

Rational design of enantioselective enzymes requires considerations of entropy

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Abstract

Entropy was shown to play an equally important role as enthalpy for how enantioselectivity changes when redesigning an enzyme. By studying the temperature dependence of the enantiomeric ratio *E* of an enantioselective enzyme, its differential activation enthalpy ($\Delta_{R-S}\Delta H^\ddagger$) and entropy ($\Delta_{R-S}\Delta S^\ddagger$) components can be determined. This was done for the resolution of 3-methyl-2-butanol catalyzed by *Candida antarctica* lipase B and five variants with one or two point mutations. $\Delta_{R-S}\Delta S^\ddagger$ was in all cases equally significant as $\Delta_{R-S}\Delta H^\ddagger$ to *E*. One variant, T103G, displayed an increase in *E*, the others a decrease. The altered enantioselectivities of the variants were all related to simultaneous changes in $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$. Although the changes in $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ were of a compensatory nature the compensation was not perfect, thereby allowing modifications of *E*. Both the W104H and the T103G variants displayed larger $\Delta_{R-S}\Delta H^\ddagger$ than wild type but exhibited a decrease or increase, respectively, in *E* due to their different relative increase in $\Delta_{R-S}\Delta S^\ddagger$.

Keywords: Enthalpy; entropy; enantiomeric ratio; lipase; *Candida antarctica*; site-directed mutagenesis

With the advances in protein engineering the vision of tailor-made proteins is becoming a reality. Modified proteins with improved properties have an enormous commercial potential (Rubingh 1997; Nixon and Firestine 2000). For instance, enzymes modified for new or improved properties would greatly improve their usability as catalysts in organic chemistry. The use of enzymes as biocatalysts has already become a popular alternative to classic chemistry because of their advantageous high selectivity and activity under mild reaction conditions (Liese and Filho 1999; Schulze and Wubbolts 1999; Carrea and Riva 2000; Faber 2000). However, finding the optimal biocatalyst and reaction conditions for a specific reaction still requires a great deal of experimental screening. Enabling rational design of enzymes

would relieve this need for screening large numbers of biocatalysts to suit a target reaction and allow the introduction of new enzymatic activities. Present-day knowledge of protein structure–function relationships is, however, insufficient to predict the effects of point mutations on proteins.

Candida antarctica lipase B (CALB) is an enzyme that has proved to be a highly useful and versatile biocatalyst. It displays high activity in organic solvents and is very enantio- and regioselective, as well as being a robust and stable enzyme (Anderson et al. 1998). A model of CALB's mechanism for resolving the enantiomers of *sec*-alcohols based on a combination of experimental and molecular modeling research has been proposed (Orrenius et al. 1998a). This model involves the binding of the two enantiomers in two different modes in transition state to allow for catalysis (Fig. 1). The slow-reacting enantiomer, usually *S*, is sterically more hindered and hence, has a much lower reaction rate, yielding the possibility to kinetically resolve the two enantiomers. However, it has been shown that differences in both the enthalpic and the entropic components of the free energy of activation are important to enantioselectivity (Pham et al. 1989; Philips 1992, 1996; Galunsky et al. 1997;

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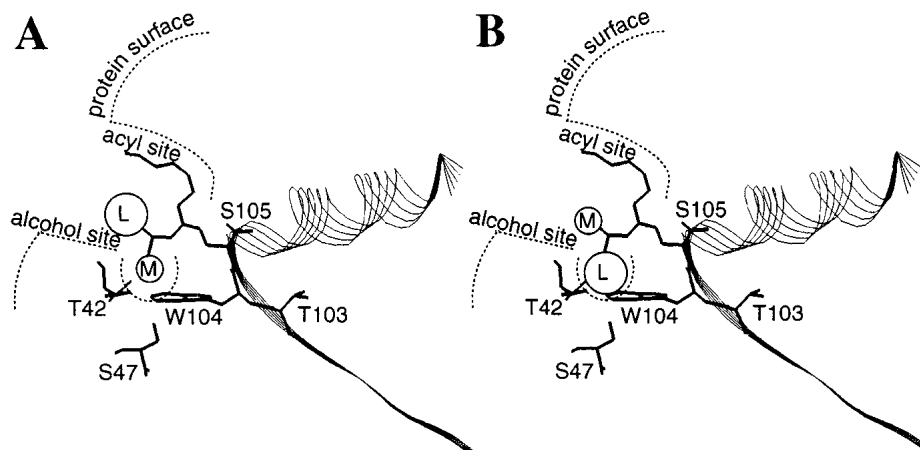


Fig. 1. Simplified scheme of the two productive binding modes in the model proposed by Orrenius et al. (1998a) that rationalizes the enantioselectivity of *Candida antarctica* lipase B. (A) Fast-reacting enantiomer. (B) Slow-reacting enantiomer. (M) medium-sized substituent; (L) large-sized substituent.

Sakai et al. 1997; Jönsson et al. 1999; Overbeeke et al. 1999; Ottosson and Hult 2001). Differential activation entropy is not accounted for in this two-mode model. Attempts have been made to modify this already popular biocatalyst to improve thermostability, oxidation stability as well as enantioselectivity through site-directed mutagenesis, and has been somewhat successful (Patkar et al. 1997, 1998; Rotticci et al. 2001). This paper presents a thermodynamic analysis, which determines the entropic as well as the enthalpic components of enantioselectivity toward 3-methyl-2-butanol of the T103G, W104H, S47A, T42V, T42VS47A variants of CALB (Scheme 1 and Fig. 1), and compares the components to those of the wild type. The aim is to determine the importance of entropy and enthalpy in enzyme enantioselectivity with the ultimate goal of understanding how these are affected by variations in the enzyme and establishing their implications for rational design of proteins.

Theory

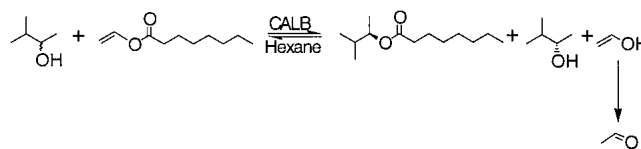
Orrenius et al. (1998a) developed a model of how *Candida antarctica* lipase B discriminates between the enantiomers of *sec*-alcohols, which involves two different productive binding modes for the substrate enantiomers. Experimental and molecular modeling research showed that the two modes were necessary to allow both enantiomers to develop the hydrogen-bonding pattern within the active site required for catalysis. In Figure 1 a schematic picture of the alcohol-binding site and the two productive binding modes are presented. The active site of CALB is deep and narrow. In the innermost part, there is a free volume designated as a stereospecificity pocket. The fast-reacting enantiomer, generally R, places its medium-sized substituent of the alcohol moiety into this pocket and its large substituent in the vol-

ume of the active site open to the surface of the protein. To keep that hydrogen-bonding pattern, the slow-reacting enantiomer, usually S, has to place its large substituent into this stereospecificity pocket. A steric difference between the transition states of the enantiomers can therefore explain the enzyme's ability to resolve enantiomers. However, the model only considers differences in activation enthalpy and not entropy.

Enantioselectivity is the result of a difference in activation free energy between enantiomers, $\Delta_{R-S}\Delta G^\ddagger$, which is related to the enantiomeric ratio E as $-RT\ln E$. Furthermore, $\Delta_{R-S}\Delta G^\ddagger$ is related to the difference in activation enthalpy and entropy as $\Delta_{R-S}\Delta G^\ddagger = \Delta_{R-S}\Delta H^\ddagger - T\Delta_{R-S}\Delta S^\ddagger$. Therefore, the enthalpic and entropic components of the enantiomeric ratio E is determined by temperature studies and equated according to equation 1 (Philips 1992).

$$\ln E = -\frac{\Delta_{R-S}\Delta H^\ddagger}{R} \cdot \frac{1}{T} + \frac{\Delta_{R-S}\Delta S^\ddagger}{R} \quad (1)$$

E will vary with temperature and a racemic temperature, T_R , at which there is no enantioselectivity, $E = 1$ and $\Delta_{R-S}\Delta G^\ddagger = 0$, can be determined as $T_R = \Delta_{R-S}\Delta H^\ddagger / \Delta_{R-S}\Delta S^\ddagger$ (Philips 1992). In the case of the resolution of 3-methyl-2-butanol, as well as most other *sec*-alcohols resolved by



Scheme 1. Kinetic resolution of the enantiomers of 3-methyl-2-butanol catalyzed by *Candida antarctica* lipase B and its T103G, W104H, S47A, T42V, T42VS47A variants.

CALB, $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ are of the same sign, resulting in a real positive T_R (Overbeeke et al. 1999; J. Ottosson and K. Hult, unpubl.). The enantiomer favored by enthalpy is disfavored by entropy and vice versa. Below T_R , E will decrease with temperature and the entropic component will counteract the enthalpic yielding a lower enantioselectivity than expected from purely enthalpic considerations. On the other hand, above T_R , the enantiopreference will change to that favored by entropy and E will increase with temperature. Generally T_R for CALB-catalyzed resolutions of *sec*-alcohols is well above (>100 K) the experimental temperature.

Several researchers have shown that differential activation entropy significantly affects the enzyme-catalyzed resolution of enantiomers (Pham et al. 1989; Philips 1992, 1996; Galunsky et al. 1997; Sakai et al. 1997; Jönsson et al. 1999); therefore, to create a complete model of the mechanism of CALB enantioselectivity, entropy considerations must be included (Overbeeke et al. 1999; Ottosson and Hult 2001).

Results

Table 1 summarizes the results of the thermodynamic analysis of enantioselectivity in the resolution in Scheme 1 catalyzed by wild-type CALB and five variants with one or two point mutations. One of the variants, T103G, displayed an increase in enantioselectivity as compared to the wild type. The other variants had a somewhat reduced enantioselectivity and, in the case of W104H, it was the most pronounced, the enantiomeric ratio E dropped from 970 in wild type to only 150. All systems had T_R s significantly above the experimental temperature and hence, the entropic component counteracts the enthalpic component to E. The changes in E were due to changes in both the enthalpic ($\Delta_{R-S}\Delta H^\ddagger$) as well as the entropic ($T\Delta_{R-S}\Delta S^\ddagger$) components of the activation free energy difference between the enan-

tiomers ($\Delta_{R-S}\Delta G^\ddagger = -RT\ln E = \Delta_{R-S}\Delta H^\ddagger - T\Delta_{R-S}\Delta S^\ddagger$). It can be noted that although the W104H mutation caused an essential decrease in the enantiomeric ratio E, both $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ were significantly greater in absolute values than for the other variants and the wild-type enzyme. The entropic component increased in number to a greater extent than the enthalpic and E was decreased, as entropy is counterproductive to enantioselectivity below T_R . The T103G variant also displayed a significant increase in both the enthalpic and the entropic component absolute values. However, in this case the entropic component did not increase enough in number to cancel the increase of the enthalpic component, yielding a more enantioselective enzyme, with an increase in E from 970 to 2140. This shows that although enthalpy and entropy displayed somewhat compensatory changes, significant changes in E can be achieved from single point mutations.

The three remaining variants, T42V, S47A, and T42VS47A, all displayed a reduction in the enantiomeric ratio E. For T42V, this change was caused by a decrease in both $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ absolute values. The lowered E for S47A and T42VS47A was mainly caused by a decreased absolute value of $\Delta_{R-S}\Delta H^\ddagger$. T42V was the most enantioselective of these three variants in the studied temperature range, whereas S47A and T42VS47A were somewhat less enantioselective. The latter two displayed a practically identical temperature dependence of E. Focusing solely on E one may wrongfully draw the conclusion that the T42V mutation caused the smallest changes in the reaction system as it displayed the smallest change in E. However, this variant was the one of the three variants that gave the largest changes in the enthalpic and entropic components of E. On the other hand, focusing solely on the thermodynamic parameters $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$, one may conclude that S47A alone was responsible for the changes seen in T42VS47A, as it is very similar to S47A. Therefore, the effects were clearly not additive when more than one point mutation was introduced.

Table 1. Thermodynamic components of E for the kinetic resolution of 3-methyl-2-butanol catalyzed by *Candida antarctica* lipase B, CALB, and variants with point mutations

Enzyme	E (296 K)	$\Delta_{R-S}\Delta G^\ddagger$ (296 K) (kJ/mole)	$\Delta_{R-S}\Delta H^\ddagger$ (kJ/mole)	$\Delta_{R-S}\Delta S^\ddagger$ (J/mole, K)	$\Delta\Delta H^\ddagger$ (kJ/mole)	$T\Delta\Delta S^\ddagger$ (296 K) (kJ/mole)	T_R (K)
Wild type	970	-16.9	-20.8 ± 0.9	-13.2 ± 3.0	0	0	1570
T103G	2140	-18.9	-31.7 ± 0.1	-43.4 ± 0.4	-10.9	-8.9	730
W104H	150	-12.3	-44.7 ± 1.0	-109.5 ± 3.3	-23.8	-28.5	410
T42V	520	-15.4	-16.7 ± 0.9	-4.5 ± 2.9	+4.1	+2.6	3740
S47A	340	-14.3	-18.3 ± 0.7	-13.4 ± 2.4	+2.6	-0.1	1360
T42VS47A	390	-14.7	-18.1 ± 0.4	-11.5 ± 1.4	+2.8	+0.5	1570

$\Delta\Delta H^\ddagger$ and $\Delta\Delta S^\ddagger$ represents the change in $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ relative wild type. Errors are ± standard error of the linear regression of $\ln E$ versus $1/T$. E at 296 K was calculated from the linear relation.

Discussion

As previously shown, for substrate effects on CALB enantioselectivity, modifications of the protein affect both the enthalpic and the entropic components of the enantiomeric ratio E (Overbeeke et al. 1999; Ottooson and Hult 2001). This fact stresses the importance of entropy in enzyme catalysis, in general, and enantioselectivity, in particular. The observed changes in enthalpy and entropy displayed a compensatory character in that, if $\Delta_{R-S}\Delta H^\ddagger$ was large, so was $\Delta_{R-S}\Delta S^\ddagger$, and vice versa. However, they were not perfectly compensatory, allowing for modifications of the enzyme to improve E . Figure 2 shows the general energy profile diagrams of enthalpy and entropy for an enzyme-catalyzed kinetic resolution. Changes in the components of enantioselectivity, $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$, caused by mutations on the enzyme must be related to one or both enantiomers shifting their individual activation energy, $\Delta H^\ddagger$ and $\Delta S^\ddagger$, up or down relative to each other.

T103G variant of CALB

T103G is an example of an enzyme variant with an improved enantioselectivity. This mutation introduces the consensus sequence G-X-S-X-G around the active serine, found in most other lipases, into CALB (Patkar et al. 1997). The consensus sequence is positioned on what is usually referred to as the nucleophilic elbow, a sharp turn between an α -helix and a β -strand, in lipases. The catalytic serine is positioned at the very tip of this elbow (Ollis et al. 1992). This mutation, T103G, has been shown to give a significant increase in thermostability (Patkar et al. 1997). They suggested that the increased thermostability is caused by an increase in the number of interactions between the helix and the sheet, either through a structural adaption or additional waters in the empty space formed by the mutation. The

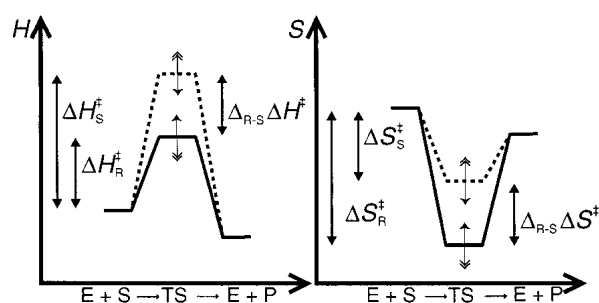


Fig. 2. Enthalpy and entropy profile diagrams of the enantioselective reaction catalyzed by *Candida antarctica* lipase B. Dashed lines represent the slow-reacting enantiomer and solid lines the fast-reacting enantiomer, generally S and R, respectively. The double-headed arrows display possible changes to obtain the increase in $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ caused by the T103G and W104H mutations. The decrease in the thermodynamic parameters as caused by the T42V, S47A, and T42VS47A mutations are shown with single-headed arrows.

increased enantioselectivity observed in the present investigation must be caused by structural changes in the active site that either enthalpically favors and entropically disfavors the R-enantiomer transition state, vice versa for the S-enantiomer transition state, or a combination to increase the absolute numbers of $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ (see energy profile in Fig. 2). When comparing the average structure of 100-ps long molecular dynamics runs of the T103G variant to the wild type with the tetrahedral intermediate enzyme–substrate structure as a model of the transition state, no significant structural changes were seen. However, there was a tendency for the angle of the nucleophilic elbow to decrease in T103G compared with wild type, causing structural changes in the innermost parts of the active site. Such a change could minimize the stereospecificity pocket and restrict the large substituent of the S-enantiomer, causing an increase in $\Delta H^\ddagger_S$.

W104H variant of CALB

The W104H mutation also caused the absolute values of the enthalpic $\Delta_{R-S}\Delta H^\ddagger$ and the entropic $\Delta_{R-S}\Delta S^\ddagger$ components of enantioselectivity to increase. As a matter of fact $\Delta_{R-S}\Delta S^\ddagger$ for this variant was more than eight times the value of the wild type and $\Delta_{R-S}\Delta H^\ddagger$ was more than doubled. In this case the entropic component, which is counterproductive for enantioselectivity, more than canceled the increase in the enthalpic component, causing, in contrast to T103G, a significant decrease in E . The residue W104 is positioned deep in the active site and makes up part of the boundary of the alcohol-binding site and its stereospecificity pocket (Fig. 1). The mutation of a tryptophan to a histidine liberates a volume of the active site in close contact with the chiral alcohol. Whether this causes structural changes or leaves an empty space within the enzyme remains unknown until the structure is solved. The fact that $\Delta_{R-S}\Delta H^\ddagger$ significantly increased for W104H would result in an erroneous prediction of a very high enantioselectivity if only enthalpic energy considerations were made, as is done in most contemporary molecular modeling of enzyme catalysis where the entropic components generally are neglected (Hæffner et al. 1998; Ke et al. 1998; Orrenius et al. 1998a,b). Attempts to calculate activation entropy in enzyme catalysis with computer modeling have recently appeared in literature (Strajbl et al. 2000; Villa et al. 2000).

T42V, S47A, and T42VS47A

Amino acid residues 42 and 47 as well as 104 constitute part of the boundaries of the active alcohol-binding site. In a successful attempt to rationally design an enzyme enantioselective toward halohydrins these residues were changed by site-directed mutagenesis to T42V, S47A, and T42VS47A (Rotticci et al. 2001). These residues were chosen as they

constitute part of the stereoselectivity pocket in the alcohol-binding site (Fig. 1). By exchanging the hydroxyl groups for a methyl group, T42V, or hydrogen, S47A, the enantioselectivity of *sec*-alcohols containing halogens on the medium substituent was hypothesized to increase due to the removal of the repulsion between the halogen and the hydroxyls. Table 2 shows how the enantiomeric ratio for 1-chloro-2-octanol and 1-bromo-2-octanol increased for the enzyme variants containing the S47A mutation. E has also decreased for the resolution of 3-methyl-2-butanol catalyzed by the same variants. This is in agreement with the model, as the variants were designed to improve E for compounds with halogenated medium substituent and as a side effect, lower E for those with alkyl substituent. However, on closer inspection of the thermodynamic components of the resolution of 3-methyl-2-butanol (Table 1) one notes that the largest changes in $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$ are found for the T42V variant, the mutation that from inspection of E only seems to yield the smallest or no effect. Although the design of the S47A and T42VS47A variants was based on a model including only enthalpic and no entropic considerations, these variants were successful in improving the enantioselectivity toward the halohydrins, as seen in Table 2. However, the results from the resolution of 3-methyl-2-butanol show that the largest changes in activation enthalpy and entropy were found for the T42V mutation. This underlines the importance of not drawing premature conclusions from an enzyme model that excludes considerations of entropy.

Conclusion

The findings in the present investigation show that variations, even minor ones, in the enzyme affect both the activation enthalpy and the activation entropy of the reaction. Enzyme models that neglect contributions by entropy will always be incomplete. In the case of enantioselectivity, the entropic contribution $\Delta_{R-S}\Delta S^\ddagger$ is just as significant to

selectivity as the enthalpic contribution $\Delta_{R-S}\Delta H^\ddagger$. We see that changes in the enzyme yield changes in $\Delta_{R-S}\Delta H^\ddagger$ and $\Delta_{R-S}\Delta S^\ddagger$, which are partly compensatory in nature. Because this compensation is not perfect it allows modifications on the protein to optimize enantioselectivity. Improvement of enzyme enantioselectivity is therefore possible through rational design, although it requires enzyme catalysis models that also consider entropy. This calls for more research to elucidate the role of entropy in enzyme catalysis.

Materials and methods

Site-directed mutagenesis

The T42V, S47A, and T42VS47A variants were obtained by using the overlap-PCR technique (Ho et al. 1989). The following primers were used to insert the desired mutations: fCALBT42V, 5'-GGAACCGCGTCACAGGTCC; rCALBT42V, 5'-GGACCTGTGACGCCGGTTCC; fCALBS47A, 5'-TCCACAGGCTTTCGACTCGAA; and rCALBS47A, 5'-GTTTCGAGTCGAAAGCCTGTGG. The CALB gene was inserted in the plasmid YpDC541 using *Xho*I and *Not*I restriction sites, placing the lipase gene directly after the α -factor secretion signal sequence, as previously described by Rotticci-Mulder et al. (2001). Concerning the W104H variant, the Transformer site-directed mutagenesis kit was used as described by the manufacturer (Clontech Laboratories Inc.). The plasmid YpCALB (Rotticci-Mulder et al. 2001) was mutagenized using the mutagenic primer 5'-GCTTCCCCGTGCTTACCCACTCCCAGGGTGG and the selection primer 5'-GCTGTTCCAGGGATCGCAGTGGTGAG. The selection primer allowed a silent mutation in the plasmid that removed the unique restriction-site *Sma*I. All different CALB variants were sequenced on both strands using Sanger sequencing reactions with dye terminators (Sanger et al. 1977) and were analyzed on an ABI Prism 377 DNA sequencer (Perkin-Elmer, Wellesley, MA). Plasmids containing the desired mutated lipase gene were transformed by electroporation into the methylotrophic yeast *Pichia pastoris*. One lipase-expressing transformant of each mutant was selected for further cultivation.

Protein production and purification

Wild-type lipase and its variants were produced in a 3-L fermentor according to instructions given by the manufacturer (Invitrogen, Carlsbad, CA). The cultivation medium had a volume of 1 L at the beginning of the fermentation. Using the fermentor the expression levels, measured after the protein purification, increased ~20 times as compared to production in shake flasks. The T42VS47A variant was produced in shake flasks. The lipase variants were purified by hydrophobic interaction chromatography and gel filtration (Rotticci-Mulder et al. 2001). Fermentor cultivation not only increased the amount of desired protein, but also drastically decreased the amount of alcohol oxidase that was released into the medium, making the gel filtration step unnecessary.

Kinetic resolution of 3-methyl-2-butanol in hexane

The enzymes, immobilized on Accurel MP (Membrana GMBH, Obernburg, Germany), were equilibrated in the reaction vessels to a water activity of 0.11 (LiCl, sat. aq.) for more than 10 h. Dried

Table 2. Enantioselectivity, *E*, of *Candida antarctica* lipase B and variants

Enzyme	1-bromo-2-octanol ^a (296 K)	1-chloro-2-octanol ^a (296 K)	3-methyl-2-butanol ^b (296 K)
Wild type	6.5 ± 0.4	14 ± 2	970
W104H	1.7 ± 0.1	2 ± 0.1	150
T42V	6.7 ± 0.3	15 ± 2	520
S47A	12.4 ± 0.4	28 ± 3	340
T42VS47A	9.4 ± 0.8	20 ± 2	390

^a Transesterified with vinyl butyrate.

^b Transesterified with vinyl octanoate.

Errors are ± one standard deviation. *E* for 3-methyl-2-butanol is calculated from the linear relation of ln*E* versus 1/*T*.

(molecular sieves) 3-methyl-2-butanol (0.43 M) and hexane were added to the reaction vessel and equilibrated in temperature. A low enzyme load and high substrate concentrations were chosen to avoid the mass transport limitations on E, as pointed out by Rotticci et al. (2000). The reaction was started by the addition of vinyl octanoate (0.43 M). Samples were removed with a syringe at regular intervals for conversions of the substrates $\leq 50\%$.

Determination of enantioselectivity

The samples were analyzed for the enantiomeric excess of the remaining substrate 3-methyl-2-butanol, ee_s , and the produced octanoate ester, ee_p , with chiral capillary GC. The column used was Chirasil-Dex CB column from Chrompack, the Netherlands. The enantiomeric ratio E was calculated as an average of 5–12 samples at conversions 0–50% according to Rakels et al. (1993) from the enantiomeric excess of both substrate ee_s and product ee_p .

Molecular modeling

Molecular dynamic calculations were performed as previously published by Raza et al. (2001).

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