A structural basis for enantioselective inhibition of *Candida rugosa* lipase by long-chain aliphatic alcohols

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Abstract

Molecular modeling showed that the enantiomers of heptyl 2-methyldecanoate are productively bound to the active site of *Candida rugosa* lipase in quite different conformations. The fast-reacting *S*-enantiomer may well occupy the previously identified acyl-binding tunnel in the active site of the lipase. By contrast, the slow-reacting *R*-enantiomer must be bound to the active site, leaving the tunnel empty to allow the formation of two catalytically essential hydrogen bonds between His 449 of the catalytic triad and the transition state of the catalyzed reaction. This information enables us to propose a molecular mechanism explaining how long-chain aliphatic alcohols act as enantioselective inhibitors of this lipase in the resolution of 2-methyldecanoic acid. Long-chain aliphatic alcohols may coordinate to the acyl-binding tunnel of the *C. rugosa* lipase, thereby selectively inhibiting the turnover of the fast-reacting *S*-enantiomer, thus resulting in a lowered enantioselectivity in the resolution.

Keywords: chiral recognition; chiral resolution; molecular modeling; structure–function relationships; substrate binding; 2-methylalkanoic acid

Triacylglycerol lipases (EC 3.1.1.3) have evolved to efficiently hydrolyze neutral lipids in aqueous emulsion, but they also catalyze acyl transfer reactions in organic media (Zaks & Klivanov, 1985). Lipases have, in recent years, become important biocatalysts in synthetic organic chemistry owing to their enantioselective properties, which constitute one of the best ways to obtain enantiomerically pure building blocks for syntheses of pharmaceuticals, pheromones, and fragrances with controlled stereochemical properties (for reviews see Cheetham, 1993; Faber & Franssen, 1993). The lipase from *Candida rugosa* has been found to be particularly well suited, compared to other lipases, to the resolution of 2-substituted carboxylic acids such as 2-methylalkanoic acids (Engel, 1991; Holmberg et al., 1991; Sonnet & Baillargeon, 1991). The active site of the *C. rugosa* lipase contains a substrate-binding tunnel, unique among the structurally characterized triacylglycerol lipases, in which an unbranched acyl moiety of a substrate is coordinated (Grochulski et al., 1994a; Cygler et al., 1994). The chiral recognition of the lipase for enantiomers of 2-substituted carboxylic acids has been

suggested to involve the binding of the acyl chain of the substrate in the identified acyl-binding tunnel (Ahmed et al., 1994). With such a binding mode of the enantiomers, the spatial geometry at the entrance of the tunnel appeared to explain the high enantioselectivity toward 2-substituted carboxylic acids.

Primary alcohols act as competitive substrate inhibitors in lipase-catalyzed acyl transfer reactions through the formation of dead-end complexes with the lipases (Chulalaksananukul et al., 1990; Rizzi et al., 1992; Martinelle & Hult, 1995). Recently, it was found that long-chain aliphatic primary alcohols act as enantioselective substrate inhibitors of the *C. rugosa* lipase. An increased alcohol concentration thus markedly reduced both the reaction rate and the enantioselectivity in the *C. rugosa* lipase-catalyzed esterification of 2-methyldecanoic acid (Berglund et al., 1995). The molecular basis for this phenomenon was previously poorly understood.

In this paper, we report the result from a molecular modeling study performed to get a detailed structural understanding of how the *C. rugosa* lipase discriminates between the enantiomers of 2-methyldecanoic acid. Different modes of binding of the enantiomers to the active site of the lipase were investigated by means of analyses of energy-minimized structures of diastereomeric lipase–transition state complexes. A structural basis for the enantioselective inhibiting effect of long-chain aliphatic al-

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cohols on the *C. rugosa* lipase is presented and discussed in relation to the unique geometry of the active site of this lipase.

Results and discussion

Transition states in the resolution of a chiral acid substrate

Lipase-catalyzed resolution of a racemic acid involves the formation and subsequent breakdown of different diastereomeric acyl-enzyme intermediates for the two enantiomers. Both the acylation and the deacylation of the enzyme may, in principle, contribute to the enantioselectivity of the lipase. However, in the *C. rugosa* lipase-catalyzed esterification of 2-methyldecanoic acid, the enantioselective step appears to be the deacylation of the lipase, because the enantioselectivity of the lipase varies with the alcohol employed in the esterification reaction (Berglund et al., 1993). To calculate the enantioselectivity of the *C. rugosa* lipase in the esterification of 2-methyldecanoic acid, the transition state involved in the deacylation of the acyl-enzyme intermediate is therefore relevant to study by means of molecular modeling. It should also be noted that the acyl moiety of the substrate is believed to be coordinated to the active site of the lipase similarly during both the acylation and the deacylation of the lipase (Grochulski et al., 1994a). Therefore, the transition state modeled in this paper should serve its purpose in the investigation of how a chiral acyl moiety of a substrate is bound to the active site of the lipase.

Modeling of transition states

The modeling of the transition states followed the protocol that was used previously to calculate the enantioselectivity of chymotrypsin (Norin et al., 1993) and lipases (Norin et al., 1994a). The docking of the transition state was guided by the known crystal structure of complexes of the *C. rugosa* lipase with transition-state analogues (Cygler et al., 1994; Grochulski et al., 1994a). The atoms around the tetrahedral carbon of the transition state, the oxyanion, and the oxygens of the leaving alcohol and the active serine were positioned so that all catalytically essential hydrogen bonds were formed. During the simulations, the positions of these atoms did not change much, whereas the other parts of the substrate adopted a conformation with lower energy. In some structures, one or several of the catalytically essential hydrogen bonds to the transition state were broken during the simulations. These structures were regarded as not representing reactive complexes. The fact that the lost hydrogen bonds to the transition state were recovered in structures collected at a later stage during the simulations shows that the modeling procedure allowed for a thorough search of the conformation space and that energy barriers separating two local energy minima could be passed. The energies of the 10 analyzed conformations of each lipase-transition state complex differed by at least 3 kJ/mol upon pairwise comparisons. Thus, the calculated lowest-energy conformation of the complexes is a well-defined conformation of the transition state that we believe represents the global energy minimum of the complex. This conclusion is further supported by results presented in previous reports, where the method used herein has been shown to give results that agree well with those of kinetic experiments (Norin et al., 1993, 1994a).

Binding of substrate in an extended conformation with the acyl moiety coordinated to the active site tunnel

The enantiomers of heptyl 2-methyldecanoate were docked into the active site of the lipase as a tetrahedral oxyanion with the acyl chain placed in the long hydrophobic tunnel, characterized as the acyl binding site (Grochulski et al., 1994a). After applying a previously developed computational method (Norin et al., 1993, 1994a) involving a combination of energy minimization and molecular dynamics simulation, we found that the *S*-enantiomer complex had a potential energy 26 kJ/mol lower than that of the *R*-enantiomer complex. This result is in qualitative agreement with those of kinetic experiments with respect to the enantioselectivity of the lipase. However, the simulation indicated a much higher enantioselectivity than found in practice, which corresponds to a difference in activation energy of $\Delta\Delta G = 11$ kJ/mol for the enantiomers (Berglund et al., 1993). Analysis of the energy-minimized structure of the *R*-enantiomer complex showed that the His 449 of the catalytic triad had been forced to move by the 2-methyl group at the chiral center of the substrate (Fig. 1 and Kinemages 1 and 2). In that complex, the two catalytically essential hydrogen bonds between the N ϵ -H proton of His 449 and the oxygen atoms of the active Ser 209 and the leaving group of the transition state had been lost. This fact suggests that the *R*-enantiomer would be an even poorer substrate compared to the *S*-enantiomer than predicted by the calculated energy difference. This idea is contradicted by experimental evidence.

Binding of substrate in a hairpin conformation leaving the active site tunnel empty

An alternative mode of binding of the substrate into the active site that would allow the formation of all catalytically crucial hydrogen bonds to the transition state of the *R*-enantiomer was sought. Simulations were performed with the 2-methyl group of the enantiomeric substrates coordinated into the presumed acyl-binding tunnel. With this binding mode, both the acyl chain and the alcohol moieties of the substrate point out of the active site (Fig. 2 and Kinemages 3 and 4). This substrate conformation resembles the hairpin conformation of lipid molecules at the lipid-water interface in aqueous emulsions (Brockman, 1984). The binding mode of the substrate tested herein seems therefore relevant to how a substrate molecule is presented to the lipase when adsorbed to a lipid particle. The calculations resulted in three-dimensional structures with an intact hydrogen bonding network in the catalytic triad and to the transition state with both enantiomers of the substrate (Fig. 2 and Kinemages 3 and 4). The free energy of the *S*-enantiomer complex was now 17 kJ/mol higher than that of the *R*-enantiomer complex. Apparently, the *R*-enantiomer is the fast-reacting substrate of the two competing enantiomers with this binding mode. The enantioselectivity of the lipase was thus opposite to the one obtained when the acyl moiety of the substrate was positioned in the acyl-binding tunnel (Fig. 1 and Kinemages 1 and 2).

A rule for predicting the enantioselectivity of the *C. rugosa* lipase toward 2-substituted carboxylic acids has been presented (Ahmed et al., 1994). This rule was based on the proposal that the largest substituent at the stereocenter of the substrate is coordinated to the active site tunnel. Although the rule was shown to have predictive power, for some substrates it predicted an enantioselectivity opposite to the *R*-enantioselectivity observed experimentally. The results obtained in the present investigation

Fig. 1. Stereo diagrams showing the active site of *C. rugosa* lipase. The three-dimensional structures represent the energy minima of the enzyme-substrate transition-state complexes of the *S*-enantiomer (top) and the *R*-enantiomer (bottom) of heptyl 2-methyldecanoate. The tetrahedral intermediate, including Ser 209, is shown in black. The acyl chain of the enantiomers is bound in the previously characterized acyl-binding tunnel (Grochulski et al., 1994a), whereas the heptyl moiety of the substrate points out of the active site. In this view, the tunnel lies in the plane of the paper. The hydrogen bonding network in the Ser-His-Glu catalytic triad and to the transition state is outlined. Gly 124 is a part of the oxyanion hole. Top: Fast-reacting *S*-enantiomer with all catalytically essential hydrogen bonds formed to the His 449 residue. Bottom: Slow-reacting *R*-enantiomer with two catalytically essential hydrogen bonds to the tetrahedral transition state lost due to the displaced His 449 residue.

Fig. 2. Stereo diagrams showing the active site of *C. rugosa* lipase with the *S*-enantiomer (top) and the *R*-enantiomer (bottom) of heptyl 2-methyldecanoate bound in a hairpin conformation with the acyl chain pointing out of the active site. The tunnel of the active site points into the plane of the paper. The tetrahedral intermediate, including Ser 209, is shown in black. The hydrogen bonding network in the Ser-His-Glu catalytic triad and to the transition state is outlined. Gly 124 is a part of the oxyanion hole. Both the fast-reacting *S*-enantiomer (top) and the slow-reacting *R*-enantiomer (bottom) form all of the catalytically essential hydrogen bonds to the His 449 residue of the catalytic triad.

might explain the reversed enantiopreference with those substrates (Fig. 3). If the largest substituent at the chiral center of the substrate is too bulky to fit into the active site tunnel, the substrate may be bound in the hairpin binding mode proposed here, which would reverse the enantiopreference as compared to substrates whose largest substituent can be accommodated in the tunnel. It has been suggested that a phenyl group fits into the tunnel (Ahmed et al., 1994), but our analysis indicates that it may be difficult for groups bulkier than that to fit into the narrow active site tunnel. Even if more of the region around the active site tunnel had been included in the molecular dynamics simulation, the tunnel geometry and the ability to incorporate the substrate would not have been affected in any significant way. In addition, X-ray crystallographic analysis has shown that the geometry of the tunnel stays the same upon binding substrates as when empty (Grochulski et al., 1994a).

Simple ability of the acyl chain of the substrate to fit into the lipase tunnel may not be the entire explanation for the observed enantioselectivity toward the substrates shown in Figure 3. When the substrate is bound in a hairpin conformation, the acyl moiety points out of the active site, and our calculations show that the *R*-enantiomer complex of 2-methyldecanoic acid is the most stable. The relative stability of the *R* and *S*-enantiomer complexes could be affected by additional substrate-lipase interactions mediated by the acyl tail part of a bound substrate. This effect could explain the quantitative differences in the *R*-enantiopreference of the lipase toward the different substrates.

The energies of the calculated structures were compared only when the enantiomers were coordinated to the active site with a similar binding mode, because the entropic contributions to the potential energy can be considered similar for the enantiomers. This approximation may not be valid when comparing the calculated energies of complexes in which the substrate is differently oriented in the active site of the lipase. Consequently, we cannot determine which mode of binding is the most favored for each enantiomer. To the best of our knowledge, no methods are available to calculate the entropy contribution to the free energy of systems such as that studied here.

2-Methylalkanoic acid	Enantiopreference	Enantiomeric ratio
<i>S</i>	35 ^a
<i>R</i>	>100 ^b
<i>R</i>	24 ^b

Fig. 3. Enantiopreference and enantiomeric ratio of *C. rugosa* lipase in the resolution of 2-methylalkanoic acids. The structure of the fast-reacting enantiomer is shown. ^a Berglund et al. (1993). ^b Ngooi et al. (1990).

Substrate bound in a hairpin conformation and heptanol coordinated to the active site tunnel

It is known that aliphatic alcohols can act as competitive substrate inhibitors of lipases in acyl transfer reactions by forming a dead-end complex with the enzyme (Chulalaksananukul et al., 1990; Rizzi et al., 1992; Martinelle & Hult, 1995). Thus, the alcohol and the acyl donor compete for the same binding site in the active site of the lipase. This means that a long-chain aliphatic alcohol substrate could bind to the tunnel of the active site of *C. rugosa* lipase. In fact, the electron density seen at the entrance of the tunnel by X-ray crystallographic analysis of the open lipase was assigned to 2-methyl-2,4-pentanediol, which was used as a precipitating agent (Grochulski et al., 1994b). In the X-ray structure of the related *Geotrichum candidum* lipase, an unidentified organic molecule was bound in the active site tunnel (Schrage & Cygler, 1993).

If heptanol is coordinated to the active site tunnel, the oxyanion transition state of heptyl 2-methyldecanoate can still be bound productively to the active site in the hairpin conformation. When the simulations were performed with heptanol positioned in the tunnel, the potential energy of the lipase-transition state complex was 38 kJ/mol lower with the *R*-enantiomer than with the *S*-enantiomer. This result indicates that coordination of heptanol to the active site tunnel may give a higher enantiopreference for the *R*-enantiomer than when the tunnel is left empty. The increased enantioselectivity relates to small structural changes of both enantiomers imposed by the alcohol coordinated into the active site tunnel. This means that the exact location of the alcohol molecule in the tunnel becomes crucial to the enantioselectivity of the lipase. Because no structural information is currently available on how an alcohol binds to the tunnel, we do not wish to speculate on further details as to how the alcohol may increase the enantiopreference for the *R*-enantiomers when the substrate is bound to the active site in a hairpin mode.

A molecular basis for enantioselective substrate inhibition

Long-chain aliphatic alcohols act as enantioselective substrate inhibitors in the *C. rugosa* lipase-catalyzed resolution of 2-methyldecanoic acid (Berglund et al., 1995). An increase in alcohol concentration has been shown to reduce both the reaction rate and the enantioselectivity in the esterification of 2-methyldecanoic acid. The apparent enantiomeric ratio of the lipase (Chen et al., 1982), $E_S = (k_{cat}/K_m)_S / (k_{cat}/K_m)_R$, decreased from $E_S = 83$ to $E_S = 37$ when the heptanol concentration was increased from 90 mM to 900 mM in the esterification reaction (Berglund et al., 1995). This change was shown to be due to a difference in the dependences of $(V_{max})_S$ and $(V_{max})_R$ of the enzyme on the alcohol concentration. The structural basis for this phenomenon was, however, previously unclear.

The results obtained in the present molecular modeling study show that only the fast-reacting *S*-enantiomer is productively bound in the previously identified acyl-binding tunnel, whereas the slower-reacting *R*-enantiomer is bound in the active site, leaving the tunnel empty. This information casts new light on the mechanism by which long-chain aliphatic alcohols may function as enantioselective inhibitors. Free lipase molecules in which the active site tunnel is accessible to the carboxylic acid substrate have an enantiopreference for the *S*-enantiomer when

the acyl moiety of the substrate is bound to the active site tunnel, and a preference for the *R*-enantiomer when the substrate is bound in a hairpin conformation. Lipase molecules with heptanol coordinated to the active site tunnel have an enantioselectivity preference for the *R*-enantiomer of 2-methyldecanoic acid. The overall apparent enantioselectivity for the *S*-enantiomer in the resolution reaction should thus depend on the relative concentrations of free enzyme and enzyme with an alcohol molecule bound in the active site tunnel. An increase in the alcohol concentration will shift the equilibrium between the two enzyme species toward the formation of the lipase-alcohol complex, which has an enantioselectivity opposite to that of the free lipase. This may explain why the apparent enantioselectivity of the lipase is lowered in the presence of excess alcohol.

The active site tunnel in the *C. rugosa* lipase is unique among the structurally characterized triacylglycerol lipases. This implies that the phenomenon of enantioselective inhibition according to the proposed mechanism is unique for this lipase. Kinetic experiments with the lipase from *Humicola lanuginosa* showed, in fact, that the reaction rate but not the enantioselectivity was affected by an increased alcohol concentration in the esterification of 2-methyldecanoic acid (M. Holmquist, unpublished data).

The chiral recognition of 2-methyldecanoic acid by the *C. rugosa* lipase seem to involve multiple binding modes of the 2-methyldecanoic acid enantiomers to the active site of the enzyme. The existence of multiple productive binding modes of unnatural artificial substrates to the active site of lipases might be of general importance in chiral resolutions. Further investigations are necessary to unravel the mechanistic and structural details of chiral recognition of lipases.

Materials and methods

Molecular modeling equipment

All calculations were performed with a Silicon Graphics Indigo2 workstation equipped with the molecular modeling software SYBYL version 6.1 (Tripos Associates, 1994).

Lipase structure

The three-dimensional structure of the *C. rugosa* lipase as determined by X-ray crystallography (Grochulski et al., 1993) was used in the calculations. Coordinates for the structure of the lipase were obtained from the Brookhaven Protein Data Bank, entry number 1CRL. All water molecules bound to the lipase according to the X-ray structure were included in the calculations, except one water molecule, which was removed to permit binding of substrate into the active site of the enzyme. The carbohydrate moieties covalently linked to Asn 314 and Asn 351 (Grochulski et al., 1994b) were excluded in the calculations.

Preparation of starting models of lipase-transition state complexes

The model of the tetrahedral oxyanion transition state, in which the O γ atom of the active Ser 209 residue is covalently bound to the carbonyl carbon atom of the substrate, was built using SYBYL. The charges of the substrate atoms were calculated by a semi-empirical method (Besler et al., 1990) using the software

MOPAC 6.0 ESP. A detailed description of the procedure used has been published previously (Norin et al., 1993). The transition-state model was docked into the active site of the lipase and oriented so as to form all catalytically essential hydrogen bonds to the active site. The negatively charged oxygen atom of the transition-state model was coordinated to the oxyanion hole defined by the backbone NH groups of Ala 210, Gly 123, and Gly 124 (Grochulski et al., 1994a). The Ne2 atom of His 449 in the catalytic triad was protonated (Kossiakoff & Spencer, 1981) and allowed to form a bifurcated hydrogen bond to O γ of the catalytic Ser 209 and to the oxygen atom of the heptanol moiety of the substrate, by analogy with the active site function of serine proteases. (For reviews of active site function in serine proteases, see Kraut, 1977; and Warshel et al., 1989.) The positions of the hydrogen atoms in the lipase were calculated by the BIOPOLYMER module of SYBYL. Amino acid residues within a 6-Å distance from any atom of the transition-state model were treated by the all-atom AMBER force field (Weiner et al., 1986), whereas the united atom AMBER force field (Weiner et al., 1984) was applied to the rest of the amino acid residues in the lipase. Water molecules were represented with use of the TIP3P water model (Jorgensen et al., 1983). Hydrogen bonds were defined as having bond lengths (X-H...Y) of 0.80–2.80 Å and angles (X-H...Y) of 120–180° between the X-H and H...Y bonds of the hydrogen bond donating (X) and accepting (Y) atoms.

Calculation of the geometry and energies of lipase-transition state complexes

The three-dimensional structures representing the energy minima of the diastereomeric lipase-substrate transition-state complexes were found by applying a computational protocol previously used for calculating the enantioselectivity of chymotrypsin (Norin et al., 1993) and lipases (Norin et al., 1994a). The free energy of the ground state is the same for the two enantiomeric substrates. Therefore, the difference in free energy between the diastereomeric lipase-transition states is a measure of the enantioselectivity (Chen et al., 1982) $E_S = (k_{cat}/K_m)_S / (k_{cat}/K_m)_R$ of the enzyme. The simulations were performed in vacuum with a distance-dependent dielectric function ($\epsilon = r$). Nonbonded interactions were truncated at $r > 8$ Å. These modeling conditions have been shown to be a good description of the environment of a lipase acting in a hydrophobic organic solvent (Norin et al., 1994b).

Energy minimizations were performed by means of the Powell minimizer in SYBYL. Initially, the transition state of the substrate was subjected to 200 steps of energy minimization, with the lipase kept in a locked conformation. In the subsequent calculations, amino acid residues and water molecules within a 6-Å distance from the substrate were allowed to move freely during the simulations. This defines a volume that well includes the active site and the bound substrate. All interactions between the substrate and the protein were thus treated in as much detail as possible to obtain the best model of the complex. In previous reports, this approach has given results that agreed well with those of kinetic experiments (Norin et al., 1993, 1994a). In a region between 6 and 8 Å away from the substrate, only the amino acid side chains and water molecules were allowed to move. The rest of the lipase was kept rigid during the simulations. The lipase-transition state complex was energy minimized for 500

steps. The initial energy minimization steps described above were done to obtain a relaxed structure of the complex before the subsequent molecular dynamics simulations. All simulations were equilibrated by raising the temperature to 300 K during 1.2 ps, followed by a simulation for 4 ps. A time step of 2 fs was used in all simulations. By periodically lowering and raising the temperature between 300 and 1 K, during molecular dynamics simulation, the conformational space was searched for local energy minima of the complex. The temperature was kept at 300 K for 6 ps and then slowly decreased to 1 K in temperature decrements of 10 K with interval length of 0.05 ps. Subsequently, the temperature was raised stepwise from 1 to 300 K in increments of 50 K with an interval length of 0.2 ps. The total simulation time comprising 10 cycles was 107 ps. Ten different conformers corresponding to energy minima of the lipase-transition state complex were collected at 1 K and subsequently refined through energy minimization for 5,000 steps or until the RMS gradient was less than 0.04 kJ/Å. The conformer with the lowest potential energy was selected and considered to represent the global energy minimum of the complex.

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