Greatly reduced amino acid alphabets in directed evolution: Making the right choice for saturation mutagenesis at homologous enzyme positions

Manfred T. Reetz* and Sheng Wu

Protein sequences alignment of BVMOs

Fig 1 Multiple sequence alignment of eight BVMOs. The sequences are: phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* (Q47PU3), steroid monooxygenase (STMO) from *Rhodococcus rhodochrous* (BAA24454), cyclohexanone monooxygenase (CHMO) from *Acinetobacter sp. NCIMB9871* (BAA86293), cyclohexanone monooxygenase 1 (CHMO1) from *Brevibacterium sp. HCU* (AAG01289), cyclohexanone monooxygenase 2 (CHMO2) from *Brevibacterium sp. HCU* (AAG012690), cyclohexanone
monooxygenase 3 (CHMO3) from *Acinetobacter* sp. (P12015), cyclopentanone monooxygenase (CPMO) from *Comamonas testosterone* (CAD10798), and cyclododecanone monooxygenase (CDMO) from *Rhodococcus ruber* (AAL114233).

**Mutant Library Preparation**

The plasmid pPAMO contains the WT PAMO gene under the control of the P_{BAD} promoter.$^1$ The saturation mutagenesis library was created by the QuikChange™ PCR method with the template pPAMO and two complementary primers PAMO Lib441-444 (5’-CAGGCCCGGGCAGCCCGKCAKBGBDCNSCAACATGCTGGTCTCTAT-3’) and rePAMO Lib441-444 (5’-ATAGAGACCACGATGTTGSNGHVCVMTGMCCGGCTGCCCCGGGCTG-3’). The reaction (25 µL final volume) contained: 10×KOD buffer (2.5 µL), MgCl$_2$ (1 µL, 25 mM), dNTP (2.5 µL, 2 mM each), primers (2.5 µL, 2.5 µM each), template plasmid (1 µL, 10 ng µL$^{-1}$) and 0.5 units of KOD hot star polymerase. The PCR cycle consisted of an initial denaturation step at 94°C for 3 min followed by cycling at 94°C for 1 min and 72°C for 14 min for 15 cycles, then a final elongation for 35 min at 72°C. The template plasmid in the PCR amplification reaction was removed by digestion with 1 unit of Dpn I (New England Biolabs) in 2.5 µL of NEB buffer 4 for 2–3 h at 37°C. The resulting PCR product was used to transform into electrocompetent *E. coli* TOP10 cells. The cells were spread on LB agar plates containing 100 µg mL$^{-1}$ carbenicilline.

**Library Screening**

Individual colonies were placed into 2.2-mL 96-deep-well plates containing 800 µL of LB media with 100 µg mL$^{-1}$ carbenicilline by a colony picker QPIX (Genetix, New Milton, UK). After cell growth at 37°C overnight with shaking at 800 rpm, 10 µL of each preculture was transferred into a new plate containing 800 µL of TB media supplemented with 0.1% L-arabinose as inducer and 100 µg mL$^{-1}$ carbenicilline. The duplicate plates were grown for additional 24 h to induce PAMO expression. The cultures were centrifuged at 4000 rpm and 4°C for 6 min and the supernatants were discarded. The original plates were stored. Each cell pellet was resuspended in 600 µL of 50 mM Tris-HCl (pH 8.0) containing 1 mg mL$^{-1}$ lysozyme and 4 units of Dnase I. Lysis were performed at 37°C and 800 rpm for 3 h. Cell debris was precipitated by
centrifugation at 4000 rpm and 4°C for 30 min and 50 µL of each cleared supernatant transferred to a 1.1-
mL 96-deep-well plate. Then in each well, 50 µL of the secondary alcohol dehydrogenase (2° ADH) crude
extract (about 10 U)\(^2\), 10 µL of 1 mM NADP\(^+\), 10 µL of 100 mM rac-1a in acetonitrile and 380 µL of 50
mM Tris-HCl (pH 8.0) containing 5 mM isopropanol were added. The reaction plates were incubated at
37 °C and 800 rpm for 24 h. 400 µL of ethyl acetate was then added to each well, and a plastic cover was
used to cover the plate tightly. The plate was vibrated vigorously to extract the substrate and product from
the solution. After centrifugation, 200 µL of organic layer in each well was transferred into a new glass-
made 96-deep-well plate, and subjected to GC analysis for medium-throughput screening. The WT PAMO
did not show any activity with regard to rac-1a under the screening conditions. Mutants with more than
20% conversion were collected and the results reproduced by measuring their activity using whole-cell
catalysis.

**Whole-Cell Catalysis**

200 µL of the precultures of WT PAMO and mutants were transferred into 20 mL TB media containing
0.1% L-arabinose and 100 µg·mL\(^{-1}\) carbenicilline in 100-mL Erlenmeyer flasks and incubated at 37 °C with
shaking at 250 rpm until the OD\(_{600}\) was between 2.5–3.0. Then 500 µL of 30% glycerol, 280 µL of 100 mM
rac-1a (or rac-1b) in acetonitrile and 300 µL of 134 mg·mL\(^{-1}\) of 2-hydroxypropyl-β-cyclodextrin were
added to the flasks and the culture continued.

**GC Analysis**

Samples (1 mL) were taken out at various time intervals by extraction with 1 equivalent of ethyl acetate
containing hexadecane (0.1 g·L\(^{-1}\)) as internal standard. The organic phase was then analyzed by GC on a
HP-5 column to determine the ketone conversion. The ee values of 1a and 2a were determined using a 30-
m BGB 176/BGB 15 G/494 column (temperature: 220/150 11 min iso 150/min 160 20 min iso 18/min 220
5 min 320; 0.6 bar hydrogen pressure). The ee values of 1b and 2b were determined using a 30-m BGB
176/BGB 15 G/494 column (temperature: 220/80 1.2/min 170 18/min 220 5 min iso 320; 0.5 bar hydrogen
pressure) and a 25-m Lipodex-G G/515 column (temperature: 220/140 25 min iso 50/min 150 56/min iso
18/220 320; 0.5 bar hydrogen pressure), respectively.

The absolute configuration of lactone 2a was made by comparison with an authentic sample. The
assignment of the absolute configuration of lactone 2b was made on the basis of analogy and by comparing
the GC analyses of 2a and 2b. All E-values were calculated using the equation of Sih.\(^4\)

**Enzyme Purification**

The wild type (WT) and mutant enzymes were purified to homogeneity as described earlier with minor
modifications\(^1\).

**Steady-State Kinetics**

The activities of the purified enzymes were determined spectrophotometrically by monitoring the decrease
in the level of NADPH over time at 25 °C and 340 nm (\(\varepsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}\)). The reaction mixture (1.0
mL) contained 50 mM Tris-HCl (pH 7.5), 100 µM NADPH, 1.0 mM phenyl acetone, 1% (v/v) acetonitrile
1 μM enzyme. For measuring the thermostability of the WT PAMO and mutants, solutions of the purified enzymes (WT PAMO and mutant 254-21, 254-60, 254-67) were incubated at 50 °C. The samples were taken out at different time intervals (1 h, 10 h, 15 h, 20.5 h, 25h, 33 h and 40 h) and put on ice for 10 min. Residual activity was determined as described above in the presence of 10 mM phenyl acetone. The results were showed in the Fig. 2.

![Figure 2](image_url)

**Fig. 2** Thermostability of wt PAMO and three mutant enzymes.

**References**

Examples of focused libraries:

(b) M. S. Warren and S. J. Benkovic, *Protein Eng.*, 1997, **10**, 63–68;