BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

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Biotransformation of β-ionone by engineered cytochrome P450 BM-3

Abstract Wild-type cytochrome P450 monooxygenase from *Bacillus megaterium* (P450 BM-3) has a low hydroxylation activity for β-ionone (<1 min^{-1}). Substitution of phenylalanine by valine at position 87 led to a more than 100-fold increase in β-ionone hydroxylation activity (115 min^{-1}). Enzyme activity could be further increased by both site-directed and random mutagenesis. The mutant R47L Y51F F87V, designed by site-directed mutagenesis, and the mutant A74E F87V P386S, obtained after two rounds of error-prone polymerase chain reaction, exhibited an increase in activity of up to 300-fold compared to the wild-type enzyme. The triple mutant R47 LY51F F87V exhibited moderate enantioselectivity, forming (*R*)-4-hydroxy-β-ionone with an optical purity of 39%. All mutants regioselectively converted β-ionone into 4-hydroxy-β-ionone. The regioselectivity is determined amongst others by the absolute configuration of the substrate.

Introduction

Ionones (α-, β-, γ-) (Fig. 1) and their derivatives, in particular, hydroxy ionones, are essential intermediates in the synthesis of many carotenoids (Eschenmoser et al. 1981). They can be used in the industrial synthesis of zeaxanthin and cantaxanthin. Ionone derivatives are virtually omnipresent and constitute substantial aroma components of floral scents (Sefton et al. 1989; Eugster and Maerki-

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Fischer 1991). They are also important components of insect lures (Donaldson et al. 1990; McQuate and Peck 2001) and improve pollinization by insects. Hydroxy-β-ionones are versatile compounds in the synthesis of the plant hormone abscisic acid (Oritani and Yamashita 1984). These characteristics make ionones and their hydroxy metabolites particularly attractive for the fragrance and flavor industry and chemical synthesis.

Several methods of preparing 4-hydroxy-β-ionone have been described. They are based either on chemical optical resolution of α-ionone (Haag et al. 1980) or on selective enzymatic hydrolysis of 4-hydroxy-β-ionone acetate using lipases (Kakeya et al. 1991). *Aspergillus niger* and *Aspergillus awamori* are able to convert β-ionone into two major products, 4-hydroxy-β-ionone and 2-hydroxy-β-ionone (Sode et al. 1989). The biotransformation involved has been explored to generate a mixture of derivatives that can be utilized for tobacco flavoring (Sode et al. 1989).
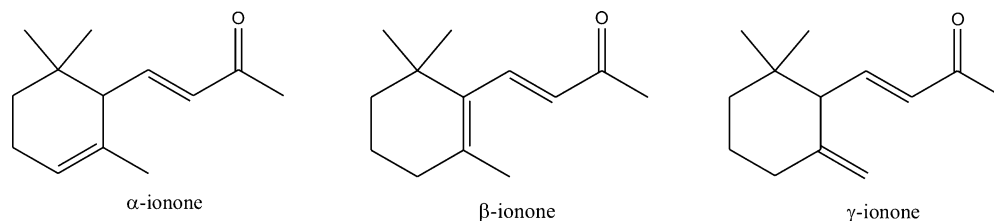
Cytochrome P450 monooxygenases (also called CYP) are a potentially very useful class of catalysts for oxidation. Cytochrome P450 monooxygenases from several *Streptomyces* strains are able to hydroxylate α- and β-ionone (Lutz-Wahl et al. 1998). A mutant of cytochrome P450 BM-3 from *Bacillus megaterium* (also referred to as CYP102A1) can also efficiently hydroxylate α-ionone, resulting in a mixture of different hydroxylated products (Appel et al. 2001).

P450 BM-3 is a catalytically self-sufficient monooxygenase, consisting of a P450-heme domain being attached to a diflavin nicotinamide adenine dinucleotide phosphate (NADPH) dependent reductase. P450 BM-3 hydroxylates and epoxidizes long-chain saturated and unsaturated fatty acids with high efficiency (Narhi and Fulco 1986; Narhi et al. 1988). To accommodate a wider range of unnatural substrates, the enzyme has been modified using methods of protein engineering (Appel et al. 2001; Carmichael and Wong 2001; Li et al. 2001a; Peters et al. 2003). Here we present P450 BM-3 mutants that are capable of the selective hydroxylation of β-ionone to 4-hydroxy-β-ionone with high activity. The mutants were produced either by rational design or by directed evolution.

Fig. 1 Structure of α-, β- and γ-ionone

α-ionone   β-ionone   γ-ionone

Materials and methods

Chemicals

All chemicals were of analytical grade or higher quality and purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). An aqueous solution (5 mg ml^{-1}) of NADPH tetrasodium salt (Jülich Fine Chemicals, Germany) was used for the experiments. β-Ionone (Merck) was prepared as a 15 mM solution in dimethyl sulfoxide (DMSO). 3-Hydroxy-β-ionone was purchased from BASF AG (Ludwigshafen, Germany). 4-Hydroxy-β-ionone was synthesized from α-ionone as previously described (Haag et al. 1980). The 4-hydroxy-β-ionone structure was confirmed by ^{1}H- and ^{13}C-NMR analyses.

Preparation of P450 BM-3 and its mutants

Mutants were generated by polymerase chain reaction (PCR) using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's protocol (Vandeyar et al. 1988) and expressed in *Escherichia coli* strain BL21(DE3) after induction with isopropyl-β-D-thiogalactopyranoside (IPTG). Protein expression and purification were carried out as previously described (Maurer et al. 2003). Concentration of correctly folded P450 enzymes was determined from the CO-binding difference spectra of the reduced heme iron as reported elsewhere (Omura and Sato 1964).

Determination of binding spectra

The substrate-induced spectral shifts of the P450 monooxygenases were recorded with UV-VIS spectrophotometer from Amersham Biosciences (Cambridge, England) at room temperature according to the method described elsewhere (Truan et al. 1999). The changes in the absorbance at 388 nm (peak) and 422 nm (trough) were determined. Binding constants K_d were calculated by plotting the maximal absorption changes calculated for each difference spectrum against the concentration of β-ionone.

Random mutagenesis and screening for hydroxylation activity

Error-prone PCR was performed on the heme domain of the gene, coding for the F87V mutant cloned into the pET22(b)+ vector. Two gene libraries were constructed under conditions that led to one to two amino acid substitutions per gene (1,340 bp). 0.05 mM MnCl$_2$ was used as mutagenic agent. PCR, subsequent restriction of PCR products and original plasmid with the *Nde*I and *Bam*HI endonucleases and ligation by T4-DNA ligase were done by standard methods (Sambook and Russell 2001). A *Bam*HI recognition site was inserted by site-directed mutagenesis between the monooxygenase and reductase domain, resulting in a silent mutation. The ligated genes were used to transform *E. coli* BL21(DE3) gold super competent cells (Novagen, CA, USA). Protein expression was performed in 1.2-ml-deep-well plates containing 400 μl Luria–Bertani medium, supplemented with ampicillin (100 μg ml^{-1}). Cells were grown at 37°C and 200 rpm and induced with 0.5 mM IPTG at an OD$_{578}$ ~0.8. After incubation at 30°C and 200 rpm for 12–16 h, cells were harvested by centrifugation. After cell lysis and centrifugation, supernatant aliquots (typically ~0.4–1 μM P450 BM-3) were transferred into microtiter plates. β-Ionone (150 μM) in DMSO (1%) was added to the lysate, and the plates were incubated for 10 min at room temperature. NADPH (450 μM) solution was added and its turnover was measured at room temperature at 340 nm in a FLUOstar 403 spectrophotometer (BMG LabTechnologie, NC, USA) and was calculated using ε_M=6,200 M^{-1} cm^{-1}. Background NADPH turnover without addition of the substrate served as negative control.

NADPH assay

The NADPH assay was performed as described elsewhere (Appel et al. 2001). The observed rates of NADPH consumption were corrected for the slow background reaction that was observed with substrate but without enzyme. The Michaelis–Menten parameters were determined by standard methods.

Product analysis

The 1-ml reaction mixture was extracted twice with 0.5 ml trichlormethane containing γ-ionone (final concentration 15 μM) as internal standard; the combined organic phases were evaporated to dryness and dissolved in 100 μl trichlormethane. Gas chromatography–mass spectrocopy (GC–MS) analysis was performed on Shimadzu GCMS-QP2010 using the following program: 150°C for 1 min, 20°C/min to 250°C. Retention times for β-ionone, 4-hydroxy-β-ionone and 3-hydroxy-β-ionone were 4.06, 5.06 and 5.23 min, respectively. Mass spectra were collected using electron

ionisation. The compounds were identified by their fragmentation pattern and compared to those of authentic β-ionone, 4-hydroxy-β-ionone and 3-hydroxy-β-ionone. β-Ionone turnover rate was calculated from the peak area ratios. Retention time for α-ionone was 3.74; its oxidized products were found after 5.98, 6.13, 6.52 and 6.62 min.

Silica gel 60 F254 (Merck) was used for preparative thin-layer chromatography. Optical rotation was recorded by a Perkin Elmer 241MC Polarimeter (Perkin Elmer, MA, USA). Specific optical rotation $[\alpha]_D$ is the observed rotation which is corrected for sample concentration (c; g ml^{-1}) and a defined path length (l; diameter), namely, $[\alpha]_D = \alpha_{obs}/(c \times l)$. Optical purity (%) was calculated as follows: $100\% \times [\alpha]_D/[\alpha]_D$ of enantiomerically pure sample.

Results

All monooxygenases, produced during this work, were successfully overexpressed in *E. coli* and showed a characteristic Soret band at 450 nm in reduced CO-bound form. The P450 BM-3 mutants demonstrated characteristic heme spin shift upon binding of β-ionone (Table 1).

Construction of mutants by rational design

Cytochrome P450 BM-3 was found capable of hydroxylating β-ionone in the presence of NADPH, although it did not show a significant heme spin state shift upon substrate binding (Table 1). A very low turnover rate of <1 min^{-1} was observed. In contrast to the natural substrates of P450 BM-3, fatty acids, β-ionone is a more bulky molecule. Therefore, we decided to enlarge the binding pocket around the heme by means of site-directed mutagenesis. The position 87 of P450 BM-3 was already previously proven to be important in the hydroxylation of fatty acids (Graham-Lorence et al. 1997) and some artificial substrates (Li et al. 2001a,b). Phenylalanine 87 is located directly above the heme and controls activity, regio- and enantioselectivity of P450 BM-3 (Graham-Lorence et al. 1997; Li et al. 2001b). We tested a set of single mutants for their activity towards β-ionone. Three mutants, F87V, F87A and F87G, were observed with significantly higher activity towards β-ionone than the wild-type enzyme. Binding of β-ionone to all three mutants resulted in changes in the absorption spectra in the Soret region, which are typical for real substrates (Table 1).

Although the mutant F87A showed a higher NADPH turnover during β-ionone hydroxylation compared to the mutant F87V (~380 vs. ~310 min^{-1}), the latter led to a higher 4-hydroxy-β-ionone production under the same reaction conditions (~90 min^{-1} compared to ~115 min^{-1}). This suggested a higher coupling between NADPH oxidation and substrate hydroxylation for the F87V (Table 1). As described previously, not all NADPH consumed by the reductase is utilized for substrate oxidation (Gorsky et al. 1984). As a result, the rates of 4-hydroxy-β-ionone formation are always lower than the corresponding NADPH turnover rates.

The introduction of glycine at position 87 had a positive effect on enzyme activity, though to a lesser extent than alanine or valine (Table 1). Valine, alanine and phenylalanine are non-polar, hydrophobic amino acids. In contrast, glycine is a small polar amino acid and has a different effect on substrate interaction. Spectral-binding titration revealed that the F87V and F87A enzymes have higher affinities to β-ionone than the F87G.

To the best of our knowledge, little is known about the metabolic fate of β-ionone. The β-ionone metabolism in rabbits leads to four oxidized derivatives (Ide and Toki 1970). *A. niger* converts β-ionone to two major products, 4-hydroxy-β-ionone and 2-hydroxy-β-ionone, and several minor ones (Sode et al. 1989). *Lasiodiplodia theobromae* transforms β-ionone into a large variety of oxidized metabolites (Krasnobajew and Helmlinger 1982). Wild-type P450 BM-3 and its mutants only produced 4-hydroxy-β-ionone (Fig. 2). This product was identified by comparing it with authentic 4-hydroxy-β-ionone and 3-hydroxy-β-ionone by GC–MS. The obtained fragmentation pattern was identical to that of 4-hydroxy-β-ionone (Fig. 2).

In this reaction, the high regioselectivity of P450 BM-3 was particularly remarkable. P450 BM-3 and its mutants are able to oxidize saturated fatty acids (Truan et al. 1999) and alkanes (Glieder et al. 2002) at subterminal (ω-1, ω-2,

Table 1 Kinetic, binding parameters and coupling of β-ionone hydroxylation by P450 BM-3 and its mutants

P450 BM-3 mutants	NADPH turnover rate (min^{-1})	Product formation rate (min^{-1})	Coupling efficiency (%)	K_M (μM)	K_d (μM)
WT	24±6	1±0.5	3.3	1139±260	nd
F87A	380±16	93±8	24	704±80	75±10
F87V	312±8	115±5	37	305±28	85±7
F87G	144±12	42±4	29	476±36	139±4
R47L Y51F	48±6	2±0.5	4	4545±380	nd
R47L Y51F F87V	816±28	268±10	31	1611±205	152±7
F87V P386S	645±7	185±7	29	350±26	143±13
A74E F87V P386S	792±10	280±13	35	769±60	100±12
R47L Y51F A74E F87V P386S	1034±21	190±8	17	540±75	115±10
F87V A74G L188Q	274±36	90±10	33	1346±185	160±11

nd Not detectable
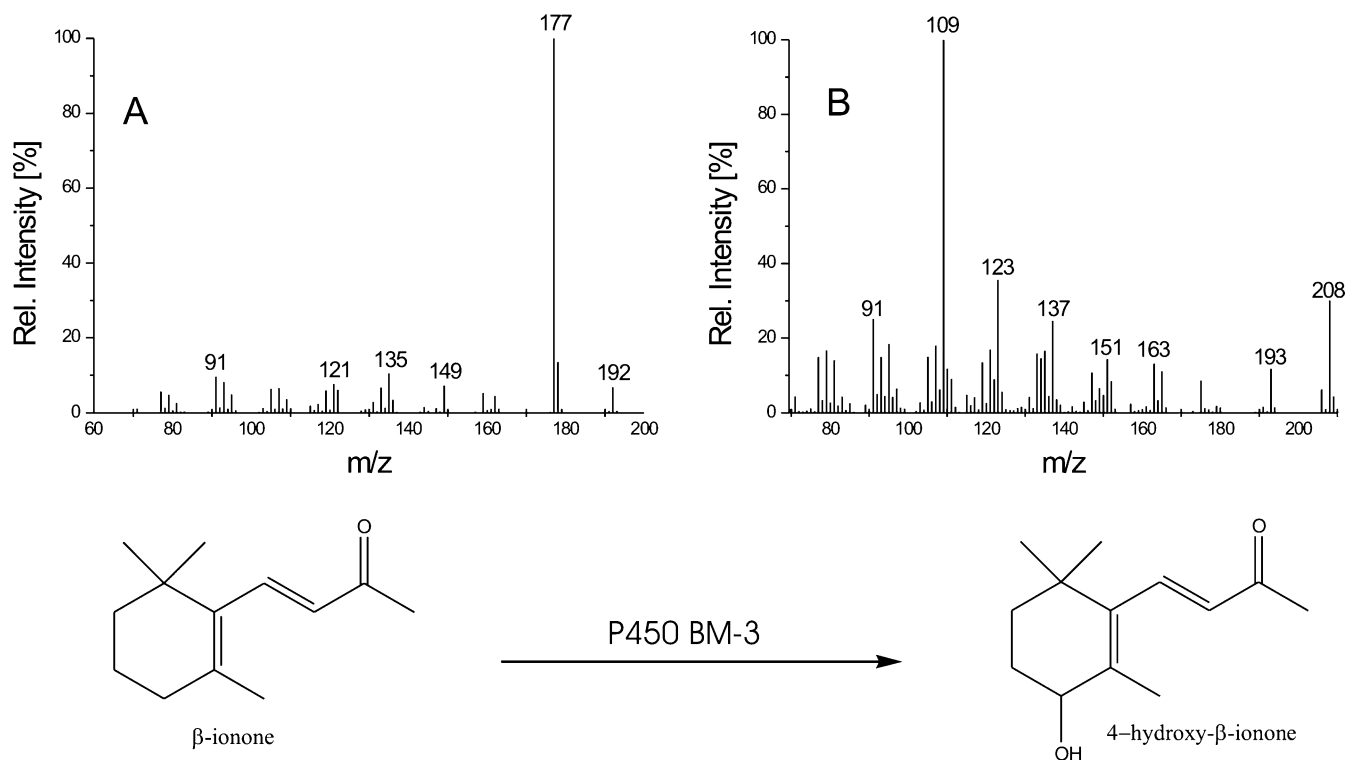
Fig. 2 Hydroxylation of β-ionone (**A** MS fragmentation pattern) yielding 4-hydroxy-β-ionone (**B** MS fragmentation pattern) by wild-type P450 BM-3 and its mutants

ω-3) positions, producing different hydroxylated products. This monooxygenase can also oxidize polycyclic aromatic hydrocarbons (Carmichael and Wong 2001) and terpenes (Wong et al. 2000) at different positions. Interestingly, the regioisomer of β-ionone, α-ionone, was hydroxylated by the triple P450 BM-3 mutant, A74G F87V L188Q, to several different products: 3-hydroxy-α-ionone (70%), 7-hydroxy-α-ionone (24%) and some other minor hydroxylated derivatives (Appel et al. 2001). When α-ionone was used as substrate for the F87V mutant, the mixture of four oxidized products was obtained.

For the further improvement in the activity of the enzyme, methods of site-directed and random mutagenesis were applied. Previous analysis of enzyme structure and comparison of substrate-bound (Li and Poulos 1997) with substrate-free (Ravichandran et al. 1993) P450 BM-3 forms revealed the importance of the Y51 and R47 residues at the entrance of the substrate channel for the catalytic activity and specificity of the enzyme (Carmichael and Wong 2001; Cowart et al. 2001). Therefore, the effect of their substitution was examined. Since β-ionone is hydrophobic and bulky, an increase in hydrophobicity at the entrance of the binding pocket was supposed to facilitate the diffusion of substrate further into the active site. Consequently, the basic arginine 47 was exchanged with nonpolar leucine, and polar tyrosine 51 was exchanged with hydrophobic phenylalanine. The double mutant R47L Y51F exhibited a very low activity towards β-ionone. After introduction of an additional F87V substitution, a new triple mutant was generated which demonstrated a 2.5-fold increased NADPH turnover rate (~820 min^{-1}) and a doubling in hydroxylation activity (~270 min^{-1}) compared to the mutant F87V (Table 1).

To produce sufficient amount of the hydroxylated product, the whole-cell oxidation of β-ionone by the triple mutant R47L Y51F F87V was performed. The product 4-hydroxy-β-ionone was purified by preparative thin-layer chromatography and its optical rotation was measured. Specific optical rotation $[\alpha]_D$ of +5.1° was compared with the corresponding literature value, reported for (R)-4-hydroxy-β-ionone ($[\alpha]_D$=+13°, c=1.6; trichlormethane). The optical purity of (R)-4-hydroxy-β-ionone was determined to be 39%, indicating moderate enantioselectivity of the triple mutant R47L Y51F F87V.

The triple mutant A74G F87V L188Q, previously reported as highly active towards α-ionone (Appel et al. 2001), was tested for β-ionone oxidation. Spectral shift in Soret region indicated that this substrate was tightly bound in the binding channel of the enzyme. NADPH oxidation rate as well as the product formation was comparable with corresponding values for the F87V mutant but not better (Table 1). This mutant also produced only 4-hydroxy-β-ionone.

Construction of a random mutant library

The F87V gene was selected as a template for the first round of random mutagenesis. Two generations of P450 BM-3 mutants were produced by error-prone PCR. Two

libraries consisting of 1,500 and 2,500 clones, respectively, were screened. The monitoring of NADPH turnover was used as an activity assay.

The screening of the first 1,500 transformants and following detailed analysis led to the identification of one mutant that had a higher activity towards β-ionone. Sequence analysis revealed that proline was substituted by serine at position 386. The NADPH oxidation rate with β-ionone was twice as fast compared to the parent enzyme F87V (~645 vs. ~310 min^{-1}) and almost 25-fold faster compared to the wild-type P450 BM-3 (~645 vs. ~25 min^{-1}). Position 386 is located on the surface of the protein molecule and cannot affect activity or substrate binding directly. The positive effect may be due to either enhanced electron transfer from flavin mononucleotide (FMN) to the heme iron or to a long-range effect on the structure of the protein.

The gene encoding the mutant F87V P386S was used as a template for generating the second error-prone PCR library. Two thousand and five hundred colonies were screened by monitoring of NADPH turnover. Four highly active variants were detected and characterized further. After protein expression, purification and the calculation of specific activity, only one mutant turned out to be more active than the F87V P386S. The new mutant retained both mutations of its parent gene F87V P386S and acquired one new substitution A74E. The new triple mutant A74E F87V P386S exhibited a further increase in activity (up to 2.5-fold faster compared to the F87V enzyme). This activity is similar to that of the R47L Y51F F87V in terms of NADPH oxidation and β-ionone hydroxylation (Table 1). GC–MS

analysis detected 4-hydroxy-β-ionone as a single hydroxylated product. After the whole-cell conversion of the substrate, the product was extracted, purified by thin-layer chromatography and analyzed with polarimeter. The optical purity of only 8% for (R)-4-hydroxy-β-ionone, calculated from the specific optical rotation, indicated very low enantioselectivity of the mutant A74E F87V P386S.

The coupling efficiencies of 30–35%, calculated for all mutants containing the mutation *F87V*, were similar to that of the F87V mutant. From this, it can be deduced that this particular substitution minimizes the uncoupling effects during the hydroxylation process.

Both triple mutants, the R47L Y51F F87V and A74E F87V P386S, could convert also α-ionone, giving rise to multiple oxidized products as revealed by GC–MS (data not shown).

Combining the best mutations in one variant

In an attempt to enhance the activity of P450 BM-3 further, all five substitutions were combined, resulting in a new variant, R47L Y51F A74E F87V P386S (Fig. 3). However, the expected improvement could not be achieved. Although the NADPH turnover rate was greater than 1,000 min^{-1}, the product formation rate was only about 200 min^{-1}. This means that the coupling efficiency of the mutant was, on average, twice as low compared to that of the parent mutants. In summary, the experiments led to a mutant displaying an increased NADPH turnover rate, but highly uncoupled β-ionone hydroxylation (Table 1).

Fig. 3 Model of P450 BM-3. The mutation sites and the heme are shown in black
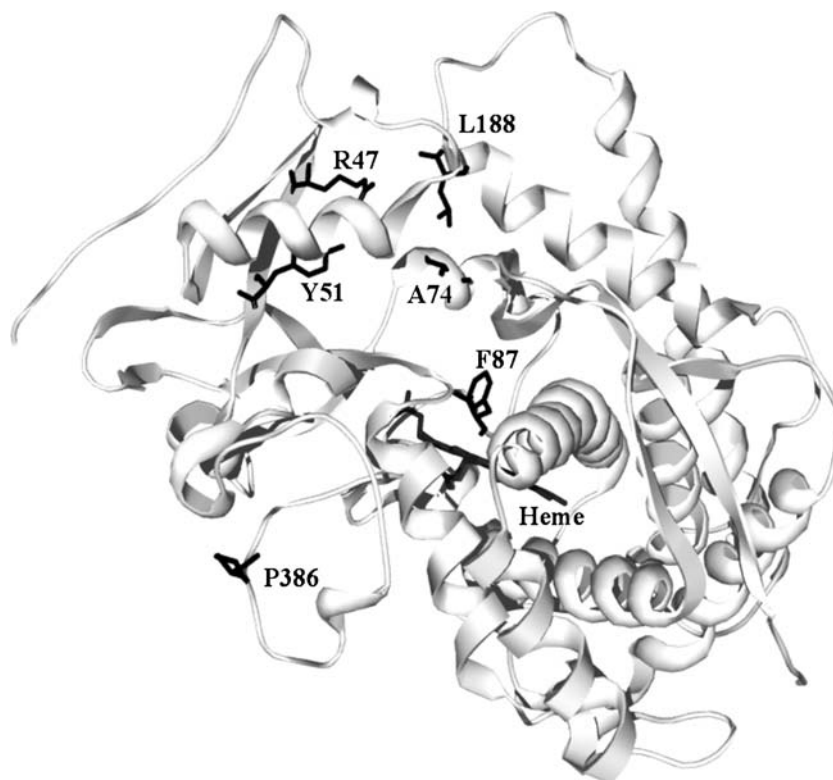
Discussion

New mutants of P450 BM-3 have been constructed for regioselective hydroxylation of β-ionone with high activity (up to 800 NADPH min^{-1}) and rather high coupling efficiency (29–37%). Although β-ionone has no similarity with fatty acids, the substrate channel of the enzyme could be "adapted" for this molecule.

It has been shown in many previous studies, that enlarging the binding pocket around the heme by a substitution of phenylalanine 87 by valine (Li et al. 2001a,c) or alanine (Carmichael and Wong 2001) is essential step in engineering new activities of P450 BM-3. The replacement of phenylalanine 87 by valine significantly improved NADPH turnover rate (up to 15-fold), the hydroxylation of β-ionone (up to more than 100-fold) as well as the coupling efficiency (up to 10-fold). Further increase in activity was achieved by the replacements R47L and Y51F, which facilitate the improved diffusion of β-ionone deeper into the active site.

Proline at position 386 is located on the surface of the enzyme molecule (Fig. 3); nevertheless, its substitution by serine resulted in a drastic increase in NADPH turnover during β-ionone hydroxylation. On the one hand, the crystal structure of the complex between the heme and FMN-containing domains of P450 BM-3 indicates that the region between proline 382 and glutamine 387 is supposed to affect the electron transfer from the FMN to the heme iron (Sevrioukova et al. 1999). Therefore, exchange at position 386 could influence the interaction between the monooxygenase domain and an FMN-binding reductase domain. On the other hand, this substitution could probably change overall dynamics of the molecule and so influence the enzyme activity.

Also remarkable is the effect of the substitution A74E that can narrow the substrate channel and provide a better orientation of the substrate in the binding pocket.

All mutants of P450 BM-3 constructed during present work hydroxylated β-ionone exclusively in position 4 to a single product. Such high regioselectivity is very unusual for this monooxygenase that is known as an enzyme with rather low product selectivity. P450 BM-3 produces mixtures of different hydroxylated and/or epoxidized products (Carmichael and Wong 2001; Sulistyaningdyah et al. 2004; Sowden et al. 2005), suggesting multiple orientations of these substrates in the binding pocket. Also the regioisomer of β-ionone, α-ionone was oxidized by all new mutants to a mixture of four oxidized products. This suggests that the more rigid β-ionone structure might cause a stronger fixation of the substrate in the P450 BM-3 binding pocket exclusively in one orientation.

The enantioselectivity of P450 BM-3 in this reaction is still a challenge for the protein engineering. The triple mutant R47L Y51F F87V that produces (R)-4-hydroxy-β-ionone with an optical purity of 39% is a good candidate for further improvement in enzyme selectivity.

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