

Laboratory Evolution of Cytochrome P450 BM-3 Monooxygenase for Organic Cosolvents

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Abstract: Cytochrome P450 BM-3 (EC 1.14.14.1) catalyzes the hydroxylation and/or epoxidation of a broad range of substrates, including alkanes, alkenes, alcohols, fatty acids, amides, polyaromatic hydrocarbons, and heterocycles. For many of these notoriously water-insoluble compounds, P450 BM-3's K_m values are in the millimolar range. Polar organic cosolvents are therefore added to increase substrate solubility and achieve high catalytic efficiency. Using P450 BM-3 as a catalyst for these important transformations requires that we improve its ability to tolerate the cosolvents. By directed evolution, we improved the activity of P450 BM-3 in the presence of dimethylsulfoxide (DMSO) and tetrahydrofuran (THF), achieving increases in specific activity up to 10-fold in 2% (v/v) THF and 6-fold in 25% (v/v) DMSO. The engineered P450 BM-3's are also significantly more resistant to acetone, acetonitrile, dimethylformamide, and ethanol as cosolvents in the reaction. © 2004 Wiley Periodicals, Inc.

Keywords: P450 BM-3; CYP102; *Bacillus megaterium*; organic solvent resistance; random mutagenesis; directed evolution

INTRODUCTION

Monooxygenases are impressive for their ability to catalyze the insertion of oxygen into nonactivated C-H bonds, a reaction that is difficult to achieve chemically with high selectivity, especially in water, at room temperature and under atmospheric pressure. For many P450-catalyzed reactions no chemical catalysts come close to the enzymes in terms of turnover rate and selectivity. The enzymes, however, are often only poorly active towards nonnatural substrates (Li et al., 2000) and cannot tolerate common bioprocess conditions, including organic cosolvents (Kuhn-Velten, 1997).

The fatty acid hydroxylase from *Bacillus megaterium*, P450 BM-3 (CYP102A1), and its Phe87→Ala (F87A) mutant (Carmichael and Wong, 2001; Cirino and Arnold, 2002; Lentz et al., 2001; Li et al., 2001; Noble et al., 1999; Oliver

et al., 1997; Rock et al., 2002) are water-soluble heme enzymes of M_r 118 kDa (Narhi and Fulco, 1986, 1987). As a natural fusion protein, P450 BM-3 contains its heme domain and reductase on a single polypeptide chain. Its catalytic efficiency is high compared to other P450s; it is also more stable and easier to handle than many other P450s, particularly the membrane-associated eukaryotic enzymes. P450 BM-3 and various engineered mutants catalyze subterminal hydroxylation of short-, medium-, and long-chain fatty acids and alkanes, epoxidation of medium- and long-chain unsaturated fatty acids, as well as hydroxylation of polyaromatic compounds and heteroarenes (Appel et al., 2001; Boddupalli et al., 1990; Capdevila et al., 1996; Carmichael and Wong, 2001; Li et al., 2000, 2001; Miura and Fulco, 1975; Ost et al., 2000). Numerous P450 BM-3 reaction products, such as indigo, catechols, and hydroxylated alkanes, have a significant market value. One serious drawback, however, is that the K_m values of P450 BM-3 for nonnatural substrates often fall in the millimolar range. High substrate concentrations are therefore required to achieve high rates of reaction, and these concentrations greatly exceed the solubilities of the nonpolar substrates. The water miscible cosolvents added to increase substrate solubility have severe negative effects on the catalytic activity of P450 BM-3 (Schwaneberg, 1999a).

To increase the utility of P450 BM-3 as an oxidative biocatalyst, we have used methods of directed evolution (Chen and Arnold, 1993) to create variants that can operate efficiently in aqueous solutions containing an organic cosolvent. We have discovered mutations that significantly improve the activities of P450 BM-3 and its F87A mutant in the presence of acetone, acetonitrile, dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol, and tetrahydrofuran (THF).

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical reagent grade or higher quality and were purchased from Fluka (Ronkonkoma, NY),

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Sigma (St. Louis, MO), or Aldrich (Milwaukee, WI). THF (Aldrich, 99.9%) and DMSO (Mallinckrodt, St. Louis, MO; 99.9%) were of highest available purity grade. Enzymes were purchased from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA), and Roche Diagnostics (Indianapolis, IN).

Cultivation and Expression in 96-Well Plates

The P450 BM-3 and P450 BM-3 F87A genes are under the control of the strong temperature-inducible P_{RPL} -promoter (Belev et al., 1991). Mutated P450 BM-3 F87A variants were cloned into the pUSC1BM3 vector (Schwaneberg, 1999b) by using *Bam*HI//*Eco*RI or *Age*I//*Eco*RI restriction sites. Transformed clones grown on LB_{amp} plates (Genetix; Queensway, Hampshire, UK) were transferred with a colony picker (QPix; Genetix) into 96-well plates (flat bottom; Rainin, Emeryville, CA) containing 120 μ l LB culture supplemented with 12 μ g ampicillin per well. After growth for 12 h at 37°C in an incubator (280 rpm), 3 μ l of each culture was transferred with a grooved 96-pin deep-well replicator tool (V&P-Scientific, San Diego, CA) into 2 ml deep-well plates (Becton Dickinson, Bridgeport, NJ) containing 400–500 μ l of enriched TB_{amp} medium. TB_{amp} solution was supplemented with 75 μ l trace element solution (0.5 g CaCl₂ · 2H₂O, 0.18 g ZnSO₄ · 7H₂O, 0.10 g MnSO₄ · H₂O, 20.1 g Na₂-EDTA, 16.7 g FeCl₃ · 6H₂O, 0.16 g CuSO₄ · 5H₂O, 0.18 g CoCl₂ · 6H₂O, add 1 l dH₂O and autoclave) and 2 mg aminolevulinic acid per 50 ml TB. *Escherichia coli* cells were grown in this medium for 6 h at 37°C then induced for 14 h at 42°C. All deep-well plates were covered with a taped lid. The original LB_{amp} plates were stored until further use at –80°C after adding to each well 100 μ l glycerol (sterile, 50% (v/v)).

Screening Procedures

All experiments were performed in organic solvent-resistant polypropylene flat-bottom 96-well plates (Greiner Bio-one, Monroe, NC). Blank pre readings were recorded for each plate prior to assay procedures.

Rapid Screen for Catalytic Activity With and Without Cosolvent

Deep-well cultures were mixed using a liquid handling machine (Multimek 96; Beckman Coulter, Fullerton, CA) and a 90 μ l cell culture was transferred to each well of reference and assay plate. Forty μ l Tris/HCl buffer (25 mM, pH 7.5, containing 200 μ M polymyxin B) and 5 μ l 12-pNCA (15 mM, dissolved in DMSO) were pipetted to each well using the liquid handling machine. Additionally, 4.5 μ l THF or 45 μ l DMSO were transferred to each well of the assay plate. After 12 min incubation, 12-pNCA conversion was initiated by adding 20 μ l of an isocitrate cofactor regeneration solution (isocitric acid sodium salt (20 mM) dissolved in dH₂O and supplemented with NADP⁺ (3 mM) and isocitric dehydrogenase (0.8 U/ml)); total reaction volume was 160 μ l for THF and 200 μ l for DMSO. The reaction was stopped after

visible color development by the addition of 50 μ l NaOH (1.5 M). After 8–12 h incubation and removal of the bubbles using a Bunsen burner, the absorption of the clear solution at 410 nm was recorded. P450 BM-3 variants with high activity in both the presence and absence of organic cosolvent were rescreened.

Rescreening Procedure

In this second screen, deep-well cultures were centrifuged at 4,400g for 10–20 min to remove the brownish TB medium. The cell pellets were frozen overnight at –20°C and resuspended in 200 μ l lysomix (pH 7.5, 25 mM Tris/HCl or 25 mM K_xPO₄, supplemented with 50 mg lysozyme (Sigma) per 100 ml). Ninety μ l of cell suspension was transferred to the reference and assay plates using a 12-channel pipettor (Eppendorf, Hamburg, Germany). To lyse the *E. coli* cells, the plates were incubated at 37°C for 1 h; 30 μ l Tris/HCl buffer (25 mM) and 5 μ l 12-pNCA (15 mM, dissolved in DMSO) were pipetted to each well using the liquid handling machine. Fifteen μ l THF solution (15% (v/v); dH₂O) was added to each well of the assay plate. After 12 min incubation, 12-pNCA conversion was initiated by adding 20 μ l of the isocitrate cofactor regeneration solution. Total reaction volume was 160 μ l. The reaction was stopped after visible color development by the addition of 100 μ l UT-buster (NaOH 1.5 M, 1.5 M urea, 50% (v/v) DMSO). After 8–12 h incubation and removal of the bubbles using the Bunsen burner, the absorption at 410 nm was recorded. P450 BM-3 variants with high activity in both the presence and absence of organic cosolvent were cultured and expressed in shaking flasks for further characterization.

Shake Flask Cultures and Enzyme Purification

Fifty and 500 ml cultures were inoculated with a 1:100 dilution of an overnight LB culture of recombinant *E. coli* XL-10 Gold cells harboring mutated P450 BM-3 F87A variants on the pT-USC1BM3 plasmid. The cultures were shaken at 300 rpm at 37°C. At an OD₅₇₈ = 0.8–1, P450 BM-3 expression was induced by increasing the temperature to 42°C. After 8 h, the cells were harvested by centrifugation at 4–8°C. The cell pellets were resuspended in Tris-HCl (5–15 ml, 25 mM, pH 7.5) or K_xPO₄ (5–15 ml, 25 mM, pH 7.5) and lysed by sonication (3 × 2 min; output control = 5, duty cycle = 40%). The lysate was centrifuged at 23,300g for 30 min. The supernatant was further cleared through a low protein binding filter (0.45 μ m; Millex-HV Syringe-driven filter unit; Millipore, Bedford, MA). The filtrate was loaded on a SuperQ650M anion exchanger column (TosoH, Montgomeryville, PA) and purified as described previously (Schwaneberg et al., 1999b).

Photometric Enzyme Assays

All photometric assays were carried out under aerobic conditions. UV/vis measurements were performed in a

Shimadzu spectrophotometer (BioSpec-1601; Pleasanton, CA). P450 BM-3 F87A concentrations were measured by CO-difference spectra, as reported by Omura and Sato (1964) using $\epsilon = 91 \text{ mM}^{-1}\text{cm}^{-1}$. Conversion of the *p*-nitrophenoxydodecanoic acid (12-pNCA) was monitored at 410 nm using a ThermomaxPlus plate reader (Molecular Devices, Sunnyvale, CA) and an $\epsilon = 13,200 \text{ M}^{-1}\text{cm}^{-1}$ (Schwaneberg et al., 1999a). The pNCA-assay system allows continuous photometric detection of the P450 BM-3 activity with almost no background reaction, even in crude cell lysates (Schwaneberg et al., 1999a).

Mutagenesis

A thermocycler PTC 200 (MJ Research, San Francisco, CA) and thin-wall PCR tubes were used in all PCRs. The total reaction volume was always 50 μl . P450 BM-3 F87A mutants were generated in the first round of error-prone PCR (94°C for 4 min 1 cycle; 94°C for 70 sec/55°C for 90 sec / 72°C for 4 min 30 cycles; 72°C for 10 min 1 cycle) employing 27 pmol primers (5'-GAACCGGATCCATGACAATTAAGAAATGC-3'//5'-CTATTCTCACTCCGCTGAAACTGTTG-3'), 0.04 mM MnCl_2 , 5 U Taq Polymerase (Roche Diagnostics), 0.2 mM dNTP, and 1 μl plasmid DNA (Miniprep; Qiagen, Valencia, CA).

At selected positions saturation mutagenesis was performed using the following PCR conditions: 94°C for 4 min 1 cycle; 94°C for 75 sec / 55°C for 75 sec / 68°C for 16 min 20 cycles; 68°C for 20 min 1 cycle. For the PCR reaction 2.5 U Pfu-Polymerase (Stratagene), 0.2 mM dNTP, 1 μl plasmid DNA (1:20 diluted with dH_2O ; Miniprep, Qiagen) and 17.5 pmol of each primer were used. The primers were: for aa-position 235 (5'-GCGATGATTATTANNNCATATGCTAAACGGA-3'//5'-TCCGTTTAGCATATGNNNTAATAAATCATCGC-3'), for aa-position 471 (5'-CAGTCTGCTAAAAAAGTANNNAAGGCACTCTGCTAAAAAAGTANNNAAGGCA-GAAAACGC-3'//5'-GCGTTTTCTGCCTTTTTNNNTACTTTTTAGCAGACTG-3'), and for aa-position 1024 (5'-GACGTTACCAAGTGNNNGAAGCAGACGCTCGC-3'//5'-GCGAGCGTCTGCTTCNNNCACTTGGTGAACGTC-3'). In the evolved P450 BM-3 variants position 87 was back-mutated to the wildtype sequence (phenylalanine) using these saturation mutagenesis conditions except for a modified annealing temperature (60°C instead of 55°C) and the primers (5'-GCAGGAGACGGGTTATTACAAGCTGGACG-3'//5'-CGTCCAGCTTGTAATAACCCGTCTCCTGC-3'). For the second mutant generation the Stratagene GeneMorph kit was used. The PCR reaction (95°C for 30 sec 1 cycle; 95°C for 30 sec / 55°C for 30 sec / 72°C for 3 min, 30 sec 30 cycles; 72°C for 10 min 1 cycle) was done in the presence of 2.5 U Mutazyme, 0.2 mM dNTP, 20 pmol of primers (5'-GAACCGGATCCATGACAATTAAGAAATGC-3'//5'-CTATTCTCACTCCGCTGAAACTGTTG-3'), and 2.5 μl plasmid DNA (Miniprep, Qiagen).

RESULTS AND DISCUSSION

Wildtype P450 BM-3 is stable at ambient temperature in the presence of cosolvents, at least within the time scale of the kinetic experiments (5 min). Its catalytic activity, however, is reduced. We therefore refer to “organic solvent resistance” when we describe the behavior of evolved and parental enzymes. Resistant enzymes retain a larger fraction of their activity in the presence of a cosolvent, and we used the ratio of specific activity in the presence of organic cosolvent to that in the absence of cosolvent to compare the resistance of different mutants. To identify the P450 BM-3 variants resistant to organic solvents, the evolutionary experiment was performed under conditions designed to preserve the stability and high total activity of the parental enzyme. Using the P_{RPL} -promoter system (Belev et al., 1991) for enzyme expression, which is induced at 42°C, imposes a minimum thermal stability requirement.

To evolve the cosolvent-resistance of P450 BM-3, we started with P450 BM-3 mutant F87A, which has improved activity (4–5-fold faster conversion, 1.5-fold lower K_m) in the photometric assay employing *p*-nitrophenoxydodecanoic acid (12-pNCA) (Li et al., 2001; Schwaneberg et al., 1999a). Terminal hydroxylation of 12-pNCA leads to an unstable hemiacetal, which spontaneously dissociates at room temperature and releases the chromophore, *p*-nitrophenolate. The relatively high activity of P450 BM-3 F87A allows accurate activity measurements in 96-well plates, even in the presence of an organic cosolvent that reduces activity.

In this evolutionary process, we used two screens—a rapid screen followed by rescreening of the positive clones. The first assay system allowed screening of large libraries by using whole cells and avoiding time-consuming steps such as centrifugation. The cell-free rescreening assay, however, was more accurate because the brownish background of the TB medium was removed. The second screen served to eliminate false-positives and identify the most promising clones for further characterization in shake-flask cultures. In both systems, a cofactor regeneration system was implemented to reduce the NADPH cost. To avoid expression mutants, kinetics were measured in the absence (reference plate) and presence of organic cosolvent (assay plate). Both plates contained same amount of cells (rapid screen) or cell extract (rescreen). Resistant mutants with high activity were used as parents in the next generation.

First Mutant Generation

Random mutations were introduced into the P450 BM-3 F87A gene coding for 1,049 amino acids (heme and reductase domains) and a His₆ tag at the C-terminal end, under PCR conditions designed to generate an average of 1–2 amino acid substitutions per sequence. In the first round, 6,520 clones were tested in DMSO (22.5%) and THF (2.8%) using the 12-pNCA assay. From the 26 confirmed positives, two types of improved clones were discovered: 1) those with increased DMSO and reduced THF resistance (65.4%), and

2) those with increased resistance to both DMSO and THF (34.6%). No clones with increased THF and reduced DMSO resistance were found. For applications in organic synthesis, mutants with multiple solvent resistances are probably most promising. Therefore, two mutants from the second category, F87AB5 and F87APEC3, were selected for detailed analysis. Sequencing revealed three nonsynonymous mutations, two in F87AB5 ($T_{(ACG)}235A_{(GCG)}$; $S_{(AGT)}1024R_{(AGA)}$) and one in F87APEC3 ($R_{(CGC)}471C_{(TGC)}$). Interestingly, position 471 was also mutated in another clone, where substitution to serine rather than cysteine occurred. Clone F87APEC3 also contained two synonymous mutations. Mutations are summarized in Table I. The purified P450 BM-3 F87AB5 mutant has increased specific activity 3.7-fold at 10% (v/v) DMSO and 5.3-fold at 2% (v/v) THF, relative to F87A (Table IIA).

Saturation Mutagenesis

Point mutagenesis at low error rates explores only a very limited set of (primarily conservative) amino acid substitutions. We and others have observed that saturation mutagenesis performed at sites identified by error-prone PCR often generates further improvements. Taking double-mutant F87AB5 as the starting point, we used saturation mutagenesis to introduce all possible amino acid changes at position 471. Screening ~576 clones revealed the triple mutant F87ASB3, which is more resistant towards organic cosolvents (Table II) and is expressed at higher levels (result not shown) than its parent, F87AB5. The specific activity of F87ASB3 is comparable to F87AB5. Hence, F87ASB3 has significantly higher total activity compared to F87AB5 in the presence of organic cosolvents. Sequencing showed that $R_{(CGC)}471$ had been exchanged for $A_{(GCT)}$ (Table I). Saturation mutagenesis of position A235 of F87ASB3 generated no further improvements and five of the most active clones still contained alanine at position 235. Saturation mutagenesis at position R1024 of F87ASB3 led to the discovery of two clones with higher activity, F87ABC1F10 ($R_{(AGA)}1024T_{(ACG)}$) and F87ABC1B6 ($R_{(AGA)}1024K_{(AAA)}$). In comparison to F87A, variant F87ABC1F10 exhibits a 4.4-fold (10% (v/v) DMSO) and 7.9-fold (2% (v/v) THF) increase in specific activity (Table IIA).

Second Generation of Random Mutagenesis

In parallel with the saturation mutagenesis, further random mutagenesis was performed on F87ASB3. Mutazyme polymerase (GeneMorph® kit; Stratagene) generates a different mutational spectrum compared to the *Taq* polymerase used in the first round of error-prone PCR (Cline and Hogrefe, 2002). Screening ~1,440 clones yielded mutant F87A5F5, which contains mutations leading to amino acid substitutions $E_{(GAA)}494K_{(AAA)}$ and $R_{(AGA)}1024E_{(GAG)}$ (Table I). The unexpected simultaneous exchange of three nucleotides at position 1024 was confirmed by sequencing this position twice in F87ASB3 and F87A5F5. Compared to F87A, the specific activity of F87A5F5 is increased 5.5-fold at 10% (v/v) DMSO and 10-fold at 2% (v/v) THF (Table IIA).

Organic Solvent Resistance Profiles

The relative activities of purified parents and evolved mutants at various DMSO and THF concentrations are shown in Figures 1 and 2. For all the evolved mutants, these profiles are shifted to higher relative activities over nearly the entire range of investigated cosolvent concentrations. The improvements are not limited to the cosolvent concentration employed in the screening, and the order corresponds well with the order of relative activities obtained under HTS conditions. Thus, 30% (v/v) DMSO seems to be a critical value for all P450 BM-3 variants; above this concentration the relative activity is drastically reduced for all variants (Fig. 1).

Organic solvent resistance profiles of lysed crude extracts and purified monooxygenase are in very good agreement for DMSO, while relative activities in THF were slightly (3–9%) reduced for the purified enzyme. We can only speculate that hydrophobic compounds or other proteins in the crude lysates somehow protect the enzyme from the effects of the organic solvent.

Back-Mutation of Position 87

Wildtype cytochrome P450 BM-3 is significantly more resistant to organic cosolvents than the F87A variant that was used as the starting point for the evolution (Fig. 1C). Whether the mutations responsible for increased cosolvent

Table I. Nucleotide and amino acid substitutions in P450 BM-3 variants with increased resistance to organic solvents.

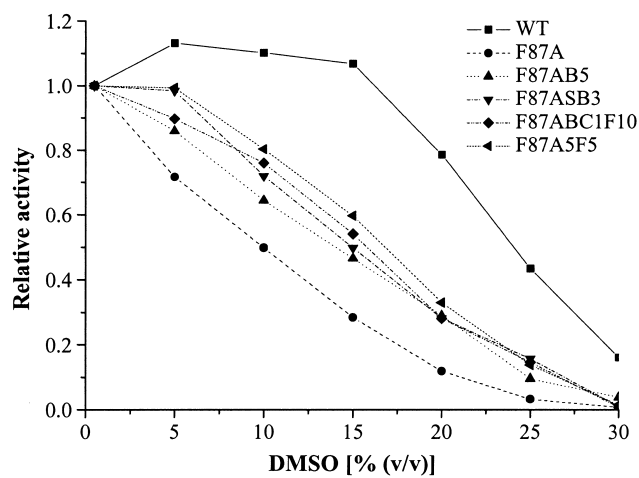
Clone	aa-Position				aa-Position		
	235	471	494	1024	97	595	884
	Nucleotide changes (amino acid changes)				Synonymous mutations		
Wildtype/F87A	ACG (T)	CGC (R)	GAA (E)	AGT (S)	AAA (K)	GAA (E)	CGC (P)
WB5/F87AB5	GCG (A)			AGA (R)			
WPEC3/F87APEC3		TGC (C)			AAG (K)	GAG (E)	
WSB3/F87ASB3	GCG (A)	GCT (A)		AGA (R)			
WBC1F10/F87ABC1F10	GCG (A)	GCT (A)		ACG (T)			
WBC1B6/F87ABC1B6	GCG (A)	GCT (A)		AAA (K)			
W5F5/F87A5F5	GCG (A)	GCT (A)	AAA (K)	GAG (E)			CCA (P)

Table II. Activities of P450 BM-3 variants towards 12-pNCA, in the absence and presence of organic cosolvents.

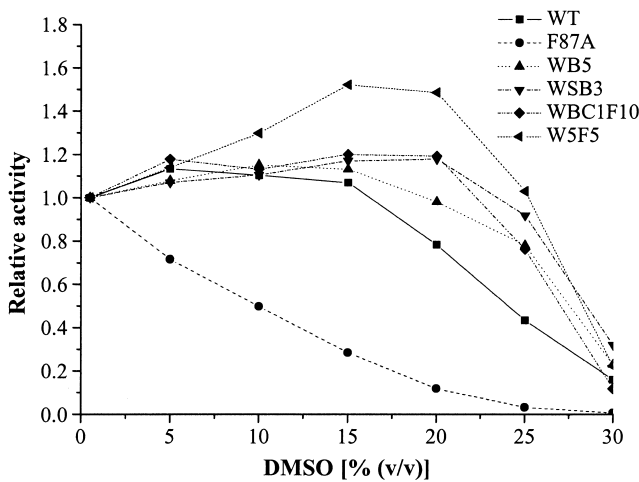
P450 BM-3 variant (purified enzyme)	F87A variants					Variants back-mutated to Phe at position 87									
	Specific activity in the absence of organic cosolvent (eq/min) ^a	Increase in specific activity in the absence of organic cosolvent	Specific activity in 10 % (v/v) DMSO (eq/min) ^a	Relative activity in 10 % (v/v) DMSO ^b	Increase in specific activity in 10 % (v/v) DMSO	Specific activity in 2 % (v/v) THF (eq/min) ^a	Relative activity in 2 % (v/v) THF ^b	Increase in specific activity in 2 % (v/v) THF	Specific activity in the absence of organic cosolvent (eq/min) ^a	Increase in activity in the absence of organic cosolvent	Specific activity in 25 % (v/v) DMSO (eq/min) ^a	Relative activity in 25 % (v/v) DMSO ^b	Increase in specific activity in 25 % DMSO	Specific activity in 2 % (v/v) THF (eq/min) ^a	Relative activity in 2 % (v/v) THF ^b
F87A	508 ± 15	1.0	253 ± 10	0.50	1.0	44 ± 3	0.09	1.0	114 ± 6	1.0	49 ± 3	0.43	40 ± 2	0.35	1.0
F87AB5	1450 ± 36	2.9	934 ± 27	0.64	3.7	231 ± 9	0.16	3.7	117 ± 6	1.0	92 ± 5	0.78	39 ± 2	0.33	1.0
F87ASB3	1290 ± 35	2.5	928 ± 27	0.72	3.7	250 ± 10	0.19	3.7	166 ± 7	1.5	153 ± 7	0.92	69 ± 4	0.41	1.7
F87ABC1F10	1460 ± 37	2.9	1110 ± 30	0.76	4.4	347 ± 12	0.24	4.4	229 ± 10	2.0	175 ± 8	0.76	92 ± 5	0.40	2.3
F87A5F5	1730 ± 35	3.4	1390 ± 35	0.80	5.5	439 ± 14	0.25	5.5	286 ± 11	2.5	294 ± 12	1.03	135 ± 6	0.47	3.4

^aAll kinetic measurements (total reaction volume 1 ml) were done in 2.5 ml cuvettes. The measurements were repeated three times. Values reported were the average of three measurements and average deviation from mean value. Specific activity is amount of product formed per unit time per unit protein (Eq/min). Eq/min is the number of molecules of 12-pNCA converted per min per molecule of P450 BM-3.

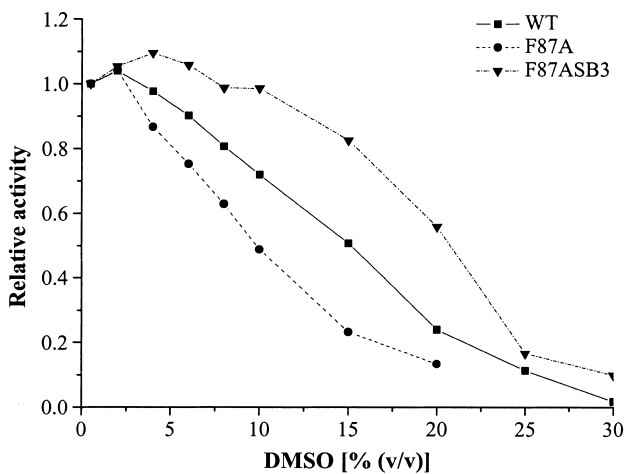
^bRelative activity is the ratio of specific activity in the presence of organic cosolvent to that in the absence of organic cosolvent. Trace amounts of DMSO used to dissolve the 12-pNCA substrate were not taken into account.



(a)



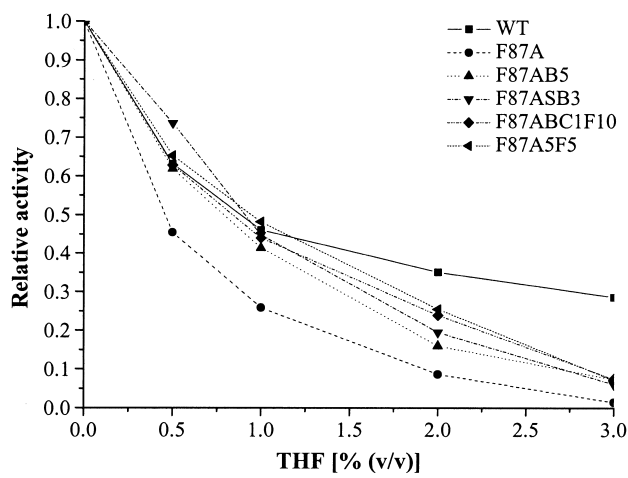
(b)



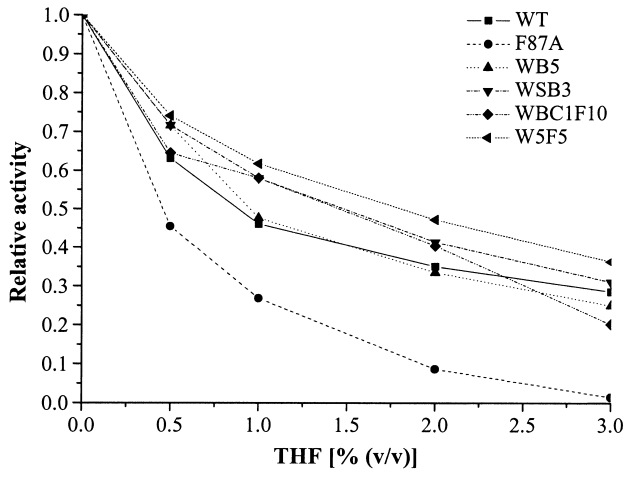
(c)

Figure 1. Relative activities of P450BM3 variants as a function of DMSO concentration; **a:** F87A variants. **b:** Variants back-mutated to Phe at position 87. **c:** Purified enzymes. Relative activity is the ratio of specific activity in the presence of organic cosolvent to that in the absence of organic cosolvent.

resistance in F87A also increase the activity of the wildtype enzyme in organic cosolvents was investigated by mutating



(a)



(b)

Figure 2. Relative activities of P450 BM-3 variants as a function of THF concentration; **a:** F87A variants. **b:** Variants back-mutated to Phe at position 87. Relative activity is the ratio of specific activity in the presence of organic cosolvent to that in the absence of organic cosolvent.

position A87 back to phenylalanine in the evolved sequences. (These back-mutated variants are labeled with a “W” instead of F87A in Table IIB.) Figures 1B and 2B show that these back-mutated enzymes have improved organic solvent resistance, especially for DMSO. Compared to wildtype, variant W5F5 (parent F87A5F5) has almost 6-fold higher specific activity in 25% DMSO (3.4-fold in 2% THF) (Table IIB). The back-mutated variants retain the same rank order of resistance as the F87A variants. However, Table IIA,B show smaller improvements for the wildtype mutants in THF.

Other Water Miscible Cosolvents

The organic solvent resistance of the parents, P450 BM-3 and P450 BM-3 F87A, and evolved mutants W5F5 and F87A5F5 at various concentrations of acetone, acetonitrile, DMF, and ethanol are shown in Figure 3. Resistance profiles of the evolved enzymes were shifted toward higher solvent concentrations for all four cosolvents. As observed for THF

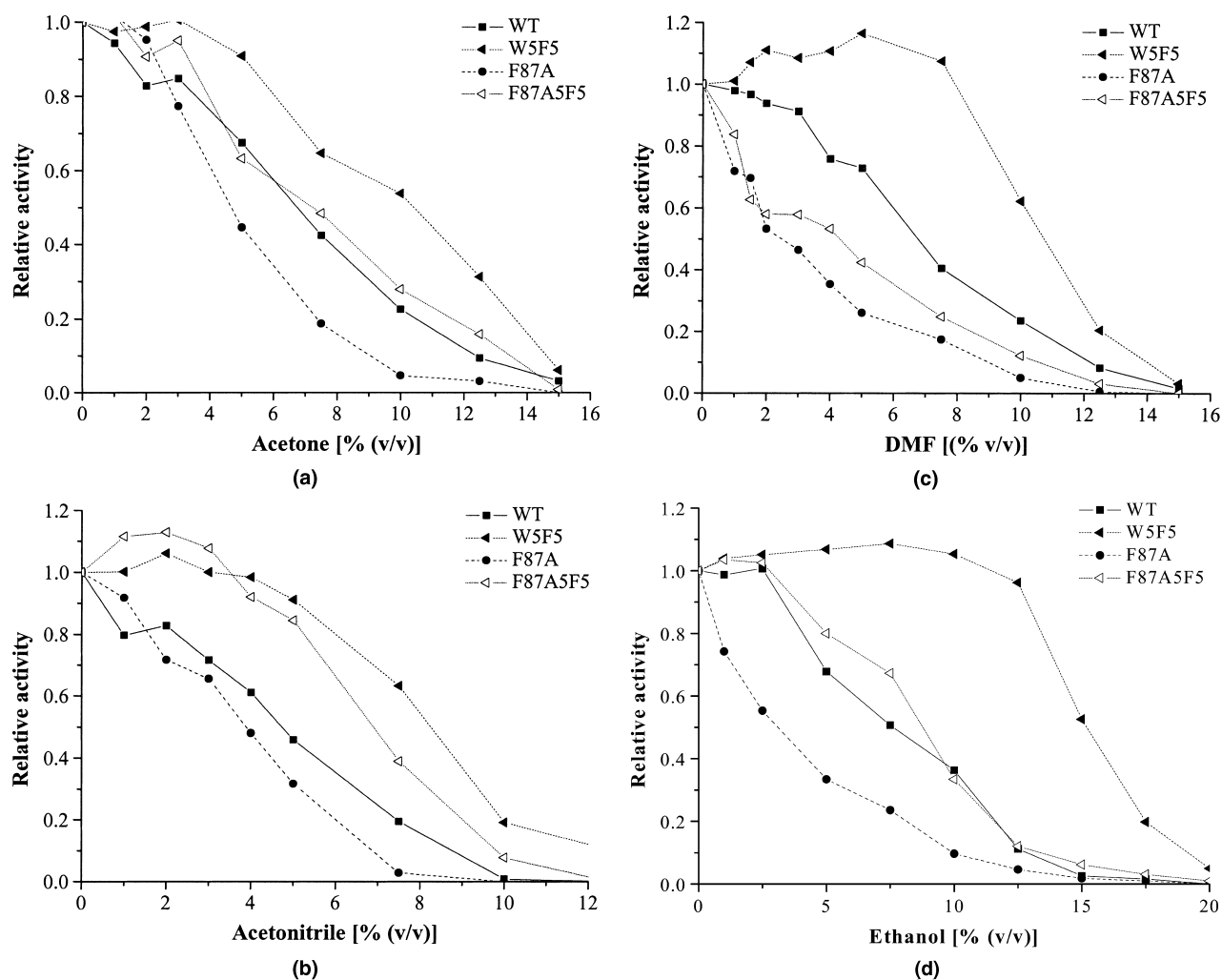


Figure 3. Relative activities of P450 BM-3 variants as a function of organic solvent concentration; **a:** Acetone. **b:** Acetonitrile. **c:** DMF. **d:** Ethanol. Relative activity is the ratio of specific activity in the presence of organic cosolvent to that in the absence of organic cosolvent.

and DMSO, variant W5F5 can tolerate significantly higher concentrations of all four cosolvents than the wildtype and F87A5F5; similarly, variant F87A5F5 resists higher cosolvent concentrations than its parent F87A. The improvements obtained for acetone, acetonitrile, DMF, and ethanol are comparable to those for THF and DMSO. Hence, evolution for resistance to DMSO and THF allowed simultaneous improvement in resistance to multiple cosolvents.

Total Activities of Evolved Enzymes

All the P450 BM-3 variants revealed increased specific activities for 12-pNCA (up to 3.4-fold without organic cosolvent; Table IIA,IIB). Three expression studies in 50-ml shake flasks, where all the mutants were expressed simultaneously in the same incubator, also showed higher expression for all mutants compared to wildtype and F87A, with the exceptions of F87AB5 and WB5. F87AB5 and WB5 reached 70–85% of the parent expression. Expression levels of W5F5 and F87A5F5 were between 1.6- and 3.8-fold higher than F87A and wildtype. Hence, the mutants evolved

for function in aqueous-organic solvent exhibit both improved organic solvent tolerance and higher expression. Activities reported in Table IIA,IIB do not include the contribution of improved expression levels because the exact values vary with cultivation conditions. Hence, total activities (amount of product formed per unit time) of mutants were not reported here even though they were significantly higher than that of wildtype.

Structure – Function Relationships

Mutants containing a small alanine residue at aa-position 87 have significantly lower organic solvent resistance compared to the P450 BM-3 variants having phenylalanine at this position (Figs. 1B, 2B). The bulky phenylalanine, which lies perpendicular to the catalytic heme center, likely restricts the access of organic cosolvent to the heme (Graham-Lorence et al., 1997). The first of the mutations discovered to improve resistance to organic solvent (aa-position 235) is on the surface of the heme domain in the H-helix; this residue protrudes towards and may interact

with the reductase domain. The linker region between the heme and reductase domains apparently plays a pivotal role in the organic solvent resistance of P450 BM-3. Amino acid position 471 was also found to be a preferred mutation site; this is the last amino acid of the P450 BM-3 heme domain and connects the heme domain to the linker region (Govindaraj and Poulos, 1995). Yet another important residue was identified in close proximity, aa-position 494, which is located in the linker region. Electron transfer and functionality requires a precise spatial orientation of the heme and reductase domains. A crystal structure of the P450 BM-3 that includes the linker region and the complete reductase on one polypeptide chain is not available; it is thus difficult to predict the influence of our mutations on the molecular level. However, we are confident in assuming that these beneficial mutations serve to preserve a functional, active orientation between the two domains.

No structural information is available for aa-position 1024 of P450 BM-3. We can only speculate that this residue is also involved in the interaction between reductase and heme domains, electron transfer, or protection, for example, of the solvent-accessible FMN domain (Murataliev et al., 1997).

CONCLUSIONS

Monooxygenases such as P450 BM-3 catalyze beautiful oxidative reactions and the results we achieved prove that laboratory evolution offers a fast and elegant way to improve the organic cosolvents resistance of this exceptional catalyst and to bring P450 BM-3 a step closer to applications in chemical syntheses.

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