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Understanding Lipase Action and Selectivity

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In this article, a survey of recent lipase research, with special emphasis on molecular structure-function relationships, is presented. Determination of crystallographic structures of lipases from microbial and mammalian origin has shed light on the molecular mechanism of lipase catalyzed acyl ester hydrolysis. A catalytic triad similar to serine proteases is responsible for the cleavage of substrate ester bonds, involving the formation of an acyl-enzyme intermediate. Comparative structural studies revealed a common three dimensional fold and a superimposable topology of the catalytic machinery in lipases, esterases, and other hydrolytic enzymes. Availability of three dimensional structures is the basis for understanding the mechanism of lipase catalysis and for elucidation of the molecular interactions that result in variant selectivities towards triacylglycerols and their analogs.

INTRODUCTION

Lipases (triacylglycerol ester hydrolases, EC. 3.1.1.3.) are lipolytic enzymes capable of hydrolyzing triacylglycerols, thus liberating fatty acids.[1] The ubiquitous distribution of lipases in nature reflects their key role for fat digestion and utilization in higher animals, plants, and microorganisms. Besides their general physiological significance for lipid metabolism, lipases exhibit special catalytic properties that make them attractive to various industrial applications.[2] Additional demand for lipase research derives from a medical viewpoint[3] since malfunction of lipolytic enzymes is related to lipid metabolism diseases (*e.g.*: obesity, atherosclerosis).

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Whether the scope of research has a pure academic background or aims at commercial exploitation, the elucidation of structure-mechanism relationships seems to be a milestone in lipase exploration. In 1981, the first amino-acid sequence of a triacylglycerol lipase was completed. Today, more than 25 complete sequences have been determined either directly or by deduction from the gene sequence.[4] In 1990, the first reports of successful X-ray structure determinations of two lipases appeared in literature.[5,6] Crystal structures of 10 different neutral lipases have been described to date. Additionally, the availability of pure lipase preparations was improved by the development of sophisticated isolation and purification schemes and the introduction of recombinant DNA-technologies.[7]

THE CATALYTIC TRIAD

Simultaneous reports of 3-D structures of lipases from human pancreas (HPL)[5] and the fungus *Rhizomucor miehei* (RML)[6] identified a catalytic Ser-Asp-His triad similar to serine proteases. Subsequently, the X-ray structures of lipases from *Pseudomonas glumae* (PGL),[8] *Humicula lanuginosa* (HLL), *Rhizopus delemar* (RDL), *Penicillium camembertii* (PCL)[9], *Candida antarctica* (CAL),[10] and a cutinase from *Fusarium solani pisi* (FSC)[11] pointed to the same catalytic machinery. However, the acidic member of the triad in acyl-ester hydrolases can be replaced by glutamic instead of an aspartic acid, as deduced from structure determinations from *Geotrichum candidum* lipase (GCL),[12] *Candida rugosa* lipase (CRL),[13] and acetylcholine esterase from *Torpedo californica*.[14] A conservative replacement of Glu by Asp in a recombinant GCL displayed similar activities with triolein, as compared to the wild-type lipase.[15] The catalytic triad residues always occur in the same order in the primary sequence: Ser, Acid (Glu/Asp), His. The active site Ser is part of a Gly-Xxx-Ser-Xxx-Gly consensus sequence, which is also observed for esterases and other serine-hydrolases.[16] However, a *Bacillus subtilis* lipase was recently found to be an exception, having an Ala-Xxx-Ser-Xxx-Gly sequence around the active site serine.[17] Interestingly, restoration of the Gly-Xxx-Ser-Xxx-Gly »lipase consensus« sequence by site directed mutagenesis resulted in specific activities, comparable to those observed with the wild-type enzyme. Apart from the active site serine consensus, there is very little overall sequence identity between different lipases, except in enzymes from closely related origins.[4,18]

In analogy to what is known for serine proteases,[19] the catalytic reaction of triacylglycerol ester hydrolysis can formally be sketched as follows (Figure 1): After the formation of a competent substrate-enzyme complex, the nucleophilic Oγ of the catalytic triad serine attacks the scissile ester bond to form a tetrahedral intermediate, while the hydrogen atom of the serine hydroxyl is transferred to the histidine. Cleavage of the substrate-ester

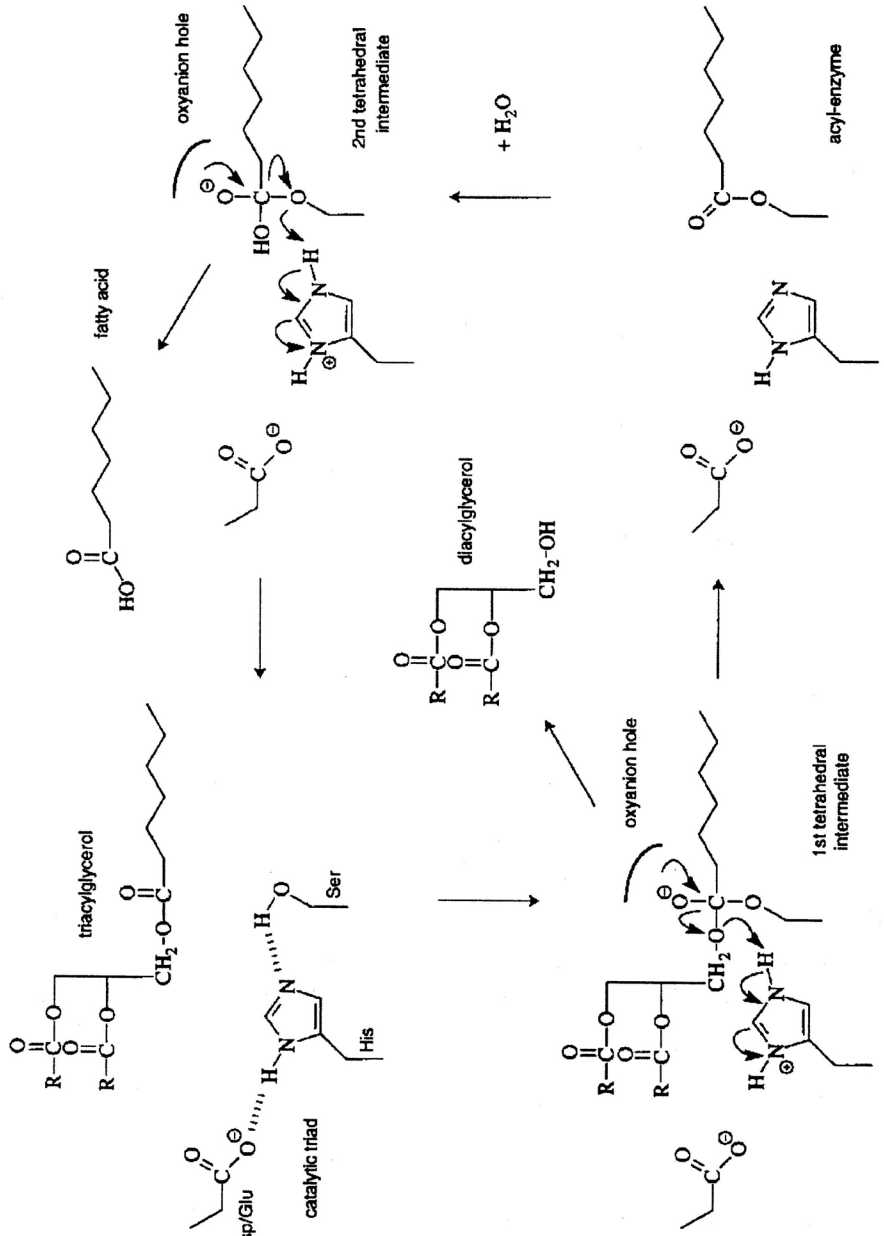


Figure 1. Schematic diagram of triacylglycerol hydrolysis by lipase, as described in text.

bond, breakdown of the intermediate, protonation, and dissociation of the diacylglycerol yield the acyl-enzyme (serine-acylester). Deacylation results from nucleophilic attack on the acyl-enzyme by an activated water molecule, again proceeding *via* formation of a tetrahedral intermediate. The active site serine is hydrogen-bonded to an imidazole nitrogen of the histidine. The second imidazole nitrogen is hydrogen bonded to the carboxylate group of the corresponding acidic part of the triad (*i.e.*: charge relay system). The spatial arrangement of the triad enables the serine or water to perform the nucleophilic attack on the carbonyl group of the substrate or acyl-enzyme, respectively. When the lipase operates in an environment of low water activity, nucleophiles other than water (*e.g.*: alcohols, amines) can act as final acyl-acceptors in lipase catalyzed acyl-transfer reactions. During acylation and deacylation steps, the charged oxygen atoms of tetrahedral intermediates are stabilized by a so called oxyanion hole, which lowers the free energy of the transition states, thereby accelerating the catalytic turnover.

THE α/β HYDROLASE FOLD

The recent X-ray structures of lipases demonstrate similar secondary structure features despite their great differences in the amino acid sequence. All the lipases that have been described so far fold in a similar way and have a superimposable topology of the catalytic triad residues and a potential oxyanion hole. Ollis and co-authors[20] summarized this protein folding pattern and named it α/β hydrolase fold. It was identified not only in lipases and esterases, but also in other hydrolytic enzymes having a catalytic triad, including a serine carboxypeptidase from wheat (Ser-Asp-His),[21] dienelactone hydrolase from *Pseudomonas sp.* B13 (Cys-Asp-His),[22] haloalkane dehalogenase from *Xanthobacter autothrophicus* (Asp-Asp-His),[23] cutinase from *Fusarium solani pisi* (Ser-Asp-His),[11] and a thioesterase from *Vibrio harveyi* (Ser-Asp-His).[24]

The α/β hydrolase fold contains a core of mainly parallel central β-sheets surrounded by α-helices. The active site serine (or the corresponding nucleophilic amino acid) rests at a sharp hairpin bend between one of the β-strands and a buried α-helix. Indeed, the lipase consensus sequence Ala/Gly-Xxx-Ser-Xxx-Gly can be explained by the steric requirements of this nucleophilic elbow. The whole catalytic triad is embedded in a hydrophobic environment. The serine is in a strained conformation with unfavorable torsion angles (β-εSer-α) and sticks out of the hydrophobic surface. However, as a result of this unique architecture, the serine hydroxyl group has access to the catalytic histidine and the carbonyl carbon of a bound substrate molecule.

Superposition of X-ray structures of lipases and esterases, coupled with sequence alignment, located the conserved residues that are involved in

maintaining the scaffold of the overall structure.[25] Besides the active site, most of the invariant residues are positioned at the edges of secondary structural elements, disulfide or salt bridges, and in the core of the proteins. On the other hand, high variability positions are found on the surface of the enzymes and in the vicinity of potential substrate binding sites. The catalytic triad (Ser-Asp/Glu-His), supported on a central β-sheet with a similar supersecondary structure around the active site serine (β-εSer-α), is postulated to be a common motif in all serine-dependent lipases and esterases.[26] Since a comparable topology of the central catalytic domain is also seen in apparently unrelated hydrolases, such as a serine carboxypeptidase, haloalkane dehalogenase, and dienelactone hydrolase, it was suggested that all α/β-hydrolase fold enzymes diverged from a common ancestor. Divergent evolution has conserved a structural framework of key catalytic components, while the lack of overall sequence similarity between obviously related enzymes like lipases and esterases is associated with domains determining different specificities.[20,25]

As pointed out before, acylester hydrolases are functional analogs of serine proteases. Superposition of the catalytic triad residues showed that the active atom of the nucleophile (Ser-Oγ) and the corresponding acid carboxyl group (Asp or Glu) are in plane with the His imidazole in both enzyme families, forming a similar hydrogen-bonding network, which is essential for catalysis.[5,6,12] However, the Ser-Oγ is presented from the opposite side of the plane in proteases and lipases, proposing an inverse handedness of the tetrahedral intermediate. In addition, it was emphasized that, besides the organization of the catalytic triad, serine-proteases and α/β hydrolase fold enzymes have no apparent similarities in the secondary structure.[20] Therefore, the mechanistic relationship between these enzymes appears as a result of convergent evolution, developed to catalyze a chemically similar hydrolytic reaction (*e.g.*: amide *versus* ester hydrolysis).

INTERFACIAL ACTIVATION

Lipases differ from classical esterasess by their ability to hydrolyze long chain triacylglycerols, which are insoluble in water. Contrary to esterases, the activity of most lipases is maximal only when the concentration of substrate exceeds the solubility limit and a water-lipid interface is formed. This unique property, first described by Sarda and Desnuelle in 1958,[27] is known as interfacial activation and does not conform to simple Michaelis-Menten kinetics. A kinetic model for the hydrolysis of insoluble lipids proposes an adsorption process of the lipase to the interface (*e.g.*: substrate emulsion, micelle, monolayer, *etc.*) prior to the catalytic turnover of a single substrate by a Michaelis-Menten fashioned enzyme-substrate complex.[28]

Two theory categories have been suggested relating to the molecular basis of interfacial activation: The »substrate theory« proposes that the quality of the lipid-water interface results in the observed activation phenomenon. This includes a steep substrate concentration gradient at the interface,[29] reduction of the water shell around the substrate ester,[30] and a better orientation of the scissile ester bond.[31] The »enzyme theory«, however, postulates conformational changes in the lipase upon adsorption to the interface.[32]

The first crystal structures of lipases strongly supported the latter theory. The structures of RML[6] and HPL[5] revealed that the respective catalytic triad is buried completely beneath a helical segment and, therefore, shielded from contact with the aqueous medium. It was assumed that reorientation of this lid or flap has to take place to allow substrate access to the active site. Similar »closed« states were subsequently reported for X-ray structures of GCL,[12] PGL,[8] and CRL.[33] Crystallographic studies of RML complexed with covalently attached inhibitors provided the first structural evidence for the hypothesis that the lid is replaced during activation.[34,35] In these studies, n-hexyl chlorophosphonate ethyl ester and diethyl *p*-nitrophenyl phosphate were used to trap the lipase in an activated state. They react covalently with the active site serine (Figure 2) and form stable phosphonyl or

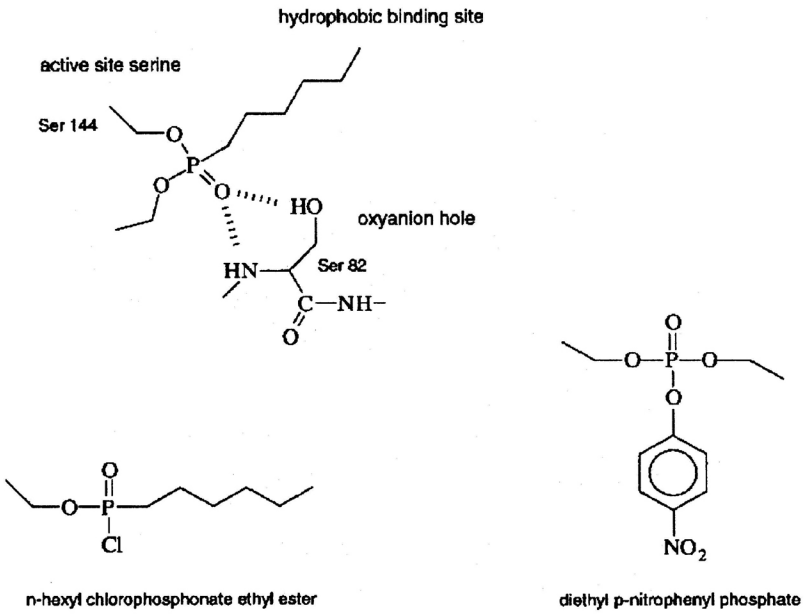


Figure 2. Schematic representation of the tetradedral intermediate of RML[34,35] covalently complexed with a phosphonate inhibitor.

phosphoryl serine esters, which mimic the first tetrahedral intermediate during acyl ester hydrolysis (Figure 1). In RML, the lid consists of a short amphiphatic helix. Superposition of the native and inhibited structures of RML[36] demonstrated that conformational changes of the lid can be described as a simple rigid body movement of this helical part. As the lid rolls back, the active site and the hydrophobic surface of the amphiphilic lid, which has previously interacted with the hydrophobic surrounding of the catalytic center, become exposed. Similar conformational changes were observed with the structure determination of a pancreatic lipase-procolipase complex, cocrystallized with mixed phospholipid-bile salt micelles,[37] when compared to structures obtained in the absence of lipid interface.[38] Although the reorganization of the HPL structure upon activation is more complex than in RML and cannot be described by the motion of a single α-helical domain, the functional consequences are the same. In both cases, the movement of a lid opens up the active site and creates a large hydrophobic surface. In the case of the HPL-colipase complex, this hydrophobic plateau is extended with hydrophobic patches of the colipase. This accessory protein, which is required for hydrolytic activity under physiological conditions, binds to the carboxy-terminal, non-catalytic domain of the lipase and also interacts with the lid stabilizing the open conformation. The extended hydrophobic face of the activated lipase-colipase complex is assumed to enable sufficient binding at lipid-water interfaces in the presence of high bile salt concentrations.[38] Mutants of HPL in which the lid domain was removed demonstrated no interfacial activation and a decreased ability to bind to an interface.[39]

Studies of the RML structures have shown that the opening of the lid and the exposure of internal hydrophobic surfaces is accompanied by correct positioning of amino acids involved in stabilization of the oxyanion intermediates (Figure 1). Upon activation, Ser 82 is placed in an orientation that allows (Figure 2) hydrogen bonding to the oxyanion intermediate, as deduced from the inhibited lipase structures.[34,35] Similar structural rearrangements of oxyanion stabilizing residues were reported for HPL,[37] HLL,[40] RDL, and PCL.[9] However, the oxyanion hole in CRL is already properly configured in the closed conformation.[33]

The importance of transition state stabilization in lipases has been demonstrated by site directed mutagenesis experiments. For example, in the case of RDL where Thr 83 occupies the same position as Ser 82 in RML, replacement of the threonine by an alanine (thr88ala) caused an almost total loss in specific activity towards triacylglycerols.[41] Similar results were obtained for PCL when the corresponding serine was replaced by glycine.[42] Interestingly, replacement of threonine with a serine (thr83ser) in RDL resulted in a lipase whose specific activities were reduced but not totally destroyed, as compared to the wild type and the thr83ala-mutant lipase, respectively.[41]

Reviewing structural and kinetic evidences, a mechanistic model of lipase interfacial activation is related to various conformational and functional changes running from the inactive »closed« to the activated »open« conformation. However, a model based exclusively on two discrete conformational states of the lipase seems to be an oversimplification. In the structures of HLL, RDL,[43] and CRL,[13] the lid was not found to be closed, even in the absence of an interface. Although a part of these open structures observed may have been induced by crystallization conditions or crystal packing factors, an intrinsic conformational lability was observed in the absence of an activating substrate phase or inhibitor, and a subtle equilibrium between the two conformations prior to the contact with an appropriate lipid interface is proposed.[44] Comparison of a closed and open structure of CRL[33,44] suggested that lid opening is triggered by a *cis/trans* isomerization of a specific proline residue. Therefore, different authors propose that both lipase conformational changes (enzyme theory) and the nature of the lipid interface (substrate theory) contribute to interfacial activation.[43,44] Conformational changes in lipase structure are thought to be essential for understanding the interfacial activation phenomenon, however, not sufficient to explain observations[45] suggesting that the composition of substrate interfaces modulates hydrolytic activity.

The idea that conformational changes of various lids are at least in part responsible for interfacial activation is also supported by X-ray structures of FSC[11,46] and the characterization of a novel pancreatic lipase subfamily.[47] Cutinase, whose natural role is the degradation of wax esters covering the surface of plants, hydrolyzes triacylglycerols as efficiently as lipases, but lacks interfacial activation. The structure determination of a native FSC[11] and the enzyme covalently inhibited by diethyl *p*-nitrophenyl phosphate[46] demonstrated structural and functional homology to lipases. However, FSC does not have a lid shielding the active site. In addition, it possesses a preformed oxyanion hole, providing an electrophilic environment that stabilizes the catalytic intermediates (Figures 1 and 2). Primary structure comparisons of pancreatic lipases from guinea pig (GPL), *Myocastor coypus* (CPL), and several classical pancreatic lipases provided rational evidence of a new pancreatic lipase subgroup with kinetic properties that are different from classical lipases. Both the guinea pig[48] and coypu[49] enzymes display no interfacial activation, lack the positive colipase effect observed with classical pancreatic lipases, and share high phospholipase A activity. GPL has a deletion in the lid domain, as observed from the sequence comparison with other mammalian pancreatic lipases,[48] and the active site is envisioned to be easily accessible. CPL possesses a full-length lid domain but its sequence is poorly conserved when compared to HPL. In particular, replacement of a specific Trp residue, which stabilizes the closed conformation in HPL[5,37] by a Leu, is rationalized to facilitate a spontaneous opening of the active site.[47] Therefore, it can be concluded that either the absence of a lid, as in case of FSC and GPL, or probably a highly flexible lid, as proposed for CPL, result in the ability to perform triacylglycerol hydrolysis without interfacial activation.

SUBSTRATE SELECTIVITY

In order to designate the stereochemistry of glycerolipids, stereospecific numbering (*sn*-nomenclature) has been introduced (IUPAC-IUB Commission on Biochemical Nomenclature, 1968). When the glycerol backbone molecule is drawn in the Fischer projection with the substituent in position 2 facing to the left, glycerol carbons are numbered *sn*-1, *sn*-2, *sn*-3 from top to bottom (see Figure 3), thereby allowing an unambiguous description of the steric configuration of any isomeric glycerides.

Four different types of lipase substrate selectivity have been observed with acylglycerols: (i) lipid class selectivity, (ii) acyl chain selectivity, (iii) regioselectivity and (iv) stereoselectivity.[50,51] For example: (i) a pronounced diacyl- and monoacylglycerol specificity was reported for a lipase isolated from the fungus *Penicillium camembertii*, which was completely inactive towards various triacylglycerols.[52] A purified monoacylglycerol lipase from adipose tissue[53] was shown to have no activity against long chain di- and triacylglycerols. (ii) A *Geotrichum candidum* B lipase is highly specific to esters of *cis*-Δ-9-unsaturated fatty acids as determined with acylglycerols and other fatty acid esters.[54] (iii) Absolute regio- or positional selectivity is observed for instance with RML, which attacks only the primary ester bonds in a triacylglycerol substrate molecule (1,3-specific, see Figure 3), while CRL hydrolyzes all three acyl ester groups at comparable rates (non-specific).[55] Interestingly, there is no report of a lipase exclusively hydrolyzing the secondary ester bond. However, some lipases from different *Geotrichum* species were recently described, which cleaved the inside ester bond of triolein (*sn*-2 position) twice as fast as the ester bonds at the primary positions.[56,57] (iv) Stereoselectivity of lipases involved in acylglycerol hydrolysis (see Figure 3) can formally be subdivided into: 1) discrimination of two separate enantiomeric substrate molecules, and 2) discrimination of two chemically identical but stereochemically different enantiotopic groups within a prochiral substrate molecule. In both cases, however, faster hydrolysis of one primary ester as compared to the other is designated as *sn*-1 or *sn*-3 stereopreference. The term stereopreference may be used to describe the preferentially attacked acylester (*i.e.* quality of chiral discrimination, *sn*-1 versus *sn*-3), while stereoselectivity in this matter assigns the quantity of this differentiation.[58] An example of a profound stereopreference is lipoprotein lipase (LPL). The purified lipase from bovine milk exhibits a clear preference towards the *sn*-1 acylester in triolein and trioctanoin, respectively.[55] On the other hand, *sn*-3 preference was observed, for example, with gastric lipase from human (HGL) and other sources, while porcine pancreatic lipase (PPL) showed no significant stereoselectivity when tested under similar conditions.[59]

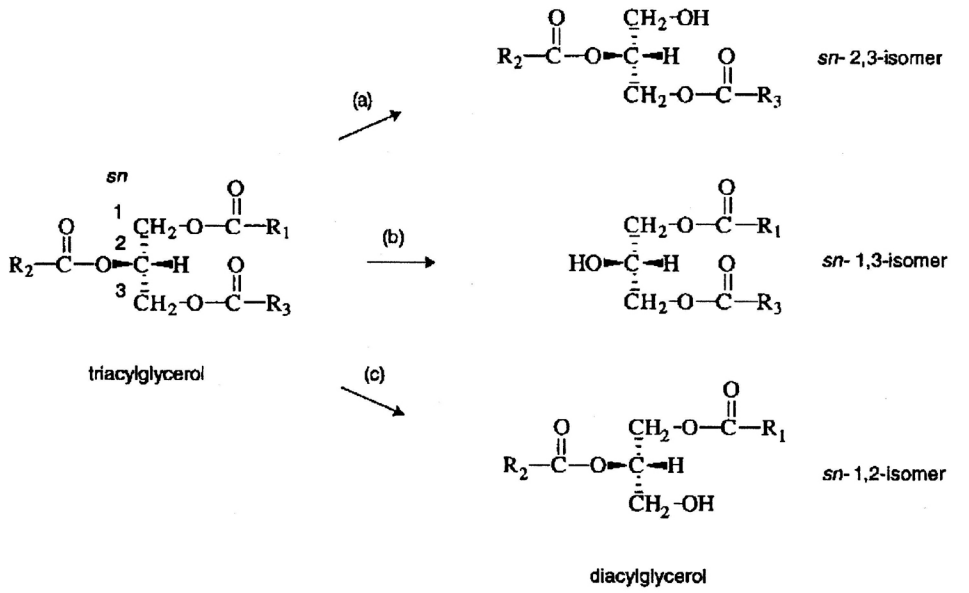


Figure 3. Regio- and stereoselective triacylglycerol hydrolysis. Lipase regioselectivity: [(a)+(c) not (b)] = regioselective hydrolysis; [(a)+(b)+(c)] = non-specific hydrolysis. Lipase stereoselectivity: 1) enantiomeric discrimination if R_1 and R_3 are different moieties and 2) enantiotopic discrimination if R_1 equals R_3 in a prochiral triacylglycerol. Stereoselective hydrolysis: [(a)>(c)] = *sn*-1 stereopreference, [(c)>(a)] = *sn*-3 stereopreference; (a), (b), and (c) are relative rates of the respective hydrolysis reactions.

In addition, combinations of various types of substrate selectivity (i–iv) are possible and sometimes may obscure the identification of discrete specificities.[51] For example, as mentioned before, several *Geotrichum* lipases were reported to have some preference for the *sn*-2 ester of triolein. When 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol was used as a substrate, this preference for the secondary ester moiety was significantly improved.[57] One could speculate whether a slight preference for the unsaturated fatty acid, as observed with different *Geotrichum* lipases, enhanced the relative rate of cleavage at position *sn*-2 in this case. An additive overlap of stereo- and acyl chain selectivity was demonstrated in the case of CRL, using synthetic enantiomeric triradylglycerols with different primary acyl ester chains.[58] In this case, introduction of an acyl moiety preferentially hydrolyzed by the lipase resulted in a significantly enhanced stereoselectivity with one enantiomer and a reversal of stereopreference with the other enantiomer, as compared to the corresponding prochiral analogs.

Whether a microorganism secretes the enzyme to degrade external substrate, an oil seed mobilizes storage lipids during germination, or dietary fats are hydrolyzed in the intestinal lumen of mammalians, the primary physiological function of lipases is to hydrolyze triacylglycerols to yield free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. In all cases, this hydrolytic reaction is the first step in the utlization of lipids as a fuel providing energy or metabolic intermediates. Therefore, the substrate selectivities observed may have biological relevance reflecting specific metabolic demands. For instance, the regioselectivity of pancreatic lipases converts triacylglycerols (and partial hydrolysis products resulting from gastric lipolysis) into 2-monoacylglycerols and fatty acids to achieve a complete intenstinal absorption.[60] The fungus *Geotrichum candidum* produces multiple isozymes with different positional and fatty acid selectivities.[54,57] Although some of the observed heterogeneity is due to differences in glycosylation, two different genes of extracellular lipases have been identified[61] and the corresponding recombinant lipases have been characterized.[62] Similar findings demonstrate the expression of, at least, five extracellular lipase isoforms in the yeast *Candida rugosa*.[63] The relative amount of the various lipases produced depends on culture conditions. Therefore, it is assumed that the existence of different adjustable selectivities enables specific adaptation to different environmental conditions. The biological significance of lipase stereoselectivity is not quite clear. Interestingly, HGL and HPL perform complementary stereoselectivity, thus probably providing an optimized two step digestive system.[55,59] The *sn*-1 stereoselectivity of LPL was sugested to avoid reutilization of *sn*-1,2-diacylglycerols as intermediates for triacylglycerol and phospholipid biosynthesis.[64,65] In general, as compared to other enzymes, lipases are considered to have rather relaxed specificities upon their natural substrates, which underlines their central role in lipid metabolism and reflects the structural variety of naturally occurring triacylglycerols.

Various industrial applications exploit the triacylglycerol hydrolyzing capability of lipases.[2] For example, dairy industry uses lipases to hydrolyze milk fats to accelerate cheese ripening and flavor enhancement. Specific flavor characteristics are generated by addition of lipases that primarily release medium and short chain fatty acids. Oleochemical industry exploits lipases for fat splitting and soap manufacture under mild conditions, avoiding degradation of highly unsaturated fatty acids. More recently, due to the trend towards lower laundering temperatures, lipases are considered as additives in household detergents to remove fat stains.[66] On the other hand, lipases are able to catalyze acylation reactions in a non-aqueous environment, where the equilibrium is shifted towards ester synthesis. Different lipase catalyzed acyl-transfer reactions, involving various esterification and transesterification methods, are used for the modification of fats and other lipids. For instance, structured triacylglycerols possessing valuable physical,

dietetic, or nutritional properties are synthesized from low quality fats using regioselective and fatty acid selective lipases.[67,68] (Poly)glycerol and carbohydrate fatty acid esters, which are used as food emulsifiers and environmentally safe detergents, are obtained from lipase based technologies. Production of various high quality isopropyl acyl esters and wax esters for personal-care products is achieved by lipase catalyzed esterification, requiring minimum downstream refining in comparison with conventional processes.[2,68] Stereoselectivity is perhaps the most interesting feature of lipase substrate specificity because it is not only observed with natural and related substrates but also with synthetic compounds. This, together with the capability of many lipases to retain activity in organic solvents, accounts for their application as valuable tools for biotransformations in organic chemistry. They are used in stereoselective acylation and hydrolysis[69] in the laboratory as well as in industrial processe. Above all, pharmaceutical industry exploits the ability of lipases for chiral discrimination in hydrolysis and acylation reactions during the preparation of optically pure synthons for various chiral drugs such as β-blockers and nonsteroidal antiinflammatory agents.[2,70]

MOLECULAR MECHANISM OF SUBSTRATE SELECTIVITY

Resolution of 3-D structures of several mammalian and microbial lipases revealed a similar overall fold and confirmed a catalytic mechanism involving a serine-protease like catalytic triad. However, despite great structural and mechanistic similarities, the substrate specificities of lipases are quite diverse. This broad substrate tolerance of lipases is exploited in the field of organic chemistry since lipases also catalyze the hydrolysis of a wide range of unnatural, non-acylglycerol like esters. In addition, reversal of the hydrolysis reaction in organic media gives rise to various acyl-transfer methodologies. Acceptance of a broad range of substrates in various types of reactions is often accompanied with pronounced regioselectivity and stereopreference, thus enabling chemists to generate isomerically pure intermediates. Lipases were, therefore, recognized as valuable chiral catalysts. However, their synthetic potential was screened empirically in the absence of any insight into the molecular mechanisms by which lipases distinguish between enantiomeric substrates or enantiotopic groups in a prochiral substrate molecule. In order to simplify the tedious task of choosing an appropriate enzyme by trial and error, the known enantioselectivities of lipases upon various substrates have been summarized in empirical rules. Common structural features of different substrates were mapped either intuitively or using computerized molecular simulation methods[71] and were correlated with the available kinetic data to develop substrate models that

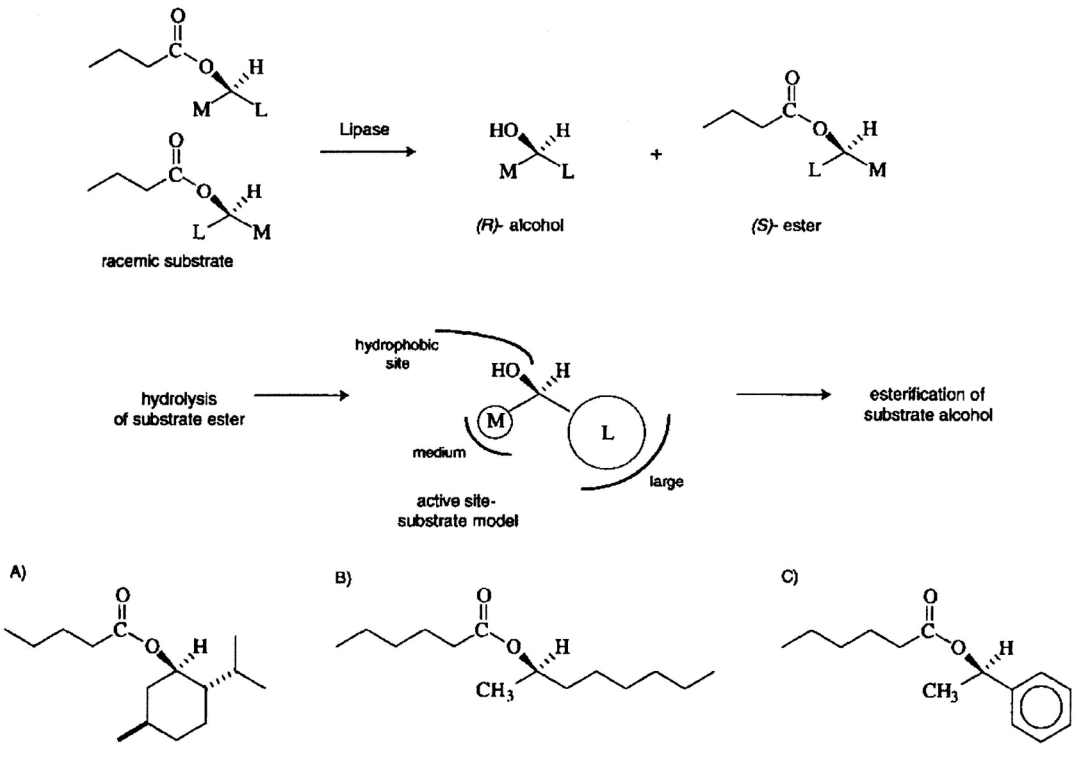


Figure 4. Model for the lipase enantiorecognition of secondary alcohols.[72] The empirical rule predicts the favored enantiomer of a secondary alcohol; L represents a large substituent; M represents a medium substituent. Assuming a Sequence Rule order of L > M, the preferably accepted enantiomer possesses (*R*)-configuration, as shown schematically for the hydrolysis reaction; e.g. A) (1*R*)-menthyl pentanoate, L = $CH[CH(CH_3)_2]$, M = CH_2-$CHCH_3$; B) (*R*-2-octyl hexanoate, L = C_6H_{13}, M = CH_3; C) (*R*)-1-phenylethyl hexanoate, L = phenyl, M = CH_3

mirror hypothetical substrate binding sites of the respective lipase (active site model). These models define multiple attachment or binding sites recognizing specific structural motifs of the substrate molecules.[69] For example, a simple rule for secondary alcohols, based on the size of the substituents at the stereocenter (see Figure 4), was formulated to predict which enantiomer reacts faster during lipase catalyzed hydrolysis and esterification reactions.[72] The ability of lipases to differentiate between enantiomeric secondary alcohols with similarly sized substituents (L = M, see Figure 4) is poor but improves when the size of one substituent is increased (L > M). Although the degree of stereopreference varies for different alcohols and enzymes, this rule seems to be predictive for various lipases and esterases.[72,73] On the other hand, models developed by different research groups for the same enzyme are not necessarily congruent[74] or only describe the steric course of a narrow range of related substrate structures. These empirical rules are used as guidelines for the development of new lipase catalyzed reactions, and provide the basic strategies for enhancing the enantioselectivity of these reactions. However, this models do not explain the details of the underlying molecular interactions that are responsible for the observed or predicted stereoselectivities of the respective lipase with its substrates.

More mechanistic answers to this intriguing problem are only expected from detailed information on the 3-D structures of enzyme-substrate complexes. To identify how substrate esters bind in the catalytic site of lipases, researchers have determined the X-ray structures of lipases with covalently linked phosphonates.[34,40,73] The crystal structure of RML covalently complexed with *n*-hexyl phosphonate ethyl ester (see Figure 2) indicated that the hexyl chain of the bound inhibitor binds in a hydrophobic groove,[34] generated upon activation by flap movement. A similar hydrophobic binding site for the scissile fatty acid during lipid hydrolysis was identified in HLL.[40] In the latter study, the corresponding n-dodecyl chlorophosphonate ethyl ester was used for inhibition, and at least the first seven to eight carbons of the alkyl phosphonate are tightly bound in a hydrophobic channel. In CRL, the alkyl chain of enantiomeric n-hexyl phosphonate menthyl esters was found to bind in a hydrophobic tunnel inside the protein.[73] The hexyl chain of the (*1R*)- and (*1S*)-menthyl inhibitor occupies the binding tunnel in an identical fashion. While phosphonates mimic the first tetrahedral intermediate prior to the formation of the acyl-enzyme during ester hydrolysis, alkylsulfonyl chlorides were used with CRL to resemble the second tetrahedral intermediate[75] preceding the deacylation step in the reaction pathway (see Figure 1). The structures of CRL complexed with dodecanesulfonyl or hexadecanesulfonyl chloride indeed demonstrated that the tunnel is occupied by the respective alkyl chains, similarly to what was observed with the phosphonates. The tunnel is already preformed in the closed conformation of CRL, L shaped with an almost perpendicular bend and can accommodate a fatty acid length of at least 18 carbons without major side chain rearrangements.

Determination of X-ray structures of two complexes with enantiomeric *n*-hexyl phosphonate menthyl esters also demonstrated how the binding of esters of two enantiomeric secondary alcohols differs and suggested why the (*R*)-enantiomer of the corresponding substrate (structure A in Figure 4) is preferred to the other.[73] The hexyl chain is inserted into the binding tunnel with the phosphonate group covalently linked to the active site serine identically in both complexes forming the same oxyanion hole hydrogen bonds. The respective menthyl moiety binds in a crevice which surrounds the catalytic triad facing the solvent. The isopropyl substituent of the alcohol points into a hydrophobic region which is open to the solvent, while the medium substituent, CH_2-$CHCH_3$, lies on the floor of the crevice. Thus, the model of the binding site for secondary alcohols (Figure 4) is consistent with the overall shape of the binding crevice observed in the CRL structures. The authors stated that the major interactions between the menthol and the binding crevice involve parts of the catalytic machinery common to many lipases, thus explaining similar enantiopreferences of lipases for secondary alcohols. In the case of the faster reacting (*R*)-enantiomer, the catalytic triad histidine hydrogen bonds to the menthol oxygen, which is essential for the protonation and subsequent dissociation of the leaving alcohol in the normal reaction sequence (see Figure 1). On the other hand, with the (*S*)-enantiomer, the alcohol oxygen is shifted away from the His due to the opposite stereochemisty and, additionally, the isopropyl substituent pushes against the catalytic imidazole. Both factors contribute to the collapse of the key hydrogen bond and thus slow down the reaction with CRL.

In a different approach, the substrate binding sites of lipases were studied by means of computer modeling methods in order to rationalize their preference for (*R*)-configured secondary esters. Molecular dynamics simulations and energy minimizations of transition state analogs of (*R*)-2-octyl hexanoate and (*R*)-1-phenylethyl hexanoate (structures B and C in Figure 4) in the active site of RML and HLL suggested that the large substituents bind similarly in both lipases but occupy different sites with the corresponding (*S*)-enantiomers. Large substituents interacted unfavorably with the enzyme in the latter case and the slow reacting (*S*)-enantiomers were found more strained as compared to the transition states of the fast reacting enantiomers.[76]

The three dimensional structures of lipase-inhibitor complexes in which diethylphosphate, alkylphosphonate or alkylsulfonate were covalently linked to the active site serine allowed description of potential enzyme-substrate interactions of the leaving fatty acid and the so-called oxyanion hole that stabilizes the tetrahedral intermediates of lipase catalyzed ester hydrolysis and esterification reactions. However, they gave no conclusive information about the acyl, regio- or stereoselectivity of lipases towards their

natural substrates. The chemical structure of the inhibitors used so far would not allow comparison with the natural acylglycerol substrate beyond the scissile acyl chain. In the absence of complexes of lipases and triacylglycerols or closely related, non-hydrolyzable triacylglycerol analogs, possible interactions of the glycerol backbone and the remaining two fatty acyl chains, besides the scissile fatty acid (see Figure 1), were studied by means of computer modeling methods based on available crystallographic data.[40,75,76] For example, a tripentanoylglycerol was modeled into the active site of CRL.[75] The authors suggested a probable binding site for a second acyl chain of the triacylglycerol. Crystallographic observations indicated that, upon inhibition with higher alkylsulfonyl chloride to enzyme ratios, a second inhibitor molecule covalently modified the catalytic His in this enzyme. Therefore, the triacylglycerol was modeled into the binding crevice in a »tuning fork« conformation, accommodating two acyl chains in a location that was observed for the respective alkyl chains in the doubly liganded structure. Similar interactions between the triacylglycerol and the enzyme were reported to be formed, regardless of whether the *sn*-1, *sn*-2, or *sn*-3 fatty acid was forced into the scissile acyl binding tunnel of CRL. The region around the active site of an inhibited structure of HLL was probed intuitively for hydrophobic patches that might be the binding sites for the remaining fatty acyl chains.[40] This analysis displayed a distinct clustering of acyl chains in at least four discrete hydrophobic sites, which may board the remaining acyl chains when one primary acyl chain is bound in the proposed scissile acyl binding cavity. Interestingly, the authors noted that, due to steric hindrances, they were not able to fit a catalytic competent *sn*-2 hydrolysis model for HLL. This is in contrast to the CRL triacylglycerol model but mirrors the different regiospecificities of these two lipases. However, a specific binding site for the leaving diacylglycerol group was not found in the HLL structure, when the active site was screened for latent hydrophobic or hydrophilic binding interactions using computerized energy potential algorithms.[76] Determination of the structure of a pancreatic lipase-procolipase complex cocrystallized with phosphatidylcholine[37] gave the first insights into the possible orientation of a glycerolipid bound into the active site of the enzyme. The primary acyl chain of the phospholipid passes through a hydrophobic channel with its carbonyl carbon in front of the catalytic serine. The *sn*-2 acyl chain was found lying in a hydrophobic groove formed by side chain residues of the flap region of the pancreatic lipase. Based on the former HPL structure, a similar model was also proposed for LPL.[77] The phospholipid carries a polar headgroup instead of an acyl group at the remaining glycerol hydroxyl and the spatial orientation may be different as compared to a triacylglycerol. However, recent crystallographic studies of HPL-colipase complexes inhibited with *n*-undecylphosphonate methyl esters indicated a

superimposable topology of the aliphatic chains, as compared to acyl moieties of the complexed phospholipid structure (Verger *et al.*, personal communication).

The mechanism of lipase selectivity towards simple esters of enantiomeric secondary alcohols is assumed to be similar for many lipases, based on the similar spatial arrangement in the immediate vicinity of the catalytic machinery, which is at least partially imposed from the similar overall protein fold.[73,78] On the other hand, comparison of structural and kinetic data suggests that, in the case of triacylglycerols, subtle interactions more distant from the catalytic serine may be important for determining substrate binding, explaining the noticed differences of lipase selectivities towards their physiological substrates. More detailed information about the molecular interactions of a triacylglycerol in the active site of a lipase are anticipated from structure determinations of complexes with transition state analogs that resemble the natural substrates with respect to the scissile fatty acid and the leaving diacylglycerol, respectively.

Different scientists have speculated whether the lid domain of lipases, which is thought to provide sufficient binding to the lipid interface upon activation prior to the formation of a catalytically competent substrate enzyme complex, is also involved in the expression of substrate selectivities.[48,77,79] This would be in line with the observations of crystallographic and molecular modeling studies suggesting that the opening of the lid does not only enable free access to the catalytic machinery but that internal lid residues are also involved in the formation of specific substrate recognition sites. Therefore, the function of the lid may be divided into interfacial interactions and substrate binding.[77]

However, it should be emphasized that enzyme selectivity must not be exclusively explained by refined description of a single substrate bound to the active site, as it is deduced from crystallographic determinations of stoichiometrical substrate analog complexes. For example, the reaction medium hydrophobicity is known to influence the specificity when enzymes are used in low water systems.[80] Since lipolysis is a heterogeneous catalysis of a water soluble enzyme at a substrate interface, physico-chemical properties of the interface may also influence the substrate selectivity. Recently, the stereopreference of various lipases towards enantiomeric triradylglycerols was demonstrated to depend on the solubilization method of the substrate.[81] Surface pressure in monolayers at the air-water interface was shown to affect both the activity and stereoselectivity of different lipases during hydrolysis of racemic diacylglycerols.[82] Decreased lipid packing at low surface pressures enhanced stereoselectivity while the catalytic activity decreased. It was assumed that surface pressure-dependent conformational changes of the enzymes shifted the residues involved in specific substrate recognition.

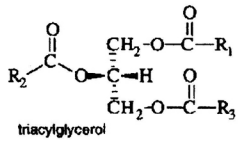
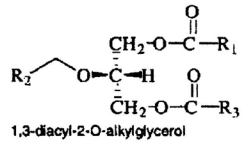
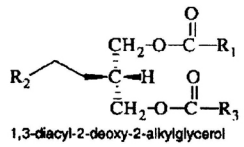
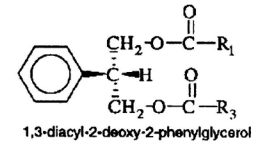
Hydrolysis of Triacylglycerols and Analogs – Effect of the sn-2 Moiety on the Stereoselectivity of Lipases

As mentioned before, stereoselectivity is regarded as the most attractive property of lipase substrate specificities since it provides preparative organic chemistry with a helpful device for asymmetric synthesis. A model describing the lipase enantioselectivity towards secondary alcohols was proposed[72] and the underlying molecular mechanism was elucidated for CRL.[73] It was further suggested that the common chiral preference of lipases and esterases for this substrate type results from the similarity of supersecondary structure features of the catalytic machinery.[44,73,78] However, for lipase catalyzed triacylglycerol hydrolysis, there is neither a simple empirical rule nor a comprehensive structural model to describe the diversity of lipase stereoselectivity towards their natural substrates. A study testing the stereoselectivity of 25 enzymes of microbial and animal origin towards triolein and trioctanoin showed wide variation between different enzymes, including switches in stereopreference (*sn*-1 *versus sn*-3; see Figure 3), when hydrolysis of two substrates by one lipase was compared.[55] There was also no obvious correlation for various closely related or highly homologous lipases. For example, HPL exhibits *sn*-1 preference while the corresponding enzyme from porcine pancreas, PPL, showed no significant stereopreference with both substrates tested. Dog pancreatic lipase (DPL) was *sn*-3 selective, and the stereopreference of GPL reversed from *sn*-3 to *sn*-1 when the oleoyl substrate was used, as compared to trioctanoylglycerol. Referring to the above mentioned crystallographic studies of lipase inhibitor complexes, the scissile fatty acid ester is assumed to be accommodated in a way that allows cleavage of the carboxyl ester bond by the catalytic machinery, involving nucleophilic attack of a competent serine and stabilization of the reaction intermediate by an oxyanion hole (Figure 1). This mechanism and the topology of the important residues are thought to be similar for all lipases. Therefore, the reason for preferential catalysis in position *sn*-1 or *sn*-3 (Figure 3) was assumed to result from specific interactions of the *sn*-2 acyl with distinct regions distant from the catalytic triad.[55]

First indications of the importance of the *sn*-2 ester carbonyl for the correct positioning of glycerol lipids by lipolytic enzymes came from experiments performed with substrate analogs containing ether bonds at certain positions of glycerol.[83,84] Different *O*-alkyl-diacyl and di-*O*-alkyl-acyl glycerols have frequently been used to study lipase specificity in order to avoid difficulties that might arise from acyl migration and subsequent hydrolysis of partial hydrolysis products (for references see Ref. 58). Using racemic mixtures of 1(3)-*O*-alkyl-2,3(1,2)-diacylglycerols, the stereopreference of various mammalian lipases was determined, showing similar results as those with the corresponding triacylglycerols.[64] However, crude LPL from bovine milk totally lost its *sn*-1 stereopreference upon replacement of the *sn*-2 acyl in the

TABLE I

Effect of the *sn*-2 moiety of prochiral triradylglycerols on the stereopreferences of microbial lipases (data from [58]).

Substrate		enzyme *sn-1/3*: ee 18:1 (ee 8:0)
 triacylglycerol	type A	RAL *sn-1*: 33 (32) CVL *sn-3*: 22 (25)
 1,3-diacyl-2-O-alkylglycerol	type B	RAL *sn-1*: i) 64 (74), ii) 67 (79), iii) 84 (87) CVL *sn-1*: i) 46 (62), ii) 40 (61), iii) 58 (69)
 1,3-diacyl-2-deoxy-2-alkylglycerol	type C	RAL *sn-1*: 44 (48) CVL sn-1: 28 (20)
 1,3-diacyl-2-deoxy-2-phenylglycerol	type D	RAL *sn-3*: 64 (71) CVL *sn-3*: 86 (76)

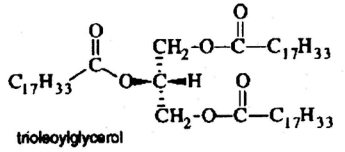
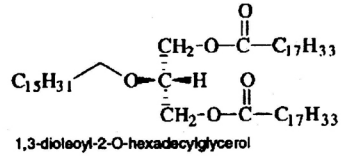
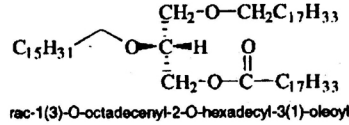
Substrate type A–D) R_1COO-, R_3COO- = oleoyl or octanoyl; type A) R_xCOO- = oleoyl or octanoyl; type B) R_2 = i) $C_{15}H_{31}$, ii) C_7H_{15}, iii) C_4H_9; type C) R_2 = C_4H_9; stereopreference indicated as preferentially hydrolyzed primary acylester *sn*-1 or *sn*-3, quantity of stereopreference expressed as enantiomeric excess values (ee) of diradylglycerols resulting from the hydrolysis of 1,3-dioleoyl-2-radylglycerol (ee 18:1) and 1,3-dioctanoyl-2-radylglycerol (ee 8:0), respectively; for details and method see.[58]

former substrate by an alkyloxy moiety.[83] Similar results were subsequently described for phospholipases C and D, which lost their strict stereospecificty towards 1,2-diacyl-*sn*-glycero-3-phosphocholine upon introduction of an ether group at position *sn*-2.[84]

Recently, we described an extensive study of stereoselectivity of four purified microbial lipases towards triacylglycerols in comparison with a variety of structural analogs.[58] Triacylglycerol analogs with distinct polarities at position *sn*-2 of the glycerol backbone (see Table I) were synthesized. Additionally, the substrate hydrophobicity and steric requirements were modified by

TABLE II

Stereo- and regioselectivity of purified bovine LPL tested with different triradylglycerols.

Substrate			Single substrate emulsion			mixed equimolar substrate emulsion			
			fatty acid %	ee	*sn*	share of total %	fatty acid %	ee	*sn*
 trioleoylglycerol	type A	1	69.2			28.9	67.5		
		2	*0.0*	34.8	1	*0.0*	*0.0*	35.0	1
		3	30.8			13.9	32.5		
 1,3-dioleoyl-2-O-hexadecylglycerol	type B	1	72.0			31.2	76.5		
			–	44.0	1	–	–	53.0	1
		3	28.0			9.6	23.5		
 rac-1(3)-O-octadecenyl-2-O-hexadecyl-3(1)-oleoylglycerol	type C	1	89.8			14.9	91.0		
			–	79.6	1	–	–	82.0	1
		3	10.2			1.5	9.0		

Lipolysis experiments were performed according to [58] in 2mM Tris/HCl pH 8.5, 1% BSA, 1 μg/μl apo CII at 37 °C. The percentage of fatty acid released from the *sn*-1/*sn*-3 position and *sn*-2 (in italic), respectively, based on single substrate species (fatty acid %) or total diradylglycerols formed during hydrolysis of equimolar mixture of substrates (share of total %).

variation of the alkyl and acyl chain length. The results show that minor structural variations at C-2 of the triacylglycerol analogs significantly affect the stereoselectivity of the lipases tested. It is noteworthy that variation of the substrate structure did not only alter the extent of stereoselectivity expressed as enantiomeric excess but in some cases, also resulted in a reversal of stereopreference. For example, *Chromobacterium viscosum* lipase (CVL) exhibited preference for the *sn*-3 ester bond of triacylglycerols (type A in Table I), but preferred position *sn*-1 in all the other substrates tested carrying an aliphatic moiety linked to C-2 (type B and C). On the other hand, *Rhizopus arrhizus* lipase (RAL) hydrolyzed all of these substrates preferentially in position *sn*-1. However, stereopreference of both CVL and RAL switched from *sn*-1 to *sn*-3 when the flexible 2-deoxy-2-alkyl moeity of substrate type C was replaced by the rigid and more bulky phenyl rest (type D).

In order to reinvestigate the previous findings obtained with a crude preparation of LPL, we determined the stereo- and regioselectivity of the purified enzyme from bovine milk (kindly provided by G. Olivecrona, Umea, Sweden) towards prochiral and enantiomeric triradylglycerols (Table II). Contrary to the results described for the crude lipase, the purified LPL did not lose its *sn*-1 preference upon introduction of an alkyloxy group at position *sn*-2. Stereoselectivity significantly increased, regardless of whether the substrate was a prochiral 1,3-diacyl-2-*O*-alkylglycerol (type B) or racemic 1(3)2-di-*O*-alkyl-3(1)-acylglycerol (type E), as compared to the corresponding triacylglycerol. Possible effects due to physico-chemical differences of single substrate emulsions that might arise as a consequence of distinct hydrophobicity of the different substrates were excluded when lipolysis was carried out with equimolar mixtures of substrates, and identical results were obtained. Inconsistent results might therefore arise from the heterogeneity of the crude preparation used in earlier studies. The presence of additional lipolytic enzymes may have obscured the results in a way that enzymes with opposite stereoselectivity were present in the crude enzyme preparation and/or the selectivity of these enzymes is oppositely affected by alteration of the substrate structure.

Our studies with microbial and mammalian lipases confirmed the decisive role of the *sn*-2 position for chiral recognition of acylglycerols. In addition, results with LPL underline the importance of the use of homogenous enzyme preparations for the determination of substrate selectivities. Furthermore, it became evident that substrates (triacylglycerols) and substrate analogs may behave quite differently during lipolysis, and that the results obtained with the substrate analogs need not necessarily reflect the behavior of the enzyme towards natural substrates. Most importantly, the results obtained with various triacylglycerol analogs provide some basic data to enable a more rationalized modeling of lipase-substrate interactions. The selectivity pattern observed for one lipase is suggested to be the net result of

various interactions (*e.g.* polar, sterically controlled, and hydrophobic) of a substrate molecule with different distinct regions of the respective enzyme. These interactions contribute to different degrees and probably at different stages of the catalytic process to the formation and stabilization of, at least, two competent substrate-enzyme complexes to yield the corresponding *sn*-1 or *sn*-3 hydrolysis products at different rates. Since substrate variations resulted also in opposite effects with different lipases, substrate binding sites are considered to vary from one lipase to another. It has already been demonstrated by site directed mutagenesis of RDL that single amino acid replacements may result in a significantly altered substrate specificity.[41] Recently, differences in the substrate selectivity pattern of two isoformic GCLs were assumed to be related to only a small number of changed residues within the substrate binding cavity.[62]

CONCLUDING REMARKS

Determination of 3-D structures of lipases, both native or complexed with various inhibitors, enabled a basic view on a molecular level to explain the catalytic mechanism of lipase catalyzed reactions. The catalytic triads of most lipases were found to be buried beneath flexible domains (lid) covering the catalytic machinery of the native (closed, inactive) enzyme, suggesting that conformational changes in the enzyme are involved in the phenomenon of interfacial activation. Recently, lipases were described that do not exhibit interfacial activation due to the absence or lability of lid structures. On the other hand, different lipases are reported to be able to hydrolyze ester bonds in both triacylglycerols and phospholipids (phospholipase A1 activity). Therefore, the classical distinction between lipases, esterases, and phospholipases will have to be reviewed critically. Selectivities of lipases towards triacylglycerols and their analogs were found to be significantly influenced by minor variations of substrate structure. Specific interactions of the substrate with the lipase that are distant from the catalytic serine are supposed to be important for the expression and modulation of diverse selectivities. In addition, lipase activity and selectivity was demonstrated to depend on conformational changes during catalysis. Therefore, selective hydrolysis must not be seen as a simple lock and key mechanism, but as a more dynamic process. In the future, crystallographic studies on enzyme-substrate complexes with triacylglycerol related inhibitors will, therefore, improve our insight into molecular interactions that govern the substrate selectivity of lipases and provide a rational basis for site directed mutagenesis to change the enzyme structure and performance.

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SAŽETAK

Djelovanje i selektivnost lipaza

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U ovom članku dan je pregled istraživanja lipaza s posebnim osvrtom na odnos molekulske strukture i funkcije. Određivanjem kristalografskih struktura lipaza iz mikroba i sisavaca objašnjen je mehanizam hidrolize acilnih estera kataliziranih s lipazama. Katalitička triada, slična onoj u serin proteaza, odgovorna je za cijepanje

esterske veze supstrata, preko međuprodukta acil-enzima. Usporedna strukturna istraživanja otkrila su zajednički put trodimenzijskog nabiranja i istovjetnu topologiju katalitičkog mjesta u lipaza, esteraza i drugih hidrolitičkih enzima. Trodimenzijska struktura osnova je za razumijevanje molekulskih interakcija koje dovode do različite selektivnosti prema triacilglicerolima i njihovim analozima.