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Synthesis of β-hydroxy-α-amino acids with a reengineered alanine racemase

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ABSTRACT

The Y265A mutant of alanine racemase (alrY265A) was evaluated as a catalyst for the synthesis of β-hydroxy-α-amino acids. It promotes the PLP-dependent aldol condensation of glycine with a range of aromatic aldehydes. The desired products were obtained with complete stereocontrol at C_α (ee > 99%, D) and moderate to high selectivity at C_β (up to 97% de). The designed enzyme is thus similar to natural D-threonine aldolases in its substrate specificity and stereoselectivity. Moreover, its ability to utilize alanine as an alternative donor suggests an expanded scope of potential utility for the production of biologically active compounds.

Aldolases are proficient catalysts for the stereoselective formation and cleavage of carbon–carbon bonds.[1–3] As a consequence, they have found wide application in the production of diverse chiral building blocks.[4] Nevertheless, natural biocatalysts are not necessarily optimized for reactions outside the cell, so there is considerable interest in expanding their scope and selectivity through enzyme engineering.[5]

We recently showed that an alanine racemase (alr) from *Geobacillus stearothermophilus*, can be converted into a retro-aldolase by a single point mutation.[6] Replacement of one of the catalytic bases, a tyrosine at position 265, with alanine decreases the starting epimerase activity by more than three orders of magnitude.[7] However, this same mutation also enables a completely new reaction, the cleavage of D-configured β-phenylserines to give glycine and benzaldehyde. The retro-aldol reaction exploits the inherent chemical reactivity of the enzyme's pyridoxal-5-phosphate (PLP) cofactor together with the altered binding properties of the enlarged active site.[8,9] In both activity and mechanism, alrY265A thus resembles threonine aldolases,[10] a completely different class of PLP-dependent enzymes.

Natural threonine aldolases have been shown to be useful catalysts for the synthesis of β-hydroxy-α-amino acids, which are valuable precursors of pharmaceuticals and agrochemicals.[10–14] In the course of the enzymatic reaction, glycine is condensed with an appropriate aldehyde to form two chiral centers. The stereoselectivity of this process is typically highly stringent at C_α, either L or D depending on the source of the enzyme, but less strict at C_β. Given the functional similarities between alrY265A and threonine aldolases, we wondered how well the reengineered racemase would compare with its natural counterparts in the biocatalytic production of β-hydroxy-α-amino acids—the microscopic reverse of the previously studied cleavage reaction. Here we describe the efficiency, selectivity and substrate scope of this catalyst.

The synthesis of β-phenylserine from glycine and benzaldehyde was chosen as a model reaction to assess the synthetic capabilities of alrY265A. The enzyme-catalyzed aldol condensations were carried out in aqueous buffer, supplemented with PLP. Although the solubility of benzaldehyde in water is limited, high concentrations of both substrates are desirable to maximize the rate of the biomolecular reaction. Moreover, as shown previously,[11] a large excess of glycine helps to shift the reaction equilibrium towards the aldol product. We therefore used a 10-fold excess of donor over acceptor (1 M of glycine, 100 mM benzaldehyde) in all cases. Typical reaction times were 24 h. Since equilibrium is reached relatively slowly in these systems (>48 h), the reactions are under kinetic control under these conditions.[15]

All reaction mixtures were monitored by analytical HPLC to assess both yield and stereoselectivity. After 24 h at pH 8 and 30 °C, D-β-phenylserine is formed in low yield but excellent de (Table 1, entry 1). To optimize production of the aldol product several reaction parameters were varied. Unlike D-threonine aldolases, which require divalent metal ions for activity,[16] addition of magnesium ions had no effect on either the activity or selectivity of alrY265A. However, the enzyme was found to be more efficient at higher temperature, as expected given its thermophilic origin (Table 1, entries 1–3). Raising the pH has an even greater effect (Table 1,

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Table 1
Effect of temperature and pH on the alrY265A-catalyzed synthesis of D-β-phenylserine

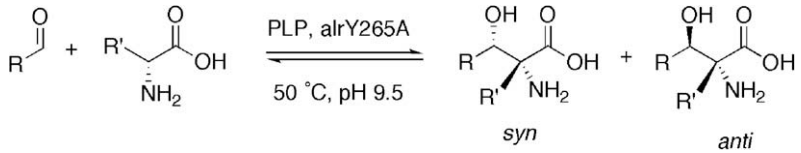
Entry	*T* (°C)	pH	Yield (%)	de (%)
1	30	8	4	97
2	40	8	5	95
3	50	8	8	87
4	50	9	15	85
5	50	9.5	17	76

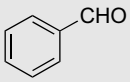
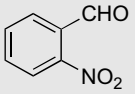
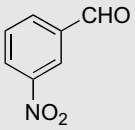
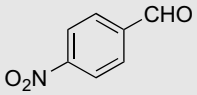
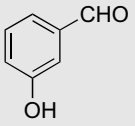
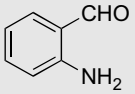
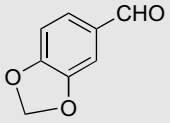
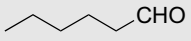
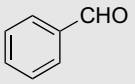
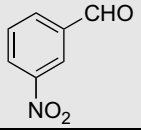
Conditions: 1 mL buffer solutions (pH 8–9.5) containing glycine (1 M), benzaldehyde (100 mM), PLP (50 μM) and alrY265A (4 U), ee > 99% for all reactions. See Supplementary Information for details.

entries 3–5). The highest yields of D-β-phenylserine were obtained at pH 9.5 and 50 °C (Table 1, entry 5), although diastereoselectivity decreases with increasing temperature and pH. For comparison, natural threonine aldolases afford up to 80% yields of β-phenylserine, reflecting their greater specific activity. Nonetheless, the diastereoselectivity of alrY265A is comparable to that of the D-specific enzyme from *Alcaligenes xylosoxidans* (98% de at 5 °C and 73% de at 40 °C) and superior to that of typical L-specific enzymes like the threonine aldolase from *Pseudomonas putida* (20% de at 25 °C).[12]

Because the Y265K variant of alanine racemase has been reported to promote the cleavage of D-β-phenylserine with some-

Table 2
Substrate specificity of alrY265A in aldol reactions



Entry	Aldehyde	R′	Reaction time (h)	Analytical yield (%)	de (%) (*syn*)
1		H	3 24	10 17	97 76
2		H	3 24	1 1	 >97
3		H	3 24	20 55	93 85
4		H	3 24	12 36	40 40
5		H	3 24	<1 3	 70
6		H	3 24	<1 <1	– –
7		H	3 24	1 5	 70
8		H	3 24	0 0	– –
9		H	3 24	0 0	– –
10[a]		CH_3	3 24	<1 <1	– –
11[a]		CH_3	3 24	5 12	80 65

Conditions: 1 mL buffer solution (pH 9.5) contains glycine or alanine (1 M), aldehyde (100 mM), PLP (50 μM), alrY265A (4 U, 0.6 mM) at 50 °C, ee > 99% (D) for all reactions. For details, see Supplementary Information.

[a] The stereochemical assignment is based on the secondary coupling constants of the C_β proton with the protons of the C_α methyl group.

what higher k_{cat} and k_{cat}/K_m parameters than alrY265A,[9] it was also tested under the optimized conditions. It proved to be less effective for the synthetic reactions, affording lower yields of the aldol product than alrY265A (data not shown). It is possible that alrY265K is less stable under the preparative conditions or that high concentrations of benzaldehyde favor non-productive reactions with Lys265 at the active site, blocking the binding pocket.

The substrate scope of the alrY265A variant was subsequently evaluated using a representative panel of aldehyde acceptors. As shown in Table 2, the enzyme accommodates a range of benzaldehyde derivatives (Table 2, entries 1–7), but aliphatic aldehydes appear to be poor substrates (Table 2, entries 8 and 9). The observed aldol products are formed with high stereoselectivity with respect to the configuration at C_α, affording exclusively D-amino acids (ee > 99%), and moderate to high selectivity at C_β (de, 40–97%). The *syn* diastereomer is the dominant product in all cases.

As seen previously for threonine aldolases,[12] the best substrates are aromatic aldehydes bearing electron-withdrawing groups. The highest conversion was obtained with 3-nitrobenzaldehyde, which gave D-3-nitro-β-phenylserine in 55% yield and 85% de (entry 3). When the nitro group is in the *para* position, both yield and diastereoselectivity decrease somewhat: D-4-nitro-β-phenylserine, a precursor of a chloramphenicol isomer,[17,18] was obtained in only 36% yield and 40% de (entry 4). 2-Nitrobenzaldehyde is an even poorer substrate (entry 2), presumably for steric reasons. Benzaldehyde derivatives with electron-donating amine or hydroxyl substituents are weak electrophiles and generally give less than one percent conversion (entries 5, 6). Judging from color changes in reaction mixtures containing 2-hydroxy-, 3-hydroxy-, 4-hydroxy- and 3,4-dihydroxybenzaldehyde (yellow to orange), oxidative side reactions are a potential problem and may compete with the desired aldol condensation. Nevertheless, reaction with piperonal (entry 7) appears promising, despite the low yield, since it provides a stereoselective route to D-3,4-methylenedioxyphenylserine (70% de), a precursor of DOPS and noradrenaline isomers.[19,20]

Interestingly, alrY265A also accepts D-alanine as a donor in the aldol reactions. Such activity has not been seen with natural threonine aldolases, but follows from the observation that the modified racemase is able to cleave D-α-methyl-β-phenylserine.[8] Although the aldol reaction between alanine and benzaldehyde did not result in detectable amounts of product under the standard reaction conditions (entry 10), the more reactive 3-nitrobenzaldehyde gave α-methyl-3-nitro-β-phenylserine in 12% yield and 65% de (entry 11).

Our results thus demonstrate that alrY265A can be used for the semi-preparative production of β-hydroxy-α-amino acids. The lower yields obtained with the reengineered racemase compared with the natural enzymes presumably reflects its 100- to 1000-fold lower specific activity. As a consequence, higher catalyst concentrations and longer reaction times are needed to achieve significant conversions. Under these conditions, catalyst stability may be an issue. Applications of natural aldolases have been optimized by continuously removing the product from the reaction mixture.[21] The unfavorable equilibrium has also been successfully shifted in the desired direction by coupling the aldol reaction with an irreversible decarboxylation catalyzed by a highly diastereoselective tyrosine decarboxylase.[22] This bienzymatic approach not only resulted in quantitative conversions but also improved C_β selectivity. Similar strategies are likely to extend the utility of alrY265A.

The stereoselectivity of the reengineered racemase (ee > 99%, de > 40%) is of particular note, since it is at least comparable to that of the best D-threonine aldolase in the literature,[12,15,23] and significantly better than typical L-specific enzymes.[12,13,15,22,24–26] These findings are consistent with genetic analyses showing that alanine racemases and D-threonine aldolases share a common evolutionary heritage, whereas L-threonine aldolases are evolutionarily unrelated.[27] Furthermore, since the related enzymes are members of the same structural class of PLP proteins, it will presumably be possible to augment the activity of alrY265A, and perhaps its selectivity, either via additional site-directed mutagenesis or by subjecting the enzyme to multiple rounds of directed evolution.[28]

The ability of the reengineered racemase to utilize alanine as a donor in the synthetic aldol reactions is even more striking, as such activity is unprecedented in other catalysts. It reflects the racemase origins of alrY265A and highlights the advantage of an engineering approach for accessing novel activities unavailable to naturally occurring catalysts. β-Hydroxy-α,α-disubstituted-α-amino acids are interesting as enzyme inhibitors[29–31] and as conformational modifiers of physiologically active peptides.[32,33] As a consequence, improved versions of the reengineered racemase could be of considerable synthetic utility, affording these densely functionalized molecules in a single step from simple starting materials, namely alanine and an aldehyde.

In summary, the Y265A mutant of alanine racemase is a viable catalyst for the synthesis of β-hydroxy-α-amino acids. Although its activity must still be optimized, its favorable stereochemical properties and unusual scope suggest promising opportunities for future applications.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.031.

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