ELECTRONIC SUPPLEMENTARY INFORMATION

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

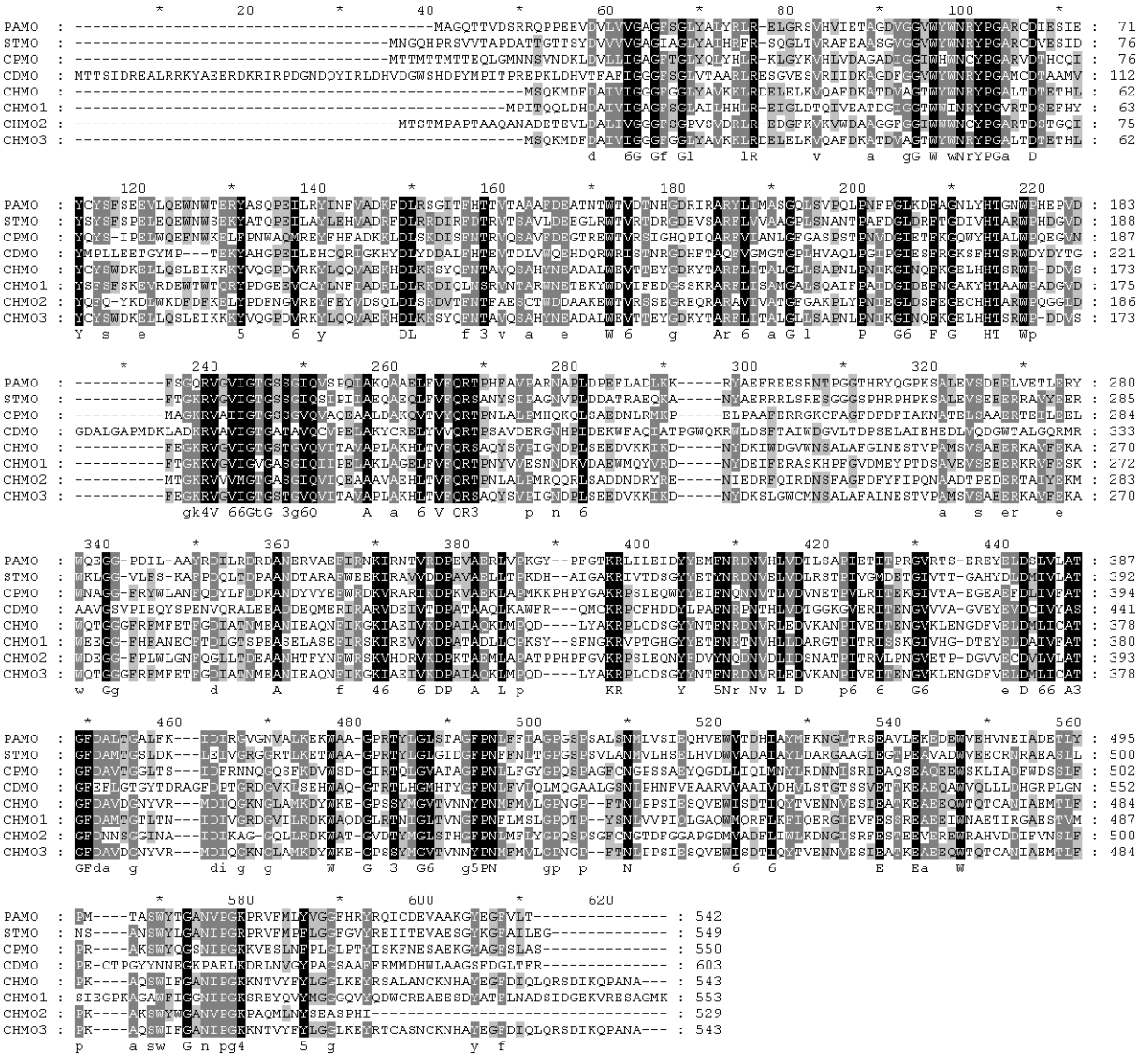

Fig.1 Multiple sequence alignment of eight BVMOs. The sequences are: phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* (Q47PU3), steroid monooxygenase (STMO) from *Rhodococcus rhodochrous* (BAA24454), cyclohexanone monooxygenase (CHMO) from *Acinetobacter sp*. NCIMB9871 (BAA86293), cyclohexanone 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'-ATAGAGACCAGCATGTTGSNGHVCVMTGMCGGGCTGCCCGGGCCTG-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℃ for 3 min followed by cycling at 94℃ for 1 min and 72℃ for 14 min for 15 cycles, then a final elongation for 35 min at 72 ℃. 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 ℃. The resulting PCR product was used to transform into elctrocompetent *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 ℃ 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℃ 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 ℃ and 800 rpm for 3 h. Cell debris was precipitated by

centrifugation at 4000 rpm and 4℃ 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^o 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 ℃ 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 $\mu g \cdot mL^{-1}$ carbenicilline in 100-mL Erlenmeyer flasks and incubated at 37 ℃ 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 $mg \cdot mL^{-1}$ 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 $g \cdot L^{-1}$) 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.3 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 ℃ and 340 nm ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$). 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 ℃. 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.

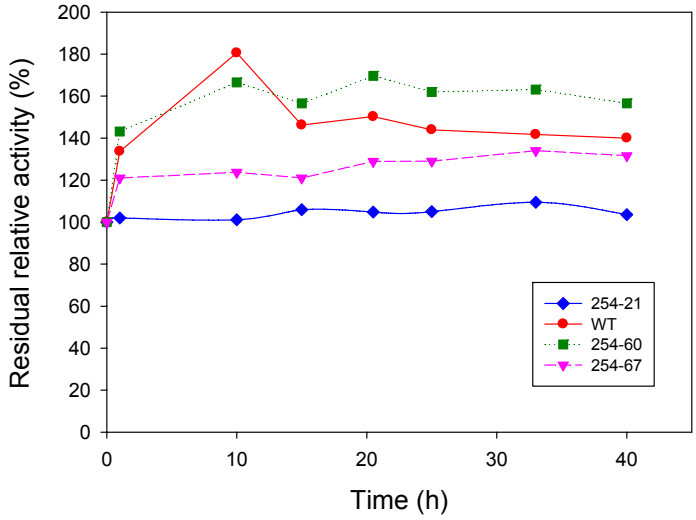

Fig. 2 Thermostability of wt PAMO and three mutant enzymes.

References

1. M. W. Fraaije, J. Wu, D. P. H. M. Heuts, E. W. van Hellemond, J. H. L. Spelberg and D. B. Janssen, *Appl. Microbiol. Biotechnol*., 2005, **66**, 393–400.
2. For preparation of 2°ADH crude extract: (*a*) D. S. Burdette, C. Vieille and J. G. Zeikus, *Biochem. J*, 1996, **316**, 115–122; *(b)* F. Schultz, F. Leca, F. Hollmann and M. T. Reetz, *Beilstein J. Org. Chem*., 2005, **1**: 10.
3. M. Bocola, F. Schulz, F. Leca, A. Vogel, M. W. Fraaije and M. T. Reetz, *Adv. Synth. Catal*., 2005, **347**, 979–986.
4. C. S. Chen, Y. Fujimoto, G. Grirdaukas and C. J. Shi, *J. Am. Chem. Soc*., 1982, **104**, 7294–7299.

Examples of focused libraries:

(*a*) M. T. Reetz, S. Wilensek, D. Zha and K.-E. Jaeger, *Angew. Chem.*, 2001, **113**, 3701–3703; *Angew. Chem. Int. Ed.*, 2001, **40**, 3589–3591;
(*b*) M. S. Warren and S. J. Benkovic, *Protein Eng.*, 1997, **10**, 63–68;
(*c*) Y. Koga, K. Kato, H. Nakano and T. Yamane, *J. Mol. Biol.*, 2003, **331**, 585–592;
(*d*) G. P. Horsman, A. M. F. Liu, E. Henke, U. T. Bornscheuer and R. J. Kazlauskas, *Chem.–Eur. J.*, 2003, **9**, 1933–1939;
(*e*) C. Nowlan, Y. Li, J. C. Hermann, T. Evans, J. Carpenter, E. Ghanem, B. K. Shoichet and F. M. Raushel, *J. Am. Chem. Soc.*, 2006, **128**, 15892–15902;
(*f*) L. Rui, L. Cao, W. Chen, K. F. Reardon and T. K. Wood, *J. Biol. Chem.*, 2004, **279**, 46810–46817;
(*g*) E. M. Gabor and D. B. Janssen, *Prot. Eng., Des. Sel.*, 2004, **17**, 571–579;
(*h*) A. Juillerat, T. Gronemeyer, A. Keppler, S. Gendreizig, H. Pick, H. Vogel and K. Johnsson, *Chem. Biol.*, 2003, **10**, 313–317;
(*i*) S. Bartsch, R. Kourist and U. T. Bornscheuer, *Angew. Chem.*, 2008, **120**, 1531–1534; *Angew. Chem. Int. Ed.*, 2008, **47**, 1508–1511;
(*j*) L. Liang, J. Zhang and Z. Lin, *Microb. Cell Fact.*, 2007, 6:36;
(*k*) N. U. Nair and H. Zhao, *ChemBioChem*, 2008, **9**, 1213–1215.