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Published in:
ChemBioChem

DOI:
10.1002/cbic.201600149

ChemPubSoc Europe

DOI: 10.1002/cbic.201600149

CHEMBIOCHEM Communications

Mutations Closer to the Active Site Improve the Promiscuous Aldolase Activity of 4-Oxalocrotonate Tautomerase More Effectively than Distant Mutations

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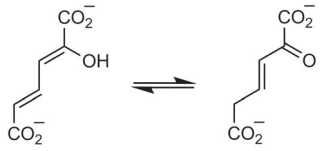
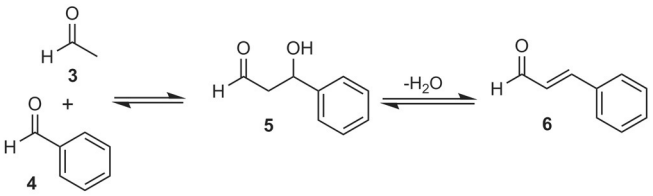
The enzyme 4-oxalocrotonate tautomerase (4-OT), which catalyzes enol–keto tautomerization as part of a degradative pathway for aromatic hydrocarbons, promiscuously catalyzes various carbon–carbon bond-forming reactions. These include the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde. Here, we demonstrate that 4-OT can be engineered into a more efficient aldolase for this condensation reaction, with a >5000-fold improvement in catalytic efficiency (k_{cat}/K_m) and a $>10^7$-fold change in reaction specificity, by exploring small libraries in which only "hotspots" are varied. The hotspots were identified by systematic mutagenesis (covering each residue), followed by a screen for single mutations that give a strong improvement in the desired aldolase activity. All beneficial mutations were near the active site of 4-OT, thus underpinning the notion that new catalytic activities of a promiscuous enzyme are more effectively enhanced by mutations close to the active site.

The homohexameric enzyme 4-oxalocrotonate tautomerase (4-OT) is a member of the tautomerase superfamily, a group of homologous proteins that share a βαβ structural fold and a unique catalytic N-terminal proline (Pro1).[1–3] 4-OT catalyzes the conversion of 2-hydroxy-2,4-hexadienedioate (**1**) into 2-oxo-3-hexenedioate (**2**; Scheme 1) as part of a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2.[4,5] In this tautomerization reaction, Pro1 functions as a general base to remove the 2-hydroxy proton of **1** for delivery to the C-5 position to yield **2**. Pro1 can function as a general base because it has a pK_a of ~6.4 and exists largely as the uncharged species at cellular pH.[6]



Scheme 1. The native proton-transfer reaction catalyzed by 4-OT.

In addition to its natural tautomerase activity, 4-OT promiscuously catalyzes several carbon–carbon bond-forming Michael-type addition and aldol condensation reactions.[7–16] This includes the aldol condensation of acetaldehyde (**3**) with benzaldehyde (**4**) to yield cinnamaldehyde (**6**; Scheme 2).[10,12] 4-OT catalyzes both the initial addition of **3** to **4** to yield 3-hydroxy-3-phenylpropanal (**5**) and the subsequent dehydration of **5** to



Scheme 2. The aldol condensation reaction promiscuously catalyzed by 4-OT.

give **6**. 4-OT also catalyzes a retro-aldol reaction with **5** as the substrate, thereby yielding **3** and **4**.[12] $NaCNBH_3$ trapping, MS, and X-ray crystallography experiments strongly suggest a mechanism in which Pro1 functions as a nucleophile, rather than a base, and reacts with the carbonyl functionality of **3** to form a covalent enamine intermediate.[10,17] This intermediate reacts with **4** by an aldol addition. After enzymatic dehydration of **5**, the final product **6** is released from the active site upon hydrolysis. Notably, mechanism-inspired engineering provided an active-site mutant (F50A) with enhanced aldol condensation activity (600-fold k_{cat}/K_m).[12]

The aldol reaction is one of the most important reactions in synthetic chemistry, and has been widely used for the production of valuable compounds.[18–22] Therefore, there is great interest in the development of novel aldolases for biocatalytic applications. In this study, we investigated whether 4-OT can be engineered into a more efficient aldolase by exploring small libraries in which only "hotspot" positions were varied.

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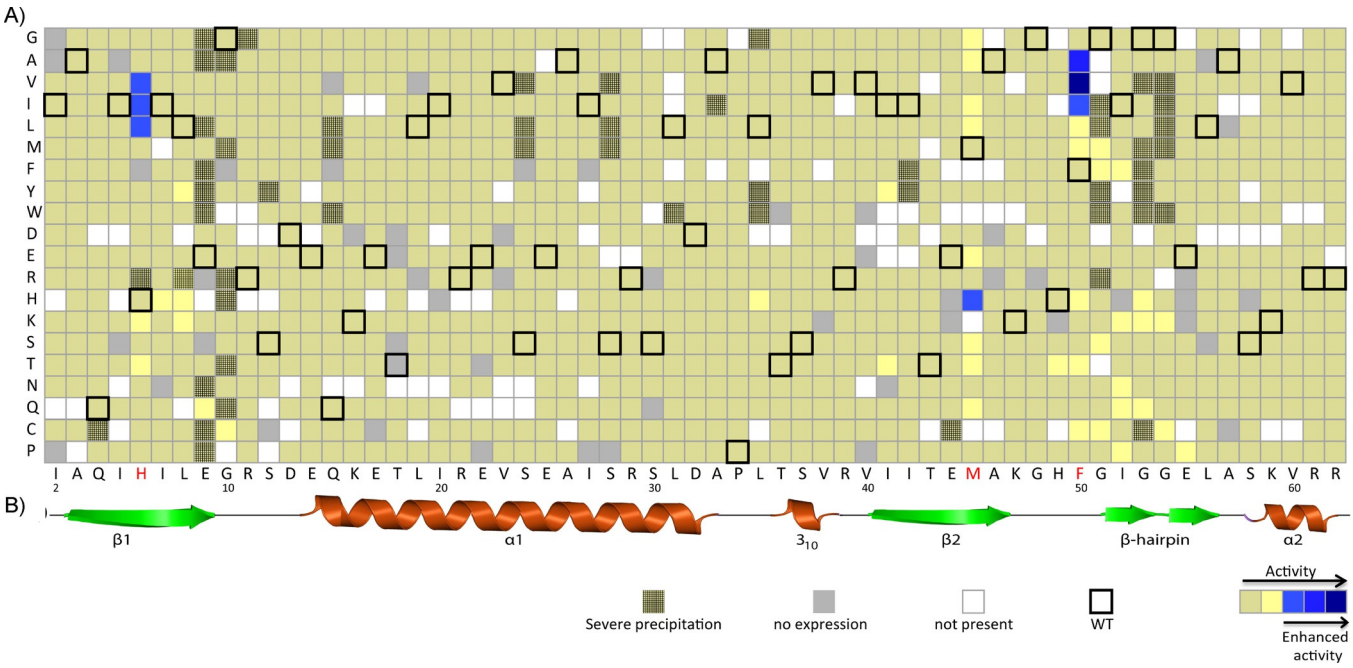

Figure 1. A) Heat map of 4-OT for the aldol condensation of acetaldehyde (**3**) with benzaldehyde (**4**) to yield cinnamaldehyde (**6**). The ability of the single mutants of 4-OT to catalyze the aldol condensation of **3** with **4** to give **6** was determined by UV-spectroscopy (absorbance at 290 nm corresponds to the formation of **6**). The horizontal axis represents the sequence of 4-OT; the vertical axis depicts the 20 possible amino acid residues. Gray boxes ("no expression") represent mutants that were not produced above the detection limit (0.5 mg mL^{-1} in cell-free extract). B) Secondary structure of 4-OT.

As residues that affect activity can be found anywhere in proteins,[23–26] we first applied a systematic mutagenesis strategy to identify residue positions at which mutations give a marked improvement in the aldolase activity of 4-OT. For this, a previously constructed collection of 1040 single mutants of 4-OT[27] was used; this covered at least 15 of the 19 possible variants at each residue from Ile-2 to Arg-62 (the 4-OT monomer has 62 residues). A heat map for the aldolase activity of 4-OT was generated by determining the effect of each mutation on the ability of the enzyme to catalyze the aldol condensation of **3** with **4** to yield **6** (Figure 1). Given the low aldolase activity of wild-type 4-OT, the activity measurements were performed under screening conditions that only allowed the detection of variants with strongly improved aldolase activity.

By using this systematic mutagenesis approach, three hotspots were identified at which a single mutation greatly improved aldolase activity (Figure 1): His6 (to Val, Ile, and Leu), Met45 (His), and Phe50 (Ala, Val, and Ile). His6, Met45, and Phe50 line the Pro1 pocket, thus illustrating that single mutations close to the active site can strongly improve the promiscuous aldolase activity of 4-OT. The single mutants with the best aldolase activity at each position (H6I, M45H, and F50V; see Figure S1 in the Supporting Information) were purified, and their catalytic performances were compared to that of wild-type 4-OT by using 150 μM enzyme and a 25-fold excess of **3** (50 mM) over **4** (2 mM). Analysis of the progress curves of these reactions showed that H6I, M45H, and F50V had strongly enhanced aldolase activity for the condensation of **3** with **4** compared to wild-type 4-OT, with F50V displaying the highest activity (Figures 2 and S2). Notably, no mutation that significantly enhanced the activity was found at a distant residue position.

In order to investigate whether the three hotspots (His6, Met45, and Phe50) are good targets for further enhancement of the promiscuous aldolase activity of 4-OT, these positions were subjected to combinatorial mutagenesis. NNK codon degeneracy (covering all 20 possible amino acids) was used for randomization of His6 and Met45; NYK codon degeneracy was used for Phe50[28–31] because it reduces the library size by covering only nine codons (for aliphatic and polar amino acid residues), including those that resulted in enhanced aldolase activity (Ala, Val, and Ile), as well as the wild-type residue (Phe). In addition to this triple-site library, a double-site library was constructed by varying just Met45 and Phe50 with NNK codon degeneracy. Met45 and Phe50 were chosen for the double-site

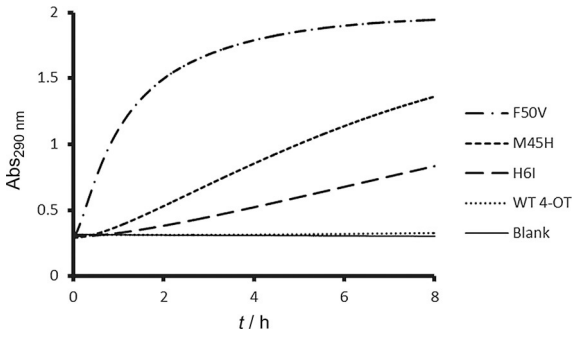

Figure 2. UV monitoring ($\lambda = 290$ nm) of the formation of cinnamaldehyde (**6**) from acetaldehyde (**3**) and benzaldehyde (**4**) in the absence of enzyme (Blank) or in the presence of the indicated purified enzyme.

library because single mutations at these positions resulted in higher aldolase activity than mutations at His6 (Figure 2).

The two libraries were transformed into *Escherichia coli*. The double-site library was screened by evaluating ~500 transformants for the aldol condensation of **3** and **4**; ~3500 transformants of the triple-site library were screened. Activity screening of the two libraries resulted in the identification of two mutants (M45T/F50A from the double-site library and H6F/M45T/F50A from the triple-site library) that showed significant improvements in activity over that of the best single mutant (F50V, which was used as a control in the screening assays). Progress curves of the aldol condensation of **3** (50 mM) with **4** (2 mM) catalyzed by purified enzymes confirmed the enhanced activities of mutants M45T/F50A and H6F/M45T/F50A over wild-type 4-OT and mutant F50V (Figure 3). ^{1}H NMR spectroscopic analysis confirmed that **6** was formed from **3** and **4**, catalyzed by mutants F50V, M45T/F50A, and H6F/M45T/F50A (Figure S3).

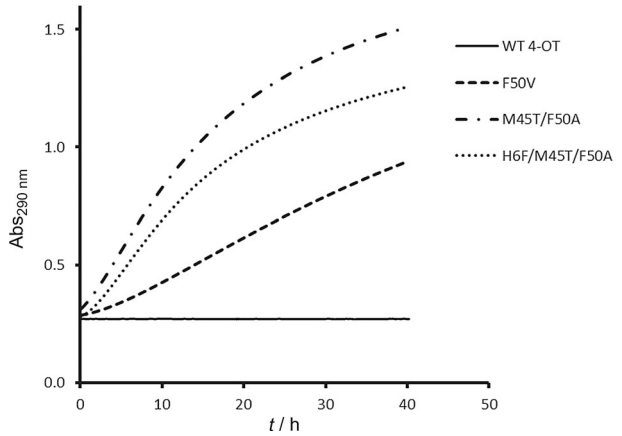

Figure 3. UV monitoring ($\lambda = 290$ nm) of the formation of cinnamaldehyde (**6**) from acetaldehyde (**3**) and benzaldehyde (**4**) in the presence of the indicated purified enzyme.

Having established that mutants F50V, M45T/F50A, and H6F/M45T/F50A had greatly improved aldolase activity, the apparent kinetic parameters were determined by using a fixed concentration of **3** (50 mM) and varying concentrations of **4** (0.1–15 mM). Catalytic efficiency (k_{cat}/K_m) of the single mutant F50V was ~640-fold higher than that of wild-type 4-OT (Table 1). Strikingly, the catalytic efficiencies of the double mutant M45T/F50A and the triple mutant H6F/M45T/F50A were 3300-fold and ~5300-fold, respectively, higher than that of wild-type 4-OT. The higher catalytic efficiencies of the double and triple mutants over that for the single mutant F50V can be attributed to the lower K_m for **4**.

Next, we chemically synthesized **5**[12,32] and assessed the ability of mutants F50V, M45T/F50A, and H6F/M45T/F50A to catalyze dehydration and retro-aldol reactions with this substrate. UV spectroscopic analysis showed increases in absorbance at 250 and 290 nm (Figure S4). The increase in absorbance at 290 nm corresponded to the formation of **6** by dehydration of **5**. The increase at 250 nm corresponded to the formation of **4** from retro-aldol cleavage of **5**.[12] Interestingly, although mutants M45T/F50A and H6F/M45T/F50A catalyzed both retro-aldol and dehydration reactions with **5** as the substrate, mutant F50V appeared to catalyze mainly the dehydration reaction (Figure S4). All three mutants showed strongly enhanced activity over wild-type 4-OT with substrate **5**. Notably, additional increases in absorbance at 227 nm (corresponding to the formation of but-2-enal, the product of self-condensation of **3**)[10] were observed for the mutant enzymes, thus indicating the formation of **3** during the retro-aldol reactions.

Table 1. Apparent kinetic parameters for the aldol condensation of **3** and **4** to yield **6**, catalyzed by wild-type 4-OT and variants

Enzyme	k_{cat} [min^{-1}]	K_m (**4**) [mM]	k_{cat}/K_m [M^{-1} min^{-1}]	Relative (k_{cat}/K_m)
wild-type	n.d.[a]	n.d.[a]	5.08×10^{-2}	1
F50V	0.27 ± 0.01	8.4 ± 0.8	32	636
M45T/F50A	0.28 ± 0.008	1.7 ± 0.1	168	3300
H6F/M45T/F50A	0.19 ± 0.005	0.7 ± 0.06	268	5276

[a] n.d.: not determined.

^{1}H NMR spectroscopic analysis confirmed the enhanced activity of the three mutant enzymes for substrate **5** when compared to wild-type 4-OT (Figure S5). After 18 h of incubation, **5** was fully converted by the three mutant enzymes, whereas only 34% conversion (26% conversion of **5** into **6**, 8% conversion of **5** into **3** and **4**) was observed for wild-type 4-OT. The ^{1}H NMR data confirmed that mutant F50V catalyzes mainly the dehydration reaction (65% dehydration, 35% retro-aldol reaction), and demonstrated that mutants M45T/F50A (28% dehydration, 72% retro-aldol reaction) and H6F/M45T/F50A (21% dehydration, 79% retro-aldol reaction) mainly catalyze retro-aldol cleavage of **5**. These results surprisingly demonstrate that the double and triple mutants have reversed specificity compared to wild-type 4-OT and the single mutant F50V.

Finally, we examined whether the engineered 4-OT variants (F50V, M45T/F50A, and H6F/M45T/F50A) still exhibited the native tautomerase activity. Kinetic parameters were determined by using varying concentrations of the natural substrate **1** (Table 2).[5] Tautomerization by F50V, M45T/F50A, and H6F/M45T/F50A showed 87-, 5750-, and 4893-fold decreases, respectively, in k_{cat}/K_m compared to wild-type 4-OT. Notably, mutations M45T/F50A and H6F/M45T/F50A resulted in enzymes with a $>10^7$-fold change in reaction specificity. Hence, the

Table 2. Kinetic parameters for the ketonization of **1** to yield **2**, catalyzed by wild-type 4-OT and variants

Enzyme	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [M^{-1} s^{-1}]	Relative (k_{cat}/K_m)
wild-type	1433 ± 35	31 ± 2	4.6×10^7	1
F50V	134 ± 1	253 ± 6	5.3×10^5	1/87
M45T/F50A	n.d.[a]	n.d.[a]	8.0×10^3	1/5750
H6F/M45T/F50A	n.d.[a]	n.d.[a]	9.4×10^3	1/4893

[a] n.d.: not determined.

large increase in promiscuous aldolase activity for these mutants was accompanied by a large decrease in (natural) tautomerase activity, thus indicating a strong tradeoff between evolved and former activity.[33–37]

In summary, we have demonstrated that the promiscuous enzyme 4-OT can be engineered into a more efficient aldolase by exploring small libraries in which only hotspots are varied. The hotspots were identified by systematic mutagenesis (covering each residue position), followed by a screen for single mutations that gave a marked improvement in the desired aldolase activity. All beneficial mutations were found to be near the active site, thus providing direct support for the notion that for new catalytic activity of a promiscuous enzyme, mutations closer to the active site improve the enzyme more effectively than distant ones.[23] In future work, we aim to explore the substrate scope of the engineered 4-OT variants, by focusing on challenging aldol reactions between two aldehydes. We also aim to design new 4-OT variants that lack dehydration activity but possess further-enhanced aldolase activity. For the latter goal and to place our engineering efforts in a structural context, work is in progress to determine the crystal structures of 4-OT and the best 4-OT variants in complex with the unnatural substrate **5** or product **6**.

Acknowledgements

This research was financially supported by ALW grant 820.02.021 from the Division of Earth and Life Sciences of the Netherlands Organisation of Scientific Research and by the European Research Council under the European Community's Seventh Framework Programme (FP7/2007–2013)/ERC grant agreement no. 242293. The authors thank Prof. Wim J. Quax for helpful discussions.

Keywords: aldolase activity · catalytic promiscuity · mutagenesis · oxalocrotonate tautomerase · protein engineering

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Manuscript received: March 10, 2016
Final article published: May 30, 2016