Structural basis of the chiral selectivity of Pseudomonas cepacia lipase

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(Received 13 February/1 April 1998) — EJB 98 0222/3

To investigate the enantioselectivity of Pseudomonas cepacia lipase, inhibition studies were performed with \( S_\text{R} \) and \( R_\text{C}-(R_\text{C},S_\text{R})-1,2\)-dialkyacylкаромолglycerol-3-O-p-nitrophenyl alkylphosphonates of different alkyl chain lengths. \( P. cepacia \) lipase was most rapidly inactivated by \( R_\text{C}-(R_\text{C},S_\text{R})-1,2\)-dioctylcarbamoylglycerol-3-O-p-nitrophenyl octylphosphonate \((R_\text{C},\text{tributyl})\) with an inactivation half-time of 75 min, while that for the \( S_\text{R}-(R_\text{C},S_\text{R})-1,2\)-dioctylcarbamoylglycerol-3-O-p-nitrophenyl octylphosphonate \((S_\text{R},\text{tributyl})\) compound was 530 min. X-ray structures were obtained of \( P. cepacia \) lipase after reaction with \( R_\text{C} \)-tributyl to 0.29-nm resolution at pH 4 and covalently modified with \( R_\text{C}-(R_\text{C},S_\text{R})-1,2\)-dibutylcarbamoylglycerol-3-O-p-nitrophenyl butyl-phosphonate \((R_\text{C},\text{tributyl})\) 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 \((S_\text{R})\) in both complexes despite the use of a racemic \((R_\text{C},S_\text{R})\) mixture at the phosphorus atom of the triacylglycerol analogues. In the structure of \( R_\text{C},\text{tributyl}-\text{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 \( R_\text{C},\text{tributyl}\)-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, covalently complexed with organosulfates [7], organophosphates [8, 9], or organophosphonates [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 \( S_\text{R} \) and \( R_\text{C}-(R_\text{C},S_\text{R})-1,2\)-dialkyacylкаромолglycerol-3-O-p-nitrophenyl alkyl-phosphonates, with the alkyl chains consisting of either four carbon atoms (tributyl) or eight carbon atoms (tributyl). Guided by kinetic studies, the binding mode of the lipase with the \( R_\text{C},\text{tributyl}\) and \( R_\text{C},\text{tributyl}\) 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 \( R_{c} \)-triocetyl- and \( R_{c} \)-tributyl-complexed Pseudomonas cepacia lipase.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for complex</th>
<th>( R_{c} )-triocetyl</th>
<th>( R_{c} )-tributyl</th>
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</thead>
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<tr>
<td>Data collection statistics:</td>
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<td></td>
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<tr>
<td>resolution (nm)</td>
<td>5.0–0.29</td>
<td>3.0–0.175</td>
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</tr>
<tr>
<td>no. observations</td>
<td>22335</td>
<td>140279</td>
<td></td>
</tr>
<tr>
<td>no. unique reflections</td>
<td>6515</td>
<td>56937</td>
<td></td>
</tr>
<tr>
<td>( R )-merge (%)</td>
<td>9.2</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>( R )-merge (%) (last shell)</td>
<td>15.8</td>
<td>5.9</td>
<td></td>
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<tr>
<td>mosaicity (*)</td>
<td>0.4</td>
<td>0.3</td>
<td></td>
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<tr>
<td>completeness (%)</td>
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<td>95.3</td>
<td></td>
</tr>
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<td>Refinement:</td>
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<td></td>
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<tr>
<td>resolution range (nm)</td>
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<td>2.0–0.175</td>
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<tr>
<td>no. reflections</td>
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<td>56677</td>
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<tr>
<td>( R_{	ext{free}} ) (%)</td>
<td>20.7</td>
<td>17.8</td>
<td></td>
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<tr>
<td>( R_{	ext{free}} ) (no. reflections)</td>
<td>25.2 (606)</td>
<td>20.2 (1721)</td>
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</tr>
<tr>
<td>Model deviations from ideality:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>bond lengths (nm)</td>
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<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>bond angles (*)</td>
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<td>1.3</td>
<td></td>
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<tr>
<td>( B )-factors:</td>
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<td></td>
<td></td>
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<tr>
<td>( B )-value model</td>
<td>group B factors</td>
<td>individual isotropic</td>
<td></td>
</tr>
<tr>
<td>Mean ( B )-values:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>main chain atoms</td>
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<td>8.27 Å²</td>
<td></td>
</tr>
<tr>
<td>side chain atoms</td>
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<td></td>
</tr>
<tr>
<td>all protein atoms</td>
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<tr>
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<td>21.64 Å² (563)</td>
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<tr>
<td>ligand (no. atoms)</td>
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<td>5.27 Å² (14)</td>
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<tr>
<td>( \text{Ca}^{2+} )</td>
<td>8.66 Å²</td>
<td>14.68 Å²</td>
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Table 2. Rmsd in Ca positions of the different Pseudomonas cepacia lipase models.

<table>
<thead>
<tr>
<th>Alkyl phosphonate</th>
<th>( R_{c} )-tributyl</th>
<th>( R_{c} )-tributyl – model I</th>
<th>( R_{c} )-tributyl – model II</th>
<th>( P ). cepacia lipase</th>
</tr>
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<tbody>
<tr>
<td>( R_{c} )-tributyl</td>
<td>0.036</td>
<td>0.036</td>
<td>0.043</td>
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<tr>
<td>( R_{c} )-tributyl – model I</td>
<td>0.019</td>
<td>0.037</td>
<td></td>
<td></td>
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<tr>
<td>( R_{c} )-tributyl – model II</td>
<td>0.041</td>
<td></td>
<td></td>
<td></td>
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</table>

on the enzyme’s surface, allowing the factors that are important for this lipase’s stereopreferences to be rationalized.

**EXPERIMENTAL PROCEDURES**

**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 octanolate and 10 mM CaCl\(_2\) at pH 8.0 (modified after [18]). Activities were calculated from the increase in absorbance at 400 nm. The inhibitors \( R_{c} \)-tetra- or \( R_{c} \)-tributyl, \( S_{c} \)-(\( R_{c} \)-tributyl), \( S_{c} \)-(\( R_{c} \)-triocetyl) and \( R_{c} \)-(\( S_{c} \)-tributyl) were synthesised according to Mannesse et al. [19].

They are diastereomeric (\( S_{c} \) vs. \( R_{c} \)) at phosphorus, but enantiopure at the C2 atom of the glycerol (either \( R_{c} \)- or \( S_{c} \)-). All inhibition experiments on an analytical scale were done at 25°C. The kinetics of inactivation was followed by incubation of P. cepacia lipase (1–5 \( \mu \)M) in 10 mM Tris/HCl pH 8.0 containing 10 mM CaCl\(_2\) and 100 mM Triton X-100. The reaction was started by addition of the inhibitor from a concentrated stock solution in acetonitrile to a final concentration of 300 \( \mu \)M. 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].

**Preparation of the complexes and crystallization.** The lipase-trialkyl complexes were prepared by adding either the \( R_{c} \)-tributyl or the \( R_{c} \)-triocetyl 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.

\( R_{c} \)-Tributyl-inhibited lipase crystals grew from 20% 2-methyl-2,4-pentanediol, 100 mM CaCl\(_2\) in 100 mM Tris/HCl pH 8.5 at 12°C. They diffracted to 0.175-nm resolution on the wiggle beamline BW6 of the Max-Planck Institute at DESY in Hamburg using cryo-conditions (90 K). They are monoclinic, spacegroup \( P2_1 \), with cell dimensions \( a = 8.404 \text{ nm}, b = 4.636 \text{ nm}, c = 8.538 \text{ nm}\) and \( \beta = 116.53^\circ \). This corresponds to a \( V_M \) of 0.00225 nm\(^3\)/Da [21] assuming 2 molecules/asymmetric unit.

\( R_{c} \)-Triocetyl-inhibited lipase crystals grew from 14% isopropanol, 20 mM CaCl\(_2\) in 100 mM sodium acetate pH 4 at 12°C. They diffracted to 0.29-nm resolution on the X-31 beamline of the EMBL outstation at DESY in Hamburg, using cryo-conditions (120 K). They have the monoclinic spacegroup \( C2 \) with cell dimensions \( a = 8.873 \text{ nm}, b = 4.642 \text{ nm}, c = 8.395 \text{ nm}\) and \( \beta = 112.23^\circ \). This corresponds to a \( V_M \) of 0.00224 nm\(^3\)/Da [21] assuming 1 molecule/asymmetric unit.

**Crystal structure elucidation.** 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 \( R_{c} \)-triocetyl-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 \( R_{	ext{free}} \) of 32.6% and an \( R_{	ext{free}} \) of 33.6%. After positional refinement, bulk solvent correction and manual building of the \( R_{c} \)-triocetyl compound into a difference Fourier density map using the program O [24], the \( R_{	ext{free}} \) and the \( R_{	ext{free}} \) had decreased significantly. The final model includes all 320 amino acids, one \( \text{Ca}^{2+} \) ion, four water molecules (located inside the protein molecule) and the \( R_{c} \)-triocetyl 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.

RESULTS AND DISCUSSION

Inhibition and X-ray structure determination. The stereo-selectivity of *P. cepacia* lipase towards phosphonate triacylglycerol analogues was investigated with two stereoisomers of the triacylglycerol compound: *R*<sub>C</sub>-triacylglycerol and *S*<sub>C</sub>-triacylglycerol. The lipase was most rapidly inactivated by the *R*<sub>C</sub>-triacylglycerol inhibitor with an observed half-time of 75 min under conditions as described in Experimental Procedures. The corresponding *S*<sub>C</sub>-triacylglycerol stereoisomer inactivated the lipase with a half-time of 530 min. Since the *R*<sub>C</sub> stereoisomer reacts faster with the enzyme, only the *R*<sub>C</sub>-enantiomers of the triacylglycerol and the tributyl compound were used in the subsequent crystallization experiments. *P. cepacia* lipase, which has an amino acid sequence identical to the *P. cepacia* M-12-33 lipase [25, 26] and Nakanishi, Y., Kurono, Y., Kolde, Y. & Beppu, T. (1989) European patent no. 0331376, could be complexed and crystallized with *R*<sub>C</sub>-tributyl at pH 8.5 and with *R*<sub>C</sub>-triacylglycerol at pH 4.0. The statistics of data collection and refinement are summarized in Table 1.

The *R*<sub>C</sub>-tributyl-complexed lipase molecules crystallized as dimers in the asymmetric unit, diffracting to 0.175-nm resolution. After completion of the refinement there was no electron
The substitution of a phosphonate ester bond by the active-site Ser but they are racemic at the phosphorus atom (R) or S moiety. Phosphonate inhibition of serine hydrolases occurs via an in-line displacement reaction results in the formation of a negatively charged monoester between the active-site Ser and the phosphonate moiety positions (Table 2). In both molecules in the asymmetric unit the inhibitor is covalently bound, but surprisingly only the R-enantiomer as defined by Cahn and co-workers [34] has a slightly different conformation from that observed in native P. cepacia lipase (0.043 nm rms for Ca atoms), probably caused by the glycine flexibility, but the remainder of the protein is unchanged. The complete inhibitor is excellently visible in the difference electron density map (Fig. 1B). The R-enantiomeric structure differs by 0.036 nm in its Ca atoms positions from those in the molecules of the R-enantiomeric complex, an rms which is slightly higher than the coordinate error of ±0.02 nm in each structure as estimated from a Luzzati plot [29].

The overall topology of each structure resembles that of the open unliganded form of P. cepacia lipase (Protein Data Bank entries 2LIP, 3LIP and 10IL), 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 Ca 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.

**Oxyanion hole and catalytic triad.** The R-tributyl- and R-enantiomeric 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 arrangement of the catalytic residues, Ser87, His286, and Asp264, is similar to that of native P. cepacia lipase. However, the N2 atom of His286 is also in close contact (±0.27 nm) with the O1 atom of the bound phosphonate inhibitors (Fig. 2). This confirms the hypothesis, put forward by Cygler et al. [30], that the active-site His of the lipase is hydrogen-bonded to both the active-site Ser and the enantiomeric substrate which, in our case, is the R compound. This interaction would facilitate bond cleavage and the departure of the leaving group.

The side chain O1 atom of the catalytic Asp264 is at hydrogen-bonding distance from the O1 atom of Glu289 (Fig. 2), as previously described for native P. cepacia lipase [16] and the lipase from *Pseudomonas glumae* [31]. This latter lipase has an amino acid sequence identical to that of the *Chromobacterium viscosum* lipase. Because of the much higher resolution of the *C. viscosum* lipase structure (1.6 Å [32]), we have used this lipase instead of *P. glumae* lipase for our structural comparisons. Asp264 in *P. cepacia* lipase makes a further hydrogen bond to one of the internally located water molecules which bridge the catalytic triad residues with the carbonyl oxygens of Gly211, a residue located at C-terminus of β-strand 7 of the central β-sheet [16] and of Val267, one of the last residues of the substrate binding domain (see below). This hydrogen bonding network presumably contributes to the stabilization of the active site of this lipase.

**Stereochemistry of the inhibitors.** The triacylglycerol inhibitors used are enantiopure at the glycerol backbone C2 atom (R), but they are racemic at the phosphorus atom (R or S). However, the R-tributyl- and the R-enantiomered P. cepacia lipase structures show that only one phosphorus enantiomer (S) is observed. It is generally assumed that the mechanism of phosphonate inhibition of serine hydrolases occurs via an in-line displacement 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 S-enantiomer.

**The binding pockets.** The three-dimensional structure of the R-enantiomeric 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 \(\theta_2\) (O5-C2-C3-O3) and \(\theta_4\) (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.

spectively [36]. This represents the so called sc/βsc 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 $0.8 \times 1.0 \text{ nm}$ 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 β 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 a6 in the direct neighborhood of the lid helix a5 (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 Cδ 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 phosphonylalkyl...
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 \( R_C \)-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 \( S_C \)-trioctyl inhibitor on the \( R_C \)-trioctyl compound to demonstrate the ligand substitutions. The \( R_C \)-trioctyl compound has its carbon atoms in light grey and bonds in darker grey, the carbon atoms of the \( S_C \)-trioctyl model are black, and the bonds are in light grey. The \( sn \)-nomenclature of the acyl chains is indicated: for the \( S_C \)-trioctyl model within square brackets, for the \( R_C \) compound without.

Chain of the \( S_C \) 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 \( S_C \) compound, but the \( sn-3 \) chain in the \( R_C \) compound. The \( sn-3 \) chain of the \( S_C \)-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 \( R_C \)-trioctyl compound would be occupied by the C3-O3-C12(O7) atoms of the \( S_C \)-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 \( S_C \)-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 \( R_C \) over the \( S_C \)-trioctyl compound. The environment of the \( sn-1 \) and \( sn-2 \) substituents near the stereocenter of the \( R_C \) compound (or of the \( sn-2 \) and \( sn-3 \) chains of the \( S_C \) compound) is different: the HB pocket is more hydrophobic, and the HH pocket is more hydrophilic. This ob-
servation underscores the notion by Studler et al. [38] that substrate engineering at the sn-2 position may change the stereoselectivity of lipases.

The results of the modelling experiments described above might explain why *P. cepacia* lipase shows a sevenfold preference for the (R)-inhibitor over the (S)-inhibitor, which corresponds to an sn-3 preference. The stereoselectivity is however dependent on many more factors. For example, Rogalska and co-workers [39] reported a clear sn-1 preference for *P. cepacia* lipase acting on trioctanoylglycerol and trioleoylglycerol emulsions. We used the (R)- and (S)-enantiomers of 2-decanoylamido-1-decanoyldecanol, which can be regarded as tridecanoylglycerol analogues containing only one hydrolysable ester bond [19]. We tested these substrates as mixed micelles in the presence of Triton X-100 essentially as described before for cutinase [19]. No stereopreference for either the (R)- or the (S)-enantiomer was found (data not shown). Thus the stereoselectivity of *P. cepacia* lipase seems to be dependent on the chemical nature and/or the physical state of the substrate, as has been observed before for other lipases [38, 40].

**Comparison with homologous lipases.** Comparison of the amino acids involved in binding of the different parts of the triacylglycerol analogue reveals important differences between members of the *Pseudomonas* lipase family [26]. Between *P. cepacia* lipase and *P. aeruginosa* lipase [41], Leu17→Met and Val266→Leu substitutions influence the size and the width of the HA pocket, where the sn-3 fatty acid chain binds (Fig. 4A). The Val266→Leu substitution is also one of the amino acid differences between *P. cepacia* lipase and *P. glumae* lipases, others being two amino acid substitutions in the HH pocket (Thr18→Ala and Tyr23→Phe) that might reduce the hydrophobicity of this pocket in *P. glumae* lipase and affect the interaction with the substrate’s sn-2 carbonyl oxygen atom (Fig. 4B).

**CONCLUSIONS**

The present crystal structures provide valuable information on the factors that are important for the stereoselectivity of *P. cepacia* lipase. A hydrophobic, well defined groove is the binding site for the sn-3 fatty acid chain of the substrate. The binding site for the sn-2 chain is subdivided into a small hydrophilic patch where, at the bottom of a cleft, the ester bond region is bound and a larger hydrophobic patch towards the surface, where the hydrophobic part of the sn-2 fatty acid chain is bound. The sn-2 chain is separated from the sn-1 acyl chain by residues from the calcium binding loop. The small sn-1 binding site is slightly hydrophobic, and has few interactions with the inhibitor. Among the members of the Pseudomomadaceae family the size and/or the ratio between hydrophobicity and hydrophilicity of these sn-2 and sn-3 binding sites vary, allowing the different regio- and enantio-specificities of these lipases to be rationalized. This result may be helpful for bacterial lipase engineering to improve their industrial applicability in bioconversion reactions.

We thank the co-workers at the European Molecular Biology Laboratory (EMBL) and the Max-Planck Institute in Hamburg for their support with data collection. We thank the European Union for support of the work at EMBL Hamburg through the European Union BIOTECH program (contract number BIO2 CT94-3013) and the Human Capital and Mobility Program Access to Large Installations Project, contract number CHGE-CT93-0040.

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