Structural basis of the chiral selectivity of Pseudomonas cepacia lipase

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To investigate the enantioselectivity of Pseudomonas cepacia lipase, inhibition studies were performed with Sn- and Rr-(Rr,Sr)-1,2-dialkylcarbamoylglycero-3-O-p-nitrophenyl alkylphosphonates of different alkyl chain lengths. P. cepacia lipase was most rapidly inactivated by Rr-(Rr,Sr)-1,2-dioctylcarbamoylglycero-3-O-p-nitrophenyl octylphosphonate (Rr-trioctyl) with an inactivation half-time of 75 min, while that for the Sr-(Sr,Sr)-1,2-dioctylcarbamoylglycero-3-O-p-nitrophenyl octylphosphonate (Sr-trioctyl) compound was 530 min. X-ray structures were obtained of P. cepacia lipase after reaction with Rr-trioctyl to 0.29-nm resolution at pH 4 and covalently modified with Rr-(Rr,Sr)-1,2-dibutylcarbamoylglycero-3-O-p-nitrophenyl butyl-phosphonate (Rr-tributyl) to 0.175-nm resolution at pH 8.5. The three-dimensional structures reveal that both triacylglycerol analogues had reacted with the active-site Ser87, forming a covalent complex. The bound phosphorus atom shows the same chirality (Sr) in both complexes despite the use of a racemic (Rr,Sr) mixture at the phosphorus atom of the triacylglycerol analogues. In the structure of Rr-tributyl-complexed P. cepacia lipase, the diacylglycerol moiety has been lost due to an aging reaction, and only the butyl-phosphonate remains visible in the electron density. In the Rr-trioctyl complex the complete inhibitor is clearly defined; it adopts a bent tuning fork conformation. Unambiguously, four binding pockets for the triacylglycerol could be detected: an oxyanion hole and three pockets which accommodate the sn-1, sn-2, and sn-3 fatty acid chains. Van der Waals’ interactions are the main forces that keep the radyl groups of the triacylglycerol analogue in position and, in addition, a hydrogen bond to the carbonyl oxygen of the sn-2 chain contributes to fixing the position of the inhibitor.

Keywords: crystal structure; transition-state analog; enantioselectivity; lipase; stereospecificity.

Lipases are lipolytic enzymes, which hydrolyze ester bonds of triacylglycerols. However, their substrate specificity is not limited to triacylglycerols. They may also hydrolyze ester bonds of other compounds such as acetyl-arylpropionic acid esters, which are precursors for the nonsteroidal anti-inflammatory agents naproxen and ibuprofen [1]. Because of this broad substrate specificity, and because of their distinct stereopreferences, lipases have found widespread application in the enantioselective synthesis of organic compounds, and in the resolution of racemic mixtures [2].

Over the years crystal structure determinations of various lipases have shown that all lipases contain the a/b-hydrolase fold, a structural motif common to a wide variety of hydrolases [3]. Their active sites consist of a catalytic triad, Ser-His-Asp/Glu, which are precursors for the nonsteroidal anti-inflammatory agents naproxen and ibuprofen [4–6]. Studies with lipases covalently complexed with organosulfates [7], organophosphates [8, 9], or organophosphonates [10–12] demonstrated that, in the presence of lipid-like compounds or organic solvents, their active-site regions may undergo drastic conformational changes, exposing the catalytic residues and the surrounding hydrophobic surface area to the solvent [13].

Although these studies provided insight into the catalytic mechanism of lipases and yielded a proposal for factors determining their enantioselectivity, none of the inhibitors used resembles a natural substrate. Only the recent investigations by Longhi et al. [14] on cutinase, an enzyme which does not show interfacial activation, made use of a triacylglycerol-like inhibitor. Unfortunately, those studies did not reveal any interactions of the inhibitor’s fatty acid chains with the protein.

Here we report crystallographic studies of the lipase from Pseudomonas cepacia. Structures of the open conformation of this lipase, from crystals grown from organic solvents, have been published recently [15, 16], but no structures are available with bound lipid analogues. We have now investigated the interaction of this enzyme with Sr- and Rr-(Rr,Sr)-1,2-dialkylcarbamoylglycero-3-O-p-nitrophenyl alkyl-phosphonates, with the alkyl chains consisting of either four carbon atoms (tributyl) or eight carbon atoms (trioctyl). Guided by kinetic studies, the binding mode of the lipase with the Rr-trioctyl and Rr-tributyl compounds was analyzed by X-ray crystallography. For the first time this resulted in a lipase structure with fatty acid chains bound.
Table 1. Statistics of data collection and refinement of R_c-trioctyl- and R_c-tributyl-complexed Pseudomonas cepacia lipase.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for complex</th>
<th>R_c-trioctyl</th>
<th>R_c-tributyl</th>
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<td>3.0−0.175</td>
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Table 2. Rmsd in Ca positions of the different Pseudomonas cepacia lipase models.

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<th>Alkyl phosphonate</th>
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<th>R_c-tributyl — model I</th>
<th>R_c-tributyl — model II</th>
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Preparation of the complexes and crystallization. The lipase-triacylglycerol complexes were prepared by adding either the R_c-tributyl or the R_c-trioctyl inhibitor dissolved in acetonitrile to the protein solution (8 mg/ml in 20 mM glycine pH 9) in a molar excess of 10:1. After a 90-h incubation at 12°C, the protein/inhibitor solutions were subjected to crystallization screens [20]. Crystals suitable for X-ray analysis grew within 4 weeks using the sitting-drop vapour-diffusion technique.

Inhibition experiments. Pseudomonas cepacia M-12-33 (Amano Pharmaceuticals Corp. Ltd) lipase was produced from Pseudomonas strain ATCC21808 as a host. The enzyme was purified as described previously [17] and its activity was determined spectrophotometrically in the presence of 100 mM Triton X-100, 0.25 mM p-nitrophenyl octanoate and 10 mM CaCl₂ at pH 8.0 (modified after [18]). Activities were calculated from the increase in absorbance at 400 nm. The inhibitors R_c-(R_c,S_c)-1,2-dibutylcarboxamylglycero-3-O-p-nitrophenyl butylyphosphonate (R_c-tributyl), S_c-(R_c,S_c)-1,2-dioctylcarboxamylglycero-3-O-p-nitrophenyl octylphosphonate (S_c-trioctyl) and R_c-(R_c,S_c)-1,2-dioctylcarboxamylglycero-3-O-p-nitrophenyl octylphosphonate (R_c-trioctyl) were synthesised according to Manesse et al. [19]. They are diastereomeric (S_c,R_c) at phosphorus, but enantiopure at the C2 atom of the glycerol (either R_c or S_c). All inactivation experiments on an analytical scale were done at 25°C. The kinetics of inactivation was followed by incubation of P. cepacia lipase (1−5 μM) in 10 mM Tris/HCl pH 8.0 containing 10 mM CaCl₂ and 100 mM Triton X-100. The reaction was started by the addition of the inhibitor from a concentrated stock solution in acetonitrile to a final concentration of 300 μM. Final acetonitrile concentrations never exceeded 5%. The residual lipase activity was measured by taking aliquots at different times and testing them spectrophotometrically. From the decrease in activity, the half-times of inactivation were determined. The activity measurements of the lipase towards the triacylglycerol analogues (R)- and (S)-2-decanoylamido-dodecyldecanoate, were performed titrimetrically as described elsewhere [19].

Experimental procedures. The structures were solved by molecular replacement using the P. cepacia lipase structure (Protein Data Bank code 3LIP [16]) without water molecules as a starting model. Calculations were done with AMoRe [22]. The structure elucidation was straightforward. For the R_c-trioctyl-inhibited lipase, the highest peak in the cross-rotation function had a correlation coefficient of 0.53 using data between 0.4−1.0-nm resolution. The translation function revealed a shift of the molecule along the a and c axes of 1/4 of the unit cell lengths. After applying the translation, the overall R-factor was 33.1% and the correlation coefficient was 0.69. With this solution an X-PLOR [23] rigid body refinement was done with data between 0.29−0.8-nm resolution which gave an R_mer of 32.6% and an R_free of 33.6%. After positional refinement, bulk solvent correction and manual building of the R_c-trioctyl compound into a difference Fourier density map using the program O [24], the R_mer and the R_free had decreased significantly. The final model includes all 320 amino acids, one Ca²⁺ ion, four water molecules (located inside the protein molecule) and the R_c-trioctyl compound. The final refinement was done with all reflections in the appropriate resolution range giving an overall R-value of 21.4% (Table 1).

For R_c-tributyl-inhibited P. cepacia lipase, we searched with the monomeric model for a dimer, expecting two solutions 180° apart. The cross-rotation function calculated with data between 0.4−1.0 nm revealed two strong peaks which were related by a
Fig. 1. Stereo-figures of the electron densities of the compounds covalently bound to Ser87 of P. cepacia lipase, produced with BOBSCRIPT [42]. (A) Fo-Fc omit map of the R<sub>C</sub>-tributyl compound, contoured at 2 σ. Only a bound butylphosphonate moiety is visible. (B) 2Fo-Fc omit map of R<sub>C</sub>-trioctyl compound, contoured at 1 σ. From left to right the sn-3, sn-1 and sn-2 chains are shown.
density visible for the first amino acid in both molecules and consequently these residues have been left out from the final model. The two molecules differ by 0.019 nm rms in their Ca atom positions (Table 2). In both molecules in the asymmetric unit the inhibitor is covalently bound, but surprisingly only the butyl phosphonate (sn-3 moiety) is visible in the electron density maps. Not even weak density is extending from the phosphonate towards the glycerol moiety. This suggests that the glycerol phosphonate ester has been cleaved (Fig. 1A). A nucleophilic substitution of a phosphonate ester bound by the active-site Ser has been observed before in serine proteases [27, 28]. This so-called aging reaction results in the formation of a negatively charged monomer between the active-site Ser and the phosphonate moiety.

The crystals of the R₆-triacyl-complexed lipase diffracted to 0.29 nm. A solvent-accessible glycine-rich loop (residues 1–10) is slightly higher than the coordinate error of 0.02 nm in each structure as estimated from a Luzzati plot [29].

Oxyanion hole and catalytic triad. The R₆-triacyl- and R₇-triacyl-complexed structures represent the putative transition-state conformation of a substrate molecule bound to the active site. In native P. cepacia lipase (Protein Data Bank entry 3LIP) the oxyanion hole has been proposed to be formed by the peptide NH-groups of Gln88 and Leu17 [16]. Indeed, in our complexed structures this site is occupied by one of the phosphonyl oxygen atoms, making hydrogen bonds to the main-chain nitrogen atoms of Gln88 and Leu17, with comparable hydrogen bonding distances in both structures (Fig. 2).

The overall topology of each structure resembles that of the open unliganded form of P. cepacia lipase (Protein Data Bank entries 2LIP, 3LIP and 1OIL), previously described in detail [15, 16]. The Ramachandran plots (data not shown) of the native and complexed lipase structures are almost identical, with Ser87 and Leu234 in the disallowed regions, as has been observed for the native lipase as well [15, 16]. The rmsd between all Cα atoms (PDB code 3LIP) and our complexed structures are around 0.04 nm (Table 2), which might be explained by the different temperatures at which the data were collected: native at 298 K and complexed at 90 K and 120 K.

Stereochemistry of the inhibitors. The triacylglycerol inhibitors used are enantiopure at the glycerol backbone C2 atom (R₆), but they are racemic at the phosphorus atom (R₇ or S₇). However, the R₆-tritubyl- and the R₇-trioctyl-complexed P. cepacia lipase structures show that only one phosphorus enantiomer (S₇) is observed. It is generally assumed that the mechanism of phosphatase inhibition of serine hydrolases occurs via an in-line displace reaction with inversion of configuration at phosphorus [33]. Because the priorities of the substituents at the phosphorus atom as defined by Cahn and co-workers [34] change as a result of the reaction of the phosphonate with serine, the fast-reacting inhibitor must have been the S₇-enantiomer.

The binding pockets. The three-dimensional structure of the R₆-trityl inhibitor complexed to P. cepacia lipase allows us to describe the different binding pockets (HA, HB and HH, see Figs 3a and 4) and the conformation of this lipid analogue. The lipid has a tuning fork shape, similar to the proposed conformation of a triacylglycerol present at an interface [35]. This conformation is characterized by the glycerol backbone dihedral angles θ₁ (O5-C2-C3-O3) and θ₂ (O1-C1-C2-O5) of 89° and 81°, re-
Fig. 3. Stereo-figures of the $R_{c}$-trioctyl inhibitor covalently linked to $O_{\gamma}$ of Ser87 (produced with the program BOBSCRIPT [42]). (A) Surface map [43] of *P. cepacia* lipase showing the inhibitor bound in a bent tuning-fork conformation. The HA pocket binds the $sn$-3 fatty acid chain, deeply buried in the enzyme’s surface, the HH pocket accommodates the $sn$-2 fatty acid chain, and the HB pocket loosely binds the $sn$-1 chain. (B) Close-up of the bound inhibitor with labeled atoms, showing the covalent link between inhibitor and enzyme. Atoms C4–C11 constitute the $sn$-3 chain, atoms N1–C20 the $sn$-1 chain, and atoms N2–C30 the $sn$-2 chain.

respectively [36]. This represents the so called sc/fb/sc conformer [35], with the extended octyl chain of the $sn$-2 moiety pointing to the outside (Fig. 3). About 8–10 fatty acid carbon atoms can be accommodated in the pockets. Longer chains probably partly stick into the solvent or micelle.

The binding cleft for the $sn$-3 moiety (HA) is a hydrophobic groove of $0.8 \times 1.0$ nm in width. The octyl group of the triacylglycerol analogue fits snugly in this cleft and is bound via van der Waals’ interactions (Fig. 4A). Pro113 closes the groove at the C-terminus of the central $\beta$ sheet and the side-chain atoms of residues Leu17, Phe119, Leu164, Leu167, Val266 and Val267 are in the wall of the cleft. These residues are part of functionally important secondary structure regions: the oxyanion loop (Leu17), helix $\alpha6$ in the direct neighbourhood of the lid helix $\alpha5$ (Leu164 and Leu167) and the active-site Asp loop (Val266 and Val267). The $sn$-3 chain of the butylphosphonate (which is the remaining part of the tributyl inhibitor) binds in a very similar way in this groove, with an rmsd of 0.023 nm compared to the equivalent atoms of the octyl chain. This indicates a well conserved mode of $sn$-3 fatty acid binding.

The binding of the $sn$-2 moiety in the HH pocket is also mainly determined by van der Waals interactions (Fig. 4B). The NH group of the carbamoyl function in this chain does not make any specific interactions with the protein. Leu287 and Leu293, both located in the calcium binding loop, make hydrophobic contacts via their $C\delta$ atoms to the carbon atoms of the $sn$-2 octyl chain. In addition, a hydrogen bond connects $O_{\gamma}$ of Thr18 to the carbonyl oxygen of the carbamoyl function of this chain. We propose that the $sn$-2 carbonyl oxygen ester of a triacylglycerol molecule can make a comparable hydrogen bond.

The $sn$-1 moiety is bound via hydrophobic interactions to Ala247 and Thr251 in the HB pocket (Fig. 4B), in which a smaller number of van der Waals’ interactions is possible. No interactions are made by the NH group of the carbamoyl function.

**Enantiomeric selectivity.** From the kinetic investigations, we know that the inactivation of *P. cepacia* lipase by the $S_{c}$-trioctyl compound is sevenfold slower than the inhibition by the $R_{c}$-trioctyl compound. To obtain information about the stereoisomeric discrimination of the enzyme between the $S_{c}$- and the $R_{c}$-trioctyl compounds, we modelled the $S_{c}$-trioctyl compound into the active site of *P. cepacia* lipase by a substituent exchange at the C2 position of the glycerol moiety. The phosphorylalkyl
Fig. 4. Schematic representation of the triacylglycerol binding mode in the active site of *P. cepacia* lipase as deduced from the observed binding mode of the *R*<sub>c</sub>-trioctyl inhibitor. The fatty acid part is abbreviated as R, the glycerol part as R'. Residues in *Pseudomonas* spec. involved in binding are indicated. (A) The HA acyl-chain pocket. Leu17 and Val266 are located at the pocket entrance; they are replaced by Met17 and Leu266 (darkened residues) in *P. aeruginosa* lipase [26]. (B) The HB and HH acyl-chain pockets. Thr18 and Tyr23 are replaced by Ala18 and Phe23 in *Chromobacterium viscosum* lipase [26] (darkened residues).

Fig. 5. Superposition of the modeled *S*<sub>c</sub>-trioctyl inhibitor on the *R*<sub>c</sub>-trioctyl compound to demonstrate the ligand substitutions. The *R*<sub>c</sub>-trioctyl compound has its carbon atoms in light grey and bonds in darker grey, the carbon atoms of the *S*<sub>c</sub>-trioctyl model are black, and the bonds are in light grey. The *sn*-nomenclature of the acyl chains is indicated: for the *S*<sub>c</sub>-trioctyl model within square brackets, for the *R*<sub>c</sub> compound without.

The presence of such unfavourable interactions might explain the observed preference for the *R*<sub>c</sub>-trioctyl compound has its carbon atoms in light grey and bonds in darker grey, the carbon atoms of the *S*<sub>c</sub>-trioctyl model are black, and the bonds are in light grey. The *sn*-nomenclature of the acyl chains is indicated: for the *S*<sub>c</sub>-trioctyl model within square brackets, for the *R*<sub>c</sub> compound without.

chain of the *S*<sub>c</sub> compound remains located in the HA pocket of the enzyme (Fig. 5). According to the stereospecific numbering (*sn*) of glycerol [37], this chain is the *sn*-1 chain in the *S*<sub>c</sub> compound, but the *sn*-3 chain in the *R*<sub>c</sub> compound. The *sn*-3 chain of the *S*<sub>c</sub>-trioctyl compound is now located in the HH pocket, and the *sn*-2 radyl part of this compound is located in the HB pocket (Fig. 5).

In the HH pocket the positions of the O5-C22(O6)-N2 atoms of the *R*<sub>c</sub>-trioctyl compound would be occupied by the C3-O3-C12(O7) atoms of the *S*<sub>c</sub>-trioctyl model. As a result the hydrophilic interaction of the *sn*-2 carbonyl oxygen (O6) with the Oγ1 atom of Thr18 would disappear. We cannot exclude that this interaction might be taken over by an interaction with the 0.36-nm-distant O3 atom. Most important, however, seems to be the orientation of the carbonyl oxygen (O7) in the *S*<sub>c</sub>-trioctyl model, since it would clash with the Cδ2 atom of the hydrophobic side chain of Leu287 (0.24 nm) and the Cδ1 atom of Ile290 (0.27 nm). Consequently, either the carbonyl oxygen (O7) of the compound or the side chains of the appropriate residues must reorient to prevent unfavourable protein–ligand interactions. The presence of such unfavourable interactions might explain the observed preference for the *R*<sub>c</sub>-trioctyl compound. The environment of the *sn*-1 and *sn*-2 substituents near the stereocenter of the *R*<sub>c</sub> compound (or of the *sn*-2 and *sn*-3 chains of the *S*<sub>c</sub> compound) is different: the HB pocket is more hydrophobic, and the HH pocket is more hydrophilic. This ob-
Design and construction of a serine protease triad forms the catalytic centre of a triacylglycerol lipase.


