SECTION C

LIPASES

CHAPTER 16

LIPASES: MOLECULAR STRUCTURE AND FUNCTION

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1. INTRODUCTION

Early reports on the production by both bacterial and eukaryotic cells of enzymes able to degrade lipid substrates date to over a century ago. Since then, research on lipolytic enzymes – that includes lipases, esterases, phospholipases – has been driven by their central roles in lipid metabolism and in signal transduction. Lipases are generally versatile enzymes that accept a broad range of substrates (*i.e.* aliphatic, alicyclic, bicyclic and aromatic esters, thioesters, activated amines) whilst maintaining high regio-, chemo- and enantioselectivity. The stability of most lipases in organic solvents paves the way for their exploitation in organic synthesis: in esterification, transesterification, aminolysis and oximolysis reactions (Drauz and Waldman, 1995). Such properties make lipases key players in the industrial enzyme sector (Schmid and Verger, 1998; Bornscheuer, 2000; Kirk *et al.*, 2002; Jaeger and Eggert, 2002; Gupta *et al.*, 2004).

In this chapter we review the fundamental knowledge available on lipases, with particular emphasis on the relationship between the sequence, structure and function of those most commonly used in industrial processes. On the basis of this knowledge, novel and improved lipases may be generated, able to meet the requirements for robustness, selectivity and catalytic performances posed by modern biocatalysis.

2. BIOCHEMISTRY, FUNCTION AND EXPRESSION

2.1. Lipases Versus Esterases. The Concept of Interfacial Activation

Lipases are hydrolases and exert their activity on the carboxyl ester bonds of triacylglycerols and other substrates. Their natural substrates are insoluble lipid compounds prone to aggregation in aqueous solution. As early as 1958 Sarda

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and Desnuelle described the sharp increase in lipase activity at substrate concentrations exceeding their solubility threshold as being the major difference to the esterases, enzymes active on ester bonds of soluble molecules that follow classical Michaelis- Menten kinetics (Sarda and Desnuelle, 1958). Since then the formation of an interface between aggregated substrates and the aqueous solution has been recognized as necessary for the activation of lipases (sometimes also referred to as "interfacial enzymes"). This behaviour – known as interfacial activation – found a structural rationale some years later when the first three-dimensional structures of lipase enzymes were elucidated (Winkler *et al.*, 1990; Brady *et al.*, 1990). These studies revealed that the enzyme active sites are shielded from the solvent by a mobile structure, the "lid" or "flap", that has to be displaced upon interaction with the substrate/water interface in order to yield an active enzyme conformation with the catalytic centre accessible to substrates. The crystal structures of lipases alone or in complexes with transition state analogues facilitated the elucidation of the conformational changes involved in the transition from the inactive closed lid conformation to the active one when the lid is open (Grochulski *et al.*, 1994; Brzozowski *et al.*, 1991). The mechanics of lid opening may vary between enzymes but in all cases leads to the creation of an open, accessible active site and a large hydrophobic lipid binding site (Fig. 1). In several lipases lid opening is also responsible for the formation of the so-called "oxyanion hole" which is involved in the stabilization of the reaction intermediates (see later). However, the classification of a lipolytic enzyme as being a *true* lipase (EC 3.1.1.3) on the basis of its activation at the interface and the presence of a lid structure does not hold in a number of cases. Lipases without a lid or with a lid but no interfacial activation have been described (Verger, 1997). To date, the broader definition of a lipase as a carboxylesterase catalysing hydrolysis and synthesis of long-chain acylglycerols is generally accepted and seems to be adequate to describe all known lipases. It specifically refers to

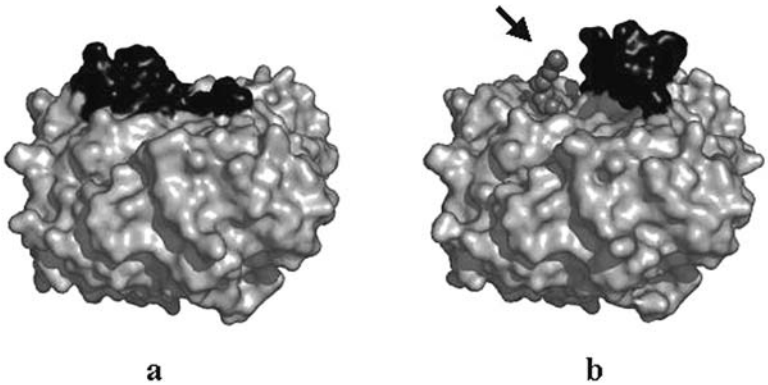

Figure 1. Lipase from *Candida rugosa* represented in the closed (a) and open conformation (b) with the lid depicted in black. In the active conformation (b) the enzyme active site is accessible to substrates here represented by an inhibitor (dark grey) and highlighted by the arrow

the behaviour of enzymes on insoluble substrates but it has to be recalled the most lipases are active also on soluble esterase substrates.

In low-water conditions, the reverse synthetic reaction is favoured, leading to esterification, alcoholysis and acidolysis. Such synthetic ability, along with the tolerance of several lipases to organic solvents (Zaks and Klibanow, 1984), is extensively exploited in organic synthesis (dealt with in depth in other chapters of this book).

2.2. General Molecular and Biochemical Features

Lipases are ubiquitous enzymes present in all types of living organisms. In eukaryotes they may be confined within an organelle (*i.e.* the lysosome), or they can be found in the spaces outside cells and play roles in the metabolism, absorption and transport of lipids. In lower eukaryotes and bacteria lipases can be either intracellular or be secreted in order to degrade lipid substrates present in the environment, and in some pathogenic organisms (*Candida albicans*, *Staphylococcus* and *Pseudomonas* species, *Helicobacter pylori*) they can even act as virulence factors. Enzymes from bacteria and fungi have the greatest potential as industrial biocatalysts since they are usually robust, easy to produce by fermentation and easy to recover from the culture broth. As a consequence, a large number of microbial enzymes can be obtained from commercial producers. Most bacterial lipases are sourced from *Pseudomonas, Burkholderia, Alcaligenes, Acinetobacter, Bacillus* and *Chromobacterium* species; widely used fungal lipases are produced by *Candida, Humicola, Penicillium, Yarrowia, Mucor, Rhizopus* and *Aspergillus* sp. Among the lipases from higher eukaryotes, porcine pancreatic lipase has been in use for several years as a technical enzyme. Other mammalian lipases are of medical interest as possible drug targets in the treatment of metabolic diseases or for direct development as drugs (Müller and Petry, 2004). In such cases, recombinant forms are favoured to overcome demanding purification protocols. For example, recombinant human gastric lipase is used in the treatment of pancreatic insufficiency caused by cystic fibrosis and pancreatitis. Plant enzymes, *e.g.* from papaya, pineapple, *Veronia*, *Euphorbia*, and in particular germinating seeds (castor bean, oil palm, oilseed rape), have interesting applications in biocatalysis as they display unusual fatty acid selectivities (Mukherijee and Hills, 1994). Such diversity in origin, cellular localization and function is reflected in an astonishing degree of biochemical variability since lipases from different organisms, or even isoenzymes produced by the same organism, may vary greatly in molecular mass, pH and temperature optima, post-translational modifications, and substrate and reaction specificities. This extensive variation is of importance to biotechnology as a potential source of biocatalysts endowed with a wide range of optima and specificities that can adapt to various process conditions. Attempts to broaden the biocatalytic power of the available lipases are taking a number of routes including the search for novel enzymes produced by organisms adapted to unusual habitats, the metagenomic approach, and rational and random mutagenesis of known enzymes.

Molecular masses of known lipases range from less than 20 kDa as in the case of the small lid-less lipolytic enzymes lipase A from *Bacillus subtilis* and cutinase from *Fusarium solani pisi*, to about 60kDa for the larger fungal lipases (*i.e. Geothricum candidum* lipase). In spite of this, almost all lipases share a common architecture and are structured in a single protein domain. Exceptions are found in lipases from higher eukaryotes where complex functions, *i.e.* interaction with other molecules and regulation, are attained through additional structural modules.

The range of temperature optima observed is wide, generally falling between 30 °C and 60 °C. However, this concerns lipases obtained from conventional sources. More recently the search for enzymes from extremophiles, *i.e.* organisms adapted to life in extreme environments, has enriched the spectrum with lipases with T_{opt} over 70 °C (*i.e. Bacillus thermocatenulatus* lipase) or those endowed with high activity at low temperature as is the case for enzymes produced by Antarctic bacteria, *i.e.* from *Pseudomonas* and *Moraxella* sp. Such extreme and unusual features open the possibility to apply these enzymes in their wild type form without the need for engineering approaches to adapt them for use in reactions carried out at high temperatures or, conversely low temperature processes such as that of detergents (low temperature washes) or in food processing (Demirhian *et al.*, 2001). Most lipases used in biocatalysis have neutral or alkaline pH optima, in some cases up to or beyond pH 9.0 (*Pseudomonas* and *Bacillus* lipases). Less common are acidic lipases active at pH as low as ca. 3.0. Interestingly, some lipases from *Bacillus* sp. are active over a broad pH range (Gupta *et al.*, 2004).

2.3. Control of Lipase Production

Lipases are involved in specific metabolic processes hence the expression of the genes encoding them is tightly regulated. The occurrence of these regulatory mechanisms has to be taken into particular account during the production of industrial lipases by fermentation, *i.e.* when dealing with bacterial or fungal producers. Expression of lipolytic proteins is often inducible and can be modulated by several parameters. Among them the carbon and nitrogen source provided during fermentation are of particular importance, as is the addition of compounds that can act as inducers, for example, fatty acids, Tweens, olive oil. Physiological parameters set during the fermentation protocol, such as the pH of the medium, temperature and oxygen supply also play roles (Gupta, 2004) since the production of lipases can be dependent on the growth phase of the culture as has been shown for *Streptomyces* and *Staphylococcus* strains as well as in *Pseudomonas aeruginosa* (Jaeger *et al.*, 1999). Knowledge about the regulation of gene expression is of particular relevance in several known cases where the source organism produces lipase isoenzymes, *i.e.* related proteins encoded by a family of paralogous genes. Usually protein isoforms are closely related in sequence and biochemical features, but not identical, and differences can be relevant from a catalytic point of view. Good examples are provided by fungal strains, as for example the asporogenic yeast *Candida rugosa* which produces at least 7 proteins differing in substrate specificity,

glycosylation, temperature and pH stability (Lotti *et al.*, 1993; Lopez *et al.*, 2004), *Yarrowia lipolytica* (Fickers *et al.*, 2005) and the opportunistic pathogen *Candida albicans* which has at least ten lipase proteins (Hube *et al.*, 2000). In such organisms the expression of isoenzymes can be subjected to complex control mechanisms. This issue has been studied in detail for the *Candida rugosa* lipases, some of which are constitutively expressed whilst others are induced by substrates present in the medium (Lotti *et al.*, 1998; Lee *et al.*, 1999). Whereas the availability of related and complementary enzymatic activities has obvious metabolic advantages for the producing strains, it can lead to enzymatic preparation of poorly reproducible composition and/or catalytic performance (Lopez *et al.*, 2004).

2.4. Occurrence and Functional Relevance of Post-Translational Modifications

Eukaryotic lipases are often glycosylated. The role of sugar chains in the activity, stability and secretion of a number of lipases has been investigated in depth using mutant proteins lacking glycosylation sites. However, determining the functional role of oligosaccharides is not always straightforward. In most cases they affect protein solubility and, as a consequence, the folding and/or the secretion of the enzyme (Miller *et al.*, 2004). Nevertheless, some specific functional roles have been elucidated. A clear role for asparagine-linked sugars has been pointed out in enzymes belonging to the acid lipase family, characterized by stability and activity under low pH conditions. Human gastric lipase (HGL) for example, which initiates the digestion of triglycerides in the stomach, is a highly glycosylated protein (up to 15% of the protein mass) with four potential N-glycosylation sites. The activity of deglycosylated recombinant HGL is affected to different extents depending on the number of sugar chains removed, but the most evident impact of deglycosylation is the increased susceptibility to pepsin degradation in acidic conditions shown by the deglycosylated enzyme (Wicker-Planquart *et al.*, 1999). An active role in enzyme activation, *i.e.* in lid opening, has been shown in two fungal lipases. Removal of an asparagine residue strictly conserved in the *Candida rugosa* lipase family resulted in a dramatic drop in enzyme activity whereas deglycosylation at other locations impacted to a much lower extent on activity (Brocca *et al.*, 2000). In this case, crystallographic analysis of the enzyme in the open and closed forms suggested that this sugar chain contributes to the stabilization of the open active form by interacting with the inner surface of the open lid (Grochulski *et al.*, 1994; Fig. 2a). The second example concerns a non-glycosylated mutant of *Thermomyces lanuginosa* lipase which displays lower binding affinity to phospholipid liposomes. This behaviour is suggested to affect the dynamics of lid movement and, as a consequence, the binding of the enzyme to the interface (Peters *et al.*, 2002). These and a number of other reports clearly indicate that the glycosylation ability of the host has to be carefully considered in heterologous expression of lipases, as sugar chains appear to impact on several issues of lipase functionality.

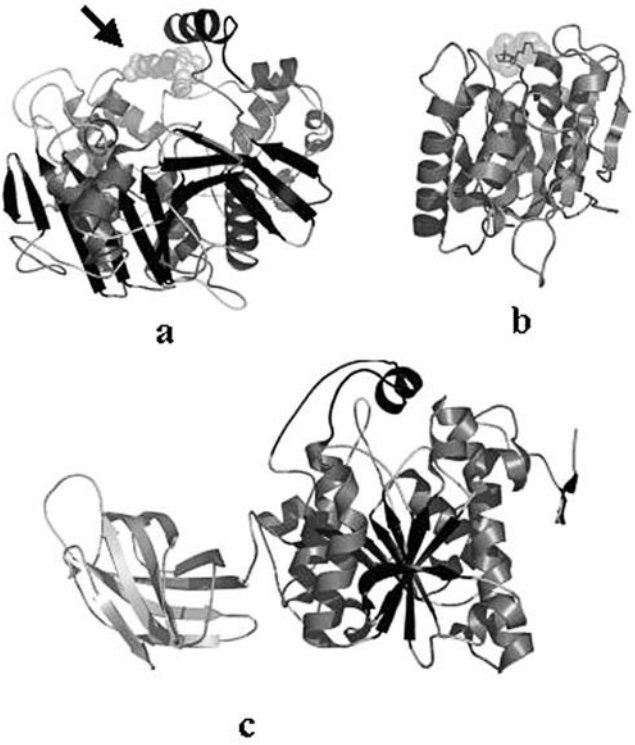

Figure 2. Variation on the α/β hydrolase fold design in lipases of different complexity: (a) the *Candida rugosa* enzyme structure where the arrow marks the oligosaccharide chain linked to Asn 351, (b) the mini-lipase from *Bacillus subtilis* distinguished by the lack of a lid structure and (c) the human pancreatic lipase with the colipase binding domain on the left side

Rare and so far unique to lipases subjected to hormonal regulation, is reversible phosphorylation. Hormone-sensitive lipase (HSL) is responsible for the mobilization of fatty acids in adipose tissue in response to hormonal stimuli and is regulated by phosphorylation by a number of protein kinases, in particular by cAMP-dependent protein kinase A. Four serine residues have been identified as kinase targets. The mechanism leading to HSL phosphorylation-mediated activation seems to involve not just conformational changes but also translocation of the protein from the cytosol to lipid droplets (for a recent review see Yeaman, 2004).

2.5. Specificity (Selectivity) of Lipase-Catalysed Reactions

The potential of lipases as biocatalysts relies on their sophisticated selectivity and specificity which permits the fine tuning of reactions. Specificity or selectivity can concern regioselectivity, *i.e.* the position in the substrate molecules of the ester bonds hydrolysed or formed; chemo-selectivity, *i.e.* the nature of the substrate

recognized; and stereoselectivity. One field of biocatalysis where such properties are successfully exploited is the modification of triglycerides where three features are relevant: i) regioselectivity *i.e.* the position of the fatty acid on the glycerol backbone; ii) fatty acid specificity concerning *i.e.* the length or unsaturation of the chain; iii) the class of acylglycerols, *i.e.* mono-, di- or triglycerides. Most known lipases are 1, 3 regiospecific with activity on the primary alcohol positions whereas only a few are able to recognize also the sn-2 position allowing for the complete hydrolysis of triglycerides to free fatty acids. Concerning fatty acid selectivity, lipases are able to convert esters of medium to long chain (C4 to C18, rarely up to C22) but with different efficiencies. Even isoforms of the same enzyme can differ in this property. This is the case for the isoforms of *Candida rugosa* lipase where isoform 1 acts mainly on medium chain (C8-C10) substrates, isoform 3 on short-chain soluble substrates, and isoforms 2 and 4 on long-chain molecules (C16-C18). Some lipases display unusual preferences towards unsaturated fatty acids. Worthy of mention in this regard are one isoform of *Geotrichum candidum* lipase selective for *cis* (Δ-9) unsaturated substrates, pancreatic lipase and some microbial lipases active on long-chain polyunsaturated substrates (PUFA), and others (from guinea pig, *S. hyicus*, *Rhizopus*) with phospholipase A1 activity. Lipolytic enzymes possessing different selectivities can therefore be used alone or in combination to obtain valuable products, such as structured triglycerides with improved nutritional value, cocoa butter substitutes, and oils enriched in PUFAs, as well as an impressive range of mono- di- and triacylglycerols, fatty acids, esters and intermediates (Bornscheuer, 2000). Another field where lipases find increasing application is in the regioselective acylation of polyfunctional molecules such as carbohydrates, amino acids and peptides - in particular in the protection/deprotection steps necessary for the generation of combinatorial libraries on carbohydrate scaffolds for the development of new drugs (Le *et al.*, 2003). Another property of lipases of paramount importance for application in fine chemistry and drug and agrochemical production, is their stereoselectivity toward a broad range of substrates which facilitates reactions on prochiral substrates and the kinetic resolution of racemates. The use of lipases in such processes extends to prochiral and chiral alcohols, carboxylic acid esters, α and β-hydroxy acids, diesters, lactones, amines, diamines, amino-alcohols, α- and β-amino acid derivatives (Schmidt *et al.*, 2001). Examples of industrial scale lipase-catalysed processes include the kinetic resolution of various amines and the production of an intermediate in the synthesis of DiltiazemTM (a calcium antagonist used to control high blood pressure) by *Serratia marcescens* lipase (Shibatani *et al.*, 1990).

3. DIVERSITY AND CONSERVATION WITHIN LIPASES: SEQUENCES AND STRUCTURES

3.1. Primary Sequences and Sequence-Based Classification of Lipases

By the end of 2005 about 2000 non-redundant sequences of lipases and related enzymes were present in protein sequence databases. No specific sequence similarity

is shared by all known lipases. On the contrary, they appear to be astonishingly variable. In the Lipase Engineering Database (LED), lipases are grouped in 16 superfamilies and 39 homologous families (Fisher and Pleiss, 2003). The lone consensus shared by all of them is the pentapetide Gly-X-Ser-X-Gly (with rare cases where glycines are substituted by other small residues). This motif, which encloses the active site serine, is denominated in the PROSITE database (Hulo *et al.*, 2004) as PS00120 ([LIV]-{KG}-[LIVFY]-[LIVMST]-G-[HYWV]-S-{YAG}-G-[GSTAC]) and identifies a proteins as a lipase.

3.2. All Lipases Share a Common Structural Fold

Despite their variability in primary sequence, all lipases display the same structural architecture, the so-called α/β hydrolase fold, and have identical catalytic machineries. Such structural conservation is a very valuable tool helping in the classification of newly identified proteins even in the absence of clear sequence similarity. Moreover, it facilitates modelling approaches prior to protein engineering experiments. The original description of this fold was based on the comparison of the three-dimensional structures of hydrolases mostly unrelated in primary sequence and active on substrates very different in structure, one of which was a fungal lipase (Ollis *et al.*, 1992). All lipases whose 3D structures were later solved were found to be members of this fold family. The design of the canonical α/β hydrolase fold is based on a central, mostly parallel β-sheet of eight strands with the only strand (β2) antiparallel. Strands β3 to β8 are connected by α-helices packed on both sides of the β-sheet. Variations from the canonical fold can affect the number of β-strands, the presence of insertions, and the architecture of the substrate binding subdomains (Fig. 2). Lipases of known 3D structure are currently classified by the SCOP database (Murzin *et al.*, 1995) into 7 families based on the elements of the basic fold that they contain: acetylcholinesterase-like, gastric lipase, lipase, fungal lipase, bacterial lipase, pancreatic lipase N-terminal domain, and cutinase-like. The small bacterial lipase A from *Bacillus subtilis* has been defined as a "minimal α/β hydrolase fold protein" as it only contains a six-stranded parallel β-sheet flanked by five α-helices (van Pouderoyen *et al.*, 2001). Additional domains can be added to this basic architecture, *i.e.* in enzymes involved in protein-protein or protein-lipid interactions or those subjected to regulation such as pancreatic lipase and hormone-sensitive lipase.

In α/β hydrolases the active site consists of a catalytic triad comprising a nucleophile, an acidic residue and a histidine, reminiscent of that of serine proteases but with a different order in the sequence: nucleophile-acid-histidine (Ollis *et al.*, 1992). The lipase catalytic triad is composed of serine, aspartate or glutamate and histidine, with the serine enclosed in the consensus motif previously mentioned which forms a sharp turn (the nucleophile elbow) in a strand-turn-helix motif in strand β5 which forces the nucleophile to adopt unusual main chain Φ and Ψ torsion angles. Due to its functional relevance, the nucleophile elbow is the most conserved feature

of the fold. Hydrolysis of the substrate follows a two-step mechanism. The nucleophilicity of the active serine is enhanced by transferring a proton to the catalytic histidine with the formation of an oxyanion that attacks the carbonyl carbon of the susceptible ester bond. A tetrahedral intermediate is formed carrying a negative charge on the carbonyl oxygen atom of the scissile bond and it is stabilized through hydrogen bonding to main-chain NH groups. Such residues build up the so-called oxyanion hole that in some lipases is preformed in the correct orientation, whereas in others it is positioned upon the opening of the lid structure. The proton on the histidine is then transferred to the ester oxygen of the bond that is cleaved and a covalent intermediate forms with the fatty acid from the substrate esterified to serine. The second step of the reaction is deacylation of the enzyme through a water molecule that hydrolyses the covalent intermediate. In this case, transfer of a proton from water to the active site serine produces a hydroxide ion that attacks the carbonyl carbon atom in the substrate–enzyme covalent intermediate. In addition the negatively charged tetrahedral intermediate is stabilized by hydrogen bonds to the oxyanion hole. Finally, histidine donates a proton to the oxygen atom of the active serine and the acyl component is released.

3.3. Complexity in Lipases From Eukaryotes: Modularity and Regulation

Some lipolytic enzymes active in eukaryotic cells are faced with demanding functions that require additional abilities, such as the interactions with lipids under unfavourable conditions, membranes, other molecules, and more rarely, regulation. The two best characterized examples are pancreatic lipase (PL) and hormone-sensitive lipase (HSL). Both enzymes are organized in modules with a catalytic domain with the functional and structural characteristics previously described, plus additional domains that confer other properties.

PL is composed of two domains connected by a flexible hinge, a large N-terminal catalytic domain and a β-sandwich C-terminal domain which is related to the peculiar physiological environment in which the enzyme has to be active. In the intestinal lumen dietary triglycerides are mixed with phospholipids, fatty acids, proteins and bile salts that act as emulsifiers. Bile salts would prevent PL from adsorbing to the lipid substrate were it not for the association with a small protein – colipase – that is co-secreted by the pancreas. Colipase is an amphiphilic protein able to anchor the lipase to the lipid interface and stabilize it in the active open conformation. Upon binding to the lipase C-terminal domain colipase exposes hydrophobic finger structures on the opposite site and brings the enzyme in contact with the interface. Colipase binding does not induce conformational changes in the lipase molecule but indirectly allows opening of the lid through contact with the interface. However the cofactor makes contact with the open lid and with it forms a large hydrophobic surface able to interact strongly with the lipid-water interface (van Tilbeurgh *et al.*, 1992).

Hormone-sensitive lipase (HSL) is an intriguing enzyme whose complex functions are still not completely unravelled. Its major and best characterized activity is the hydrolysis of triacylglycerols stored in adipose tissue, the first and rate-limiting step in the mobilization of fatty acids. HSL is composed of two structural domains with the active site in the C-terminal module. Phosphorylation sites are located in an extra module that interrupts the sequence of the catalytic domain. In the tertiary structure this module protrudes from the core of the domain which can therefore assume the canonical α/β hydrolase fold. In addition, HSL contains an N-terminal domain involved in protein-protein and protein-lipid interactions, as the enzyme has to make contact with lipid droplets accumulated in tissues. The main interactor of this docking domain has been shown to be the fatty acid-binding protein (FABP) that facilitates the release of fatty acids and their intracellular diffusion (Jenkins-Kruchten *et al.*, 2003). HSL, which is subjected to several levels of regulation including reversible phosphorylation, translocation and association with regulatory proteins, provides an interesting example showing that new properties can be introduced in a lipase without interfering with its fold and conformation (Yeaman, 2004).

4. DETERMINANTS OF LIPASE SPECIFICITY

Lipase selectivity has been studied from several points of view with the aim of understanding its molecular and conformational basis on the one hand, and to be able to modulate enzyme performances on the other. The molecular features of the enzyme, the chemical structure of the substrate and the reaction conditions are the three major factors affecting specificity. With regard to the latter, several studies have been devoted to assess the influence of the solvent, the quality of the substrate interface and the matrix used to immobilise the biocatalyst (Cernia and Palocci, 1997; Villeneuve *et al.*, 2000). Medium engineering has explored the effects of different organic and non-conventional solvents and water activity conditions and, more recently, the influence of ionic liquids has been examined (Park and Kazlauskas, 2003). However, understanding the molecular basis of lipase selectivity is a prerequisite for modifying the properties of the enzyme, hence this has been investigated in depth during recent years making use of synergic and complementary approaches: i) X-ray analysis of lipases in complex with substrates or their analogues; ii) the generation of site-specific and random mutants; iii) modelling of the available experimental results to extrapolate general rules and acquire predictive capabilities. From such investigations two structural elements came into focus as being major determinants of lipase specificity: the substrate binding site and the lid.

4.1. The Substrate Binding Site

The active site in lipases is buried within the protein structure and substrate access to it is through a binding site located in a pocket on the top of the central β-sheet. Although lipases share the same structural fold their substrate binding regions are

considerably different in size, structure and physico-chemical features, in particular regarding the hydrophobicity of residues lining the pocket. The length, shape and hydrophobicity of the binding pocket has been related to chain length preference, obtaining good agreement with experimental results (Pleiss *et al.*, 1998). Based on this information and X-ray determinations of the structures of complexes to substrate analogues, several mutant enzymes have been created by introducing bulkier or more hydrophilic residues at the entrance, along the walls and at the bottom of the binding pocket in lipases from *Mucor miehei*, *Rhizopus*, *Humicola lanuginosa* and *Candida rugosa* (see for example Klein *et al.*, 1997; Schmitt *et al.*, 2002). In most cases this results in a change in the relative activity toward ester or lipid substrates of different chain lengths. These results confirmed the central role of the substrate binding site and showed that specific shifts in selectivity can be planned based on the analysis of structural and docking data.

It has been more difficult to define general rules explaining the stereopreference of lipases toward chiral and prochiral substrates. This appears to depend on both the substrate structure and on the lipase used, and is strongly influenced by the reaction conditions (Ransac *et al.*, 1990). Attempts to rationalize the structural bases of stereopreference aimed at the identification of the binding regions of the acyl and alcohol portions of substrates. This was approached by crystallographic analysis of complexes of lipases with transition state analogues of fast- and slow-reacting enantiomers. A detailed structural analysis of the binding of a lipid analogue to *Burkholderia cepacia* lipase led to the identification of four binding pockets for the substrate: the oxyanion hole and three pockets lined by hydrophobic amino acids that accommodate the *sn*-1, *sn*-2 and *sn*-3 fatty acid chains. A central role is played by hydrogen bonding between the ester oxygen atom of the *sn*-2 chain and the histidine of the active site, and the sn-2 pocket is identified as the major determinant of the enzyme's stereopreference (Lang *et al.*, 1998). This is in good agreement with experiments pointing to the importance of the substituent at the *sn*-2 position of the substrate (Kovac *et al.*, 2000), and found further support from site-directed mutagenesis performed on the residues lining the binding pockets. A general conclusion can be drawn from the studies reported in this section, *i.e.* that the size, shape and hydrophobicity/hydrophilicity of the various substrate binding pockets are key players in determining lipase enantio- and regio-preferences and are therefore obvious targets for mutagenesis aiming to improve/modify these properties. Based on rational design, the enantioselectivity of the *Candida antarctica* B lipase catalysed resolution of 1-chloro-2-octanol was improved from E=14 to 28 by a single amino acid exchange as predicted by molecular modelling (Roticci *et al.*, 2001).

4.2. The Lid

Lipases occur in alternative conformational states stabilised by the interaction with water/substrate interfaces. In the closed conformation the lid covers the enzyme active site, making it inaccessible to the substrate molecules, whereas transition to

the open conformation opens the entrance of the catalytic tunnel. In recent years it has become clear that the function of this lid is not simply to act as a gate that regulates access to the active site. Lids are amphipathic structures: in the closed enzyme structure their hydrophilic side faces the solvent and the hydrophobic face is directed towards the protein core. As the enzyme shifts to the open conformation, the hydrophobic face becomes exposed and contributes to the formation of a larger hydrophobic surface and the substrate binding region (Fig. 1). Studies by several groups have pointed to the lid as being a major molecular determinant of lipase activity and selectivity. Thus, for example, two members of the lipase gene family, human pancreatic lipase and guinea pig pancreatic lipase-related protein 2 differ in specificity in that the former enzyme shows high activity only on triglycerides whereas the latter has additional phospholipase and galactolipase activities. The main structural difference between the two enzymes concerns the presence in the guinea pig protein of a lid of extremely reduced size (5 amino acids). Site-directed mutagenesis and the creation of chimeras with exchanged lids revealed the role of the lid domain in the selectivity towards triglycerides, phospholipids and galactolipids (Carrière *et al.*, 1998). Other examples pointing to a crucial role of the lid in substrate selectivity are *Candida rugosa*, *Pseudomonas* and *Bacillus* lipases, among others. *Candida rugosa* produces isoenzymes of differing substrate specificities, of which only isoforms 2 and 3 hydrolyse cholesterol esters. Replacement of the lid of isoform 1, which is completely inactive on such substrates, was sufficient to improve activity on cholesteryl linoleate by 200 fold (Brocca *et al.*, 2003). The lipase from *Pseudomonas fragi* is highly specific for short-chain substrates whereas closely related enzymes from *Pseudomonas* and *Burkholderia* sp. prefer medium- or long-chain substrates. Mutagenesis of specific residues of the lid produced a shift in chain length preference towards medium-chain molecules (Santarossa *et al.*, 2005). Whether the effect on specificity can be directly attributed to the sequence of the lid structure is not always clear, and possible effects on the flexibility and conformation of this structure that might be of importance for enzyme-substrate interactions cannot be excluded. This region of the protein is therefore a good target for protein engineering, since the lid is a surface loop and is likely to tolerate amino acid substitutions, insertions and deletions easier than structures buried in the core of the protein (Eggert *et al.*, 2004).

5. PERSPECTIVES FOR LIPASE RESEARCH

In recent years the importance of lipases as industrial catalysts has grown steadily, raising interest in finding new enzymes endowed with novel and often non-natural properties. It is well recognized that the catalytic ability and specificity of lipases can be considerably influenced by the experimental conditions and therefore methods to modulate catalytic behaviour through, for example, reaction engineering are exploited in several laboratories. However, direct manipulation of the biocatalyst appears to be the most straightforward approach. Several ways are open to

researchers, among which two are considered below: i) the search for novel enzymes from organisms adapted to unusual and poorly explored environments or organisms that cannot be cultured in the laboratory, and ii) engineering of already known enzymes by rational engineering or random mutagenesis.

5.1. Search for Novel Enzymes by Exploiting Biodiversity

Organisms exploited as enzyme producers represent just a tiny fraction of those existing in nature. Environments extreme for temperature, pH or salt concentration are sources of adapted organisms that often produce proteins with unusual properties (Demirhian, 2001). A large number of micro-organisms are not amenable to laboratory cultivation and therefore completely unexplored regarding their catalytic repertoire. The so-called metagenomic approach aims to isolate genes of interest from non-characterized samples without any need to cultivate and/or isolate them. It relies on the construction of gene libraries from samples directly taken from the environment (soil, water) enriched in the organisms/activities of interest followed by the screening of the library obtained (Henne *et al.*, 2000; Voget *et al.*, 2003). Additionally, the list of genomes completely sequenced is constantly growing and sequences available in public databases can be screened by bioinformatic methods to identify putative lipase genes that are then amplified by PCR (Kim *et al.*, 2004).

5.2. Construction of New Enzymes by Protein Engineering

Several recombinant lipases have been expressed in bacterial, fungal, plant and insect systems and can therefore be subjected to mutagenesis. Rational protein design is applied to lipases whose 3D structure has been solved (Table 1) or can be modelled by homology. A large number of site-specific mutants or chimeric proteins has been generated with the purpose of addressing lipase stability and specificity (for a review see Svendsen, 2000). Several successful cases can be cited, among them the enhancement of the enantioselectivity of *Candida antarctica* B lipase in the resolution of 1-chloro-2-octanol by virtue of a single amino acid substitution, or the expansion of the range of secondary alcohols it accepts by rational redesign of the stereospecificity pocket (Roticci *et al.*, 2001; Magnusson *et al.*, 2005). An ambitious goal of protein engineering is to obtain so-called “enzyme promiscuity”, which refers to the ability of an enzyme to catalyse more than one chemical transformation, such as the formation of carbon-carbon bonds by *C. antarctica* lipase B (Kazlauskas, 2005).

Directed evolution relies on the generation of libraries of random mutants followed by selection of those variants with improved qualities on which further rounds of mutagenesis can be performed (Arnold and Georgiou, 2003). In recent years directed evolution has been applied to a large number of proteins and enzymes for the purpose of improving activity, stability and specificity. This is especially useful when structural data are not available for rational protein engineering or in those cases where the determinants of the required feature are complex, *e.g.* for

Table 1. Lipases of known 3D structure

Organism	Reference
Bacteria	
Burkholderia glumae	Noble *et al.,* 1993
Burkoholderia cepacia	Scharg *et al.,* 1997
Pseudomonas aeruginosa	Nardini *et al.,* 2000
Bacillus subtilis	van Pouderoyen *et al.,* 2001
Streptomyces exfoliatus	Wei *et al.,* 1998
Bacillus stearothermophilus	Tyndall *et al.,* 2002
Fungi	
Candida rugosa 1	Grochulski *et al.,* 1993
Candida rugosa 2	Mancheno *et al.,* 2003
Candida rugosa 3	Ghosh *et al.,* 1995
Thermomyces lanuginosa	Brzozowski *et al.,* 2000
Candida antarctica	Uppenberg *et al.,* 1994
Rhizopus niveus	Kohno *et al.,* 1996
Rhizomucor miehei	Brady *et al.,* 1990
Geothricum candidum	Scharg *et al.,* 1993
Penicillium camembertii	Derewenda *et al.,* 1994
Fusarium solani cutinase	Martinez *et al.,* 1992
Higher Eukaryotes	
Human pancreatic	van Tilbeurgh *et al.,* 1992
Horse pancreatic	Lombardo, 1989
Bile-salt activated	Terzyan *et al.,* 2000
Human gastric	Roussel *et al.,* 1999
Dog gastric	Roussel *et al.,* 2002
Rat pancreatic lipase-related pr2	Roussel *et al.,* 1998

Table 2. Recent examples of lipases modified by directed evolution

Organism	Modification	Reference
Pseudomonas aeruginosa	enantioselectivity	Liebton *et al.,* 2000
Pseudomonas aeruginosa	inversion of enantioselectivity	Zha *et al.,* 2001
Pseudomonas aeruginosa	range of substrate accepted	Reetz *et al.,* 2005
Pseudomonas aeruginosa	amidase activity	Fujii *et al.,* 2005
Bacillus thermocatenulatus	phospholipase activity	Kauffmann and Schmidt-Dannert, 2001
Bacillus subtilis	inversion of enantioselectivity	Funke *et al.,* 2003
Bacillus subtilis	thermostability	Acharya *et al.,* 2004
Bacillus subtilis	enantioselectivity	Eggert *et al.,* 2005
Burkholderia cepacia	inversion of enantioselectivity	Koga *et al.,* 2003
Candida antarctica B	activity and thermostability	Suen *et al.,* 2004
Candida antarctica B	enantioselectivity, secondary alcohols	Qian and Lutz, 2005
Acinetobacter sp	hydrolytic activity	Han *et al.,* 2004
Rhizopus oryzae	reaction specificity	Shibamoto *et al.,* 2004
Metagenome esterase	lipase activity	Reyes-Duarte *et al.,* 2005

enantioselectivity or stability (Table 2). The most extensive and successful example reported so far is the evolution of the enantioselectivity of a *Pseudomonas aeruginosa* lipase towards 2-methyldecanoate. Rounds of directed evolution followed by saturation mutagenesis on the positions identified as "hot spots" for selectivity enhanced selectivity from E=1 to E=50 and produced mutants with reverse stereopreference (Liebton *et al.*, 2000; Zha *et al.*, 2001). The same approach has been also applied for improving the phospholipase A1 activity of two bacterial lipases (Kauffmann and Schmidt-Dannert, 2001; van Kampen and Egmond, 2000).

6. CONCLUSIONS

Despite their broad diffusion in biotransformation reactions the use of lipases (and of most enzymes) in industrial processes is still limited by intrinsic weaknesses of the biological catalyst, in particular low stability under operational conditions and low activity or specificity on particular or non-natural substrates. In this chapter, the potential of lipases has been emphasized and attention has been drawn to recent developments that are expected to expand the natural abilities of these proteins. Knowledge of the molecular determinants of enzyme properties has accumulated allowing the rational choice or creation of the "right catalyst" for a given process. On the other hand, the cloning of genes encoding as yet unknown enzymes from non-conventional sources and the modification of those already available by a combination of molecular techniques are very promising as potential sources of novel catalysts with improved or completely new properties.

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