Aldolase Optimization

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# Enhancing Activity and Controlling Stereoselectivity in a Designed PLP-Dependent Aldolase**

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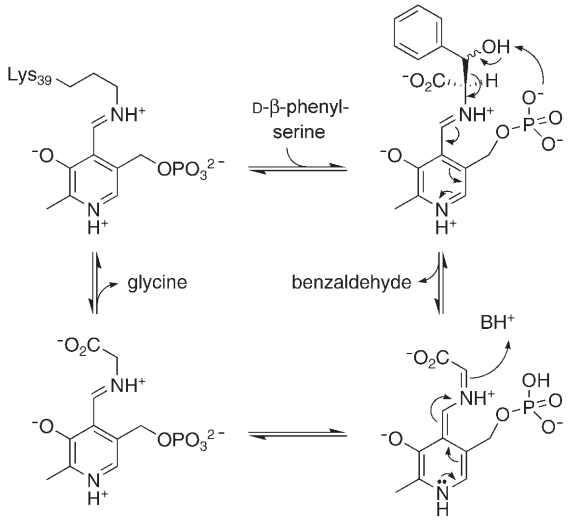
Biocatalysis is increasingly seen as a viable option for performing chemical transformations in the laboratory and on an industrial scale.[1] Although nature provides a wealth of catalysts for such applications, natural enzymes may be unavailable or otherwise unsuitable for specific reactions of interest. For this reason, tailoring the properties of existing enzyme scaffolds to access altered or completely new activities has attracted considerable attention.[2]

Previously, we showed that a single active-site mutation is sufficient to convert a pyridoxal phosphate (PLP)-dependent alanine racemase from *Geobacillus stearothermophilus* into an aldolase.[3] The substitution of tyrosine at position 265 in this protein (Figure 1a) with alanine removes a catalytic residue that is essential for racemase activity and, at the same time, creates a cavity that accommodates D-configured β-phenylserine isomers (Figure 1b). Native racemase activity is decreased by greater than $10^3$-fold at the modified active site, whereas retroaldol cleavage of the new substrate to give benzaldehyde and glycine (Scheme 1) is accelerated by five orders of magnitude. The Tyr265Ala variant also cleaves α-disubstituted β-hydroxy amino acids, an activity that is not reported for natural PLP-dependent aldolases.[4]

Molecular modeling shows that the side chain of D-β-phenylserine in the aldimine complex can fit comfortably in the space left vacant by the Tyr265Ala mutation, oriented so that its Cα–Cβ bond is orthogonal to the PLP plane and hence activated for scission (Figure 1b).[4] Although the alanine substitution engenders the desired activity, this amino acid may not be the optimal replacement for tyrosine. Consequently, we systematically varied the residue at position 265. As summarized in Table 1, introduction of serine, valine, or glutamate at this site is detrimental for retroaldol activity, whereas arginine has opposing effects on $k_{cat}$ and $k_{cat}/K_m$. In contrast, the Tyr265Lys substitution results in 9- and 2-fold increases in $k_{cat}$ and $k_{cat}/K_m$, respectively, for the cleavage of D-β-phenylserines.[5] Although this result cannot be fully rationalized in the absence of a structure or pre-steady-state kinetic data, the flexible lysine side chain presumably




***Scheme 1.*** Mechanism of the enzyme-catalyzed retroaldol reaction of D-β-phenylserine. B = base.

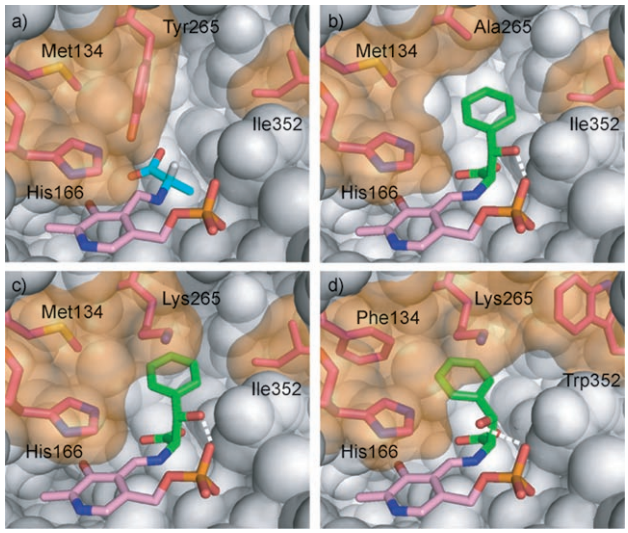



***Figure 1.*** Active-site models of *G. stearothermophilus* alanine racemase variants.[14] Structures of the external aldimines between L-alanine (cyan) and PLP (pink) complexed to the wild-type active site (a), the (2*R*,3S)-β-phenylserine-PLP aldimine (green) complexed to the Tyr265-Ala (b) and Tyr265Lys (c) variants, and the (2*R*,3*R*)-β-phenylserine-PLP aldimine bound at the Met134Phe/Tyr265Lys/Ile352Trp active site (d) are shown. Mutations at positions 134, 265, and 352 (red) reduce the free space surrounding the aldimine intermediate, improving the surface complementarity of the active site. The hydrogen bond between the Cβ-hydroxy group of the substrate and the phosphate group of PLP (the proposed catalytic base) is shown as a white dashed line.

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***Table 1:*** Steady-state parameters for the enzymatic conversion of D-β-phenylserine isomers to benzaldehyde and glycine.[a]

| Enzyme | (2*R*,3*S*)-β-phenylserine | | | (2*R*,3*R*)-β-phenylserine | | | Selectivity[b] |
|---|---|---|---|---|---|---|---|
| | $k_{cat}$ [$min^{-1}$] | $K_m$ [mM] | $k_{cat}/K_m$ [$M^{-1}min^{-1}$] | $k_{cat}$ [$min^{-1}$] | $K_m$ [mM] | $k_{cat}/K_m$ [$M^{-1}min^{-1}$] | |
| Alr-WT[c] | | | 0.0029[c] | – | – | – | |
| Y265A | 5.7[c] | 8.5[c] | 670[c] | 0.044 | 0.60 | 73 | 9.2 |
| Y265S | 1.2 | 43 | 28 | n.d. | n.d. | n.d. | |
| Y265V | – | – | – | n.d. | n.d. | n.d. | |
| Y265E | – | – | – | n.d. | n.d. | n.d. | |
| Y265R | 13 | 73 | 180 | n.d. | n.d. | n.d. | |
| Y265K | 52 | 35 | 1500 | 0.43 | 2.3 | 190 | 7.9 |
| M134F/Y26K | 31 | 17 | 1800 | 3.4 | 5.5 | 620 | 2.9 |
| Y265K/I352W | 48 | 15 | 3200 | 0.83 | 0.60 | 1400 | 2.3 |
| M134F/Y26K/I352W | 8.4 | 5.9 | 1400 | 2.7 | 1.2 | 2300 | 0.6 |

[a] Assays were performed at 30°C in 100 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) buffer solution (pH 8.0). The standard error for all kinetic parameters is less than 20%. (– indicates that no activity was detected above background, n.d. = not determined). For comparison, *E. coli* L-threonine aldolase[5] catalyzes the conversion of (2*S*,3*R*)-β-phenylserine with $k_{cat} = 225\ min^{-1}$, $k_{cat}/K_m = 1.9\times10^6\ M^{-1}min^{-1}$, and the conversion of (2*S*,3*S*)-β-phenylserine with $k_{cat} = 337\ min^{-1}$, $k_{cat}/K_m = 1.4\times10^6\ M^{-1}min^{-1}$; *A. xylosoxidans* D-threonine aldolase[6] catalyzes the conversion of (2*R*,3*S*)-β-phenylserine with $k_{cat} = 1900\ min^{-1}$, $k_{cat}/K_m = 1.9\times10^6\ M^{-1}min^{-1}$, and the conversion of (2*R*,3*R*)-β-phenylserine with $k_{cat} = 814\ min^{-1}$, $k_{cat}/K_m = 1.4\times10^6\ M^{-1}min^{-1}$. The $k_{cat}$ values were estimated from the $V_{max}$ ($U\,mg^{-1}$) values reported in references [5] and [6] assuming 1 U catalyzes the formation of 1 μmol $min^{-1}$ of product, and $M_r = 36\,495$ Da for the *E. coli* enzyme and 42 195 Da for the *A. xylosoxidans* enzyme; [b] The selectivity was calculated as $[k_{cat}/K_m\ (2R,3S)]/[k_{cat}/K_m\ (2R,3R)]$; [c] Reference [3].

improves the packing and surface complementarity of the substrate binding pocket; its cationic terminus may also engage in favorable cation–π interactions with the aryl ring of the substrate or help stabilize developing negative charge at the more distant β-alcohol in the transition state (Figure 1c). Whatever the ultimate origin of the improvement, it is notable that the $k_{cat}$ value for the optimized enzyme is only five times smaller than that of L-threonine aldolase from *Escherichia coli*[6] and 40-fold smaller than that of a promiscuous D-threonine aldolase from *Alcaligenes xylosoxidans*[7] for conversion of β-phenylserines. These findings highlight the potential of single active-site mutations for remodeling the chemical properties of existing enzymes while underscoring the fact that the "obvious" substitution is not necessarily the best.

Because the catalytic base that initiates the retroaldol reaction is most likely the phosphate group of the cofactor,[4] C–C bond cleavage should be sensitive to the configuration of the alcohol at the Cβ atom as well as to rotation around the Cα–Cβ bond. We therefore tested (2*R*,3*R*)- and (2*R*,3*S*)-β-phenylserine as substrates for our engineered aldolases.[8] The relative $k_{cat}/K_m$ values for the starting Tyr265Ala variant indicate a 9:1 preference for the D-*threo* isomer, and this preference is not significantly eroded in the kinetically superior Tyr265Lys variant (8:1; Table 1). With β-phenylserine, the engineered aldolases are thus substantially more diastereoselective than many natural PLP-dependent aldolases, which exhibit stringent stereoselectivity at the Cα atom in the cleavage of β-hydroxy amino acids but poor stereochemical control at the Cβ atom.[6,7,9] The *E. coli* and *A. xylosoxidans* aldolases, for instance, achieve *threo*:*erythro* selectivities of only about 1.4 with their respective L- and D-β-phenylserine substrates.[6,7] Interestingly, the presence of an additional methyl group at Cα diminishes the ability of the reengineered racemase to discriminate between the different β-isomers,[4] probably because interactions with this extra substituent stabilize productive orientations of the normally less-favored aldimine complex.

The preferred conformations of the two β-phenylserine aldimine diastereomers differ mainly in a 30° rotation around the Cα–Cβ bond.[4] Formation of a productive hydrogen bond between the β-hydroxy group and the catalytically important phosphate group of the cofactor requires swinging the aryl ring of the less-favored (2*R*,3*R*)-aldimine isomer closer to His166 than in the case of the (2*R*,3*S*) isomer. Given this, we wondered whether the inherent *threo* selectivity of the engineered aldolase could be inverted by altering residues 134 and 352, which flank the pocket into which the aryl group docks. For example, binding of the (2*R*,3*R*) isomer might be enhanced by altering the packing interactions on one side of the pocket (Met134Phe) while increasing steric bulk on the other (Ile352Trp; Figure 1d). In fact, when combined with the Tyr265Lys mutation, both changes improve retroaldol cleavage of the (2*R*,3*R*) diastereomer relative to that of the (2*R*,3*S*) diastereomer (Table 1). The triple mutant that contains all three changes exhibits the highest overall catalytic efficiency for (2*R*,3*R*)-β-phenylserine (a 12-fold increase over Tyr265Lys and a 31-fold increase over Tyr265Ala, which are achieved mostly through an improved $k_{cat}$ parameter). Because these substitutions increase $k_{cat}/K_m$ for the D-*threo* substrate only slightly when compared with the Tyr265Ala variant (approximately twofold), they effectively reverse selectivity and lead to preferential retroaldol cleavage of the *erythro* isomer by a factor of approximately 2:1. The diastereoselectivity of this catalyst is thus directly responsive to the residues that line the active site and therefore subject to rational manipulation. Nevertheless, further enhancement of the *erythro* selectivity of the triple mutant, or the inherent *threo* selectivity of the starting catalyst, will likely require more extensive mutagenesis at sites distant from the binding pocket.[10]

These experiments illustrate the adaptive potential of the alanine racemase scaffold. Modification of the first shell of active-site residues generates significant retroaldol activity that compares favorably in terms of efficiency and selectivity with natural enzymes that have evolved specifically to promote this transformation. Given the importance of β-hydroxy-α-amino acids as bioactive agents[11] and building blocks for pharmaceutically important natural products, such

  

as vancomycin[12] and thiamphenicol,[13] and the relatively poor diastereoselectivity of many natural PLP-dependent aldolases,[6,7,9] further optimization of the properties of these engineered aldolases may afford practical catalysts for kinetic resolutions or, in the synthetic direction, stereocontrolled C–C bond formation.

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