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Altering the regioselectivity of cytochrome P450 BM-3 by saturation mutagenesis for the biosynthesis of indirubin

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ABSTRACT

Engineering the regioselectivity of enzymes to fulfill application needs is an important goal of protein engineering. To create biocatalysts suitable for the biosynthesis of indirubin (a drug for chronic myelogenous leukemia and a novel promising anticancer agent), cytochrome P450 BM-3 was engineered by site-directed saturation mutagenesis at position D168 to alter its hydroxylation regioselectivity towards indole. One mutant, D168W, was created. It primarily produces indirubin (~90%) whereas the parent enzyme primarily forms indigo (~85%). Docking calculations showed that the mutation altered the orientation of indole, and that the C-2 of the indole pyrrole ring was closer to the heme iron of P450 BM-3 than the C-3. The mutation possibly shifted the hydroxylation preference of P450 BM-3 for indole from the C-3 to C-2, which may be responsible for the reversal of distribution of the product yield. This mutant yielded high-purity indirubin and may be a good starting point for the biosynthesis of indirubin.

1. Introduction

Modulating (or even controlling) the catalytic selectivity of enzymes to meet the special requirements of diverse industrial applications is an important task in protein engineering. High chemo-, regio-, and stereoselectivity make enzymes superior to chemical catalysts and attractive for industrial applications [1]. Nevertheless, not all wild-type enzymes have the desired selectivity for specific applications and they often need to be tailored to fulfill these requirements by optimizing process conditions or, in particular, by protein engineering. Protein engineering has recently become an important tool to overcome the limitations of natural enzymes as practical biocatalysts [1,2].

One major area of recent advances has mainly focused on applying directed evolution as well as rational design to improve the regioselectivity of oxygenases [2–4]. Oxygenases are very useful in the chemicals business. They can catalyze the hydroxylation of a wide variety of organic substrates and are inexpensive, environmentally friendly oxidants [5,6]. Although stereoselectivity for the production of enantiomerically pure compounds is important for the fine chemicals and the pharmaceutical industry, regioselectivity is more vital because the main industrial applications of oxygenases make use of their regioselectivity [7]. Therefore, many oxygenases have been engineered to create the desired regioselectivity [2–6].

Among these engineered oxygenases, cytochrome P450 BM-3 (CYP102A1) from *Bacillus megaterium* is considered to be one of the most promising monooxygenases for biotechnological applications [8]. As a natural fusion of a P450 to its P450 reductase redox partner, it possesses efficient electron transfer within itself and an unusually high catalytic efficiency unmatched in the P450 system [9,10]. It is also readily prepared in bulk through heterologous expression as a water-soluble and relatively stable protein [11,12].

The hydroxylation regioselectivity of P450 BM-3 towards various substrates has been engineered [13–20]. The hydroxylation of indole has received great interest because it can produce indigo and indirubin. The latter is an active constituent of a traditional Chinese medicine (TCM) and has been used to treat chronic leukemia in China for decades [21]. Recently, indirubin and its derivatives have become novel promising anticancer compounds because they can inhibit several cyclin-dependent kinases (CDKs) and induce apoptosis of cancer cells [22,23]. Such findings make preparation of indirubin through indole hydroxylation very attractive.

Attempts have been made to prepare indirubin by biotransformation. Indirubin is traditionally extracted from the roots or leaves of *Baphicacanthus cusia*, *Polygonum tinctorium*, *Isatis indigotica*, *Indigofera suffruticosa*, or *Indigofera tinctoria* with chloroform or ethyl acetate [24–26]. Unfortunately, the extraction method con-

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Table 1
Characteristics of some enzymes that produce indirubin.

Enzyme	Color of the product	Indirubin yield (mg/L)	Indirubin ratio (%)	Reference
TOM A113S[a]	Purple	38.0	64	[38]
TOM A113F[a]	Purple	53.2	60	[38]
TOM A113I[a]	Purple	41.9	64	[38]
TOM V106F[a]	Purple	47.8	55	[38]
Oxygenase in pJEC54[b]	Purple	64	65	[42]
Human P450 2E1Human P450 2A6Human P450 2C19	Blue	–[d]	–[d]	[33,40]
P450 BM3 3X[c]	Blue	–[d]	28	[41,43]
P450BM3 3X/D168H	Blue	–[d]	7	[43]
P450 BM3 3X/E435T	Blue	–[d]	15	[43]
P450 BM3 3X/E435T/D168W	Pink	46.5	90	Present study

[a] TOM: toluene *ortho*-monooxygenase.
[b] NDO: naphthalene dioxygenase.
[c] 3X: triple mutant A74G/F87V/L188Q.
[d] No available data in the corresponding articles.

sumes a great amount of organic solvent and time because these materials have a low content of indirubin (less than 0.1%) [24]. Although techniques for the chemical synthesis of indirubin have been developed [27], demands for an environmentally friendly, economical biosynthesis method have increased.

Various biosynthesis routes have been explored. At first, they were dedicated to the *in vitro* production of indirubin in *Polygonum tinctorium* cell cultures [28–31]. Later, with the discovery of oxygenases and bacteria catalyzing indole into indigo and indirubin [32–34], the biosynthesis of indirubin with these biocatalysts became another alternative. Unfortunately, indirubin is usually a minor byproduct in their indole-hydroxylated products, with indigo being the major compound [34–41]. For example, many biocatalysts (including naphthalene-degrading bacteria [34], naphthalene dioxygenase (NDO) [37], toluene *ortho*-monooxygenase (TOM) variants (A113V, V106P, V106Q/A113G) [38], toluene-4-monooxygenase (T4MO) [39], human cytochrome P450 mutants [40], and cytochrome P450 BM-3 mutants [41]) have been reported to react with indole. However, most of them produced much more indigo than indirubin, with the exception of variants of TOM (A113S, A113F, and A113I) [38] and a forest soil metagenome clone [42], which formed indirubin as the major product (Table 1). The postulated reason is that they are apt to hydroxylate indole at the C-3 of indole pyrrole ring [38]. Shifting the hydroxylation regioselectivity of enzymes towards indole may be necessary to selectively produce indirubin with high-purity.

Our previous work [43] on directed evolution of P450 BM-3 aiming to increase indigo yield prompted us to ask further questions about the relationship between the structure of P450 BM-3 and indirubin formation. Noting that substitution at the D168 site (D168H) lowered the percentage of indirubin in the products from 28% to 7% [43], we wonder if this site is related to the regioselectivity of indole hydroxylation? Is it possible to increase the yield of indirubin (or even to make indirubin become the dominant part) by introducing an appropriate mutation at this site?

In the present study, we aimed to alter the hydroxylation regioselectivity of P450 BM-3 towards indole and to create mutants that could preferentially transform indole into indirubin. We carried out saturation mutagenesis at the site D168 of P450 BM-3 to produce such mutants. As expected, one such mutant, D168W, was obtained. It predominantly produced indirubin (90% of products) and resulted in a red product (most of the enzymes mentioned above gave rise to products that were colored blue or purple). Docking analysis suggested that the D168W substitution could alter the model of indole binding and orienting towards the active site of P450 BM-3, and therefore change the profile of regioselectivity and the products. These findings provided not only a novel promising biocatalyst for the preparation of indirubin, but also new information for study of the regioselectivity of P450 BM-3.

2. Materials and methods

2.1. Materials

Pfu DNA polymerase, the UNIQ-10 Column Plasmid Mini-preps Kit and the EZ Spin Column DNAgel Extraction Kit, as well as tryptone powder and yeast extract powder, were from Bio Basic Inc. (Ontario, Canada). *Dpn* I was from Fermentas International Inc. (Ontario, Canada). The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) was from Roche Diagnostics GmbH (Mannheim, Germany). Unless specified, all other chemicals were of analytical grade or higher quality, and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), Amresco (Solon, OH, USA) or Fluka (Buchs, Switzerland). *Escherichia coli* BL21 (DE3) and pET 28a (+) were from Novagen (Madison, WI, USA).

2.2. Saturation mutagenesis of P450 BM-3 at position D168

The saturation mutagenesis library of P450 BM-3 at position D168 was created following the Stratagene Quick Change™ site-directed mutagenesis protocol [44]. The template was pET 28a (+)-P450 BM-3 (A74G/F87V/L188Q/E435T). The degenerate primers used were: forward primer, 5′-CAGCTTTTACCGANNNCAGCCTCATCC-3′; reverse primer, 5′-GGATGAGGCTGNNNTCGGTAAAAGCTG-3′ (the target amino-acid positions coded by NNN are underlined where N = A, G, C, or T).

The reaction was carried out in a total volume of 50 μL containing 1 μL template DNA (about 10 ng), 10 nmol each dNTP, 15 pmol each primer, 2.5 U *Pfu* polymerase and 5 μL 10 × *Pfu* reaction buffer. The reaction mixture was heated at 95 °C for 2 min, followed by 20 cycles of incubation at 95 °C for 30 s, annealing at 53 °C for 2 min, and extension at 72 °C for 16 min, and a final incubation at 72 °C for 2 min on a thermal cycler (Mastercycle personal, Eppendorf). The polymerase chain reaction (PCR) product was digested with 10 U of *Dpn* I at 37 °C for 3 h and transformed into competent *E. coli* BL21 (DE3) cells using the heat-shock method [45]. Transformed cells were plated onto LB agar plates containing 30 μg mL^{-1} kanamycin to form single colonies.

2.3. Screening of mutant libraries

Positive clones were directly selected according to their ability to primarily produce indirubin, which resulted in the development of a red or pink color. Positive clones could be readily detected visually in 96-deep-well plates. First, each clone on the transformation plates was selected and inoculated into 96-deep-well plates, where incubation in 1 mL of LB liquid medium containing 30 μg mL^{-1} kanamycin was carried out at 37 °C. When cells grew

to an optical density of 0.8–1.0 (600 nm), P450 BM-3 expression was induced with 0.5 mmol L^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG). After an additional incubation of 8 h at 30 °C, 10 μL of 50 mmol L^{-1} indole dissolved in dimethylsulfoxide (DMSO) was added to the culture. The subsequent indole hydroxylation was conducted at 30 °C for 12 h with shaking at 150 rpm. The product generated in each well was extracted once with an equal volume of methenyl chloride. Suspicious mutants were selected based on the intensity and hue of their extracts. To confirm the existence of indirubin, the absorbance of the extracts for these suspicious mutants was measured using a spectrophotometer (Ultrospec3300 Pro; Amersham Biosciences, Amersham, UK) over the wavelength range of 400–710 nm.

2.4. Identification of catalysate composition by high-performance liquid chromatography (HPLC)

The selected mutants were cultured and grown as described above, except the culture volume was scaled up to 100 mL. After expression of P450 BM-3, cells were collected with centrifugation (8000 × *g*, 10 min, 4 °C) and diluted in 10 mL of 100 mmol L^{-1} Tris–HCl buffer (pH 8.2). Whole-cell catalysis of indole was carried out at 30 °C for 12 h after the addition of 100 μL of 50 mmol L^{-1} indole dissolved in DMSO. The resulting products were extracted as described in Section 2.3 and analyzed by HPLC using an Agilent 1100 Series Liquid Chromatography system with an Agilent C18 reverse-phase column (ø5 μm, 4.6 mm × 250 mm). A gradient system of CH_3CN (solvent A) and water (solvent B) was used: 0–5 min: 15–40% A; 5–15 min: 40–50% A; 15–25 min: 50–25% A; 25–30 min: 25–15% A. The flow rate was 1 mL min^{-1}. The UV detector was set at 240 nm. The concentration of each compound was determined using calibration curves established with authentic standards under identical HPLC conditions.

2.5. Expression and purification of proteins

The P450 BM-3 parent enzyme and the selected mutant, both with a 6-His tag, were expressed in *E. coli* BL21 (DE3) by the procedure described above. Purification was carried out by affinity chromatography with Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose (Qiagen GmbH, Hilden, Germany) according to the protocol of batch purification under native conditions. Briefly, centrifuged cells were lysed by sonication in lysis buffer (10 mmol L^{-1} imidazole, 50 mmol L^{-1} NaH_2PO_4, pH 8.0, 300 mmol L^{-1} NaCl, and 15% glycerol). After centrifugation (12,000 × *g*, 30 min, 4 °C), the crude enzyme solution was gently mixed with 50% Ni-NTA slurry by shaking at 4 °C for 60 min, and loaded into a column for washing and elution. P450 BM-3 concentrations were determined by the CO-difference spectra method using an extinction coefficient of 91 mM^{-1} cm^{-1} [46].

2.6. Enzyme activity assay

The activity of P450 BM-3 for indirubin formation was assessed by a series of reactions containing 2–200 mmol indole and 0.6 nmol purified enzyme in 100 mmol L^{-1} Tris–HCl buffer (pH 8.2) in a quartz cuvette (final volume, 1 mL). After incubation at 25 °C for 5 min, 0.2 mg of NADPH was added to start the reaction. Indirubin was measured according to its absorbance at 610 nm. In a parallel test, NADPH consumption was monitored at 340 nm and calculated with an extinction coefficient of 6.2 mM^{-1} cm^{-1} [47]. All values were measured in triplicate and kinetic parameters calculated by standard methods.

2.7. Computational modeling analyses

The homology model of the P450 BM-3 mutant was created based on the crystal structure of wild-type P450 BM-3 from PDB (PDB ID: 1BVY) using the program Swiss-PdbViewer 4.0.1 (Swiss Institute of Bioinformatics, Geneva, Switzerland) [48]. The indole data of the mol2 format was obtained from ZINC (ZINC ID: 14516984) [49]. Receptors and ligands were processed by Chimera alpha 1.3 (RBVI, University of California, San Francisco, CA, USA) [50]. Rigid ligand docking was carried out with UCSF Dock 6.1 (UCSF Molecular Design Institute, University of California, San Francisco, CA, USA), the results of which were also analyzed with Chimera alpha 1.3.

3. Results and discussion

3.1. Saturation mutagenesis

Saturation mutagenesis was undertaken at position 168 of the P450 BM-3 (A74G/F87V/L188Q/E435T) mutant because it has better catalytic efficiency to indole than its parent P450 BM-3 (A74G/F87V/L188Q) (sixfold higher activity than its parent) [43].

3.2. Screening of the target mutant

Screening was carried out in liquid micro-cultures. In theory, it would be easy to select the mutants favoring the formation of indirubin on LB agar plates because such mutants will endow their corresponding colonies with a red or pink color. However, the colonies on the LB plates did not present a particular color. This was different from previous reports stating that hydroxylation of endogenous indole gave colonies of various colors [51]. At first, we attributed the absence of color to the lack of indole in the LB plates, but supplementation of various doses of indole into the LB agar plates did not elicit a color. However, in LB liquid cultures, colors gradually appeared after the expression of P450 BM-3, independent of the addition of indole. Considering that the formation of pigments requires oxygen to drive the condensation of hydroxyindole [52], we attributed it to the difference in oxygen concentration between the solid medium and the liquid culture. That is, there was insufficient oxygen in the LB plate but there was a lot of oxygen in the shaking liquid broth. We therefore undertook primary screening with 96-deep-well plates. We screened ~500 colonies to ensure with a probability higher than 99.9% that all 64 possible outcomes of single-site random mutagenesis had been checked [51].

The screening of target mutants was based on the color and composition of the extracts. The extracts of whole-cell transformation products of indole displayed various colors. Those that looked pink or red suggested a dominant proportion of indirubin. In this way, suspicious mutants were selected and subjected to further examination by spectrophotometry. We wanted to find those mutants that their absorbance values at 550 nm (the characteristic absorption peak of indirubin in methenyl chloride) are much higher than their values at 610 nm (the characteristic absorption peak of indigo in methenyl chloride). Surprisingly, we found one mutant which had only one absorption peak at 550 nm and nearly no absorption peak at 610 nm (Fig. 1). The mutant was selected for further validation by analyzing its product composition with HPLC. We found that the corresponding indole hydroxylation product comprised 90% indirubin and 10% indigo, remarkably different to that of the parent enzyme and other enzymes capable of being used to synthesize indirubin (Table 1). The mutant was therefore selected as our target.

By gene sequencing, the mutation to the mutant was corroborated as being D168W and there was no any other base change.

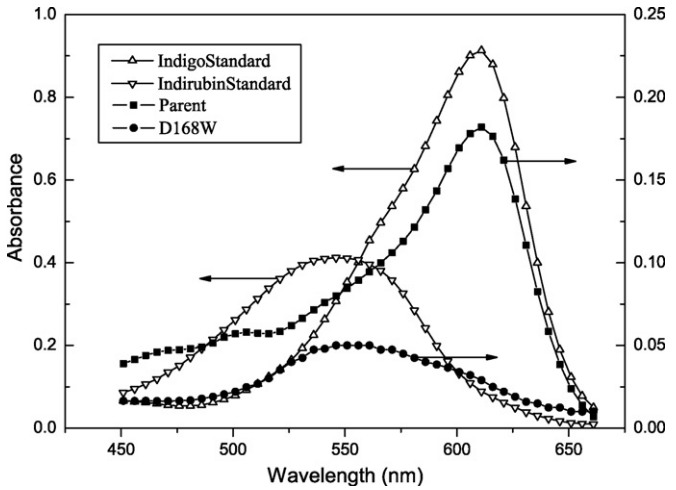


Fig. 1. Absorption spectra of indigo and indirubin, as well as the indole hydroxylation products of the D168W mutant and the parent in methenyl chloride. In methenyl chloride, the absorption peaks of indigo and indirubin are 550 nm and 610 nm, respectively.

3.3. Characterization of the mutant

To ascertain the characteristics of the D168W variant, its kinetic constants were determined with a purified enzyme. Indigo was formed in much lower quantity than indirubin, so its formation was neglected. Indirubin was assayed at 610 nm (the absorption peak of indirubin in Tris–HCl buffer). With average results of three independent experiments (standard deviation <10%), the corresponding Michaelis–Menten parameters were calculated using standard methods (Table 2). We were surprised to find that the catalytic efficiency of D168W was decreased by nearly fivefold compared with that of the parent (P450 BM-3 (A74G/F87V/L188Q/E435T)). This was mainly because its K_m was increased by fivefold compared with that of the parent. The lowered affinity of the mutant towards indole suggests that the mutation D168W may have an adverse effect on the binding and orientation of indole, and influence the catalytic efficiency.

3.4. Effects of the mutation on the binding of indole to P450 BM-3

To discover the structural effects of the substitution of D168W added to the P450 BM-3 quaternary mutant (F87V/L188Q/A74G/E435T), the model of indole binding into the substrate pocket of P450 BM-3 was analyzed by rigid ligand docking with UCSF dock 6.1 [53]. As opposed to the docking model of D168 whereby the C-3 of the indole pyrrole ring is oriented closer to the heme iron than the C-2 (Fig. 2a) [43], the replacement of aspartic acid with tryptophan probably makes the C-2 of indole orient nearer to the heme iron than the C-3 (Fig. 2b).

What is the relationship between the change in orientation of indole binding with the alteration of product composition? It may relate to the mechanism of formation of indigo and indirubin.

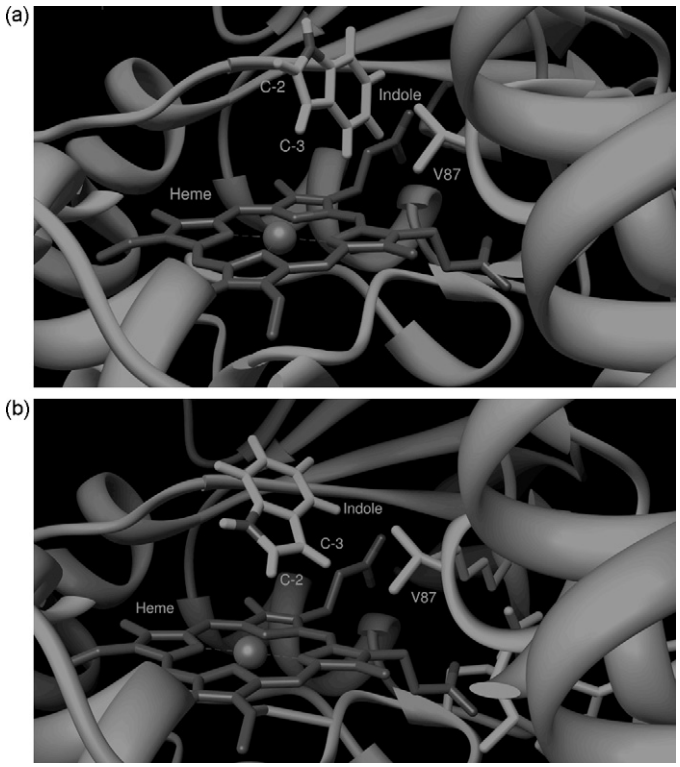


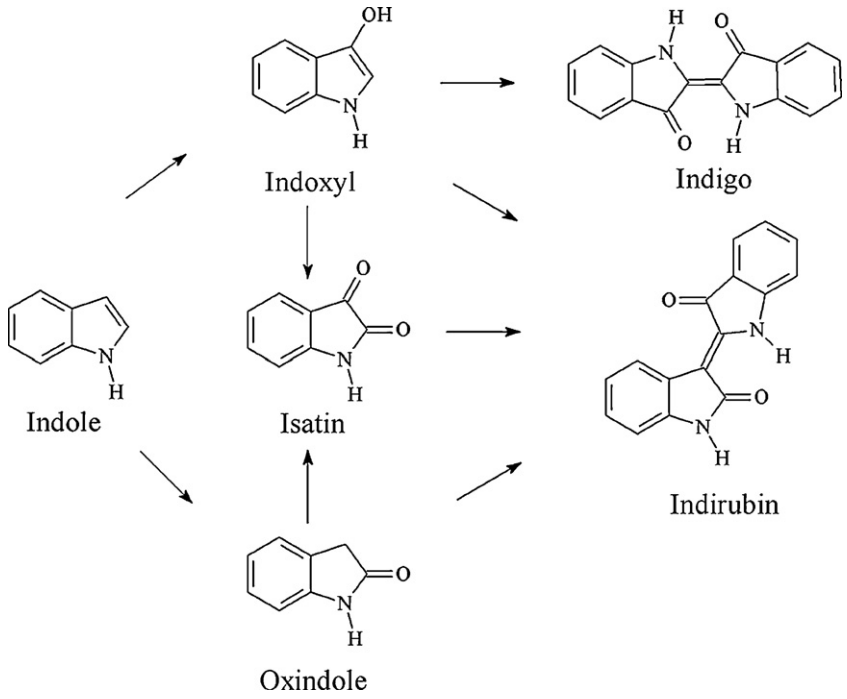
Fig. 2. Docking of indole into the active sites of P450 BM-3 mutants. (a): P450 BM-3 (A74G/F87V/L188Q/E435T) [40]; (b): P450 BM-3 (A74G/F87V/L188Q/E435T/D168W). The mutation of D168W makes the C-2 of indole closer to the heme iron than the C-3. Without detailed structural information of the mutant, this model should be regarded only as a suggestion of the position and orientation of indole in the binding site of the mutant enzyme. V87 is a key active residue.

Indigo and indirubin arise from the oxidation of indole (Scheme 1): indole is oxidized into variant hydroxyindole intermediates, which then dimerize to form pigments. The C-3 carbon of indole in the pyrrole ring is the most reactive site towards oxygenating reagents, so the hydroxylation preferentially takes place at the C-3 position rather than the C-2 position [54]. Consequently, 3-hydroxyindole (indoxyl) is the major product from indole in biological systems whereas the 2-hydroxylated compound (oxindole, the more stable carbonyl tautomer of 2-hydroxyindole) and the 2,3-hydroxylated compound (indole-2,3-dione, or isatin) are minor products [38]. Indoxyl, as an unstable intermediate, is spontaneously oxidized by oxygen to yield indigo. Indirubin, resulting from the combination of indoxyl and isatin (or oxindole), thus becomes a trace byproduct of indigo biosynthesis. For the mutant D168W, with the shorter distance towards the heme iron, the C-2 may be more readily hydroxylated than the C-3 to the extent that much more isatin or oxindole is produced than indoxyl. If so, few indoxyl molecules will be left to be transformed into indigo after its combination with isatin or oxindole.

Table 2
Activity of P450 BM-3 parent and mutant for indole hydroxylation.

P450 BM-3 enzyme	Kinetic parameters[a] (min^{-1} $mmol^{-1}$)			Product composition[b] (%)[2]	
	K_m (mmol)	k_{cat} (s^{-1})	k_{cat}/K_m (mol^{-1} s^{-1})	Indigo	Indirubin
PT[c]	0.8	1.6	2000	85	15
D168W	4.0	1.55	390	10	90

[a] The kinetic parameters of PT were determined based on the formation of indigo whereas those of D168W were based on the formation of indirubin (see Section 2.6).
[b] Determined by HPLC (see Section 2.4).
[c] P450 BM-3 (A74G/F87V/L188Q/E435T).



Scheme 1. Proposed mechanism for the formation of indirubin and indigo.

To test this speculation, we intended to use HPLC to monitor changes in the levels of indoxyl, oxindole, isatin, indigo, and indirubin with time during the indole oxidation catalyzed by the mutant. However, a commercial standard of indoxyl was not available, so only the other four materials were assayed.

Oxindole emerged as the major compound at the early stage of the reaction; the slowdown of oxindole accumulation followed the increase in the amount of indirubin. Isatin was a minor product, the amount of which accumulated slowly with time without an obvious correlation with indirubin. Indigo appeared mainly at the late stage, and was a minor part in the final product. One remarkable finding was that a lot of indole did not undergo transformation (Fig. 3). This information could lead to two conclusions. First, D168W mutant hydroxylation may take place preferentially at the C-2 position, so oxindole is the major product. Indirubin is therefore dominant in the final product because there is insufficient indoxyl to turn into indigo. Second, the mutation lowered the conversion ratio of indole.

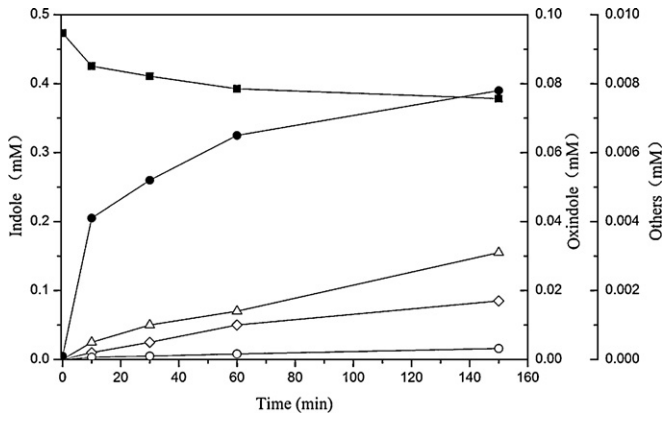


Fig. 3. Time course of the concentrations of various indigoids during the indole hydroxylation catalyzed by D168W. Close square (■), indole (the left axis); close circle (●), oxindole (the middle axis); open triangle (△), isatin; open diamond (◊), indirubin; open circle (○), indigo.

Why did the substitution of only one residue cause such a radical change in the product composition (regioselectivity) and substrate affinity of P450 BM-3 towards indole? Similar examples in which a single-point mutation strongly altered regioselectivity [13,14,55–58,62–64] or the binding properties of substrates [59–61] have been described for many enzymes. For instance, substitution of F87A affected the regioselectivity of P450 BM-3 towards variant substrates. Similarly, a single-point mutation on the A113 of TOM presumably altered the oxidation regiospecificity towards indole [38]. It is worth noting that these residues are usually located in or near active sites or substrate access channels [61–63]. For example, the F87 of P450 BM-3 is in close contact with the heme on the distal side of the binding pocket, so it was considered to be a critical regulatory residue controlling the regioselectivity of substrate oxygenation [60]. It is likely that the A113 of TOM is close to the diiron center [38], that the F366 of tetrachlorobenzene dioxygenase is in the active sites [64], and that the amino-acid positions affecting the regioselectivity of T4MO for indole oxidation are adjacent to the ligands of the diiron center and comprise part of the substrate-binding pocket [39]. They may therefore restrict the access of substrates to the active site or limit reasonable substrate binding [65]. Conversely, D168 is far from the active site and unlikely to directly act on substrates. However, it is located at a loop connecting two α-helices, one of which is a substrate recognition sequence and involved in defining the substrate channel. We speculate that D168W substitution may alter the spatial orientation of the α-helix related to the substrate access channel, and influence the recognition and orientation of indole, just as residues A58 and P59 do on the T104 IpoA enzyme [66]. This is supported by the discovery that the only big difference between the parent and the mutant was substrate affinity, whereas the reaction velocities (k_{cat}) were approximately identical. However, this inference needs to be validated by further structural analysis on the mutant.

Although the substitution of single amino-acids can alter the regioselectivity of certain enzymes, it may not be sufficient to satisfy the application requirements because altering the regioselectivity is usually achieved at the expense of activity and coupling efficiency [8]. In the present study, the D168W mutation decreased

the catalytic efficiency and conversion ratio of indole. Similar phenomena have been reported in the directed evolution of P450 BM-3 (with one exception in [13]). For example, the F87A mutation reduced the reaction rate when it modified regiospecificity and the hydroxylation site [17]; the P25Q mutation greatly decreased the binding affinity of palmitate when the hydroxylation specificity was changed [60]. Nevertheless, the reasons may be different. For the F87A and P25Q mutation, their influence may be mainly attributed to steric hindrance because they are located in the substrate pocket or near active sites, and can interact with the substrate directly. However, for D168W, considering its location far from active sites, the mutation probably caused a conformational change in the substrate access that may be disadvantageous for indole access into the substrate pocket. Confirming this hypothesis by exploring if and how the mutation provokes the conformational rearrangement of the substrate access channel is difficult, because the analysis of protein structure is complex. Nevertheless, we are undertaking mutagenesis on residues near the substrate access entrance to help clarify the mechanism as well as to improve the affinity of indole binding.

4. Conclusion

Overall, a novel P450 BM-3 mutant capable of transforming indole principally into indirubin was created by saturation mutagenesis at position D168, possibly owing to a shift in the regioselectivity of indole hydroxylation caused by an alteration of enzyme conformation. Different from most studies of alteration of the hydroxylation regioselectivity of P450 BM-3 in which the modified residues are usually located in or near the active sites or the substrate access channel, the position of the point mutation is far from the active sites and the substrate access. As a result, the D168W mutation may affect the binding and orientation of indole by inducing conformational rearrangement of substrate access. Identification of the functionally important amino-acid residue expands the understanding of the correlation between non-active site residues and the regiospecificity of P450 BM-3. Further, the high-purity of the indirubin produced with D168W may be a valuable observation, and could be a good starting point for developing potent biocatalysts for the biosynthesis of indirubin in the future.

The D168W mutation decreased the reaction efficiency mainly because of the severe decline in substrate affinity. The catalytic activity needs to be improved and such work is underway by our research team.

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