Protein Engineering

An *S*-Selective Lipase Was Created by Rational Redesign and the Enantioselectivity Increased with Temperature**

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Lipases have characteristics that make them attractive tools for the synthesis of fine chemicals. They catalyze acyl-transfer reactions and are stable in organic solvents and at high temperatures. The possibility of modifying the performance of enzymes by biomolecular methods gives the opportunity to create tailor-made catalysts for each desired application. The high enantioselectivity of lipases toward secondary alcohols is a property often used in organic synthesis.[1] The enantioselectivity follows the Kazlauskas rule which normally predicts an *R* selectivity.[2] Structural explanations now exist for the strong chiral preference of many lipases.[3] Efforts to modify their properties have resulted in large improvements of the catalyst, such as increased stability and activity, but few articles report large effects on the enantioselectivity.[4,5] The largest changes in enantioselectivity of lipases concern substrates with a chiral center on the acyl chain of the substrate.[6] We redesigned the active site of *Candida antarctica* lipase B (CALB) to modify its specificity toward secondary alcohols. The enantioselectivity was greatly changed and an *S*-selective enzyme was created, mainly as a result of the increased activity toward the slow-reacting enantiomer. The mutant had very unusual behavior: the enantioselectivity increased strongly with temperature, and thermodynamic analysis showed that the altered enantioselectivity was dominated by entropy. In dynamic kinetic resolution, lipases can be used to produce close to 100% of the *R* enantiomer.[7] The creation of an *S*-selective lipase affords the possibility of achieving high yields of the *S* enantiomer.

CALB is a very robust and efficient catalyst that shows a high regio- and enantioselectivity.[8] The active site contains the catalytic triad Ser-His-Asp, an oxyanion hole that stabilizes the transition states, and a cavity called the stereospecificity pocket.[9] The strong *R* selectivity of CALB toward secondary alcohols is explained by the different binding modes of the enantiomers in the stereospecificity pocket.[10,11] The fast-reacting enantiomer positions its medium-sized substituent in the stereospecificity pocket and its large substituent toward the active-site entrance. For the slow-reacting enantiomer, the medium-sized substituent points toward the active-site entrance and the large substituent is placed in the stereospecificity pocket. Although the large substituent is not easily fitted in the stereospecificity pocket, this is the catalytically active binding mode for the slow-reacting enantiomer. The largest substituent that is well accommodated in the stereospecificity pocket is an ethyl group. This steric limitation makes CALB very selective toward secondary alcohols with a medium-sized substituent not larger than an ethyl group and a large substituent bigger than an ethyl group. When both substituents are larger than an ethyl group, the selectivity is low and the catalytic activity drops drastically.[11] To overcome this limitation in size and to increase the number of secondary alcohols that are good substrates for CALB, we earlier redesigned the volume of the stereospecificity pocket. Trp 104, which forms the bottom of the stereospecificity pocket, was replaced by smaller amino acids. The redesigned stereospecificity pockets were shown to readily accommodate much larger substituents than the stereospecificity pocket of the wild-type lipase. The mutant with the largest stereospecificity pocket, Trp 104 Ala, had a specificity constant (k_{cat}/K_M) of 830 $s^{-1}M^{-1}$ toward 5-nonanol, 5500 times greater than that of the wild-type enzyme.[12]

A larger stereospecificity pocket should affect the enantioselectivity of the lipase. Chiral secondary alcohols might easily position their large substituent in the redesigned stereospecificity pocket of the Trp 104 Ala mutant, which would lead to a low enantioselectivity. Computer modeling was used to compare the binding of secondary alcohols in the active site of the Trp 104 Ala mutant and wild-type CALB. Energy minimizations were performed on the tetrahedral intermediate of both lipase variants with the butanoate ester of (*R*)- and (*S*)-1-phenylethanol covalently bound to the catalytic serine (Figure 1). The *R* enantiomer was similarly positioned in both wild-type CALB and the Trp 104 Ala mutant; the large substituent (phenyl group) was oriented toward the active-site entrance and the medium-sized substituent (methyl group) was positioned in the stereospecificity pocket. The *S* enantiomer had different orientations in the two enzyme variants. In the wild-type lipase the slow-reacting enantiomer could not be accommodated in a catalytically active tetrahedral intermediate, as the phenyl group was too large to fit in the stereospecificity pocket. This finding is in accordance with a low reaction rate for the *S* enantiomer and a high enantioselectivity favoring the *R* enantiomer. However, the redesigned stereospecificity pocket of the Trp 104 Ala mutant comfortably accommodated the phenyl group.

We tested the low enantioselectivity expected for the Trp 104 Ala mutant by performing acyl-transfer reactions from vinyl butanoate to four secondary alcohols in cyclohexane. With 3-methyl-2-butanol, the selectivity for the *R* enantiomer dropped by a factor of 160 as a result of the mutation (Table 1, entries 6 and 7). With 2-hexanol, the *R* selectivity of several hundred for the wild-type enzyme changed into a low *S* selectivity for the mutant (Table 1, entry 5). Thus methyl, isopropyl, and butyl groups are almost equally well-accommodated in the stereospecificity pocket of the Trp 104 Ala

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[**] This work was supported by the Swedish Research Council and the Erasmus program. The graphical material presenting the active sites of the lipases was prepared by Linda Fransson.

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

  DOI: 10.1002/ange.200500971

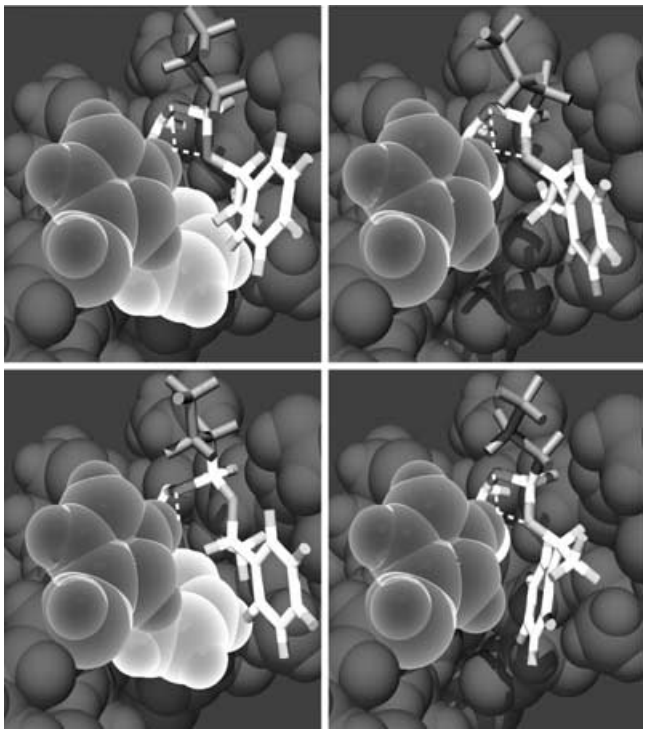


Figure 1. The active site of wild-type CALB (left) and Trp104Ala mutant (right) with the butanoate ester of (*R*)-1-phenylethanol (top) and (*S*)-1-phenylethanol (bottom) covalently bound to the catalytic serine in the tetrahedral reaction intermediate. The substrate is presented with a stick model and amino acid 104 with a space-filling model in white. The *R* enantiomer has a similar configuration in the wild-type CALB and Trp104Ala mutant: the large substituent (phenyl) points toward the active-site entrance and the medium-sized substituent (methyl) is positioned in the stereospecificity pocket. In the wild-type CALB, the *S* enantiomer cannot position its phenyl group in the stereospecificity pocket, and not all the hydrogen bonds required for catalysis can be formed. In the Trp104Ala mutant, the phenyl group is comfortably accommodated in the space liberated by the mutation in the stereospecificity pocket.

mutant. Interestingly, while wild-type CALB has a very high *R* selectivity, the mutant has a moderate *S* selectivity toward the two secondary alcohols with the bulky phenyl group as the large substituent (Table 1, entries 3 and 4). The phenyl group can probably fit well in the space liberated by the Trp104Ala mutation.

The enantioselectivity of wild-type CALB is very sensitive to the solvent used in the incubation.[13] The effects of the solvent and temperature on the enantioselectivity of the Trp104Ala mutant were investigated. We resolved 1-phenylethanol in three solvents and at temperatures between 10 and 70°C (Figure 2). Both the solvent and the temperature had

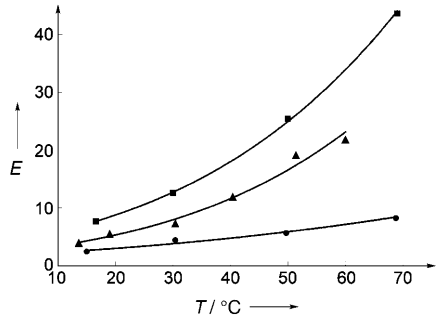
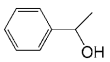
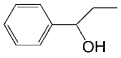
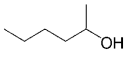


Figure 2. The *S* selectivity increased with temperature in the resolution of 1-phenylethanol catalyzed by the Trp104Ala mutant of CALB in acetonitrile (●), cyclohexane (▲), and *cis*-decalin (■). Trend lines are plotted according to Equation (1) using the values of the entropy and enthalpy terms in Table 1.

large effects on the enantioselectivity. The *S* selectivity increased to a large extent with the molecular size of the solvent. An increase in the temperature also favored the *S* enantiomer, which resulted in the unusual behavior of an increasing enantioselectivity with temperature. These effects gave an *S* selectivity of 44 for the Trp104Ala mutant toward 1-phenylethanol at 69°C in *cis*-decalin. Wild-type CALB is

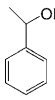
Table 1: Thermodynamic components for acyl-transfer reactions from vinyl butanoate to secondary alcohols in various solvents catalyzed by wild-type CALB and the Trp104Ala mutant. Differential entropy ($\Delta_{S-R}\Delta S^{\ddagger}$) and enthalpy ($\Delta_{S-R}\Delta H^{\ddagger}$) and their standard errors were determined from the linear regression of $R\times\ln E$ versus T^{-1}. The entropic contribution ($T\Delta_{S-R}\Delta S^{\ddagger}$), Gibbs free energy ($\Delta_{S-R}\Delta G^{\ddagger}$), and the enantioselectivity (E) were calculated for 303 K. The preferred enantiomer (Pe) and the racemic temperature (T_R) are also presented.

Entry	CALB variant	Sec. alcohol	Solv.	Pe	E[a]	$\Delta_{S-R}\Delta G^{\ddagger}$[a] [kJ mol^{-1}]	$T\Delta_{S-R}\Delta S^{\ddagger}$[a] [kJ mol^{-1}]	$\Delta_{S-R}\Delta H^{\ddagger}$ [kJ mol^{-1}]	$\Delta_{S-R}\Delta S^{\ddagger}$ [J K^{-1} mol^{-1}]	T_R [K]
1	Trp104Ala		CH_3CN	*S*	3.8	−3.3	21	18±2	70±8	250
2	Trp104Ala		Deca[b]	*S*	13	−6.4	34	27.5±0.3	112±1	250
3	Trp104Ala		cHex[c]	*S*	7.9	−5.2	35	30±2	116±5	260
4	Trp104Ala		cHex[c]	*S*	12	−6.3	26	20±1	87±4	230
5	Trp104Ala		cHex[c]	*S*	2.2	−1.9	23	21±2	77±5	280
6	Trp104Ala		cHex[c]	*R*	2.8	2.6	26	28±2	84±5	330
7	wild-type		cHex[c]	*R*	470	15	7.8	23±2	26±7	900

[a] Calculated for 303 K from the linear relation of Equation (1). [b] *cis*-Decalin. [c] Cyclohexane.

used in dynamic kinetic resolution to achieve the *R* enantiomer,[14] and by using the Trp104Ala mutant in the same processes a high yield and enantiomeric excess could be achieved for the *S* enantiomer. The CALB mutant also has a higher activity (Table 2) and stability compared to those of subtilisin, which has been used in dynamic kinetic resolution to achieve the *S* enantiomer.[15]

Table 2: Apparent kinetic constants, specificity constants, preferred enantiomer (Pe), and *E* value of the wild-type CALB and Trp104Ala mutant for the acylation of the pure enantiomers of 1-phenylethanol in cyclohexane at 30°C with 500 mM vinyl butanoate.

CALB variant	Substrate enantiomer		k_{cat}^{app} [s^{-1}]	K_M^{app} [mM]	k_{cat}/K_M [$s^{-1}\,M^{-1}$][a]	Pe	E[a]
Wild-type		*R*	570	61	9300	*R*	1300000
		S	0.00053	71	0.0075		
Trp104Ala		*R*	4.4	29	150	*S*	6.6
		S	34	34	1000		

[a] Calculated from k_{cat}^{app} and K_M^{app}.

The Trp104Ala variant showed the very unusual behavior of increasing enantioselectivity with temperature for all the substrates tested, except for 3-methyl-2-butanol where the selectivity changed from the *R* to the *S* enantiomer. The selectivity between the two enantiomers depends on the difference in activation free energy ($\Delta_{S-R}\Delta G^{\ddagger}$), which can be divided into its enthalpic ($\Delta_{S-R}\Delta H^{\ddagger}$) and entropic components ($\Delta_{S-R}\Delta S^{\ddagger}$) according to Equation (1), where *R* is the gas constant and *T* is the temperature in Kelvin.

$$E = e^{-\Delta_{S-R}\Delta G^{\ddagger}/RT} = e^{(-\Delta_{S-R}\Delta H^{\ddagger} + T\Delta_{S-R}\Delta S^{\ddagger})/RT} \quad (1)$$

The value of the thermodynamic components can be calculated by determining the enantioselectivity (*E*) at several temperatures. This was done for the Trp104Ala mutant with 1-phenylethanol in three solvents (see Figure 2) and with three other secondary alcohols in cyclohexane. The enantioselectivity of the wild-type enzyme was determined for only one of the substrates at several temperatures; with the other substrates the *R* selectivity was too large to be accurately determined by resolution. Equation (1) was rearranged into Equation (2) and, if one assumes that the thermodynamic components are constant within the experimental temperature range, there is a linear correlation between $R\ln E$ and T^{-1}.[16] A good linearity was also seen, which justified this assumption. The differential entropy and enthalpy terms and their standard errors were calculated (Table 1) by linear regression to Equation (2).

$$R\ln E = -\frac{\Delta_{S-R}\Delta G^{\ddagger}}{T} = -\frac{\Delta_{S-R}\Delta H^{\ddagger}}{T} + \Delta_{S-R}\Delta S^{\ddagger} \quad (2)$$

In all the resolutions tested, the *R* enantiomer was favored by the enthalpy ($\Delta_{S-R}\Delta H^{\ddagger} > 0$) and the *S* enantiomer by the entropy ($\Delta_{S-R}\Delta S^{\ddagger} > 0$). The resolution of 3-methyl-2-butanol catalyzed by wild-type CALB afforded the *R* enantiomer, as the enthalpic contribution was larger than the entropic one (Table 1, entry 7). The magnitudes of the thermodynamic components agree well with the values obtained for the resolution of other secondary alcohols catalyzed by the same enzyme.[17] For the resolutions catalyzed by the Trp104Ala mutant, the enthalpic contribution was not significantly different from that of the wild-type enzyme. However, the entropic contribution increased so much that the enantioselectivity was eliminated or reversed. An enantioselectivity dominated by the entropy afforded a very unusual temperature dependence that has rarely been seen before.[18] The enantioselectivity of CALB Trp104Ala increased strongly with temperature: in the resolution of 1-phenylethanol in *cis*-decalin an *E*-value of 44 in favor of the *S* enantiomer was reached at 69°C (Figure 2). The racemic temperature ($T_R = \Delta_{S-R}\Delta H/\Delta_{S-R}\Delta S$),[19] at which the enantioselectivity is 1, was 330 K for this mutant toward 3-methyl-2-butanol; below this temperature the mutant was *R*-selective and above it, *S*-selective. A racemic temperature outside the experimental range should only be used to compare enthalphic and entropic contributions. Resolutions catalyzed by the Trp104Ala mutant have a much lower racemic temperature (Table 1) than those catalyzed by the wild-type lipase. This corresponds to the three to four times higher entropy contribution of the mutant.

The enantioselectivity toward 1-phenylethanol changed from a strong *R* selectivity with wild-type CALB to an *S* selectivity with the Trp104Ala mutant. The Trp104Ala mutation increases the size of the stereospecificity pocket, which should facilitate the binding of the *S* enantiomer in an orientation conducive to catalysis. This would lead to a higher ratio of productive binding of the *S* enantiomer in the Trp104Ala mutant compared to that in the wild-type CALB, and accordingly to a higher reaction rate. The apparent kinetic constants (k_{cat}^{app} and K_M^{app}) were determined for wild-type CALB and Trp104Ala mutant for the acylation of (*R*)- and (*S*)-1-phenylethanol with vinyl butanoate (500 mM) in cyclohexane at 30°C (Table 2). The specificity constant (k_{cat}/K_M) and the enantioselectivity (*E*) were calculated using the kinetic constants determined for both enzyme variants (Table 2).

The enantioselectivity was changed by a factor of 8300000 by the carefully selected point mutation in the stereospecificity pocket of CALB, which is more than any other example found in the literature. Rational design has been used to create variants of a phosphotriesterase with 6900000-fold difference in enantioselectivity, but the largest difference compared to the wild-type enzyme was 18000-fold.[5] The huge change in enantioselectivity by the Trp104Ala mutation of CALB was mainly achieved by increasing the apparent catalytic constant (k_{cat}^{app}) toward the slow-reacting enantiomer. The apparent catalytic constant toward (*S*)-1-phenylethanol was 64000 times larger for the mutant than for the wild-type CALB, while the same constant decreased by a factor of 130 toward the *R* enantiomer. However, the mutation did not affect the apparent Michaelis constant (k_M^{app}) to a large extent. The apparent Michaelis constants for the Trp104Ala mutant

were almost equal toward the *R* and *S* enantiomers, and they both decreased by a factor of two compared to those of the wild-type enzyme.

In summary, the redesigned stereospecificity pocket of *Candida antarctica* lipase B (CALB) was able to accommodate much larger groups than that of the wild-type lipase. This change transformed the strongly *R*-selective wild-type CALB into an *S*-selective mutant. The *S* selectivity increased with temperature and it was dominated by entropy. For 1-phenylethanol the enantioselectivity changed by a factor of 8300000 as a result of the mutation. This was mainly achieved by an increased reaction rate toward the *S* enantiomer, which resulted in a very effective catalyst with a high specificity constant of $1000\ s^{-1}M^{-1}$. The *S* selectivity of the Trp104Ala mutant could be increased to a respectable value of $E = 44$ when the reaction was conducted in *cis*-decalin at 69 °C. The altered enantioselectivity of CALB is a demonstration of the possibilities offered by protein redesign and shows the importance of the entropy contribution in enzyme catalysis.

Received: March 16, 2005
Published online: June 23, 2005

Keywords: enantioselectivity · enzyme catalysis · hydrolases · protein engineering · thermodynamics

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