Structural determinants defining common stereoselectivity of lipases toward secondary alcohols

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REVIEW / SYNTHÈSE

Structural determinants defining common stereoselectivity of lipases toward secondary alcohols[1]

Miroslaw Cygler, Pawel Grochulski, and Joseph D. Schrag

Abstract: In this review we summarize some aspects of the enantiopreference of the lipase from *Candida rugosa* following structural analysis of complexes of this lipase with two enantiomers of an analog of a tetrahedral intermediate in the hydrolysis of simple esters. The analysis of the molecular basis of the enantiomeric differentiation suggests that these results can be generalized to a large class of lipases and esterases. We also summarize our experiments on identification of the key regions in the lipases from *Geotrichum candidum* lipase responsible for differentiation between fatty acyl chains.

Key words: lipases, stereoselectivity, three-dimensional structure, conformational rearrangement.

Résumé : Nous résumons ici certains aspects de l'énantiopréférence de la lipase de *Candida rugosa* suite à l'analyse de la structure de complexes de cette lipase avec deux énantiomères d'un analogue d'un intermédiaire tétraédrique lors de l'hydrolyse d'esters simples. L'étude de la base moléculaire de la différenciation des énantiomères permet de généraliser ces résultats à une vaste classe de lipases et d'estérases. Nous présentons aussi nos expériences portant sur l'identification des régions-clés des lipases de *Geotrichum candidum* responsable de la différenciation entre les chaînes grasses acyclées.

Mots clés : lipases, stéroéosélectivité, structure tridimensionnelle, réarrangement de conformation.
[Traduit par la Rédaction]

Introduction

Despite spectacular successes of modern synthetic methods in creating a myriad of molecular shapes, the synthesis of compounds with a predefined chirality still presents a major challenge. While standard synthetic reactions usually result in racemic mixtures, chirality of products is typical for enzyme-catalyzed reactions. Many enzymes have been investigated for a broad range of hydrolytic and synthetic reactions. Among the enzymes most widely used by chemists are proteases and lipases (Klibanov 1990). The latter group of enzymes is of special interest for synthetic reactions because of their stability in nonaqueous media resulting from their natural site of action residing at the water–lipid interface.

The biological function of lipases is the hydrolysis of triacylglycerides. These enzymes display, however, a very broad specificity and catalyze hydrolysis of many esters, albeit with various efficiencies (Macrae and Hammond 1985; Jensen et al. 1990). In organic media, with very low water activity, lipases catalyze reactions in the synthetic direction (Klibanov 1990). The great potential for commercial applications of lipases in organic syntheses (Boland et al. 1991; Margolin 1993) and polymer syntheses (Dordick 1992) has stimulated detailed investigations of the mechanism of lipase-catalyzed reactions and the factors determining their stereopreferences.

Determination of the three-dimensional (3-D) structures of a number of lipases and cloning and sequencing of many others, showed that they all possess serine-protease-like catalytic triads consisting of Ser-His-Asp/Glu (Brenner 1988; Cygler et al. 1992). In all of them the catalytic serine is embedded in a signature pentapeptide sequence, Gly-X-Ser-X-Gly, and is located in a unique supersecondary structure, at a tight bend between a β-strand and an α-helix (Cygler et al. 1992; Derewenda and Derewenda 1991). The postulated mechanism for lipase-catalyzed reactions is rather similar to that proposed for serine proteases and is schematically shown in Fig. 1. The reaction progresses through two tetrahedral intermediates, with the nucleophilic, active-site serine attacking the ester carbonyl carbon, followed by an attack by an activated water molecule on the covalent intermediate (e.g., Grochulski et al. 1994*a*). To the best of our knowledge the only enzyme from the lipase superfamily that is a possible exception to the catalytic triad

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Fig. 1. A postulated mechanism for the lipase-catalyzed reaction with two tetrahedral intermediates.

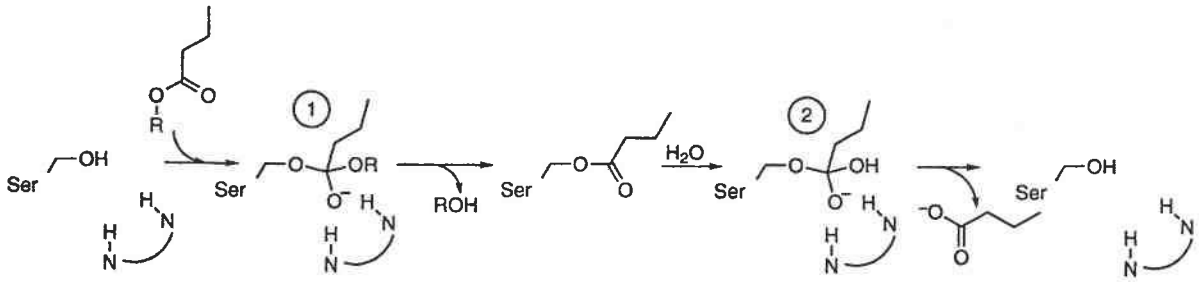
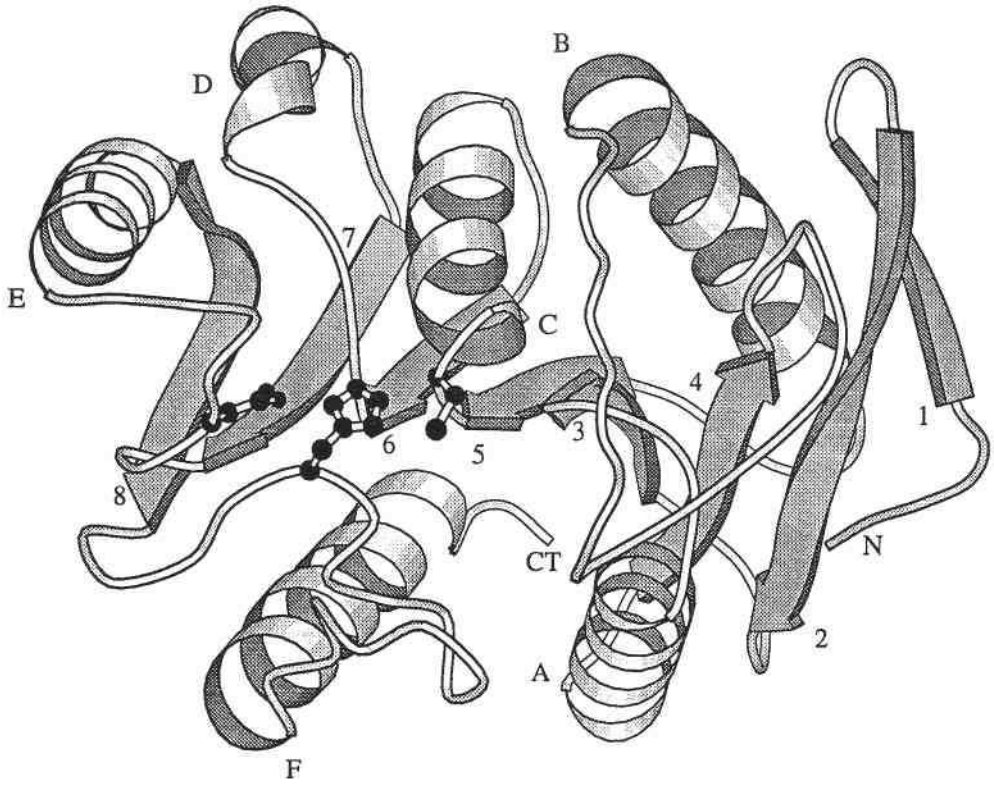


Fig. 2. Elements of the α/β hydrolase fold as exemplified by the structure of dienlactone hydrolase (Pathak and Ollis 1990). The eight β-strands and six α-helices identified as conserved elements of the fold are marked. The positions of catalytic residues are shown in full.



paradigm, and which may use a different mechanism to activate a nucleophilic serine, is a lipase/acyltransferase from *Aeromonas hydrophila* (Hilton et al. 1990). Although this enzyme contains two histidines in the catalytically competent fragment, it was shown that their mutations to Asn did not affect significantly the enzymatic activity (Hilton et al. 1990; Hilton and Buckley 1991).

On the basis of sequence homologies, lipases can be divided into several families (e.g., Cygler et al. 1992). However, the 3-D structures of lipases from different families show striking similarities in their folds, strongly suggesting that they are all evolutionarily related. Their folds conform in full or in part to the α/β hydrolase fold identified in a number of hydrolases with unrelated sequences (Ollis et al. 1992; Cygler et al. 1992). This fold is built around a scaffold formed by a mixed β-sheet (with parallel strands in the central part) and a few well-conserved helices running approximately parallel to the β-strands on either side of the sheet (Fig. 2). The sheet is significantly twisted, to the extent that the strands on the opposite ends form an angle between 50° (for smaller sheets) and 90° (for longer sheets, Schrag and Cygler 1993). Following the nomenclature of Ollis et al. (1992), the catalytic serine is located at the end of strand 5, the acid comes from a short loop after strand 7 (pancreatic lipase and homologous enzymes are an exception, with the acid following strand 6, Schrag et al. 1992), and the histidine is embedded in a rather long loop after strand 8.

One of the striking characteristics of lipases, which is most likely a consequence of the similarities of their 3-D structures, is the observed preference of many lipases in a broad range of reactions for the same enantiomer of the substrate molecule (Sih and Wu 1989; Crout and Christen 1989). These common enantiopreferences have been summarized in empirical rules that allow prediction of which of the two enantiomers of a given substrate reacts faster. For example, a rule for secondary alcohols predicts the enantiopreference on the basis of the size of the substituents at the stereocenter (Kazlauskas et al. 1991) and the preferred enantiomer is shown in Fig. 3. A similar rule was recently proposed for the hydrolysis of carboxylic acid esters catalyzed by the lipase of *Candida rugosa* (Ahmed et al. 1994).

In our investigations of lipases we have concentrated on a family of high molecular weight enzymes, represented by the lipases of *Geotrichum candidum* and *C. rugosa* (GCL and CRL, respectively). These lipases show significant amino acid homology to a large class of esterases, especially cholinesterases, and are considered to be members of the lipase/esterase superfamily (Cygler et al. 1993). This is the first identified enzyme family with a catalytic triad in which the role of the acid member of the triad is played by glutamic acid.

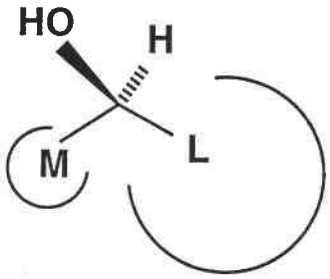
Structures of *G. candidum* and *C. rugosa* lipases

GCL and CRL are globular, single-domain proteins built around an 11-stranded mixed β-sheet. Their approximate dimensions are 45 × 60 × 65 Å (1 Å = 0.1 nm). The connections on the N-terminal side of the sheet are rather short, whereas the connections on the C-terminal side are rather long, containing a total of 17 helices and forming a cap on the top of the β-sheet. The active site, formed by Ser-209–His-449–Glu-341 in CRL and Ser-217–His-463–Glu-354 in GCL, is located near the center of the sheet at the C-terminal side of the strands.

A characteristic feature of lipases is their increased activity at the lipid–water interface, a phenomenon known as interfacial activation (Sarda and Desnuelle 1958). This has long been thought to be associated with a conformational change in the enzyme that occurs near or at the interface, and this idea has been confirmed in recent years for at least three lipases. They were each crystallized in two different conformations: one in which the active site triad is occluded from the solvent and another in which the active site is available to the substrate (Brzozowski et al. 1991; Grochulski et al. 1994*b*; van Tilbeurgh et al. 1993). The access to the active site is regulated by a conformational change in one or two loops called the flap or the lid. In some of the lipases, residues that participate in the formation of the oxyanion hole come from the flap region. The flap reorganization contributes to the formation of a catalytically competent oxyanion hole and thus helps to create a fully functional active site.

In CRL the flap is formed by an omega loop that is 38 amino acids long, anchored at the bottom by a disulfide bridge and a salt bridge (Grochulski et al. 1994*b*). The part of this loop that undergoes a major conformational change involves 27 residues. Both the open and the closed conformations have been observed in the crystalline state (Grochulski et al. 1994*b*). They are shown in Fig. 4 together with the position of

Fig. 3. The preferred enantiomer of the alcohol leaving group in reactions catalyzed by many lipases. M indicates the medium, L the large substituent at the stereocenter.



the catalytic triad. For this lipase the oxyanion hole is already preformed in the closed conformation. In the open conformation the active site is located at the bottom of a large, mostly hydrophobic depression. Examination of the solvent-accessible surface in the open form of CRL revealed that the depression narrows down near the catalytic serine and connects to a long tunnel leading toward the center of the protein. This tunnel is sealed off from the solvent by the flap in the closed, inactive form of CRL.

GCL has only been observed in the inactive form, with the flap in a closed conformation. Nevertheless, comparison with the CRL structure suggests an activation mechanism of this enzyme similar in general features to that of CRL. As in CRL, there is a long tunnel in the interior of GCL, partly filled with ordered solvent molecules (Schrag and Cygler 1993). The shape of this tunnel is more complex than in CRL, and taken together with the observed difference in the conformation of the loop-forming oxyanion hole, it suggests that the activation of GCL involves rearrangement of one or more loops in addition to the flap (Schrag and Cygler 1993).

Substrate binding site

The inhibitor complexes are analogs of the first and the second tetrahedral intermediates along the reaction pathway (Fig. 1). They allowed us to identify the location of the scissile fatty acyl chain and to propose a binding mode for the triglyceride substrate (Grochulski et al. 1994*a*). Recently, more comprehensive calculations by the Monte Carlo method were used to identify the potential locations of the two other fatty acyl chains of the triglyceride substrate (H. Zuegg, E. Purisima, and M. Cygler, unpublished results).

The fatty acyl chains of both transition state analogs were found inside the long, hydrophobic tunnel described above. This tunnel, observed in CRL and GCL, is unique among the lipases studied to date. The tunnel in CRL, which starts near active-site Ser-209, is L-shaped and can accommodate in its observed form a fatty acyl chain at least 20 carbon atoms long. The tunnel is lined by the side chains of Met-213, Pro-246, Leu-302, the aliphatic part of Arg-303, Leu-304, Phe-362, Phe-366, and Val-534. The end of the tunnel is closed off by Tyr-361 and Ser-365 (Grochulski et al. 1994*a*).

The alcohol leaving group is bound at the mouth of the tunnel. This binding crevice is formed on the top of the β-sheet

Fig. 4. The C_α tracing of CRL with both conformations of the flap shown. The open conformation, in which the flap extends nearly perpendicularly to the protein surface, is stabilized by hydrophilic interactions with the rest of the protein and by the interactions with a carbohydrate at the nearby glycosylation site. In this conformation a large hydrophobic patch becomes exposed. The open conformation of the flap is shown with thick lines whereas the closed conformation is shown with stippled lines.

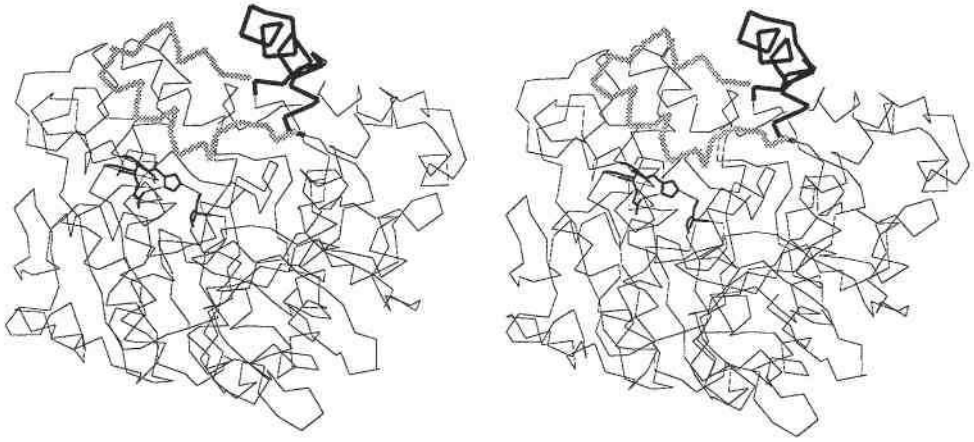
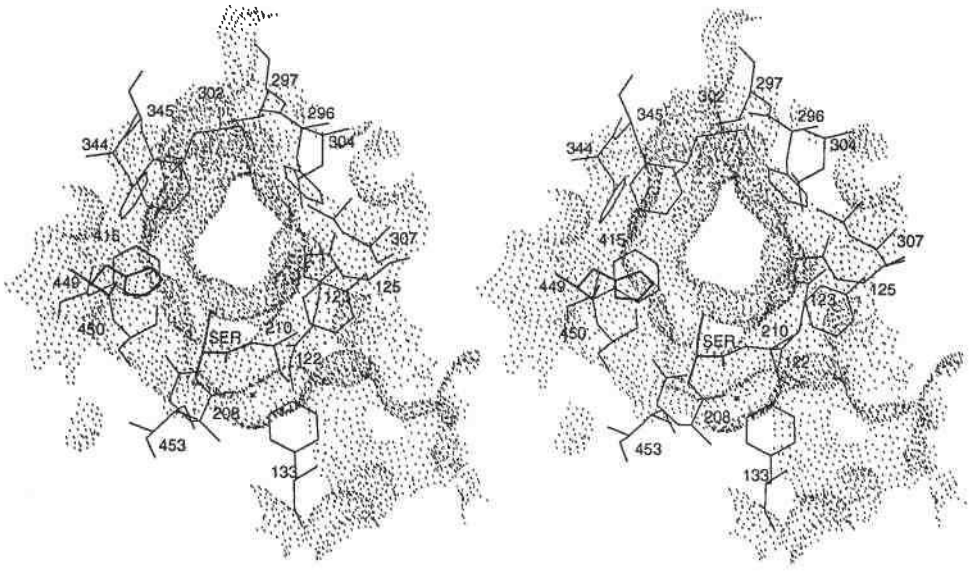


Fig. 5. The mouth of the tunnel near the active site with the binding site for the leaving group. The dots represent the molecular surface (calculated using the MS program; Connolly 1983).



and has an oval shape, with the long axis following the direction of β-strands. This site is created by the residues from several loops. The bottom of the crevice is formed by Glu-208, Gly-122, Phe-133, and Ile-453. One of its sides is lined by Gly-123, Gly-124, and Phe-296, while the other side is lined by His-449 (catalytic), Ser-450, Phe-344, and Phe-345. The crevice is more open at the top (Fig. 5) and can accommodate larger groups there. Importantly, the side chains forming the crevice come mostly from the loops that are also used for assembling the catalytic machinery. The loop 119–134 is involved in forming of the oxyanion hole (see below).

Oxyanion hole

As in other serine and cysteine proteases, the lipases have found a way to stabilize an oxyanion that develops in each of the tetrahedral intermediates in the reaction. The crystal structures of the complexes confirmed the predicted location of the oxyanion hole that is formed by the amide NH groups of Gly-124 and Ala-210 (Grochulski et al. 1994*a*). The distances between these nitrogen atoms and the oxyanion analog are in the range of 2.7–3.0 Å. It is also likely that the amide of Gly-123 contributes to the oxyanion stabilization. The α-helix

following the active site serine provides an additional stabilization of the oxyanion through the helix dipole (Hol et al. 1978).

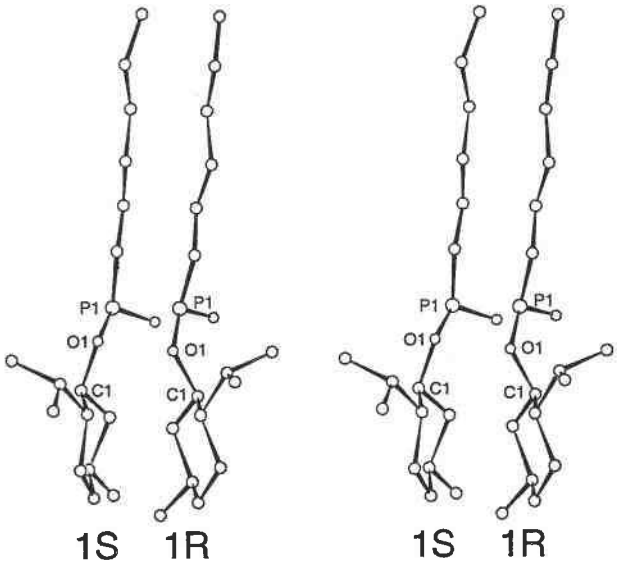
Enantioselectivity

In reactions that involve a secondary alcohol as a leaving group, various lipases show chiral preference for the same enantiomer of the alcohol (Yamaguchi et al. 1976; see also references cited in Cygler et al. 1994). These observed enantiopreferences of lipases have been expressed in an empirical rule that allows the preferred enantiomer to be predicted on the basis of the size of the substituents at the stereocenter (Kazlauskas et al. 1991) and they are reflected in Fig. 3.

As a first step to identify the molecular basis for this general rule we selected CRL for detailed structural studies. This enzyme hydrolyzes both enantiomers of menthyl esters (2-isopropyl-5-methyl-cyclohexan-1-yl ester) with a high preference for the (1*R*,2*S*,5*R*) enantiomer. Enantiomeric ratios of 10 and greater have been reported for such reactions (Cygler et al. 1994 and references cited therein). We selected phosphonate analogs of the first tetrahedral intermediate of the two enantiomers of menthyl hexanoate (*O*-(1*R*,2*S*,5*R*)-menthyl hexylphosphonyl chloride (**1R**) and *O*-(1*S*,2*R*,5*S*)-menthyl hexylphosphonyl chloride (**1S**)), reacted them with CRL to form covalent complexes, and determined their crystal structures (Cygler et al. 1994). Both **1R** and **1S** were found covalently bound to O_γ of Ser-209, and the reaction proceeded through nucleophilic displacement of the chlorine atom from phosphorus, most likely with inversion of the configuration (Corriu et al. 1980).

Despite different configurations at the menthyl group stereocenters, the two enantiomers are bound in the active site in a somewhat analogous way. Their hexyl chains and phosphonyl groups superimpose very well on each other (Fig. 6). In each case the oxygen at the phosphorus is located in the oxyanion hole, forming hydrogen bonds to the appropriate amides. The menthyl rings of **1R** and **1S** adopt similar orientations in the binding site, with the mean plane of the cyclohexane ring being perpendicular to the central part of the β-sheet, the small substituent (methyl group) pointing toward the bottom of the crevice, and the large substituent to the ring (the isopropyl group) pointing upwards. To attain a similar orientation of the rings relative to the enzyme, **1R** and **1S** differ in the torsion angles along the P-O1 and O1-C1 bonds (O1 corresponds to the hydroxyl group of the leaving alcohol; see Fig. 6). Such orientations of the rings are dictated by the oval shape of the crevice and by the limited space at the bottom of the crevice, too small to accommodate the isopropyl substituent. As a result, the isopropyl groups of the two enantiomers are located in a more open part of the crevice, extend from the cyclohexane ring in opposite directions, and contact different residues (Fig. 7). There are small but important adjustments in the positions of the side chains contacting the isopropyl group in comparison with the free enzyme (Cygler et al. 1994). In **1R** the isopropyl group is directed toward Phe-296 and causes movements of the side chains of Phe-296 and Ile-297 of up to 1 Å away from the inhibitor. The isopropyl group in **1S** is directed toward Phe-344 and His-449 and causes both of these rings to rotate by approximately 60° (Cygler et al. 1994). These side chain

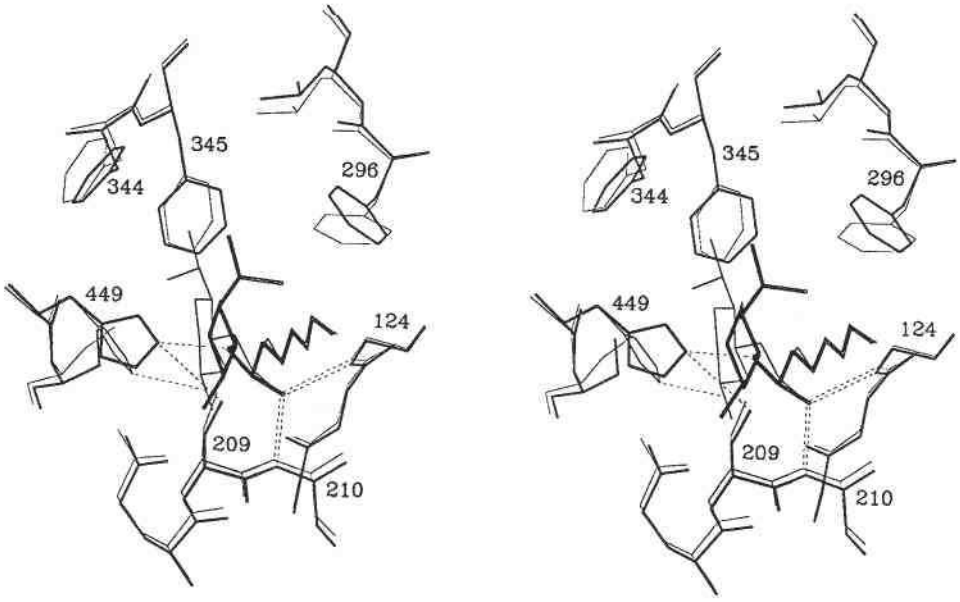
Fig. 6. The conformations of the two enantiomers, **1R** and **1S**, of menthyl hexylphosphonate as observed in the complexes with CRL. The molecules were first displayed from the same viewpoint and then rotated slightly in opposite directions along the vertical axis to provide a better view of the menthyl rings.



movements, which correlate with the position of the isopropyl substituents, indicate intrinsic flexibility of this hydrophobic pocket and its capacity for small adjustments to accommodate different substrates.

What do the structures of CRL–inhibitor complexes tell us about enantiomeric preference? Superposition of the crystal structures provides two possible explanations for the differences in the catalytic rates of the two enantiomers of menthyl esters. First, since the two enantiomeric molecules are bound with different conformations along P-O1 and O1-C1 bonds, it is possible that one of them (**1S**) is of higher energy. However, we performed molecular mechanics calculations on the phosphonate inhibitor molecules and on the corresponding ester substrates and found that the two conformations observed in the crystal structures correspond to local energy minima with a very small difference in energies (Cygler et al. 1994). Second, we observed a distortion in the orientation of the His-449 imidazole ring in the **1S** complex from the orientation maintained in the uncomplexed enzyme (both open and closed conformations) and in the complex with **1R**. While in the **1R** complex His-449 forms a bifurcated hydrogen bond to O_γ of Ser-209 and to O1 of menthol, in **1S** only the hydrogen bond to O_γ is formed. We believe that it is this distortion of His-449 in the **1S** complex that impairs the hydrolysis of this enantiomer. This bifurcated hydrogen bond, seen in the **1R** complex and in other CRL–inhibitor complexes (Grochulski et al. 1994*a*), is consistent with the proposed role of the imidazole ring in the catalysis, during which the histidine mediates the transfer of a proton from O_γ of Ser-209 to the oxygen of the alcohol leaving group. In the **1S** complex the imidazole ring of His-449 rotates by ~60° owing to the steric hindrance of the large isopropyl substituent, and the alcohol oxygen moves slightly away from the histidine to allow for the

Fig. 7. Close-up of the inhibitor binding site. **1R**-CRL is drawn in thicker lines, **1S**-CRL in thinner lines. The inhibitors are drawn in thicker lines than those for CRL. Residues are numbered. The hydrogen bonds between His-449 and the inhibitors and between the oxygen located in the oxyanion hole and the NH groups are shown as broken lines.



different conformation of **1S**. As a result, the hydrogen bond between $N_{\varepsilon}2$ of the imidazole ring and O1 of the menthol is no longer formed. Molecular mechanics calculations indicate that the energy required to reorient the imidazole ring to form the additional hydrogen bond to the alcohol oxygen is ~1 kcal/mol, which corresponds well with the observed enantiomeric ratio for the menthyl pentoate ester (Cygler et al. 1994).

Generalization for other lipases

Can this explanation of the enantiomeric preference of CRL be extended to other lipases and esterases? We believe that this is the case. Structural similarities between these enzymes are most pronounced near the catalytic site. To re-emphasize, in all lipases with known 3-D structures the catalytic site is located at the C-terminal end of a section of parallel strands of a β-sheet, the catalytic serine is embedded in a tight bend between a β-strand and an α-helix (Ollis et al. 1992; Derewenda and Derewenda 1991), the catalytic triad histidine approaches the active site serine from the same direction, and the positions of the inhibitors in different lipase–inhibitor complexes seem to be similar (Grochulski et al. 1994*a*). The oxyanion in the transition state is stabilized in an oxyanion hole that is positioned in a comparable location relative to the active site in all of these lipases. These similar arrangements of the essential elements of the catalytic machinery are likely to translate into similarities in the orientation of the substrate in the active site of various lipases. From the description of the leaving group binding site given above, it is clear that the binding crevice is formed by many of the same loops that assemble the catalytic machinery of the enzymes: the catalytic triad and the oxyanion hole. That conveys many of the same space limitations for all the lipases, namely the restrictions at the bottom of the crevice from the supporting β-sheet, from the catalytic residues at one side, and from the oxyanion hole-forming loop from the other side. The largest differences between the various lipases are expected at the top of the crevice, where the crevice is most open. The nature of the side chains of the residues forming the binding crevice determines the detailed shape of the binding site and provides for the observed individual differences in the enantiomeric ratio, but does not change the enantiopreference of the enzymes.

Fatty acyl chain preferences

Lipases present us with another challenging question: what are the molecular determinants of fatty acyl chain selectivity? To address this question we turned to the lipases of *Geotrichum candidum*. This fungus expresses two closely related lipases (Shimada et al. 1989, 1990) with 86% amino acid identity. Despite such high similarity these lipase isoforms display different specificity profiles (Charton and Macrae 1992) and present an excellent system for the identification of key residues involved in the differentiation between fatty acyl chains. We cloned both isoforms in yeast (Vernet et al. 1993; Bertolini et al. 1994) and characterized their activity profiles with a wide variety of triglyceride substrates (Bertolini et al. 1995). Isoform I (GCL I) shows a distinct preference for long, unsaturated fatty acyl chains (C_{18}), especially those with a *cis*-9 double bond. Isoform II (GCL II), on the other hand, displays a much broader specificity and effectively hydrolyzes a range of medium-length triglycerides (C_8-C_{14}) as well as those with long, unsaturated fatty acyl chains.

Since the 3-D structure of GCL II is known (Schrag et al. 1991), we have located positions corresponding to sequence differences between GCL I and GCL II. These residues can be

grouped into three categories: *(i)* surface-exposed residues relatively far away from active site, *(ii)* residues contributing to the formation of a large internal cavity (in analogy to CRL this is a presumed binding site of the scissile acyl chain), and *(iii)* the residues in the flap and those covered by the flap. The residues belonging to the first group are unlikely to be in contact with the triglyceride substrate and therefore are unlikely to affect the specificity. Residues from the other two groups seem to be equally likely to contribute to specificity. The structure of the enzyme alone, without a detailed knowledge of the position of the bound substrate, is insufficient to uniquely identify the residues most important for differences between the GCL I and GCL II substrate specificities.

In the first attempt to identify the regions of the lipases of *G. candidum* that might be involved in substrate differentiation we constructed a GCL I–II hybrid molecule that contained the N-terminal portion of GCL I (residues Glu-1–Leu-194) and the C-terminal portion of GCL II (residues Glu-195–Gly-544, Bertolini et al. 1995). Consequently, the GCL I–II hybrid included the flap region of GCL I (Schrag et al. 1991; Schrag and Cygler 1993). Comparison of the specific activities of the hybrid molecule with those of GCL I and GCL II for various substrates indicated clearly that the hybrid molecule behaves like GCL II with respect to the specific activities for the different substrates studied. These results provide strong evidence that the flap is not involved in substrate differentiation and that, if any contacts are made between the fatty acyl chains of the triglyceride and the flap, they must be rather nonspecific. The most likely candidates for the key residues in the recognition of the fatty acyl type seem to reside along the internal cavity, which, as in CRL, likely becomes connected to the solvent in the open, active conformation of GCL. Yet, even with this knowledge and the 3-D structure of inactive GCL, it is presently not clear which of the possible residues are the most important. We are hoping to identify these key residues through further mutational analysis.

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