Laboratory evolution of P450 BM3 for mediated electron transfer yielding an activity-improved and reductase-independent variant

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One of the main obstacles in employing P450 monooxygenases for preparative chemical syntheses in cell-free systems is their requirement for cofactors such as NAD(P)H. In order to engineer P450 BM3 from Bacillus megaterium for cost-effective process conditions in vitro, a validated medium throughput screening system based on cheap Zn dust as an electron source and Cobalt(III) sepulchrate (Co(III)sep) as a mediator was reported. In the current study, the alternative cofactor system Zn/Co(III)sep was used in a directed evolution experiment to improve the Co(III)sep-mediated electron transfer to P450 BM3. A variant, carrying five mutations (R47F F87A V281G M354S D363H), Table I), P450 BM3 M5 was identified and characterized with respect to its kinetic parameters. P450 BM3 M5 achieved for mediated electron transfer a 2-fold higher \( k_{\text{cat}} \) value and a 3-fold higher catalytic efficiency compared with the starting point mutant P450 BM3 F87A (\( k_{\text{cat}}/K_m \) of 28 min\(^{-1} \), \( k_{\text{cat}}/K_m \) of 19 μM\(^{-1}\) min\(^{-1}\)). For obtaining first insights on electron transfer contributions, three reductase-deficient variants were generated. The reductase-deficient mutant of P450 BMP M5 exhibited a catalytic efficiency of 69% and a \( k_{\text{cat}} \) value of 89% of the values obtained for P450 BM3 M5.

**Keywords:** cofactor/directed evolution/high-throughput screening/mediator/monooxygenase

Introduction

The ability of the heme containing ‘superfamily’ of P450 monooxygenases to oxygenate unactivated C–H bonds is one of the most attractive reactions from a catalysis point of view. The need of P450 monooxygenases for expensive cofactors, such as NADPH or NADH, limits their applications in cell-free organic syntheses. The use of whole cells for cofactor regeneration is efficient (Wolberg et al., 2000; Appel et al., 2001; Stamper et al., 2002), but restricted in terms of solvent choices and to substrates able to translocate through cell membranes without affecting membrane integrity. In addition, cofactor and substrate concentrations have to be balanced to achieve high catalytic efficiencies, because many P450s are inhibited in the presence of cofactors and absence of substrate (Murataliev et al., 1997).

Approaches to provide reduction equivalents to P450s comprise reports regenerating NAD(P)H (Adlercreutz, 1996; Wolberg et al., 2000; Appel et al., 2001; Drazu et al., 2002; Hollmann et al., 2002; Stamper et al., 2002; van der Donk and Zhao, 2003), the use of peroxides as alternative electron sources (Joo et al., 1999a, 1999b; Cirino and Arnold, 2003), mediators for shuttling electrons (Estabrook et al., 1996; Reipa et al., 1997, 2002; Mayhew et al., 2000; Shumyantseva et al., 2000; Gilardi et al., 2002) and direct electron transfer on electrodes (Lvov et al., 1998; Munge et al., 2003; Bistolas et al., 2004; Fantuzzi et al., 2004; Shumyantseva et al., 2004, 2007; Shukla et al., 2005; Matsumura et al., 2006; Udité et al., 2006). NAD(P)H regeneration has been achieved electrochemically (Hollmann et al., 2002), enzymatically (Adlercreutz, 1996; Drazu et al., 2002; van der Donk and Zhao, 2003) and with in vivo recycling systems (Wolberg et al., 2000; Appel et al., 2001; Stamper et al., 2002). The use of peroxides as alternative electron sources (Joo et al., 1999a, 1999b; Cirino and Arnold, 2003) is scientifically interesting, but suffered from low tolerance of monooxygenases toward peroxides, which resulted in rapid inactivation (Cirino and Arnold, 2003).

Previously, we reported an alternative cofactor system based on cheap zinc dust as an electron source and Cobalt(III) sepulchrate (Co(III)sep) as a mediator (Nazor and Schwaneberg, 2006). The alternative cofactors systems have been shown successful in driving the monooxygenase P450 BM3 from Bacillus megaterium (Estabrook et al., 1996; Schwaneberg et al., 2000) and a Zn/Co(III)sep-driven screening system based on the Schwaneberg pNCA assay (Schwaneberg et al., 1999a) was developed and downscaled to the 96-well microtiter plate format (Nazor and Schwaneberg, 2006).

P450 BM3 has, from an application point of view, several beneficial properties compared with other P450s: (i) reductase and heme domains are on a single polypeptide chain (Narhi and Fulco, 1987; Ruettinger et al., 1989), (ii) \( k_{\text{cat}} \) values are often 10–1000 times higher compared with other fatty acid hydroxylases (Zimmer et al., 1995), (iii) production and purification in gram scale has been achieved (Schwaneberg et al., 1999b) and (iv) validated high-throughput screening systems to improve properties exist, for instance, to broaden substrate spectra (Schwaneberg et al., 2001) and to improve organic solvent resistance (Wong et al., 2004).

Here, we report a first directed evolution experiment of a monooxygenase (P450 BM3) for mediated electron transfer (Co(III)sep) in which a whole P450 BM3 gene was randomized using error-prone polymerase chain reaction (epPCR). Individual contributions of P450 BM3’s reductase to the Co(III)sep-mediated electron transfer rates were further quantified by generating three reductase-deficient variants (P450 BMP; Table I) in which a stop codon was introduced after the heme domain by site-directed mutagenesis.
Given are the chosen names for the mutants used in the manuscript, positions of amino acid substitutions, mutations in the corresponding codons, and the method used for diversity generation.

### Results

Table I summarizes the P450 BM3 variants generated by site-directed mutagenesis and random mutagenesis.

#### Table I. List of all P450 BM3 variants generated by site-directed mutagenesis and random mutagenesis

<table>
<thead>
<tr>
<th>Name of mutant</th>
<th>Position of amino acid substitution</th>
<th>Amino acid change</th>
<th>Codon change</th>
<th>Method for diversity generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450 BM3 M87A</td>
<td>47</td>
<td>F→A</td>
<td>TTT→GCC</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>P450 BM3 M87A</td>
<td>47</td>
<td>F→A</td>
<td>TTT→GCC</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>P450 BM3 M1</td>
<td>47, 87</td>
<td>R→F, F→A</td>
<td>CGT→TTT, TTT→GCC</td>
<td>Site-directed saturation mutagenesis</td>
</tr>
<tr>
<td>P450 BM3 M2</td>
<td>47, 87, 354</td>
<td>R→F, F→A, M→S</td>
<td>CGT→TTT, TTT→GCC, ATG→TCT</td>
<td>Site-directed saturation mutagenesis</td>
</tr>
<tr>
<td>P450 BMP M2</td>
<td>47, 87, 354</td>
<td>R→F, F→A, M→S</td>
<td>CGT→TTT, TTT→GCC, ATG→TCT</td>
<td>Site-directed saturation mutagenesis</td>
</tr>
<tr>
<td>P450 BM3 M3</td>
<td>47, 87, 354, 363</td>
<td>D→H</td>
<td>GAC→CAC</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>P450 BM3 M4</td>
<td>47, 87, 354, 281</td>
<td>V→G</td>
<td>GTG→GGG</td>
<td>Random mutagenesis (two rounds of epPCR)</td>
</tr>
<tr>
<td>P450 BM3 M5</td>
<td>47, 87, 281, 354, 363</td>
<td>M→S, D→H</td>
<td>ATG→TCT, GAC→CAC</td>
<td>Site-directed mutagenesis</td>
</tr>
</tbody>
</table>

cpPCR mutagenesis of P450 BM3 M2 and library screening

P450 BM3 M2 was taken as a template for a directed evolution experiment. Random mutations were inserted into the whole gene of P450 BM3 M2 under PCR conditions designed to introduce one to two amino acid changes per gene. In the first round of screening (~1920 variants), five mutants with increased activity were discovered and used as template for a second round of mutagenic PCR under identical conditions. Screening of another 1920 clones resulted in a clone named P450 BM3 M5 (Table I) with three times improved activity under the screening conditions. P450 BM3 M5 was analyzed in detail. Sequencing results revealed that the P450 BM3 M5 mutant contained an additional two mutations which resulted in two amino acid substitutions: V281G (GTG to GGG) and D363H (CAC to GAC).

Site-directed mutagenesis studies

The P450 BM3 mutants obtained in the first round of epPCR were not sequenced; the individual amino acid exchanged from P450 BM3 M5 were therefore introduced subsequently to P450 BM3 M2 (V281G: P450 BM3 M3; D363H: P450 BM3 M4) for elucidating their individual influence on the kinetic parameters. Reductase-deficient variants were generated by site-directed mutagenesis from P450 BM3 F87A, P450 BM3 M2 and P450 BM3 M5. These reductase-deficient variants were used for quantifying the reductase contribution of the Zn/Co(III)sep-mediated electron transfer.

Characterization of P450 BM3 mutants by 12-pNCA conversion employing NADPH and Zn/Co(III)sep as ‘electron’ donors

Figure 1 shows the $K_m$, $k_{cat}$/values and $k_{cat}/K_m$ of the starting point P450 BM3 F87A and all P450 BM3 variants which have been improved for the alternative cofactor system Zn/Co(III)sep. For activity measurements, the 12-pNCA assay and NADPH (Fig. 1, left part) or Zn/Co(III)sep (Fig. 1, right part) were employed as an ‘electron’ donor.
Employing the natural reduction equivalent, NADPH resulted in an increase of $K_m$ values for all P450 BM3 mutants compared with the starting point mutant, P450 BM3 F87A (4.0 to 22–39 μM). $k_{cat}$ values of P450 BM3 mutants dropped from 170 min$^{-1}$ (P450 BM3 F87A) and 171 min$^{-1}$ (P450 BM3 M1) to 124 and 100 min$^{-1}$ for P450 BM3 M2 and P450 BM3 M5. The catalytic efficiencies of the P450 BM3 mutants dropped progressively compared with P450 BM3 F87A (42 μM$^{-1}$ min$^{-1}$ to 5.0, 4.3 and 4.5 for P450 BM3 M1, M2 and M5; Fig. 1, left part).

Opposite results were obtained when using the Zn/Co(III)sep electron donor system. All P450 BM3 mutants had lower $K_m$ values; the $K_m$ values ranged from 1.6 μM (P450 BM3 F87A) to 0.6, 1.0 and 1.1 μM (P450 BM3 M1, M2 and M5). The $k_{cat}$ values of the starting point mutant, P450 BM3 F87A, and of the double and triple mutants, P450 BM3 M1 and P450 BM3 M2, were in the same range (28 min$^{-1}$ for P450 BM3 F87A, 19 min$^{-1}$ for P450 BM3 M1 and 37 min$^{-1}$ for P450 BM3 M2). The $k_{cat}$ value of the P450 BM3 M5 mutant increased by 2.3-fold compared with the starting point mutant P450 BM3 F87A (28–62 min$^{-1}$). Notably, only one of the quadruple mutants, P450 BM3 M3, had the same level of activity as P450 BM3 M5. The level of activity of the other quadruple mutant, P450 BM3 M4, was comparable with that of the triple mutant P450 BM3 M3 and M2. The catalytic efficiencies for all P450 BM3 mutants, except the starting point mutant P450 BM3 F87A, were higher when switching from NADPH to the Zn/Co(III)sep electron donor system. Catalytic efficiencies values were in the same range, for the starting point mutant (P450 BM3 F87A, 19 μM$^{-1}$ min$^{-1}$), the double mutant (P450 BM3 M1, 30 μM$^{-1}$ min$^{-1}$) and the triple mutant (P450 BM3 M2, 26 μM$^{-1}$ min$^{-1}$). For the P450 BM3 M5 and the quadruple mutant P450 BM3 M3, a 3.0- and 2.3-fold higher catalytic efficiency was achieved.

When switching from NADPH to the Zn/Co(III)sep, one can observe a catalytic efficiency which is 12.9-fold (P450 BM3 M5) and 14.8-fold (P450 BM3 M3) higher for the Zn/Co(III)sep electron donor system (Fig. 1).

Figure 2 shows the $K_m$, $k_{cat}$, and $k_{cat}/K_m$ of reductase-deficient variants (P450 BMP F87A, P450 BMP M2 and P450 BMP M5). For activity measurements, the 12-pNCA assay and Zn/Co(III)sep were employed as an ‘electron’ donor.

The kinetic parameters of reductase-deficient P450 BMP variants could not be determined when employing NADPH as reduction equivalent. This is quite expectable since the NADPH-binding site is located in the removed reductase domain of P450 BM3.

On the other hand, all of the reductase-deficient P450 BMP variants were active when employing the Zn/Co(III)sep system as an electron source. The $K_m$ values of all the reductase-deficient mutants were in the same range: 1.7 μM (P450 BMP F87A), 1.2 μM (P450 BMP M2) and 1.3 μM (P450 BMP M5). The $k_{cat}$ values increased from 28 min$^{-1}$ (P450 BMP F87A) to 31 min$^{-1}$ and to 57 min$^{-1}$ (P450 BMP M2 and M5). Catalytic efficiencies also increased progressively from 14 μM$^{-1}$ min$^{-1}$ (P450 BMP F87A) to 26 μM$^{-1}$ min$^{-1}$ and to 43 μM$^{-1}$ min$^{-1}$ (P450 BMP M2 and M5).

**Discussion**

Biocatalysts are by natural design not optimized for applications in bioelectrocatalysis since random electron transfer would in living organisms result in wasting energy and radical formation, which reduces the fitness of living organisms. Redox-active centers are therefore often embedded deeply in an ‘insulating’ protein shell and sophisticated control mechanisms, such as the thermodynamic switch mechanism in P450 BM3 (Daff et al., 1997), regulate electron transfer upon substrate binding from FMN to the heme. Successful protein engineering strategies, for improving mediated electrical communication between biocatalyst and the electrode, are, to our best knowledge, rational design approaches that have been summarized in a review (Wong **Fig. 1.** Kinetic parameters of P450 BM3 variants for hydroxylation of 12-pNCA in the presence of two different electron sources (left: NADPH and right: Zn/Co(III)sep). Each P450 BM3 variant is colored in a specific color for both electron sources: White, P450 BM3 F87A; very light gray, P450 BM3 M1; gray, P450 BM3 M2; light gray, P450 BM3 M3; dark gray, P450 BM3 M4; black, P450 BM3 M5. Values reported are the average of three measurements; average deviations from the mean values are shown as error bars. Min$^{-1}$ is the number of 12-pNCA molecules converted per min per molecule of P450 monoxygenase.

**Fig. 2.** Kinetic parameters of reductase-deficient P450 BM3 mutants for hydroxylation of 12-pNCA in the presence of Zn/Co(III)sep as an electron source. White, P450 BMP F87A; gray, P450 BMP M2; black, P450 BMP M5. Min$^{-1}$ is the number of 12-pNCA molecules converted per min per molecule of P450 monoxygenase.
and Schwaneberg, 2003). One exception is the recent improvement of the mediated electron transfer between glucose oxidase from Aspergillus niger employing ferrocenemethanol as mediator (Zhu et al., 2006).

Fundamental design principles for efficiently redesigning biocatalyst for bioelectrocatalytical applications are not understood yet. Directed protein evolution allows improving biocatalyst properties without understanding targeted properties and thereby offers opportunities to generate hypotheses for redesigning biocatalysts by studying improved biocatalyst variants.

We improved mediated electron transfer properties by directed evolution of P450 BM3 for an alternative cofactor system, Zn/Co(III)sep. P450 BM3- and reductase-deficient P450 BM3 variants that have been generated in this study are summarized in Table I. P450 BM3 M1 was found in a previous research effort (Nazor and Schwaneberg, 2006). In the current study, the screening of further saturation mutagenesis libraries resulted in P450 BM3 M2 which was subsequently subjected to two rounds of random mutagenesis using the classical MnCl₂ epPCR method. In two rounds of random mutagenesis and screening, two additional amino acid substitutions were obtained (position 281 and 363; P450 BM3 M5).

On the basis of the results for P450 BM3 M1, we hypothesized in the initial report on the Zn/Co(III)sep-mediated screening system (Nazor and Schwaneberg, 2006) that favorable association of Co(III)sep at the entrance of the substrate access channel might improve electron transfer rates and/or substrate binding because R47 plays a key role in modulating substrate binding because R47 plays a key role in modulating electron transfer rates and/or substrate binding when Co(III)sep is present. Therefore, several amino acid positions in close vicinity of the substrate access channel (L188, P329, M354S and L437) were saturated and screened for improved activity. Only saturation of position M354 in P450 BM3 M1 and analysis of the eight most active clones resulted in amino acid substitution from M354S (position 281 and 363; P450 BM3 M5).

Position 354 is located within a 12 Å amino acid substitution from M354S (eight out of eight variants are stable to an extent of 80% in a 15 ns simulation of the wild-type P450 BM3 and the P450 BM3 F87A mutant (Roccatano et al., unpublished results). Replacing D363 with a histidine residue could lead to the weakening of D363–Q55 interactions might alter the electron transfer mechanism proposed by Servioukova et al., 1999), the weakening of D363–Q55 interactions might alter the structure of the complex between the FMN and the heme binding domains (pdb code: 1bvy), ~50 α-bonds were identified that could provide a pathway for electron transfer from FMN to heme (via indol ring conjugated σ-orbitals of W574 to the stretch of amino acids N381–Q387 located on the heme binding loop). This stretch of amino acids is located on the same K–L loop as D363 (Fig. 4). In the light of this proposed electron transfer mechanism (Servioukova et al., 1999), the weakening of D363–Q55 interactions might alter the structure of the region. Hence, an altered loop orientation could expose the N381–Q387 peptide stretch to the solvent, making it more accessible for the mediator Co(III)sep and thereby leading to an enhanced electron transfer rate.

The comparison of reductase-deficient variants (P450 BMP F87A, P450 BMP M2 and P450 BMP M5) with the P450 BM3 M5 mutant shows that the reductase-deficient variants are not active with NADPH. For P450 BMP M5 using Zn/Co(III)sep as an electron source, a $k_{cat}$ value and catalytic efficiency of 89 and 69% were achieved compared with P450 BM3 M5 (Figs. 2 and 1, black part). These results show that the electron transfer from the reductase to the heme contributes only to a minor extent to the overall electron transfer rates of the Co(III)sep-mediated electron transfer. In the light of the electron transfer mechanism proposed by Servioukova et al., one could hypothesize that Co(III)sep interfaces in P450 BM3 M5 with the proposed electron pathway from
Materials and methods

All chemicals used were of analytical reagent grade or higher quality and purchased from Sigma-Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany). Zn dust of p.a. grade was obtained from Riedel-de Haen (Seelze, Germany) and Carl Roth (Karlsruhe, Germany). Zn dust of p.a. grade (Steinheim, Germany), AppliChem (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany). Zn dust of p.a. grade (Steinheim, Germany), AppliChem (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany).

FMN to the N381–Q387 peptide stretch. Saturation mutagenesis and focused randomized mutant libraries in the K′–L loop (Fig. 4) are currently being generated to understand possible interfacing mechanisms. These studies are complemented by crystallization efforts in the presence of Co(III)sep.

Out of curiosity, the total turnover numbers (TTNs) for Zn/Co(III)sep were determined under non-optimized conditions to be 936 for P450 BM3 M5 and 670 for P450 BMP M5. These TTN are still one to two orders of magnitude below any industrial consideration. However, directed evolution of P450 BM3 for Zn/Co(III)sep-mediated electron transfer demonstrates that it is possible to improve bioelectrocatalytic properties that were never required for biological function. Directed evolution raises, therefore, hopes that redox proteins in general can be engineered for bioelectrochemical applications in industrial biotechnology through introducing or redesigning interfaces between biocatalyst and an electrochemical setup.

Site-directed mutagenesis

Site-directed mutagenesis was employed to generate reductase-deficient variants of the P450 BM3 M5 mutant (P450 BMP F87A, P450 BMP M2 and P450 BMP M5), and two quadruple P450 BM3 mutants (P450 BM3 M3 and P450 BM3 M4). The quadruple mutants were generated by site-directed mutagenesis using the triple mutant, P450 BM3 M2, as template. P450 BMP M5 was generated by insertion of a stop codon in the gene, upstream of the SacI restriction site. In all cases, a modified Quick-Change method was carried out (Zheng et al., 2004).
For insertion of the stop codon, 35 ng of P450 BM3 M5 template DNA was employed and 17.5 pmol of each mutagenic primer were used: forward primer: 5'-TACAAAC TACGAGCTGATATATGAACTCA-3'; reverse primer: 5'-AGCTGCTAGTTGTATGATCCTCA AGTCAAAG-3', under the following PCR conditions: 94°C for 4 min 1 cycle; 94°C for 1.5 min/55°C for 1 min/68°C for 17 min 20 cycles; 68°C for 20 min 1 cycle, employing 2.5 U Pfu polymerase (Fermentas) and 200 nM dNTP. P450 BMP F87A and P450 BMP M2 were prepared using the same procedure.

For inserting the V281G and D363H mutations, 50 ng of P450 BM3 M2 template DNA and 17.5 pmol of each mutagenic primer were used: forward primer: 5'-GCTGTATTCT TAGGCAAAAATCCACATGATTACA-3'; reverse primer: 5'-ATTTTTTGCTTAAGAAATACGGCAAATGATAAA AG-3' (in case of V281G mutation) and forward primer: 5'-CTCAGCTTCACCGTCACAAAACAATTTGGGGA-3'; reverse primer: 5'-GTITTTGTGACGGTAGCTGAGG AATCAGAAACC-3' (in case of D363H mutation), under the following PCR conditions: 94°C for 4 min 1 cycle; 94°C for 1.5 min/55°C for 1 min/68°C for 17 min 20 cycles; 68°C for 20 min 1 cycle, employing 2.5 U Pfu polymerase (Fermentas) and 200 nM dNTP.

Subsequently, 40 U of DpnI (New England Biolabs) were supplemented to each PCR mix and incubated for 2–3 h at 37°C. The PCR products were purified using NucleoSpin Extract kit (Machery-Nagel). Subcloning of the mutated P450 BMP genes from pCYTEXP1 to pCWori and transformation in E. coli DH5α was carried out as described previously (Wong et al., 2005).

Cultivation and expression in 96-well plates
Colonies grown on LBamp agar plates were transferred, using toothpicks, into 96-deep-well microtiter plates (2.2 ml polypropylene plates; Brand GmbH, Wertheim, Germany), containing 150 µl of LB media supplemented with 15 µg ampicillin per well. After growing overnight in a microtiter plate shaker (Multitron II, Infors GmbH, Einsbach, Germany; 37°C, 900 rpm, 70% humidity), ~5 µl culture volume per well was transferred with the System Duetz tool (Kühner, Birrsfelden, Switzerland) into 2 ml deep-well plates containing 600 µl of enriched TBamp medium (isopropyl-β-D-thiogalactoside IPTG 100 µM; β-α-methyleneacrylic acid anhydride, ALA, 0.5 mM) and separately autoclaved trace element solution (0.6 µl in 600 µl enriched TB) consisting of 0.5 g CaCl2·2H2O, 0.18 g ZnSO4·7H2O, 0.10 g MnSO4·H2O, 20.1 g Na-EDTA, 16.7 g FeCl3·6H2O, 0.16 g CuSO4·5H2O, 0.18 g CoCl2·6H2O dissolved in 1 l of dH2O). Clones were purified by anion exchange chromatography as described previously (Schwaneberg et al., 1999a) at 410 nm using a microtiter plate reader (FlashSCAN S12, Analytik Jena AG, Jena, Germany).

Expression and purification of P450 BM3 mutants
Shaking flasks (1 l) containing 250 ml of TBamp media, supplemented with 250 µl trace element solution, were inoculated with a 1:100 dilution of an LBamp-overnight culture (E. coli DH5α harboring pCWori). After reaching an OD780 value of 0.8–1 during cell cultivation (35°C, 250 rpm, Multitron II), ALA (0.5 mM, final concentration) was added and expression was induced by supplementing IPTG (100 µM; final concentration). E. coli cells were harvested, after 20 h of expression by centrifugation (Eppendorf 5810 R 4°C, 3220 g, 15 min) and resuspended in phosphate buffer (25 ml; KH2PO4/K2HPO4, 25 mM, pH 7.5). E. coli cells were subsequently lysed using a high-pressure homogenizer (1500 bar, two cycles; Avestin Emulsiflex, Mannheim, Germany). The lysate was centrifuged (Eppendorf 5417R, 4°C, 16 000 g, 15 min) and further clarified by filtration through a low protein binding filter (0.45 µm; Celtron 30/0 Syringe-driven filter unit; Schleicher & Schuell, Dassel, Germany). All kinetically characterized P450 BM3 mutants were purified by anion exchange chromatography as described previously (Schwaneberg et al., 1999b). Monoxygenase concentrations were determined by CO-difference spectra as reported by Omura and Sato using e = 91 mM⁻¹ cm⁻¹ (Omura and Sato, 1964).

Determination of Km and kcat of P450 BM3 mutants
NADPH consumption assays and the Zn/Cot(III)sep activity assays were performed as described previously (Schwaneberg et al., 1999a, 2000) except that the buffer was changed in the NADPH consumption assays (Tris–HCl 50 mM, KH2PO4/K2HPO4 50 mM, KCl 0.25 M, pH 8.0) and an Eppendorf Thermomixer comfort (Eppendorf AG, Hamburg, Germany) was used for incubating 1.5 ml Eppendorf tubes at 1200 rpm, in the Zn/Cot(III)sep activity assay. The amount of P450 BM3 variants used in each experiment was 0.17 nmol and 12-pNCA substrate concentrations varied between 0.6 µM and 114 µM. Kinetic constants were calculated by fitting the experimental results into a
hyperbolic function using the Origin 7.0 software and by Eadie-Hofstee plots.

**Determination of TTNs of P450 BM3 mutants**

Total turnover numbers were determined for 0.1 nmol of P450 BM3 variants with 75 μM 12-pNCA in a total volume of 1 ml. The conversions were preformed in 1.5 ml Eppendorf tubes as described previously (Schwaneberg et al., 2000), except that the reactions were continued for 13 h. Product generation and TTNs were calculated using the following calibration curve: y (μM) = 0.00848x + 0.03819 \( (r^2 = 0.9982) \), which was obtained by measuring the absorbance at 410 nm of p-nitrophenol concentrations ranging from 0.03 to 0.3 mM.

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**References**


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