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Structural investigations of the regio- and enantioselectivity of lipases

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Abstract

Although lipases are widely applied for the stereospecific resolution of racemic mixtures of esters, the atomic details of the factors that are responsible for their stereospecificity are largely obscure. We determined the X-ray structures of *Pseudomonas cepacia* lipase in complex with two enantiopure triglyceride analogues, that closely mimic natural substrates. This allowed an unambiguous view of how the two wings of the boomerang-shaped active site accommodate the acyl and alcohol parts of the triglyceride. The binding groove for the hydrophobic *sn*-3 fatty acid chain is large and hydrophobic. The cleft for the alcohol moiety is divided in two parts, one tightly binding the *sn*-2 acyl chain with hydrophilic and hydrophobic interactions, the other more weakly binding the *sn*-1 fatty acid. The enantioselectivity of *Pseudomonas cepacia* lipase seems therefore to be predominantly determined by the size and interactions of the *sn*-2 chain and by the size of the *sn*-3 chain.

Keywords: Lipase; Enantioselectivity; Stereospecificity; Crystal structure; Active site architecture; X-ray crystallography

1. Introduction

Over the years the use of enzymes as biodegradable and stereospecific catalysts has been increasing significantly, with world-wide sales volumes of industrial enzymes expected to be doubling in the next 10 years (Godfrey and West, 1996). Seventy five percent of the enzymes of industrial interest are hydrolases. After proteases and carbohydrases, lipases (E.C. 3.1.1.3) have the third largest sales volume. Their major application is the hydrolysis of fat stains in laundry, but they are also extensively used to synthesize a wide variety of esters, or to hydrolyze esters other than triglycerides (Theil, 1995). For example, lipases are used

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to stereospecifically hydrolyze ester bonds of compounds such as acetyl-arylpropionic acid esters, which are precursors for the nonsteroidal anti-inflammatory agents naproxen and ibuprofen (Bando et al., 1997).

To tailor lipases for specific applications knowledge of their three-dimensional structures and the factors that determine their regio- and enantiospecificity are essential. Crystal structure determinations of a number of lipases and related esterases (Sussman et al., 1988; Brady et al., 1990; Winkler et al., 1990; Schrag et al., 1991; Martinez et al., 1992; Grochulski et al., 1993; Noble et al., 1993; van Tilbeurgh et al., 1993; Uppenberg et al., 1994; Lang et al., 1996; Kim et al., 1997; Schrag et al., 1997) have shown that these enzymes share the folding pattern of the α/β hydrolases (Ollis et al., 1992), with an active site consisting of a Ser–His–Asp/Glu catalytic triad.

Subsequent structural investigations concerned the catalytic mechanism of lipases. Lipolytic enzymes are characterized by their drastically increased activity when acting at the lipid-water interface of micellar or emulsified substrates (Desnuelle, 1972), a phenomenon called interfacial activation. This increase in enzymatic activity is triggered by structural rearrangements of the lipase active site region, as witnessed from crystal structures of lipases complexed with small transition state analogues (Brzozowski et al., 1991; Derewenda et al., 1992; van Tilbeurgh et al., 1993). The movement of a single helix (Brzozowski et al., 1991; Derewenda et al., 1992), or two helices (Kim et al., 1997; Schrag et al., 1997), as well as a loop region (Grochulski et al., 1994b) exposes a large hydrophobic surface that is presumed to interact with the lipid interface. Another feature shown by all members of the α/β hydrolase superfamily is the presence of an oxyanion hole, which stabilizes the transition state via hydrogen bonds with at least two main chain nitrogen atoms.

Further research on the action of lipases was directed towards the identification of the binding regions of the acyl and alcohol portions of the substrate and the rationalization of the observed enantioselectivity of various lipases. The X-ray structures of *Rhizomucor miehei* lipase complexed with a C6 phosphonate inhibitor (Derewenda et al., 1994), of *Candida rugosa* lipase with a long sulfonyl chain (Grochulski et al., 1994a), of the human pancreatic lipase/colipase complex covalently inhibited by the two enantiomers of a C11 alkyl chain phosphonate (Egloff et al., 1995) and of porcine pancreatic lipase covalently inhibited by ethylene glycol monooctylether (Hermoso et al., 1996) represent important steps to mimic the natural tetrahedral intermediates. However, none of those compounds resembled a true triglyceride. A first structural view of lipase stereoselectivity towards secondary alcohols was obtained by Cygler et al. (1994) who succeeded in complexing (*R*)- and (*S*)-menthyl ester transition state analogues to *Candida rugosa* lipase. In the fast-reacting, (*R*)-enantiomer a hydrogen bond is present between the alcohol oxygen of the substrate and the NE2 atom of the active site histidine. This hydrogen bond is absent in the slow-reacting, (*S*)-enantiomer and therefore it was suggested that this hydrogen bond would be responsible for the stereospecificity of the enzyme. In contrast, Uppenberg et al. (1995) carried out X-ray and modeling studies on *Candida antarctica* type B complexed with a long-chain polyoxyethylene detergent. They found from molecular dynamics calculations that the hydrogen bond between substrate and active site histidine, first found by Cygler et al. (1994), is also present in *C. antarctica* lipase, but not only in the fast reacting (*R*)-enantiomer but also in the slow reacting (*S*)-enantiomer. Therefore those authors concluded that the enzyme's enantioselectivity cannot simply be explained by the presence or absence of this hydrogen bond. Finally Longhi et al. (1997) made a complex of cutinase, a small, non interfacially active lipolytic enzyme, with an enantiopure triglyceride analogue with three C4 alkyl chains, R_C-(R_P, S_P)-1,2-dibutylcarbamoylglycero-3-O-*p*-nitrophenyl butylphosphonate (TC4, (Mannesse et al., 1995)). Although this inhibitor was expected to reveal the stereospecific substrate interactions with the protein, it unfortunately was bound in the active site in an exposed position, with the alkyl chains not interacting with any

amino acid residues of the enzyme. Thus, despite all this work, there is not yet an agreement on the factors that are responsible for the stereospecificity of these lipases.

Among the bacteria that secrete lipases the members of the family of *Pseudomonadaceae* (Gilbert, 1993) show distinct differences in regio- and enantioselectivity (Rogalska et al., 1993), despite a high amino acid sequence homology. In addition these lipases are widely used in industry, especially for the production of chiral chemicals which serve as basic building blocks in the synthesis of pharmaceuticals, pesticides and insecticides (Theil, 1995). Rogalska et al. (1993) found that the lipase from *Chromobacterium viscosum* (which is identical to the *Pseudomonas glumae* lipase (Taipa et al., 1995)) reacts unspecifically (*sn*-3 (*R*)/*sn*-1 (*S*)) with trioctoin, whereas the lipases from *Pseudomonas cepacia* (PCL) and *Pseudomonas aeruginosa* (Jaeger et al., 1994) are absolutely specific for *sn*-1 fatty acid chains of natural substrates.

To resolve how a triglyceride binds in the active site and to determine the factors which are responsible for the stereospecificity of *Pseudomonas* lipases we have determined the X-ray structures of PCL complexed with the TC4 transition state analogue of Mannesse et al. (1995), as well as with an analogue of medium alkyl chain length, R_C-(R_P,S_P)-1,2-dioctylcarbamoyl-glycero-3-O-*p*-nitrophenyl octylphosphonate (TC8). These compounds are enantiopure at the *sn*-2 position, but are racemic at the phosphorous atom. Our structural results allow us to describe in detail all the interactions between enzyme and triglyceride analogues and the properties of the alkyl binding pockets. This knowledge allows the rationalization of the stereospecificity of various homologous lipases.

2. Experimental procedures

The lipase from *Pseudomonas spec*. ATCC 21808 (recently renamed as *Burkholderia cepacia*) is identical to the lipase from PCL. It was obtained from the American Type Culture Collection (Rockville, MD) and purified as described previously (Kordel et al., 1991). Preparation of the complexes with TC4 and TC8, co-crystallization and structure elucidation will be published elsewhere (Lang et al., 1998). The PCL-TC4 complex was crystallized at pH 8.5 with two molecules in the asymmetric unit, whereas the PCL-TC8 complex forms crystals at pH 4 with one molecule per asymmetric unit. The diffraction limit of the two crystal forms is different: the PCL-TC4 complex diffracts to 1.75 Å and the PCL-TC8 complex to 2.9 Å. The difference in the diffraction limit might be caused by the crystal packing and/or the pH of crystallization. The structure elucidation was done in both cases by molecular replacement using AMoRe (Collaborative Computational Project Number 4, 1994), using native PCL (PDB entry 3LIP (Schrag et al., 1997)) as the starting model (Table 1). Initial (2Fo–Fc) and (Fo–Fc) maps showed clear electron density connected to the side chain of the catalytic Ser 87 in each molecule, indicating the presence of a covalently bound phosphonate inhibitor. Only the S_P enantiomer of both the TC4 and TC8 inhibitor could be fitted into the electron density map, indicating that only one enantiomer at the phosphorous atom of the inhibitors had reacted.

3. Results and discussion

3.1. The PCL-TC4 complex

The PCL-TC4 complex crystallizes in space group $P2_1$ with two molecules in the asymmetric unit. In both molecules, no density was observed for the N-terminal residue, Ala 1, which was excluded from the model. The overall structure of both molecules of the PCL-TC4 complex is very similar to that of the native *P. cepacia* lipase (Protein Data Bank entry 3LIP), as indicated by the low Cα root-mean square (r.m.s.) difference of 0.39 Å between both non-crystallographic symmetry-related molecules and the 3LIP coordinates (Table 2). The final 1.75 Å model did not reveal any differences in the amino acid sequences of the lipases from *Pseudomonas spec*. strain ATCC 21808 and PCL.

Table 1
Results of the molecular replacement (data between 4 and 10 Å)

	α	β	γ	t1	t2	t3	CC	R-factor
Pseudomonas cepacia lipase-TC4 complex								
Rotation (°)	23.6	45.5	10.1					
Translation				0.26	0.0	0.27	0.64	0.35
Refined	24.9	46.3	9.1	0.26	0.0	0.25	0.69	0.33
Pseudomonas cepacia lipase-TC8 complex								
Rotation (°)								
Molecule 1	49.2	156.5	74.0					
Molecule 2	130.8	23.5	253.9					
Translation								
Molecule 1				0.24	0.0	0.49		
Molecule 2				0.26	0.0	0.01	0.82	0.35
Refined								
Molecule 1	48.7	157.2	74.0	0.24	0.0	0.49		
Molecule 2	128.3	23.5	255.9	0.26	0.0	0.01	0.84	0.33

The two protein molecules in the asymmetric unit are related by a 2-fold axis (Fig. 1a). Several water molecules are present in the interface between the two molecules, mediating hydrogen bonds between them. The most conspicuous residue in this interface is Gln 191. Its OE1 atom makes a direct hydrogen bond to the other molecule (Gln 191 OE1… 2.76 Å… NH 198 Thr), while its NE2 atom is part of a contact via a bridging water molecule (Fig. 1b). As a consequence of the close contacts between the two molecules the antiparallel part of β-strand 8 (residues 196–198, Schrag et al. (1997)) has shifted somewhat and the r.m.s. difference of Cα-positions compared with those in the native structure is more than 1.0 Å in this region. This conformational flexibility is probably facilitated by the presence of several glycine residues in this region.

Table 2
Interactions of water molecules in the interface between the non-crystallographically related molecules in the PCL-TC4 complex

Thr D192 CO	2.74 Å	W562	2.73 Å	CO E182 Ala
Ala D182 CO	2.68 Å	V565	2.78 Å	CO E192 Thr
Gln E191 NE2	2.98 Å	W568	2.73 Å	OG1 D196 Thr
			2.65 Å	OG1 D198 Thr
Gln D191 NE2	2.99 Å	V571	2.62 Å	OG1 E198 Thr
			2.76 Å	OG1 E196 Thr
Thr D198 CO	2.50 Å	W671	3.14 Å	OE1 E191 Gln
			2.43 Å	CO E189 Ser

Like native PCL the active site of the TC4 complex is in the open conformation. The hydrophobic substrate binding site is made up of side chains in the loop formed by residues 228 to 237 and of side chains of the putative lid helix α5 (residues 142 to 149, Schrag et al. (1997)). The open conformation is stabilized by crystal contacts of this α5-helix to a symmetry related α5-helix, interacting via the side chains of Phe 142, Val 145 and Leu 149. This is different from other lipases, where the large hydrophobic surfaces, which are created around the active sites when the lids open, are orientated face-to-face to each other, thereby locking the symmetry related lids in the open conformation (Brzozowski et al., 1991; Grochulski et al., 1993; Lawson et al., 1994; Longhi et al., 1997).

To our surprise only the butyl phosphonate part (*sn*-3 moiety) of the TC4 inhibitor was visible in the electron density; no density was present for the glycerol moiety with the two C4-alkyl chains. As no indications were present for even weak density for the glycerol moiety extending from the

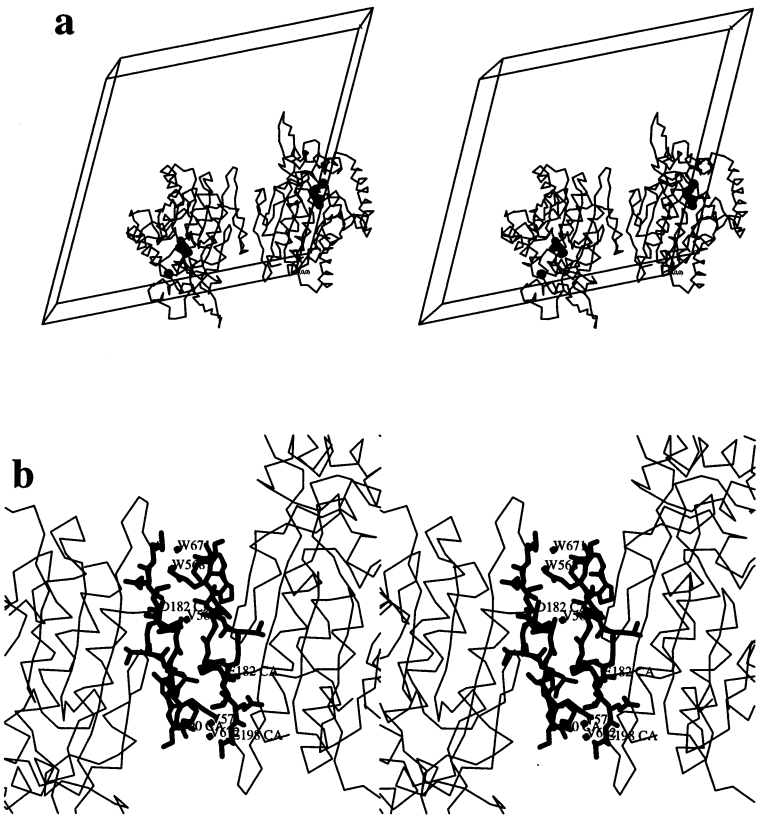

Fig. 1. (a) Stereo-figure of the Cα traces of the two non-crystallographic related molecules of the PCL-TC4 complex in the asymmetric unit. The atoms of the inhibitors are shown as filled spheres. The Ca^{2+} ion is represented as a black ball located adjacent to the active site pocket. (b) Stereo-figure of a close-up of the interface between the two molecules of the PCL-TC4 complex related by non-crystallographic symmetry. Water molecules are indicated by black crosses. The amino acid side chains involved in intermolecular contacts are high-lighted (see Table 2).

phosphonate group, this suggests that the glycerol phosphonate ester has been cleaved. Since the PCL-TC4 crystals grew very slowly (30 days), this could easily have happened during the crystallizations. Phosphonate cleaving reactions have been observed before in serine proteases, where a nucleophilic substitution of a phosphonate ester bond by the active site serine can occur (Kovach et al., 1991; Bencsura et al., 1995). Nevertheless, the butyl phosphonate moiety could be unambiguously positioned in the electron density. It defines the acyl pocket of the enzyme. The oxyanion hole is occupied by one of the phosphorous oxygen atoms, which is 2.6 Å from the NH group of Leu 17 and 2.8 Å from the NH group Gln 88. The C4 atom of the inhibitor contacts the CB atom of Leu 17 (4.1 Å) and the C5 and C6 atoms are in close van der Waals contact to Val 266 (4.0 and 3.9 Å, respectively).

3.2. The PCL-TC8 complex

The PCL-TC8 complex crystallizes in the monoclinic space group *C*2 with one molecule per asymmetric unit. The enzyme is in the open conformation, similar to that observed for the PCL-TC4 complex. Density for the complete TC8 inhibitor is visible (Fig. 2). The substrate binding region of PCL has good density for the loop region (228–237), but less and weaker interactions for the two interacting symmetry related $\alpha 5$ helices.

The presence of the complete TC8 inhibitor allows us to unambiguously define the substrate binding mode of *P. cepacia* lipase. The boomerang shaped active site (Kim et al., 1997; Schrag et al., 1997) is divided into a large hydrophobic groove in which the acyl chain snugly fits and a part that embeds the inhibitor's alcohol moiety. The alcohol binding pocket can be subdivided into a mixed hydrophilic/hydrophobic cleft for the *sn*-2 moiety of the substrate and a smaller hydrophobic groove for the *sn*-1 chain. The bound lipid analogue assumes the bent tuning fork conformation preferred by lipids at an interface (Pascher, 1996). As the *sn*-2 pocket provides the most intimate interactions with the substrate, we propose that this pocket is the one that predominantly determines the enzyme's stereopreferences. A further pocket constitutes the oxyanion hole. It is occupied by a phosphonate oxygen, which has the same interactions as in the TC4 complex.

Superposition of the TC8 conformation in PCL with the cutinase-TC4 complex (Longhi et al., 1997) shows that, except for the oxyanion hole, the two complexes do not very well match. This is probably caused by the absence in cutinase of interactions of the acyl chains with the enzyme (Fig. 3).

3.3. Enantiomeric selectivity

PCL is one of the most widely used enzymes for the enantiomeric resolution of esters of secondary alcohols (Theil, 1995). It has a preference for the R_C-trioctyl compound (R_C-TC8) over the S_C-trioctyl compound (S_C-TC8): it is inhibited seven times faster by R_C-TC8 than by S_C-TC8 (Lang et al., 1998). This demonstrates that also the less preferred enantiomer can productively be bound in the active site. Since no structure is available of a PCL–S_C–TC8 complex, we modeled the S_C compound in the active site of PCL by a simple substituent exchange at the glycerol C2 atom position in the PCL–R_C–TC8 complex. In this modeled PCL–S_C–TC8 complex the acyl chain bound to the primary hydroxyl group of the glycerol moiety clashes with the hydrophobic side chains of Leu 287 and Ile 290. To prevent this unfavorable interaction either the amino acid side chains have to be moved out of the way or the substrate should undergo a conformational change, for instance via a rotation about a single C–C bond.

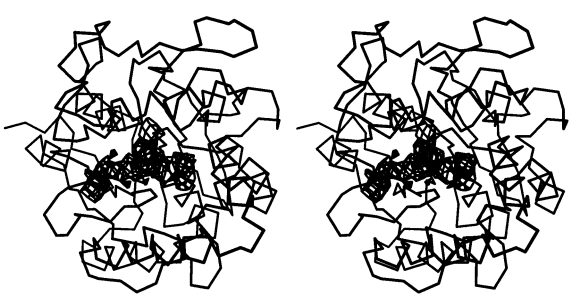

Fig. 2. SigmaA weighted (Fo–Fc)—electron density map of the PCL-TC8 complex calculated without contributions of the inhibitor to the Fc's and phases. The electron density defines the various active site pockets that are responsible for the enzyme's stereopreferences.

Fig. 3. Superposition of Cα traces of the PCL-TC8 complex (thick lines) with the cutinase-TC4 complex (thin lines). The binding modes of the inhibitors do not very well agree, probably because the acyl chains of the inhibitor in the cutinase-TC4 structure do not contact any protein residues. The Ca^{2+} ion is represented as a single black sphere.

Hirose et al. (1995) have carried out site-directed-mutagenesis experiments to probe the importance of various amino acid residues for the stereoselectivity of PCL. They succeeded in changing the enzyme's enantioselectivity from an R_C to S_C specificity by introducing a combination of three mutations, Val266Leu, Leu287Ile and Phe221Leu. Val 266 is located at the entrance of the acyl pocket (*sn*-3 pocket), while Leu 287 is at the beginning of the *sn*-2 pocket. Phe 221 is at the surface of the enzyme, about 20 Å away from the inhibitor. While the Leu287Ile and Val266Leu

substitutions can be envisaged to affect the size and width of the *sn*-2 and *sn*-3 pockets, respectively, Phe 221 seems too far away to directly influence the enzyme's stereospecificity. Nevertheless, the Phe221Leu mutation on its own was reported to slightly decrease the enzyme's enantioselectivity (Hirose et al., 1995). Clearly, further research is required to elucidate the role of Phe 221 as an enantioselectivity determining factor.

In the past, it has been found that PCL shows the largest enantioselectivity if one of the substituents differs significantly in size from the other (Kazlauskas et al., 1991). This observation can now be rationalized, the enzyme contains a large hydrophobic groove in which the *sn*-3 acyl chain fits, a mixed hydrophilic/hydrophobic cleft for the *sn*-2 moiety of the substrate and a smaller hydrophobic groove for the *sn*-1 chain. The differences in size and the hydrophilicity/hydrophobicity of the various pockets determine the enzyme's enantio- and regiopreferences.

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