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 $S_{c}$- and $R_{c}-(R_{c},S_{s})$-1,2-dialkylcarbamoylglcyero-3-$O$-$p$-nitrophenyl alkylphosphonates of different alkyl chain lengths. P. cepacia lipase was most rapidly inactivated by $R_{c}-(R_{c},S_{s})$-1,2-dioctylcarbamoylglcyero-3-$O$-$p$-nitrophenyl octylphosphonate ($R_{c}$-trioctyl) with an inactivation half-time of 75 min, while that for the $S_{c}-(R_{c},S_{s})$-1,2-dioctylcarbamoylglcyero-3-$O$-$p$-nitrophenyl octylphosphonate ($S_{c}$-trioctyl) compound was 530 min. X-ray structures were obtained of P. cepacia lipase after reaction with $R_{c}$-trioctyl to 0.29-nm resolution at pH 4 and covalently modified with $R_{c}-(R_{c},S_{s})$-1,2-dibutylcarbamoylglcyero-3-$O$-$p$-nitrophenyl butyl-phosphonate ($R_{c}$-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 ($S_{c}$) in both complexes despite the use of a racemic ($R_{c},S_{s}$) mixture at the phosphorus atom of the triacylglycerol analogues. In the structure of $R_{c}$-tributyl-complexed P. cepacia lipase, the diacylglycerol moiety has been lost due to an aging reaction, and only the butyl phosphate remains visible in the electron density. In the $R_{c}$-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 α/β hydrolase fold, a structural motif common to a wide variety of hydrolases [3]. Their active sites contain a catalytic triad, Ser-His-Asp/Glu, similar to those of serine proteases [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 $S_{c}$- and $R_{c}-(R_{c},S_{s})$-1,2-dialkylcarbamoylglcyero-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 $R_{c}$-trioctyl and $R_{c}$-tributyl compounds was analyzed by X-ray crystallography. For the first time this resulted in a lipase structure with fatty acid chains bound...
on the enzyme’s surface, allowing the factors that are important for this lipase’s stereopreference 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 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 Rₐ-(Rₛ,Rₛ)-1,2-dibutylcarbamoylglycerol-3-O-p-nitrophenyl butylyphosphonate (Rₛ-tributyl), Sₛ-(Rₛ,Sₛ)-1,2-dioctylcarbamoylglycerol-3-O-p-nitrophenyl octylphosphonate (Sₛ-trioctyl) and Rₛ-(Sₛ,Sₛ)-1,2-dioctylcarbamoylglycerol-3-O-p-nitrophenyl octylphosphonate (Rₛ-trioctyl) were synthesised according to Mannesse et al. [19]. They are diastereomeric (Sₛ,Rₛ) at phosphorus, but enantiopure 0.8-nm resolution which gave an overall agreement factor of 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_cryst (38) of 21.64 Å (563) and Rₐ-(Rₛ,Sₛ) (38) of 28.47 Å (41) and Rₛ-(Sₛ,Sₛ) (38) of 14.68 Å (2). Rₐ-(Rₛ,Sₛ)-tributyl-inhibited lipase crystals grew from 20% 2-methanol, 20 mM CaCl₂ in 100 mM Tris/HCl pH 8.5 at 12°C. They diffracted to 0.175-nm resolution on the wiggler beamline BW6 of the Max-Planck Institute at DESY in Hamburg using cryo-conditions (90 K). They are monoclinic, spacegroup P₂₁, with cell dimensions a = 8.404 nm, b = 4.636 nm, c = 8.395 nm and β = 116.53°. This corresponds to a Vₜₐₐₛ of 0.00225 nm³/Da [21] assuming 2 molecules/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 Rₛ-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_cryst of 32.6% and an R_mol of 33.6%. After positional refinement, bulk solvent correction and manual building of the Rₛ-trioctyl compound into a difference Fourier density map using the program O [24], the R_cryst and the R_mol had decreased significantly. The final model includes all 320 amino acids, one Ca²⁺ ion, four water molecules (located inside the protein molecule) and the Rₛ-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 Rₛ-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 \textit{P. cepacia} lipase, produced with BOBSCRIPT [42]. (A) F\textsubscript{o}-F\textsubscript{c} omit map of the \textit{R}\textsubscript{C}-tributyl compound, contoured at 2 \(\sigma\). Only a bound butylphosphonate moiety is visible. (B) 2F\textsubscript{o}-F\textsubscript{c} omit map of \textit{R}\textsubscript{C}-trioctyl compound, contoured at 1 \(\sigma\). From left to right the \textit{sn}-3, \textit{sn}-1 and \textit{sn}-2 chains are shown.

RESULTS AND DISCUSSION

Inhibition and X-ray structure determination. The stereo-selectivity of \textit{P. cepacia} lipase towards phosphonate triacylglycerol analogues was investigated with two stereoisomers of the triacylglycerol compound: \textit{R}\textsubscript{C}-triacylglycerol and \textit{S}\textsubscript{C}-triacylglycerol. The lipase was most rapidly inactivated by the \textit{R}\textsubscript{C}-triacylglycerol inhibitor with an observed half-time of 75 min under conditions as described in Experimental Procedures. The corresponding \textit{S}\textsubscript{C}-triacylglycerol stereoisomer inactivated the lipase with a half-time of 530 min. Since the \textit{R}\textsubscript{C} stereoisomer reacts faster with the enzyme, only the \textit{R}\textsubscript{C}-enantiomers of the triacylglycerol and the tributyl compound were used in the subsequent crystallization experiments. \textit{P. cepacia} lipase, which has an amino acid sequence identical to the \textit{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 \textit{R}\textsubscript{C}-tributyl at pH 8.5 and with \textit{R}\textsubscript{C}-triacylglycerol at pH 4.0. The statistics of data collection and refinement are summarized in Table 1.

The \textit{R}\textsubscript{C}-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...
Oxyanion hole and catalytic triad. The \(R_e\)-tributyl- and \(R_e\)-trioctyl-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 phosphoryl 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 \(N_e\) atom of His286 is also in close contact (\(\approx 0.27\) nm) with the \(O_1\) 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 enantio-preferred substrate which, in our case, is the \(R_e\) compound. This interaction would facilitate bond cleavage and the departure of the leaving group.

The side chain \(O_1\) atom of the catalytic Asp264 is at hydrogen-bonding distance from the \(O_1\) atom of the complexed lipase structure (\(0.043\) nm rms for \(C_{\alpha}\) atoms), 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 \(C_2\) atom (\(R_e\)), but they are racemic at the phosphorus atom (\(R_p\) or \(S_p\)). However, the \(R_e\)-tributyl- and the \(R_e\)-trioctyl-complexed \(P. cepacia\) lipase structures show that only one phosphorus enantiomer (\(S_p\)) is observed. It is generally assumed that the mechanism of phosphonate inhibition of serine hydrolases involves the formation of a phosphonate monoester between the active-site Ser and the phosphonate moiety as defined by Cahn and co-workers [34]. Nevertheless, this interaction would facilitate bond cleavage and the departure of the leaving group.

The crystals of the \(R_e\)-trioctyl-complexed lipase differ by 0.29 nm. A solvent-accessible glycerine-rich loop (residues 199-202) has a slightly different conformation from that observed in native \(P. cepacia\) lipase (0.043 nm rms for \(C_{\alpha}\) 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 \(R_e\)-tributyl-inhibited structure differs by 0.036 nm in its \(C_{\alpha}\) atom positions from those in the molecules of the \(R_e\)-tributyl complex, an rmsd 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 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 \(C_{\alpha}\) 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.

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Enantiomeric selectivity. From the kinetic investigations, we know that the inactivation of *P. cepacia* lipase by the \( S \)-trioctyl compound is sevenfold slower than the inhibition by the \( R \)-trioctyl compound. To obtain information about the stereoisomeric discrimination of the enzyme between the \( S \) and \( R \)-trioctyl compounds, we modelled the \( S \)-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 \textit{P. cepacia} lipase as deduced from the observed binding mode of the \textit{R}_{C}-triacyl inhibitor. The fatty acid part is abbreviated as \( R \), the glycerol part as \( R' \). Residues in \textit{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 \textit{P. aeruginosa} lipase [26]. (B) The HB and HH acyl-chain pockets. Thr18 and Tyr23 are replaced by Ala18 and Phe23 in \textit{Chromobacterium viscosum} lipase [26] (darkened residues).

Fig. 5. Superposition of the modeled \textit{S}_{C}-triacyl inhibitor on the \textit{R}_{C}-triacyl compound to demonstrate the ligand substitutions. The \textit{R}_{C}-triacyl compound has its carbon atoms in light grey and bonds in darker grey, the carbon atoms of the \textit{S}_{C}-triacyl model are black, and the bonds are in light grey. The \textit{sn}-nomenclature of the acyl chains is indicated: for the \textit{S}_{C}-triacyl model within square brackets, for the \textit{R}_{C} compound without.

The presence of such unfavourable interactions might explain the observed preference for the \textit{R}_{C}- over the \textit{S}_{C}-triacyl compound. The environment of the \textit{sn}-1 and \textit{sn}-2 substituents near the stereocenter of the \textit{R}_{C} compound (or of the \textit{sn}-2 and \textit{sn}-3 chains of the \textit{S}_{C} compound) is different: the HB pocket is more hydrophilic, and the HH pocket is more hydrophobic. This ob-
ervation 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-decanoylamilido-1-dodecanoyldecanol, 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 lipase, 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 Pseudomonadaceae 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.

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