Esterases

DOI: 10.1002/anie.200704606

Complete Inversion of Enantioselectivity towards Acetylated Tertiary Alcohols by a Double Mutant of a *Bacillus Subtilis* Esterase**

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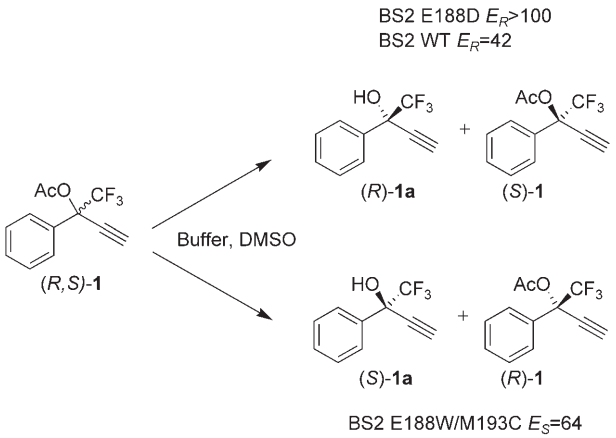
Enantiomerically pure compounds are playing a rapidly growing role as building blocks in organic chemistry. Biocatalysts are frequently used for their synthesis owing to their high chemo-, regio-, and stereoselectivity, making them often superior to chemical catalysis.[1] However, the wild-type (WT) enzyme often does not show broad substrate specificity in combination with sufficiently high selectivity. These limitations can be overcome by using rational protein design or directed evolution. Whereas several examples have been reported in which the enantioselectivity could be increased by these methods, a switch in enantiopreference could be achieved only rarely.[2] Most importantly, only E values >50 are of synthetic importance, and this threshold is difficult to surpass starting from an enzyme with opposite natural selectivity. For instance, May et al. converted a D-selective hydantoinase into an L-selective variant, but this gave only 20% *ee*,[2d] and Zha et al.[2e] inversed the enantioselectivity of a lipase from *Ps. aeruginosa* to $E=30$. In addition, the variants might lose considerable activity as reported for an arylmalonate decarboxylase with inverted selectivity.[3] Thus, changing the enantioselectivity of an enzyme is still a major challenge in protein engineering.

An esterase from *Bacillus subtilis* (BS2) was identified as a GGG(A)X-hydrolase[4] showing activity towards the acetates of tertiary alcohols. Esterases containing the more common GX motif in the oxyanion pocket do not show activity towards the esters of sterically more demanding tertiary alcohols because of the smaller active site. With the mutants G105A and E188D two variants with excellent R enantioselectivity ($E>100$) towards 1,1,1-trifluoro-2-phenyl-but-3-yn-1-yl acetate (**1**) were created, while the wild-type gave $E_R=42$.[5] Optically active tertiary alcohols are important building blocks for organic synthesis[6] and have been recently applied for the synthesis of an orally active A_{2A} receptor antagonist in a mouse catalepsy model.[7] Furthermore, fluorine-containing optically active alcohols are also of great interest owing to their potential use as ferroelectric liquid crystals and drugs.[8]

Here we report a focused directed-evolution approach based on a previous rational-protein-design study[5a] for the inversion of the enantioselectivity of BS2 towards acetylated tertiary alcohols (Scheme 1).



Scheme 1. Kinetic resolution of *rac*-**1** using the wild-type esterase and E188D of *Bacillus subtilis* (BS2) yields the *R* enantiomer (top) while the double-mutant E188W/M193C yields the *S* enantiomer (bottom).

Recently,[5a] we identified, based on computer modelling, residue E188 as having a strong influence on the enantioselectivity of esterase BS2 towards acetylated tertiary alcohols: While mutant E188D had an E value of $E_R>100$ towards **1**, mutant E188F had an inversed enantiopreference ($E_S=3$). Encouraged by this observation, we decided to apply focused directed evolution based on saturation mutagenesis inspired by the recently proposed CASTing[9] method developed by the Reetz group. From the crystal structure of the highly homologous BsubpNBE esterase (pdb entry: 1QE3[10]), three adjacent amino acids (E188, A190, and M193) were selected as target residues as they all point into the active site. Furthermore these residues are located around the catalytic triad. Hence, a focused library with simultaneous saturation mutagenesis at these three positions was created using degenerate primers bearing NNK codons, which theoretically should lead to $20^3=8000$ variants.

The resulting library of 2800 mutants was first submitted to a prescreening on agar plates in which only $\approx 40\%$ of all clones still showed activity. To facilitate high-throughput screening of the remaining variants, we applied a previously developed spectrophotometric assay based on the quantification of the released acetic acid, which is converted to NADH in an enzyme cascade.[11] Using enantiomerically pure (*S*)-**1** and (*R*)-**1** in separate wells of microtiterplates, we determined the apparent enantioselectivity E_{app} for approximately 1100 clones. The best variant turned out to be the

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[**] We are very grateful to Prof. Karl Hult, Linda Fransson, and Martin Veld (KTH, Stockholm, Sweden) for fruitful discussions and introduction to the docking program.

double-mutant E188W/M193C, which exhibited an inversed enantioselectivity ($E_S = 64$) towards **1**; this result was also confirmed in a preparative-scale experiment. Furthermore, this mutant still had acceptable specific activity towards *p*-nitrophenyl acetate (pNPA; 46.5 U mg^{-1}; 14% of the WT activity). Hence, the enantioselectivity towards **1** was switched from $E_R > 100$ (E188D) to $E_S = 64$.

Next, the corresponding single mutants were created to identify which mutations contributed to the substantial inversion in selectivity. Surprisingly, only the double mutant was highly *S*-selective: E188W showed modest *S* enantioselectivity ($E_S = 26$) and very low activity, while M193C still had *R* preference with $E_R = 16$, but similar activity. Thus, only the simultaneous saturation mutagenesis allowed the creation of an esterase variant with inverted *and* synthetically useful enantioselectivity and activity.

To understand the molecular basis of the inversion of the enantiopreference, we performed molecular dynamic simulations of the first tetrahedral intermediate (TI1) in esterase BS2. In the WT three sterically possible orientations for each enantiomer were found. As the orientations did not show differences in stability, we assumed that in the conversion of acetylated tertiary alcohols by BS2 esterase the TI1 is not the decisive intermediate for enantioselectivity. A similar observation was already described for the GGG(A)X-hydrolase *C. rugosa* lipase.[12] Alternatively, automated docking experiments were performed. One productive orientation for each enantiomer was found by docking **1** into the active site of BS2 WT. The observed *R* preference of the WT was supported by the fact that the successfully docked (*R*)-**1** in the active site had a lower energy (−4.31 kcal mol^{-1}) than the *S* enantiomer (−3.92 kcal mol^{-1}). In addition, the successfully docked states of the enantiomers of **1** perfectly matched one of our previously discovered orientations from the molecular dynamic simulations.

Docking experiments with a model of the double-mutant E188W/M193C showed that the substitution of Glu188 to Trp caused a steric repulsion of the preferred WT orientation of the *R* enantiomer of **1**, leading to an *S*-selective enzyme (Figure 1). Also, no productive orientation for the *R* enantiomer of **1** in the double mutant could be identified. The *S* enantiomer showed the same productive orientation as the *S* enantiomer in the WT but with a lower free energy than that with the WT. However, the synergistic effect of M193C cannot be explained yet, but this mutation seems to stabilize the preferred *S* orientation.

Interestingly, the double mutant also had a wider substrate range than the wild-type enzyme (Scheme 2). We reported earlier the influence of the mutants E188D and G105A[13] on the substrate specifity of BS2 and found nearly no activity for substrates **5** and **6**. Substrate **4** was only converted by the G105A variant. With the double-mutant E188W/M193C all substrates **1**–**6** were converted with inversed enantioselectivity compared to reactions with E188D and G105A (Table 1). Still, compounds **7** and **8** were not converted by any mutant. Furthermore, the double mutant showed high enantioselectivity towards **5** and good enantioselectivity towards **6** and is thus the first known enzyme showing activity towards these substrates.

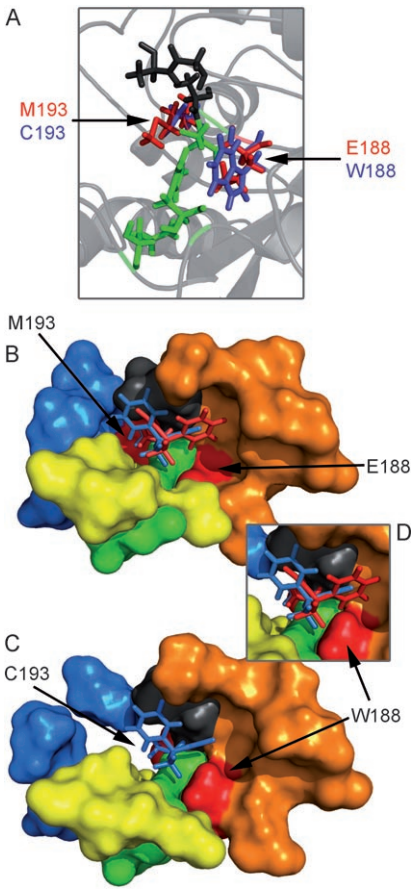
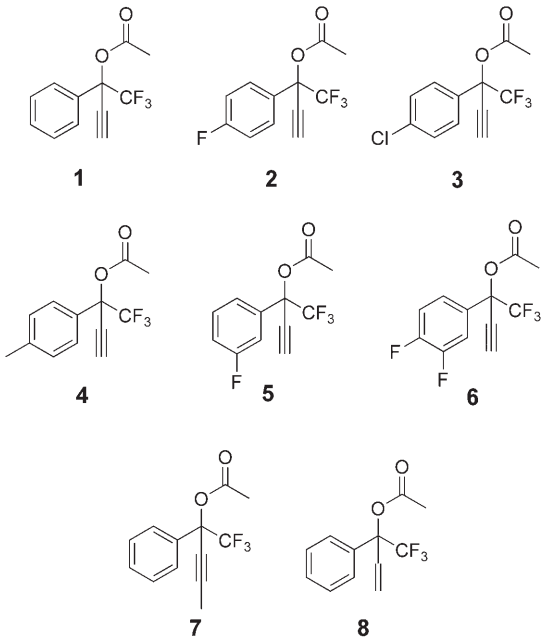


Figure 1. A) Comparison of the active sites of BS2 WT and double-mutant. WT residues (E188/M193) are highlighted in red, double-mutant residues (W188/M193) in blue. B) Comparison of preferred orientations of (*R*)-**1** (blue) and (*S*)-**1** (red) docked in the active site of BS2-WT. C) (*S*)-**1** in the active site of the double-mutant E188W/M193C. D) Comparison of preferred orientation of (*S*)-**1** (blue) and superimposed (*R*)-**1** (red) in E188W/M193C. Phenyl-binding site: orange; ethynyl binding site: blue; trifluoromethyl binding site: yellow; oxyanion hole: black; catalytic triad: green.

The unexpected change in substrate range by replacing glutamate with tryptophane at residue 188 could again be explained by computer modeling: The phenyl group of the *S* enantiomer points out of the active site (Figure 1 B), allowing the incorporation of larger substitutions. The phenyl group of the *R* enantiomer, however, is oriented inside the phenyl-binding pocket of the WT and therefore the size of substituents is limited. In all cases the trifluoro group points towards His399 of the catalytic triad.

In conclusion, the highly enantioselective double-mutant E188W/M193C of BS2 esterase created by focused directed evolution exhibits an enantiopreference opposite to that of the WT and the highly selective variants E188D and G105A created earlier. These findings could be rationalized by docking experiments, and exploration of the substrate range led to an improved understanding of the molecular basis of the enantioselectivity of BS2 towards acetylated tertiary alcohols. Furthermore, position Glu188 turns out to be a very crucial residue in BS2 as it controls promiscuous amidase[14] activity, and most importantly determines the sense and



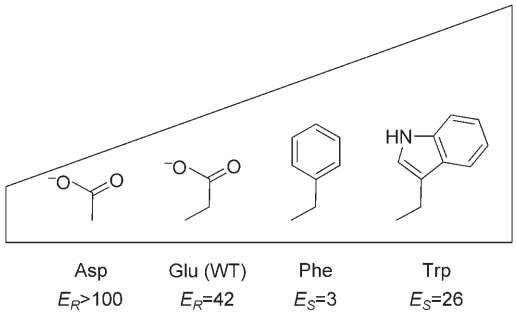
Scheme 2. Tertiary alcohol acetates **1**–**8** used for substrate mapping.

Table 1: Properties of E188W/M193C in the kinetic resolution of **1**–**8**.

Substrate	ee_S [%][a]	ee_P [%][a]	C [%][b]	E[c]
1	67	93	42	64
2	68	93	42	53
3	63	94	40	65
4	91	99	48	>100
5	58	98	37	>100
6	29	89	24	23
7	n.d.[d]	n.d.[d]	<1	n.d.[d]
8	n.d.[d]	n.d.[d]	<2	n.d.[d]

[a] Determined by GC analysis on a chiral phase. [b] Conversion determined with 200 U of the BS2 double-mutant after 20 min. [c] Determined from ee_S and ee_P. [d] Not determined.

degree of enantioselectivity. This becomes clear when the size of the residues at position 188 is increased stepwise from Asp to Trp leading to a stepwise change in enantioselectivity *and* -preference of BS2 (Scheme 3).



Scheme 3. Influence of residue 188 on the enantioselectivity and -preference in BS2. Small residues yield *R* preference and bulky residues *S* preference. *E* values for E188D and E188F were published previously.[5a]

Experimental Section

Tertiary alcohol acetates were prepared from the commercially available ketones as described previously.[13] The activity of BS2 WT and double-mutant was determined towards hydrolysis of *p*-nitrophenyl acetate (1 U = 1 μmol min^{-1}). The specific activities of purified enzymes were determined to be 333 U mg^{-1} (WT) and 46.5 U mg^{-1} (double-mutant E188W/M193C). Preparative biocatalysis to obtain (*S*)-**1** and (*R*)-**1a** was performed as described[13] using 500 mg of **1** and 1500 U of the BS2 mutant G105A. Alcohol (*R*)-**1a** was then acetylated using acetyl chloride. The mutant libraries were created by site-directed mutagenesis using degenerate primers containing NNK codons. For the overlay agar activity staining, mutant libraries were transferred to agar plates (supplemented with Luria Bertani (LB) media, ampicillin, and rhamnose) and, after incubation at 37 °C for 5 h, covered by an agar-agar solution containing Fast Red and α-naphthyl acetate. Active clones having a red color were transferred, cultivated, and expressed in microtiterplates (MTP), and cells were disrupted using lysis buffer. The acetate assay was performed as described,[13] but substrates (*R*)-**1** and (*S*)-**1** (1 mM) (dissolved in DMSO, 10 % v/v) were dissolved in the kit buffer prior to the addition of the other kit components. To 170 μL acetate assay kit solutions containing the acetates, 20 μL of the soluble fraction from the disrupted cells was added. The absorption at 340 nm was measured over 30 min. Apparent *E* values were calculated from the slope of NADH formation. Biocatalysis in small and preparative-scale analysis as well as GC analysis was performed as described.[11] A preparative-scale reaction using 700 U of BS2 E188W/M193C for 125 mg of **1** gave 30 % (*R*)-**1** (64 % *ee*) and 17 % (*S*)-**1a** (94 % *ee*) at $E_S = 59$ and $C = 41$ %. In another experiment 3600 U of E188W/M193C was used for kinetic resolution of 200 mg of **6** yielding 41 % **6** (58 % *ee*) and 33 % **6a** (92 % *ee*) at $E = 48$ and $C = 39$ %.

Molecular dynamic studies were performed in a periodic water box using the YASARA software (version 7.4.22) with AMBER99 forcefield using long-range electrostatics with a cutoff at 7.86 Å (Particle-Mesh-Ewald).[15] The force field of the substrate was obtained using AutoSMILES force field parameter assignment.[16] After addition of the solvent, cell neutralization and pK_s prediction,[17] simulations of 500 ps were performed at 35 °C, pH 7 and a solvent density of 0.997 g L^{-1}. Each simulation step contained a 1.25-fs step for inter- and intramolecular forces.

The docking experiments were performed by using AutoDock4[18] and AutoGrid4 in combination with AutoDock Tools.[19] In the first step, a grid box was defined (45 × 45 × 45 points with a grid spacing of 0.375 Å), defining the space for the docking experiment. The box was centered on the catalytic active Ser189. In the second step, the Larmackian-Genetic algorithm was used with a maximum of 15 million energy evaluations and maximum 2700 generations. Starting with a population of 150 individuals, 100 independent runs were performed. Three of the five possible torsions of the substrate were active with 20°/step. All other options were set on program defaults.

Received: October 5, 2007
Published online: January 18, 2008

Keywords: alcohols · directed evolution · enantioselectivity · enzymes · esterases

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