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The mutagenesis of a single site for enhancing or reversing the enantio- or regiopreference of cyclohexanone monooxygenases†

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The mutagenesis of a "second sphere" switch residue of $CHMO_{Acineto}$ could control its enantio- and regiopreference. Replacing phenylalanine (F) at position 277 of $CHMO_{Acineto}$ into larger tryptophan (W) enabled a significant enhancement of enantio- or regioselectivity toward structurally diverse substrates, moreover, a complete reversal of enantio- or regiopreference was realized by mutating F277 into a range of smaller amino acids (A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V).

Directed evolution, as a method generally exploited to engineer the binding properties of proteins or the catalytic profiles of enzymes, has been utilized for manipulating their regio- or enantioselectivity, substrate scope and stability.[1] However, huge screening efforts are still the bottleneck of directed evolution. One of the efficient mutagenesis strategies is scrutinizing the potential sites for enzyme engineering to achieve the desired catalytic properties, which can significantly reduce the number of mutants to be investigated. Computational modeling and crystallography technique provide useful information on the structures of enzymes and reaction mechanisms, and allow for the rational selection of potential residues to be mutated. In the last decade, enormous efforts in the rational engineering of enzymes have been reported where the residues lining the binding pocket were selected smartly to tune the enzymatic properties.[2] Generally, the selected residues were usually in direct contact with the substituents of substrates to reshape the binding pocket for the desired properties. However, several advances focusing on mutagenesis of the residues distant from substrate-binding sites for tuning enzymatic properties have also been reported.[3] For example, a possible "second sphere" residue P440 of phenylacetone monooxygenase from *Thermobifida fusca* (PAMO) was mutated for the transformation of several substituted cyclohexanone derivatives with high enantioselectivities.[3a] The mutation of Q93 and P94 of PAMO situated remote from the active site induced domain movements to reshape the binding pocket, leading to an expansion of the substrate scope.[3b]

Cyclohexanone monooxygenases (CHMO, EC 1.14.13.22) belong to the subfamily of Baeyer–Villiger monooxygenases (BVMOs) and transform a multitude of reactions selectively.[4] The reaction catalyzed by a CHMO generally forms a tetrahedral Criegee intermediate through a nucleophilic attack of the C4a-peroxyflavin intermediate on the substrate carbonyl carbon, and then the rearrangement of the Criegee intermediate yields the ester or lactone product.[5] A previous theoretical investigation reported that the insertion of an oxygen atom has a certain directionality due to the requirement of anti-periplanarity, and as a result, the orientation of the substituent groups of ligands ultimately determines their enantioselectivity.[6]

Natural CHMOs often gave one specific enantio- or regiopreference toward an array of substrates. Recently, reversal of regio- or enantioselectivity of products was reported by engineering CHMOs from different sources,[7–10] such as *Acinetobacter* sp. NCIMB 9871 ($CHMO_{Acineto}$),[7] *Thermocrispum municipale* ($CHMO_{Thermo}$),[8] *Arthrobacter* sp. BP2 ($CHMO_{Arthro}$)[9] and *Acinetobacter* calcoaceticus ($CHMO_{Acineto\text{-}cal}$).[10] Notably, most studies were focused on mutating hot spots lining the binding pocket in apparently direct contact with the substrates, such as L143, P431–L435 and F505 (numbered in $CHMO_{Acineto}$). Exploring distinct residues that are far away from the most-studied substrate-binding sites would open a new pathway to manipulate its enantio- or regiopreference of CHMOs.

Here, we found mutating a "second sphere" switch residue F277 of $CHMO_{Acineto}$ could control the enantio- or regiopreference toward four-membered, five-membered, and six-membered cycloketones (Scheme 1), and even sulfides. Until now, mutating only

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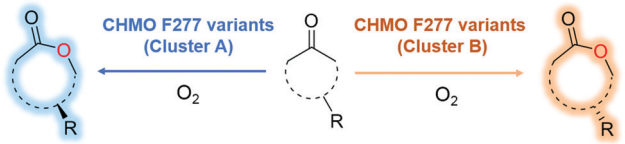


Scheme 1 Enantiocomplementary Baeyer–Villiger oxidation of various cyclic ketones.

one residue F277 of $CHMO_{Acineto}$ for regulating its regio- or enantiopreference toward an array of substrates, has never been reported. Furthermore, the knowledge obtained from $CHMO_{Acineto}$ could be transferred to other CHMOs.

It is a big challenge to identify potential residues in an enzyme, the mutagenesis of which could trigger a reversal of enantio- or regioselectivity with a reshaped binding pocket. $CHMO_{Acineto}$[7,11] was selected as a starting enzyme. Previous experimental results revealed that WT PAMO exhibits different catalytic properties in some cases from CHMO, such as opposite stereopreference toward a same substrate.[7f,13] Comparison of the structures of CHMO[12a,c] and PAMO[12d] (Fig. S1, ESI†), disclosed a small bulge at F277 connecting helix 260–276 and helix 278–283 (numbered in $CHMO_{Acineto}$) in CHMO, while the bulge is absent in PAMO. Considering that the structural difference of enzymes is undoubtedly responsible for the specificity of substrates, we were curious to test position F277, since mutagenesis of this residue may modulate the properties of CHMO. It should be noted that the slight bulging at F277 is located at the entrance of a compact ligand-binding site composed of L143, L144, F246, F277, R327, L426, P428, F432, T433, L435, W490, and F505, and is in proximity to the catalytic center (Fig. S2, ESI†). The residue F277 forms a π–π stacking interaction with the side chain of F246 and a cation–π interaction with R327 that participates in the stabilization of the Criegee intermediate as well as in shifting throughout the catalytic cycle to accommodate NADPH binding.[12a,b] Inspired by this, we speculated that mutating the bulky phenyl side chain of F277 into smaller sized ones may induce the reshaping of the binding pocket, which only allows one specific configuration of substrates to reach the proximity of the flavin, thus altering the enantio-preference or catalytic activity of this enzyme.

To gain a deeper insight into the effect of position 277 on the catalytic properties, saturation mutagenesis at F277 was performed. 3-Phenylcyclobutan-1-one **1a** (Fig. 1) was selected as a model substrate, since **1a** has a four-membered ring and the reversal of enantioselectivity by protein engineering for such a small cyclic non-natural substrate with high ring strain was never realized. As shown in Fig. 1 and Table S2 (ESI†), modest stereoselectivity was observed in the oxidation of **1a** by WT $CHMO_{Acineto}$, which gave an *R* product with 60% ee. Strikingly, the mutation of phenylalanine (F) at 277 into larger-sized tryptophan (W) enabled the enhanced enantioselectivity up to 99% ee (*R*) (WT, F277W classified into Cluster A). It is noteworthy that mutating F277 into all smaller-sized amino acids (F277A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V classified into Cluster B) led to a

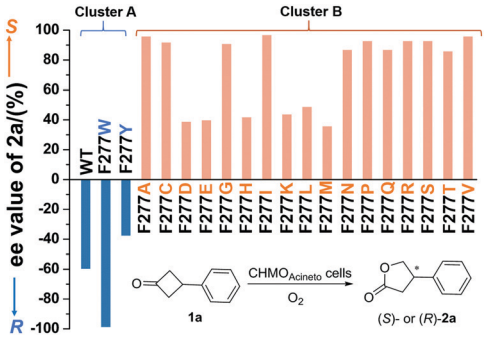
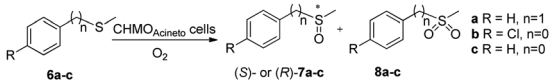


Fig. 1 Enantioselectivity of WT $CHMO_{Acineto}$ and its mutants in the oxidation of ketone **1a**.

distinct reversal of enantioselectivity in the conversion of **1a** (up to 97% ee (*S*)). The catalytic efficiency of mutants, determined by kinetic parameters (Table S3, ESI†), was still retained relative to WT. To verify the potential application of these mutants in synthetic chemistry, we also performed the amplified reactions using 0.5 L cell culture and 2.9 g L^{-1} of **1a**. Both WT $CHMO_{Acineto}$ and F277W achieved complete conversions toward **1a**, and mutant F277V showed 85% conversion, providing similar stereoselectivity as analytical grade reactions. To our knowledge, F277W and F277V are by far the best biocatalysts with the highest enantiopreference for offering (*R*)- and (*S*)-**2a** among all BVMOs reported.

To understand the source of distinctly enhanced and reversed enantioselectivity, molecular dynamics (MD) simulations were performed. It was found that the substituent groups of ligands do not form direct contact with position 277 (Fig. S3, ESI†). Considering the orientation of the substituent groups of ligands determines the enantioselectivity,[6] it is difficult to find out how the orientation of the substituent groups is affected by protein environment. A careful inspection of the structures disclosed that the side chain of W277 still forms a π–π stacking interaction with F246, while F277 was mutated into V, and the corresponding π–π stacking interaction was lost. Notably, loop 243–247 (in blue) adjacent to loop 431–435 (in red) was concomitantly moved (Fig. S3A and B, ESI†). As a result, loop 431–435 (in red), which was suggested to be a selectivity-controlled region,[7a,c,e,8a,b] may be induced to move inward into or outward from the binding pocket (Fig. S3A and B, ESI†). (*S*)-**2a** with a "down" substituent may be hampered by the steric effect introduced by the inward movement of loop 431–435; in contrast, the prefered (*R*)-**2a** with an "up" substituent forms the interaction with the side chain of F432 and F505, thus resulting in an enhanced enantioselectivity of (*R*)-**2a** (Fig. S3A, ESI†). Since CH–π hydrogen bonds between the Ph substituent and the side chain of L143, L426 and L435 may be formed in the reshaped protein environment, (*S*)-**2a** would be more energically preferred, thus resulting in enhanced (*S*)-selectivity of **2a** (Fig. S3B, ESI†). It could be speculated that position 277 may act as a "second sphere" switch residue, mutagenesis of which could cause the concomitant movement of adjacent loops, and induce the reshaping of the binding pocket (Fig. S4, ESI†), so as to control the preference of specific stereo- or regio-isomers of the products.



6a-c (S)- or (R)-7a-c 8a-c

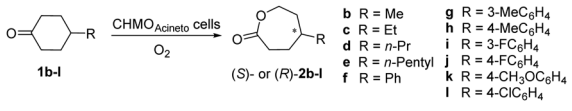
Scheme 2 The sulfoxidation of sulfides.

Next, to explore the catalytic potential of enzymes, the stereoselectivity-enhanced mutant (F277W) and three selected stereoselectivity-reversed mutants (F277P/V/I) evolved for **1a** were tested in the oxidation of various substrates including five-membered cycloketones (**3a**), six-membered cycloketones (**1b–1l**, **3b–3c**) and sulfides (**6a–6c**) (Scheme 2).

In the desymmetrization of prochiral substrates (**1b–1f**), F277W gave the corresponding products with enhanced ee values (up to >99%) in comparison to WT (Table 1). In particular for **1e**, an ee value up to 99% (−) was obtained by F277W, while only 33% (+) by WT. Strikingly, the single-site mutants (F277P/V/I) completely reversed the stereopreference in the transformation of almost all tested substrates except **1b–1c** with a small methyl or ethyl group (Table 1).

We further evaluated the interesting influence of the F277 switch on the regioselectivity in the conversion of asymmetric ketones. WT only exhibited moderate regio- and stereoselectivity toward **3a** to yield the corresponding mixture of lactone products **4a** and **5a** (**4a**/**5a** = 82/18, ee_{4a} 87%(*R*)) (Table 2). It is undoubtedly a challenge to override traditional electronic effects for switching regioselectivity from normal products to abnormal products. In sharp contrast, the single-site mutants F277P/V/I enabled the complete switch of regiopreference, leading to the dominated products **5a** (**4a**/**5a** < 5/95, ee_{5a} > 96%(*S*)) with high ee values. Similar results with completely reversed regioselectivity and enhanced stereoselectivity displayed by F277P/V/I mutants were also observed for **3c** (Table 2). These results clearly implied the important role of the selectivity "switch" of F277. Interestingly,

Table 1 Desymmetrization of ketones **1a–1l** by WT $CHMO_{Acineto}$ and variants[a]



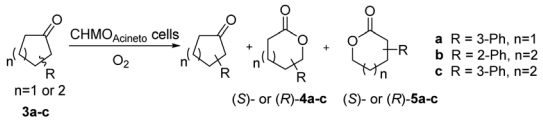
1b-l (S)- or (R)-2b-l

Enzyme	1a	1b	1c	1d	1e	1f
WT	99[b], 62[c](*R*)[d]	99, 99(*S*)	99, 98(*S*)	99, 94(*S*)	89, 33(+)[e]	99, 98(*S*)
F277W	99, 99(*R*)	99, 99(*S*)	99, 99(*S*)	99, 97(*S*)	50, 99(−)	68, 99(*S*)
F277P	99, 93(*S*)	99, 99(*S*)	99, 94(*S*)	99, 88(*R*)	84, 97(+)	99, 97(*R*)
F277V	99, 96(*S*)	99, 99(*S*)	99, 78(*S*)	99, 98(*R*)	81, 99(+)	99, 99(*R*)
F277I	99, 97(*S*)	99, 92(*S*)	99, 94(*S*)	99, 91(*R*)	79, 99(+)	99, 98(*R*)

Enzyme	1g	1h	1i	1j	1k	1l
WT	79, 88(−)	87, 97(−)	81, 96(−)	84, 93(−)	88, 60(−)	91, 85(−)
F277P	91, 93(+)	92, 96(+)	93, 99(+)	93, 99(+)	86, 99(+)	97, 99(+)
F277V	93, 98(+)	95, 96(+)	91, 99(+)	90, 87(+)	91, 99(+)	99, 99(+)
F277I	84, 89(+)	85, 95(+)	89, 99(+)	95, 99(+)	83, 99(+)	90, 99(+)

[a] The whole cell experiments are described in the Experimental section. [b] The conversion was determined using HPLC or GC. [c] ee values of lactones were calculated using HPLC data or GC data. [d] The absolute configurations of lactones were confirmed by comparison with the literature.[14] [e] The optical rotations.

Table 2 Oxidative kinetic resolution of ketones *rac*-**3a–3c** by WT $CHMO_{Acineto}$ and variants[a]



n=1 or 2 3a-c (S)- or (R)-4a-c (S)- or (R)-5a-c

Sub.	Enzyme	Conv.[b]/%	Product 4	ee_p[cd]/%	Product 5	ee_p[cd]/%	Regio-ratio 4 : 5
3a	WT	35	4a	87(*R*)	5a	9(*S*)	82 : 18
3a	F277P	26	4a	11(*R*)	5a	96(*S*)	3 : 97
3a	F277V	30	4a	22(*R*)	5a	99(*S*)	5 : 95
3a	F277I	20	4a	34(*R*)	5a	96(*S*)	4 : 96
3b	WT	45	4b	97(*R*)	5b	—	>99 : 1
3b	F277P	40	4b	96(*R*)	5b	—	>99 : 1
3b	F277V	43	4b	95(*R*)	5b	—	>99 : 1
3b	F277I	46	4b	95(*R*)	5b	—	>99 : 1
3c	WT	36	4c	45(*S*)	5c	—	>99 : 1
3c	F277P	45	4c	—	5c	99(*S*)	<1 : 99
3c	F277V	41	4c	—	5c	99(*S*)	<1 : 99
3c	F277I	43	4c	—	5c	99(*S*)	<1 : 99

[a] The whole cell experiments are described in the Experimental section. [b] Calculated by HPLC data. [c] Calculated by HPLC data. [d] The absolute configurations of lactones were confirmed by comparison with the literature.[14]

in the kinetic resolution of 2-phenyl cyclohexanone **3b** by mutants F277P/V/I, high stereopreference was still retained as WT (**4b**/**5b** > 99/1, ee_{4b} > 95%(*R*)).

The regulation of stereoselectivity in the sulfoxidation of sulfides by using F277 mutants was also investigated (Table S4, ESI†). A reversal in enantioselectivity toward **6a** and **6b** was achieved by F277P/V/I (Table S4, entries 1–8, ESI†). For substrate **6c**, compared with WT, the decreased ee_p value of product **7c** also implied an increased tendency of the reversed enantio-preference toward (*S*)-**7c** by F277P/V/I, though the enantioselectivity was not reversed (Table S4, entries 9–12, ESI†). The results revealed that mutagenesis of the single site 277 can manipulate the selectivity for various structurally diverse substrates.

After demonstrating the stereoselectivity-"switch" role played by F277 of $CHMO_{Acineto}$, we next turned to extend the generality of our approach in other CHMOs. Multiple-sequence alignment of $CHMO_{Acineto}$ with 94 homologues revealed that the residue F277 exhibited high conservation (Fig. S5, ESI†). $CHMO_{Thermo}$, $CHMO_{Rhodo}$, and CHMO from *Rhodococcus* sp. HI-31 (chnb2) ($CHMO_{Rhodo2}$) were selected as targets (Fig. S6, ESI†). Phenylalanine (F) at the corresponding position of CHMOs was mutated into larger-sized tryptophan (W) and smaller-sized valine (V), isoleucine (I) and proline (P). The stereocontrolled experiments catalyzed by corresponding mutants were carried out using **1a–1f** as substrates (Table 3 and Table S5, ESI†). As expected, in the conversion of **1a**, **1d–1f** with medium- or large-sized substituents, all CHMOs mutants displayed highly enhanced or reversed enantioselectivities. These results implied that this stereoselectivity-"switch" role of F277 ($CHMO_{Acineto}$) may be generally used for engineering other CHMOs that share sequence similarity with $CHMO_{Acineto}$. Moreover, it is usually very challenging to reverse the enantioselectivity in the conversion of

Table 3 Baeyer–Villiger oxidation of ketones **1a–1f** by WT CHMOs and variants

CHMOs	Enzyme	1a	1b	1c	1d	1e	1f
$CHMO_{Thermo}$	WT	51(*R*)	99(*S*)	99(*S*)	94(*S*)	34(−)	89(*S*)
	F279W	98(*R*)	99(*S*)	99(*S*)	97(*S*)	99(−)	98(*S*)
	F279P	91(*S*)	99(*S*)	88(*S*)	98(*R*)	94(+)	98(*R*)
	F279V	94(*S*)	99(*S*)	74(*S*)	96(*R*)	93(+)	98(*R*)
	F279I	95(*S*)	99(*S*)	50(*S*)	98(*R*)	94(+)	98(*R*)
$CHMO_{Rhodo}$	WT	34(*R*)	99(*S*)	98(*S*)	74(*S*)	21(+)	15(*S*)
	F279W	89(*R*)	99(*S*)	99(*S*)	98(*S*)	99(−)	99(*S*)
	F279P	97(*S*)	98(*S*)	47(*S*)	98(*R*)	99(+)	99(*R*)
	F279V	96(*S*)	97(*S*)	34(*S*)	94(*R*)	99(+)	98(*R*)
	F279I	95(*S*)	98(*S*)	20(*S*)	94(*R*)	99(+)	98(*R*)
$CHMO_{Rhodo2}$	WT	56(*R*)	99(*S*)	99(*S*)	98(*S*)	33(−)	95(*S*)
	F282W	98(*R*)	99(*S*)	99(*S*)	99(*S*)	99(−)	99(*S*)
	F282P	91(*S*)	94(*S*)	80(*R*)	99(*R*)	99(+)	94(*R*)
	F282V	94(*S*)	94(*S*)	84(*R*)	99(*R*)	99(+)	96(*R*)
	F282I	88(*S*)	94(*S*)	73(*R*)	99(*R*)	99(+)	84(*R*)

small-sized substrates by mutating only a single site, such as **1b** and **1c**. Remarkably, for **1c** with a small ethyl group, we found the reversal of enantioselectivity was achieved by $CHMO_{Rhodo2}$ from an ee value of 99% (*S*) (WT) to 84% (*R*), other CHMO variants from different species also gave an increased ratio of (*R*)-products in comparison to WT. In the future, the mutation of F277 combined with other known active sites (*e.g.*, L143, P431–L435 and F505) would be implemented to verify if there are any cooperative or additive effects for the reversal of enantioselectivity toward substrates with small substituents.

In conclusion, the ''designed'' directed evolution of enzymes is usually focused on the mutation of ligand-binding sites, especially the sites in direct contact with the substituents of ligands. Here, we discovered that a ''second sphere'' site of enzymes acts as a stereo- and regiocontrolled switch, mutagenesis of which may induce the reshaping of active sites so as to tune the preference of chiral products. Furthermore, the knowledge obtained from engineering $CHMO_{Acineto}$ could be efficiently transferred to other CHMOs.

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Conflicts of interest

There are no conflicts to declare.

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