# Creation of an Enantioselective Hydrolase by Engineered Substrate-Assisted Catalysis

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We have engineered a hydrolase scaffold to perform enantioselective substrate-assisted catalysis. First, a transition-state stabilizing residue in the enzyme active site was removed by site-directed mutagenesis, causing reduced activity. The catalytic activity was then partially and enantioselectively restored by a substrate containing the missing catalytic functional group. By this approach we converted the wild-type lipase with very poor enantioselectivity ($E = 1.6$) into an enzyme with remarkably improved enantioselectivity ($E = 22$) toward ethyl 2-hydroxypropanoate. Enzymes offer one of the best routes to enantiomerically pure building blocks for the synthesis of bioactive compounds such as pharmaceuticals, pheromones, fragrances and fine chemicals.[1] Hydrolases such as lipases, esterases, and proteases are commonly used catalysts for the preparation of optically active carboxylic acids, alcohols, amino acids, and amines.[2] Yet, it has remained a challenging task to rationally tailor or improve the substrate specificities of these enzymes. To date, only a few examples of improvements in enantioselectivities of lipases/esterases have been achieved using site-directed mutagenesis,[3] directed evolution,[4] or chemical modification.[5]

Here we describe a new means to create enantioselective hydrolases via engineered substrate-assisted catalysis (SAC). Examples of SAC[6] include naturally occurring enzymes as well as engineered serine proteases[7] and glycosidases.[8] Our goal was to engineer an enzyme to performe SAC toward 2- or 3-hydroxyacid esters. As a target enzyme lipase B was identified from *Candida antarctica* (CALB) which has found numerous synthetic applications.[9] Our hypothesis was that the hydroxyl group in such substrates could substitute for an active-site residue, replaced by site-directed mutagenesis in the enzyme. The catalytic machinery of CALB consists of a Ser-His-Asp triad.[10] The transition state of the catalyzed ester hydrolysis reaction is an oxyanion (Figure 1). The oxyanion hole is a spatial arrangement of hydrogen-bond donors in the active site that is the major factor for lowering the free energy of the transition state.[11] The 3D-structures of CALB/inhibitor-complexes suggest that the oxyanion is stabilized by two backbone amide hydrogen atoms and the side-chain hydroxyl group of Thr40.[12] Our hypothesis was that the Thr40 side-chain hydroxyl group of the enzyme could be replaced with one placed at the 2-position in the acyl moiety of the substrate (Figure 1). The steric requirements of the active site suggested that one enantiomer of the substrate would be much more reactive compared to the other.

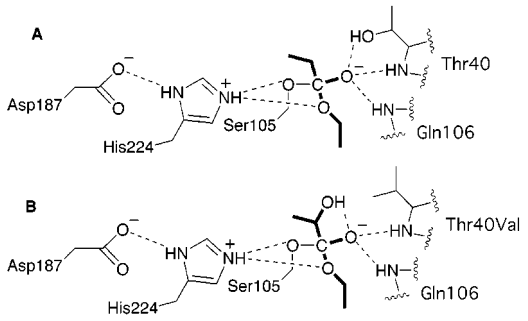


**Figure 1.** Active-site close-up of *Candida antarctica* lipase B. (A) Transition-state stabilization in wild-type enzyme. (B) Substrate-assisted transition state stabilization in a Thr40Val mutant.

We targeted Thr40 in CALB by site-directed mutagenesis to create enzyme variants with a compromised oxyanion hole. Wild-type lipase and the Thr40Val and Thr40Ala mutants were produced in recombinant yeast *Pichia pastoris* in high yield[13] and purity.[14] The produced lipases were purified by means of hydrophobic interaction chromatography followed by gel filtration.[14] Specificity constants ($k_{cat}/K_M$) for wild-type and mutated lipase were determined toward ethyl propanoate and ethyl butanoate (Table 1). The enzymes did not show saturation kinetics within the solubility range of the investigated substrates. The Thr40Val and Thr40Ala mutants showed 3 orders of magnitude lower $k_{cat}/K_M$ values than wild-type enzyme, demonstrating the crucial role of Thr40 in catalysis (Table 1). The lowered $k_{cat}/K_M$ values correspond to increased activation energies by 15−19 kJ/mol. These data agree well with the loss of one stabilizing hydrogen bond to the transition state.[15] In a few hydrolases the importance of a side chain involved in oxyanion hole stabilization has been investigated by site-directed mutagenesis and enzyme kinetics. Such mutants of the serine protease subtilisin,[16] the cysteine protease papain,[17] and the esterase cutinase[18] showed reduced $k_{cat}/K_M$ values corresponding to reduced transition-state stabilization by 10−16 kJ/mol. In all cases the effect was attributed to a large decrease in $k_{cat}$ and a minor change in $K_M$. It was thus concluded that the role of the oxyanion hole is to stabilize the transistion state and not to facilitate binding of the substrate.

Having established the essential role of Thr40 in transition-state stabilization in CALB, we now investigated if a hydroxyl group positioned in the substrate could substitute for the side chain of Thr40 replaced by site-directed mutagenesis in the mutant lipases. The wild-type lipase showed very low substrate specificity[19] for either enantiomer of ethyl 2-hydroxypropanoate compared

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(19) Substrate specificity is here defined by the ratio between the specificity constants ($k_{cat}/K_M$) of the enzyme for competing substrates.

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**Table 1.** Kinetic Constants for Wild-Type and Mutated *Candida antarctica* Lipase B in the Hydrolysis of Chiral and Achiral Ethyl Esters[a]

| Catalyst | $k_{cat}/K_M$ propanoate ($s^{-1}$ $M^{-1}$) | $k_{cat}/K_M$ (*S*)-2-hydroxy-propanoate ($s^{-1}$ $M^{-1}$) | $k_{cat}/K_M$ (*R*)-2-hydroxy-propanoate ($s^{-1}$ $M^{-1}$) | $k_{cat}/K_M$ butanoate ($s^{-1}$ $M^{-1}$) | $k_{cat}/K_M$ (*S*)-3-hydroxy-butanoate ($s^{-1}$ $M^{-1}$) | $k_{cat}/K_M$ (*R*)-3-hydroxy-butanoate ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|---|---|
| wild-type | 830 ± 13 | 190 ± 4 | 120 ± 5 | 6700 ± 530 | 54 ± 3 | 1700 ± 85 |
| Thr40Ala | 0.33 ± 0.07 | 2.0 ± 0.1 | 0.20 ± 0.03 | 1.7 ± 0.1 | 0.14 ± 0.02 | 1.1 ± 0.2 |
| Thr40Val | 0.60 ± 0.01 | 5.0 ± 0.3 | 0.23 ± 0.02 | 2.0 ± 0.3 | 0.059 ± 0.004 | 0.12 ± 0.02 |
| nonenzymatic | 0.22 | 2.0 | 2.0 | 0.15 | 0.36 | 0.36 |

[a] Kinetic constants were obtained from initial rates determined at 1−20 mM substrate concentrations, in the presence of 0.125 mM *p*-nitrophenol in 2.5 mM MOPS buffer at pH 7.2 and 25 °C. Enzyme activities were measured spectrophotometrically at 400 nm in triplicates or more. Second-order rate constants for non-enzymatic alkaline hydrolysis of the esters were determined at pH 10.

to ethyl propanoate (Table 1). By contrast, the Thr40Ala and Thr40Val mutants showed remarkably improved (*S*)-2-hydroxypropanoate/propanoate specificity[19] by factors of 26 and 36, respectively (Table 1). In the engineered lipases the hydroxyl group with *S*-configuration in the substrate acyl chain lowers the activation energy by 4−5 kJ/mol compared to the corresponding straight chain substrate. These data clearly suggest that the hydroxyl group with *S*-configuration is oriented to allow for improved transition-state stabilization. To quantify differences in reactivity between substrates we determined the second-order rate constants for alkaline hydrolysis. These experiments showed that ethyl 2-hydroxypropanoate is 9 times more reactive than ethyl propanoate (Table 1). Thus, only the reengineered enzymes can take full advantage of the inherent chemical reactivity difference between the substrates. By contrast, with the *R*-enantiomer of ethyl 2-hydroxypropanoate the Thr40Ala and Thr40Val mutants were not able to perform substrate-assisted catalysis. The (*R*)-2-hydroxypropanoate/propanoate specificities of the mutants were only marginally improved compared to wild-type enzyme (Table 1).

The wild-type lipase showed very low enantioselectivity ($E = 1.6$) toward ethyl 2-hydroxypropanoate. The Thr40Ala and Thr40Val mutants showed substantially improved enantioselectivities, $E = 9.8$ and $E = 22$, respectively (Figure 2). The *E*-values of wild type and Thr40Val mutant enzymes correspond to an enantiomeric excess of the product of 20 and 89%, respectively, at 25% conversion. This clearly demonstrates that engineered substrate-assisted catalysis is a powerful means to create highly enatioselective hydrolases. The higher *E*-value of the Thr40Val mutant compared to that of the Thr40Ala variant is due to its higher $k_{cat}/K_M$ value for the fast-reacting (*S*)-2-hydroxypropanoate ester (Table 1). The size of the oxyanion hole is therefore also an important factor controlling enantiorecognition. The key role of residue 40 in catalysis and enantioselectivity was further demonstrated with ethyl 3-hydroxybutanoate. The enantioselectivity decreased from $E = 31$ for wild-type lipase to $E = 7.8$ and $E = 2.0$ as a result of the Thr40Ala and Thr40Val mutations, respectively (Table 1, Figure 2). In the mutated enzymes the enantioselectivities were thus altered toward improved *S*-enantiomer selectivity for both ethyl 2-hydroxypropanoate and ethyl 3-hydroxybutanoate (Figure 2).

The oxyanion hole is a universal feature in the catalytic function of serine and cysteine proteases[20] and $\alpha/\beta$-hydrolase fold enzymes.[21] Large parts of the oxyanion hole anatomy are defined by backbone amide protons. CALB, subtilisin, papain, cutinase, and some lipases also have a side chain contributing to transition-state stabilization. The current approach is therefore generally applicable to all of these enzymes, representing three different protein folds. Also, subtilisin has the mirror-image organization of the catalytic triad members and the oxyanion hole compared to CALB. Thus, engineered substrate-assisted catalysis applied to these different scaffolds provides novel opportunities for the creation of tailored enzymes with opposite and complementary enantiopreference.

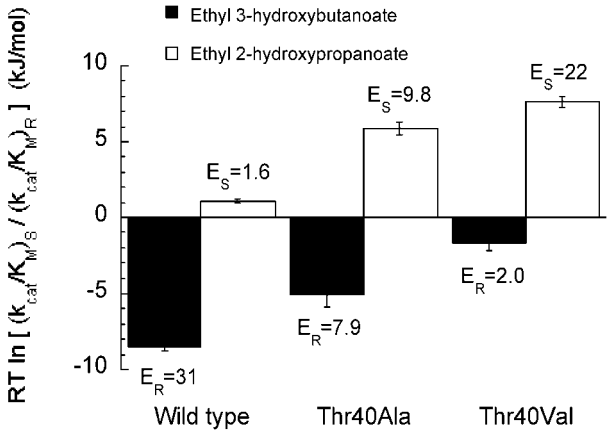


**Figure 2.** Comparison of $k_{cat}/K_M$ values and enantioselectivities, $E_S = [(k_{cat}/K_M)_S/(k_{cat}/K_M)_R]$, of wild type *Candida antarctica* lipase B and oxyanion hole mutants.

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