of hydroxylated PAHs for industrial purposes as well as for the assessment of their carcinogenic activity in mammals.

Keywords: cytochrome P450 BM3 · polycyclic aromatic hydrocarbons · chrysene · pyrene · directed evolution

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hazardous hydrophobic compounds with increased persistence in ecosystems. They originate from natural and anthropogenic pyrolysis of organic matter, such as forest fires and oil industry-related processes [1]. Because of their hydrophobicity and thermal stability, they are used as antioxidants, organic diluters, plasticisers and heat thermal fluids in capacitors. Four-rings containing PAHs, for example chrysene and pyrene, show increased hydrophobicity, low bioavailability, greater environmental persistence and stronger toxicity compared to smaller PAHs and have been characterised as priority pollutants due to their mutagenic, endocrine-disrupting and carcinogenic properties [2]. In mammals, these compounds undergo oxidative activation by cytochromes P450, such as P450 1A1, generating molecules capable to irreversibly react with tissues and biological macromolecules exerting mutagenic effects [3].

On the other hand, oxidation of PAHs by microbial cytochromes P450 represents the first and often rate-limiting step for the biodegradation of these compounds [4,5]: hydroxylated PAHs are more water-soluble and can then be used as substrates for oxidases, such as peroxidase and laccase that decompose phenolic compounds [6]. The rate of degradation of PAHs is inversely proportional to the number of rings in the molecule [4,7]. The most recalcitrant PAHs are therefore molecules containing polyaromatic rings, such as chrysene and pyrene.

The availability of an engineered enzyme able to efficiently hydroxylate these molecules could therefore provide a tool to accelerate the synthesis of PAHs metabolites for the assessment of their toxicity and carcinogenic activity. Furthermore, the biocatalyst would provide a green tool for the first step of biodegradation of PAHs in bacteria in a sustainable manner.

Cytochrome P450 BM3 (CYP102A1) is a soluble, catalytically self-sufficient monooxygenase from Bacillus megaterium consisting of a N-terminal haem domain (BMP) fused to a C-terminal FAD/FMN-contanining reductase domain (BMR) in a single polypeptide chain [8]. It performs the subterminal hydroxylation of long chain (~ C12-C20) fatty acids, their alcohols and amides, and the epoxygenation of unsaturated fatty acids [9] with the highest catalytic activity determined for a P450 monooxygenase, due to efficient electron transfer between the reductase and haem [10,11]. Because of the availability of the crystal structure of P450 BMP also in complex with substrate [12,13], site-directed mutagenesis has been used to generate mutants of P450 BM3 that oxidise PAHs including naphthalene, phenanthrene, fluoranthene and pyrene [6,14-17].

Directed evolution on P450 BM3 has already been reported as powerful tool to improve activities toward drugs or drug-like molecules [18-20], aliphatic and aromatic compounds [21], styrene [22], indole [23,24] and medium chain fatty acids [25]. It has also been exploited to engineer mutants able to hydroxylate alkybenezenes [26-29].

Here we report the directed evolution of P450 BM3 for the recognition and oxidation of chrysene and pyrene. Three different mutants were selected for their improved activities and coupling efficiencies toward these two compounds. The sequencing of these mutants revealed the presence of point mutations in positions otherwise unpredicted to affect the enzyme functionality by using a rational approach.

2. Materials and methods.

2.1 Chemicals

Chemicals were purchased from Sigma-Aldrich Inc., Supelco and 6-hydroxychrysene from AccuStandard. Enzymes for DNA manipulation were from New England Biolabs respectively. The *E. coli* BL21(pT7Bm3Z) clone containing the CYP102A1 gene encoding P450 BM3 was donated by Dr. T. L. Poulos (University of California at Irvine, Irvine, USA).

2.2 Random mutagenesis

PCR was performed on the pT7Bm3Z plasmid containing the *CYP102A1* gene encoding P450 BM3 to amplify part of the haem domain between the *BamHI* and *MscI* (an isoschizomer of *BalI*) restriction sites. The reaction mixture contained 1x Taq buffer, 0.2 mM dNTPs, 100 pmol μl^{-1} primer (forward: CTTAACAAGTGAAGGAGGGATCCTATG and reverse: ATACCGGTTGACGAGGACGCAAAAGGG), 5 ng of template DNA and 2 U Taq polymerase to a total volume of 40 µl. The purified DNA fragment was used as a template in error-prone PCR. The reaction mixture contained: 1x Taq buffer, 0.5 mM $MnCl_2$, 1.0 mM of three dNTPs with the fourth at 0.2 mM, 100 pmol μl^{-1} of each primer, 5 ng of template DNA and 2 U Taq polymerase to a total volume of 40 µl. Another EP-PCR approach was attempted in which the reaction mixture was supplemented with $MgCl_2$ to a final concentration of 3.5 mM. For backcrossing, the fragment of the haem domain of the mutant M3 was shuffled with 40-fold excess of the corresponding fragment of the WT. The four reactions were ran in parallel and pooled following recovery from a 2% agarose gel. The purified fragment was

digested with *BamHI* and *MscI*, purified and ligated into the pT7Bm3Z vector. The ligation mixture was used to transform the *E. coli* BL21(DE3) strain and the transformants were plated on LB agar medium supplemented with 100 μg ml^{-1} ampicillin.

2.3 Screening of P450 BM3 library: the alkali assay

Single colonies were picked and inoculated into 1 ml Luria-Bertani (LB) medium supplemented with 100 μg ml^{-1} ampicillin overnight at 37°C. 50 μl of the pre-cultures were inoculated into 5 ml LB supplemented with 100 μg ml^{-1} ampicillin and incubated at 37°C to an OD_{600}~ 1.0 followed by induction with 0.5 mM IPTG and further growth under the same conditions for 20 hr. The cells were centrifugated, the supernatant discarded and the cells resuspended in 100 mM KPi pH 8.0. Their OD_{600} were made comparable prior to aliquoting 200 μl to 96-well organic-solvent resistant microtitre plates. Wild-type was also included. Initially PAH mix containing 18 compounds was added to the cells (100 μM) at room temperature (~20°C) and following incubation for 2 hrs, 150 μM NADPH was added for 4 hrs. Control reactions with 800 μM of lauric acid and without the addition of substrate and cells were also included. Once the reactivity towards the mix was confirmed, the individual components of the mixes were dissolved in organic solvents (acetonitrile, benzene, ethanol) and the assay was repeated with them (100 μM final concentration). The volume of the solvent did not exceed 20% of the total volume. Additional controls with solvents to the same volume of the corresponding substrate were carried out. The alkali product was then developed as described by Tsotsou et al. [30]. Briefly, 0.3 M HCl was added to the reaction mixture for 10 minutes at room temperature. In this way, the pH was lowered to 2 in order to stop the reaction and eliminate the excess NADPH. Then, 80 μl of this

sample were transferred in a new microtitre plate well containing 270 μl of 9 M NaOH and incubated at the dark for 2.5 hours at room temperature. In this way, the pH was increased to a value of 14.8 allowing the developement of the alkali product derived from the $NADP^+$ generated by enzyme turnover. The plates were scanned on a SPECTRA 340c microplate spectrophotometer (Molecular Devices, California) and the mutants that showed improved activity compared to the wild type were sequenced, expressed and purified. The assay was repeated on purified protein on the individual components of the mixes to verify the results of the whole cell assay. Protein (up to 1 μM) was incubated with substrate for 15 min and with NADPH for 1 hr. Protein concentration was determined by the difference between the oxidised and CO-bound ferrous BM3, using a Δε of 91.0 mM^{-1} cm^{-1} for the ferrous/CO haem adduct at 450 nm with a Hewlett-Packard 8452A diode array spectrophotometer [31].

2.4 Protein expression and purification

Wild type P450 BM3 and mutants were expressed in *E. coli* BL21(DE3) cells transformed with the IPTG-inducible pT7Bm3Z vector and purified as previously described [32,33]. The protein was stored in 50 mM HEPES pH 8.0 and for the NADPH consumption, substrate turnover and alkali assay experiments was in 100 mM KPi pH 8.0 following buffer exchange through a PD10 size exclusion column.

2.5 NADPH consumption studies

The rate of NADPH oxidation was measured by incubating the enzyme (μM range, > 0.1 μM) with substrate up to saturating concentrations in 100 mM KPi pH 8.0 at 20°C. After 2 minutes of incubation, 150 μM NADPH was added to start the reaction and

NADPH oxidation was followed spectrophotometrically at 340 nm (ε_{340}=6.22 mM^{-1} cm^{-1}). Fittings of the data were performed by using Sigma Plot 8.0 Software.

2.6 GC-MS analysis

GC analysis was performed with a GC-MS instrument (Trio 1000 GC 8060 MS, Fisons Instruments) in the splitless mode on a DB5-MS capillary column (20 m, GE. Ringwood, Australia) using helium as the carrier gas. The column temperature was initially 100°C and was raised linearly to 320°C at a rate of 20°C min^{-1} and maintained at that temperature for 3 min. The injector and detector were operated at 240°C. The GC flow was routed into the ion source of the MS, which was operated in the electron impact mode with electron energy set at 70 eV and a source temperature of 180°C.

Standards for chrysene-3,4-trans-dihydrodiol and 1-hydroxychrysene were kindly donated from Dr Grete Jonsson (RF-Akvamiljø, Norway). Standard of chrysene-3,4-trans-dihydrodiol was used to obtain 3- and 4-hydroxychrysene GC-MS profiles.

These compounds arose from water elimination in the injector resulting in the breaking down of the polar chrysene-3,4-trans-dihydrodiol to 3- and 4-hydroxychrysene, as verified by selected ion minitoring at m/z 406 and 316.

Enzyme (0.54 μM wild type or 0.43 μM for each mutant) was incubated with 1 μM chrysene or 20 μM pyrene for 5 min at room temperature prior to adding 500 μM NADPH in 100 mM KPi pH 8.0. The reactions were repeated in the presence of catalase, or superoxide dismutase or both (50 U each) and controls with enzyme and substrate alone were also included. The reaction was stopped 80 min later by extracting with an equal volume of benzene, evaporating to dryness and treating with an excess of bis(trimethyl-silyl)trifluoro-acetamide (BSTFA) (50 μl) for 18 hrs at room

temperature. Derivatisation with BSTFA replaced the hydrogen in the hydroxyl group of the metabolite with trimethyl silyl [$Si(CH_3)_3$] that decreases the polarity of the analytes allowing improved detection sensitivity [34]. Excess reagents in the samples and standards were dried under N_2, dissolved in 10 µl decane and 2 µl were loaded onto the GC column. 1-hydroxypyrene and 6-hydroxychrysene were used as internal standards for chrysene and pyrene respectively to correct differences in total volume and variations in injection volume. Quantitative determination was performed on selected ion monitoring mode (SIM). The dwell time for each ion was 0.08 sec with a mass span of 0.1 u. Integrated peak areas of each metabolite were expressed as ratios to the internal standard peak area and plotted against metabolite concentration to generate calibration curves.

3. Results and Discussion

3.1 Production, expression and purification of P450 BM3 mutants

The first 324 amino acids containing four regions aligning with the substrate recognition sites (SRS) of human cytochromes P450 [35] and helix I were selected as target for random mutagenesis. The mutant named A2, generated after a first round of error-prone PCR, showed a slight increase in activity toward a mixture of PAH compared to the wild type (WT) protein, and it was therefore selected for a second round of error-prone PCR (EP-PCR) to generate a library of mutants of P450 BM3 with improved activity toward PAHs. The library of mutants obtained after the two rounds of EP-PCR was screened towards chrysene and pyrene by using the alkali assay previously described [30]. This assay is based on the measurement of the fluorescent NADP+-adduct formed under alkaline conditions following enzyme

turnover in the presence of the target substrate using E. coli cells expressing a library of P450 BM3 mutants [30]. The screening procedure led to the identification of two mutants, named M3 and P2, that showed an improved activity towards chrysene and a new activity for pyrene in comparison to the WT.

DNA sequencing of the mutants revealed the presence of 6 mutations in M3 (Asp251Gly, His266Ala, Glu267Arg, Thr269Asn, Ser270Glu, Gln307His) and 4 mutations in P2 (Asp208Arg, Ile209Tyr, Asp251Gly, Gln307His); both mutants were found to carry the mutations Asp251Gly and Gln307His present in the starting DNA template of mutant A2.

A backcross of mutant M3 with the parental (WT) gene, led to mutant K4 that maintained an improved activity toward chrysene and pyrene compared to the WT. Interestingly its DNA sequence revealed the presence of only one single point mutation, Val317Cys.

The three mutants were successfully expressed and purified with yields of 8-9 mg pure protein per litre of liquid culture. The visible spectrum of the three mutants displayed an absorbance maximum at 418 nm that shifted at 450 nm upon reduction with sodium dithionite and bubbling with CO. Furthermore, the ratio A_{280}/A_{418} resulted similar for the variants respect to WT, suggesting the same purity degree and the same heme content (estimated >96% for all the proteins). The thermal stability of the variants, measured by far UV circular dichroism spectroscopy, was also similar to the one measured for WT, ranging from 58.6°C for P2 and 60°C for both WT and K4.

The purified mutants were further tested with the alkali assay toward chrysene and pyrene using lauric acid as positive control (Figure 1).

They showed improved activity in comparison to WT with M3 having the highest amount of alkali product derived from NADPH oxidation in the presence of chrysene

and pyrene. The NADPH consumption towards lauric acid resulted in a slightly decreased activity for the three mutants with respect to the WT. It is well known that cytochromes P450 can consume NADPH for product formation (coupling) but also for the formation of reactive oxygen species (uncoupling). Since the alkali assay measures NADPH consumption, it can be affected by different levels of coupling/uncoupling in the mutants. For this reason, further characterisation in terms of quantification of product formation was carried out.

3.2 NADPH consumption studies

NADPH consumption was measured for WT and its mutants as decrease in absorbance at 340 nm in presence of different concentrations of chrysene and pyrene. Experiments carried out with chrysene showed a typical hyperbolic trend for the WT and the mutants, allowing the fitting of the experimental points to the Michaelis-Menten model with the K_M and k_{cat} values reported in Table 1. It has to be taken into account that K_M and k_{cat} are "apparent" values, as they can be affected by uncoupling. In any case, kcat and k_{cat}/K_M values follow the order M3 > P2 > K4 > WT, suggesting an increase in the affinity and in the catalytic efficiency of all the mutants in comparison to the WT, with M3 being by far the most efficient catalyst for chrysene hydroxylation.

Pyrene was found to induce NADPH consumption, but it was not possible to calculate kinetic parameters for this substrate because of its poor solubility within the range required for the construction of a Michaelis-Menten curve.

3.3 GC-MS analysis of chrysene turnover

GC-MS analysis was carried out to identify the metabolites derived from chrysene oxidation by the WT and mutants M3, P2 and K4, and to determine reaction rates and coupling efficiencies.

Standard solutions of chrysene, 6-hydroxychrysene and 1-hydroxychrysene were used to determine the retention time and the mass spectrum. The retention times were 10.9, 11.8 and 12.4 minutes for chrysene, 6-hydroxychrysene and 1-hydroxychrysene respectively, while the molecular masses were 228 for chrysene and 316 for the 1- and 6-hydroxychrysene products. Standards for 3- and 4-hydroxychrysene were obtained from chrysene-3,4-trans-dihydrodiol as described in the Experimental section and their retention times in the GC analysis were 11.75 min for 4-hydroxychrysene and 12.25 min for 3-hydroxychrysene. The mass spectra showed a major ion at m/z 316, corresponding to the derivatised monohydroxychrysene [36].

Chrysene was oxidized by the WT and the mutants to four products, corresponding to 1-, 3-, 4- and 6-hydroxychrysene. The mass spectra of these products all showed a major ion at m/z 316. Other prominent ions were detected at m/z 301 and 228 which corresponded to the loss of CH3• and [Si(CH3)3]OH used for the derivatization of the hydroxyl groups of the products.

Comparison of the GC chromatograms of incubations with the WT and the three mutants show differences in the ratios between the four metabolites, suggesting a change in the region-specificity of hydroxylation due to the introduction of the mutations (Figure 2).

Although the low activity of the WT for chrysene led to very small amounts of products and made the quantitative analysis very difficult, qualitative analysis of the GC chromatograms of the incubations with the WT show that 6-hydroxychrysene is

the main product, followed by 1-hydroxychrysene and traces of 3- and 4-hydroxychrysene. On the other hand, the GC chromatograms of the incubations with M3 and P2 show that 1-hydroxychrysene is the main product, followed by comparable amounts of 3- and 6-hydroxychrysene and traces of 4-hydroxychrysene. The product profile of K4, the mutant obtained by backcrossing with the WT gene and carrying only a single point mutation in its sequence, is very similar to that of the WT. In all cases, further oxidation of the metabolites to other compounds, such as dihydrodiols, was not observed and control reactions performed without the addition of the enzymes resulted in no detectable products.

Measurement of the performance in terms of the reaction rate and relative coupling efficiency of the mutants and WT was carried out in reference to the production of 6-hydroxychrysene. The reactions were performed in presence of catalase and superoxide dismutase to avoid possible interference by oxygen reactive species. The relative coupling efficiency was calculated from the ratio of 6-hydroxy produced and NADPH consumed (Table 1). The mutant M3 showed the highest activity and coupling efficiency. The differences in catalytic rates and coupling efficiency found for the different mutants could be due to different orientations of chrysene in the active site of the proteins [37].

The structure of chrysene and the positions that are hydroxylated by the mutants are illustrated in Scheme 1. The formation of multiple hydroxylated products suggests flexible binding and increased mobility of chrysene in the active site, allowing the oxidising species to encounter different hydroxylation sites of this substrate [38]. M3 and P2 also oxidise chrysene to the non K-region metabolites 1-hydroxy and 3-hydroxychrysene to a much greater extent than K4 and the WT. This indicates a more efficient binding of chrysene in the active site in M3 and P2 compared to WT,

as suggested by k_{cat}/K_M values. Furthermore, literature data suggested that substrates that are uncoupled show high mobility in the active site [39]. Therefore, the oxidation of chrysene to non-K-region metabolites by the mutants was in agreement with their higher coupling efficiencies of M3 and P2 compared to K4 and WT.

3.4 GC-MS analysis of pyrene turnover

The GC-MS analysis was carried out to identify and quantify the pyrene metabolites. The chromatographic profiles resulting from the incubation of WT and mutants with pyrene are shown in Figure 3. A standard of 1-hydroxypyrene was injected on GC column, resulting in a retention time of 10.45 min and a mass spectrum with a major ion at m/z 290; 6-hydroxychrysene was used as internal standard. As previously reported no product was detected for the WT [17], whereas for the mutants produced a peak at 10.45 min retention time corresponding to 1-hydroxypyrene, as confirmed by mass spectrometry.

Table 2 summarises the data from the catalysis with pyrene by WT and mutants M3, P2 and K4.

The results reported in Table 2 show that mutants M3, P2 and K4 give 1-hydroxypyrene (scheme 2) with mutant M3 giving the highest level of product and mutant K4 showing the highest coupling efficiency (98.4%). The purity of the product formed combined with the high level of coupling of these mutants offer a clear advantage over the data available to date in the literature. Bacterial metabolism of pyrene generates mainly dihydrodiols [40] and the mutants of P450 BM3 obtained by rational design of the active site have been reported to oxidise pyrene to 1-hydroxypyrene and 1,8-pyrenequinone, with traces of 2-hydroxypyrene and 1,6-

pyrenequinone. Further oxidation of 1-hydroxypyrene led to 1,6- and 1,8-pyrenequinone [17]. The highest coupling efficiency was 6.39% for the active site mutant Arg47Leu/Tyr51Phe/Phe87Ala/Ala264Gly with a product formation rate of 19.5 nmol product min^{-1} $nmol^{-1}$ P450 [17]. These findings suggest that it is very difficult to improve at the same time both the turnover rate and the coupling efficiency of P450 BM3.

The turnover rates obtained for the mutants toward chrysene and pyrene are, in general, quite low. It has to be taken into account that the PAHs studied are very unreactive compounds and structurally very different from the fatty acids substrates of the enzyme. However, significant differences were found between the variants, giving important information about which mutations can affect chrysene and pyrene catalysis.

Comparison between the data obtained from mutants M3, P2 and K4 with chrysene and pyrene show that both the rate of product formation and relative coupling efficiencies were higher for pyrene than chrysene, but in both cases the mutants can be used as biocatalysts for the NADPH-dependent production of hydroxylated forms of these PAH. Interestingly, the coupling efficiency observed with pyrene are much higher than those measured in the presence of chrysene, suggesting differences in the substrate-bound structures of the mutants that lead to different performances in the radical-rebound mechanism of the P450 monoxygenation reaction.

3.5 Structural analysis of mutants M3, P2 and K4

The crystal structure of the haem domain of P450 BM3 (BMP) in complex with palmitoleic acid (PDB code: 1FAG) was analysed to identify the positions of the mutations found in M3, P2 and K4 (Figure 4).

All mutations were found to be in regions far from the substrate binding and the active site. Substitutions of amino acids can indeed alter enzyme specificity and activity also when they are not in direct contact with the substrate, and this can occur via subtle long-range conformational perturbations of the 3D structure (domino effect) or by affecting electron transfer efficiency from the reductase to the haem [41,42]

Mutant M3 shows non-conservative substitutions at His266Ala, Glu267Arg, Thr269Asn and Ser270Glu that lie in the groove of helix I. The oxygen binding pocket in P450 enzymes is defined by a well-conserved five-residue string that corresponds to residues 264-268 in P450 BM3. The string comprises an alanine and a glycine at the N-terminus, followed by an acidic residue in position 267, a conserved threonine in 268 and a serine or threonine at position 269 [12]. Interestingly none of the mutants showed substitutions in the crucial Thr268 position. It has been proposed that Glu267, which is solvent-exposed, is indirectly involved in proton transfer to Thr268, which then donates the proton to the iron-bound oxygen during catalysis in P450 BM3. The structure of the WT enzyme shows that substrate binding causes a 1.7 Å shift of helix I away from the haem iron, which in turn allows the haem water ligand to shift towards the side chain of Thr268 and interact with it, resulting in a planar hydrogen bond network [43]. The movement also affects interhelical non-bonded contacts between helices F and I [13]. Literature data show that mutation Glu267Gln in P450 BM3 decreases the hydroxylation activity on palmitate and myristic acid, and it alters the substrate binding region, as evidenced by changes in regio-specificity of hydroxylation and the production of water by uncoupling [44]. In M3 this position is substituted by an Arg and this change can be at the basis of the different catalytic properties shown by this mutant towards chrysene and pyrene.

Mutant P2 containes mutations Asp208Arg and Ile209Tyr on helix G, that is known to be involved in substrate binding and that can be responsible for its catalytic properties.

Mutant K4 contains the non-conservative substitution Val317Cys on helix K that is buried in the protein interior. Helix K belongs to the P450 core structure and it is involved in haem binding and protein folding. Val317 is in close proximity (~ 3 Å) to Glu320, which is part of a conserved sequence in helix K (ExxR) that is involved in holding the meander in place via hydrogen bonding to Asn, Arg and His.

4. Conclusion

The data presented in this work demonstrate that a random protein engineering approach resulted in a new activity of P450 BM3 variants toward chrysene and improved activity toward pyrene. The improved mutants can produce different hydroxylated forms of chrysene and 1-hydroxypyrene and can therefore be used as biocatalysts. One way could be their introduction at genic level in microbial hosts in conjunction with other engineered enzymes to achieve microbial degradation of recalcitrant compounds such as PAHs. The other way is to use them to mimic the functionality of the human P450 counterpart for the synthesis of molecules that are suspected to be toxic for human health.

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Tables.

Table 1. Binding and catalytic parameters of P450 BM3 WT and mutants M3, P2 and K4 for the hydroxylation of chrysene in the presence of catalase and superoxide dismutase. The amount of 6-hydroxychrysene formed by the WT was too small to allow a precise quantification (n.d. = not determined).

	k_{cat} (nmol NADPH min^{-1} $nmol^{-1}$ protein)	K_M (μM) for chrysene (measured from NADPH consumption)	k_{cat} / K_M (min^{-1} μM^{-1})	6-hydroxychrysene formed (nM)	NADPH consumed (nM)	Coupling efficiency (%)
WT	0.16 ± 0.01	0.620 ± 0.120	0.26	n.d.	278	n.d.
M3	7.42 ± 0.59	0.040 ± 0.010	185	12.8	84	15.2
P2	2.54 ± 0.51	0.033 ± 0.020	77	14.2	266	5.3
K4	0.52 ± 0.03	0.029 ± 0.005	18	3.2	80	4.0

Table 2. Result from the quantification of 1-hydroxypyrene formed from the turnover of mutants M3, P2 and K4 in the presence of catalase and superoxide dismutase.

	1-hydroxypyrene formed (nM)	NADPH consumed (nM)	Coupling Efficiency (%)
WT	0.2	314	0.07
M3	300	380	78.9
P2	260	380	68.4
K4	120	122	98.4

Figure and schemes legends.

Figure 1. Results of the alkali assay performed with purified P450 BM3 WT and mutants M3, P2 and K4 with chrysene (grey bars) and pyrene (white bars). Lauric acid (black bars) was used as positive control. The signal of the alkali product (360 nm) was normalized for the protein concentration measured from the absorbance of the Soret peak at 418 nm.

Figure 2. GC chromatograms of the oxidation products resulting from incubations of WT (0.54 μM) and mutants M3, P2 and K4 (0.43 μM) with chrysene (1 μM), NADPH (500 μM), catalase (50 U) and superoxide dismutase (50 U). Peak assignments: (I) 4-hydroxychrysene, (II) 6-hydroxychrysene, (III) 3-hydroxychrysene and (IV) 1-hydroxychrysene.

Scheme 1. Structures of chrysene (A) and its hydroxylated products (B) generated by mutants M3, P2 and K4.

Figure 3. GC chromatograms of the oxidation products resulting from incubations of WT (0.54 μM) and mutants M3, P2 and K4 (0.43 μM) with pyrene (20 nM), NADPH (500 μM), catalase (50 U) and superoxide dismutase (50 U). Peak assignments: (I) 1-hydroxypyrene, (II) internal standard (6-hydroxychrysene).

Scheme 2. Structure of pyrene (A) and of the product 1 hydroxypyrene (B), generated by mutants M3, P2 and K4.

Figure 4. A) Crystal structure of the heam domain of P450 BM3 in complex with palmitoleic acid (PDB 1FAG). The α-helices where mutations were found in M3, P2

and K4 are: helix I (green), helix G (light blue), helix K (magenta) and helix J' (brown). The heme is shown in red and palmitoleic acid in yellow. The position of the Gln307 mutated in His in M3 and P2, is also shown. B) View of the protein core showing the positions of the mutations Asp251Gly, His266Ala, Glu267Arg, Thr269Asn, Ser270Glu present on helix I in mutant M3 and Asp208Arg, Ile209Tyr, Asp251Gly in mutant P2, and the only Val317Cys mutation present in K4.