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Exploiting Cofactor Versatility to Convert a FAD Dependent Baeyer−Villiger Monooxygenase into a Ketoreductase

Jian Xu[+], Yongzhen Peng[+], Zhiguo Wang, Yujing Hu, Jiajie Fan, He Zheng, Xianfu Lin, Qi Wu*

Abstract: Cyclohexanone monooxygenases (CHMOs) show very high catalytic specificity for natural Baeyer−Villiger (BV) reactions, but promiscuous reduction reactions have not been reported to date. In order to reach this goal, we focused mechanistically on the flavin adenine dinucleotide (FAD) as the cofactor of CHMOs, which can switch between oxidation and reduction states. We achieved such a novel promiscuous reduction activity of CHMOs by converting $CHMO_{Acineto}$ into a ketoreductase. Rational structure-guided engineering of $CHMO_{Acineto}$ was implemented in order to drastically improve the catalytic reduction activity (yield up to 99%) and stereoselectivity (e.e. up to 99%).

Enzymes are known to be powerful catalysts in organic synthesis and pharmaceutical chemistry because of their high efficiency and selectivity.[1] In contrast to traditional organometallic catalysts, they offer attractive alternatives due to their low toxicity and mild reaction conditions. However, in most cases, enzymes catalyze only the respective natural reaction types. Inducing additional reactivities as “enzymatic promiscuity” remains a great challenge.[2] Driven by the development of protein engineering and advances derived from mechanistic studies, new notable cases of catalytic promiscuity have been reported recently, leading to higher synthetic utility.[3-6] For example, nicotinamide-dependent ketoreductases were employed to catalyze asymmetric dehalogenation by irradiating with light.[5] Cytochrome P450 monooxygenases and hemoglobins have been engineered into new artificial enzymes for constructing a series of useful chemical bonds, such as chiral C-C, C-N, C-Si and C-B bonds.[6] Inspired by these significant advances, we report herein an unprecedented promiscuous case of a Baeyer−Villiger monooxygenase (BVMO) as a mechanistically novel biocatalyst in enantioselective carbonyl reduction. To date such reactivity has not been observed in any other FAD-dependent monooxygenases known in nature.

As one of the most studied BVMOs,[7] cyclohexanone monooxygenase (CHMO), which can catalyze *in vivo* the BV transformation of cyclohexanone to caprolactone, among many other reactions, has been successfully engineered to increase its activity, thermal stability, and enantioselectivity by directed evolution.[8] However, CHMO is quite specific for its natural reaction type. Other catalytic activities such as the oxidations of thioethers or boronic acid were performed in the similar mechanism with the natural one.[9] In addition, Fraaije and coworkers converted a BVMO into a NADPH oxidase by protein engineering.[10] As the active site of CHMO, flavins are extremely versatile molecules containing multiple states, such as oxidized (quinone; Fl_{ox}), reduced (hydroquinone; Fl_{red}) and peroxidized (peroxyflavin; FlOOH).[11] As outlined in Figure 1a, traditional oxygenation reactions catalyzed by CHMO begin with the combination of Fl_{red} and O_2 to form FlOOH. After one atom of oxygen is incorporated into ketones, the FlOOH is converted into FL_{ox} by water elimination, and then FL_{ox} undergoes reduction by NADPH leading to the regeneration of Fl_{red}. In nature, enoate reductases from the family of old yellow enzymes (OYEs) are a class of flavin-containing enzymes that can catalyze asymmetric reduction of activated C=C bonds by Fl_{red} (Figure 1b).[12] Recently, Hyster and coworkers have discovered phenylacetone monooxygenase (PAMO) could be converted into a reductase to catalyze an enantioselective radical dehalogenation reaction.[3a] Inspired by these advances, we hypothesized that the catalytic cycle of CHMO could be broken, enabling Fl_{red} to be capable of catalyzing ketone reduction reactions of suitable substrates by hydride transfer from N5.

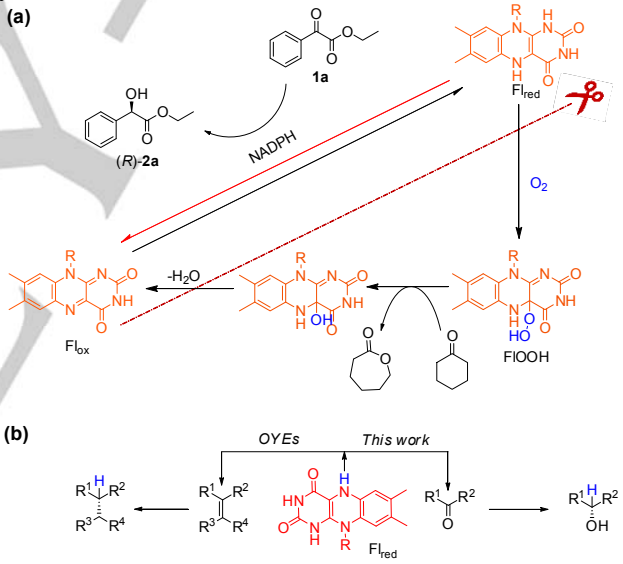

Figure 1. (a) The catalytic cycle of a Baeyer-Villiger reaction and the proposed mechanism of flavin-dependent carbonyl reduction. (b) Fl_{red}-catalyzed reduction reaction.

Optically active α-hydroxy carbonyl compounds are important chiral building blocks in pharmaceutical chemistry and chemical biology.[13] We therefore chose the α-keto ester **1a** as an ideal model substrate for our study. CHMO from *Acinetobacter* sp. NCIMB 9871 ($CHMO_{Acineto}$)[7, 14] was first tested under anaerobic conditions with *E. coli* as host organism. We were delighted to find that the reduction of **1a** appeared to be feasible, although the observed reaction efficiency was poor (15% yield and 84% e.e. in favor of (*R*)-**2a**, Table 1, entry 1,). Due to the low activity of $CHMO_{Acineto}$, we turned our attention to PAMO,[15] and found the whole-cell system of PAMO displayed a significant increase in promiscuous activity toward **1a** (Table 1, entry 2). However, the control experiment of *E. coli* (TOP 10) in the absence of PAMO also afforded the reduced product **2a** with the same

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yield and e.e. value as PAMO (Table 1, entry 3). This significant background reaction prevented us from investigating the reduction activity of PAMO in the whole-cell system. The influence of the host cells of $CHMO_{Acineto}$ (BL21-DE3 or BL21-DE3 with plasmid pET-22b (+)) was also evaluated. Only trace amounts of reduced product **2a** with low stereoselectivity were observed (Table 1, entry 4-5). Upon isolating, purifying and testing the enzyme, the e.e. value improved significantly (up to 99%), pointing to the influence of whole-cell system (Table 1, entry 6). In yet another control experiment with $CHMO_{Acineto}$ which was devoid of FAD cofactor, no reduced product was observed.[16] GDH was also tested separately in the model reaction providing no reduction product.

Table 1: Screening the conditions for the promiscuous reduction of **1a** [a]

Entry	Condition	Yield (%)	e.e. (%)
1	$CHMO_{Acineto}$-WT, BL21-BE3[a]	15	84
2	PAMO-WT, Top 10[a]	99	12
3	Top 10[a]	99	12
4	BL21-DE3[a]	10	45
5	BL21-DE3-pET 22b (+)[a]	11	51
6	Purified-$CHMO_{Acineto}$, GDH[b]	6	99
7	Purified-$CHMO_{Acineto}$ devoid of FAD, GDH[b] [c]	0	0
8	GDH[d]	0	0

[a] **1a** (0.01 mmol) was dissolved in 20 µL acetonitrile, then added to 2 mL cell cultures (OD_{600} ≈ 3.0) under anaerobic condition for 4 h at 20 °C; the yields and e.e values were determined by chiral HPLC; [b] 0.01 mmol **1a**, 20 µL acetonitrile, 1% $NADP^+$, 10 mg glucose, 0.01 µmol CHMO and 1 µmol GDH were dissolved in 2 mL PBS buffer (50 mM, pH=7.4) under anaerobic condition for 20 h at 20 °C; [c] treated with urea and KBr, supporting information; [d] without Purified-CHMO.

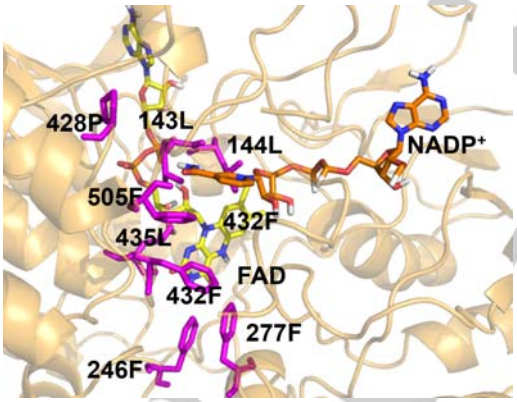

Figure 2. Active site of $CHMO_{Acineto}$, homology model based on the X-ray structure of CHMO from *Rhodococcus* sp. (PDB code: 3UCL[19])

With the aim of increasing product formation, we sought to engineer $CHMO_{Acineto}$ by rational design[17] rather than directed evolution.[18] If successful, large mutant libraries as in directed evolution would not have to be screened. As the crystal structure of $CHMO_{Acineto}$ is not available, we built a homology model (named as $CHMO_{homo}$) using the X-ray structure of CHMO from *Rhodococcus sp*. (PDB code: 3UCL),[19] which shows high sequence similarity around the active sites with $CHMO_{Acineto}$. A

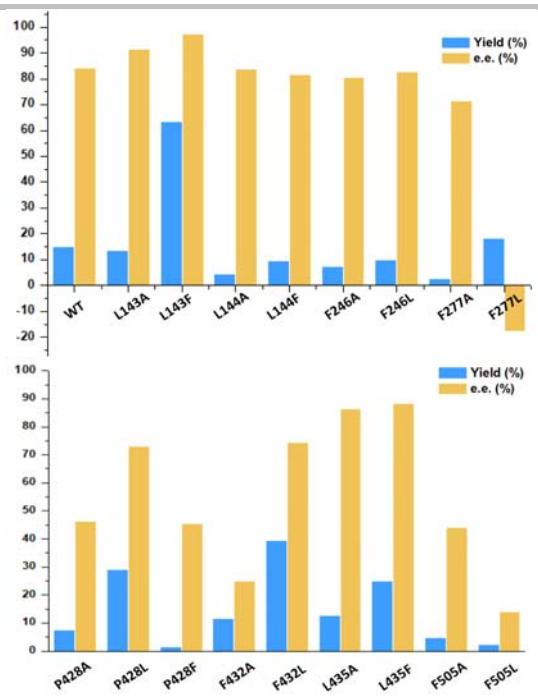

Figure 3. Assessing the introduction of volume-based rationally designed mutagenesis at the chosen hot spots. The experiment details were shown in supporting information.

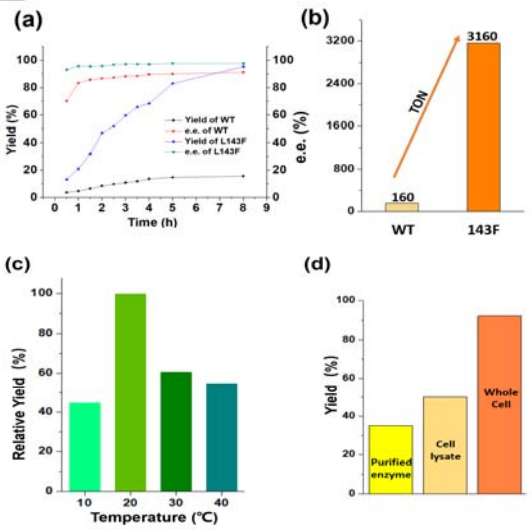

Figure 4. $CHMO_{Acineto}$-catalyzed reduction of α-Keto esters. (a) The progress curve of reduction catalyzed by WT-CHMO and L143F. (b) TON of WT and L143F for **1a** reduction. (c) The Influence of temperature with L143F. (d) Reaction yields catalyzed by whole-cell catalyst, cell lysate, or purified enzyme of L143F. The experiment details were shown in supporting information.

series of residues around the binding pocket including L143, L144, F246, F277, A428, F432, L435 and F505 were chosen as hotspots for protein engineering to improve the reduction activity (Figure 2). A focused rationally designed library was constructed based on a volume-scanning strategy using alanine (A), leucine (L), and phenylalanine (F). These were regarded as small, medium and large amino acids, replacing the amino acids individually at hotspots in the anticipation that this would optimally modify the space of the substrate-binding pocket. As

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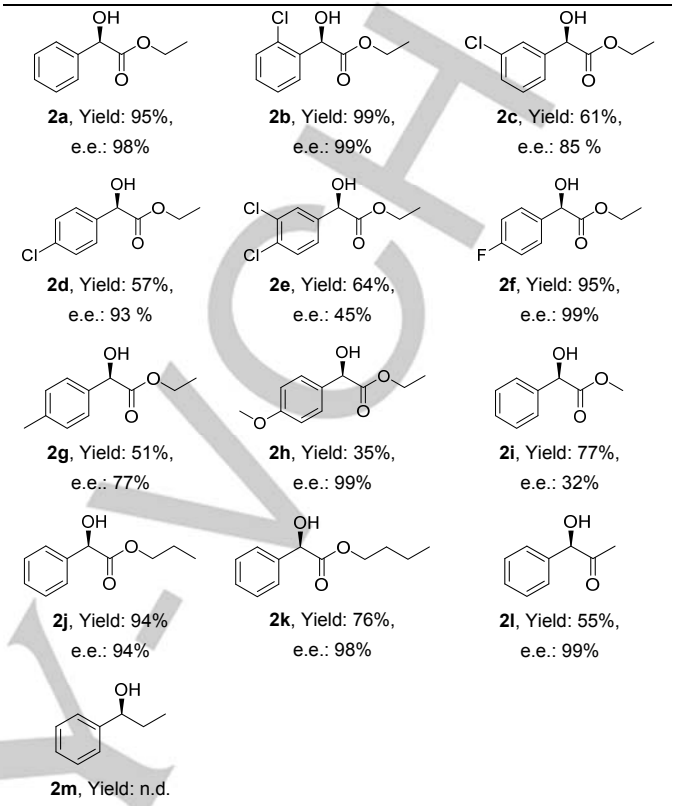
shown in Figure 3, several mutants in the volume-based library were found to have noticeable effects on the activity and stereoselectivity. Under the same reaction conditions, L144A, F246A and F246L displayed slightly increased reduction yields (25-30%) with high stereoselectivity (80-90% e.e.) compared with the WT. Remarkably, the stereoselectivity of L277A was reversed from (*R*)- into (*S*)-configurational preference. Among the hot positions tested, residue L143 was considered as a crucial spot for improving activity, with the best mutant L143F exhibiting a notable yield of 62% and an e.e. of 98% (*R*).

With the best mutant in hand, the properties of the biocatalytic system were assessed. The time-reaction curves showed that the reaction catalyzed by L143F proceeds much faster than WT (95% *vs.* 18% conversion in 8 hours, Figure 4a). The catalytic efficiency of L143F, measured by kinetic parameters (Table S3), was improved remarkably relative to WT-CHMO$_{Acineto}$ (36.30 $s^{-1}mM^{-1}$ *vs* 0.30 $s^{-1}mM^{-1}$). And L143F exhibited a 20-fold improvement in TON compared with WT (Figure 4b). We then investigated the influence of temperature on the formation of (*R*)-**2a** in the whole-cell systems. The results showed that the structure of CHMO$_{Acineto}$ is unstable with increasing temperature, the reduction activity diminishing considerably over 30 °C (Figure 4c). Notably, although the stereoselectivity achieved by purified CHMO$_{Acineto}$ is excellent, the reaction rates catalyzed by purified protein or cell lysate proved to be much slower than those in the whole-cell system (Figure 4d, Figure S3). We speculate that the cell membrane periplasm may protect enzyme from possible inactivation by substrate, or keep the enzyme in a more reducing environment preventing the formation of Fl-OOH species.[6a,f] Surprisingly, this transformation could proceed both in aerobic and anaerobic conditions (Table S5). However, the catalytic activities in aerobic conditions are slightly lower than anaerobic conditions. Additional experiment (Table S6) ensured that **1a** is hardly to be oxidized by L143F. Thus, the reaction was considered to be implemented under air. The decrease of reduction activity may be caused by the formation of FLOOH and the transformation of uncoupling.

Next, we explored the substrate scope of this unnatural reduction reaction catalyzed by CHMO$_{Acineto}$ in the whole-cell system. As illustrated in Table 2, a wide range of differentially substituted aromatic α-keto esters accommodating electron-rich and electron-deficient groups were readily converted into the corresponding alcohols in moderate to good yields and seteroselectivity. Notably, substrates bearing chlorine substituents at different positions (*ortho*-, *meta*-, *para*-) of the phenyl ring were all accepted by L143F and displayed excellent (*R*)-selectivity (**2b**-**2d**). α-Keto esters characterized by a different length of the alcohol parts also underwent reduction with high yield and satisfactory stereoselectivity (**2a, 2j**, **2k**), while the methyl ester is relatively poor with respect of stereoselectivity (**2i**). The reduction of the 1,2-diketone **1l** into the α-hydroxy ketone **2l** occurs with moderate yield and high e.e. value (99%). It is noteworthy that propiophenone (**1m**) could not be reduced by CHMO$_{Acineto}$ (**2m**), probably due to the absence of some interactions between the *β*-carbonyl function and enzyme binding pocket. Large scale reactions of **1a** and **1l** (100 mg) were also implemented with good yields (**2a**: 93%, **2l**: 50%) and high stereoselectivity (**2a**: 98% e.e., **2l**: 90% e.e.).

Table 2: Substrate scope of the promiscuous reduction reaction[a][b]



[a] Reaction conditions: **1** (0.01 mmol) was dissolved in 20 μL acetonitrile, then added to 2 mL cell cultures under anaerobic condition for 8-12 h at 20 °C; [b] the ee values of **2** were determined by chiral HPLC.

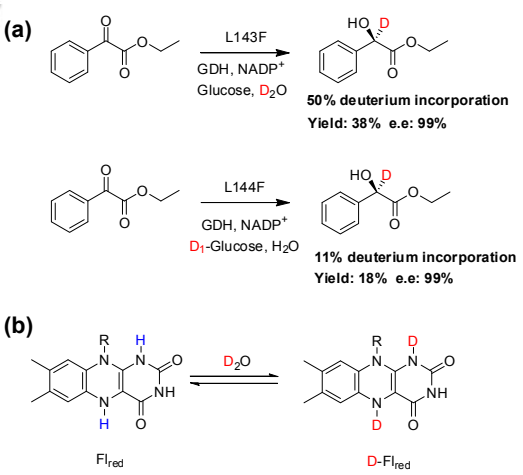


Figure 5. Kinetic isotope effect studies.

Mechanistic investigations were first performed by isotope incorporation (Figure 5). When D_1-glucose was used to generate D-Fl$_{red}$, only 18% yield was observed with 99% e.e. value and 11% deuterium incorporation. We considered that the absence of complete deuterium incorporation was due to the H-D exchange between N5 of D-Fl$_{red}$ and H_2O.[20] This was also confirmed with the transformation in which D_2O was used as a solvent under otherwise standard conditions: 38% yield of **2a**

was obtained with 99% e.e. and 50% deuterium incorporation. Rate study of deuterium incorporation experiments was implemented, and the results showed the reducation rate in the isotope incorporation reaction with D_1-glucose proceeds significantly slower compared with that of D_2O (Figure S4). Thus, the reduction rate with D_1-glucose might be much slower than H-D exchange between the reduced flavin with the solvent (H_2O), leading to less deuterated product (11%). On the other hand, in the isotope incorporation reaction with D_2O, the higher reactive rate resulted in more deuterated product (50%). This result supports a mechanism in which FI_{red} is regenerated *in situ* and acts as a hydrogen atom donor to enable the reduction of appropriate carbonyl compounds. Further insight into the reduction mechanism was obtained by using density functional theory (DFT) calculations. These results indicate that the mechanism we propose is also thermodynamically favorable ($\Delta Go = -13.96$ kcal·mol^{-1}, Figure S5 and Table S8).

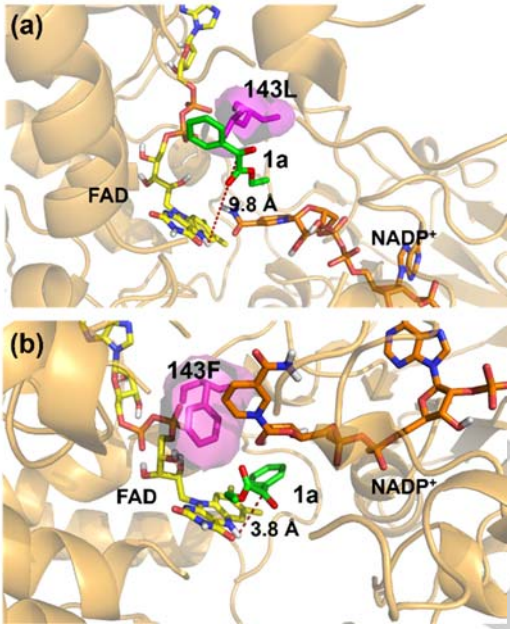


Figure 6. MD simulations for gaining insight into the origins of activity improvement with WT-CHMO (a) and L143F (b).

Finally, the docking and MD experiments were implemented to gain insight into the origins of high activity of L143F in comparison with WT. As shown in Figure 6, when compared with WT-CHMO, the nature of the binding pocket is dramatically altered by mutation L143F, especially the possible T-shaped π-π interaction between L143F and $NADP^+$.[19] The average distance between the carbon atom in carbonyl moiety of **1a** and the hydrogen atom on N5 of FI_{red} is clearly shorter in L143F than in WT (3.8 Å in L143F *vs* 9.8 Å in WT), according to the analysis of 25 ns trajectory of MD simulation (Figure S6). This is a requirement for the substrate to undergo hydride transfer from FI_{red} with formation of the reduction product **2a**.

In summary, we report a mechanistically novel biocatalytic carbonyl reduction catalyzed by a Baeyer−Villiger monooxygenase (BVMO). It is a new case of a promiscuous enzyme-catalyzed transformation, enabled by the cofactor FAD that has not been observed in any other FAD-dependent monooxygenase known in nature. Whereas WT-$CHMO_{Acineto}$ displayed only a very limited ability to catalyze the model reaction **1a**→**2a**, rational design based on a volume-scanning strategy at hotspots was successfully implemented to improve this promiscuous function with minimal screening effort. The origin of enhanced reduction activity of $CHMO_{Acineto}$ mutants was rationalized by computational simulation. Future research will focus on extending the application of this new biocatalytic reaction.

Acknowledgements

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Keywords: Cyclohexanone monooxygenases • Catalytic promiscuity • Protein engineering • Reductase • Volume-based library

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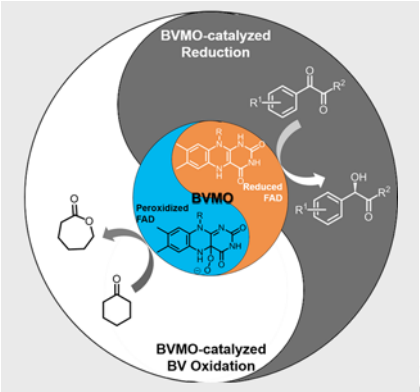
COMMUNICATION

Baeyer−Villiger Monooxygenase-catalyzed carbonyl reduction that has not been observed in any other FAD-dependent monooxygenase known in nature, was demonstrated in this work. Rational structure-guided engineering of $CHMO_{Acineto}$ was implemented in order to drastically improve the catalytic reduction activity (yield up to 99%) and stereoselectivity (e.e. up to 99%).



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Exploiting Cofactor Versatility to Convert an FAD Dependent Baeyer−Villiger Monooxygenase into a Ketoreductase