Communications

selective C–C bond making and breaking. Indeed, natural aldolases have been used to catalyze a wide range of aldol reactions on synthetically useful scales.^[2] Furthermore, enzyme-engineering methods have been applied to generate catalysts with higher activities or altered substrate specificities to match a growing demand for molecular diversity in the chemical and pharmaceutical industries.^[3]

In a previous report, we showed that aldolase activity can also be recruited from unexpected origins: a single active-site mutation (Y265A) in a pyridoxal 5'-phosphate (PLP)-dependent alanine racemase from *Geobacillus stearothermophillus* (alr) was found to increase retro-aldolase activity against phenylserine **1** (Scheme 1) by five orders of magnitude.^[4] The



Engineered Aldolase

DOI: 10.1002/anie.200602529

Stereoselectivity and Expanded Substrate Scope of an Engineered PLP-Dependent Aldolase**

Florian P. Seebeck, Angelo Guainazzi, Celine Amoreira, Kim K. Baldridge, and Donald Hilvert*

Biocatalysis has become an invaluable tool for industrial scale chemical synthesis.^[1] Aldolases are particularly interesting for practical applications owing to their proficiency at enantio-

[*]	Dr. F. P. Seebeck, A. Guainazzi, Prof. Dr. D. Hilvert Laboratorium für Organische Chemie
	ETH Zurich
	Fax: (+41) 44-632-1486
	E-mail: hilvert@org.chem.ethz.ch
	C. Amoreira, Prof. Dr. K. K. Baldridge
	Organisch-Chemisches Institut
	Universität Zürich
	Winterthurstrasse 190, 8057 Zürich (Switzerland)
[**]	We thank the ETH Zürich, the University of Zürich, and the Schweizerischer Nationalfonds for financial support. PLP=pyridoxal 5'-phosphate.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

the β -phenylserine derivatives 1 and 2. The substrate initially reacts with the enzyme-bound PLP 3 to form the external aldimine adduct 4. Cleavage of the C α -C β bond affords benzaldehyde and quinonoid intermediate 5. Subsequent protonation to give 6, followed by hydrolysis, liberates glycine or alanine and regenerates the catalyst. The cofactor phosphate is the likely base (B) for the retroaldol reaction (see text), and either Lys39 or water may protonate 5 (B'H).

conversion of the racemase into an aldolase profited from the similarity of the central PLP-quinonoid intermediates formed in the course of both reactions. Interestingly, natural evolution appears to have exploited this chemical relationship as well. Genetic analyses have demonstrated that alanine racemases and natural D-threonine aldolases share a common evolutionary origin.^[5] However, alanine racemase evolved to accommodate an alanyl-PLP quinonoid intermediate (**5**, R = CH₃), whereas D-threonine aldolases stabilize a glycyl-PLP quinonoid species (**5**, R = H). In the case of our alrY265A variant, one could imagine that the residual binding pocket



6824

for the alanine methyl group might be exploited together with the new aldolase activity to catalyze the cleavage (or, in the synthetic direction, formation) of quaternary α -amino acids like **2** (Scheme 1). Such activity has not been reported for natural aldolases but would have considerable synthetic utility.^[6]

To examine the ability of alrY265A to process β -hydroxy amino acids bearing an additional α substituent, we synthesized (2*R*,3*S*)- and (2*R*,3*R*)- α -methyl- β -phenylserines (**2a** and **2b**, respectively) following published procedures,^[7] and measured the kinetic parameters associated with their alrY265A-catalyzed conversion into alanine and benzalde-hyde. As shown in Table 1, the two diastereomers are

Table 1: Steady-state parameters for the conversion of β -phenyl-serines into benzaldehyde and glycine/alanine.^[a]

Substrate	k _{cat} [min ⁻¹]	К _т [тм]	$k_{\text{cat}}/K_{\text{m}}$ [M ⁻¹ min ⁻¹]	$k_{uncat}^{[c]}$ [min ⁻¹]
(2 <i>R</i> ,3 <i>S</i>)-β-phenyl-serine (1) ^[b]	5.7	8.5	670	0.0015
$(2R,3S)$ - α -methyl- β -phenylserine (2 a)	2.8	2.8	1000	0.00033
$(2R, 3R)$ - α -methyl- β -phenylserine (2b)	0.16	0.14	1100	-

[a] The enzymatic reactions were performed with alrY265A at 30 °C in 100 mM HEPES buffer (pH 8). [b] See Ref. [4]. [c] The rate constant for the pyridoxal-dependent retroaldol reaction of β -phenylserines in the absence of enzyme was determined following procedures described in Refs.[4, 13]; the standard error on all kinetic parameters is \leq 20%.

consumed with an even higher catalytic efficiency $(k_{cat}/K_m > 10^3 \text{ m}^{-1} \text{min}^{-1})$ than the previously examined (2R,3S)- β -phenylserine (1; $670 \text{ m}^{-1} \text{min}^{-1})$. Although the individual steady-state parameters k_{cat} and K_m are likely to be complex amalgams of different microscopic rate and binding constants, the fact that the K_m values observed for 2a and 2b are lower than that for 1 can be attributed to favorable interactions of the extra α -methyl group with the binding pocket. Furthermore, while its k_{cat} parameter is only twofold lower than that of 1, substrate 2a shows a k_{cat}/k_{uncat} ratio that is twofold higher (Table 1).

Natural D-threonine aldolases exhibit stringent stereoselectivity at C $\!\alpha$ in the cleavage of β -hydroxy amino acids but relaxed selectivity at CB.[8] alrY265A is similarly selective with respect to $C\alpha$, accepting only D-configured amino acids.^[4] However, the fact that **2a** and **2b** afford similar k_{cat} $K_{\rm m}$ values (Table 1) indicates poor stereochemical control at the β carbon. Nor does the enzyme exhibit strict stereochemical fidelity in the final stages of the reaction. In principle, the quinonoid intermediate 5 ($R = CH_3$) could be protonated either from the si face of the imine to give D-alanine, or from its *re* face to give L-alanine.^[9] The partitioning of $\mathbf{5}$ (R = CH₃) was quantified by measuring the amount of D-alanine formed in the alrY265A-catalyzed cleavage of 2b relative to benzaldehyde. D-Alanine-dependent oxidation of NADH, using Damino acid oxidase and lactate dehydrogenase as coupling enzymes, showed that 80% ($\pm 10\%$) of the alanine product has the D configuration. Preferential formation of D- over L-

alanine is consistent with the participation of Lys39, the active-site residue that protonates the quinonoid intermediate from the *si* face in native alr, and the absence of Tyr265, which normally protonates **5** from the opposite face. The small amount of L-alanine that is formed presumably arises by direct protonation of the intermediate by water from the same face as the departing benzaldehyde.

To rationalize the stereochemical preferences of alrY265A, we performed hybrid docking and ab initio investigations $^{[10]}$ with the aldimines of all four $\alpha\text{-methyl-}\beta\text{-}$ phenylserine diastereomers (2a and 2b, and the corresponding L-amino acids) bound at the enzyme active site.^[11] In accord with our experimental findings, the modified binding pocket readily accommodates both 2R diastereomers, whereas neither of the 2S isomers fits without clashes with active-site residues. Moreover, energy minimization of the models reveals that the 2R addimines exploit very similar interactions with the binding pocket and the cofactor (Figure 1). As expected, the carboxylate and α -methyl groups lock these substrates in an orientation that places the benzyl side chain perpendicular to the plane of the PLP cofactor, with the aryl ring inserted into the cavity created by the Y265A substitution. The preferred conformations of the two diastereomers differ by a 30° rotation around the C α - β bond, so that the aryl ring of the 2R, 3R isomer (2b) is a little closer to the side chain of His166. As a consequence of this difference in dihedral angle, the β -hydroxy group of both isomers points toward the cofactor phosphate. The resulting O…O distance is 2.70 and 3.05 Å for **2a** and **2b**, respectively, which corresponds to a favorable hydrogen bond.

Although His166 was originally proposed as a possible candidate for the base that initiates proton abstraction from the substrate hydroxy group (Scheme 1, $4\rightarrow$ 5),^[4] the modeling results indicate that its imidazole ring adopts the wrong tautomeric form and is too far from the alcohol to assume this role. Instead, as shown in Figure 1, the PLP phosphate group is well positioned to serve as the catalytic base for both diastereomers. The postulated recruitment of the cofactor phosphate for catalysis has some precedent in the reaction catalyzed by serine dehydratase.^[12] If this mechanistic hypothesis can be confirmed, it may be possible to exploit subtle steric changes within the active site to disrupt the crucial hydrogen bond for one but not the other substrate epimer, thereby enhancing the C β stereoselectivity of the enzyme.

In conclusion, we have shown that an engineered alr catalyzes a biologically unprecedented retroaldol reaction of α -substituted β -phenylserines with high stereoselectivity at the α carbon but low stereochemical control at the β carbon. Not only is this reaction catalyzed some 10⁴-fold more efficiently than by the cofactor in solution, but our computational model provides a basis for further optimization of the alrY265A active site by means of site-directed mutagenesis or directed evolution. Such improvement may yield practical catalysts for the enantioselective synthesis of novel amino acids and their derivatives.

Received: June 23, 2006 Published online: September 26, 2006

Communications



Figure 1. Energy-minimized structures of (2R,3S)-α-methyl-β-phenylserine (top) and (2R,3R)-α-methyl-β-phenylserine (bottom) bound to the active site of alrY265A as external aldimines with PLP. The β-hydroxy group of both substrates is within hydrogen-bonding distance of the phosphate group of PLP. A hydrogen bond between Arg219 and His166 (dotted line to the protein), which is also present in the parent enzyme,^[20] dictates the tautomeric form of the imidazole.

Keywords: β -hydroxy amino acids \cdot aldolases \cdot enzyme models \cdot stereoselectivity

- A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* 2001, 409, 258; H. E. Schoemaker, D. Mink, M. G. Wubbolts, *Science* 2003, 299, 1694.
- [2] T. Kimura, V. P. Vassilev, G.-J. Shen, C.-H. Wong, J. Am. Chem. Soc. 1997, 119, 11734; T. D. Machajewski, C.-H. Wong, Angew. Chem. 2000, 112, 1406; Angew. Chem. Int. Ed. 2000, 39, 1352; J.-Q. Liu, T. Dairi, N. Itoh, M. Kataoka, S. Shimizu, H. Yamada, J. Mol. Catal. B 2000, 10, 107; W. D. Fessner, V. Helaine, Curr. Opin. Biotechnol. 2001, 12, 574; W. A. Greenberg, A. Varvak, S. R. Hanson, K. Wong, H. J. Huang, P. Chen, M. J. Burk, Proc. Natl. Acad. Sci. USA 2004, 101, 5788; H. J. Lamble, M. J. Danson, D. W. Hough, S. D. Bull, Chem. Commun. 2005, 124.

- [3] S. Fong, T. D. Machajewski, C. C. Mak, C.-H. Wong, *Chem. Biol.* 2000, 7, 873; N. Wymer, L. V. Buchanan, D. Henderson, N. Mehta, C. H. Botting, L. Pocivavsek, C. A. Fierke, E. J. Toone, J. H. Naismith, *Structure* 2001, 9, 1; G. DeSantis, J. J. Liu, D. P. Clark, A. Heine, I. A. Wilson, C.-H. Wong, *Bioorg. Med. Chem.* 2003, 11, 43; J. S. Griffiths, M. Cheriyan, J. B. Corbell, L. Pocivavsek, C. A. Fierke, E. J. Toone, *Bioorg. Med. Chem.* 2004, 12, 4067; G. J. Williams, S. Domann, A. Nelson, A. Berry, *Proc. Natl. Acad. Sci. USA* 2003, 100, 3143; D. Franke, C.-C. Hsu, C.-H. Wong, *Methods Enzymol.* 2004, 388, 224.
- [4] F. P. Seebeck, D. Hilvert, J. Am. Chem. Soc. 2003, 125, 10158.
- [5] A. Paiardini, R. Contestabile, S. D'Aguanno, S. Pascarella, F. Bossa, *Biochim. Biophys. Acta* 2003, 1647, 214.
- [6] C. Cativiela, M. D. Diaz-de-Villegas, *Tetrahedron: Asymmetry* 1998, 9, 3517.
- [7] U. Schöllkopf, *Tetrahedron* 1983, 39, 2085; U. Schöllkopf, U. Groth, W. Hartwig, *Liebigs Ann. Chem.* 1981, 2407; U. Schöllkopf, U. Groth, K.-O. Westphalen, C.-Z. Deng, *Synthesis* 1981, 969.
- [8] M. Kataoka, M. Ikemi, T. Morikawa, T. Miyoshi, K. Nishi, M. Wada, H. Yamada, S. Shimizu, *Eur. J. Biochem.* **1997**, *248*, 385; J. Q. Liu, M. Odani, T. Yasuoka, T. Dairi, N. Itoh, M. Kataoka, S. Shimizu, H. Yamada, *Appl. Microbiol. Biotechnol.* **2000**, *54*, 44.
- [9] M. A. Spies, J. J. Woodward, M. R. Watnik, M. D. Toney, J. Am. Chem. Soc. 2004, 126, 7464.
- [10] Computational methods: Binding of the four diastereomeric aldimine adducts of α -methyl- β -phenylserine at the alrY265A active site was investigated using ab initio and docking methods. Ab initio calculations were performed using GAMESS^[14] at the B3LYP/DZV + (2d,p) level of theory ($\varepsilon = 4$),^[15] with CHELPG charges^[16] and standard radii. Initial docking was performed with QMView,^[17] and energy maps were generated by varying the torsional angles corresponding to rotation around the C α -C β and C β -C γ bonds of the intermediates in 30° increments. Electrostatic binding energies were calculated with APBS.^[18] Following molecular dynamics simulations with AMBER,^[19] the most favorable conformers were optimized with ab initio methods. See the Supporting Information for details.
- [11] A. Watanabe, T. Yoshimura, B. Mikami, H. Hayashi, H. Kagamiyama, N. Esaki, J. Biol. Chem. 2002, 277, 19166.
- [12] T. Yamada, J. Komoto, Y. Takata, H. Ogawa, H. C. Pitot, F. Takusagawa, *Biochemistry* 2003, 42, 12854.
- [13] K. Tatsumoto, A. E. Martell, J. Am. Chem. Soc. 1978, 100, 5549.
- [14] M. W. Schmidt, K. K. Baldridge, J. A. Boatz, S. T. Elbert, M. S. Gordon, J. H. Jensen, S. Koseki, N. Matsunaga, K. A. Nguyen, S. J. Su, T. L. Windus, M. Dupuis, J. A. Montgomery, Jr., J. Comput. Chem. 1993, 14, 1347.
- [15] C. T. Lee, W. T. Yang, R. G. Parr, Phys. Rev. B 1988, 37, 785.
- [16] C. M. Breneman, K. B. Wiberg, J. Comput. Chem. 1990, 11, 361.
- [17] K.K. Baldridge, J.P. Greenberg, J. Mol. Graphics 1995, 13, 63.
- [18] N. A. Baker, D. Sept, M. J. Holst, J. A. McCammon, *IBM J. Res. Dev.* 2001, 45, 427.
- [19] J. M. Wang, P. Cieplak, P. A. Kollman, J. Comput. Chem. 2000, 21, 1049.
- [20] J. P. Shaw, G. A. Petsko, D. Ringe, Biochemistry 1997, 36, 1329.