Directed Evolution of the Fatty-Acid Hydroxylase P450 BM-3 into an Indole-Hydroxylating Catalyst

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Abstract: The self-sufficient cytochrome P450 BM-3 enzyme from *Bacillus megaterium* catalyzes subterminal hydroxylation of saturated long-chain fatty acids and structurally related compounds. Since the primary structure of P450 BM-3 is homologous to that of mammalian P450 type II, it represents an excellent model for this family of enzymes. During studies on the directed evolution of P450 BM-3 into a medium-chain fatty-acid hydroxylase, several mutants, in particular the triple mutant Phe87Val, Leu188Gln, Ala74Gly, were observed to hydroxylate indole, producing indigo and indirubin at a catalytic efficiency of $1365\,\text{M}^{-1}\text{s}^{-1}$ ($k_{cat} = 2.73\ \text{s}^{-1}$ and $K_m = 2.0\,\text{mM}$). Both products were unequivocally characterized by NMR and MS analysis. Wild-type P450 BM-3 is incapable to hydroxylate indole. These results demonstrate that an enzyme can be engineered to catalyze the transformation of substrates with structures widely divergent from those of its native substrate.

Keywords: *bacillus megaterium* · cytochrome P450 · heme proteins · heterocycles · oxygenations

Introduction

Enzymes with novel functions and properties may be obtained by screening natural samples. Alternatively, genetic engineering techniques such as protein engineering[1] or directed evolution[2] of known enzymes may lead to properties, for example solvent stability, which are unlikely to be generated by natural selection. Among the numerous engineered enzymes, however, there are only few examples where enhanced catalytic efficiency of the mutant enzyme for a specific substrate was actually achieved.[3–12]

We describe herein cytochrome P450 BM-3 mutants, engineered by a combination of rational protein design and saturation PCR ("rational evolution"), that are capable of hydroxylating indole, resulting in indigo and indirubin production. P450 BM-3 normally catalyzes subterminal hydroxylation of long-chain saturated acids and amides, as well as of the respective alcohols, or epoxidation of medium- and long-chain unsaturated fatty acids.[13–15] For saturated fatty acids, the optimum chain length is C_{14-16}; fatty acids with a chain length $< C_{12}$ are not hydroxylated at all.[13]

The structure of the P450 BM-3 heme domain has been determined by X-ray[16, 17] as well as by NMR spectroscopy analysis.[18] The substrate binding site extends, as a large, funnel-shaped shaft, from the molecular surface down to the heme and is lined almost exclusively with hydrophobic residues. The two charged moieties on the surface of the heme domain, Arg47 and Tyr51, are assumed to bind the carboxylate group of the substrates via hydrogen bonds.[16] Mutation of Arg47 to Glu renders the enzyme inactive towards arachidonic acid,[15] while increasing its activity towards C_{12-14} alkyltrimethylammonium compounds.[19]

In the course of an investigation on P450 BM-3 mutants, engineered for hydroxylation of shorter-chain fatty acids,[20] a few colonies were observed to produce a water-insoluble blue pigment several hours after induction. This pigment was tentatively identified as indigo, by comparison of its UV/Vis absorption spectrum with that of authentic commercial material. We suspected that it was formed from indole, which was included in the complex growth medium or by degradation of L-tryptophan through the action of tryptophanase[21–23] via indole hydroxylation. However, there is no structural homology between indole and the native substrates of P450 BM-3; more specifically, indole has no functionality by which it could bind to the charged amino acid residues mentioned above. Thus, indigo formation must be correlated with changes in the hydroxylating activity of the mutant enzyme. In an attempt to exploit this unexpected finding, we subjected P450 BM-3 to further evolution, now directed towards more efficient indigo production.

At the time of submission of this manuscript, the formation of indigo by cytochrome P450 2E1, a mammalian monooxygenase, was reported.[24] As native P450 2E1, a microsomal liver enzyme involved in the detoxification of xenobiotic

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compounds, exhibits a wide substrate specificity which includes hydroxylation of aromatic and heterocyclic compounds such as thiobenzamide and iproniazid, the oxidation of indol by this enzyme is less surprising and, in view of its low activity and stability, less promising from the perspective of synthetic organic chemistry.

Results and Discussion

In the course of the directed evolution[1, 2] of P450 BM-3 mutants towards a catalyst for the hydroxylation of shorter-chain fatty acids,[20] some colonies were observed to produce a water-insoluble blue pigment several hours after induction. Since *E. coli* DH5α, the host strain used for the expression of P450 BM-3 wild type, does not form this pigment, its formation had to be correlated with the modified catalytic activity of the mutant enzyme. All mutants of P450 BM-3 producing the blue pigment were sequenced and found to contain mutations at either one of the three sites Phe87, Leu188, and Ala74. We therefore selected these three sites for another round of site-specific randomization mutagenesis, now directed towards higher productivity for the blue pigment.

Increasing blue pigment productivity by mutagenesis of P450 BM-3: Positive effects in this respect can be accumulated either by simultaneous saturation mutagenesis of all three sites, or by successive, site-specific randomization mutagenesis of each site starting, respectively, with the best mutant of the previous step. As the first procedure leads to a relatively large library of mutant enzymes (20^3), we decided to use the latter protocol where the choice of the position for the first mutation is critical: The wrong decision may direct the process along a pathway not leading to an optimal multiple mutant. From the structure of the cytochrome P450 BM-3 heme domain, complexed with palmitoleic acid, it is apparent that Phe87 hinders the substrate from approaching the heme any closer.[16] For the mutant Phe87Val, high regio- and stereoselectivity in arachidonic acid (14*S*,15*R*)-epoxidation has been reported;[15] hydroxylation of saturated fatty acids is shifted from the subterminal chain positions ω-1, ω-2, and ω-3 to the terminal ω carbon.[25] We have therefore selected this site for the first site-specific randomization mutagenesis PCR.

In tube culture, seven colonies produced a minimal amount of blue pigment after induction. The colony where most of the blue pigment was formed was selected for DNA sequencing which revealed substitution once more of Phe87 by Val. This Phe87Val mutant was used as template for the second round of site-specific randomization mutagenesis directed at Leu188. As the structure of the palmitoleic acid/P450 BM-3 heme domain complex shows, the Leu188 site is brought into direct contact with the substrate upon repositioning of the Phe/Gly loop,[16] and therefore probably plays an important role in either binding or orienting the substrate.

After this second screening round, blue pigment was formed in 31 colonies. The mutant producing most of the pigment contained the substitution Leu188Gln besides Phe87Val. This Phe87Leu188 mutant was finally mutated at the position Ala74 in a third round of site-specific randomization mutagenesis PCR. One of the triple mutants thus obtained, Phe87Leu188Ala74 (Phe87Val, Leu188Gln, Ala74Gly), produced several milligrams of blue pigment in a two liter flask with 300 mL tryptone broth (TB) medium, sufficient for isolation and purification of the pigment(s) for NMR and other analyses (see Table 1).

Table 1. Kinetic parameters of P450 BM-3 mutants for indole hydroxylation.

Mutants	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
WT	–[a]	–[a]	–[a]
Leu188Gln	n.d.[b]	n.d.[b]	n.d.[b]
Phe87Val	2.03 (0.14)	17.0 (1.0)	119
Phe87Leu188	2.28 (0.16)	4.2 (0.4)	543
Phe87Leu188Ala74	2.73 (0.16)	2.0 (0.2)	1365

[a] No activity was detected. [b] No value could be determined since activity was too low to be quantified.

Isolation and characterization of the blue and red pigment: The blue pellet remaining after washing out the cells was extracted with THF. Upon TLC analysis, the blue pigment separated into a rapidly moving blue and a slower red component; both showed exactly the same mobility pattern as the components of a commercial indigo sample.

After painstaking separation and purification of the two pigments, absorption spectra were recorded for both in DMSO. The spectrum of the blue component proved identical with that of a commercial indigo sample. No authentic indirubin sample was available for comparing the absorption spectrum with the red pigment. The purified blue and red components were therefore analyzed by mass spectrometry. The mass spectra of both pigments (EI, 70 eV) are charac-

Abstract in German: *Cytochrom P450 BM-3, ein natürliches Fusionsprotein aus Bacillus megaterium, katalysiert die subterminale Hydroxylierung von gesättigten langkettigen Fettsäuren und strukturverwandten Verbindungen. Da seine Primärstruktur eine hohe Homologie zu P450-Monooxygenasen vom Typ II aus Säugetieren aufweist, ist das Bakterien-Enzym ein interessantes Modell für diese Enzymklasse. Während unserer Untersuchungen zur gerichteten Evolution von P450 BM-3 zu einer Hydroxylase, die mittelkettige Fettsäuren zu hydroxylieren vermag, beobachteten wir einige Mutanten, die auch Indol hydroxylieren und damit die Bildung von Indigo und Indirubin auslösen, wie wir durch NMR- und MS-Analyse der Produkte eindeutig nachweisen konnten. Die katalytische Wirksamkeit der Hydroxyindol-Bildung einer optimierten Dreifachmutante (Phe87Val, Leu188Gln, Ala74Gly) beträgt 1365 $M^{-1}s^{-1}$ ($k_{cat} = 2.73\ s^{-1}$ und $K_m = 2.0$ mM). Das Ausgangsenzym P450 BM-3 ist zu dieser Reaktion nicht in der Lage. Unsere Ergebnisse zeigen, dass P450-Monooxygenasen durch gerichtete Evolution in einem Mass modifiziert werden können, dass sie auch Substrate umzusetzen vermögen, deren Struktur vom natürlichen Substrat sehr verschieden ist.*

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Table 2. MS data for the red pigment (indirubin; (z)-1*H*,1′*H*-2,3′-biindolylidene-3,2′-dione)[a] and the blue pigment (indigo; 1*H*,1′*H*-2,2′-biindolylidene-3,3′-dione)[b].

Ion	Elemental composition	*m*/*z* [Dalton] (rel. intensity)		
		red pigment (indirubin)		blue pigment (indigo)
		calcd	exptl[c]	exptl
$[M]^{+\cdot}$	$C_{16}H_{10}N_2O_2$	262.0742	262.0746 (100)	262 (100)
$[M-CO]^{+\cdot}$	$C_{15}H_{10}N_2O$	234.0793	234.0798 (40)	234 (15)
$[M-CO/OCH]^{+\cdot}$	$C_{14}H_9N_2$	205.0766	205.0767 (20)	205 (15)

[a] Source temperature 390 K, probe 490 K. [b] Source temperature 435 K, probe 520 K. [c] Exact mass determined by peak matching against PFK as reference.

terized by a prominent molecular ion peak at *m*/*z* 262, with only two significant fragment peaks at *m*/*z* 234 and 205; this pattern is typical of indigoid compounds. For the red pigment, the elemental composition of these ions was established by high resolution mass spectrometry as $C_{16}H_{10}N_2O_2$, $C_{15}H_{10}N_2O$, and $C_{14}H_9N_2$, respectively, which again is characteristic of indigo-type structures (see Table 2).

The NMR spectra of both the blue and red pigment in [D$_6$]DMSO (at 500.13 MHz) unequivocally confirm the respective structures, that is indigo and indirubin. The ^{1}H-NMR spectrum of the red pigment displays two four-spin systems in the aromatic proton region ($\delta = 7-9$), and two uncoupled signals at $\delta = 10.9$ and 11.0 (1-/1′-NH; see Table 3). Both aryl systems are amenable to first-order analysis and represent the four mutually adjacent protons of a 1,2-disubstituted benzene ring each. The only serious complication is that two triplets of the two independent subsystems appear virtually superimposed, with a chemical shift difference of 0.002 ppm, that is just 1 Hz even at 500.13 MHz (5-H and 5′-H, cf. Table 3). The chemical shift of $\delta = 8.77$ can be assigned straightforwardly, by virtue of its >1 ppm downfield shift, to 4′-H in the 3-indolylidene-2-one moiety. In the (*Z*) configuration of indirubin, this proton dips in deep into the C=O anisotropic deshielding cone of the carbonyl group in 3-position of the 2-indolylidene half of the molecule.[26] Starting with this definite assignment, the relative position of the four protons in each set is established unequivocally on the basis of the coupling pattern (P. Fischer et al., forthcoming detailed NMR paper). The ^{13}C-NMR resonances of indirubin which so far have only been listed numerically in the literature,[27] can now be assigned completely from a ^{1}H,^{13}C-COSY spectrum (see Table 3).

Table 3. NMR chemical shift data[a] (11.74 T) for indirubin and indigo (0.05 M and <0.005 M, respectively, in [D$_6$]DMSO, 298 K).

Position	Indirubin		Indigo
	δ (^{13}C)	δ (^{1}H)	δ (^{1}H)
N-1	–	11.02_5	10.50_3
C-2	140.81	–	–
C-3	188.60	–	–
C-3a	121.38	–	–
C-4	124.30	7.66_0	7.61_5
C-5	121.20	7.02_5	6.95_1
C-6	137.06	7.58_2	7.51_6
C-7	113.38	7.42_4	7.33_5
C-7a	152.45	–	–
N-1′	–	10.89_8	
C-2′	170.85	–	
C-3′	106.46	-	
C-3a′	118.94	-	
C-4′	124.60	8.77_0	
C-5′	121.20	7.02_7	
C-6′	129.22	7.26_0	
C-7′	109.51	6.90_8	
C-7a′	138.27	–	

[a] Nominal frequency 500.13 MHz for ^{1}H, 125.76 MHz for ^{13}C; digital resolution 0.04 Hz per pt for ^{1}H, 0.6 Hz for ^{13}C.

The blue pigment is scarcely soluble even in DMSO. Nevertheless, we have obtained a ^{1}H-NMR spectrum which in this case shows just one four-spin aryl proton set. This is identified, in a straightforward manner, with the aryl proton set of indigo since all chemical shifts and coupling constants are virtually identical with those of the *1H*-2-indolylidene-3-one subsystem of indirubin (see Table 3). The N-H resonance is located at $\delta = 10.5$.

Production of indigo with isolated enzymes: It is well-established that indigo may be prepared from indole[28] or indole-2-carboxylate[29] by microbial transformation (see Figure 1). We therefore tested the catalytic activity for indole hydroxylation first with the pure enzyme. When the purified mutant enzyme Phe87Leu188Ala74 and indole alone were mixed, no color formation was observed. Only after NADPH was added, blue pigment was formed. When the pH of the reaction mixture after 20 min was adjusted to 11, the reaction mixture turned deep blue within a few seconds. Control experiments with native P450 BM-3 were always negative, even at unnaturally high concentrations of enzyme, indole, and NADPH.

All pigment, formed in these transformations, was extracted with ethyl acetate. In TLC analysis, it separated into a rapidly moving blue and a slower red component, with R_f-values and absorption spectra identical with those obtained for fermentation broth extracts. Wild-type P450 BM-3 thus has successfully been processed, in the form of its Phe87-Leu188Ala74 mutant, into an indole hydroxylase. Two pathways have been reported for the enzymatic transformation of indole to indigo, one catalyzed by a dioxygenase, the other by a styrene monooxygenase.[28,29] All transformations reported here showed a 2:1 stoichiometry NADPH to indigo. The assumption seems justified, therefore, that the Phe87-Leu188Ala74 mutant acts as a monooxygenase which hydroxylates the substrate indole at just one position, thereby producing either oxindole or indoxal.

Kinetic parameters for indole hydroxylation: These were determined for pure samples of wild-type P450 BM-3 and for the four mutant enzymes Leu188Gln, Phe87Val, Phe87-Leu188, and Phe87Leu188Ala74 (Table 1). Even with an excess of purified enzyme and at high indole concentrations,

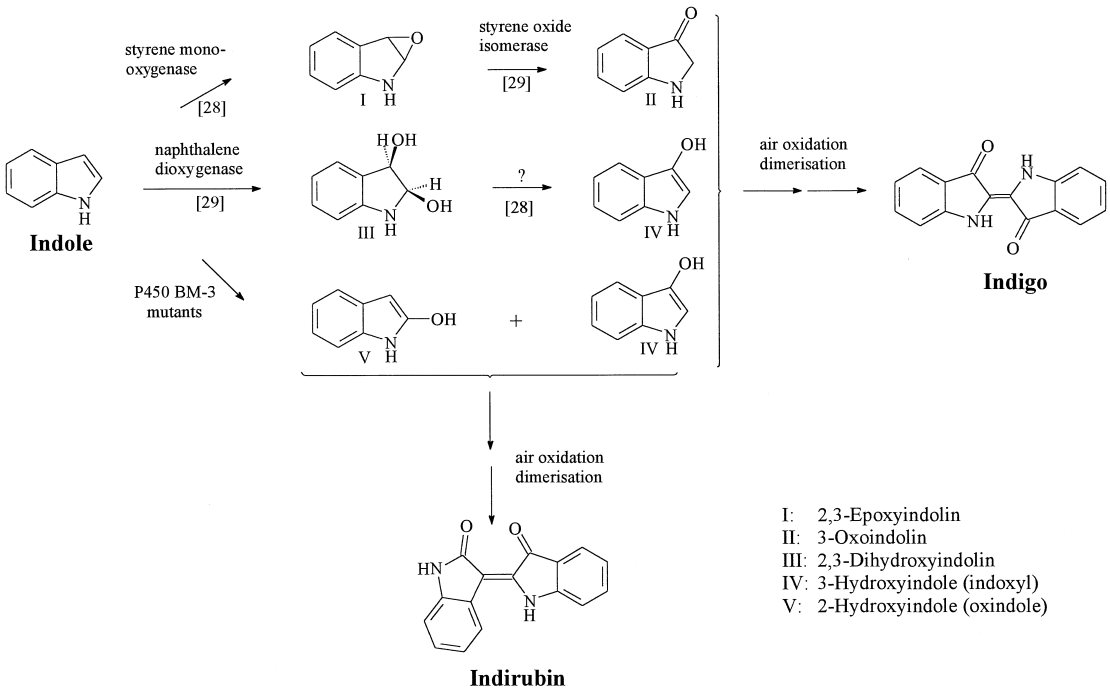

Figure 1. Conversion of indole to indigo by a styrene monooxygenase,[28] a naphthalene dioxygenase,[29] and the three P450 BM-3 mutants Phe87Val, PheF87Leu188 and Phe87Leu188Ala74.

the wild-type enzyme proved unable to oxidize indole. The activity of the single mutant Leu188Gln was still very low. For the other single mutant, Phe87Val, the catalytic efficiency for indole hydroxylation was already $119\,\text{M}^{-1}\text{s}^{-1}$, increasing fivefold to $543\,\text{M}^{-1}\text{s}^{-1}$ for the double mutant Phe87Leu188. After the final substitution Ala74Gly, the triple mutant Phe87Leu188Ala74 showed an efficiency value of $1365\,\text{M}^{-1}\text{s}^{-1}$. The overall increase in k_{cat} from Phe87Val to Phe87Leu188Ala74 is approximately 35 % while the K_m value decreases roughly sevenfold. These results suggest that the two sites, Ala74Gly and Leu188Gln, are involved primarily in substrate binding.

The catalytic efficiency of the mutant Phe87Leu188Ala74 for indole hydroxylation is rather low compared with that of the wild-type for subterminal hydroxylation of long-chain saturated fatty acids. Its K_m value for indole, on the other hand, is more than 10^3 times that for the wild-type enzyme. Also, with $k_{cat} = 2.73\ \text{s}^{-1}$, the turnover rate of Phe87Leu188Ala74 for indole is more than ten times as high as that of many P450s.

Protein engineering: As outlined in the introduction, the substrate binding site in the P450 BM-3 heme domain is a large, funnel-shaped shaft, extending from the molecular surface down to the heme, and lined almost exclusively with hydrophobic residues.[16–18] The only charged residues on the heme domain surface (Arg47 and Tyr51) are assumed to bind the carboxylate function of the respective substrates via hydrogen-bond formation. Indole lacks both a carboxylate or similar group for Coulomb interaction, and a long alkyl chain for binding by dispersive effects. The kinetic parameters for the respective mutants show that the substitutions Leu188Gln and Ala74Gly largely reduce the K_m value of Phe87Leu188Ala74 for indole while slightly increasing k_{cat}. Both amino acids influence the binding site of the fatty acids; it seems reasonable, therefore, to suppose indole and fatty acids to bind to the enzyme in a similar manner.

Despite these arguments, it is not clear how substitution of merely three amino acids in P450 BM-3 can enable the mutant enzyme to effectively catalyze hydroxylation of a chemical structure so different from that of its native substrates. Future investigations thus will have to concentrate on the way in which this enzyme actually recognizes indole as a substrate. Since the primary structure of P450 BM-3 is homologous to that of mammalian P450s,[30,31] observations of substrate recognition by P450 BM-3 mutants will be valuable for understanding the chemistry and mechanism of the P450 family in general. Indigo formation may be used to monitor changes in the hydroxylating potential since the deep blue color of this pigment becomes immediately apparent even at low concentrations. Otherwise, this new feature would probably have been overlooked in our investigation aimed at a quite different target, namely the hydroxylation of shorter-chain fatty acids.

The hypothesis is tempting that P450 BM-3 may be engineered to hydroxylate a wide spectrum of substrates. Since this enzyme is far more stable than most other P450s, and has a 100 – 1000 times higher turnover rate,[31] it seems ideally suited for creating new substrate specificities by mutagenesis. Also, P450 BM-3 may be produced in sufficiently large quantities, using a recombinant *E. coli* expression system.[24]

Work is now in progress to transfer our test-tube experiments to a bioreactor scale in which an inexpensive, inorganic substitute to the expensive NADPH cofactor system[32] is being used.

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Experimental Section

Randomization of specific codons of P450 BM-3:[33] Three sites (Phe87, Leu188, Ala74) were randomized by site-directed mutagenesis, using the Stratagene QuikChange kit (La Jolla, CA, USA). The following PCR primers were employed for the respective sites: for Phe87, 5′-gcaggagacgggttgnnnacaagctggacg-3′ and 5′-cgt-ccagcttgtnnncaacccgtctcctgc-3′; for Leu188, 5′-gaagcaatgaacaagnnncagcgagcaaa-tccag-3′ and 5′-ctggatttgctcgctgnnnncttgttcattgcttc-3′; for Ala74, 5′-gctttgataaaaaa-cttaagtcaannnctaaatttgtacg-3′ and 5′-cgtacaaatttaagnnnttgacttaagtttttatcaaagc-3′. Identical polymerase chain reaction (PCR) conditions were employed for all three sites. The 50 μL reaction volume contained 17.5 pmol of each primer, 20 pmol of the template plasmid DNA, 3 U of *Pfu* polymerase, and 3.25 nmol of each dNTP. PCR reaction was started at 94 °C for 1 min and run through 20 thermocycles (94 °C for 1 min, 46 °C for 2.5 min, 72 °C for 17 min). After completion of the 20 cycles, the reaction medium was held at 72 °C for 15 min. The template DNA was then digested with 20 U *Dpn*I for 3 h at 37 °C before transformation into *E. coli* DH5α. The transformed *E. coli* DH5α cells were plated on LB agar plates, containing 100 $\mu g\,mL^{-1}$ ampicillin, and incubated for 18 h at 37 °C.

Expression and purification of native and mutant P450 BM-3; **production of the blue pigment**: The P450 BM-3 gene and its mutants were expressed, as described previously,[34] under the control of the strong temperature-inducible P_RP_L-promoter of *pCYTEXP1* in *E. coli* strain DH5α. Colonies were picked with sterile toothpicks and transferred to 96-well plates, containing 200 μL TB medium and 100 $\mu g\,mL^{-1}$ ampicillin per well, and incubated overnight at 37 °C. Aliquots of the cell culture from each well (40 μL) were transferred to test tubes, containing 2 mL of TB medium supplemented with 100 $\mu g\,mL^{-1}$ ampicillin, and cultured first for 2 h at 37 °C, then for 6 h at 42 °C. For production of the blue pigment, the tubes were incubated overnight at 37 °C.

Large-scale preparations of enzymes, as well as of the blue pigment, were carried out accordingly, starting with 300 mL cell culture ($OD_{578\,nm} = 0.8$–1.0). For enzyme preparations, the cells were centrifugated at 4000 rpm for 10 min, resuspended in 0.1 M potassium phosphate buffer (pH 7.4), and broken up under ice-cooling with a Branson Sonifier W25 (Dietzenbach, Germany; output level 80 W, three 2 min duty cycles of 20% each). After centrifugation of the resulting suspension (20 min, 32570 g), the crude extracts were used directly for the activity assay or for enzyme purification; this was carried out as described previously.[35] Concentration of the purified enzyme was calculated from the difference in absorption between the carbonyl complex of the ferrous form (at 450 nm) and the ferrous form (at 490 nm), with an extinction coefficient $\varepsilon = 91\,mM^{-1}\,cm^{-1}$.[13]

Isolation of mutants producing substantial amounts of blue pigment: From the mutants of each site, created by randomization mutagenesis of the codon for the respective site, 100 colonies were isolated and cultured in test tubes for production of blue pigment. The cells were washed with water and subjected to several slow centrifugation steps (500 rpm). The blue pigment was extracted with dimethylsulfoxide (DMSO), and absorption was determined at 670 nm. The mutant producing the largest amount of blue pigment (on the basis of the 670 nm extinction) among all mutants from the same site was chosen for DNA sequencing (ABI DNA sequencing kit, ABI Prism™ 377 DNA sequencer), and then employed as template for further site-specific randomization mutagenesis. All mutations described here have been confirmed by DNA sequencing.

Activity assay for indole hydroxylation: This assay was carried out in preparations containing 10–500 mM indole solution in DMSO (8 μL), 0.1 M Tris/HCl buffer (pH 8.2; 850 μL), and wild-type or mutated P450 BM-3 (0.6 nmol) in a final volume of 1 mL. The mixtures were preincubated for 9 min, and transformation started by adding 1 mM aqueous NADPH solution (50 μL). After 20 min, the enzyme reaction was stopped by addition of 1.2 M KOH (60 μL). Within 5 to 30 s, the enzymatic hydroxylation products had been completely converted into indigo and indirubin. Indigo formation was determined from the absorption at 670 nm (with an extinction coefficient $= 3.9\,mM^{-1}\,cm^{-1}$, obtained from a calibration for pure indigo). With both wild-type or mutated P450 BM-3, indigo production is linear with time within 40 s in an indole concentration range of 0.05–5.0 mM. Formation of indirubin, which has only a weak absorption at 670 nm and is produced in much less amount than indigo, was neglected in determining the kinetic parameters. NADPH consumption was monitored at 340 nm, and calculated with an extinction coefficient for this wavelength of $\varepsilon = 6.2\,mM^{-1}\,cm^{-1}$.[19]

Isolation and purification of indigo and indirubin: Cells were washed with water and subjected to repeated slow centrifugation (500 g). The residual blue pellet was extracted with tetrahydrofuran (THF, 200 mL). The extract was evaporated to near dryness, and the red pigment extracted from the residue with absolute ethanol (4–5 times, 50 mL each). The remaining blue solid was dissolved in THF; thin-layer chromatographic (TLC) analysis showed only one spot ($R_f = 0.65$; THF/petrol ether 1:2 *v*/*v*). The ethanol solution was concentrated by evaporation, the residue applied to a silica gel column (DC 60, Merck, Darmstadt, Germany; 2 × 30 cm), and eluted with THF/petrol ether 1:2. The red fractions were collected, evaporated to dryness, and the purity of the residue tested by TLC ($R_f = 0.39$; THF/petrol ether 1:2 *v*/*v*).

Analytical methods: Absorption spectra (400–800 nm) of the blue and red pigments were recorded with an Ultraspec 3000 spectrophotometer (Pharmacia, Uppsala, Sweden). NMR spectra were obtained from [D_6]DMSO solutions on a Bruker (Karlsruhe-Rheinstetten, Germany) ARX spectrometer (11.74 T; nominal frequency 500.13 MHz for ^1H, 125.77 MHz for ^{13}C). Both low and high resolution mass spectra were run on a Finnigan MAT (Bremen, Germany) MAT 95 spectrometer.

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[1] Z. Shao, F. H. Arnold, *Curr. Opin. Struct. Biol.* **1996**, *6*, 513–518.
[2] J. C. Moore, F. H. Arnold, *Nature Biotechnol.* **1996**, *14*, 458–467; F. H. Arnold, A. A. Volkov, *Curr. Opin. Chem. Biol.* **1999**, *3*, 54–59.
[3] R. Chen, A. Greer, A. M. Dean, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12171–12176.
[4] C. N. Cronin, *J. Biol. Chem.* **1998**, *273*, 24465–24469.
[5] L. Hedstrom, L. Szilagyi, W. J. Rutter, *Science* **1992**, *255*, 1249–1253.
[6] A. F. A. Marsden, B. Wilkinson, J. Cortes, N. J. Dunster, J. Staunton, P. F. Leadlay, *Science* **1998**, *279*, 199–202.
[7] E. Quemeneur, M. Moutiez, J. B. Charbonnier, A. Menez, *Nature* **1998**, *391*, 301–303.
[8] C. L. Tucker, J. H. Hurley, T. R. Miller, J. B. Hurley, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5993–5997.
[9] L. Wan, M. B. Twitchett, L. D. Eltis, A. G. Mauk, M. Smith, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 12825–12831.
[10] H. M. Wilks, K. W. Hart, R. Feeney, C. R. Dunn, H. Muirhead, W. N. Chia, D. A. Barstow, T. Atkinson, A. R. Clarke, J. J. Holbrook, *Science* **1988**, *242*, 1541–1544.
[11] T. Yano, S. Oue, H. Kagamiyama, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5511–5515; S. Oue, A. Okamoto, T. Yano, H. Kagamiyama, *J. Biol. Chem.* **1999**, *274*, 2344–2349.
[12] J.-H. Zhang, G. Dawes, W. P. C. Stemmer, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4504–4509.
[13] S. S. Boddupalli, R. W. Estabrook, J. A. Peterson, *J. Biol. Chem.* **1990**, *265*, 4233–4239.
[14] J. H. Capdevila, S. Wie, C. Helvig, J. R. Falck, Y. Belosludtsev, G. Truan, S. E. Graham-Lorence, J. A. Peterson, *J. Biol. Chem.* **1996**, *271*, 22663–22671.
[15] S. Graham-Lorence, G. Truan, J. A. Peterson, J. R. Flack, S. Wei, C. Helvig, J. H. Capdevilla, *J. Biol. Chem.* **1997**, *272*, 1127–1135.
[16] H. Li, T. L. Poulos, *Nat. Struct. Biol.* **1997**, *4*, 140–146.
[17] K. G. Ravichandran, S. Sekhar, S. Boddupalli, C. A. Hasemann, J. A. Peterson, J. Deisenhofer, *Science* **1993**, *261*, 731–736.
[18] S. Modi, M. J. Sutcliffe, W. U. Primrose, L. Y. Lian, G. C. K. Roberts, *Nat. Struct. Biol.* **1996**, *3*, 414–417.
[19] C. F. Oliver, S. Modi, W. U. Primrose, L. Y. Lian, G. C. K. Roberts, *Biochem. J.* **1997**, *327*, 537–544.

  0947-6539/00/0609-1535 $ 17.50+.50/0

[20] Q.-S. Li, U. Schwaneberg, M. Fischer, J. Schmitt, J. Pleiss, R. D. Schmid, unpublished results.
[21] C. C. Hyde, E. W. Miles, *Biotechnol.* **1990**, *8*, 27 – 32.
[22] D. Murdock, B. D. Ensley, C. Serdar, M. Thalen, *Bio/Technol.* **1993**, *11*, 381 – 385.
[23] S. Hart, K. R. Koch, D. R. Woods, *J. Gen. Microbiol.* **1992**, *138*, 211 – 216.
[24] E. M. J. Gillam, A. M. A. Aguinaldo, L. M. Notley, G. Kim, R. G. Mundkowski, A. A. Volkov, F. H. Arnold, P. Soucek, J. J. DeVoss, F. P. Guengerich, *Biochem. Biophys. Res. Commun.* **1999**, *265*, 469 – 472.
[25] C. F. Oliver, S. Modi, M. J. Sutcliffe, W. U. Primrose, L. Y. Lian, G. C. K. Roberts, *Biochemistry* **1997**, *36*, 1567 – 1572.
[26] H. Laatsch, H. Ludwig-Köhn, *Liebigs Ann. Chem.* **1986**, 1847 – 1853.
[27] A. R. Katritzky, W.-Q. Fan, A. E. Koziol, G. J. Palenik, *J. Heterocycl. Chem.* **1989**, *26*, 821 – 828.
[28] K. E. O'Connor, A. W. Dobson, S. Hartmans, *Appl. Environ. Microbiol.* **1997**, *63*, 4287 – 4291.
[29] R. W. Eaton, P. J. Chapman, *J. Bacteriol.* **1995**, *177*, 6983 – 6988.
[30] T. D. Porter, M. J. Coon, *J. Biol. Chem.* **1991**, *266*, 13 469 – 13 472.
[31] F. G. Guengerich, *J. Biol. Chem.* **1991**, *266*, 10 019 – 10 022.
[32] U. Schwaneberg, D. Appel, J. Schmitt, R. D. Schmid, *Biochem. Biophys. Res. Commun.*, submitted.
[33] J. R. Cherry, M. H. Lamsa, P. Schneider, J. Vind, A. Svendsen, A. Jones, A. H. Pedersen, *Nat. Biotechnol.* **1999**, *17*, 379 – 384.
[34] U. Schwaneberg, C. Schmidt-Dannert, J. Schmitt, R. D. Schmid, *Anal. Biochem.* **1999**, *269*, 359 – 366.
[35] U. Schwaneberg, A. Sprauer, C. Schmidt-Dannert, R. D. Schmid, *J. Chromatogr. A* **1999**, *848*, 149 – 159.

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  0947-6539/00/0609-1536 $ 17.50+.50/0