

Biotransformation of ionones by engineered cytochrome P450 BM-3

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Abstract

Wild type cytochrome P450 monooxygenase from *Bacillus megaterium* (P450 BM-3) has low activity for the hydroxylation of β -ionone ($>1 \text{ min}^{-1}$). Substitution of phenylalanine by valine at position 87 increased the β -ionone hydroxylation activity up to 100-fold (115 min^{-1}). For further activity improvement methods of site-directed and random mutagenesis were applied. The R47L Y51F F87V mutant, designed by site-directed mutagenesis and the A74E F87V P386S mutant, obtained after two rounds of error-prone PCR, exhibit an increase in activity up to 300-fold compared to the wild type enzyme. All mutants converted β -ionone regioselectively to 4-hydroxy- β -ionone.

Key words: Monooxygenase; P450 BM-3; Hydroxylation; β -ionone; Regioselectivity; Mutagenesis, Gene library

Introduction

Ionones (α -, β - and γ -) and their derivatives, first of all hydroxy ionones, are essential intermediates in the synthesis of a number of carotenoids (Eschenmoser et al. 1981; Oritani and Yamashita 1984) and can be used in the industrial synthesis of e.g. zeaxanthin and cantaxanthin. Ionone derivatives are found in many sources and with a trimethylcyclohexane building block constitute substantial aroma components of floral scents (Sefton et al. 1989; Eugster and Maerki-Fischer 1991; Winterhalter and Rouseff 2002). They form important components of insect lures (Donaldson et al. 1990; McQuate and Peck 2001) and can favor insect pollinization. Hydroxy- β -ionones are versatile compounds in synthesis of abscisic acid, a phytohormone. All these useful features make ionones and their hydroxy metabolites attractive for the fragrance and flavor industry.

A few methods of preparing 4-hydroxy- β -ionone have been described. They are based either on chemical optical resolution of α -ionone (Haag et al. 1980a) or on selective enzymatic hydrolysis of the 4-hydroxy- β -ionone acetate with lipases (Oritani and Yamashita 1984; Kakeya et al. 1991). *Aspergillus niger* was found capable to convert β -ionone into two major products 4-hydroxy- β -ionone and 2-hydroxy- β -ionone (Sode et al. 1989). *A. awamori* strain has also been shown to achieve hydroxylation of β -ionone in the same manner (Kakeya et al. 1991). The process has been explored in order to afford a mixture of derivatives that is utilized as an essential oil of tobacco, used for tobacco flavoring.

Cytochrome P450 monooxygenases from several *Streptomyces* strains were proven to hydroxylate α - and β -ionone (Lutz-Wahl et al. 1998). Also, a mutant of cytochrome P450 BM-3 (also referred to as CYP102A1) from *Bacillus megaterium* can efficiently hydroxylate α -ionone, producing a mixture of different hydroxylated products (Appel et al. 2001).

P450 BM-3 is a catalytically self-sufficient monooxygenase, containing in a single polypeptide chain a P450-heme domain attached to a diflavin NADPH-oxidoreductase. This

enzyme hydroxylates and epoxidizes long chain saturated and unsaturated fatty acids with high efficiency (Narhi and Fulco 1986; Narhi et al. 1988). P450 BM-3 was successfully modified by methods of protein engineering to accommodate a wider range of unnatural synthetic substrates (Appel et al. 2001; Carmichael and Wong 2001; Li et al. 2001a; Peters et al. 2003).

Here we present P450 BM-3 mutants, capable to hydroxylate β -ionone selectively to the single product 4-hydroxy- β -ionone with high activity. The mutants were produced either by rational design or by directed evolution.

Materials and methods

Chemicals

All chemicals were of analytical grade or higher quality and were purchased from Fluka (Neu-Ulm, Germany) and Merck (Darmstadt, Germany). In all experiments aqueous solution (5 mg ml⁻¹) of tetrasodium salt of NADPH (Julich Fine Chemicals, Julich, Germany) was used. β -ionone and α -ionone (Merck, Darmstadt, Germany) were prepared as a 15 mM solution in DMSO. 3-hydroxy- β -ionone was purchased from BASF AG (Ludwigshafen, Germany). 4-hydroxy- β -ionone was synthesized from α -ionone as described previously (Haag et al. 1980b). Structures of other products were confirmed by NMR analyses. NMR spectra were run in CDCl₃ solution on a Bruker (Karlsruhe-Forchheim, Germany) ARX 500 spectrometer (normal frequencies of 500.13 MHz for ¹H and 125.77 for ¹³C).

Preparation of P450 BM-3 and its mutants

Mutants have been constructed by PCR with appropriate primers using the QuikChangeTM site-directed mutagenesis kit from Stratagene (Vandeyar et al. 1988) according to the manufacturer's protocol and expressed in *Escherichia coli* strain BL21(DE3) under induction of isopropylthiogalactoside (IPTG).

Mutant R47L Y51F A74E F87V P386S was prepared by digestion of two existing plasmid clones pT-458_BM3 (R47L Y51F F87V) and pT-7838_BM3 (A74E F87V P386S) with *Afl*III and *Mlu*I, followed by purification and ligation of the smaller fragment (1143 bp) from pT-458_BM3 and the larger fragment (7376 bp) from pT-7838_BM3. All mutations were confirmed by sequencing the complete gene.

Cloning of P450 BM-3, recombinant protein expression in *E. coli* and purification have been described previously (Maurer et al. 2003). Concentration of correctly folded P450 enzymes was determined from the CO-binding difference spectra of the reduced heme iron, as described (Omura and Sato 1964).

Random mutagenesis

Mutagenic PCR was performed on the heme domain of the gene pT-F87V_BM3, coding for the F87V mutant using following primers: 5'-atagaagcggccgcatgacaattaagaaatg-3' and 5'-cgggatccgcgtacttttttagcagactgttcag-3'. Two gene libraries were constructed under conditions to achieve 1-2 amino acid substitution per gene (1340 bp) by standard methods using 0.05 mM MnCl₂ as mutagenic agent. PCR, subsequent restriction of PCR products 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, UK). The colonies were inoculated into microtiterplates containing 150 μ l Luria-Bertani medium, supplemented with DMSO (5 %) and ampicillin (100 μ g ml⁻¹). The plates were incubated overnight at 37°C, 200 rpm and used further as stock plates. Protein expression was performed in 1.2 ml deep-well plates with square wells containing 400 μ l LB medium, supplemented with ampicillin (100 μ g ml⁻¹). Cells were grown at 37°C, 200 rpm and induced with 0.5 mM IPTG at an OD_{578nm} ~0.8. After incubation at 30°C and 200 rpm for 12-

16 h, cells were harvested by centrifugation. The cell pellet was resuspended in the lysobuffer (50 mM potassium phosphate buffer, pH 7.5, containing lysozyme 1 mg ml⁻¹, DNase 1 ng ml⁻¹ and 40 mM NaCl). After incubation at room temperature for 30 min cells were frozen at -70°C for 1 h. After one freeze-thaw cycle plates were centrifuged at 3000g for 30 min at 4°C and the supernatant was used in further experiments.

Screening for hydroxylation activity

Aliquots of cell lysate (typically 180 µl (~0,4-1 µM P450 BM-3)) were transferred into microtiterplates. β-ionone (150 µM) in DMSO (1%) was added to the lysate and the plates were incubated 10 min at room temperature. Then NADPH (450 µM) solution was added and NADPH turnover was measured at room temperature at $A_{340\text{nm}}$ in spectrophotometer FLUOstar Model 403 (BMG LabTechnologie, Offenburg, Germany) and calculated using $\epsilon_M = 6200 \text{ M}^{-1} \text{ cm}^{-1}$. Background NADPH turnover without addition of the substrate was observed and used as a blank.

In all experiments the concentration of β-ionone was maintained higher than their K_d values (Truan and Peterson 1998) to get a maximum reaction rate.

Product analysis

After full consumption of NADPH in the reaction and before extraction, α-ionone in DMSO at final concentration of 15 µM was added to the reaction mixture as an internal standard. The entire mixture was extracted twice with 0,5 ml dichlormethane and the combined organic phases were evaporated to dryness and dissolved in 100 µl dichlormethane. GC-MS analysis was performed on Shimadzu GCMS-QP2010 (column: FS-Supreme-5, length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 µm) using helium as the carrier gas. Mass spectra were collected using electron ionisation. The column oven was programmed as follows: 70°C for one minute, 10°C per minute to 350°C. The compounds were identified by their

fragmentation pattern according 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. The exact product configuration was not detected.

The Michaelis-Menten parameters were determined by standard methods. All experiments were repeated at least three times.

Results

Construction of mutants by rational design

Cytochrome P450 BM-3 was found capable to hydroxylate β -ionone in the presence of NADPH, however with a very low turnover rate of $<1 \text{ min}^{-1}$ (Table 1). As β -ionone is a bulky molecule compared to the natural substrates of P450 BM-3 fatty acids, we decided to enlarge the binding pocket around the heme by means of site-directed mutagenesis. Position 87 of P450 BM-3 has already been proven to be important in the hydroxylation not only of fatty acids (Oliver et al. 1997) but also of several artificial substrates (Graham-Lorence et al. 1997; Li et al. 2001b). Phenylalanine 87 is located directly above the heme and controls activity, regio- (Graham-Lorence et al. 1997) and enantioselectivity of P450 BM-3 (Li et al. 2001b). We tested a set of mutants (Appel et al. 2001; Lentz et al. 2001) on their activity towards β -ionone, and have identified three single mutants – F87V, F87A and F87G, with significantly higher activity towards β -ionone compared to the wild type enzyme.

While the F87A mutant showed a higher NADPH turnover during β -ionone hydroxylation compared to the F87V mutant ($\sim 380 \text{ min}^{-1}$ vs $\sim 300 \text{ min}^{-1}$), the latter led to a higher 4-hydroxy- β -ionone production under the same reaction conditions ($\sim 93 \text{ min}^{-1}$ compared to $\sim 115 \text{ min}^{-1}$). This suggested a higher coupling between NADPH oxidation and substrate hydroxylation for the F87V mutant (Table 1). As described previously, not all the NADPH consumed by the reductase is utilized for substrate oxidation (Gorsky et al. 1984). On this

reason, the rates of 4-hydroxy- β -ionone formation are always lower than corresponding NADPH turnover rates.

Glycine at position 87 influenced the enzyme activity positively, but not to such a high extent as alanine or valine; the biggest difference is observed for coupling efficiency (Table 1). Valine and alanine like phenylalanine belong to the same group of non-polar hydrophobic amino acids as, in contrast to glycine, that is a small polar amino acid and demonstrates a different effect on the interaction with a substrate.

To our knowledge, relatively little is known from literature about the metabolic fate of β -ionone. The metabolism of β -ionone in rabbits leads to four oxygenated derivatives (Ide and Toki 1970). *Aspergillus niger* converts β -ionone to two major products – 4-hydroxy- β -ionone and 2-hydroxy- β -ionone and several minor ones. Wild type P450 BM-3 and its mutants produced 4-hydroxy- β -ionone as a single product (Fig. 1). This product was identified by comparison with authentic 4-hydroxy- β -ionone and 3-hydroxy- β -ionone on GC-MS. Retention time for 4-hydroxy- β -ionone is 5.06 min and for 3-hydroxy- β -ionone 5.2 min. The obtained fragmentation pattern corresponds completely to that of 4-hydroxy- β -ionone. The high regioselectivity of P450 BM-3 in this reaction is remarkable. This monooxygenase as well as its mutants are known to oxidize saturated fatty acids (Truan et al. 1999) and alkanes (Glieder et al. 2002) in subterminal (ω -1, ω -2, ω -3) positions, producing different hydroxylated products. Also polycyclic aromatic hydrocarbons (Carmichael and Wong 2001) and terpenes (Wong et al. 2000) are oxidized by the P450 BM-3 enzymes at different positions. Interestingly the regioisomer of β -ionone – α -ionone, was hydroxylated by the P450 BM-3 triple mutant A74G F87V L188Q to four different products – 2-; 4-; 3- and 7-hydroxy- α -ionone (Appel et al. 2001). When α -ionone was used as a substrate for the F87V mutant, the corresponding mixture of four product was obtained. β -ionone molecule has more rigid structure than α -ionone. This might cause a stronger fixation of β -ionone in the access

channel of the enzyme and different binding orientations for these two isomers. As a consequence – different product patterns.

For further activity improvement methods of site-directed and random mutagenesis were applied. The analysis of the structure and the comparison of substrate-bound (Li and Poulos 1997) and substrate-free (Hoch et al. 2000) forms of P450 BM-3 revealed the importance of the Y51 and R47 residues at the entrance of the substrate channel. The substitution of these amino acids (in combination with other mutations) alters the enzyme's activity and specificity as described previously (Carmichael and Wong 2001; Cowart et al. 2001). Because the side chains of the R47 and Y51 might interact with substrate molecules, the effect of their substitution has been examined. As β -ionone is highly hydrophobic the main idea was to increase hydrophobicity at the entrance of the binding pocket, making the diffusion of substrate further into the active site easier. For this purpose we changed the basic side chain arginine 47 to non-polar leucine and polar tyrosine 51 to hydrophobic phenylalanine. The double mutant R47L Y51F exhibited a very low activity towards β -ionone, but after introduction of an additional F87V substitution a 2,5-fold increased NADPH turnover rate ($\sim 820 \text{ min}^{-1}$) and a 2-fold increased hydroxylation activity ($\sim 270 \text{ min}^{-1}$) compared to the F87V mutant were observed (Table 1).

3.2. Construction of 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 and two libraries of 1500 and 2500 clones, correspondingly, were screened. As activity assay a monitoring of NADPH turnover was used. The following detailed activity analysis revealed an uncoupling for all mutants.

After screening 1500 transformants in the first library one mutant was identified with higher activity towards β -ionone. The sequence analysis revealed, that proline at position 386 was

substituted by serine. The for this double mutant evolved NADPH oxidation rate with β -ionone was 2-fold faster compared to the parent enzyme F87V ($\sim 645 \text{ min}^{-1}$ vs $\sim 310 \text{ min}^{-1}$) and almost 25-fold faster compared to the wild type P450 BM-3 ($\sim 645 \text{ min}^{-1}$ vs $\sim 25 \text{ min}^{-1}$). 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 FMN to the heme iron or to a long-range effect on protein structure.

The gene encoding for the mutant F87V P386S was used as a template for generating the second error-prone PCR library. Two thousands five hundred colonies were screened by monitoring of NADPH turnover, revealing four highly active variants. These four candidates were selected for further detailed characterization. After protein expression, purification and the calculation of specific activity only one mutant was found to be more active than the parent. 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 compared to the F87V enzyme, what is comparable with the R47L Y51F F87V mutant activity in terms of both the NADPH oxidation and the β -ionone hydroxylation (Table1). GC-MS analysis revealed in both cases only 4-hydroxy- β -ionone as a hydroxylated product.

Coupling efficiencies of 30-35%, calculated for all mutants containing the mutation F87V are similar to that of the F87V mutant. This observation allows a strong suggestion that this substitution minimizes the uncoupling effects during hydroxylation.

3.3. Combining the best mutations in one variant

Two mutants were constructed with improved activity towards β -ionone: the R47L Y51F F87V designed rationally and the A74E F87V P386S obtained after error-prone PCR. In an attempt to enhance the activity of P450 BM-3 further all five substitutions were combined together, resulting in a new variant R47L Y51F A74E F87V P386S. However, an expected

improvement has not been observed. Although the NADPH turnover rate was more than 1000 min^{-1} , the rate of product formation was approximately 200 min^{-1} . This means, that coupling efficiency was in average double as low as for the parent mutants. Taken together, the results indicate that the new mutant demonstrates an increased NADPH turnover rate, however the β -ionone hydroxylation is highly uncoupled.

The results of this work demonstrate, that P450 BM-3 can be engineered by methods of either rational design or directed evolution leading to the enzyme variants capable the oxidation of β -ionone with high activity and rather high coupling efficiency. Although β -ionone has no similarity with fatty acids, the substrate channel of the enzyme can be “adapted” for such small molecules.

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Table 1

Rate and coupling of β -ionone hydroxylation by P450 BM-3 and its mutants

P450 BM-3 Mutants	NADPH turn. rate (min^{-1})	Rate of product formation (min^{-1})	Coupling to NADPH (%)
WT	24 \pm 6	1 \pm 0,5	3,3
F87A	380 \pm 16	93 \pm 8	26
F87V	312 \pm 8	115 \pm 4	37
F87G	144 \pm 12	42 \pm 4	30
R47L Y51F	48 \pm 6	2 \pm 0,5	4
F87V R47L Y51F	816 \pm 8	268 \pm 10	31
F87V P368S	645 \pm 7	190 \pm 4	29
F87V A74E P368S	792 \pm 10	280 \pm 13	35
F87V A74E P368S R47L Y51F	1034 \pm 21	180 \pm 8	17

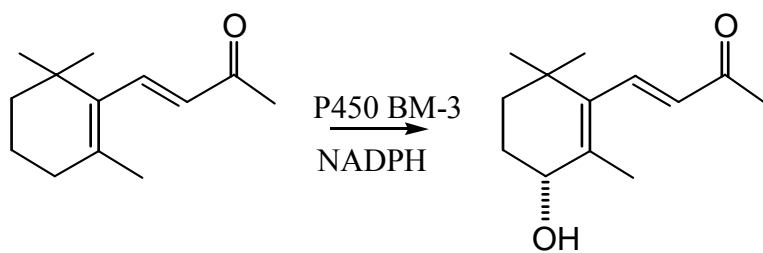


Fig. 1. Hydroxylation of β -ionone to 4-hydroxy- β -ionone by P450 BM-3 and its mutants.