

Anatomy of lipase binding sites: the scissile fatty acid binding site

Jürgen Pleiss, Markus Fischer, Rolf D. Schmid *

Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany

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Abstract

Shape and physico-chemical properties of the scissile fatty acid binding sites of six lipases and two serine esterases were analyzed and compared in order to understand the molecular basis of substrate specificity. All eight serine esterases and lipases have similar architecture and catalytic mechanism of ester hydrolysis, but different substrate specificities for the acyl moiety. Lipases and esterases differ in the geometry of their binding sites, lipases have a large, hydrophobic scissile fatty acid binding site, esterases like acetylcholinesterase and bromoperoxidase have a small acyl binding pocket, which fits exactly to their favorite substrates. The lipases were subdivided into three sub-groups: (1) lipases with a hydrophobic, crevice-like binding site located near the protein surface (lipases from *Rhizomucor* and *Rhizopus*); (2) lipases with a funnel-like binding site (lipases from *Candida antarctica*, *Pseudomonas* and mammalian pancreas and cutinase); and (3) lipases with a tunnel-like binding site (lipase from *Candida rugosa*). The length of the scissile fatty acid binding site varies considerably among the lipases between 7.8 Å in cutinase and 22 Å in *Candida rugosa* and *Rhizomucor miehei* lipase. Location and properties of the scissile fatty acid binding sites of all lipases of known structure were characterized. Our model also identifies the residues which mediate chain length specificity and thus may guide protein engineering of lipases for changed chain length specificity. The model was supported by published experimental data on the chain length specificity profile of various lipases and on mutants of fungal lipases with changed fatty acid chain length specificity. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Modeling; Structure; Chain length specificity; Mutant; Esterase; Protein engineering

1. Introduction

Abbreviations: CALB, *Candida antarctica* lipase B; PCL, *Pseudomonas cepacia* lipase; HuPL, human pancreatic lipase; RML, *Rhizomucor miehei* lipase; CRL, *Candida rugosa* lipase.

* Corresponding author. Tel.: +49 711 6853193; fax: +49 711 6853196; e-mail: Rolf.D.Schmid@rus.uni-stuttgart.de

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) catalyze hydrolysis of long chain, insoluble triglycerides and other insoluble esters of fatty acids (Schonheyder and Volqvartz, 1945; Woolley

and Petersen, 1994; Schmid and Verger, 1998) with a varying chain length specificity (Rangheard et al., 1989; Berger and Schneider, 1991; Kirk, et al., 1992). In contrast, esterases are restricted to water soluble esters of carboxylic acids.

X-ray structure investigations have shown that all lipases are members of the α/β hydrolase fold family (Brzozowski et al., 1991; Derewenda et al., 1992; Martinez et al., 1992; Ollis et al., 1992; Grochulski et al., 1994; Egloff et al., 1995; Uppenberg et al., 1995; Schrag et al., 1997). Other members of this family (Murzin et al., 1995) are *Pseudomonas sp.* diene lactone hydrolase (Pathak and Ollis, 1990), the cholinesterases (Sussman et al., 1991), *Vibrio harveyi* thioesterase (Lawson et al., 1994a), *Streptomyces aureofaciens* bromoperoxidase A2 (Hecht et al., 1994) and *Streptomyces scabies* esterase (Wei et al., 1995). The α/β hydrolase fold consists of a central hydrophobic eight-stranded β -sheet packed between two layers of amphiphilic α -helices. α/β hydrolases have a common catalytic mechanism of ester hydrolysis, which consists of five subsequent steps (Cyglér et al., 1994): after binding of the ester substrate, a first tetrahedral intermediate is formed by nucleophilic attack of the catalytic serine, with the oxyanion stabilized by two or three hydrogen bonds¹, the so-called oxyanion hole. The ester bond is cleaved and the alcohol moiety leaves the enzyme. In a last step, the acyl enzyme is hydrolyzed. The nucleophilic attack by the catalytic serine is mediated by the catalytic histidine and aspartic (or glutamic) acid. Exceptions are diene lactone hydrolase, where the nucleophile is cysteine instead of serine and *Streptomyces scabies* esterase, where a main chain carbonyl group replaces the carboxylic acid. The so-formed catalytic triad is placed on top of the β -sheet. The geometry of the catalytic machinery is highly conserved. In most lipases, a mobile element covers the catalytic site in the inactive form of the lipase. This 'lid' consists of one or two short α -helices linked to the body of the lipase by flexible structure elements. In the open, active form of the lipase, the lid moves away and makes the binding

site accessible to the substrate. The lid is not found in some lipases and in both esterases.

All lipases accept esters of medium (C4) to long chain (C16) fatty acids, some of them efficiently hydrolyze fatty acid esters as long as C22. Others have esterase activity, thus accepting short acetyl moieties. In order to understand the specificity of lipases, they have been investigated by X-ray crystallography, computer-assisted modeling and protein engineering. For five lipases, complexes with substrate analogous phosphonate, phosphate or sulfonate inhibitors have been published in the Protein Data Bank (Bernstein et al., 1977), which help to identify the binding site for the scissile fatty acid. Binding of substrates has been modeled for lipases from *Rhizopus*, *Rhizomucor miehei* and *Humicola lanuginosa* (Vasel et al., 1993; Norin et al., 1994; Lawson et al., 1994b; Haalck et al., 1997; Holzwarth et al., 1997). The location of the scissile fatty acid binding site has been supported by mutants of lipases from *Rhizopus* and *Humicola lanuginosa* with an altered fatty acid chain length profile (Joerger and Haas, 1994; Atomi et al., 1996; Martinelle et al., 1996; Klein et al., 1997).

In order to understand the molecular basis of substrate specificity of lipases and esterases, we have systematically compared geometry and properties of the scissile fatty acid binding site of six representative lipases and of two esterases. This model also supports design of lipase catalyzed reactions, for a given reaction, the appropriate lipase may be selected based on its desired specificity and second generation biocatalysts with improved properties can be engineered.

2. Methods

2.1. Geometry of the binding site

2.1.1. Superposition of protein structures

Protein sequences were obtained from the SWISS-PROT sequence database (Bairoch and Boeckmann, 1991), protein structures from the Protein Data Bank (Bernstein et al., 1977). Only structures of the open form of lipases were selected. If inhibitor complexes were available, the

¹ Only one oxyanion hole residue in thioesterase (Lawson et al., 1994a).

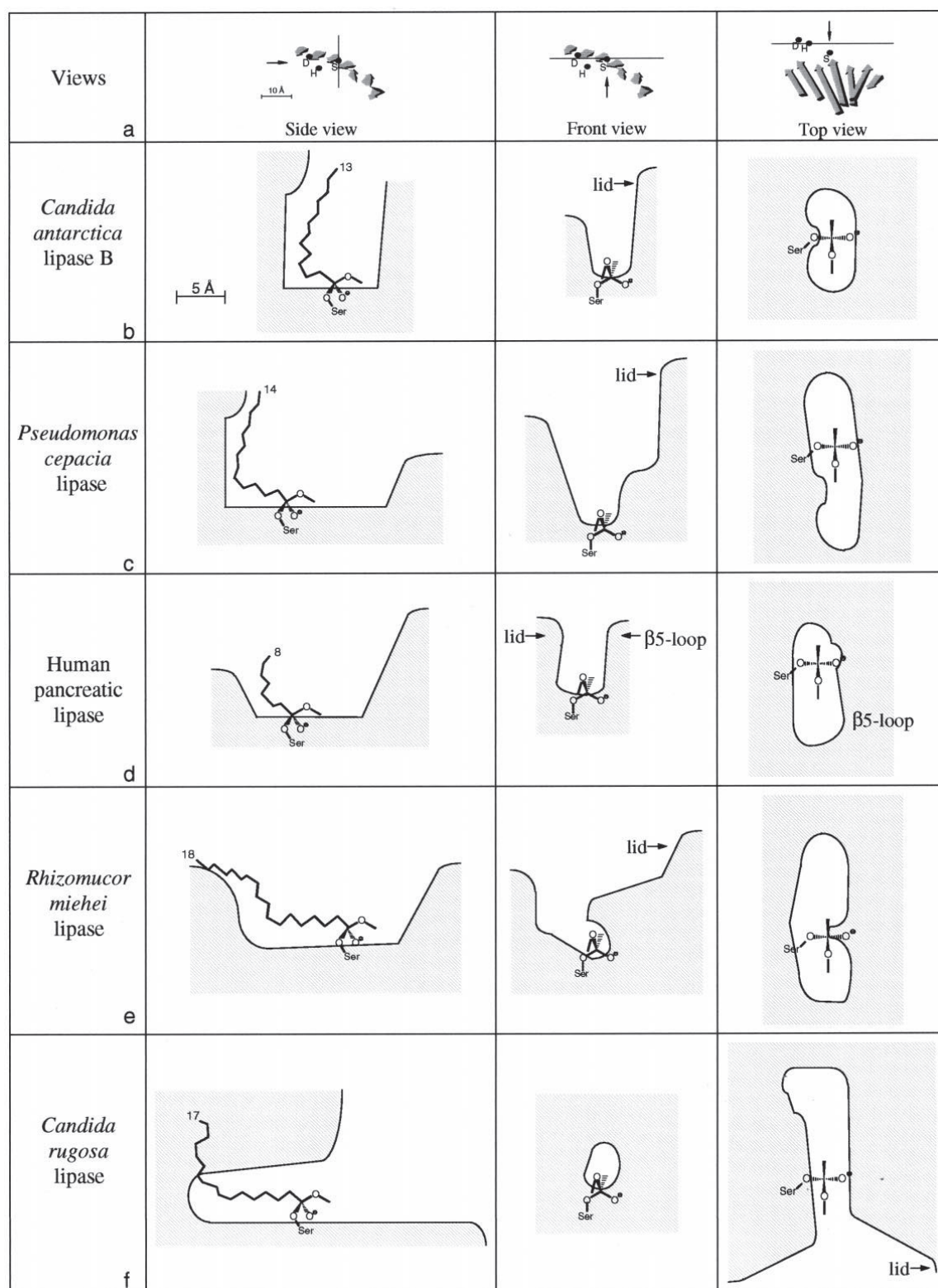


Fig. 1. Shape of the binding site of eight serine esterases and lipases. (a) Orientation of the cross-sections which are planes perpendicular to the paper plane and indicated by a straight line. The direction of the view is indicated by an arrow. The example is given for CALB, (b)–(i) shape of the binding sites in side, front and top view. In (b)–(g), only a model of the fatty acid is displayed, the alcohol moiety of an ester substrate is not shown for clarity. A number indicates the length of the longest fatty acid which completely binds inside the binding pocket.

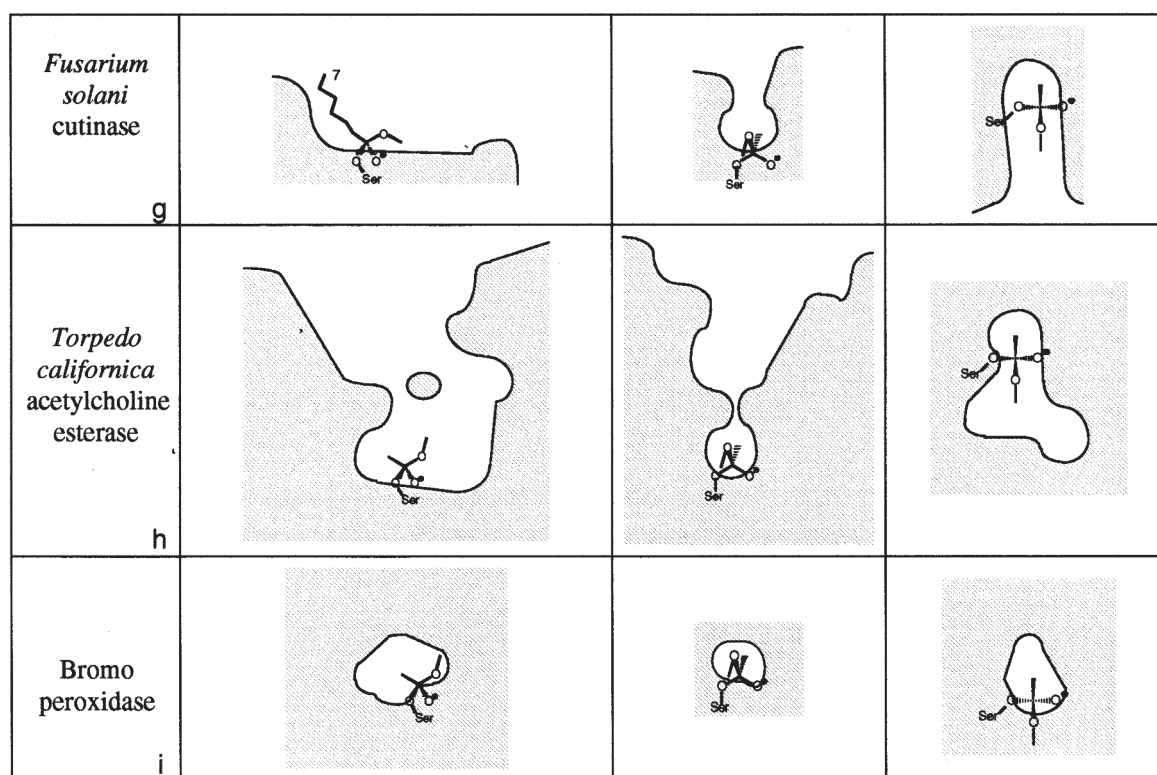


Fig. 1. (Continued)

structure with the longest scissile fatty acid analog was chosen (Table 1). The structures were superimposed using ProExplore (Oxford Molecular Group, Oxford, UK). In a first round, the following atoms were used as reference: C_{α} atoms of a pentapeptide with the catalytic serine in the center (e.g. G142–G146 for catalytic S144 in *R.miehei* lipase), of the catalytic histidine and its nearest neighbors (e.g. D256–L258 in *R.miehei* lipase for H257 as catalytic histidine) and of the catalytic aspartic or glutamic acid (e.g. D203 in *R.miehei* lipase), as well as the N–H atoms of the additional residue of the oxyanion hole (S82 in *R.miehei* lipase). Based on this superposition, the most structurally conserved four residues of the central β -strand were identified (Y172–G175 in *R.miehei* lipase). In a second round, the superposition was refined taking as reference the C_{α} atoms of these four residues, two additional residues of the catalytic serine-containing β -strand (e.g. V140, T141 in *R.miehei* lipase) and the set of atoms of the first round.

2.1.2. Shape of the binding sites

To compare the shape of the binding sites, the Connolly surface (Connolly, 1983) of the proteins was calculated excluding the inhibitor (if present) at a probe radius of 1.5 Å and visualized by InsightII (Molecular Simulations, San Diego, CA). For each protein, three perpendicular ‘views’ were generated, which are cross-sections of 2 Å thickness containing the phosphorus atom (sulfur in the case of *Candida rugosa* lipase) of the bound inhibitor. For all serine esterases and lipases, the same planes were used, which were derived from the structure of *Candida antarctica* lipase B.

For the ‘side view’, a plane was defined by three inhibitor atoms (phosphorus and two neighboring atoms, carbon and ester oxygen) (Fig. 1a). This view visualizes the inhibitor atoms near the catalytic site. The ‘front view’ is a plane perpendicular to the ‘side view’ plane and includes the phosphorus, the serine side chain oxygen atom and the oxyanion analogue. This view goes along the axis of the inhibitor or substrate molecule, looking

Table 1
Representative serine esterases and lipases investigated in this study: PDB entry, substrates and catalytic machinery

Enzyme	PDB entry	Inhibitor (in PDB)	Substrate	Catalytic triad	Oxyanion hole	Reference
<i>Candida antarctica</i> lipase	1LBS	<i>N</i> -hexylphosphonate-ethylester	Triglycerides	S105, H224, D187	T40, Q106	Uppenberg et al., 1995
<i>Pseudomonas cepacia</i> lipase	2LIP	None	Triglycerides	S87, H286, D264	L17, Q88	Schrag et al., 1997
Human pancreatic lipase	1LPB	Undecanephosphonatemethylester	Triglycerides	S153, H264, D177	F78, L154	Egloff et al., 1995
<i>Rhizomucor miehei</i> lipase	5TGL	<i>N</i> -hexylphosphonate-ethylester	Triglycerides	S144, H257, D203	S82, L145	Brzozowski et al., 1991
<i>Candida rugosa</i> lipase	1LPO	Hexadecanesulfonate	Triglycerides	S209, H449, E341	G124, A210	Grochulski et al., 1994
<i>Fusarium solani</i> cutinase	1XZM	<i>N</i> -undecyl- <i>O</i> -methyl-chlorophosphonate	Cutin triglycerides	S120, H188, D175	S42, Q121	Longhi et al., 1996
<i>Torpedo californica</i> acetylcholinesterase	1AMN	M-(<i>N,N,N</i> -Trimethyl-ammonio)	Acetylcholine	S200, E327, H440	G119, A201	Harel et al., 1996
<i>Streptomyces aureofaciens</i> bromoperoxidase	1BRO	None × trifluoro-acetophenone	Acetate	S98, H257, D228	F32, M99	Hecht et al., 1994

from the alcohol moiety toward the acyl moiety. The ‘top view’ is perpendicular to the side and the front view and presents a view down the entrance to the binding site. Schematic views were generated by manually fitting the Connolly surfaces by straight lines and circular segments.

2.2. Modeling of fatty acid complexes

2.2.1. Probing the binding sites

The properties of the lipase binding sites were investigated using the program GRID (Goodford, 1985) using the METHYL and DRY probes to identify narrow and hydrophobic patches, respectively. If an inhibitor molecule was present in the experimental structure, it was removed from the binding site prior to calculation. The interaction potential calculated by GRID is a combination of steric repulsion, electrostatic interaction and hydrogen bonding. Thus, GRID allows a quantitative analysis of the binding properties of protein surfaces. Isopotential surfaces at -2.2 kcal/mol (METHYL probe) and -0.22 kcal/mol (DRY probe) were visualized by the graphical interface InsightII. Schemes of the property of the putative scissile fatty acid binding site were generated by manually fitting the predicted binding areas by straight lines.

2.2.2. Substrate docking

Models of saturated fatty acid methyl esters in the tetrahedral intermediate state were built using the molecule builder of SYBYL (Tripos, St. Louis, MO). The models were placed in the binding site of all lipases by superposing the tetrahedral carbon atom of the polarized carbonyl group to the phosphorus or sulfur atom of the inhibitor in the X-ray structure, which were available in all lipases but *P.cepacia* lipase. In the latter, the ester group was placed in a similar orientation as in *R.miehei* lipase. The geometry of the fatty acid chain was manually adjusted guided by the conformation of the complexed inhibitor. Fatty acids longer than the inhibitor were placed along the inhibitor, then in the region predicted by the METHYL probe directing toward the region predicted by the DRY probe and finally following the DRY probe (Fig. 2). If possible, we assigned

the torsion angles to *trans*. The build-up process of the substrate model was stopped when the end of the hydrophobic binding site, as determined by GRID, or the end of the binding pocket was reached.

3. Results

3.1. Representative serine esterases and lipases

At the time of this publication, 54 structures of 25 serine esterases and lipases have been published in the Protein Data Bank. They can be grouped in 11 families of homologous proteins: (1) *Candida antarctica* lipase B; (2) *Pseudomonas* lipases (*P. cepacia*, *P. glumae*); (3) mammalian pancreatic lipases (human, porcine, horse, guinea pig); (4) filamentous fungi lipases (*Rhizomucor miehei*, *Rhizopus delemar*, *Rhizopus niveus*, *Humicola lanuginosa*, *Penicillium camembertii*); (5) other yeast lipases (*Candida rugosa*, *Geotrichum candidum*) and the structurally similar *Candida cylindracea* cholesterol esterase; (6) *Fusarium solani* cutinase; (7) acetylcholinesterases (*Torpedo californica*, *Electrophorus electricus*, mouse)²; (8) *Streptomyces aureofaciens* bromoperoxidase A2; (9) *Pseudomonas* sp. dienelactone hydrolase; (10) *Vibrio harveyi* thioesterase; and (11) *Streptomyces scabies* esterase. One representative enzyme structure of families 1 to 8 was selected for comparison (Table 1). For all lipases, with the exception of *Pseudomonas* lipases, at least one structure of a complex with a substrate-analogous inhibitor is available, which was selected for our study. Families 9, 10 and 11 were excluded from in this study, since for dienelactone hydrolase and thioesterase, the location of the scissile fatty acid can not be deduced from the structure of the free enzyme, in the structure of dienelactone hydrolase, the putative scissile fatty acid binding site is blocked by F173 and W88, while in thioesterase it is probably located in a tunnel inside the enzyme (Lawson et al., 1994a). The catalytic machinery of

² Interestingly, family 5 is homologous to family 7, although the substrate specificity of lipases and cholinesterases are completely different.

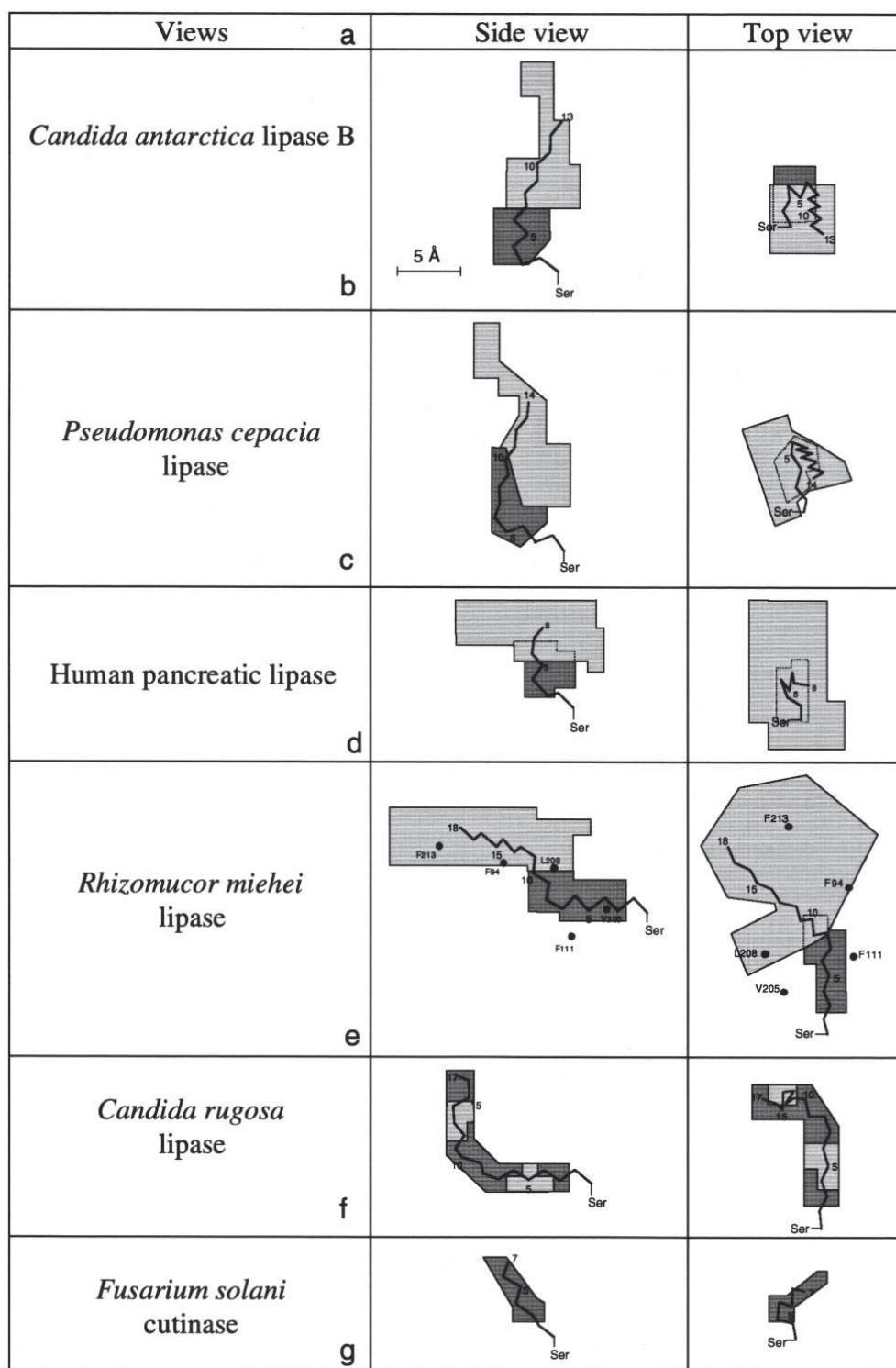


Fig. 2. Schematic view of GRID data (METHYL probe: dark grey, DRY probe: light grey) with position of modeled fatty acid chains, views as indicated in Fig. 1. In RML (e) the position of five residues is marked as dots which indicate residues in the scissile fatty acid binding site which have been shown to mediate chain length specificity in the homologous RDL and ROL.

Streptomyces scabies esterase deviates from the other serine esterases and thus cannot be compared directly.

3.2. The scissile fatty acid binding sites

In all serine esterases and lipases investigated here, the binding site is located inside a pocket on top of the central β sheet. In lipases, the protein surface at the border of this pocket is hydrophobic and is supposed to interact with the hydrophobic substrate interface. Esterases and lipases differ in size of this hydrophobic interaction area as well as in shape, deepness and physico-chemical properties of their substrate binding pocket. In this study, we have compared the binding pockets of eight serine esterases and lipases and modeled binding of the scissile fatty acid chain (Fig. 1). We have not modeled the ω -end of long-chain fatty acids exceeding the binding pocket.

The substrate binding pocket of *Candida antarctica* lipase B (CALB) is an elliptical, steep funnel of 9.5×4.5 Å. The substrate is oriented parallel to its long axis. The walls of the tube differ in height, viewed along the alcohol–acid axis (Fig. 1b, front view), the wall on the left-hand side is 6 Å high. The lid constitutes the right-hand wall (10.5 Å). The experimentally determined phosphonate inhibitor of CALB fits with its ethanol moiety to the scissile fatty acid binding site, which is also predicted by the METHYL probe (Fig. 2b). Thus, up to C4, the position of the scissile fatty acid can be deduced from the position of the inhibitor. It binds to a narrow cleft (as determined by the METHYL probe) at the hydrophilic bottom of the funnel, which is formed by D134 and the catalytic S105. It is lined by T138, I189 and V190 on its left-hand side, by Q157 and the oxyanion hole residue T40 on its right-hand side (Fig. 1b, front view). At the end of this cleft, near Q157, the fatty acid kinks sharply and follows the left-hand wall (Fig. 1b, side view). Up to C7, the binding site is a narrow cleft, from C7 to C13 it becomes smooth and hydrophobic. This hydrophobic binding site is formed by V154, I285, L144 and V149. Its width decreases with the distance from the active site with its minimum at C13 of the fatty acid chain.

Here, at 13.5 Å above the bottom, it reaches the end of the funnel and turns into the hydrophobic surface of the enzyme, which probably is in contact with the hydrophobic substrate interface. The height of the wall at the alcohol binding site is 10.5 Å. The substrate accesses the binding pocket from the upper right direction in the front view.

The binding pocket of *Pseudomonas cepacia* lipase (PCL) is an elliptical funnel with a length of 17 Å. Its width at the base is the same as in CALB (4.5 Å) and increases to 10.5 Å at the entrance to the binding site. The left and the right-hand walls viewed along the alcohol–acid axis are 10.5 and 16.5 Å, respectively (Fig. 1c, front view). The lid constitutes the right-hand wall. The substrate binds up to C6 to a narrow cleft (Fig. 2c) at the hydrophilic bottom of the funnel, which is formed by P113 and the catalytic S87. It is lined by V266/V267 and L167/L17 (oxyanion hole) on its left and right-hand side, respectively (Fig. 1c, front view). At the end of this cleft near S117 and A120, the fatty acid kinks sharply and binds to the left-hand wall (Fig. 1c, side view) in a cleft up to C9 and then to a hydrophobic binding site from C10 to C14, which is formed by F119, V123 and L164. In contrast to CALB, the hydrophobic binding site does not narrow. At 10.5 Å, it reaches the end of the funnel and follows an overhang onto the top of the protein surface. The alcohol binding site is more shallow and gently rises up to 5 Å. Access of the substrate to the binding pocket is from the top.

The funnel-like binding pocket of human pancreatic lipase (HuPL) (13×4.5 Å at its base) is less deep (7 Å) than in PCL. Its left-hand wall (Fig. 1d, side view) is the lowest of all lipase binding sites (4.5 Å). In addition, HuPL is the only lipase where this left-hand wall is lower than both the left and the right-hand walls in the front view. The lid forms the left-hand wall at the alcohol binding site (Fig. 1d, front view) instead of the right-hand wall in the other lipases. The β 5-loop lies opposite to the lid and constitutes the right-hand wall of the alcohol binding site (Fig. 1d, front view). The position of the alkyl moiety of the experimentally determined undecanephosphonate inhibitor is used to model a fatty acid

chain of length C8. C2–C4 of the fatty acid binds a cleft at the bottom of the funnel, as predicted by the METHYL probe (Fig. 2d). The bottom of this cleft is formed by A179 and the catalytic S153. At the end of this cleft, the fatty acid kinks and follows a hydrophobic binding site starting with C6 along the left-hand wall in the side view (Fig. 1d), which is formed by P181, until it reaches at C7 the top of this wall. Both the left and right-hand walls in the front view are hydrophobic. C8 binds to the right-hand wall in the front view, formed by Q157 and the oxyanion hole residue F78, where the funnel ends. The left-hand wall is built by F216 and I210. The wall at the alcohol binding site is higher (10.5 Å) than in PCL. Access of the substrate to the binding pocket is from the top.

The binding pocket of *Rhizomucor miehei* lipase (RML) is a shallow bowl with a long axis of 18 Å and a width of 4.5 Å at its base and 6 Å at the protein surface. The left-hand wall (Fig. 1e, front view) is 8 Å high. The right-hand wall, where the lid is located, rises up to 12 Å. The alkyl moiety of the hexylphosphonate inhibitor in the experimental structure fits to the region predicted by the METHYL probe. The scissile fatty acid up to C8 binds to a cleft at the bottom of the binding site (Fig. 2e). Its bottom is formed by the side chains of residues P177, H108 and the catalytic S144. It is lined by V205 and D91 on its left and right-hand side, respectively. From C8 to C10, the fatty acid chain rises at the left-hand wall (Fig. 1e, side view) and enters a hydrophobic crevice up to C18 (length 9.5 Å), which runs parallel to the surface of the protein. Its width of 5.5 Å at the bottom gives the fatty acid room for movement. Its bottom is formed by residues P209 and P210, its left and right-hand wall by L208 and F94/F213, respectively. The height of the alcohol binding site wall is 8 Å. The substrate accesses the binding pocket from the top.

The binding pocket of *Candida rugosa* lipase (CRL) is exceptional and completely differs from those of the other lipases. Here, the scissile fatty acid binding site is located in a tunnel inside the protein with a wide entrance at the right-hand side (Fig. 1f, side view). This tunnel is at least 22 Å long with a diameter of about 4 Å. In the free

CRL, this tunnel can hardly be predicted, since it is blocked by side chains. It is formed by G124 (oxyanion hole), F125, the catalytic S209, A210 (oxyanion hole), M213, V245, P246, F296, S301, L302, R303, L304, L307, F345, Y361, F362, S365, F366, V409, L410, L413, G414, F415, F532 and V534. The length of the hexadecyl moiety of the inhibitor, which has been crystallized with CRL, corresponds to a fatty acid of chain length C17. The active site serine lies just behind the entrance to the tunnel, which narrows near C3 and closely contacts the fatty acid chain up to its ω -end. The most hydrophobic parts of the tunnel are located from C4 to C7 and C12 to C14. In contrast to the other lipases, the alcohol binding site is not limited by a wall (right-hand side in Fig. 1f, side view). The lid is located at the lower right-hand side (Fig. 1f, top view) and not at the right-hand wall (front view) like in the other lipases. The substrate accesses the binding pocket from the right-hand side (Fig. 1f, side view).

The substrate binding pocket of cutinase is 16 Å long and 4.5 Å wide at its base and narrows to 2 Å at the protein surface. The left and right-hand walls are 6 and 8 Å high, respectively. A fatty acid chain of length C7 is modeled according to the experimentally determined undecanephosphonate inhibitor. Up to C3, it binds to a cleft at the bottom of the binding site (METHYL probe), formed by T150, the catalytic S120 and the catalytic H188. It is lined by V177/V184 and N84/S42 (oxyanion hole) on the left and right-hand side, respectively (Fig. 1g, front view). The fatty acid chain follows the left-hand wall (Fig. 1g, side view) built by L182. Here, the binding funnel ends. The binding funnel is of moderate hydrophobicity. The major hydrophobic binding region is found at the protein surface, which probably is in contact with the substrate interface. The alcohol binding site is flat.

In spite of the high homology between acetylcholinesterase and CRL, the shape of their binding sites differ considerably. The substrate binding site of acetylcholinesterase is a deeply buried cave in the protein (Fig. 1h, side and front view) with a small acyl and a large alcohol binding site at a width of 3.5 and 7.5 Å, respectively (Fig. 1h, top view). Its length is 10 Å as measured along the

alcohol–acid axis. The substrate accesses the binding site from the lower right direction (Fig. 1h, top view) through a narrow channel.

The binding site of bromoperoxidase is a small, ellipsoid-like cavity inside the protein. It is 7 Å long (measured along the alcohol–acid axis), 4 Å wide and 6.5 Å high. The alcohol binding site is very small. The acid binding site is also short, kinks to the upper left (Fig. 1i, front view) and opens into a narrow channel which leads to the protein surface. However, estimations derived from the structure of the free enzyme may underestimate the size of the binding site.

4. Discussion

Since the first biochemical characterization of lipases (Schonheyder and Volqvartz, 1945; Sarda and Desnuelle, 1958), they have been defined as long chain triglyceride hydrolyzing enzymes, thereby being distinguished from esterases which hydrolyze soluble esters. They have a higher activity toward insoluble substrates than toward the same substrates in monomeric form, which has been termed ‘interfacial activation’. However, the capability of lipid hydrolysis does not always coincide with the occurrence of interfacial activation (Verger, 1997).

All serine esterases and lipases are remarkably similar in structure and function (Ollis et al., 1992; Murzin et al., 1995). They use a common enzymatic mechanism, with the catalytic machinery located at the bottom of a deep, elliptical binding pocket, where the ester substrates binds with its acid–alcohol axis parallel to the long axis of the binding pocket. Its short axis has the same width of 4–4.5 Å in all serine esterases and lipases investigated here. It has the appropriate size to fix the ester substrate. The length of the acyl binding site varies from 3.5 Å in acetylcholinesterase and bromoperoxidase to 22 Å in *Candida rugosa* and *Rhizomucor miehei* lipase. Thus the enzymes investigated here can be grouped in two classes by the size of their acyl binding sites: (1) Esterases, which hydrolyze short chain acyl moieties like acetyl, propionyl and butyryl moieties; and (2) lipases, which accept medium and long chain fatty acids.

Substrate specificity is reflected in the shape of the acyl binding site. Esterases have a small acyl binding pocket which optimally fits to the acyl moiety of their substrates. Decreasing its size would lead to steric conflicts with the substrate, whereas increasing its size would leave free space leading to sub-optimal binding of substrates and thus decreasing $k_{\text{cat}}/K_{\text{m}}$, which has been shown recently for a mutant of acetylcholinesterase (Pleiss et al., 1998). Lipases have a long, hydrophobic scissile fatty acid binding site located inside the binding pocket (in a crevice, at the wall of a binding funnel or in a tunnel). Cutinase has a much less hydrophobic fatty acid binding site inside the binding pocket, thus resembling the esterases. The shape of its pocket, however, is lipase-like. Thus, the properties of the cutinase binding pocket are a combination of lipases and esterases.

These findings have two consequences, which are described in more detail below: (1) Only a small number of residues are lining the scissile fatty acid binding site inside the binding pocket. Mutation of these residues is expected to change chain length specificity in a predictable way; and (2) Length and hydrophobicity of this binding site should correlate with fatty acid chain length profile of the respective lipase. Therefore, the chain length specificity could be predicted from the structure of the lipase, as far as the chain length of the substrate under investigation is shorter than the scissile fatty acid binding site inside the binding pocket. As the chain length exceeds the binding pocket, the conformation of the fatty acid chain cannot be predicted, since it may bind to the hydrophobic protein surface, stick into the hydrophobic substrate interface or even fold back into the binding pocket. If such complexes are crystallized, the conformation of the exterior part of the substrate may be mediated by crystal contacts.

4.1. Mutants of the scissile fatty acid binding site in lipases of filamentous fungi

Table 2 summarizes previous experimental data on mutants of lipases from *Rhizopus delemar* and *Rhizopus oryzae* with changed chain length specifi-

Table 2

Mutations in the scissile fatty acid binding site of lipases from *Rhizopus delemar* and *Rhizopus oryzae* and their effect on chain length specificity. Equivalent residues in the homologous RML are given in parentheses

Lipase source	Mutation	Structural effect of the mutation (Fig. 2e)	Experimental observation	Reference
<i>R. oryzae</i>	F95Y (F94)	Blocks binding at C12	60% (30%) Increase of caproic acid methyl ester relative to oleic (stearic) acid	Atomi et al., 1996
<i>R. delemar</i>	F95D (F94)	Hydrophilic at C12	2-Fold decrease of hydrolysis	Joerger and Haas, 1994
<i>R. delemar</i>	F95D (F94) F214R (F213)	Right wall of hydrophobic crevice becomes hydrophilic (C10–C16)	3-Fold increase of tricaprylin relative to triolein	Klein et al., 1997
<i>R. delemar</i>	F112W (F111)	Blocks binding at C6	50% Increase of tributyrin relative to triolein	Joerger and Haas, 1994
<i>R. delemar</i>	F112Q (F111)	Hydrophilic at C6	no activity	Klein et al., 1997
<i>R. delemar</i>	V206T (V205)	Hydrophilic at C4	10–20% activity	Joerger and Haas, 1994
<i>R. delemar</i>	V209W (L208)	Blocks binding at C10	2-Fold increase of tributyrin relative to triolein	Joerger and Haas, 1994
<i>R. delemar</i>	V209W (L208) F112W (F111)	Blocks binding at C10 and C6	80-Fold increase of tributyrin relative to tricaprylin; no triolein hydrolysis	Klein et al., 1997
<i>R. oryzae</i>	F214Y (F213)	Blocks binding at C16	20% Increase of caproic acid methyl ester relative to oleic and stearic acid	Atomi et al., 1996

city. Comparison to our model of substrate binding to the scissile fatty acid binding site (Fig. 2e) demonstrates that the experimental effect in all mutants can be explained by the position of the residue and its interaction with the fatty acid chain. V205 lines the cleft at the bottom of the binding pocket and is positioned near C4 of the scissile fatty acid chain. Replacing it by the hydrophilic threonine decreases activity 5- to 10-fold. Replacing F111, which is near to C6, by tryptophan increases the relative specificity for short-chain triglycerides. L208 and F94 line the hydrophobic crevice near C10 and C12. Replacing them by bulkier and more hydrophilic residues decreases activity and increases relative specificity for fatty acids of short and medium chain length. F213 is located at the end of the hydrophobic crevice near C16. Replacing it by the more bulky and hydrophilic tyrosine reduces relative specific-

ity for oleic and stearic acid.

As a strategy to predict mutants with specificity for chain lengths below a given cutoff, binding of longer chains can be inhibited by increasing size and polarity of a residue positioned at the ω -end of the longest chain to be accepted.

4.2. Other factors which may influence fatty acid chain length specificity

The scissile fatty acid binding site is not the only determinant of chain length specificity. Mutations near the active site and the lid region have also shown to mediate chain length specificity. Replacing the oxyanion hole residue T83 by serine in *R. delemar* decreases tributyrin activity 4-fold relative to triolein (Joerger and Haas, 1994). Replacing the lid residue W89 in *H. lanuginosa* lipase by smaller and more polar residues increases tri-

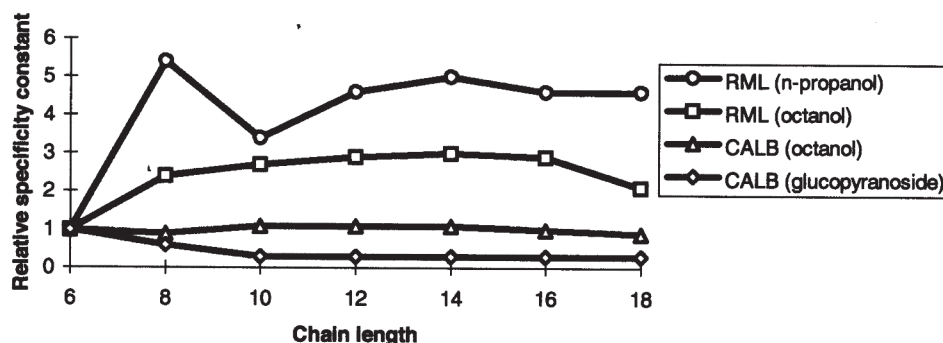


Fig. 3. Relative specificity constants k_{cat}/K_m of RML and CALB (normalized to C6) for fatty acid chains of varying length; RML, alcoholysis of monoester by n-propanol (Rangheard et al., 1989) and esterification of octanol in hexane (Kirk et al., 1992); CALB, esterification of octanol in hexane (Kirk et al., 1992) and esterification of ethyl D-glucopyranoside (Kirk et al., 1992).

acetin and trioctanoin activity relative to tributyrin (Martinelle et al., 1996). These findings are in agreement with experimental observations, that lipase activity and specificity can be influenced by many factors like water activity (Halling, 1994), immobilization support (Adlercreutz, 1991) and quality of the substrate interface (Verger, 1997). Mutations of the active site and of the lid may interfere with one or several of these factors by changing the interaction of the lipase with the substrate interface, when activity is measured at a water/oil interface. However, experimental setups which measure competitive factors α in organic solvent (Rangheard et al., 1989) have been shown to exclude these secondary effects. For a pair of substrates, α corresponds to the ratio of the specificity constants k_{cat}/K_m . In alcoholysis and esterification experiments, α depends on the fatty acid moiety rather than on reaction conditions and the chemical structure of the alcohol (Rangheard et al., 1989).

4.3. Fatty acid chain length specificity of RML and CALB

The fatty acid chain length specificity of RML and CALB in alcoholysis and esterification reactions of monoesters has been studied in organic solvent (Rangheard et al., 1989; Kirk et al., 1992) by measuring the competitive factor (Fig. 3). The chain length profile of these two lipases clearly differs. Under these experimental conditions, the scissile fatty acid binding site is the primary deter-

minant for chain length specificity, while the choice of the reaction conditions is of minor importance. CALB has high activity for short and medium chain length fatty acids and decreasing activity for long-chain fatty acids. RML, on the contrary, has relatively low activity for short chain fatty acids, but increasing activity for longer fatty acids. These findings are in accordance with shape and properties of the respective scissile fatty acid binding sites (Figs. 1 and 2). The scissile fatty acid binding site of CALB is relatively short (C13) and has a small hydrophobic area located at the wall of the binding funnel. In RML it is located in a long (C18), well-defined hydrophobic crevice.

If, however, assays are used which involve activity at a water-substrate interface, chain length dependency may change drastically, as it has been shown for the hydrolysis of triglycerides by RML (Berger and Schneider, 1991). In addition to the scissile fatty acid binding site, other structural elements will play a role in mediating chain length specificity, like the alcohol binding site and the lid.

References

- Adlercreutz, P., 1991. On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. *Eur. J. Biochem.* 199, 609–614.
- Atomi, H., Bornscheuer, U., Soumanou, M.M., Beer, H.D., Wohlfahrt, G., Schmid, R.D., 1996. Microbial lipases-from screening to design. In: *Oils-Fats-Lipids, Proceedings of*

- the Twenty-first World Congress of the International Society on Fat Research, PJ Barnes and Associates, Bridgewater, pp. 49–50.
- Bairoch, A., Boeckmann, B., 1991. The SWISS-PROT protein sequence data bank. *Nucleic Acids Res.* 19, 2247–2249.
- Berger, M., Schneider, M.P., 1991. Lipases in organic solvents: The fatty acid chain length profile. *Biotechnol. Lett.* 13, 641–645.
- Bernstein, F.C., Koetzle, T.F., Williams, G.J., Meyer, E.E. Jr., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T., Tasumi, M., 1977. The Protein Data Bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.* 112, 535–542.
- Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A., Thim, L., 1991. A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* 351, 491–494.
- Connolly, M.L., 1983. Solvent-accessible surfaces of proteins and nucleic acids. *Science* 221, 709–713.
- Cygler, M., Grochulski, P., Kazlauskas, R.J., Schrag, J.D., Bouthillier, F., Rubin, B., Serreqi, A.N., Gupta, A.K., 1994. A structural basis for the chiral preferences of lipases. *J. Am. Chem. Soc.* 116, 3180–3186.
- Derewenda, U., Brzozowski, A.M., Lawson, D.M., Derewenda, Z.S., 1992. Catalysis at the interface: The anatomy of a conformational change in a triglyceride lipase. *Biochemistry* 31, 1532–1541.
- Egloff, M.P., Marguet, F., Buono, G., Verger, R., Cambillau, C., van Tilbeurgh, H., 1995. The 2.46 Å resolution structure of the pancreatic lipase–colipase complex inhibited by a C11 alkyl phosphonate. *Biochemistry* 34, 2751–2762.
- Goodford, P.J., 1985. A computational procedure for determining energetically favorable binding sites on biologically important macromolecules. *J. Med. Chem.* 28, 849–857.
- Grochulski, P., Bouthillier, F., Kazlauskas, R.J., Serreqi, A.N., Schrag, J.D., Ziomek, E., Cygler, M., 1994. Analogs of reaction intermediates identify a unique substrate binding site in *Candida rugosa* lipase. *Biochemistry* 33, 3494–3500.
- Haalck, L., Paltauf, F., Pleiss, J., Schmid, R.D., Spener, F., Stadler, P., 1997. Stereoselectivity of lipase from *Rhizopus oryzae* towards triacylglycerols and analogs: computer aided modeling and experimental validation, *Methods Enzymol.* 284, 353–376.
- Halling, P.J., 1994. Thermodynamic predictions for biocatalysis in nonconventional media: theory, tests and recommendations for experimental design and analysis. *Enzyme Microbiol. Technol.* 16, 178–206.
- Harel, M., Quinn, D.M., Nair, H.K., Silman, I., Sussman, J.L., 1996. The X-ray structure of a transition state analog complex reveals the molecular origins of the catalytic power and substrate specificity of acetylcholinesterase. *J. Am. Chem. Soc.* 118, 2340–2346.
- Hecht, H.J., Sobek, H., Haag, T., Pfeifer, O., van Pee, K.H., 1994. The metal-ion-free oxidoreductase from *Streptomyces aureofaciens* has an alpha/beta hydrolase fold. *Nat. Struct. Biol.* 1, 532–537.
- Holzwarth, H.-C., Pleiss, J., Schmid, R.D., 1997. Computer-aided modeling of stereoselective triglyceride hydrolysis catalyzed by *Rhizopus oryzae* lipase. *J. Mol. Catal. B: Enzym.* 3, 73–82.
- Joerger, R.D., Haas, M.J., 1994. Alteration of chain length selectivity of a *Rhizopus deleamar* lipase through site-directed mutagenesis. *Lipids* 29, 377–384.
- Kirk, O., Bjoerkling, F., Godtfredsen, S.E., Larsen, T.O., 1992. Fatty acid specificity in lipase-catalyzed synthesis of glucoside esters. *Biocatalysis* 6 (2), 127–134.
- Klein, R.R., King, G., Moreau, R.A., Haas, M.J., 1997. Altered acyl chain length specificity of *Rhizopus deleamar* lipase through mutagenesis and molecular modeling. *Lipids* 32, 123–130.
- Lawson, D.M., Derewenda, U., Serre, L., Ferri, S., Szittner, R., Wei, Y., Meighen, E.A., Derewenda, Z.S., 1994a. Structure of a myristoyl-ACP-specific thioesterase from *Vibrio harveyi*. *Biochemistry* 33, 9382–9388.
- Lawson, D.M., Brzozowski, A.M., Rety, S., Vemag, C., Dodson, G.G., 1994b. Probing the nature of substrate binding in *Humicola lanuginosa* lipase through X-ray crystallography and intuitive modelling. *Protein Eng.* 7, 543–550.
- Longhi, S., Nicolas, A., Creveld, L., Egmond, M., Verrips, C.T., de Vlieg, J., Martinez, C., Cambillau, C., 1996. Dynamics of *Fusarium solani* cutinase investigated through structural comparison among different crystal forms of its variants. *Proteins* 26, 442–458.
- Martinelle, M., Holmquist, M., Clausen, I.G., Patkar, S., Svendsen, A., Hult, K., 1996. The role of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase. *Protein Eng.* 9, 519–524.
- Martinez, C., De Geus, P., Lauwereys, M., Matthyssens, G., Cambillau, C., 1992. *Fusarium solani* cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. *Nature* 356, 615–618.
- Murzin, A.G., Brenner, S.E., Hubbard, T., Chothia, C., 1995. SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* 247, 536–540.
- Norin, M., Haefner, F., Achour, A., Norin, T., Hult, K., 1994. Computer modeling of substrate binding to lipases from *Rhizomucor miehei*, *Humicola lanuginosa* and *Candida rugosa*. *Protein Sci.* 3, 1493–1503.
- Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., et al., 1992. The alpha/beta hydrolase fold. *Protein Eng.* 5, 197–211.
- Pathak, D., Ollis, D., 1990. Refined structure of diene lactone hydrolase at 1.8 Å. *J. Mol. Biol.* 214, 497–525.
- Pleiss, J., Mionetto, N., Schmid, R.D., 1998. Probing the acyl binding site of acetylcholinesterase by protein engineering. *J. Molec. Catal. B.* In press.
- Rangheard, M.S., Langrand, G., Triantaphylides, C., Baratti, J., 1989. Multi-competitive enzymatic reactions in organic media: a simple test for the determination of lipase fatty acid specificity. *Biochim. Biophys. Acta* 1004, 20–28.

- Sarda, L., Desnuelle, P., 1958. Action de la lipase pancréatique sur les esters en émulsion. *Biochim. Biophys. Acta* 30, 513–521.
- Schmid, R.D., Verger, R., 1998. Lipases: interfacial enzymes with attractive applications, *Angew. Chem.* in press.
- Schonheyder, F., Volqvartz, K., 1945. On the affinity of pig pancreas lipase for tricaproin in heterogeneous solution. *Acta Physiol. Scand.* 9, 57–67.
- Schrag, J.D., Li, Y., Cygler, M., Lang, D., Burgdorf, T., Hecht, H.J., Schmid, R., Schomburg, D., Rydel, T.J., Oliver, J.D., Strickland, L.C., Dunaway, C.M., Larson, S.B., Day, J., McPherson, A., 1997. The open conformation of a *Pseudomonas* lipase. *Structure* 5, 187–202.
- Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., Silman, I., 1991. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science* 253, 872–879.
- Uppenberg, J., Ohrner, N., Norin, M., Hult, K., Kleywegt, G.J., Patkar, S., Waagen, V., Anthonsen, T., Jones, T.A., 1995. Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols. *Biochemistry* 34, 16838–16851.
- Vasel, B., Hecht, H.J., Schmid, R.D., Schomburg, D., 1993. 3D-structures of the lipase from *Rhizomucor miehei* at different temperatures and computer modelling of a complex of the lipase with triaurylglycerol. *J. Biotechnol.* 28, 99–105.
- Verger, R., 1997. Interfacial activation of lipases: facts and artifacts. *Trends Biotechnol.* 15, 32–38.
- Wei, Y., Schottel, J.L., Derewenda, U., Swenson, L., Patkar, S., Derewenda, Z.S., 1995. A novel variant of the catalytic triad in the *Streptomyces scabies* esterase. *Nat. Struct. Biol.* 2, 218–223.
- Woolley, P., Petersen, S.B. (Eds.), *Lipases: Their Structure, Biochemistry and Application*. Cambridge University Press, Cambridge, 1994.