Heterotropic and homotropic cooperativity by a drug metabolising mutant of Cytochrome P450 BM3

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In this chapter, additional data on three steroids is presented that has not been included in the published manuscript.
ATYPICAL KINETICS IN A P450 BM3 MUTANT

Abstract

Recently we described a triple mutant of the bacterial cytochrome P450 BM3 as the first mutant with affinity for drug-like compounds. In this paper we show that this mutant, but not wild-type P450 BM3, is able to metabolise testosterone and several drug-like molecules such as amodiaquine, dextromethorphan, acetaminophen and 3,4-methylenedioxyamphetamine that are known substrates of human P450s. Interestingly, the metabolism of 3,4-methylenedioxyamphetamine and acetaminophen could be stimulated up to 70-fold by the addition of caffeine, a known activator of rat P450 3A2. With testosterone metabolism, homotropic cooperativity was observed. This shows that heterotropic and homotropic cooperativity, known to occur in the P450 3A family, can also take place in P450 BM3. P450 BM3 therefore can be used as a model system to study atypical kinetics in mammalian P450s. Secondly, this study shows that P450 BM3 can be engineered to a drug metabolising enzyme, making it a promising candidate to use as biocatalyst in drug discovery and synthesis.
Introduction

Cytochromes P450 (P450s) constitute a large superfamily of monooxygenases that are involved in the oxidation and reduction of a wide variety of compounds [1]. Next to their important endogenous functions, they are well known for their role in the metabolism of xenobiotics such as drugs, food components and environmental contaminants [2, 3]. Drug metabolising P450s generally have a broad substrate selectivity and are able to perform a wide variety of reactions. Because of this catalytic diversity, the application of P450s in biotechnology, for example as biocatalysts in the production of fine chemicals such as pharmaceuticals, may have good perspectives [4-6]. Bacterial P450s are especially interesting as biocatalysts because they are soluble, and, compared to their membrane-bound mammalian counterparts, are more stable and catalytically more active. One of the most extensively studied bacterial P450s is cytochrome P450 BM3 (P450 BM3) from *Bacillus megaterium* [4, 7]. P450 BM3 has the highest catalytic activity ever detected for a P450 monooxygenase [7, 8]. Its natural substrates are long-chain fatty acids. Very recently it was stated that the limited substrate diversity of bacterial P450s is a major disadvantage for industrial purposes [9]. Several reports, however, have demonstrated that by site-directed and random mutagenesis the substrate specificity of P450 BM3 can be altered strongly to include a wide variety of compounds, such as indole, polycyclic aromatic hydrocarbons and alkanes [10-12]. Very recently it was shown that a mutant of the P450 BM3 heme domain is able to metabolise the beta-blocker propranolol using a peroxide shunt pathway [13].

Recently, we demonstrated that a rationally designed triple mutant of P450 BM3, R47L/F87V/L188Q, has affinity for several drug-like compounds [14]. This mutant of P450 BM3 seems to share some characteristics with P450 3A4: it converts benzylxoyresorufin, which is a well-established substrate for P450 3A4, whereas ketoconazole, a potent inhibitor of P450 3A4, was identified as the most potent inhibitor when screening inhibition of dealkylation of benzylxoyresorufin by a library of 45 drug-like compounds. Comparison of the crystal structures of P450 3A4 and wild-type P450 BM3 reveals that both enzymes contain a relatively large active site composed of mainly hydrophobic residues [15, 16], and seem to be able to accommodate two substrate molecules in the active site simultaneously [17, 18]. The presence of two substrate binding sites in the active site may explain the non-Michaelis-Menten kinetic behaviour of P450 3A4 [19]. For human P450s, the occurrence of atypical kinetics is clinically relevant because non-Michaelis-Menten kinetics complicate the extrapolation of in vitro data to in vivo in drug discovery and development [22]. Cytochrome P450 eryF was the first bacterial P450 to display both heterotropic and homotropic cooperativity, and therefore was proposed as a convenient model protein to investigate the fundamental mechanisms...
underlying atypical enzyme kinetics of P450s [20]. The availability of crystal structures, the higher stability and the ease of overexpression in E. coli are big advantages that bacterial P450s have over mammalian P450s. However, bacterial P450 eryF belongs to the class I P450s which have different redox partner systems than the class II P450s to which the human drug metabolising P450s belong. For this reason, bacterial P450 BM3, which has a class II-type redox system, may be considered a more appropriate model for the mammalian P450s [8]. In contrast to its closely related homologues CYP102A2 and CYP102A3 from Bacillus subtilis, nonhyperbolic enzyme kinetics has not yet been reported for the well-studied P450 BM3 [21].

In this study, we characterised the P450 BM3 triple mutant R47L/F87V/L188Q with respect to metabolism of testosterone and the drugs acetaminophen, dextromethorphan, amodiaquine and 3,4-methylenedioxymethylamphetamine (MDMA) (Figure 1). Because these substrates are known to be metabolised by mammalian P450s to multiple metabolites these compounds are very suitable as diagnostic substrates, because the metabolite profiles obtained give valuable information on the differences in substrate orientation between mutants [21, 22]. To investigate whether heterotropic cooperativity in the presence of an effector molecule could be observed for the P450 BM3 triple mutant, similar to what has been described for the P450 BM3 triple mutant ([21, 22]).

**Figure 1:** Chemical structures of the compounds used in this study. A: amodiaquine; B: dextromethorphan; C: MDMA; D: acetaminophen; E: testosterone. Known sites of metabolism are indicated by arrows, and for acetaminophen, the structure of the metabolite NAPQI is also displayed.
3A family, two compounds that are known activators of the P450 3A family, α-naphtoflavone (α-NF) and caffeine [23], were tested for their ability to influence the reaction rates and product distribution.

**Materials and Methods**

**Chemicals**

The plasmid pT1-P450BM3 containing the wild-type P450 BM3 cDNA under control of the temperature-inducible PPr promoter was a kind gift from R. D. Schmid (Institut für Technische Biochemie, Universität Stuttgart). The construction of the triple mutant of P450 BM3 was described earlier [14]. Expression and isolation of P450 BM3 and P450 BM3 R47L/F87V/L188Q was performed as described previously [14]. BD Supersomes, containing P450 1A1 with human NADPH P450 reductase, and P450 2C8 with human NADPH P450 reductase and cytochrome b5, were obtained from BD Gentest. The plasmids pCWh3A4 and pLCMhOR containing P450 3A4 and human NADPH P450 reductase, respectively, were a kind gift from M. Kranendonk (Department of Genetics, Faculty of Medical Sciences, Universidade Nova de Lisboa, Portugal) and were expressed as described earlier [24]. All chemicals were of analytical grade and obtained from standard suppliers.

**Metabolism of drug-like compounds by P450 BM3 and P450 BM3 R47L/F87V/L188Q**

Although it was reported previously that 50 µM amodiaquine did not significantly inhibit benzyloxyresorufin O-dealkylation (BROD) activity of the P450 BM3 triple mutant [14], at 500 µM significant inhibition (40%; data not shown) was observed. Therefore, in this paper metabolism was studied at a substrate concentration of 500 µM. The amodiaquine incubations were performed in 100 mM potassium phosphate (KPi) buffer pH 7.4, with *E. coli* cytosolic fraction containing 500 nM P450 BM3 wild-type or R47L/F87V/L188Q. The final volume of the incubation was 250 µl.

The reaction was initiated by addition of an NADPH regenerating system resulting in final concentrations of 0.2 mM NADPH, 0.3 mM glucose-6-phosphate and 0.4 units/ml glucose-6-phosphate dehydrogenase. The reaction was allowed to proceed for 45 minutes at 24 °C before it was terminated by the addition of 25 µl of a 10% (v/v) HClO4 solution. Precipitated protein was removed by centrifugation (15 minutes, 4000 g), and the supernatant was analysed by HPLC. Metabolites were separated using a C18 column (Phenomenex Luna 5µ, 150 × 4.6 mm), using the following gradient: 0-2 minutes 0% B; 2-24 minutes increase to 15% B; 24-27 minutes 15% B; 27-28 minutes decrease to 0% B; 28-35 minutes 0% B. Eluens A consisted of 5% acetonitril, 10 mM ammonium acetate and 0.3% (v/v) formic acid, and eluens B consisted of 90% acetonitril, 10 mM ammonium acetate and 0.3% (v/v) formic acid. The flow rate was 0.6 ml/minute. Metabolites were detected with a UV detector at 254 nm and with MS. An LCQ Deca mass spectrometer (Thermo Finnigan) was used, with positive ion Electro Spray Ionisation (ESI). The vapouriser temperature was 450 °C, N2 was used as sheath gas (60 psi) and auxiliary gas (20 psi), the needle voltage was 5000 V and the heated capillary was at 200 °C.

The dextromethorphan incubations were performed as described for amodiaquine.
Metabolites were separated using a C18 column (Phenomenex Luna 5µ, 150 × 4.6 mm) with a flow rate of 0.6 ml/minute. The mobile phase consisted of 30% acetonitril with 0.1% triethylamine, set to pH 3.0 with HClO. Metabolites were detected with a fluorescence detector (λ<sub>excitation</sub> = 280 nm and λ<sub>emission</sub> = 311 nm), and also with LC-MS as described in [22]. The testosterone incubations were evaporated under nitrogen gas, and the metabolites were taken up in 250 µl 42% methanol. Metabolites were analysed by HPLC using a C18 column (100 x 3 mm, 5µ, Chromspher Chrompack). Metabolites were eluted using the following gradient: 0-5 minutes 20% B; 5-25 minutes increase to 50% B; 25-27 minutes 50% B; 27-28 minutes decrease to 20% B; 28-35 minutes 20% B. Eluens A consisted of 15% methanol and 500 mM ammonium acetate, and eluens B consisted of 99% methanol. The flow rate was 0.6 ml/minute. Metabolites were detected with a UV detector at 254 nm and with MS. An LCQ Deca mass spectrometer (Thermo Finnigan) was used, with positive ion Atmospheric Pressure Chemical Ionisation (APCI). The vaporiser temperature was 450 °C, N<sub>2</sub> was used as sheath gas (20 psi) and auxiliary gas (20 psi), the needle voltage was 6000 V and the heated capillary was at 200 °C.

For MDMA, incubations were performed as described for amodiaquine, but with 2 mM NADPH instead of an NADPH regenerating system. Metabolites were separated using a C18 column (Phenomenex Luna 5µ, 150 × 4.6 mm) with a flow rate of 0.6 ml/minute. The mobile phase consisted of 20% acetonitril and 0.1% triethylamine, set to pH 3.0 using HClO. Metabolites were detected with a fluorescence detector (λ<sub>excitation</sub> = 280 nm and λ<sub>emission</sub> = 320 nm).

For acetaminophen, incubations were performed as described for MDMA. Because the metabolite N-acetyl-p-benzoquinoneimine (NAPQI) is very reactive, 5 mM reduced glutathione (GSH) was added to form a stable NAPQI-SG conjugate. The acetaminophen incubation was separated using a C18 column (Phenomenex Luna 5µ, 150 × 4.6 mm) with a flow rate of 0.5 ml/minute. The gradient used was as follows: 0-5 minutes 0% B; 5-32 minutes increase to 68% B; 32-33 minutes increase to 100% B; 33-45 minutes 100% B; 45-40 minutes 0% B. Eluens A consisted of 20 mM ammonium acetate, set to pH 6.0 with acetic acid. Eluens B was composed of 20 mM ammonium acetate and 50% acetonitril, set to pH 6.0 with acetic acid. Metabolites were detected with a UV detector at 254 nm.

To test if all three mutations in the triple mutant are required for activity, the incubations with testosterone and MDMA were also performed with the single mutants R47L, F87V and L188Q, and the double mutants R47L/F87V, R47L/L188Q and F87V/L188Q. For metabolite identification and comparison, incubations with various human P450s were performed for all substrates as described above, with 50 nM P450, 500 µM substrate, and an incubation time of 20 minutes at 37 °C. The human P450s used were heterologously expressed P450 2D6 and P450 2D6[120A], cultured as described previously [22], and heterologously expressed P450 3A4, as described in [24], and P450 1A1 and 2C8 in BD Supersomes (Gentest). The BD Supersomes contained P450 and human NADPH P450 reductase, and in the case of P450 2C8 also Cytochrome b5. P450 2D6, P450 2D6[120A] and P450 3A4 were all co-expressed with human NADPH P450 reductase.
**Determination of kinetic parameters**

First, the ranges were determined where the enzyme activity is linear with enzyme concentration and incubation time. Based on these experiments (data not shown), the suitable conditions were chosen for determination of the kinetic parameters. For MDMA, the enzyme concentration was 200 nM and the incubation time was 45 minutes, and 9 substrate concentrations were used in the range of 0-4 mM. For acetaminophen, the enzyme concentration was 200 nM and the incubation time was 45 minutes, and 9 substrate concentrations were used in the range of 0-6 mM. For testosterone the enzyme concentration was 240 nM and the incubation time was 45 minutes, and 11 substrate concentrations were used in the range of 0-600 μM. Additionally, enzyme kinetic parameters were determined for the three steroids progesterone, androstenedione and nandrolone. For progesterone the incubations were carried out as described for testosterone, with the following modifications: 480 nM P450 BM3 R47L/F87V/L188Q was used; progesterone concentrations were in the range of 0-500 μM (11 concentrations); incubation times were 30 minutes. The HPLC gradient was as follows: 0-5 minutes 0% B; 5-29 minutes increase to 80% B; 29-30 minutes decrease to 0% B; 30-35 minutes 0% B. For nandrolone and androstenedione the incubations were carried out as described for testosterone, with the following modifications: 200 nM P450 BM3 R47L/F87V/L188Q was used; substrate concentrations were in the range of 0-500 μM (11 concentrations); incubation times were 20 minutes. The HPLC gradient was as follows: 0-2 minutes 5% B; 2-22 minutes increase to 50% B; 22-23 minutes increase to 100% B; 23-25 minutes 100% B; 25-26 minutes decrease to 5% B; 26-30 minutes 5% B.

Peak areas of all the metabolites were determined using the Shimadzu Class VP 4.3 software package. Formation of the MDMA metabolite 3,4-methylenedioxyamphetamine (MDA) was quantified using a synthesised reference compound [22]. N-hydroxy-3,4-methylenedioxy-methylamphetamine (N-OH-MDMA) was quantified using the MDA standard curve, assuming that the fluorescence of MDA and N-OH-MDMA are comparable. NAPQI-SG was quantified using a standard curve of acetaminophen, assuming that the extinction coefficients of acetaminophen and NAPQI-SG are comparable. The steroid metabolites were quantified using a standard curve of the parent compounds, assuming that the extinction coefficients of the parent compounds and their metabolites are comparable. Reaction rates were calculated and plotted against the substrate concentrations. The data of MDMA and acetaminophen were fitted with the Michaelis Menten equation in GraphPad Prism 4.0 to obtain the kinetic parameters $K_m$ and $V_{max}$. For the steroids, the $V/[S]$ curves were sigmoidal rather than hyperbolic, therefore the data were fitted with the Hill equation: $V = V_{max} \times S^n / (K_{0.5}^n + S^n)$ where $V$ is the reaction rate, $S$ is the substrate concentration, $K_{0.5}$ is the substrate concentration that gives a velocity of $0.5 \times V_{max}$, and $n$ is the Hill coefficient.

**Activation of P450 BM3 R47L/F87V/L188Q activity by caffeine and α-Naphtoflavone**

To study heterotropic cooperativity, the known P450 3A activators caffeine (5 mM) or α-NF (50 μM) were added to various incubations with wild-type P450 BM3 and P450 BM3 R47L/F87V/L188Q. The incubations were performed as described above. For acetaminophen and MDMA, 1 mM substrate was used, for testosterone, 100 μM substrate was used, and for dextromethorphan and amodiaquine, 500 μM substrate was used.
For MDMA and acetaminophen, the caffeine concentration dependence of the activation was studied by varying the caffeine concentration from 0 to 10 mM (6 concentrations). Enzyme kinetic parameters were subsequently determined in the presence of caffeine. For MDMA, 5 mM caffeine was used, and for acetaminophen 9 mM caffeine.

Results

Metabolism of drug-like compounds by P450 BM3 and P450 BM3 R47L/F87V/L188Q

Wild-type P450 BM3 did not metabolise any of the drug-like compounds tested. In contrast, the triple mutant metabolised all compounds studied. P450 BM3 R47L/F87V/L188Q converted amodiaquine into two metabolites (Figure 2A). The first metabolite had a retention time (t<sub>r</sub>) of 10.1 minutes, and the second metabolite had a t<sub>r</sub> of 12.5 minutes. To identify these metabolites, incubations with P450 1A1, 2C8 and 2D6 were performed, which are known to N-desethylate amodiaquine [25]. The metabolite of t<sub>r</sub> = 12.5 minutes was formed by P450 1A1, P450 2C8 and P450 2D6 and could thus be identified as N-desethylamodiaquine. This was confirmed by MS (m/z 328; MS/MS m/z 283). N-desethylamodiaquine was formed with the highest specific activity by P450 2C8, which was 125 times higher than that of P450 BM3 R47L/F87V/L188Q (Table 1). The metabolite of t<sub>r</sub> = 10.1 minutes was not formed by P450 1A1, P450 2C8 and P450 2D6; it was identified by MS as a mono-hydroxylated metabolite (m/z 372; MS/MS m/z 299).

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Figure 2: HPLC chromatograms of P450 BM3 wild-type (black line) and P450 BM3 R47L/F87V/L188Q (grey line) incubations with amodiaquine (A), dextromethorphan (B), testosterone (C) and MDMA (D). For all substrates, the chromatograms of the incubations with P450 BM3 wild-type were identical to those of the t=0 minutes control incubations (not shown), indicating that no metabolism took place. In the incubations with P450 BM3 R47L/F87V/L188Q, several metabolites were formed for all substrates (indicated by their names and by retention times).
Dextromethorphan was N-demethylated by the P450 BM3 triple mutant to form 3-methoxymorphinan (Figure 2B). This metabolite was identified by LC-MS as described previously (m/z 258; MS/MS m/z 215) [22]. The specific activity of dextromethorphan N-demethylation by the P450 BM3 triple mutant was 12.5-fold lower than that of P450 2D6 (Table 1).

Testosterone was metabolised by P450 BM3 R47L/F87V/L188Q to three metabolites (Figure 2C). The major metabolite had a t_r of 10.0 minutes, the second highest metabolite had a t_r of 16.1 minutes and the third metabolite had a t_r of 19.1 minutes. All three metabolites were identified by LC-MS as monohydroxylated metabolites (m/z 305). In addition, trace amounts of a dihydroxylated metabolite (m/z 321) at t_r= 2.6 minutes were observed with MS. The metabolite at t_r= 16.1 minutes co-eluted with a reference standard of 6β-hydroxytestosterone. Of the double and single mutants, only the two double mutants F87V/L188Q and R47L/F87V showed metabolism of testosterone. The same metabolites were formed as by the triple mutant, but the amounts were 4- and 10-fold lower, respectively (data not shown). P450 BM3 triple mutant was 12.5-fold lower than that of P450 2D6 (Table 1).

MDMA was metabolised by P450 BM3 R47L/F87V/L188Q to three metabolites (Figure 2D). The major metabolite had a t_r of 8.8 minutes and was identified as MDA by coinjection with a reference standard. The second metabolite, with t_r= 17.2 minutes, was identified as N-OH-MDMA by coinjection with a reference standard [22]. The minor metabolite with t_r= 3.8

Table 1: Activity of P450 BM3 R47L/F87V/L188Q compared to several human P450s.

<table>
<thead>
<tr>
<th>Substrate (metabolite)</th>
<th>P450 BM3 R47L/F87V/L188Q</th>
<th>P450 2D6</th>
<th>P450 3A4</th>
<th>P450 1A1</th>
<th>P450 2C8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amodiaquine (desethyl)</td>
<td>100%</td>
<td>2631%</td>
<td>1953%</td>
<td>12752%</td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan (N-demethyl)</td>
<td>100%</td>
<td>1250%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (t_r 10.0)</td>
<td>100%</td>
<td></td>
<td>45%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDMA (3,4-OH-MA)</td>
<td>100%</td>
<td></td>
<td>3682%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaminophen (NAPQI)</td>
<td>100%</td>
<td></td>
<td></td>
<td>1500%</td>
<td></td>
</tr>
</tbody>
</table>

Values are in % product formation/min/nmol enzyme relative to incubations with P450 BM3 R47L/F87V/L188Q. For amodiaquine, N-desethylamodiaquine was compared with N-desethylamodiaquine formed by P450 1A1, 2C8 and 2D6. For dextromethorphan, 3-methoxymorphinan was compared with 3-methoxymorphinan formed by P450 2D6. For testosterone, the major metabolite was compared with 6β-hydroxytestosterone formed by P450 3A4. For MDMA, 3,4-OH-MA was compared with 3,4-OH-MA formed by P450 2D6. For acetaminophen, NAPQI formation was compared with P450 3A4.
minutes was identified previously as 3,4-dihydroxy-methylamphetamine (3,4-OH-MA) using
electrochemical detection [22]. Comparison with an incubation with P450 2D6 showed that
the specific activity of 3,4-OH-MA formation by P450 BM3 R47L/F87V/L188Q was 35-fold lower
than that of P450 2D6 (Table 1). Of the double and single mutants, only the double mutant
F87V/L188Q was able to metabolise MDMA. The amount of metabolite formed was so low
that only MDA was detectable (data not shown).

Acetaminophen was metabolised by P450 BM3 R47L/F87V/L188Q to one metabolite with a t
of 17.5 minutes (Figure 3). This peak was identified as the NAPQI-SG conjugate by coinjection
with a reference standard synthesised as described [26, 27]. The specific activity of NAPQI
formation by P450 BM3 R47L/F87V/L188Q was approximately 15-fold lower than that of P450
3A4 (Table 1).

Table 2: Kinetic parameters of MDMA and acetaminophen metabolism by P450 BM3 R47L/F87V/L188Q in
the presence and absence of caffeine.

<table>
<thead>
<tr>
<th>Substrate (product)</th>
<th>Without caffeine</th>
<th>With caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} )</td>
<td>( K_m )</td>
</tr>
<tr>
<td>MDMA (MDA)</td>
<td>0.35 ± 0.01</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>MDMA (N-OH)</td>
<td>0.14 ± 0.02</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>Acetaminophen (NAPQI)</td>
<td>0.05 ± 0.01</td>
<td>7.3 ± 0.1</td>
</tr>
</tbody>
</table>

Data are for MDA and N-OH-MDMA formation from MDMA, and NAPQI formation from acetaminophen.
All values are the means of at least 3 independent experiments performed on separate days ± S.D. Because
in the absence of caffeine no saturation of enzyme activity was observed, \( K_m \) and \( V_{\text{max}} \) values could not
be determined; instead, the slope of the \( V/S \) curve was used to determine the catalytic efficiency \( V_{\text{meas}}/K_m \).
\( V_{\text{meas}} \) values are expressed in nmol product/minute/nmol enzyme; \( K_m \) values are expressed in mM.
Determination of kinetic parameters

For MDMA metabolism, the 3,4-OH-MA peak was too small to determine accurate kinetic parameters. No saturated \( V/S \) curve could be obtained for MDA- and N-OH-MDMA formation with the substrate concentration range used for this experiment (Figure 4). Therefore, the slope of the linear part of the \( V/S \) plot was used to determine the catalytic efficiency \( V_{\text{max}}/K_m \) (Table 2). Compared to MDA formation by P450 2D6[F120A], the catalytic efficiency of P450 BM3 R47L/F87V/L188Q was approximately 700-fold lower [28].

For acetaminophen metabolism, also no saturated \( V/S \) curve could be obtained for NAPQI formation with the substrate concentration range used for this experiment (Figure 4). The catalytic efficiency \( V_{\text{max}}/K_m \) was determined using the slope of the linear part of the \( V/S \) plot (Table 2). From Figure 4 it can be concluded that the \( K_m \) value is >> 2 mM. In literature, \( K_m \) values of 300 µM have been described for P450 3A4, and of 1 mM and 3 mM for P450 2E1 and P450 1A2, respectively [29]. \( V_{\text{max}} \) values could not be compared, because in reference [29] the amount of P450 in the incubations was not quantified.

For testosterone, \( V/S \) curves were constructed for all three mono-hydroxylated metabolites. All curves were sigmoidal, which suggests the occurrence of positive homotropic cooperativity. This was confirmed by the Hill coefficients (Table 3). If the Hill coefficient \( n > 1 \), Michaelis Menten kinetics apply, whereas with \( n > 1 \) or \( n < 1 \) positive or negative cooperativity applies, respectively. For all the mono-hydroxylated testosterone metabolites formed by P450 BM3 R47L/F87V/L188Q, a Hill coefficient of 1.7 was found, indicating that positive cooperativity occurs. This has also been observed previously for testosterone 6β-hydroxylation by human P450 3A4 [30]. In addition to testosterone, the three steroids androstenedione, nandrolone and progesterone were also investigated. The kinetic parameters and the retention times of the metabolites of all four steroids are summarised in Table 3, and a \( V/S \) curve of the major metabolite is shown in Figure 5. Homotropic cooperativity was observed for all steroids.
### Table 3: Kinetic parameters of steroid metabolism by P450 BM3 R47L/F87V/L188Q.

<table>
<thead>
<tr>
<th>Substrate (retention time of metabolite)</th>
<th>$K_{0.5}$ (µM)</th>
<th>$V_{max}$ (nmol/min/nmol)</th>
<th>$V_{max}/K_{m}$ ($x 10^3$)</th>
<th>$n$</th>
<th>LC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>androstenedione (t, 19.7)</td>
<td>136.0 ± 31.7</td>
<td>40.5 ± 6.2</td>
<td>97.8 ± 83.0</td>
<td>1.4 ± 0.2</td>
<td>mono-OH</td>
</tr>
<tr>
<td>androstenedione (t, 21.9)</td>
<td>216.0 ± 68.5</td>
<td>2.0 ± 0.3</td>
<td>9.3 ± 3.2</td>
<td>1.6 ± 0.1</td>
<td>mono-OH</td>
</tr>
<tr>
<td>androstenedione (t, 15.3)</td>
<td>139.5 ± 29.1</td>
<td>0.6 ± 0.1</td>
<td>43 ± 1.1</td>
<td>2.2 ± 0.6</td>
<td>di-OH</td>
</tr>
<tr>
<td>androstenedione (t, 17.7)</td>
<td>244.7 ± 56.6</td>
<td>0.13 ± 0.01</td>
<td>0.6 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>mono-OH</td>
</tr>
<tr>
<td>nandrolone (t, 16.4)</td>
<td>252.0 ± 18.1</td>
<td>25.6 ± 0.6</td>
<td>101.6 ± 7.7</td>
<td>1.7 ± 0.2</td>
<td>mono-OH</td>
</tr>
<tr>
<td>nandrolone (t, 20.2)</td>
<td>253.0 ± 8.7</td>
<td>10.9 ± 1.0</td>
<td>43.1 ± 4.2</td>
<td>1.6 ± 0.3</td>
<td>mono-OH</td>
</tr>
<tr>
<td>nandrolone (t, 21.8)</td>
<td>270.3 ± 23.4</td>
<td>2.1 ± 0.1</td>
<td>7.8 ± 0.8</td>
<td>1.8 ± 0.2</td>
<td>mono-OH</td>
</tr>
<tr>
<td>progesterone (t, 27.6)</td>
<td>43.9 ± 12.8</td>
<td>4.6 ± 0.5</td>
<td>104.8 ± 32.6</td>
<td>1.9 ± 0.3</td>
<td>mono-OH</td>
</tr>
<tr>
<td>progesterone (t, 29.6)</td>
<td>51.9 ± 11.6</td>
<td>1.0 ± 0.2</td>
<td>19.3 ± 5.8</td>
<td>2.1 ± 0.3</td>
<td>mono-OH</td>
</tr>
<tr>
<td>progesterone (t, 18.9)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>testosterone (t, 10.0)</td>
<td>85.9 ± 15.2</td>
<td>4.5 ± 1.2</td>
<td>52.4 ± 17.2</td>
<td>1.7 ± 0.3</td>
<td>mono-OH</td>
</tr>
<tr>
<td>testosterone (t, 16.3)</td>
<td>103.6 ± 11.1</td>
<td>1.4 ± 0.4</td>
<td>13.5 ± 4.1</td>
<td>1.7 ± 0.2</td>
<td>mono-OH</td>
</tr>
<tr>
<td>testosterone (t, 19.3)</td>
<td>92.0 ± 5.2</td>
<td>0.4 ± 0.1</td>
<td>4.3 ± 1.1</td>
<td>1.7 ± 0.2</td>
<td>mono-OH</td>
</tr>
<tr>
<td>testosterone (t, 1.6)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

All values are the means of at least 3 independent experiments performed on separate days ± S.D. All data were fitted with the Hill equation. $K_{0.5}$ values are in µM. $V_{max}$ values are nmol product/minute/nmol enzyme. —: metabolite only present in trace amounts, no kinetic parameters could be determined.

### Table 4: Effect of $\alpha$-NF and caffeine on the metabolism of drug-like compounds by P450 BM3.

<table>
<thead>
<tr>
<th>Substrate (concentration)</th>
<th>control</th>
<th>+ $\alpha$-NF (50 µM)</th>
<th>+ caffeine (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amodiaquine (500 µM)</td>
<td>100%</td>
<td>65%</td>
<td>104%</td>
</tr>
<tr>
<td>Testosterone (100 µM)</td>
<td>100%</td>
<td>40%</td>
<td>55%</td>
</tr>
<tr>
<td>Dextromethorphan (500 µM)</td>
<td>100%</td>
<td>70%</td>
<td>250%</td>
</tr>
<tr>
<td>MDMA (1 mM)</td>
<td>100%</td>
<td>102%</td>
<td>213%</td>
</tr>
<tr>
<td>acetaminophen P450 BM3 (1 mM)</td>
<td>100%</td>
<td>104%</td>
<td>203%</td>
</tr>
<tr>
<td>acetaminophen P450 BM3 (1 mM)</td>
<td>100%</td>
<td>105%</td>
<td>280%</td>
</tr>
</tbody>
</table>

Values are in % product formation relative to incubations without activator. Only the data for the major metabolites are displayed. The other metabolites were similarly affected.
Three monohydroxylated and one dihydroxylated metabolites were formed from androstenedione, three monohydroxylated metabolites were observed for nandrolone, and two monohydroxylated and one dihydroxylated metabolites were identified for progesterone. Androstenedione was converted with the highest activity, approximately 10-fold higher than testosterone.

**Activation of P450 BM3 R47L/F87V/L188Q activity by caffeine and α-naphtoflavone**

To investigate whether heterotropic cooperativity occurs in P450 BM3, two known activators of P450 3A, α-naphtoflavone (α-NF) and caffeine, were tested. Single concentrations of these activators (derived from reference [23]) were added to incubations with amodiaquine, dextromethorphan, testosterone, acetaminophen and MDMA. Wild-type P450 BM3 did not metabolise any of the substrates in the absence or presence of activator. For P450 BM3 R47L/F87V/L188Q, amodiaquine metabolism was not influenced by the presence of caffeine, but α-NF inhibited the product formation to 65% (Table 4). Dextromethorphan metabolism was slightly inhibited by α-NF, whereas caffeine stimulated 3-methoxymorphinan formation 2.5-fold. Testosterone metabolism was inhibited to approximately 50% by both caffeine and α-NF; all peaks were inhibited to the same extent. For acetaminophen and MDMA metabolism, α-NF did not have a significant effect on the amount of product formed. However, in the
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presence of caffeine a significant increase in specific activity was observed. For MDMA, the specific activity increased 2-fold for all three metabolites, and for acetaminophen a dramatic increase of a factor 20 was observed (Figure 3). Acetaminophen metabolism by hP450 3A4 was activated to 150% by α-NF, and to 280% by caffeine (Table 4).

The activation of acetaminophen and MDMA metabolism by P450 BM3 R47L/F87V/L188Q in the presence of caffeine was investigated further. Varying the caffeine concentration showed that maximal activation occurred at 5 mM for MDMA and at 9 mM for acetaminophen (data not shown). To determine the effect of this activator on \( K_m \) and \( V_{max} \), the kinetic parameters for acetaminophen and MDMA metabolism were determined in the presence of 9 or 5 mM caffeine, respectively. In the presence of caffeine, enzyme saturation did take place for both substrates within the substrate range used (Figure 4), and the data could be fitted with the Michaelis-Menten equation. Kinetic parameters \( K_m \) and \( V_{max} \) could be determined (Table 2). For MDMA, the presence of caffeine increased the catalytic efficiency \( V_{max}/K_m \) approximately 4-fold for MDA- and N-OH-MDMA formation. The \( V_{max} \) value for MDA formation was only 2.7-fold lower than that of MDA formation by P450 2D6[F120A] [28]. For acetaminophen, the presence of caffeine dramatically increased the catalytic efficiency \( V_{max}/K_m \) of NAPQI formation 70-fold. In comparison, a 2- to 10-fold increase in catalytic efficiency is usually observed for activation of P450 3A by α-NF or caffeine [23, 31].

Caffeine itself was not metabolised by P450 BM3 R47L/F87V/L188Q in either the absence or presence of acetaminophen or MDMA (data not shown).

Discussion

Although the limited substrate diversity of bacterial P450s has been mentioned previously as a major disadvantage for its use as a biocatalyst for the production of pharmaceuticals [9], the present study demonstrates that several drug-like compounds were readily metabolised by P450 BM3 R47L/F87V/L188Q. Enzyme kinetics analysis of this drug metabolising triple mutant of P450 BM3 revealed positive homotropic cooperativity with testosterone as substrate, with a Hill coefficient of 1.7. With three other steroids, similar behaviour was observed. For the other substrates tested hyperbolic kinetic curves were observed. Similar behaviour has been described for P450 3A4 [30]. In addition, strong heterotropic cooperativity was shown to occur in the presence of caffeine. The specific activity of acetaminophen metabolism increased 70-fold, which is much higher than the 2- to 10-fold activation described in literature for P450 3A. Furthermore, addition of caffeine strongly increased affinity towards MDMA. In contrast, the P450 3A4 activator α-NF does not act as an activator for P450 BM3 R47L/F87V/L188Q. α-NF does not influence acetaminophen and MDMA metabolism, while it inhibits the metabolism of the other substrates (Table 4). The latter implies that α-NF is able to bind to P450 BM3 R47L/F87V/L188Q, and it remains to be investigated whether it can also be metabolised.

A possible explanation for the stimulation of P450 BM3 R47L/F87V/L188Q activity by caffeine could be that caffeine binds in the active site and forces the substrates into an orientation that results in increased metabolism by reducing the substrate mobility. However, for MDMA metabolism, the metabolite ratio is not influenced by the addition of caffeine. This implies
that all the orientations required for the formation of the three metabolites are still possible and the substrate is still mobile. Alternatively, it is possible that caffeine binds to an allosteric site of the protein, resulting in a conformational change in the active site with an increased binding affinity for the substrate [32, 33]. The observed activation is substrate dependent, caffeine did not increase the metabolism of some of the substrates tested. Activation of P450 3A4 is also known to be substrate dependent: some compounds activate the metabolism of one substrate, but inhibit the metabolism of another substrate [34].

The fact that the P450 BM3 triple mutant displays homotropic and heterotropic cooperativity suggests that multiple substrate molecules can bind to this triple mutant simultaneously, possibly to multiple binding sites with distinct characteristics. P450 BM3 is easier to study than mammalian P450s, because it is a soluble protein with higher stability and activity. It can be used as a model to increase our understanding of the mechanisms of atypical kinetics in mammalian P450s. We also propose a perspective for the use of heterotropic cooperativity in biotechnology: instead of mutagenesis of the enzyme, the presence of a second ligand could be used to increase the reaction rate or alter the product distribution of an enzymatic reaction to make it more suitable for biocatalysis. Cases have been reported where the presence of a second ligand in the active site of a P450 not only enhanced the amount of metabolite produced by increasing the reaction rate and binding affinity, but also altered the product distribution of the reaction [17, 35]. Together with mutagenesis techniques, this type of allosteric kinetics can be employed as a novel tool in the further development of P450 BM3 as a fast and clean catalyst for biotechnology.

P450 BM3 has a high potential for the use in biotechnology, because it is a highly active, stable, single-component enzyme that can be easily engineered to convert a wide variety of substrates into (therapeutically) useful products. In this study it was shown that a mutant of P450 BM3 can be used to produce various metabolites from several different drug-like compounds. In addition to C-hydroxylation (steroids), also N-hydroxylation (MDMA), N-dealkylation (MDMA, dextromethorphan, amodiaquine), O-demethylation (MDMA) and dehydrogenation (acetaminophen) were observed. The rate of testosterone hydroxylation, and acetaminophen dehydrogenation in the presence of caffeine, by the P450 BM3 triple mutant is similar to the reaction rate of P450 3A4. However, the activity of the P450 BM3 triple mutant towards MDMA, amodiaquine and dextromethorphan is still 20 to 125-fold lower than the activity of mammalian P450s, which leaves room for improvement. This triple mutant will be used as the starting point for random mutagenesis, to obtain series of P450 BM3 mutants with further improved ability to convert a wide variety of drug-like compounds with higher activities. Such mutants can prove very useful in drug-discovery and drug synthesis.
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References


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