Creating Space for Large Secondary Alcohols by Rational Redesign of *Candida antarctica* Lipase B

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The active site of Candida antarctica *lipase B (CALB) hosts the catalytic triad (Ser-His-Asp), an oxyanion hole and a stereospecificity pocket. During catalysis, the fast-reacting enantiomer of secondary alcohols places its medium-sized substituent in the stereospecificity pocket and its large substituent towards the active-site entrance. The largest group to fit comfortably in the stereospecificity pocket is ethyl, and this restricts the number of secondary alcohols that are good substrates for CALB. In order to overcome this limitation, the size of the stereospecificity pocket was redesigned by changing Trp104. The substrate specificity of the Trp104Ala mutant compared to that of the wild-type lipase increased 270 times towards heptan-4-ol and 5500 times towards nonan-5-ol; this resulted in the high specificity constants 1100 and 830 $s^{-1}\,M^{-1}$, respectively. The substrate selectivity changed over 400 000 times for nonan-5-ol over propan-2-ol with both Trp104Ala and the Trp104Gln mutations.*

Introduction

Candida antarctica was selected as an interesting lipase producer in a microbial screen performed in the late 1980s, and the B lipase (CALB) was found to have interesting properties.[1] Because of its high activity, stability and selectivity in both aqueous and organic solutions, CALB has been well studied and used in numerous applications.[2] CALB was first crystallized in 1994 and was found to belong to the α/β-hydrolase family.[3,4] The active site hosts the catalytically important oxyanion hole, which stabilizes the transition state, and a catalytic triad consisting of Ser105, His224 and Asp187.[3] CALB follows the same reaction mechanism as serine hydrolases, and, during a typical reaction, an ester is bound in the acyl- and alcohol-binding sites.[5] The acyl-binding site does not confer high substrate selectivity on the enzyme. On the other hand, the alcohol-binding part, which contains the stereospecificity pocket, gives CALB high substrate selectivity towards secondary alcohols.[5] For enantiomers, the selectivity follows the rule postulated by Kazlauskas et al.[6] The fast-reacting enantiomer positions its medium-sized substituent in the stereospecificity pocket, while it orients the large substituent towards the entrance of the active site. The slow-reacting enantiomer orients its medium-sized substituent towards the entrance of the active site and its large substituent into the stereospecificity pocket. Although the large substituent is not easily fitted into the stereospecificity pocket, this binding mode allows the formation of the hydrogen bonds essential for catalysis. Other binding modes do not allow for all the essential hydrogen bonds to be formed in the transition state, and do therefore not contribute to catalysis.[7] The stereospecificity pocket (defined by Thr42, Ser47 and Trp104) is only large enough to comfortably accommodate an ethyl or a smaller substituent (Figure 1 A). This leads to a very good enantioselectivity for CALB towards secondary alcohols with a medium group equal to an ethyl or smaller and a large group bigger than an ethyl. On the other hand, the utility of CALB for larger secondary alcohols as substrates is constrained by the limited size of the stereospecificity pocket. A drastic drop in both activity and selectivity is seen when both substituents are larger than an ethyl.[8]

In order to increase the range of secondary alcohols that are good substrates for CALB, rational redesign was used to increase the size of the stereospecificity pocket of the lipase. Mutation of Trp104, which forms the bottom of the stereospecificity pocket, to a smaller amino acid, as suggested by Uppenberg et al., should open up the pocket and allow for larger substituents than an ethyl group to be comfortably accommodated there.[5] To test this hypothesis, six lipase variants with mutations in the stereospecificity pocket were produced, three variants with mutation of Trp104 and three variants with nearly isosteric mutations elsewhere in the stereospecificity pocket. To map the size of the stereospecificity pocket of the mutants and of the wild-type lipase, substrate specificities were determined for the acylation of a series of symmetrical secondary alcohols. With those alcohols, the size of the substituent bound in the stereospecificity pocket was well defined.

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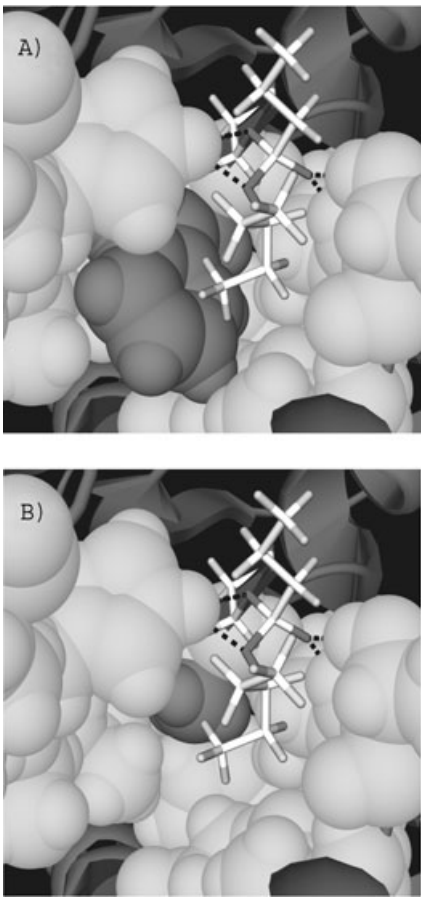

Figure 1. The active site in A) the wild-type lipase and B) the Trp104Ala mutant. The reaction intermediate is shown with the catalytic serine covalently attached to the carbonyl carbon of 3-pentyl butanoate (stick model). Hydrogen bonds essential for catalysis from the catalytic histidine and in the oxyanion hole are shown as dashed lines. Amino acid 104 (Trp in the wild-type and Ala in the mutant) is in dark grey and space-filled in the centre of the pictures. One ethyl group on the alcohol part of the substrate points into the stereospecificity pocket and the other towards the active-site entrance. The ethyl group in the stereospecificity pocket of the wild-type lipase (A) seems to reach the end of this pocket, while, in the redesigned Trp104Ala mutant (B), there is enough space for larger groups to be oriented into the stereospecificity pocket.

Results

Six variants of lipase B from *Candida antarctica* (CALB) with point mutations in the stereospecificity pocket were created (Thr42Val, Ser47Ala, Thr42Val/Ser47Ala, Trp104His, Trp104Gln and Trp104Ala). The mutants and the wild-type enzymes were expressed, purified and immobilized.

The immobilized lipase variants were characterized for acyltransfer reactions by using vinyl butanoate as the acyl donor and symmetrical achiral secondary alcohols as acyl acceptors. Initial reaction rates (v) were determined within 5% conversion, with the alcohols competing as acyl acceptors at known concentrations. From the initial reaction rates and the known substrate concentrations, the relative specificity constants $(k_{cat}/K_M)_{S1}/(k_{cat}/K_M)_{S2}$ can be calculated according to Equation (1), where S_1 and S_2 are any of the acyl acceptors. For the alcohols to be compared, the initial reaction rates have to be determined during the same time course.[9]

$$\frac{(k_{cat}/K_M)_{S1}}{(k_{cat}/K_M)_{S2}} = \frac{v_{S1}}{v_{S2}} \times \frac{[S_2]}{[S_1]} \quad (1)$$

In order to map the size of the stereospecificity pocket in the wild-type enzyme and the Trp104 mutants (Trp104His, Trp104Gln and Trp104Ala), relative specificity constants for the acylation of a series of symmetrical secondary alcohols (propan-2-ol, pentan-3-ol, heptan-4-ol, nonan-5-ol and cyclohexanol) were determined. For the mutants this was done with all alcohols in the same incubation; however, for the wild-type lipase, three separate incubations had to be performed due to the large differences in reaction rates. For each variant, the relative specificity constants were calculated compared to heptan-4-ol (Table 1).

Table 1. Relative specificity constants of the CALB wild-type and Trp104 mutants for the acylation of symmetrical secondary alcohols compared to heptan-4-ol.

CALB variant	Acyl acceptor				
	propan-2-ol	pentan-3-ol	heptan-4-ol	nonan-5-ol	cyclohexanol
wild-type	12000	100	1.0	0.036	200
Trp104His	2.9	0.36	1.0	0.12	0.23
Trp104Gln	0.20	0.10	1.0	0.26	0.088
Trp104Ala	0.54	0.067	1.0	0.74	0.079

The wild-type enzyme had a strong preference for propan-2-ol, with a more than 300000-fold decrease in specificity when the size of the alcohol was increased. On the other hand, the Trp104Gln and Trp104Ala mutants preferred heptan-4-ol and nonan-5-ol over propan-2-ol.

Relative specificity constants were also determined for the variants with nearly isosteric mutations in the stereospecificity pocket (Thr42Val, Ser47Ala and Thr42Val/Ser47Ala) by using propan-2-ol, pentan-3-ol and heptan-4-ol as acyl acceptors. Like the wild-type enzyme, these mutants showed such a diverse substrate selectivity that the relative specificity constants had to be determined in two separate incubations for each variant. The relative specificity constants were calculated compared to heptan-4-ol according to Equation (1) and are presented in Table 2. The substrate selectivity of these mutants was very similar to that of the wild-type enzyme. Thus the

Table 2. Relative specificity constants of the CALB wild-type and mutants with nearly isosteric mutations for the acylation of symmetrical secondary alcohols compared to heptan-4-ol.

CALB variant	Acyl acceptor		
	propan-2-ol	pentan-3-ol	heptan-4-ol
Wild-type	12000	110	1.0
Thr42Val	12000	100	1.0
Ser47Ala	12000	110	1.0
Thr42Val/Ser47Ala	10000	110	1.0

change in hydrophobicity of the stereospecificity pocket caused by the mutations did not result in different substrate selectivities.

To better understand how the mutations of Trp104 changed the selectivity, the apparent kinetic constants (k_{cat} and K_M) for the acylation of heptan-4-ol were determined. This alcohol was chosen as the best candidate to see the changes for the mutants without losing too much of the activity of the wild-type lipase. To determine the kinetic constants, the active-site concentration of each variant has to be known. Active-site titration was performed on the immobilized Trp104 variants and the wild-type enzyme in organic solvent by using the irreversible active-site inhibitor methyl 4-methylumbelliferyl hexylphosphonate (Table 3).

Table 3. Active-site concentrations of the immobilized CALB wild-type and Trp104 mutants.

CALB variant	Active-site concentration [nmol per g of carrier]
wild-type	230
Trp104His	1.4
Trp104Gln	5.7
Trp104Ala	3.4

The apparent k_{cat} and K_M values for the acylation of heptan-4-ol were determined, at a vinyl butanoate concentration of 500 mM, for the CALB wild-type and Trp104 mutants (Table 4).

Table 4. Apparent kinetic constants of the CALB wild-type and Trp104 mutants for the acylation of heptan-4-ol at 500 mM vinyl butanoate.

CALB variant	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [$s^{-1}\,M^{-1}$]
wild-type	0.53	130	4.2
Trp104His	12	21	550
Trp104Gln	10	18	560
Trp104Ala	28	24	1100

From the data it is clear, that the different point mutations of Trp104 have a large effect on the specificity constant (k_{cat}/K_M) towards heptan-4-ol, with an increase of 130 to 270 times compared to the wild-type lipase. From the determination of each component (k_{cat} and K_M) it is clear that the largest effect of the mutations of Trp104 was on the catalytic constant (k_{cat}). However, the increased size of the stereospecificity pocket also improved the Michaelis constant (K_M) for the bulky substrate heptan-4-ol.

Discussion

Candida antarctica lipase B (CALB) displays a very high enantioselectivity towards most secondary alcohols when one of the substituents is an ethyl group or smaller and the other is a propyl group or larger. The smaller of the substituents is believed to be placed in the stereospecificity pocket, which is only large enough to comfortably accommodate an ethyl group.[8] This specificity is also a limit for the number of secondary alcohols that are good substrates for CALB. When both substituents of a secondary alcohol are larger than an ethyl, there is a drastic decrease in both activity and selectivity. In order to increase the number of secondary alcohols being efficiently acylated by CALB, the size of the stereospecificity pocket was increased. The large amino acid (tryptophan) at position 104 forms the bottom of the stereospecificity pocket (Figure 1 A). Site-specific mutagenesis at this position was the strategy chosen to increase the size of the stereospecificity pocket. Thr104 was replaced by a histidine, found in most lipases at this position, and as suggested by Uppenberg et al.[5] The largest redesigned stereospecificity pocket was created by a mutation to alanine (Figure 1 B). The alteration to a glycine, which would lead to an even larger redesigned stereospecificity pocket, was discarded due to the increased risk of protein misfolding commonly associated with such a mutation. In the wild-type lipase, the side chain of Trp104 forms a hydrogen bond to the backbone of the catalytic histidine.[5] A glutamine mutant was therefore constructed since it also offers the possibility of forming a hydrogen bond from its side chain to the backbone of the catalytic histidine. Changing other amino acids lining the stereospecificity pocket could lead to only a small increase of the pocket size. The three Trp104 mutants of CALB (Trp104His, Trp104Gln and Trp104Ala) were designed to have progressively increasing sizes of the stereospecificity pocket compared to the wild-type lipase. In addition, three mutants with a stereospecificity pocket that was less polar but of almost the same size were studied.

In order to map the size of the stereospecificity pocket of the Trp104 mutants and the wild-type lipase, they were characterized for the acylation of secondary alcohols. In order to react, secondary alcohols have to bind in the active site by placing one substituent in the stereospecificity pocket and the other substituent towards the entrance of the active site (Figure 1). As the size of the substituent of an asymmetric secondary alcohol bound in the stereospecificity pocket would be ambiguous, symmetrical secondary alcohols were used as substrates for the characterization. Comparison of the reaction rates of a series of symmetrical secondary alcohols gives an estimate of the size of the substituent that can be comfortably accommodated in the redesigned stereospecificity pocket of the mutants.

In Table 1 it can be seen that the Trp104 mutants had very different substrate selectivities from that of the wild-type lipase. The wild-type lipase had a selectivity 300 000 times larger for propan-2-ol than for nonan-5-ol. In contrast, the redesigned Trp104 mutants had similar specificities for all the alcohols tested. The largest difference in substrate selectivity for the mutants was as low as 24-fold, obtained with the Trp104His variant for propan-2-ol over nonan-5-ol. The mutants with the smaller amino acids at position 104 (Trp104Gln and Trp104Ala) differed even more compared to the wild-type lipase, showing the highest preference for heptan-4-ol followed by nonan-5-ol.

The three variants with nearly isosteric mutations have a more hydrophobic stereospecificity pocket than the wild-type lipase, while the sizes of the stereospecificity pocket should be very similar and not affect the substrate selectivity. In spite of the differences in hydrophobicity, these variants had very similar selectivities between the tested alcohols (Table 2). Thus, the hydrophobicity of the stereospecificity pocket did not have a large influence on the selectivity between these symmetrical secondary alcohols.

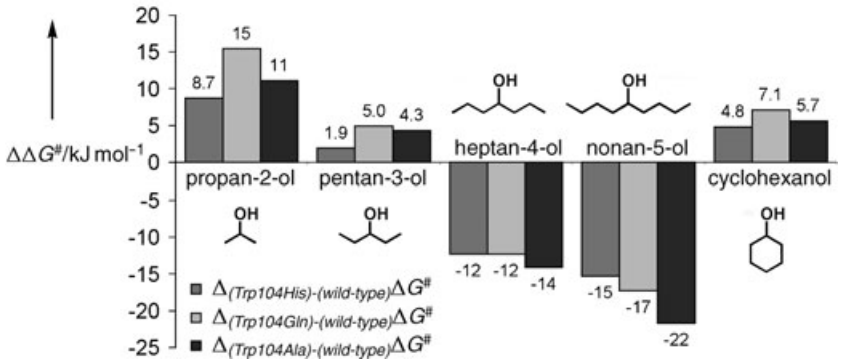

Figure 2. Differences in activation free energy for the acylation of symmetrical secondary alcohols between the Trp104 mutants and the wild-type lipase.

The apparent kinetic constants (k_{cat} and K_M) determined for the acylation of heptan-4-ol (Table 4) show that the mutation of Trp104 decreased the Michaelis–Menten constant (K_M) 5–7 times. An improvement in K_M is in agreement with the rational redesign of the lipase, the aim of which was to increase the size of the stereospecificity pocket. Interestingly, the improvement in the catalytic constant (k_{cat}) was much larger, 19–52 times. This could be explained by a higher ratio of productive binding, that is, the Trp104 mutants allowed heptan-4-ol to bind in an orientation that facilitates catalysis to a greater extent than the wild-type lipase.

The relative specificity constants compared to heptan-4-ol (Table 1) were multiplied by the specificity constant determined for each variant towards heptan-4-ol (Table 4). This gave the specificity constants for the CALB wild-type and Trp104 mutants for the acylation of each of the symmetrical secondary alcohol tested (Table 5).

Table 5. Specificity constants of the CALB wild-type and Trp104 mutants for the acylation of symmetrical secondary alcohols.

CALB variant	k_{cat}/K_M [$s^{-1}\,M^{-1}$] of acyl acceptor				
	propan-2-ol	pentan-3-ol	heptan-4-ol	nonan-5-ol	cyclohexanol
wild-type	50000	420	4.2	0.15	840
Trp104His	1600	200	550	67	130
Trp104Gln	110	59	560	150	49
Trp104Ala	610	76	1100	830	89

The mutants and the wild-type lipase can be compared by calculating the difference in activation free energy ($\Delta_{(mutant)-(wild\text{-}type)}\Delta G^{\#}$) according to Equation 2, where R is the gas constant and T is the absolute temperature.

$$\Delta_{(mutant)-(wild\text{-}type)}\Delta G^{\#} = -RT \times \ln \frac{(k_{cat}/K_M)_{mutant}}{(k_{cat}/K_M)_{wild\text{-}type}} \qquad (2)$$

The differences in activation free energy for the acylation of the symmetrical secondary alcohols between the Trp104 mutants and the wild-type lipase ($\Delta_{(mutant)-(wild\text{-}type)}\Delta G^{\#}$) are presented in Figure 2.

The rational redesign of CALB, changing Trp104 for a smaller amino acid, proved to be a successful approach to making lipase variants that accepted larger groups in the stereospecificity pocket. The mutants had an activation free energy towards heptan-4-ol that was 12–14 kJ mol^{-1} lower than that of the wild-type lipase. This shows that the redesigned stereospecificity pockets could accommodate a propyl group much better than the wild-type lipase could, and that the Trp104 mutants were similar. The difference in activation free energy for the acylation of nonan-5-ol of the mutants compared to the wild-type lipase was even larger, 15–22 kJ mol^{-1}. There was a larger difference between the Trp104 mutants for the acylation of nonan-5-ol than heptan-4-ol. Thus, it was ever more beneficial to have a smaller amino acid at position 104, the larger the symmetrical secondary alcohol was. The stereospecificity pocket of the wild-type lipase can fit an ethyl group well,[8] while the enlarged stereospecificity pockets of the Trp104His and Trp104Gln variants can fit a propyl group, and the even larger stereospecificity pocket of the Trp104Ala mutant can readily fit a butyl group. For the acylation of heptan-4-ol and nonan-5-ol, the activation free energy decreased for the best mutant (Trp104Ala) compared to that of the wild-type lipase with 14 and 22 kJ mol^{-1}, respectively. This corresponds to 270 and 5500 times increases of the specificity constants, making Trp104Ala a very good catalyst with specificity constants of 1100 $M^{-1}s^{-1}$ and 830 $M^{-1}s^{-1}$ towards heptan-4-ol and nonan-5-ol, respectively. The substrate selectivity changed over 400000 times and switched for nonan-5-ol over propan-2-ol for both the Trp104Ala and the Trp104Gln mutants, compared to the wild-type lipase.

Experimental Section

Chemicals: Cyclohexane and propan-2-ol were purchased from Labscan (Dublin, Ireland); pentan-3-ol, nonan-5-ol, cyclohexanol, dodecane and vinyl butanoate were purchased from Fluka Chemika (Buchs, Switzerland); heptan-4-ol was from TCI, Tokyo Kasei Kogyo (Tokyo, Japan); 4-methylumbelliferone was from Aldrich;

Tris-HCl and calcium chloride were purchased from Merck KGaA (Darmstadt, Germany). All chemicals were used without further purification.

Site-directed mutagenesis, protein production and purification, and lipase immobilization

Variants with nearly isosteric mutations: The Thr42Val, Ser47Ala and Thr42Val/Ser47Ala mutants and the wild-type lipase were created, and the proteins were produced, purified and immobilized as described previously.[10]

Variants with redesigned size of the stereospecificity pocket (Trp104 mutants): These variants were prepared essentially as the variants with nearly isosteric mutations. The Trp104Gln and Trp104Ala mutations were introduced by using the overlap extension polymerase chain-reaction technique,[11] with the forward primers fTrp104Ala 5′-CCGTGCTTACCGCTTCCCAGGGTG and fTrp104Gln 5′-TCCCGTGCTTACCCAATCCCAGGG together with their complementary reverse primers (Thermo, Ulm, Germany). Mutated genes were introduced in the plasmid pPIC9 (Invitrogen, Carlsbad CA, USA) by using the *Xho*I and *Not*I restriction sites. The mutations were confirmed by DNA sequencing (ABI 3700 sequencer, Applied Biosystems, Foster City, CA, USA) with the BigDye terminators RRMIX (Applied Biosystems). The plasmids carrying the correct mutations were introduced into the *Pichia pastoris* strain SMD1168 (Invitrogen) by electroporation. Yeast colonies secreting the lipase variants were selected in a colony blotting assay by using CALB specific antibodies. The Trp104His mutant was constructed and transformed to *P. pastoris* strain SMD1168 as described earlier.[10]

The wild-type enzyme and the Trp104-mutants were expressed by cultivations of *P. pastoris* essentially according to the instructions from the supplier (Invitrogen). The production of the Trp104His mutant was performed in a fermentor (Belach, Sweden), and the other variants were produced in shake-flask cultivations.

Purification of the Trp104-mutants and the wild-type enzyme was done by hydrophobic interaction chromatography, as described previously.[12]

The lipase variants were immobilized on polypropylene beads from aqueous solutions (20 mM 3-(*N*-morpholino)propanesulfonic acid–NaOH, pH 7.5).[13] From the protein concentrations determined by absorbance (280 nm), 3% (*w*/*w*) of the wild-type lipase, the Trp104Gln and the Trp104Ala variants were immobilized on Accurel MP1000 (<1500 μm bead diameter), and 0.5% (*w*/*w*) of the Trp104His mutant was immobilized on Accurel EP100 (350–1000 μm bead diameter).

Active-site titration: The active-site inhibitor methyl 4-methylumbelliferyl hexylphosphonate was synthesized according to the procedure described previously by our group,[14] by using the fluorophore 4-methylumbelliferone (4MU) in place of the chromophore *para*-nitrophenol.[15] Preparative TLC was used as the purification method. The purified inhibitor was dissolved in acetonitrile (9 mM) and stored in a freezer (−20 °C).

The inhibitor stock was diluted 3000 times with acetonitrile and added to the dry immobilized lipase. The inhibition reaction was allowed to continue until the concentration of the leaving group 4MU was stable (7 days). An aliquot of the inhibition reaction mixture (50 μL) was diluted with Tris-HCl buffer (450 μL, 100 mM, pH 8.0, 1 mM $CaCl_2$), and the concentration of the released 4MU was determined by measuring the fluorescence intensity on a Perkin–Elmer LS50B instrument (excitation 360 nm, emission 445 nm). No significant background hydrolysis of the inhibitor was detected. Four different amounts of each immobilized enzyme variant were inhibited. The amount of released 4MU had a linear correlation to the amount of carrier, loaded with enzyme, added to the inhibition reactions.

Activity measurements: The immobilized enzyme variants were equilibrated to a water activity of 0.1 against saturated aqueous lithium chloride. The solvent, internal standard and all substrates were dried over molecular sieves (3 Å). All reactions were performed with cyclohexane as solvent, dodecane as internal standard, vinyl butanoate as acyl donor and symmetrical secondary alcohols as acyl acceptors. The reactants were mixed by using a magnetic stirrer and kept at 30 °C with a water bath. The reactions were started by the addition of vinyl butanoate.

Relative reaction rates for the wild-type lipase and the Trp104 variants were determined for the acylation of propan-2-ol, pentan-3-ol, heptan-4-ol, nonan-5-ol and cyclohexanol by using vinyl butanoate (250 mM) as acyl donor. The alcohol concentrations (5–250 mM) were chosen so that the initial reaction rates could be determined within the same time interval for the competing alcohols and within 5% conversion. For the wild-type lipase, there were large differences in the reaction rates, and the alcohols had to be divided into three separate incubations (propan-2-ol + pentan-3-ol, pentan-3-ol + heptan-4-ol + cyclohexanol and heptan-4-ol + nonan-5-ol).

Apparent kinetic constants were determined for the wild-type lipase and the Trp104 variants towards heptan-4-ol. To determine the apparent kinetic constants the reaction rate was determined at five or more different heptan-4-ol concentrations (5 to 400 mM with the wild-type lipase and 5 to 80 mM with the Trp104 mutants). The vinyl butanoate concentration (500 mM) was the same in all incubations.

Relative reaction rates with the wild-type lipase and the variants with the nearly isosteric mutations were determined in two separate incubations for each variant, first comparing propan-2-ol (0.10 M) and pentan-3-ol (1.0 M) and then comparing pentan-3-ol (0.10 M) and heptan-4-ol (1.0 M). The vinyl butanoate concentration (2.0 M) was not varied.

Analysis: Samples were taken at at least five reaction times in order to determine the initial reaction rates. The samples were analyzed by using the gas chromatograph HP 5890 Series II (Hewlett–Packard, Palo Alto, CA, USA) with the capillary column CP-Sil 5CB (length 25 m, i.d. 0.32 mm, Chrompack, the Netherlands) connected to a flame ionization detector.

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