**QUEEN'S
UNIVERSITY
BELFAST**

Customizing the Enantioselectivity of a Cyclohexanone Monooxygenase by a Strategy Combining “Size-probes” with in silico Study

Hu, Y., Xu, J., Cen, Y., Li, D., Zhang, Y., Huang, M., Lin, X., & Wu, Q. (2019). Customizing the Enantioselectivity of a Cyclohexanone Monooxygenase by a Strategy Combining “Size-probes” with in silico Study. *CHEMCATCHER*, 11(20), 5085-5092. <https://doi.org/10.1002/cctc.201901200>

Published in:
CHEMCATCHER

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

© 2019 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. This work is made available online in accordance with the publisher's policies. Please refer to any applicable terms of use of the publisher.

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Customizing the Enantioselectivity of a Cyclohexanone Monooxygenase by a Strategy Combining “Size-probes” with *in silico* Study

Yujing Hu,^[a, b] Jian Xu,^[a] Yixin Cen,^[a] Danyang Li,^[a] Yu Zhang,^[a] Meilan Huang,^{*,[b]} Xianfu Lin,^[a] Qi Wu^{*,[a]}

Abstract: Enzymatic Baeyer-Villiger oxidation provides a promising green route utilizing molecular oxygen as the oxidant to produce chiral lactones. Wild-type (WT) CHMO_{Acineto} leads to enantioselectivity up to 99% ee (S) in the synthesis of substituted ϵ -caprolactones. To reverse the inherent enantiopreference of CHMO_{Acineto} toward an array of cyclohexanones with various chain length, we herein reshaped the binding pocket with a minimal number of mutations by a rational design strategy combining “size-probes” with *in silico* study, which drastically reduces the screening effort. By probing the binding pocket of variants with different-sized 4-substituted cyclohexanones substrates, single, double and triple mutants were identified as the best mutants providing highly reversed enantioselectivity for these probing molecules, respectively. The successful demonstration of the strategy combining “size-probes” with *in silico* study in the protein engineering of CHMO_{Acineto} may provide a valuable guidance for facile engineering other BVMOs with customized enantiopreference for the same classified substrates with their substituents on the chiral or prochiral central atom.

Introduction

Natural enzymes often displayed high enantiopreference toward one specific configuration of the products.^[1] When it is demanded to reverse the enantioselectivity of the products, directed evolution or rational design of wide type enzymes would be introduced to control the enantiopreference.^[2] When the bioinformatics analysis of the mechanical information of enzymes is not accessible, directed evolution through such standard techniques as the error prone polymerase chain reaction (epPCR), saturation mutagenesis, and DNA shuffling can in principle be employed to engineer the enzymes.^[3] While the structural and mechanical information of enzymes is in hand, rational design would provide a promising alternative for site-specific mutagenesis. Recently, researchers opted for performing mutagenesis based on *in silico* study and bioinformatics analysis of enzymes.^[4] The dynamic aspects of structural information of enzymes obtained from advanced

computational simulations has been used in the identification of the positions and potential residues for mutation, leading to a significant reduction of screening effort. Up to now, one of the main bottlenecks in engineering enzymes for stereo-controlled reactions is still the huge screening effort needed in the construction of mutagenesis libraries.

In the process of rational design, the selection of benchmark substrates is also of great importance, besides the atomic understanding of the enzyme systems based on computational simulations. Some examples revealed that there was an interesting relationship between the size of substrates and the reshaped active pockets of enzymes.^[5] Hult and coworkers reported that the mutant W104A of *Candida antarctica* lipase B had increased enantioselectivity toward various secondary alcohols with increasing chain length of the alkyl moiety on the chiral central atom.^[5a] It would be expected that in the restricted space of the reshaped pocket, an array of compounds with the increasing chain length would face the decreasing degrees of freedom. If a settled small compound is used as the model substrate in directed evolution, some key positions essential for accommodating diverse substrates may be omitted in the screening process, or the optimal mutants are only positive toward the small model substrate, but poorly selective for the cases of some larger compounds. Inspired by this, we proposed to utilize benchmark substrates acted as “size-probes” with varied size from large to small. Compared with a single model substrate, such size-probes may be more preferable for evaluating the size effect of catalytic pocket, enabling it to accurately select the prioritized residues for mutagenesis to control the enantioselectivity of an enzyme, so as to significantly reduce the search scope and thus the screening effort.

Scheme 1. Biotransformation of 4-substituted cyclohexanones with growing chains catalyzed by CHMO_{Acineto}.

As one of the most frequently used BVMOs for the transformation of structurally diverse ketones, cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 (CHMO_{Acineto})^[6] was studied here. In the first successful case pioneered by Reetz, a mutant provided by an epPCR technique achieved a reversal of enantioselectivity from 9% ee (R) of WT

[a] Y. Hu, J. Xu, Y. Cen, D. Li, Y. Zhang, Prof. X. Lin, Dr. Q. Wu
Department of Chemistry, Zhejiang University
Hangzhou 310027 (China)
E-mail: llc123@zju.edu.cn

[b] Dr M. Huang
School of Chemistry and Chemical Engineering, Queen's University
Belfast, BT9 5AG (U.K.)
E-mail: m.huang@qub.ac.uk

Supporting information for this article is given via a link at the end of the document.

to 79% ee (*S*) in the desymmetrization of 4-hydroxycyclohexanone.^[7] Recently, a combination of Combinatorial Active-Site Saturation Test/Iterative Saturation Mutagenesis (CAST/ISM) was used to engineer CHMO from *Thermocrisum municipal* (CHMO_{Thermo}), and successfully reverse the enantioselectivity in BV oxidation of 4-methylcyclohexanone from 99% ee (*S*) of WT to 91% ee (*R*)^[8]. However, the limitation still laid in the huge screening effort in engineering a stereo-controlled enzyme.

Here, we applied a strategy combining “size-probes” and computational analysis to customize the enantioselectivity of CHMO_{Acineto} mutants by screening a minimal amount of mutations from one mini-library for the production of an array of enantiocomplementary lactones with increasing alkyl substituents (Scheme 1). Our research offers a green synthesis route for the preparation of optically pure chiral lactones as the intermediates for pharmaceutical drugs or the precursors for chiral polyesters^[9], which are of particularly biomedical significance.

Results and Discussion

Single mutation for reversed enantioselectivity of cyclohexanone with large-sized substituents

WT CHMO_{Acineto} showed (*S*)-selectivity for 4-substituted cyclohexanones **1a-1c** with increasing substituents (Table 1) in the whole cell screening system using molecular oxygen and D-glucose as sustainable co-substrates. Additionally, in the conversion of **1d**, the preferred product is (+)-**2d** with 33% ee, which is possibly *R* configuration compared with the optical rotation data of **2a-2c**.^[1d] It appears a big challenge to engineer this enzyme for reversing or enhancing the enantioselectivity through the minimal screening of a mini-library.

To better understand how the key residues affect the enantioselectivity toward an array of cyclohexanones, molecular dynamics (MD) simulation herein was conducted on CHMO complexed with **2b** as an example (Figure 1). RMSD was performed on C α of the protein backbone (Figure S5 in ESI). The enzyme probably exhibits a certain directionality when inserting the oxygen into the substrate so that the oxygen will always be entered at the same direction.^[11a] The orientation of the substrate will then decide which enantiomer is formed.^[11a, 12] When the substituent at the C4 atom of cyclohexanone is positioned at the equatorial orientation, the (*S*)-enantiomer should be formed; Likewise, if the substituent at C4 is positioned at the axial orientation in a new variant, (*R*)-enantioselectivity would be observed (Figure 1). From the MD simulation result (Figure 1A), we can see that these residues F432 and L435 lining the top and bottom binding sides are possible to affect the enantioselectivity in the production of **2b**. However, four single mutants F432L/I and L435A/G suitable to reverse the enantioselectivity in the transformation of bulky 4-phenylcyclohexanone^[10] failed to convert **1b** into **2b** with (*R*)-configuration. The mutants F432L/I improved the enantioselectivity significantly in favor of (+)-**2d**. Interestingly, the mutants A435 and G435 displayed opposite enantioselectivity

toward **1d**, although the amino acids alanine and glycine at position 435 differ only by one methylene group in structure. We considered that a compound with a large substituent may be more preferable to fill the active pocket and thus would enable identifying the prioritized residues for mutagenesis.

Figure 1. (A) MD simulated structures of WT CHMO_{homo} in complex with (*S*)-**2b** (light blue); (B) CHMO_{homo} mutant F432I/L435A/P431L in complex with (*R*)-**2b** (yellow); (C) CHMO_{homo} mutant F432I/L435A/T433L in complex with (*R*)-**2b** (yellow); (D) The estimated volumes of enclosed cavities of WT and selected mutants calculated by Discovery studio software. The mutated residues (L431, I432, L433 and A435 in B/C) are shown in magenta. Cofactors (FAD and NADP⁺) are represented by sticks in white. The homology model (named as CHMO_{homo}) is constructed on the basis of the crystal structure of CHMO from *Rhodococcus* sp. strain HI-31 (PDB code: 4RG3, contained the product ϵ -caprolactone).^[11a]

The enclosed cavity of WT CHMO_{homo} (Figure 2A) is defined by residues L143-L144, F246, F277, R327, P431-T433, L435, W490, and F505, as well as the FAD and NADP⁺, and has a volume of ~150 Å³ calculated by Discovery studio (Figure 1D). We selected substrate **1d** (a calculated volume of ~161 Å³ in Figure 2B) as a first “size-probe” with a long chain on the prochiral central atom for engineering of stereo-controlled CHMO_{Acineto}, instead of settling a model substrate throughout the whole screening process. By using the large-sized “probe” **1d** to probe the active pocket, both positions 432 and 435 were identified to affect the enzyme’s enantioselectivity in the BV oxidation of the 4-position substituted cyclohexanones (Table S2). However, if only small-sized “probe” **1b** was applied initially in the screening process, the position F432 could be omitted.

To fully investigate the effect of mutating L435 on the conversion of substrate **1d**, saturation mutagenesis (SM) was performed. Most L435X mutants displayed medium enantioselectivity ((-) or (+)) (Table S3). The mutant L435M enhanced the enantioselectivity up to 86% (+) from 33% (+) by WT (Table 1). Remarkably, the mutant L435V gave the complete (-)-selectivity (99% ee) (entry 11 in Table 1). However, L435V and L435M still preserved (*S*)-selectivity in the transformation of

4-phenylcyclohexanone compared with the single mutants L435A/G. These results indicated that achieving the customized enantioselectivity requires a strictly controlled space created by the mutations.

Table 1 WT and selected single, double, triple CHMO_{Acineto} mutants as catalysts in the desymmetrization of prochiral cyclohexanones **1**^[a]

entry	substrate	variants	conv.(%) ^[b]	yield (%) ^[b]	ee _p (%) ^[c]
1	1a	WT	99	98	99(S)
2	1a	F432I/L435A/P431L	99	96	84(R)
3	1a	F432L/L435A/T433L	99	97	82(R)
4	1b	WT	99	98	98(S)
5	1b	F432I/L435A/P431L	99	97	96(R)
6	1b	F432I/L435A/T433L	99	96	94(R)
7	1c	WT	99	98	94(S)
8	1c	F432I/L435A	99	97	94(R)
9	1c	F432I/L435G	99	97	98(R)
10	1d	WT	99	97	33(+)
11	1d	L435V	99	95	99(-)
12	1d	L435M	99	96	86(+)
13	1d	F432I/L435A	99	97	80(+)
14	1d	F432I/L435G	99	96	97(+)

^[a] The whole cell screening experiments are described in Experiment section.

^[b] Determined by chiral GC. ^[c] The optical rotation data were listed in ESI and the absolute configuration was confirmed by comparison with the literature^[1d].

Figure 2. (A) The enclosed cavity of WT defined by sites 143-144, 246, 277, 327, 431-433, 435, 490, and 505; (B) The volume of "size-probes".

The next question was how the space created by the mutation at the critical position 435 would affect the enantioselectivity toward the substrates with different-length chains. To reduce screening effort, we selected mutants G435, A435, V435, I435,

F435 with smaller or larger side chains compared with WT (L435) (G<A<V<M<I/L<F), and examined their performance on 4-substituted cyclohexanones **1a-1c** (Table S2). As the chain length of compounds **1a-1c** increased, the ratio of (S)-products catalyzed by these mutants (L435G/A/V/M/I) decreased, but without reserved enantioselectivity (Figure 3A). The replacement of position 435 with bulky phenylalanine encountered the abolished activity for **1a-1c** (Table S2). These results indicated that small residues (A or G) at position 435 were more favorable for the improvement of (R)-selectivity than larger residues, and the enantiopreference for large substrates (**1c**) was affected more significantly than small substrates (**1a** and **1b**) (Figure 3A). We speculated that concomitant increase in volume (~ 160 Å³ in Figure 1D) of the active site pocket vacated by the mutation of leucine to small amino acids (G or A) at position 435 is favorable for the nest of axial substituent of (R)-enantiomers, especially for long axial-substituent which may bind more tightly with the reshaped pocket, while 4-substituted cyclohexanones with small substituents may be too small to fill the pocket.

Figure 3. Comparison of ee values of **2a-2d** under the catalysis of selected mutants. (A) single mutants; (B) double and triple mutants.

Double mutation for reversed enantioselectivity of cyclohexanone with medium-sized substituents

For 4-substituted cyclohexanones with small substituents, single mutation at position 432 or 435 to reshape the active pocket may be not enough to manipulate the preference for certain enantiomer products. Considering that the phenyl group of the site 432 located above the substituent of the substrate would form CH- π hydrogen bonds^[13] with equatorial group of (S)-enantiomer, which may facilitate to stabilize (S)-enantiomer. When F at position 432 was mutated into L or I, these CH- π hydrogen bonds (Figure S1) were disrupted and severe geometric changes were caused (Figure 1D). The concomitant shrunken active site pocket is possibly more liable to block a substrate with large equatorial substituents such as propyl and pentyl (**1c** and **1d**) than that with small methyl and ethyl substituent (**1a** and **1b**) (Table S2). Hence, position 432 was chosen for the subsequent iterative mutagenesis study based on position 435. To disrupt the CH- π hydrogen bonds which may facilitate to stabilize (S)-enantiomer, the aforementioned F432L mutation with flexible leucine was introduced to the L435A plasmid. The compound **1c** (a calculated volume of ~ 133 Å³) with a medium substituent was chosen as a second "size-probe" to probe the reshaped pocket. As expected, the mutant F432L/L435A reversed the enantioselectivity in favor of (R)-**2c**

with 86% ee (Table S4). Motivated by this result, we selected other nonpolar residues (V, M, I) to mutate F432 in combination with L435A, namely F432V/L435A, F432M/L435A and F432I/L435A. The best (*R*)-selective variant for the conversion of **1c** was F432I/L435A with 94% ee (Table 1), significantly higher than 60% ee by single mutant L435A. While alanine at the position 435 in the best double mutant F432I/L435A was further mutated into glycine, the (*R*)-product was also obtained with higher ee value (98%) (Table 1).

We also tested the best double mutants F432I/L435A and F432I/L435G for ketones **1a**, **1b** and **1d**. For **1a**, the mutant F432I/L435A delivered the reversed enantioselectivity with 20% ee value, but F432I/L435G still preserved (*S*)-enantioselectivity toward **1a** (Table S4). Notably, medium enantioselectivity (73% ee and 55% ee) with preference for (*R*)-products was obtained by both double mutants in the conversion of **1b** (Table S4). In the conversion of **1d**, the double mutants also increased the ee value notably (97% ee by F432I/L435G, 80% ee by F432I/L435A) from 33% ee by the WT enzyme (Table 1). The screening experiments showed that the double mutants F432I/L435A or F432I/L435G gave higher ee values for the 4-substituted cyclohexanone substrates **1c** or **1d** with relatively larger substituents than **1a** or **1b** (Figure 3B).

Triple mutation for reversed enantioselectivity of cyclohexanone with small-sized substituents

Up to now, we have successfully achieved complete reversion of enantioselectivity by mutating only one residue for cyclohexanones with large-sized substituent (**1d**) and realized the reversion of enantioselectivity for substrates with medium substituent (**1c**) by double mutation. There is still room for further improvement in the reversion of enantioselectivity for substrate **1a** or **1b** with a small methyl or ethyl group. The compound **1b** (a calculated volume of $\sim 118 \text{ \AA}^3$) with a small substituent was chosen as a third "size-probe" to probe the reshaped pocket. It may require smaller binding pocket to fit substrate **1a** or **1b** well, since the variant F432I/L435A has an estimated cavity volume of $142 \text{ \AA}^3$ (Figure 1D). We carefully inspected the MD simulated structure of the variant F432I/L435A, while introducing a third residue for attaining the higher preference of (*R*)-**2a** and (*R*)-**2b**. Considering that potential synergistic conformational effects lays in two spatially close amino acids arising from side-chain orientations,^[14] we proposed that mutating P431 or T433 adjacent to the site 432 may cause an alteration of the active pocket (Figure S2). Initially we introduced a third mutation at position 431 into the double mutant F432I/L435A. To reduce the screening effort, only alanine, leucine and phenylalanine representing amino acids with different sizes were assessed. It was revealed that F432I/L435A/P431L notably improved (*R*)-selectivity to 96% ee of **2b** and 84% ee of **2a** (Table 1), better results than the double mutant F432I/L435A (Figure 3B), whereas mutating P431 into alanine or phenylalanine led to significantly diminished activity (Table S5). The volume of enclosed cavity of F432I/L435A/P431L caused by mutating rigid proline at 431 into flexible leucine was slightly shrunk compared with F432I/L435A (Figure 1D). The shrunken active site pocket of

F432I/L435A/P431L allowed the binding of (*R*)-**2b** with the substituent nested in an axial orientation (Figure 1B).

It was found that in the double mutant template F432I/L435A, the side-chain of T433, the other site adjacent to the site 432, points away from the binding pocket and forms an obvious hydrogen bond with S438 (The distance between O(T433) and H(S438) being $1.74 \text{ \AA}$, see Figure S3A). To elucidate the effect caused by T433, we mutated threonine to hydrophobic (I/L/M/V/A) and hydrophilic residues (S/C) for comparison. Mutating T433 into small hydrophobic alanine (F432I/L435A/T433A) or a hydrophilic residue cysteine (F432I/L435A/T433C) resulted in poor (*R*)-selectivity (Table S5), while F432I/L435A/T433L (Table 1) exhibited the best reversed enantioselectivity with 94% ee (*R*) for **1b** and 82% ee (*R*) for **1a**, similar to that of F432I/L435A/P431L (Figure 3B). Interestingly, the MD simulated structure of F432I/L435A/T433L complexed with (*R*)-**2b** showed the compound was positioned with the substituent nested in an axial orientation adopting a catalytically capable pose (Figure 1C).

However, these two triple mutants gave decreased activity for **1d** (Table S5), which may be caused by that the shrunken space of the active pocket cannot accommodate the large-sized substrate **1d** well. It indicated that the best mutants selected by using small-sized substrate as a benchmark, may not be suitable to the large-sized substrates. Therefore, customizing the enzymes individually for different-sized compounds to control the enantioselectivity is necessary.

It was interesting to find that F432I/L435A/T433S changed the (*R*)-selectivity back to complete (*S*)-selectivity of **2b** (Table S5). To understand the effects of T433S and T433L in the triple mutants, we compared their MD simulation structures using the double mutant F432I/L435A as the reference. It was found that similar to threonine, a strong hydrogen bond formed between S433 and S438 with the distance being $1.93 \text{ \AA}$ (H(S433) and O(S438)) (Figure S3B). This interaction actually pulls the mobile loop (431-435) away from the binding pocket in the mutant F432I/L435A/T433S so that the binding space is enlarged, favorable for the substituent of the substrate positioned at equatorial orientation (Figure S3), leading to (*S*)-selectivity of **2b**. Interestingly, when T433 was mutated into a hydrophobic leucine residue, the hydrogen bond with S438 is lost and steric clash from L433 causes the mobile loop in the mutant F432I/L435A/T433L to move inward to the binding pocket (Figure S4). The decreased volume of the reshaped pocket ($\sim 133 \text{ \AA}^3$) would result in a stronger steric clash between the adjacent residue I432 and the equatorial ethyl group in **2b**, thus an axial orientation is preferred, forming the product with highly reversed enantioselectivity (Table 1).

Finally, we have achieved completely reversed stereoselectivity with high ee-values for all tested 4-substituted cyclohexanone derivatives by using different mutants, respectively. Hence, the rational design based on combining "size-probes" with computational analysis makes it possible to accurately select the prioritized site for mutagenesis, and thus a highly focused library was constructed successfully, which drastically reduced the screening effort (Scheme 2).

Scheme 2. A strategy for the design of a focused CHMO_{Acineto} mini-library. Amino acid residues in WT CHMO_{Acineto} and selected mutations are shown with hazel and grey blocks, respectively.

Identifying limited concentration of substrates and Scaling-up transformation

Considering the potential application in industry, the scaling-up reaction was performed in whole cell system. The use of the optimal limited concentration at which the conversion was up to 95%, maximized the benefits in the scaling-up biocatalytic process. Hence, we focused our attention on the limited concentration and first examined the substrates **1a-1d** at different concentrations in 100 mL of reaction system under the catalysis of WT and best mutants. As shown in Figure 4, the limited concentration of **1a-1d** decreased as the length of the substituents increased under the catalysis of WT (25 mM→22 mM→10 mM→4 mM). Compared with WT, the limited concentration of **1d** for the single mutants L435V and L435M which produced (**-**)-**2d** (99% ee) and (**+**)-**2d** (86% ee) respectively was enhanced (10 mM vs. 4 mM). The double mutant F432I/L435G also gave increased concentration 8 mM of **1d** (97%, **+**) and 18 mM of **1c** (98%, **R**) compared with WT. As for **1a** and **1b**, the limited concentration was 8 mM by the triple mutant F432I/L435A/P431L, while the other triple mutant F432I/L435A/T433L tolerated higher limited concentration 14 mM.

We further tested the best mutants and WT in a 1.3-2.8 gram-scale conversion of **1a-1d** with a final concentration corresponding to the limited concentration. All the conversions were completed within 36 h as analyzed by GC, and the isolated yields were obtained in the range from 59% to 74% (Table S6). The results demonstrate the synthetic significance of these selected mutants.

Figure 4. The limited concentration of **1a** (A), **1b** (B), **1c** (C) and **1d** (D) in the Baeyer-Villiger oxidation by WT and selected mutants.

Conclusions

A strategy combining “size-probes” with computational analysis has been applied to engineer the cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 (CHMO_{Acineto}) individually to control the enantioselectivity toward an array of cyclohexanones, and thus a highly focused mini-

library was constructed successfully, which drastically reduced the search scope and thus the screening effort. Completely reversed stereoselectivity with high ee-values for all tested 4-substituted cyclohexanone derivatives was achieved by using different mutants, respectively. The strategy combining “size-probes” with *in silico* study in the protein engineering of CHMO_{Acineto} will provide a valuable guidance for facile engineering of other BVMOs with customized enantioselectivity.

Experimental Section

Chemicals and materials

All commercial chemicals were purchased from Energy Chemical, Aladdin, or Sigma-Aldrich. Hot Start DNA polymerase was purchased from TOKYO (Japan); *Dpn* I was purchased from Thermo Fischer Scientific Inc. All solvents and other reagents were analytical grade and used without further purification.

Generation of mutant libraries

CHMO gene was obtained from *Acinetobacter* sp. NCIMB 9871 and synthesized by Sangon BioTech (Shanghai). The gene was subcloned into pET-22b (+) using *Nde* I and *Bam*HI cutting sites.^[15] PCR was performed using CHMO gene as the template for mutagenesis. Table S1 (Supporting Information) provides the oligonucleotide primers used for the generation of mutant libraries. PCR mixtures (50 μ L final volume) contained: ddH₂O (25 μ L), 10 $\times$ KOD buffer (5 μ L), MgSO₄ (3 μ L, 25 mM), dNTP (5 μ L, 2 mM each), forward and reverse primers (5 μ L, 2.5 μ M each), template plasmid (1 μ L, 50 ng μ L⁻¹) and 1 μ L of KOD Hot Start DNA polymerase. The PCR was initially subjected to 94°C for 5 min, followed by 18 cycles of denaturing step at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 8 min. And final extension step at 72°C for 10 min was performed. To ensure the elimination of template plasmid, PCR mixtures were digested at 37°C overnight after adding 2 μ L *Dpn* I (10 U/ μ L). The digested product was purified with an Omega PCR purification spin column, and then an aliquot of 20 μ L was used to transform electrocompetent *E. coli* BL21 (DE3) cells. The transformation mixture was shaken with 1 mL of LB medium at 37°C for 1 h, and spread on LB-agar plates containing 100 μ g mL⁻¹ ampicillin.

Expression of CHMO_{Acineto} variants

Single colony was picked into 5.0 mL LB media with 100 μ g mL⁻¹ ampicillin, and then incubated at 37°C under shaking of 200 rpm for 12 h. After DNA sequencing, the target mutants were conserved at -80°C with 30% glycerol aliquot. A fresh 20 mL of TB media in 50 mL erlenmeyer flasks containing 100 μ g mL⁻¹ ampicillin mixed with 200 μ L preculture was inoculated at 37°C, 200 rpm until the OD₆₀₀ reached between 0.6 and 0.7. Then isopropyl β -thiogalactopyranoside (IPTG) used to induce CHMO_{Acineto} expression was added to a final concentration of 0.2 mM and the incubation was continued for additional 16 h at 20°C, 200 rpm. Then the cells were harvested by centrifugation (30 min, 5000 rpm, 4°C) and were flushed by 50 mM PBS (pH 7.4). The weighing wet cells were resuspended in the fresh 50 mM PBS (pH 7.4) to obtain a final concentration of 0.1 g mL⁻¹.

Mutant library screening

In the whole cell screening protocol, the reaction system contained 1 mL cell culture (0.1 g mL⁻¹), 4 μ L of a stock solution of 0.5 M ketones in acetonitrile and D-glucose (3 equiv). The mixture in 10 mL glass tube with a sealed cap was shaken at 200 rpm and 22°C for desymmetrization, and the reaction time is 32 h. The reaction was stopped by adding sodium chloride and the mixture was extracted with 1 mL ethyl acetate three times. The sample was analyzed by chiral gas chromatographic

analyses (GC) (CP-chirasil-DEX CB 25 cm $\times$ 0.25 cm) to determine the conversion and the enantiomeric excess of the residues and products.

Identifying the limited concentration in Baeyer–Villiger oxidation

The reaction system contained 100 mL cell culture (0.1 g mL⁻¹), different concentrations of ketones and D-glucose (3 equiv). The mixture was shaken at 200 rpm and 22 °C for 32 h. The reaction was stopped by adding sodium chloride. Samples (1 mL) were taken with a pipette and extracted with ethyl acetate three times. The sample was analyzed by chiral GC (CP-chirasil-DEX CB 25 cm $\times$ 0.25 cm) to determine the conversion and the enantiomeric excess of the residues and products.

General procedure for large-scale Baeyer–Villiger oxidation

The wet cells were resuspended in the fresh 1 L PBS (pH 7.4, 50 mM) to obtain a final concentration of 0.1 g mL⁻¹. To reduce substrate inhibition, batch-fed method was adopted. Equal amounts of total ketones **1a–1d** were added per 8 h three times. The 1 L reaction system containing ketones **1a–1d** (at the limited concentration) and D-glucose (3 equiv) were shaken at 200 rpm and 22 °C for 36 h separately. The reaction was stopped by adding sodium chloride. The system was extracted with ethyl acetate (3 $\times$ 500 mL), dried over MgSO₄ and a sample was collected for GC analysis. Then the organic layers were concentrated *in vacuo* and the crude reaction products were purified directly by column chromatography on silica gel (petroleum ether/EtOAc = 4/1) to afford **2a–2d** as light-yellow oils.

Protein preparation and molecular docking

Homology modeling was conducted by Modeler software A homology model (named as CHMO_{homo}) based on the crystal structure of CHMO from *Rhodococcus* sp. strain HI-31 (PDB code: 4RG3, contained the product ϵ -caprolactone), which exhibits 55% sequence similarity. The CHMO mutants were generated using Chimera (version 1.11.2). Molecular docking was performed using the AutoDock 4.2 suite with the Lamarckian genetic algorithm (LGA).^[16] A grid box was centered on C4X atom and the size of the grid box was set as 40 Å in each dimension. A total of 100 LGA runs were carried out for each ligand: protein complex. The population was 300, the maximum number of generations was 27 000, and the maximum number of energy evaluations was 2 500 000. The top-ranked structure corresponds to the lowest binding energy structure of the most populated cluster, was used to be analyzed.

Parameter calculations

The geometries of flavin, product, NADP⁺ were optimized employing the Gaussian09 program^[17], using the density functional theory (DFT) method with the exchange-correlation functional B3LYP and the 6-31g(d) basis set^[18]. RESP charges^[19] were obtained based on the charges calculated using HF/6-31G(d) single point energy calculations. These point charges were subsequently used in the MD simulations.

Molecular dynamics

All complex systems were subsequently subjected to energy minimization and MD simulations. The Amber MD program (AMBER14)^[20] with the parm99SB^[21] and GAFF^[22] force fields was used. The protein complexes were soaked within a truncated octahedral box of TIP3P waters and sodium ions were added to neutralize the system. The systems were subjected to two energy minimizations, using the steepest descent and conjugate gradient algorithms. The minimized systems were subsequently slowly heated slowly from 0 to 300 K for 250 ps using the Langevin dynamics with a collision frequency of 1.0 ps⁻¹. An equilibration simulation with no harmonic restraints applied was carried out at 300 K with a *NVT* ensemble and a periodic boundary condition for a further 50 ps. A cut-off distance of 8 Å was set for non-bonded Van der Waals force while the Particle Mesh Ewald (PME) method was used to account for the long-range electrostatic interactions.^[23] The SHAKE method was utilized to fix the covalent bonds associated with the hydrogen atoms

within the system.^[24] 20-ns Production simulation was performed for the protein complex. *NPT* ensemble was used in the MD simulations with a time step of 2 fs and a randomly assigned initial velocity at 300 K and a pressure of 1 atm. For each system analyzed, MD reference structure from the cluster analysis corresponds to the most populated structure of the simulation.

Acknowledgements

This work was supported by National Natural Science Foundation of China (21574113, 21472169), Zhejiang Provincial Natural Science Foundation (LY19B020014) and the Fundamental Research Funds for the Central Universities (2018QNA3010). The authors are also grateful for the computing resource from the high-performance computing cluster at Queen's University Belfast.

Keywords: Baeyer–Villiger monooxygenase • directed evolution • reversed enantioselectivity • chiral lactones • biotransformation

- [1] a) M. D. Mihovilovic, G. Chen, S. Wang, B. Kyte, F. Rochon, M. M. Kayser, J. D. Stewart, *J. Org. Chem.* **2001**, *66*, 733–738; b) M. J. Taschner, D. J. Black, *J. Am. Chem. Soc.* **1988**, *110*, 6892–6893; c) S. Wang, M. M. Kayser, V. Jurkauskas, *J. Org. Chem.* **2003**, *68*, 6222–6228; d) M. J. Taschner, D. J. Black, Q. Z. Chen, *Tetrahedron: Asymmetry* **1993**, *4*, 1387–1390.
- [2] Selected reviews about directed evolution: a) N. J. Turner, *Nat. Chem. Biol.* **2009**, *5*, 567–573; b) K. Balke, A. Beier, U. T. Bornscheuer, *Biotechnol. Adv.* **2018**, *36*, 247–263; c) J. Dong, E. Fernández-Fueyo, F. Hollmann, C. E. Paul, M. Pesic, S. Schmidt, Y. Wang, S. Younes, W. Zhang, *Angew. Chem. Int. Ed.* **2018**, *57*, 9238–9261; d) H. Leisch, K. Morley, P. C. K. Lau, *Chem. Rev.* **2011**, *111*, 4165–4222; e) D. E. Torres Pazmiño, H. M. Dudek, M. W. Fraaije, *Curr. Opin. Chem. Biol.* **2010**, *14*, 138–144; f) J. B. Wang, G. Y. Li, M. T. Reetz, *Chem. Commun.* **2017**, *53*, 3916–3928; g) A. Currin, N. Swainston, P. J. Day, D. B. Kell, *Chem. Soc. Rev.* **2015**, *44*, 1172–1239; h) C. Zeymer, D. Hilvert, *Annu. Rev. Biochem.* **2018**, *87*, 131–137; i) G. Qu, A. Li, C. G. Acevedo-Rocha, Z. Sun, M. T. Reetz, *Angew. Chem. Int. Ed.* **2019**, *58*, doi: 10.1002/anie.201901491.
- [3] M. T. Reetz, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 5716–5722.
- [4] a) G. W. Zheng, Y. Y. Liu, Q. Chen, L. Huang, H. L. Yu, W. Y. Lou, C. X. Li, Y. P. Bai, A. T. Li, J. H. Xu, *ACS Catal.* **2017**, *7*, 7174–7181; b) A. T. P. Carvalho, D. F. A. R. Dourado, T. Skvortsov, M. de Abreu, L. J. Ferguson, D. J. Quinn, T. S. Moody, M. Huang, *Phys. Chem. Chem. Phys.* **2017**, *19*, 26851–26861; c) M. Voss, D. Das, M. Genz, A. Kumar, N. Kulkarni, J. Kustos, P. Kumar, U. T. Bornscheuer, M. Höhne, *ACS Catal.* **2018**, *8*, 11524–11533.
- [5] a) M. Vallin, P. O. Syrén, K. Hult, *ChemBioChem* **2010**, *11*, 411–416; b) T. K. Hyster, L. Knörr, T. R. Ward, T. Rovis, *Science* **2012**, *338*, 500–503; c) S. Bartsch, R. Kourist, U. T. Bornscheuer, *Angew. Chem. Int. Ed.* **2008**, *47*, 1508–1511.
- [6] C. T. Walsh, Y. C. Chen, *Angew. Chem., Int. Ed.* **1988**, *27*, 333–343.
- [7] M. T. Reetz, B. Brunner, T. Schneider, F. Schulz, C. M. Clouthier, M. M. Kayser, *Angew. Chem., Int. Ed.* **2004**, *43*, 4075–4078.
- [8] G. Y. Li, M. J. L. J. Fürst, H. R. Mansouri, A. K. Ressmann, A. Ilie, F. Rudroff, M. D. Mihovilovic, M. W. Fraaije, M. T. Reetz, *Org. Biomol. Chem.* **2017**, *15*, 9824–9829.
- [9] a) D. d. S. Pisoni, J. S. da Costa, D. Gamba, C. L. Petzhöld, A. C. d. A. Borges, M. A. Ceschi, P. Lunardi, C. A. S. Goncalves, *Eur. J. Med. Chem.* **2010**, *45*, 526–535; b) S. Shoda, H. Uyama, J. Kadokawa, S. Kimura, S. Kobayashi, *Chem. Rev.* **2016**, *116*, 2307–2413; c) R. Ligny, M. M. Hanninen, S. M. Guillaume, J. F. Carpentier, *Chem. Commun.* **2018**, *54*, 8024–8031.
- [10] Y. J. Hu, J. Wang, Y. X. Cen, H. Zheng, M. Huang, X. F. Lin, Q. Wu, *Chem. Commun.* **2019**, *55*, 2198–2201.
- [11] a) B. J. Yachnin, M. B. McEvoy, R. J. D. MacCuish, K. L. Morley, P. C. K. Lau, A. M. Berghuis, *ACS Chem. Biol.* **2014**, *9*, 2843–2851; b) E. Romero, J. R. G. Castellanos, A. Mattevi, M. W. Fraaije, *Angew. Chem., Int. Ed.* **2016**, *55*, 15852–15855; c) I. A. Mirza, B. J. Yachnin, S. Wang, S. Grosse, H. Bergeron, A. Imura, H. Iwaki, Y. Hasegawa, P. C. K. Lau, A. M. Berghuis, *J. Am. Chem. Soc.* **2009**, *131*, 8848–8854; d) B. J. Yachnin, T. Sprules, M. B. McEvoy, P. C. K. Lau, A. M. Berghuis, *J. Am. Chem. Soc.* **2012**, *134*, 7788–7795.
- [12] a) I. Polyak, M. T. Reetz, W. Thiel, *J. Am. Chem. Soc.* **2012**, *134*, 2732–2741; b) I. Polyak, M. T. Reetz, W. Thiel, *J. Phys. Chem. B* **2013**, *117*, 4993–5001.
- [13] M. Nishio, Y. Umezawa, J. Fantini, M. S. Weiss, P. Chakrabarti, *Phys. Chem. Chem. Phys.* **2014**, *16*, 12648–12683.
- [14] M. T. Reetz, M. Bocola, J. D. Carballera, D. X. Zha, A. Vogel, *Angew. Chem., Int. Ed.* **2005**, *117*, 4264–4268.
- [15] G. Chen, M. M. Kayser, M. D. Mihovilovic, M. E. Mrstik, C. A. Martinez and J. D. Stewart, *New J. Chem.* **1999**, *23*, 827–832.
- [16] a) R. Huey, G. M. Morris, A. J. Olson, D. S. Goodsell, *J. Comput. Chem.* **2007**, *28*, 1145–1152; b) G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639–1662.
- [17] Gaussian 09, Revision A.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, Gaussian, Inc., Wallingford CT, 2009.
- [18] a) C. Lee, W. Yang, R. G. Parr, *Phys. Rev. B* **1988**, *37*, 785–789; b) A. D. Becke, *J. Chem. Phys.* **1993**, *98*, 5648–5652.
- [19] C. I. Bayly, P. Cieplak, W. Cornell, P. A. Kollman, *J. Phys. Chem.* **1993**, *97*, 10269–10280.
- [20] D. A. Case, J. T. Berryman, R. M. Betz, D. S. Cerutti, T. E. Cheatham, III, T. A. Darden, R. E. Duke, T. J. Giese, H. Gohlke, A. W. Goetz, N. Homeyer, S. Izadi, P. Janowski, J. Kaus, A. Kovalenko, T. S. Lee, S. LeGrand, P. Li, T. Luchko, R. Luo, B. Madej, K. M. Merz, G. Monard, P. Needham, H. Nguyen, H. T. Nguyen, I. Omelyan, A. Onufriev, D. R. Roe, A. Roitberg, R. Salomon-Ferrer, C. L. Simmerling, W. Smith, J. Swails, R. C. Walker, J. Wang, R. M. Wolf, X. Wu, D. M. York, P. A. Kollman, AMBER 2015, University of California, San Francisco, CA, 2015.
- [21] V. Hornak, R. Abel, A. Okur, B. Strockbine, A. Roitberg, C. Simmerling, *Proteins* **2006**, *65*, 712–725.
- [22] J. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollman, D. A. Case, *J. Comput. Chem.* **2004**, *25*, 1157–1174.
- [23] T. Darden, D. York, L. Pedersen, *J. Chem. Phys.* **1993**, *98*, 10089–10092.
- [24] J.-P. Ryckaert, G. Ciccotti, H. J. C. Berendsen, *J. Comput. Phys.* **1977**, *23*, 327–341.

FULL PAPER

Yujing Hu, Jian Xu, Yixin Cen, Danyang Li, Yu Zhang, Meilan Huang,* Xianfu Lin, Qi Wu*

Page No. – Page No.

Customizing the Enantioselectivity of a Cyclohexanone Monooxygenase by a Strategy Combining “Size-probes” with *in silico* Study

Customizing the enantioselectivity: WT CHMO_{Acineto} leads to enantioselectivity up to 99% ee (S) in the Baeyer-Villiger oxidation of 4-substituted cyclohexanones. By probing the binding pocket of rationally designed variants with different-sized 4-substituted cyclohexanones, single, double and triple mutants were identified as the best mutants providing highly reversed enantioselectivity for these probing molecules.