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Light-Driven Kinetic Resolution of α-Functionalized Carboxylic Acids Enabled by Engineered Fatty Acid Photodecarboxylase

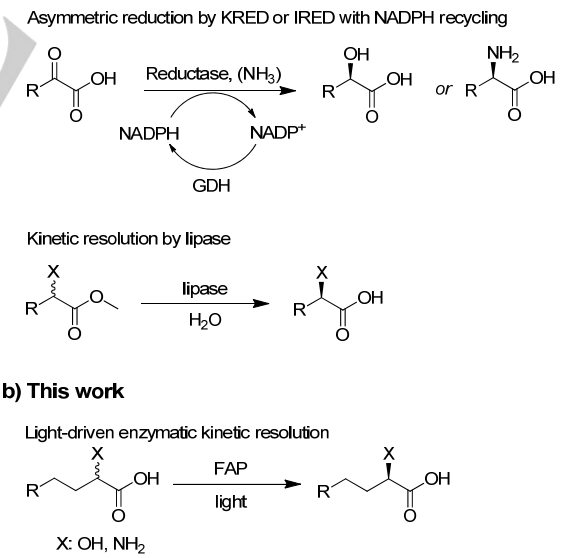
Jian Xu, Yujing Hu, Jiajie Fan, Mamatjan arkin, Danyang Li, Yongzhen Peng, Weihua Xu, Xianfu Lin & Qi Wu*

Abstract: Chiral *α*-functionalized carboxylic acids are valuable precursors for a variety of medicines and natural products. Herein, we described an engineered fatty acid photodecarboxylase (*Cv*FAP)-catalyzed kinetic resolution of *α*-amino acids and *α*-hydroxy acids providing the unreacted (*R*)-configured substrates with high yields and excellent stereoselectivity (ee up to 99%). This efficient light-driven process requires neither NADPH recycling nor prerequisite preparation of esters, which are required in previous biocatalytic approaches. The structure-guided engineering strategy is based on large-sized amino acid scanning at hotspots to narrow the substrate binding tunnel. To the best of our knowledge, this is the first example of asymmetric catalysis by an engineered *Cv*FAP.

Multifunctional chiral molecules, such as unnatural *α*-amino acids and *α*-hydroxy carboxylic acids, are important building blocks in pharmaceutical chemistry and chemical biology.[1] Over the past years, the development of new methods for the synthesis of chiral α-functionalized carboxylic acids has received considerable attention. The common chemical synthesis routes heavily depend on transition metal catalysts and complex chiral ligands, which suffer from high costs and environmental problems.[2] Therefore, the development of new methods to synthesize chiral α-functionalized carboxylic acids through an eco-friendly approach is still a topic of continuous scientific interest.

In contrast to traditional chemical methods, biocatalysis provides in many cases a greener and more sustainable process to obtain these classes of compounds. For example, keto reductases (KRED) and imine reductases (IRED) have been successfully used to convert *α*-keto acids into *α*-hydroxy/amino acids.[3] These reduction processes require a stoichiometric amount of an expensive cofactor (e.g. NADPH) as a hydrogen donor. One way to overcome this limitation is to introduce another oxidoreductase, such as glucose dehydrogenase (GDH), to recycle the cofactors.[4] However, a series of reaction conditions, such as temperature and pH, have to be optimized to ensure that each enzyme works with acceptable activity in the multi-enzyme system. Another widely used method is kinetic resolution (KR) using lipases.[5-6] However, there are still some limitations that need to be addressed, such as the requirement of prerequisite ester or amide preparation. Accordingly, exploring other highly efficient and versatile biocatalytic methods to access chiral α-functionalized carboxylic acids is certainly desirable.

Due to the advantages of low consumption of energy and being a clean process, photocatalysis is becoming a popular research topic, particularly in combination with biocatalysis for cascade reactions.[7] Although light-dependent enzymes that directly utilize visible light as an energy source are highly sought after, photoenzymes have seldom been found in nature. The activities of these type of enzymes depend on the irradiation of the cofactor in the active site with light, such as DNA-repair enzymes[8] and protochlorophyllide oxidoreductases[9]. Very recently, Beisson and co-workers discovered a fatty acid photodecarboxylase from *Chlorella variabilis* NC64A (*Cv*FAP) which they used to convert long-chain fatty acids into hydrocarbons.[9] In another key study, Hollmann's group reported the synthesis of alkanes from triglycerides through a designed lipase/*Cv*FAP-catalyzed cascade reaction.[11a] The different activity of *cis*- and *trans*-oleic acid mentioned in these reports strongly aroused our interest. We envisioned that *Cv*FAP could recognize not only *cis/trans* isomers, but stereoisomers as well. To the best of our knowledge, no examples of enantioselective reactions catalyzed by *Cv*FAP have been reported to date.[10-11] Herein, we demonstrate a new KR method catalyzed by engineered *Cv*FAP variants for the synthesis of chiral *α*-hydroxy/amino carboxylic acids. This photo-activated biotransformation proceeds without NADPH circulation or any other reaction pretreatment, thus providing a more efficient route to chiral compounds with high activity and stereoselectivity.



Scheme 1. Enzymatic asymmetric synthesis of α-hydroxy/amino acids

Initially, we studied the catalytic ability of wild-type (WT) *Cv*FAP to promote the KR of 2-hydroxyoctanoic acid (**1a**). After irradiation with a 450 nm light for 12 hours, only 20% of the substrate was converted into decarboxylation products by WT *Cv*FAP with low substrate stereoselectivity (ee_s=22%) having (*R*)-configuration. The yield and stereoselectivity failed to increase upon prolonging the reaction time (Figure 1). In

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an effort to tackle this problem, *Cv*FAP was engineered by directed evolution which had been identified as a versatile strategy to improve the targeted feature of an enzyme, such as catalytic activity or selectivity.[12] We assumed that the improvement of the activity might also influence substrate stereoselectivity.[13] As shown in Figure 2, the geometry of the active site of WT *Cv*FAP reveals a narrow substrate-binding channel for long-chain fatty acids such as palmitic acid (PLM) (PDB:5NCC[10]), and the substrate positioning is mainly stabilized by the hydrophobic interaction of Y466 with the 9th and 10th carbon atom of PLM.[10] However, when **1a** (chain length of C8) was docked into WT *Cv*FAP, we found that Y466 does not effectively influence the substrate. Thus, to improve the activity and stereoselectivity of *Cv*FAP for medium-chain fatty acids, we targeted other residues between Y466 and the flavin adenine dinucleotide (FAD) binding region for genetic modification. We aimed to modify the substrate binding tunnel so that it becomes narrower, thus better stabilizing the substrate *via* hydrophobic interactions. Residues A384, L386 and G462 were considered to be the largest potential contributors in influencing the space of the binding tunnel due to their short side chains. Therefore, site-specific mutagenesis of large size amino acids including Q, K, F and Y was used to scan these hot positions. As indicated by the screening results in Table 1, the effect of residue L386 on the activity was evident, while stereoselectivity of L386 variants remained sub-optimal (ee=55%-67%). As anticipated, mutagenesis at the other two positions led to a moderate enhancement of the activity, but to a remarkable influence on enantiopreference for the (*S*)-substrate. The best hit in the present study proved to be variant G462Y, showing the highest ee-value of the unreacted (*R*)-**1a** (up to 99%) under an ideal conversion (51%). The kinetic parameters were then measured for WT *Cv*FAP and G462Y to compare their catalytic activity (see supporting information). Variant G462Y displayed a catalytic efficiency (k_{cat}/K_m = 6.99 $s^{-1}mM^{-1}$) which is 30-fold higher than that of WT (k_{cat}/K_m = 0.23 $s^{-1}mM^{-1}$) (Table S1). Moreover, the enantiopreference of variant G462Y for the (*S*)-substrate is much better than that of WT *Cv*FAP ($(k_{cat}/K_m)_S/(k_{cat}/K_m)_R$=205 *vs.* $(k_{cat}/K_m)_S/(k_{cat}/K_m)_R$=5.7) (Table S1). Noteworthy, a little heptaldehyde was observed as by-product when screening the model reaction. We speculated that the hydroxy ions in water can also attack the reaction intermediate, and then spontaneously dehydrate to produce such aldehyde by-product.

As our goal was to develop an efficient KR method of α-functionalized carboxylic acids, the substrate scope was next investigated. To our delight, although biocatalysts can hardly be expected to be universal, mutant G462Y displayed a satisfactory substrate scope. As shown in Figure 3, a series of *α*-hydroxy acids with different chain length from C6 to C12 were all accepted by this mutant with excellent stereoselectivity (*E* up to 211). Besides compounds with different alkyl chain length, *α*-hydroxy acids bearing functional groups (**1g**, **1h, 1j**) also proved to be good substrates for G462Y, and afforded (*R*)-isomers in good yields and high stereoselectivity (ee up to 99%). Particularly noteworthy is the observation that *α*-hydroxy acids with thioether-groups, which can easily be oxidized to sulfoxide or sulfone by FAD, were also suitable for this reaction. In the case of substrate **1i**, however, the carbon chain is too short to be accepted by G462Y, leading to low activity. Interestingly, the bulky tertiary carboxylic acid (**1k**), which we thought could hardly enter the binding tunnel, also reacted to give the (*R*)-isomer with excellent enantioselectivity (ee=99%).

We next turned our attention to the KR of *α*-amino acids. As an extension of the substrate scope, *α*-amino acids underwent smooth decarboxylation with the best mutant G462Y, and produced the expected (*R*)-stereoisomers of unnatural amino acids (*R*)-**1l** with satisfactory stereoselectivity. Unexpectedly, the enantioselectivity of **1m** was reversed to (*S*)-configuration with a moderate ee value. Surprisingly, no reactions of **1n** and **1o** were observed under the catalysis of WT *Cv*FAP or G462Y mutant, probably due to

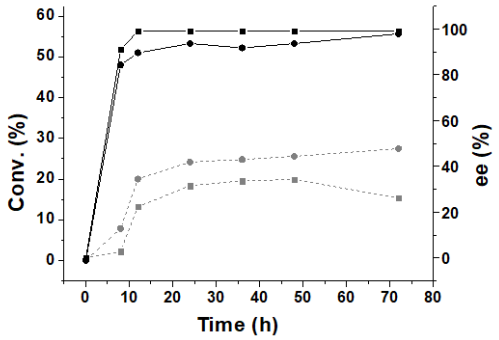


Figure 1. The progress curve of the KR of **1a** with WT and variant G462Y. Dotted gray lines are for WT-*Cv*FAP, and black straight lines for G462Y. (●) conversion; (■) ee.

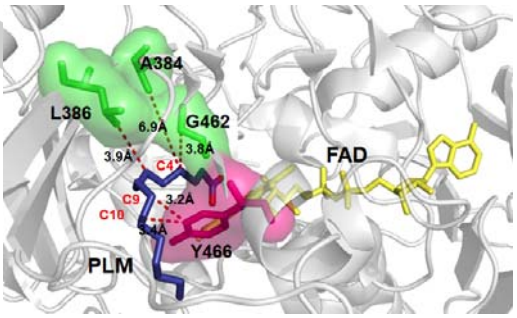
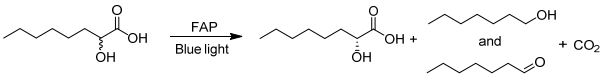


Figure 2. Active site of *Cv*FAP with palmitic acid (PLM), showing the minimum distance between PLM and hot positions (A384, L386, G462, Y466).

Table 1*:* Assessing the introduction of large-sized amino acids (K, Q, F, Y) at the chosen hot spots [a]



rac-**1a** (*R*)-**1a**

Entry	Mutant	Conv. (%)	ee_s(%) [b]
1	WT	20	22
2	A384K	53	91
3	A384Q	50	93
4	A384F	29	36
5	A384Y	38	33
6	L386K	48	55
7	L386Q	60	67
8	L386F	65	57
9	L386Y	63	60
10	G462K	70	87
11	G462Q	45	36
12	G462F	35	56
13	G462Y	51	99

[a] Reaction conditions: *rac*-**1a** was dissolved in 20 μL DMSO, then added to 2 mL crude enzyme solution (final concentration: 10 mM, see supporting information), under the irradiation of blue LEDs for 12 h at 20 °C; [b] the ee values of (*R*)-**1a** were determined by chiral GC after methyl ester derivatization.

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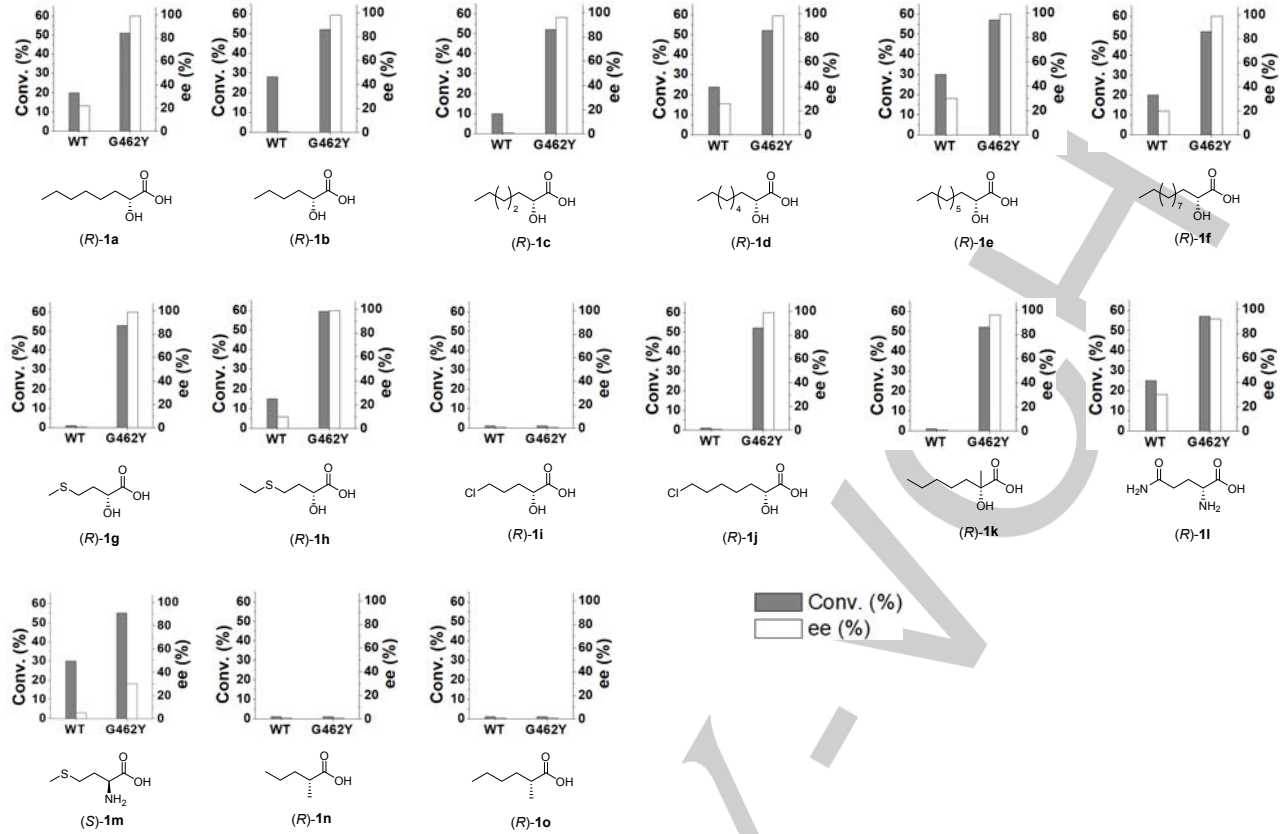
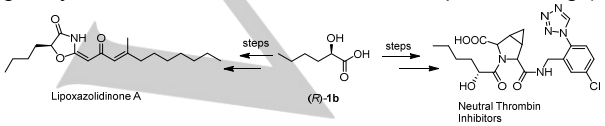


Figure 3. Substrate scope of light-driven kinetic resolution enabled by WT *Cv*FAP and variant G462Y.

the weak electron withdrawing ability of the methyl substituent. To assess the scalability of these light-driven KR processes, large scale reactions were carried out with *rac*-**1a** and *rac*-**1b** (100 mg) in 50 mL enzyme solutions, separately. After irradiation at 450 nm at 20 ºC for 12 hours, simple extraction and purification steps were performed, and the desired (*R*)-isomers were obtained in good isolated yields (45% for (*R*)-**1a**, 40% for (*R*)-**1b**) and high stereoselectivity (99% ee for (*R*)-**1a**, 98% ee for (*R*)-**1b**).

The enantioenriched products can be transformed into a variety of complex biologically active molecules. For example, (*R*)-**1b** is the chiral precursor of the antimicrobial natural product Lipoxazolidinone A[14] or the essential building block of a series of novel neutral thrombin inhibitors[15] (Scheme 2).

To gain insight into the source of high stereoselectivity, we performed docking and molecular dynamics (MD) simulation with (*R*)- and (*S*)-isomers of 2-hydroxyoctanoic acid (**1a**), respectively, using the best mutant G462Y. Two representative structures from the cluster analysis are shown in Figure 4. The hydrogen bonded network of the residues near the active site such as 451R, 572H, 575N, enable the stabilization of the (*R*)-isomer and prevents it from approaching FAD (Figure 4B). In contrast, this effect was greatly reduced in the case of the favored quick-reacting (*S*)-



Scheme 2. Derivatization of enantioenriched products.

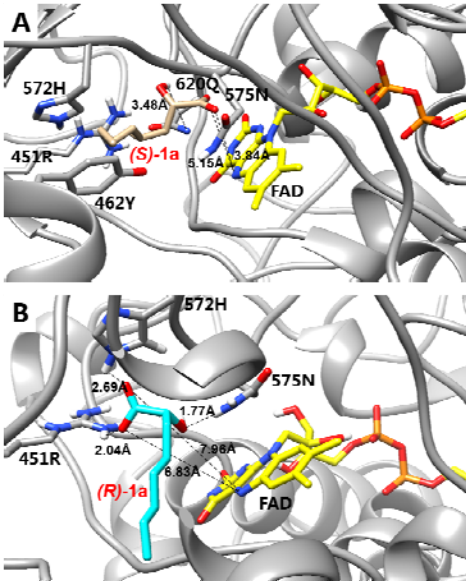


Figure 4. Models of variant G462Y with (*S*)-**1a** (A), and (*R*)-**1a** (B) docked into the active site. The overall structure (PDB: 5NCC) is shown as a gray cartoon and the FAD, and (*S*)-**1a** (tan) and (*R*)-**1a** (blue) are depicted by a stick representation.

isomer, resulting in a shorter distance between **1a** and FAD (Figure 4A). Furthermore, the average distance between the two oxygen atoms in the carboxyl moiety of **1a** and the N5 atom in FAD is clearly shorter for the (*S*)-isomer than for the disfavored (*R*)-isomer (5.08 Å and 5.60 Å for (*S*)-isomer *vs*

8.04 Å and 8.09 Å for (*R*)-isomer), according to the analysis of the 10 ns trajectory of the MD simulation (Figure S8). Based on these results, we proposed that the excited FAD should easily undergo facile single electron transfer (SET) with carboxylate of the (*S*)-isomer after irradiation by light, while the (*R*)-isomer probably remains unchanged.

In summary, our study demonstrates a new strategy of direct photoinduced KR of α-functionalized carboxylic acids by engineered *Cv*FAP without dependence on electron-transfer by NADPH or prerequisite preparation of esters, which are required in previous biocatalytic approaches. Under the guidance of rational design, the mutation strategy was performed by introducing large-sized amino acids via site-specific mutagenesis to improve the hydrophobic interaction between substrates and the binding tunnel. Rather than using saturation mutagenesis involving large amino acid alphabets, the present strategy drastically reduces the screening effort. MD simulation was implemented to gain insight into the origin of the selectivity of the best mutant. We believe that this photoinduced enantioselective biocatalytic process provides a greener and more sustainable approach to obtain chiral α-functionalized carboxylic acids, which are useful as building blocks in the synthesis of important pharmaceuticals.

Acknowledgements

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Keywords: photoenzyme • biocatalysis • α-functionalized carboxylic acids • kinetic resolution • rational design

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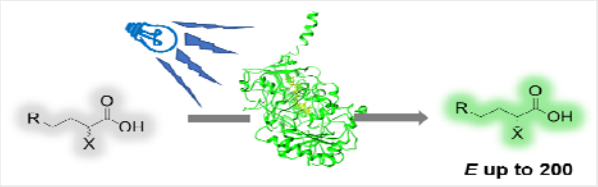

Photoenzyme-catalyzed Kinetic Resolution of *α*-amino acids and *α*-hydroxy acids was developed for the first time by using engineered *Cv*FAP variants, which were generated with a site-specific mutagenesis based on large-sized amino acid scanning. A series of (*R*)-configured *α*-functionalized carboxylic acids were obtained with *ee* up to 99% and E >200.

*Jian Xu, Yujing Hu, Jiajie Fan, Mamatjan arkin, Danyang Li, Yongzhen Peng, Weihua Xu, Xianfu Lin & Qi Wu**

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