Blocking the tunnel: engineering of Candida rugosa lipase mutants with short chain length specificity

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Abstract

The molecular basis of chain length specificity of Candida rugosa lipase 1 was investigated by molecular modelling and site-directed mutagenesis. The synthetic lip1 gene and the lipase mutants were expressed in Pichia pastoris and assayed for their chain length specificity in single substrate assays using triglycerides as well as in a competitive substrate assay using a randomized oil. Mutation of amino acids at different locations inside the tunnel (P246F, L413F, L410W, L410F/S300E, L410F/S365L) resulted in mutants with a different chain length specificity. Mutants P246F and L413F have a strong preference for short chain lengths whereas substrates longer than C10 are hardly hydrolyzed. Increasing the bulkiness of the amino acid at position 410 led to mutants that show a strong discrimination of chain lengths longer than C14. The results obtained can be explained by a simple mechanical model: the activity for a fatty acid sharply decreases as it becomes long enough to reach the mutated site. In contrast, a mutation at the entrance of the tunnel (L304F) has a strong impact on C4 and C6 substrates. This mutant is nevertheless capable to hydrolyze chain lengths longer than C8.

Introduction

Lipase from Candida rugosa (formerly Candida cylindracea, CRL) is a versatile biocatalyst which catalyzes hydrolysis, alcoholysis, esterification and transesterification of triacylglyerols and other hydrophobic esters. It is widely applied in a variety of biotechnological applications as diverse as production of carbohydrate esters of fatty acids, stereoselective synthesis of pharmaceuticals and a multitude of applications in food and flavour making (reviewed in Pandey et al., 1999).

Candida rugosa expresses a mixture of lipase isoforms which differ in substrate specificities (Shaw and Chang, 1989, Rua et al., 1993, Chang et al., 1994) . The genes of five lipases have been cloned and sequenced, consisting of 534 amino acids and an apparent MW of 60 kDa (Lotti et al., 1994). The major product of C. rugosa is encoded by the LIP1 gene. To get access to one pure isoform, LIP1 has been successfully expressed in Saccharomyces cerevisiae (Lotti et al., 1994) and, as synthetic gene, in Pichia pastoris (Brocca et al., 1998).

Like other microbial lipases, CRL preferentially hydrolyzes triglycerides in sn-1/3 position. Only few lipases including CRL also hydrolyze to a minor extent in sn-2 (Rogalska et al., 1993). CRL has a broad chain length profile ranging from short chain esters to long, saturated and even polyunsaturated fatty acids (Osada et al., 1990) with a distinct preference for fatty acids of chain lengths C4, C8, C10 and C12 (Janssen and Halling, 1994). Since CRL is an unspecific lipase towards a broad range of fatty acid chain lengths but has low activity towards long, polyunsaturated fatty acids, it has been used for the enrichment of these valuable food ingredients. CRL has been used for the enrichment of eicopentaenoic and docosahexaenoic acids from fish oil (McNeill et al., 1996, Moore and McNeill, 1996) or of gamma-linolenic acid from borage oil (Shimada et al., 1998, Schmitt-Rozieres et al., 1999). To understand the structural basis of substrate specificity, the structure of complexes of CRL with substrate-analogous covalent inhibitors was studied by X-ray crystallography (Cygler et

al., 1994). Like other microbial lipases, CRL is a member of the α/β hydrolase fold family (Ollis et al., 1992). The α/β hydrolase fold consists of a central hydrophobic eight-stranded β-sheet packed between two layers of amphiphilic α-helices. α/β hydrolases have a common catalytic mechanism of ester hydrolysis which consists of five subsequent steps (Cygler et al., 1994): the ester substrate binds to two well defined binding sites for the scissile fatty acid and the alcohol moiety; then, a first tetrahedral intermediate is formed by nucleophilic attack of the catalytic serine with the oxyanion stabilized by two or three hydrogen bonds, the so-called oxyanion hole. The ester bond is cleaved and the alcohol moiety leaves the enzyme. In a last step the acyl enzyme is hydrolyzed. Like in most lipases, a mobile element covers the catalytic site in the inactive form of the lipase. This 'lid' consists of two short α-helices linked to the body of the lipase by flexible structure elements. In the open, active form of the lipase the lid moves away and makes the binding site accessible to the substrate.

Atomic details of the interaction of the scissile fatty acid binding site and the scissile fatty acid moiety of the first tetrahedral intermediate structure are assumed to mediate fatty acid specificity. While the geometry of the catalytic machinery is highly conserved, size and shape of the scissile fatty acid binding site vary considerably among lipases. They have been assigned to three classes (Pleiss et al., 1998): (1) lipases with a hydrophobic, crevice-like binding site located near the protein surface (lipases from Rhizomucor and Rhizopus), (2) lipases with a funnel-like binding site (lipases from Candida antarctica, Pseudomonas and cutinase), and (3) lipases with a tunnel-like scissile fatty acid binding site (lipases from Candida rugosa and Geotrichum candidum). Properties of the scissile fatty acid binding site and the role of individual residues for chain length specificity have been studied by structure determination and protein engineering of lipases from Geotrichum candidum (Holmquist et al., 1997), Rhizopus and Humicola lanuginosa (Joerger and Haas, 1994; Atomi et al., 1996; Martinelle et al., 1996; Klein et al., 1997).

Based on X-ray structure, we have modeled the binding of fatty acids of different length to the scissile fatty acid binding tunnel of CRL. Residues which were assumed to mediate chain length specificity were replaced by more bulky amino acids, and the properties of resulting mutants were determined in a competitive hydrolysis assay.

Materials and methods

Strains, plasmids and media

E. coli DH5α and Epicurian coli XL2-blue (Stratagene GmbH, Heidelberg, Germany) were used as hosts for plasmid amplification, and Pichia pastoris GS115 (his4) (Invitrogen Corporation, San Diego, CA) for the expression of recombinant lipases using the vector pGAPZαB.

E. coli was grown at 37°C in low salt Luria-Bertani medium (LB) containing 25 µg/ml zeocin for selection of clones transformed with pGAPZαB derivatives. P. pastoris was grown in shaking flasks at 30°C in rich buffered medium containing 1 % glycerol and 0.1 M phosphate buffer pH 6 (BMGY, Invitrogen Manual "Pichia Expression Kit").

YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) was used for maintaining yeast cultures. YPD plates containing 2 % sorbitol or 1 % tributyrin and zeocin (100 µg/ml) were used for the selection of P. pastoris transformants.

Construction of Lip1 expression vector

The procedures for cloning the synthetic lip1 (slip1) gene preceded by the S. cerevisiae prepro-α-factor leader sequence to give the plasmid pPIC-pp-slip1 have been described previously (Brocca et al., 1998). To express the slip1 gene under the constitutive GAP promoter, the expression vector pGAPZαB was used. The 1316 bp SfuI-BamHI/blunt fragment from plasmid pPIC-pp-slip1, containing a 5' truncated form of the slip1 gene, was ligated into the SfuI-XbaI/blunt treated pGAPZαB vector giving plasmid pGAP-3'-slip1. In order to reconstitute the sequence encoding the mature lipase preceded by the α-factor prepro signal sequence, the 324 bp SfuI fragment from pPIC-pp-slip1, coding for the prepro-α-factor followed directly by the 5' part of the mature lipase, was inserted into pGAP-3'-slip1

linearized with the same enzyme. The resulting plasmid pGAP-slip1 was used as template for the site directed mutagenesis of slip1.

DNA mutagenesis and sequencing

Site-directed mutagenesis was performed using the QuikChange™ Site Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, Heidelberg, Germany) using 5-15 ng of pGAP-slip1 DNA as template and couples of mutagenic, full length complementary oligonucleotides as primers. To simplify screening for mutant slip1 genes, in some mutants diagnostic restriction sites as silent mutations were introduced by the mutagenic primers (Table I). The amplification products were treated for 2-3 hrs at 37°C with DpnI endonuclease in order to remove traces of template DNA and were directly used to transform Epicurian coli ultracompetent and supercompetent cells (Stratagene) according to Stratagene's instructions. For the construction of the double mutant L410F/S365L, plasmid pGAP-slip1-L410F containing the mutation L410F was used as template with the appropriate primer pair in a second site-directed mutagenesis reaction. Mutant plasmid pGAP-slip1-L410F/S300E was obtained by ligation of a 0.7 kb SpeI/SspI fragment from plasmid pGAP-slip1-S300E into the 3.8 kb fragment derived from pGAP-slip1-L410F after digestion with the same enzymes.

The generated mutations were checked by restriction analysis using the introduced restriction sites and by automated sequencing. To exclude any mistake introduced into the plasmid by PCR, DNA fragments containing the desired mutations were back-cloned into pGAP-slip1 using standard procedures (Sambrook et al., 1989). These plasmid reconstructions were checked by restriction analysis and by partial DNA sequencing around the junction sites. DNA sequencing was carried out using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and a Abi Prism 377 DNA Sequencing System (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions.

Transformation of P. pastoris cells, plate assay of lipase activity and expression of Lip1

Pichia pastoris GS115 cells were transformed by electroporation (Scorer et al., 1994) using a Bio-Rad Gene Pulser with 10 µg of BspHI-linearized plasmid pGAP-slip1 carrying wild-type or mutated slip1 genes and plated onto YPDS plates containing zeocin. Positive transformants were checked for lipase activity by transferring colonies onto YPD plates containing 1 % of emulsified tributyrin and incubated overnight at 30°C. Lipolytic activity was detected by the formation of a clear halo.

200 ml cultures of the wild-type or mutant slip1 expressing clones were carried out in 1 l flasks at 30°C and 250 rpm in BMGY medium at pH 6.0 for 5 days. The cultures were maintained at constant pH by adding 1 M phosphate buffer pH 6.0 and fed with 1 ml 86 % glycerol every 24 hours. After removing the cells by centrifugation, 0.1 % of sodium azide was added to the culture supernatants to prevent bacterial contamination. This was followed by filtration through cellulose nitrate (0.45 µm and 0.2 µm) and concentration of the supernatant to 5 % - 3 % of the original volume using Pall Filtron membrane type omega with a cut-off of 50 kDa. The concentrated lipase containing supernatants were used without further purification.

Determination of lipase activity

The lipolytic activity of the supernatants was routinely measured with tributyrin as substrate in a pH-stat assay at 30°C and pH 7.2 as described previously (Schmidt-Dannert et al. 1996). One unit (U) of lipase activity was defined as the amount of enzyme that liberates 1 µmol of fatty acids per minute under assay conditions. For determination of substrate specificity, 20 mM emulsions of triacetin, tricaproin and tricaprylin were used under the same conditions. The results shown represent mean values of two or three separate measurements, resulting in an error rate below 15%.

Competitive assay for determination of chain length selectivity

For the determination of chain length selectivity, a randomized oil was used. It consists of synthetic triglycerides which contain fatty acids of different chain lengths (saturated fatty acids C8:0 to C20:0, unsaturated fatty acids C18:1, C18:2, C18:3) randomly distributed over the sn-1, sn-2 and sn-3 position (John Bosley, Unilever, personal communication). As the different chain lengths were not present in equal amounts within the randomized oil (from 0.4 to 16.4 mole%), the content of the different chain lengths was calculated in relation to oleic acid and the results were multiplied with the factor obtained for each fatty acid. The values presented are referred to as "standardized".

1 g (1.3 mmol) of randomized triglycerides were emulsified in 20 ml of distilled water containing 2 % (w/v) gum arabicum and used as substrate solution in a pH stat assay. 500 U of lipase (determined using tributyrin as substrate) were incubated at 30°C and pH 7.2. Liberated fatty acids were titrated automatically with 0.1 M NaOH. After 10 % and 20 % of hydrolysis, indicated by the amount of NaOH needed to maintain the pH, 2 ml of the reaction mixture were withdrawn, and the lipolytic reaction was stopped by the addition of 0.1 ml of 85 % ortho-phosphoric acid. As 1.3 mmol of triglycerides can liberate 3.9 mmol of free fatty acids, 10 percent of hydrolysis are reached after consumption of 0.4 mmol NaOH.

For gas chromatographic analysis, free fatty acids were extracted 3 times with diethylether:n-hexane (1:1). The extract was evaporated under nitrogen and resuspended in 0.5 ml of n-hexane. 0.1 ml of this solution were again evaporated under nitrogen and resuspended in 50 µl of MSHFBA (N-methyl-N-trimethylsilyl-heptafluorbutyramide) (Millqvist Fureby et al., 1996) and incubated for 15 min at room temperature to convert the free fatty acids into the corresponding silyl-ethers before stopping the reaction by adding 100 µl of dichloromethane and analyzing 1 µl by GC (gas chromatograph: Fison 800, column: Optima 5 (25 m x 0.25 mm) Macherey & Nagel), temperature program: 50°C, 5°C/min 300°C 2 Min, injector 370°C,

flame ionisation detector 370°C, 100 KPa. A negative control consisting of the substrate emulsion without lipase was included for each measurement. As C18:3 and C20:0 were only present at 1.9 and 0.4 mole%, respectively, detection of the appropriate derivatives by GC was not possible. Experiments were performed in duplicate or triplicate, resulting in an error rate below 15%.

Modelling

The structure of CRL in complex with inhibitor hexadecanesulfonate was retrieved from the Protein Databank (Bernstein et al., 1977), PDB entry 1LPO. The inhibitor binds inside a tunnel which is flanked by mostly hydrophobic residues. Previous modeling studies (Peters and Bywater, 2001; Kahlow et al., 2001; Schulz et al., 2001) have shown that the scissile fatty acid and substrate-analogous inhibitors bind similarly. Thus, the position of a fatty acid chain was estimated by superposing C1 of the fatty acid with the sulfur atom of the inhibitor, C2 of the fatty acid with C1 of the inhibitor, and so forth. Thus, the inhibitor mimicks binding of a C17 fatty acid chain. Residues were considered as suitable mutation sites if (1) located at the inner wall of the tunnel, (2) their side chains pointed towards the hexadecanesulfonate, and (3) the size of their side chain was small or medium sized (thus phenylalanine, tyrosine and tryptophan were excluded). They were classified by the position of the fatty acid carbon atom which they contacted.

Results

Selection of mutations

The fatty acid binding site of CRL is located in a tunnel inside the protein with a wide entrance near the catalytic serine. It has a length of more than 22 Å and a diameter of 4 Å. 18 residues at its inner wall are hydrophobic (M213, V245, P246, F296, L302, L304, L307, F345, Y361, F362, F366, V409, L410, L413, G414, F415, F532, V534), two are polar and neutral (S301, S365), and one is positively charged (R303). The position of the inhibitor hexadecane sulfonate in the tunnel of CRL (Grochulski et al., 1994) allows to assign to each side chains a distance from the catalytic serine measured along the scissile fatty acid: the active site serine lies near the entrance to the tunnel which narrows near C3 of the bound scissile fatty acid, and closely contacts the fatty acid chain up to its ω-end (Figure 1). The most hydrophobic parts of the tunnel are located between C4 and C7, and between C12 and C14. Near the end of the tunnel at a position corresponding to C20 to C22, there is a small cavity near the protein surface with the side chains of Q364, Y299, and S300 pointing toward the center of the cavity. This cavity could bind the ω-end of long fatty acids. Alternatively, long fatty acids might reach out to the surface of the protein via a narrow gap which is flanked by S300, Q364, and R303.

To alter the substrate specificity profile of the Candida rugosa lipase, the following mutations were performed:

(1) Blocking near the entrance at fatty acid position C4 (L304F)

The hydrophobic leucine side chain is positioned near C4 of the scissile fatty acid; it was replaced by a bulky, hydrophobic phenylalanine. Since the side chain points toward the tunnel, the phenylalanine side chain is expected to accommodate without disturbing the protein structure.

(2) Blocking at fatty acid position C6/C8 (P246F, L413F)

The hydrophobic side chains of P246 and L413 point toward the tunnel. They were replaced by more bulky, hydrophobic phenylalanines. Since the C_α-C_β bonds of both residues point toward the tunnel, the mutation should not influence binding of scissile fatty acids shorter than C6 or C8.

(3) Blocking at C16/C18 (L410 W, L410F/S300E, L410F/S365L)

The medium sized, hydrophobic L410 is replaced by the most bulky, slightly hydrophilic tryptophan. In addition, two double mutants were investigated, with L410 replaced by phenylalanine combined to a mutation of a serine near the C20 position of the fatty acid.

(4) Blocking the exit at fatty acid position C18/C20 (S300E)

S300 is located near the surface of CRL and was replaced by the bulky, hydrophilic glutamic acid.

Characterization of mutants

To determine the substrate specificity of the CRL mutants for fatty acids of short and medium chain length, concentrated supernatants of recombinant Pichia pastoris cultures expressing CRL (wild-type) or the appropriate lipase mutant were used in a pH stat assay at 30°C and pH 7.2 using triacetin (C2), tributyrin (C4), tricaproin (C6) and tricaprylin (C8) as substrates. Neither the wild-type nor the lipase mutants hydrolyzed triacetin in substantial amounts. The relative activity of CRL wild-type and the mutants toward tributyrin, tricaproin and tricaprylin is shown in Figure 2, except for mutant L304F which showed no activity for the three substrates under these assay conditions. The activity toward tricaprylin (C8) was set as 100 %. Wild type and mutants S300E, L413F, L410W, L410F/S300E, and L410F/S365L have similar relative activity towards these three substrates, whereas mutant P246F has very high

activity towards tributyrin and tricaproin (67 and 14 fold, respectively, compared to wild type).

To determine the substrate specificity toward medium and long chain fatty acids, a randomized oil was used in a competitive pH stat assay. The activity of the concentrated supernatants of the lipase expressing Pichia clones was determined using tributyrin, and 500 units of the wild-type and each lipase variant were used for the competitive pH stat assay with the randomized oil as substrate, except for mutant L304F which showed no activity against tributyrin. For this mutant, a known amount of concentrated supernatant was used (2 ml).
Reaction rates were determined by automatic titration of the released fatty acids. Reactions were terminated after 10 % and 20 % of hydrolysis, and analyzed by GC after extraction and derivatization of the liberated fatty acids. Reaction rates were similar for the wild-type and all mutants except for mutants L304F and P246F. Whereas the reaction time necessary to reach 10 % of hydrolysis for the wild-type and mutants L413F, L410W, L410F/S300E, L410F/S365L, and S300E was about 0.5 to 1 h, mutants L304F and P246F needed 6 h and 20 h, respectively, to reach 10 % of hydrolysis. No time-dependent change of the substrate specificity profile was observed after 20 % of hydrolysis (data not shown). The substrate specificity of the mutants in comparison to the wild-type after 10 % of hydrolysis is shown in Figure 3 :

(1) Blocking near the entrance at fatty acid position C4 (L304F, Figure 3a)

In comparison to the wild-type the percentage of hydrolysis for C8 is about 2-3 fold higher, but very similar for C10. In contrast, the release of fatty acids with a chain length longer than C12 is decreased.

(2) Blocking at fatty acid position C6/C8 (P246F, L413F, Figure 3b)

Blocking at C6/C8 reduced the activity toward medium and long chain fatty acids as expected. Compared to L413F, the reaction times were much longer for mutant P246F. The hydrolysis of C8 for mutants P246F and L413F is about 4 fold and 2.5 fold, respectively, higher than for the wild-type, whereas for both mutants a discrimination against long chain fatty acids is observable, being even more pronounced for mutant P246F, where no hydrolysis of C14, C16 and C18:1 chains could be detected.

(3) Blocking at C16/C18 (L410W, L410F/S300E, L410F/S365L, Figure 3c)

As expected, for all three mutants chain lengths of C16 and longer are only poor substrates. The profile was most pronounced for mutant L410W, as its activity toward C14 is also strongly decreased. L410F in combination with mutation of S300 or S365 seems to have a weaker influence on the chain length profile.

(4) Blocking the exit at fatty acid position C18/C20 (S300E, Figure 3d)

The chain length profile of mutant S300E and wild-type are similar, with a slight decrease of selectivity toward long chain fatty acids.

To obtain a specific substrate specificity profile for the CRL variants for all chain lengths, the two profiles of Figures 1 and 2 were combined by calculating the ratios for the appropriate chain lengths referred to tricaprylin (C8) (Figure 4). The ratios for C4/C8 and C6/C8 of mutant P246F (66.7 and 14.1, respectively), demonstrate the high activity of this mutant toward tributyrin and tricaproin. Apart from this mutant and mutant L304F which shows no activity at all on tributyrin, tricaproin and tricaprylin in the pH stat assay, all other tested mutants and the wild-type show nearly the same ratios for the C4 and C6 substrates. In contrast, the behaviour of the lipase variants toward the substrates with acyl chains from C10 to C18 differs quite strongly. Whereas the wild-type shows the highest ratios for all chain lengths >C10, the mutants designed to block at C4 to C8 show a rapid decrease of the C10/C8

to C18/C8 ratio below 0.3 (closed square, triangle and circle). The ratios for the mutants to block at C16/C18 decrease less steep, reaching values lower than 0.1 for chain lengths longer than C14 (open symbols). Mutant S300E (stars) is very similar to the wild-type, differing only strongly in the ratios for the unsaturated fatty acids C18:1 and C18:2 from the wild type.

Discussion

Chain length profile of CRL and mutants

The previously reported expression of the major Candida rugosa lipase isoform, Lip1, from a synthetic gene in the yeast Pichia pastoris (Brocca et al., 1998) raised the possibility to produce this isoform in high purity. In this work, the production of mutant lipases with altered substrate specificities is reported. Molecular modelling led to the creation of several CRL Lip1 mutants by site-directed mutagenesis and their characterization in a single substrate assay as well as in a competitive substrate assay. It could be shown that blocking at a specific position located in the substrate binding region by a bulky, hydrophobic amino acid as phenylalanine results in a sharp decrease of activity toward chain lengths as long as or longer than the blocking distance.

Crude lipase containing supernatants were used for the determination of substrate specificity. As reported previously (Brocca et al., 1998), Pichia cultures transformed with the empty vector never showed lipolytic activity using tributyrin plates, as there is no endogenous lipase in Pichia. Gaskin et al. mention the presence of nonspecific esterases in Pichia pastoris and postulate that experiments with crude supernatants of Rhizomucor miehei lipase expressing Pichia pastoris cells are not sufficiently reliable due to the contribution of the nonspecific esterases to the rate of hydrolysis (Gaskin et al., 2001). In contrast, recombinant expression of the complete mature but inactive pig liver esterase (PLE) in Pichia pastoris never resulted in supernatants showing esterolytic activity using the p-nitrophenyl acetate assay (Lange et al., 2001). Therefore, we consider the use of crude extract as sufficiently reliable for screening purposes.

The use of the randomized oil allows a competitive assay for the determination of the CRL chain length specificity towards triglyceride substrates independent of regio- and stereoselectivity of the lipase. The random distribution of the different fatty acids in the oil

had been verified by the comparison of the liberated fatty acid profile of CRL and the sn-2 specific porcine pancreatic lipase, resulting in a very similar overall fatty acid composition (J. Bosley, Unilever, personal communication). In contrast to mixed substrate assays with fatty acid methyl esters as substrates, the liquid, randomized oil has the advantage that the mixture is not influenced by the lower solubility of the longer chain saturated fatty acids such as palmitic and stearic acid. The melting point of the randomized oil is at 29°C and therefore it stays in solution at assay conditions (30°C). As the amounts of the different chain lengths present in the oil differ, a "standardized" value was calculated by comparing the relative hydrolysis of the single chain lengths (see Material and methods section). While the profiles of hydrolysis were not dependent on the rate of conversion (10, 20 or 30%), the hydrolysis profiles of the mutants clearly differed from wild-type lipase. For mutants L304F and P246F, much more time was necessary to achieve 10% total hydrolysis in the random oil assay. This indicates a lower specific activity of these mutants in comparison to the wild-type lipase.

We did not detect substantial amounts of triacetin hydrolysis, although other groups report the hydrolysis of triacetin by CRL Lip1 (Pernas et al., 2001). The amounts of triacetin we used were low, according to the results of Pernas et al. below the concentrations needed for interfacial activation.

Chain length specificity follows a simple mechanical model

By engineering amino acids located in the substrate binding region of the Candida rugosa lipase, CRL mutants with different chain length specificities were obtained. As expected from the molecular modelling studies, the appropriate mutants only poorly hydrolyze chain lengths longer than the envisaged cutoff, whereas chain lengths shorter than the cutoff are hardly influenced.

For mutants inside the tunnel (P246F, L413F, L410W, L410F/S300E, L410F/S365L), the observed chain length profile can be explained by a simple mechanical model. If a side chain which is oriented towards the tunnel is replaced by a more bulky side chain, two effects are observed: for fatty acids which are short enough and do not reach the mutated side chain, activity is not changed by the mutation; for longer fatty acids, a sharp decrease of activity is observed as it becomes long enough to reach the mutated side chain. The most substantial change in chain length profile is observed for mutant P246F. As P246 is located near atom C6/C8, exchanging proline by phenylalanine is expected to block chains longer than C6/C8, which is confirmed by experimental data. Compared to wild-type CRL, mutant P246F is highly specific towards C4 and C6 chains: compared to activity towards C8 chains, it has a 67 and 14 fold relative activity towards C4 and C6 chains, respectively. In contrast, for wild-type CRL relative activity towards C4 and C6 chains is 0.78 and 0.16, respectively. Mutant L413F, also designed to block near atom C6/C8, shows a similar but less pronounced profile.

A similar effect is observed for mutants L410W, L410F/S300E, and L410F/S365L. Their chain length profile is similar. Replacing L410 which is located near C16/C18, by a more bulky residue (tryptophan, phenylalanine) nearly abolishes activity towards chain lengths longer than C14.

Changes in chain length profile caused by mutations in the tunnel of CRL are more pronounced than those observed previously by mutation of lipases from filamentous fungi. In contrast to CRL, their scissile fatty acid binding site is a long, hydrophobic crevice near the surface of the protein (Pleiss et al., 1998). Mutation of F112 to tryptophan in Rhizopus delemar lipase resulted in an increase of specificity for short-chain (C4) to long-chain fatty acids (C18:1) as shown by a 3-fold decrease in the preference for tricaprylin, whereas the relative hydrolysis of tributyrin was elevated (Joerger and Haas, 1994). A double mutant F112W/V209W of the same lipase also led to reduced activities towards chain lengths longer

than C4 (Klein et al., 1997). However, some mutation cannot be interpreted by a simple blocking model: reducing the size of F94 in Rhizomucor miehei lipase by mutation to glycine also reduced relative activity towards long chain fatty acids (Gaskin et al., 2001), which was interpreted as a change in activation of the lipase. Mutation of W89 in Humicola lanuginosa also caused changes in chain length profile, although is not contacting the scissile fatty acid (Martinelle et al., 1996).

Blocking the entrance to the tunnel

Mutations inside the tunnel demonstrated the role for chain length discrimination which can be explained by a simple model of blocking side chains from binding. In contrast, mutations at the entrance of the tunnel introduce challenging properties. Mutant L304F no longer accepted C4 and C6 chains but hydrolyzed longer chain lengths ≥8. These observations could be explained by assuming an alternative binding site outside the tunnel. Short chain scissile fatty acids preferably bind to the tunnel while medium and long-chain scissile fatty acids can bind either to the tunnel or to an alternative scissile fatty acid binding site outside the tunnel. The equilibrium is shifted towards the alternative binding site as the fatty acids get longer and unsaturated, or the tunnel is blocked. According to this model, the mutant has no activity towards short chain fatty acids but long chain fatty acid activity is maintained by binding to the alternative scissile fatty acid binding site. Hydrolysis experiments with mixtures of fish oil (containing long and highly unsaturated chains from C10 to C22) showed that even for longer, saturated or unsaturated, chain lengths the profile does not change by several blocking mutations at the end of the tunnel (data not shown). Mutants at position 410 blocking C16/C18 show a relatively low long chain activity, which seems to contradict the existence of an alternative binding site. However, the existence of an intact tunnel for short and medium

chain length fatty acids leads to high activity towards these substrates, thus the relative contribution of the alternative binding site may be small.

The existence of an alternative binding site for the scissile fatty acid has previously been suggested to explain the switch of enantioselectivity for chiral acids which are too bulky to bind into the tunnel of the homologous lipase from Geotrichum candidum (Holmquist et al., 1996). It also explains the effect of mutations at the entrance of Geotrichum candidum lipase (Holmquist et al., 1997): replacing L358 by phenylalanine in the cis (Δ-9) specific lipase I from Geotrichum candidum lowered the triolein/trioctanoin activity ratio 5-fold while the reverse mutation F358L in the non-specific lipase II did not transfer cis (Δ-9) specificity. Thus, by blocking the entrance to the cis (Δ-9) specific tunnel of lipase I leads to a non-specific lipase: either access is more hampered for C18:1 than for C8, or C8 and C18:1 bind preferably to an alternative binding site which has no specificity.

In conclusion, we showed that molecular modelling studies identified Candida rugosa lipase mutants with altered substrate specificities, especially preference towards short and medium and discrimination against longer chain lengths. This property is useful for industrial production of fats and oils enriched in saturated fatty acids with chain lengths longer than C16 and mono- or polyunsaturated fatty acids longer than C18 (Moore et al., 1995, Smith et al., 1996). Enriched margarines or spreads would be advantageous in preventing diseases such as arteriosclerosis.

Starting with CRL, a toolbox of mutants with modified chain length profile has been created. By combination of single mutations which have been shown to block the tunnel, CRL mutants with an even more pronounced profile may be engineered. Such optimized biocatalysts could be efficiently used for the production of tailor-made fats and oils.

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Tables

Table I: Primers used for mutagenesis. All reverse primers used were complementary to the sequences shown below. Introduced restriction sites are in italics, nucleotides different from the synthetic CRL gene are bold face.

mutation	forward primer (5'→ 3')	restriction site
P246F	caatctggtgc**c**at*gg*tt**ttc**tctgacgccgtcgacgg	*Sty*I
S300E	ggtttcttggcttac**gag**tccttaagattgtct	
L304F	cctccttaagatt**c**tcttacttgcc	*Hin*fI
S365L	gaatatttcaagcaat**tg**tttgtccacgctagc	*Mun*I
L410F	ctctgctgtttt**c**ggtgacttggg	
L410W	caagagaatctc**c**gctgttt**g**gggtgacttggg	*Bsi*YI
L413F	gaatctctgc**a**gttttgggtgactt**c**ggttttactttggc	*Pst*I

Figure legends

Fig. 1: Structure of the scissile fatty acid binding site of CRL in complex with the inhibitor hexadecane sulfonate (Grochulski et al., 1994). The inhibitor (atoms corresponding to C1, C4, C8, C16 are labeled), hydrophobic side chains pointing into the tunnel (L304, L410, S300, L365, L413, P246), and side chains of the catalytic triad (H449, E341,S209) are displayed.

Fig. 2: Activity of CRL mutants toward short chain triacylglycerides. Relative activities were determined using pH stat assay at 30°C and pH 7.2 using the amount of lipase corresponding to 500 U measured with tributyrin. The activity toward tricaprylin was set as 100 %. Mutant L304F did not show any activity using these assay conditions.

Fig. 3: Substrate specificity of CRL mutants using a randomized oil. Hydrolysis of the randomized oil was carried out in a pH stat at 30°C and pH 7.2 until titration of 0.4 mM free fatty acids (10% of hydrolysis). After derivatization of the fatty acids to the corresponding silyl ether, the samples were analyzed by GC analysis. The standardized values were calculated as described in the material and methods section.

Fig. 4: The ratio Cn:C8 was calculated from the same values as in Figures 1 and 2. Wild-type (diamond), short chain (closed square and triangle), medium chain (closed circle), C16/18 (open symbols), C18/20 (star).

Figure 1

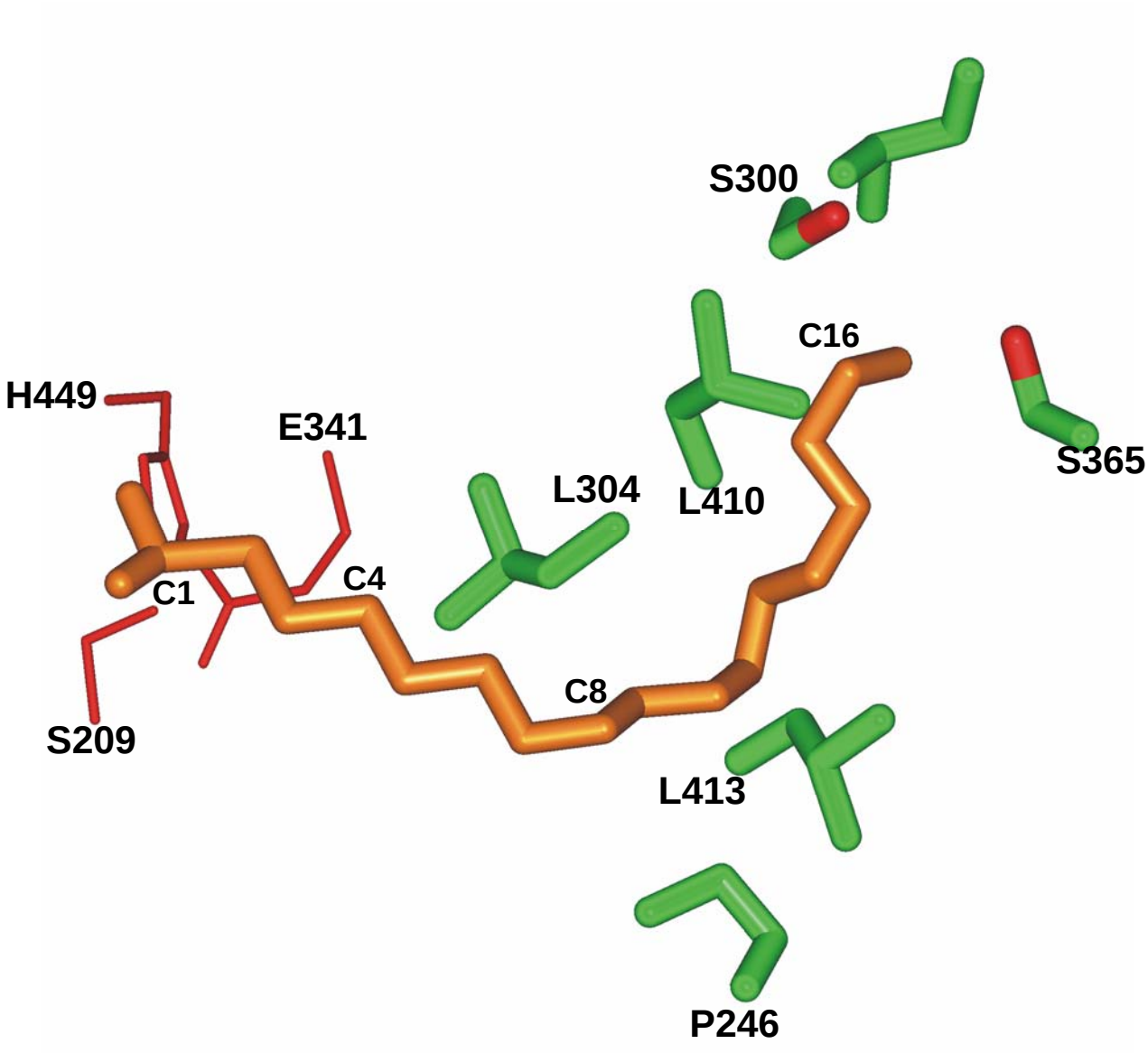


Figure 2

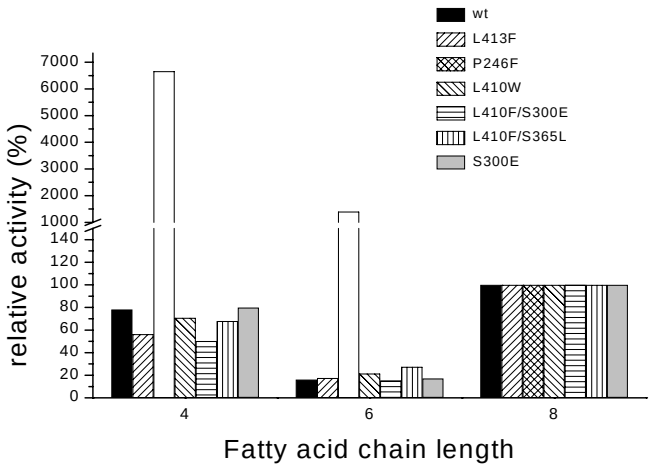

wt
L413F
P246F
L410W
L410F/S300E
L410F/S365L
S300E
relative activity (%)
7000
6000
5000
4000
3000
2000
1000
140
120
100
80
60
40
20
0
4
6
8
Fatty acid chain length

Figure 3:

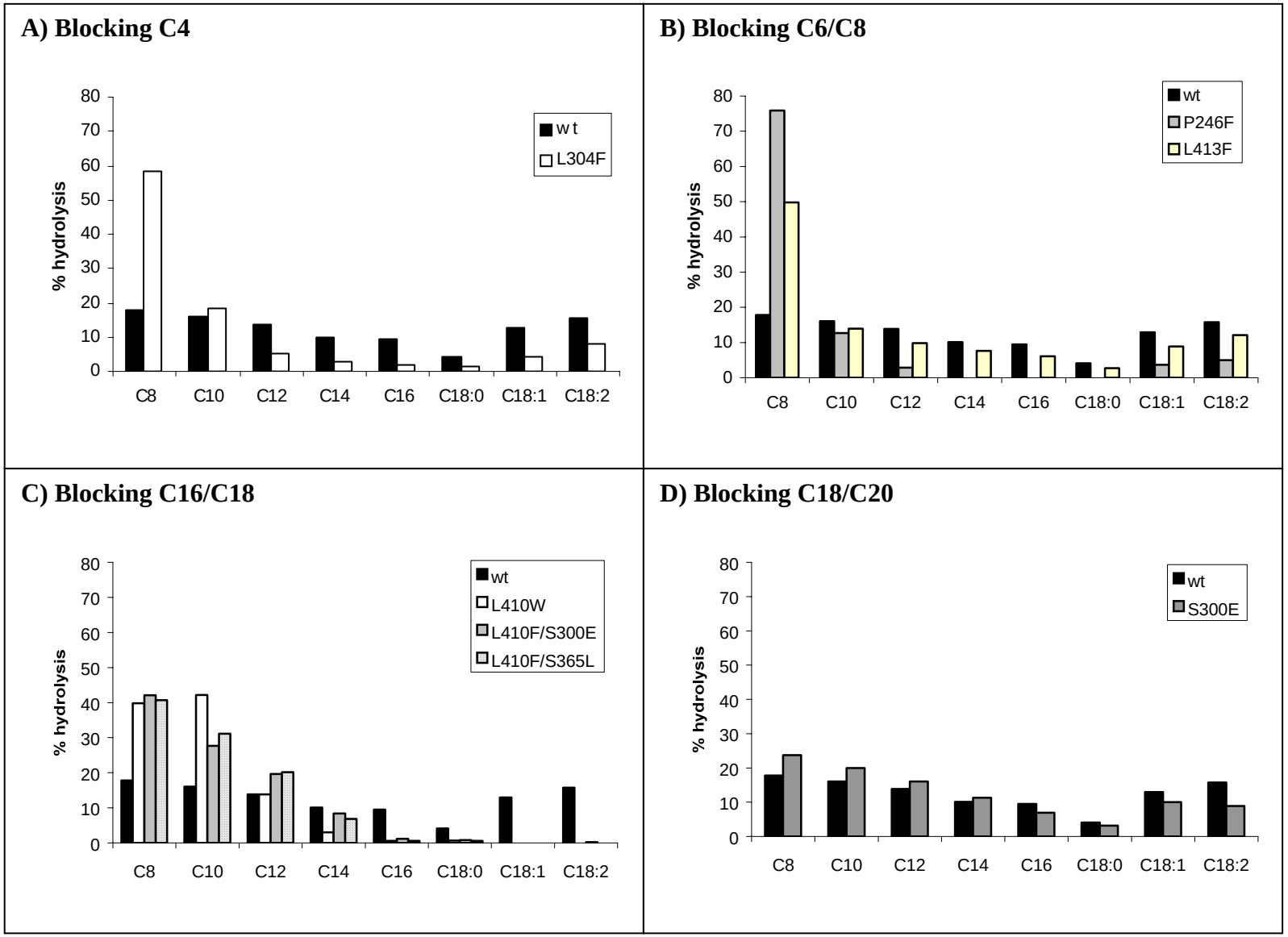
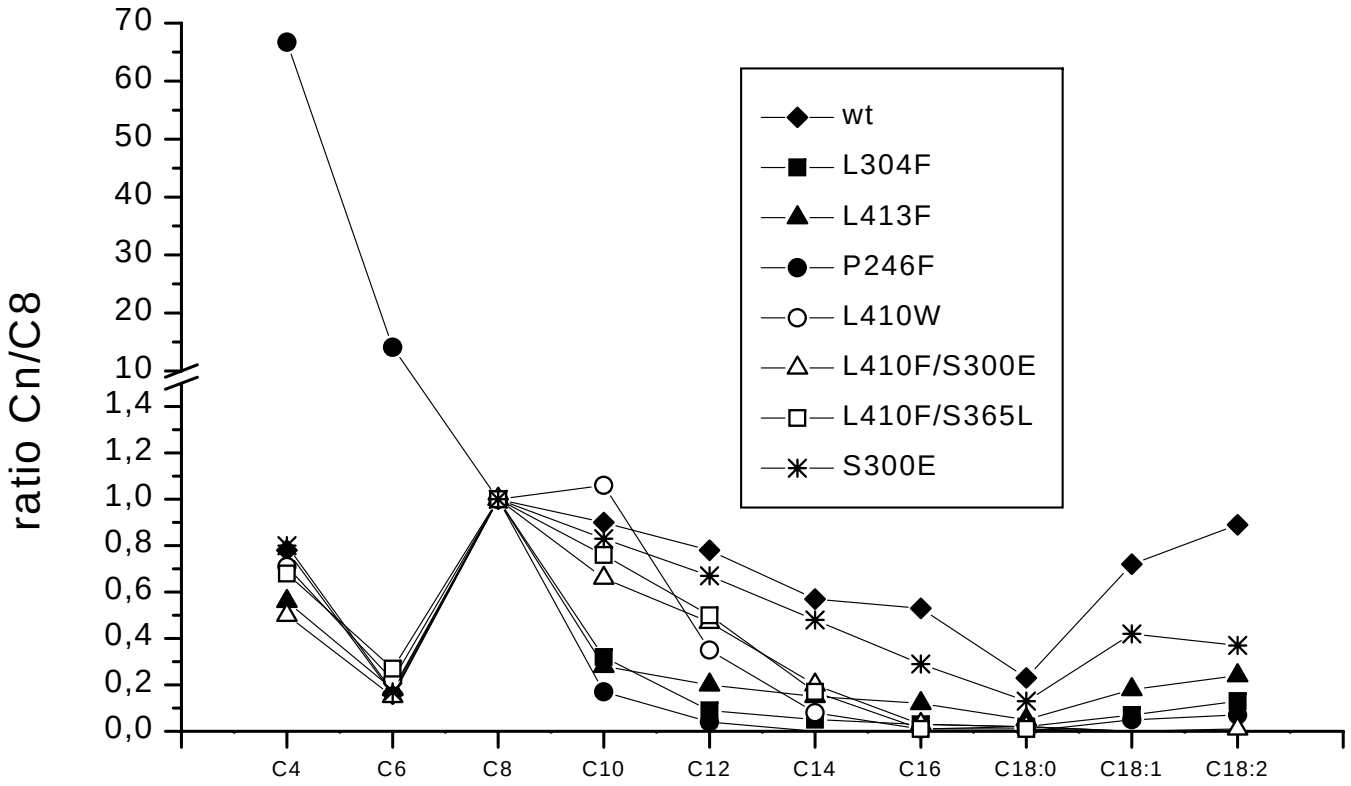

A) Blocking C4
% hydrolysis
80
70
60
50
40
30
20
10
0
C8
C10
C12
C14
C16
C18:0
C18:1
C18:2
wt
L304F
B) Blocking C6/C8
% hydrolysis
wt
P246F
L413F
C) Blocking C16/C18
% hydrolysis
wt
L410W
L410F/S300E
L410F/S365L
D) Blocking C18/C20
% hydrolysis
wt
S300E

Figure 4:


ratio Cn/C8
70
60
50
40
30
20
10
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
C4
C6
C8
C10
C12
C14
C16
C18:0
C18:1
C18:2
wt
L304F
L413F
P246F
L410W
L410F/S300E
L410F/S365L
S300E