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Conversion of a PLP-Dependent Racemase into an Aldolase by a Single Active Site Mutation

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Pyridoxal 5′-phosphate (PLP) serves as a cofactor for a wide range of enzyme-catalyzed reactions, including racemizations, transaminations, β/γ-eliminations, and aldol condensations. This functional diversity can be attributed to the modulation and enhancement of the cofactor's intrinsic chemical properties at the respective enzyme active site. Previous work has established that subtle modifications of such an environment are often sufficient to change either substrate[1,2] or reaction[3−6] specificity. In this report, we present an example of a single amino acid substitution that changes both substrate preference and reaction profile, turning an isomerase into a lyase. We find that alanine racemase (Alr) [EC 5.1.1.1] from *Geobacillus stearothermophilus*[7] is provided with novel aldolase activity, and greatly decreased racemase activity, when Tyr265 is mutated to alanine.

Alanine racemase from *G. stearothermophilus* and L-threonine aldolase from *Thermatoga maritima*[8] are evolutionarily unrelated and have completely different tertiary and quaternary structures, substrate preferences, and reaction specificities. Nevertheless, both initiate their catalytic cycles by forming aldimine intermediates between PLP and their respective substrates, alanine or a β-hydroxy-α-amino acid.[9] The aldimine is subsequently converted to a quinoid intermediate by abstraction of the C_α-hydrogen in the case of alanine racemase and by general base-promoted cleavage of the $C_\alpha-C_\beta$ bond in L-threonine aldolase. Protonation of the quinoid followed by aminolysis liberates alanine or glycine and regenerates an internal aldimine between PLP and an active site lysine.

The crucial carbon−carbon bond cleavage step in threonine aldolase is initiated by proton abstraction from the β-hydroxyl group of the cofactor-bound substrate. A histidine, His83, which π-stacks against the cofactor, has been assigned this role (Figure 1).[8] Alanine racemase also has an active site histidine, His166, which stacks against the other face of the cofactor, but this residue does not interact with the substrate directly (Figure 1). Instead, it forms a hydrogen bond with the side chain of Tyr265 to lower the pK_a of the phenol.[10] The latter has been postulated to be the base needed to convert L- to D-alanine, and its replacement by alanine has been shown to decrease racemase activity by more than 4×10^3-fold.[11] We surmised that removal of this tyrosine would also allow an appropriately substituted β-hydroxy-α-amino acid to bind in the enlarged active site and form an aldimine with PLP; His166 might then serve as a general base to initiate a retroaldol reaction (Scheme 1). To test this hypothesis, we prepared and purified wild-type alanine racemase together with the Alr Y265A variant.[12]

In accord with our expectations, alanine racemase gains considerable aldolase activity when Tyr265 is replaced by alanine. Table 1 contains a summary of the kinetic data obtained for several variants of the racemase and a natural threonine aldolase. At 30 °C, Alr Y265A converts β-phenylserine to benzaldehyde and glycine with multiple turnovers. The steady-state parameters for this reaction are $k_{cat} = 5.7$ min^{-1} and $K_M = 8.5$ mM.[13] Ten-fold higher activities are observed at 60 °C, as expected for an enzyme of thermophilic origin. The apparent second-order rate constant (k_{cat}/K_m) for the Alr Y265A-catalyzed retroaldol reaction is 2.3×10^5-fold higher than that for wild-type alanine racemase. By this criterion, the variant is still 10^3-fold less efficient than L-threonine aldolase from *Escherichia coli* (eTA), but it has a rather large K_m value for substrate. Indeed, Alr Y265A has a k_{cat} value that is only 50-times smaller than that of eTA. Furthermore, comparison of k_{cat} with the first-order rate constant for decomposition of PLP-β-phenylserine to PLP-glycine in solution[14] shows that the modified active site enhances the rate of carbon−carbon bond cleavage by a factor of at least 4×10^3.

To test whether His166 is required for the retroaldol reaction, the double mutant Alr H166N/Y265A was prepared and character-

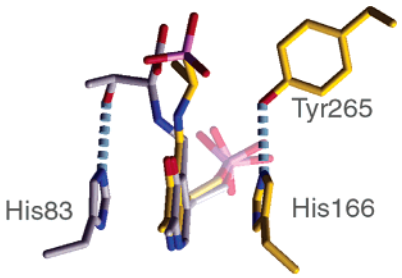
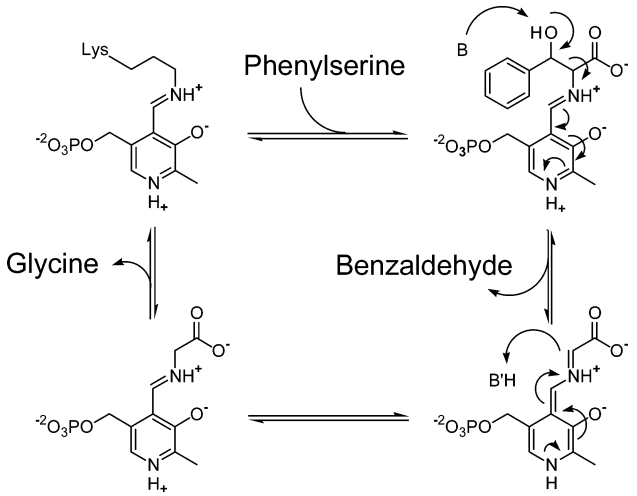


Figure 1. Superposition of the PLP cofactor at the active sites of alanine racemase[7] (yellow) and L-threonine aldolase[8] (gray). The alanine racemase structure shows PLP complexed as a Schiff base with the inhibitor (*R*)-1-aminoethylphosphonic acid and residues His166 and Tyr265. In L-threonine aldolase, PLP-L-allothreonine forms a hydrogen bond with His83.

Scheme 1 [a]



[a] PLP-dependent conversion of β-phenylserine to glycine and benzaldehyde. The general base B may be the imidazole side chain of His166 (see text).

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Table 1. Steady-State Parameters for the Conversion of β-Phenylserine to Benzaldehyde and Glycine[a]

enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}min^{-1}$)
Alr-WT			0.0029[b]
Alr-Y265A	5.7	8.5	670
Alr-H166N/Y265A	0.34	30	11
eTA[c]	278	0.38	7.3×10^5

[a] The proteins were assayed at 30 °C in 100 mM HEPES buffer (pH 8) with β-phenylserine (i.e., a racemic mixture of (2*S*,3*R*)-phenylserine and (2*R*,3*S*)-phenylserine). The K_m values were calculated assuming that only D-threo-β-phenylserine is a substrate for the enzyme (see text). [b] No saturation was observed up to 200 mM β-phenylserine. The k_{cat}/K_m parameter was therefore estimated from initial rate measurements using the equation $k_{cat}/K_m = v_0/[S][E]$. [c] Data for *E. coli* L-threonine aldolase measured under similar conditions with β-phenylserine are from Contestabile et al.[24] The standard error on all kinetic parameters is ≤21%.

ized. The kinetic parameters for this protein (Table 1) show that removal of histidine reduces catalytic efficiency by a factor of 60, stemming mostly from a decrease in k_{cat} (17-fold). This result is consistent with a possible role for the imidazole side chain as a general base in catalysis.[15] Residual activity of the double mutant presumably reflects other features of the alanine racemase active site which have evolved to stabilize a dianionic transition state.[16]

In contrast to the reaction promoted by PLP in solution, the Alr Y265A-catalyzed retroaldol reaction is stereoselective. The rate of reaction decreases to background level after 50% of the β-phenylserine sample has been transformed to product. Analysis of the product mixture by circular dichroism spectroscopy revealed that (2*R*,3*S*)-phenylserine, a D-amino acid, is the preferred substrate, whereas its enantiomer is not processed by the modified enzyme. This preference is consistent with a binding mode that places the $C_\alpha-C_\beta$ bond of the β-hydroxy-α-amino acid orthogonal to the PLP plane, allowing the aromatic side chain to bind in the newly created cavity. In the case of the wild-type racemase, the $C_\alpha-H$ bond of L-alanine occupies an analogous position (Figure 1). The specificity of the reengineered enzyme thus supports the proposal[17] that some naturally occurring D-threonine aldolases, in contrast to L-threonine aldolases, are evolutionarily related to alanine racemases.

Our data show that a single point mutation is able to convert an alanine racemase into an aldolase, changing both substrate specificity and reaction profile simultaneously. While the starting enzyme abstracts the C_α-hydrogen of either D- or L-alanine, the mutant selectively cleaves the $C_\alpha-C_\beta$ bond of a D-β-hydroxy-α-amino acid with a bulky aromatic side chain. The 2.3×10^5-fold increase in aldolase activity coupled with the 4×10^3-fold decrease in racemase activity underscores the ease with which the intrinsic catalytic promiscuity of the PLP cofactor can be redirected to access a new reaction manifold, while suppressing another. As previous studies on both natural[18,19] and directed evolution[1−6,20] have shown, conservative redesign of an enzyme active site is far from uncommon in nature. Rather, it represents a fundamental strategy for creating a new function that can be readily mimicked in the laboratory. Optimization of Alr Y265A through further mutagenesis and selection may yield useful catalysts for the enantioselective synthesis of β-hydroxy-α-amino acids[21,22] and their α-alkylated analogues.[23]

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Supporting Information Available: Experimental details for the preparation and characterization of the proteins, kinetic measurements, and product analysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Watanabe, A.; Yoshimura, T.; Mikami, B.; Esaki, N. *J. Biochem. (Tokyo)* **1999**, *126*, 781−786. In our hands, the Y265A variant exhibited 3×10^3-fold lower racemase activity than wild-type alanine racemase.
(12) The Alr gene was cloned by PCR into a pET22b vector using genomic DNA from *G. stearothermophilus* as template. Recombinant protein was produced as a C-terminal $(His)_6$ fusion protein in *E. coli* BL21 cells and purified to homogeneity using Ni^{2+}-ion affinity chromatography, followed by anion-exchange on a monoQ column. Mutations were introduced using standard overlap-extension PCR methods (Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: A laboratory manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001). The proteins were analyzed by electrospray ionization mass spectroscopy (ESI-MS) and shown to have the expected mass (wild-type Alr, found 44 648 ± 5 Da, expected 44 644 Da; Alr Y265A, found 44 561 ± 5 Da, expected 44 566 Da; Alr Y265A/H166N, found 44 539 ± 5 Da, expected 44 543 Da).
(13) Retroaldol activity was assayed at 30 °C in 100 mM HEPES (pH 8) using β-phenylserine (i.e., a racemic mixture of (2*S*,3*R*)-phenylserine and (2*R*,3*S*)-phenylserine). Formation of benzaldehyde was monitored at 279 nm using a molar extinction coefficient of 1.4×10^3 M^{-1} cm^{-1}.[23] Benzaldehyde production was confirmed by HPLC, ^1H NMR, and enzymatic reduction with NADH and horse liver alcohol dehydrogenase (Liu, J. Q.; Odani, M.; Yasuoka, T.; Dairi, T.; Itoh, N.; Kataoka, M.; Shimizu, S.; Yamada, H. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 44−51).
(14) The first-order rate constant for decomposition of pyridoxal-β-phenylserine (0.1 M) to pyridoxal-glycine and benzaldehyde was measured by ^1H NMR in H_2O at pH 8, 30 °C, as previously described (Tatsumoto, K.; Martell, A. E. *J. Am. Chem. Soc.* **1978**, *100*, 5549−5553). The value we obtained, 2.5×10^{-5} s^{-1}, is twice the reported value. This discrepancy presumably reflects a solvent kinetic isotope effect, because the earlier measurements were performed in D_2O rather than in H_2O.
(15) This assignment is supported by the sigmoidal k_{cat} versus pH profile for the Y265A variant ($pK_a = 7.1 \pm 0.3$) and the bell-shaped pH dependence of k_{cat}/K_m ($pK_{a1} = 6.8 \pm 0.3$; $pK_{a2} = 10.0 \pm 0.3$) (see Supporting Information). Reliable pH-rate data for the double mutant could not be obtained because of its low activity and instability at the pH extremes.
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