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Advances in Structural Understanding of Lipases

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Introduction

Our knowledge concerning the structure and function of lipases has increased dramatically over the last decade. Advances in molecular biology and biochemical techniques have facilitated the determination of many lipase gene and protein sequences and have allowed the purification to homogeneity of lipases from various organisms. New methods of characterizing lipase activities and specificities have been developed (Rangheard *et al.*, 1989; Jensen, Galluzzo and Bush, 1990; Sonnet and Gazzillo, 1991). Increased industrial interest in the use of lipases in process development and recognition of potential new applications (Björkling, Godtfredsen and Kirk, 1991), as evidenced by the world-wide increase in patent applications for lipase use, has emphasized the need for a fundamental understanding of lipase structure–function relationships and has spurred major research efforts to provide this knowledge. In the last few years, rapid progress in this area has been made; this will undoubtedly continue.

One long recognized feature of lipases is the phenomenon termed 'interfacial activation' (reviewed by Desnuelle, 1972; Macrae, 1983), which refers to the fact that lipases achieve their maximum catalytic rate when the substrate is presented at an interface. Generally, low rates of catalysis are observed for soluble substrates, but the enzymatic activity increases sharply as the substrate concentration exceeds its critical micelle concentration. Even catalytic rates toward small, soluble substrates and sensitivity to inhibitors can be increased in the presence of an interface (Shimada *et al.*, 1983; Nishioka *et al.*, 1991). Inert surfaces can also provide the interface required to activate

Abbreviations: AChE, acetylcolinesterase; CRL, *Candida rugosa* lipase; DFP, diisopropyl fluorophosphate; E_{600}, diethyl *p*-nitrophenylphosphate; GCL, *Geotrichium candidum* lipase; HEAR, high erucic acid rapeseed; HPL, human pancreatic lipase; HSL, hormone-sensitive lipase; NHPEE, *n*-hexylphosphonate ethyl ester; PMSF, phenylmethanesulphonyl fluoride; PNPA, *p*-nitrophenylacetate; RML, *Rhizomucor miehei* lipase.

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the enzymes (Chapus and Sémériva, 1976). Kinetic characterization of catalysis and the determination of equilibrium constants are complicated by the requirement for an interface (Brockman, 1984; Macrae and Hammond, 1985).

The Nomenclature Committee of the International Union of Biochemistry has classified hydrolases according to the substrates recognized. The term 'lipase' commonly refers to triacylglycerol hydrolases (EC 3.1.1.3). However, as pointed out by the Committee, many of the enzymes in this group hydrolyse a wide variety of ester substrates and classification may be somewhat arbitrary. Enzymes classified as diacylglycerol, and monoacylglycerol hydrolases are listed under EC 3.1.1.34 and EC 3.1.1.23, respectively. Some of the cholesterol esterases, listed as EC 3.1.1.13, are known to hydrolyse triacylglycerols. All of these enzymes can be regarded as lipases.

Lipases from many sources, including plants, microbes, and mammalian tissues, have been purified and characterized. The biochemical properties of many of these enzymes have been the subjects of previous reviews (Desnuelle, 1972; Macrae, 1983; Borgström and Brockman, 1984; Wang, Hartsuck and McConathy, 1992). Their physicochemical properties vary greatly. The molecular weights of the various enzymes range from 20 000 to >80 000. Some, such as pancreatic, lipoprotein, and hepatic lipases, are multi-domain proteins, whereas most microbial lipases and some mammalian lipases consist of a single domain. Oligomerization may be important for the activity of some lipases. For example, rat pancreatic cholesterol esterase reportedly forms hexamers in the presence of bile salts, which stimulate lipolytic activity (Rudd and Brockman, 1984 and references therein). Lipoprotein lipase exists in aqueous solution as a dimer and appears to remain a dimer when adsorbed at a lipid interface (Olivecrona *et al.*, 1985). The mechanisms by which lipase activities are modulated are also quite varied. The activity of hormone-sensitive lipase from adipose tissue is modulated by phosphorylation/dephosphorylation (see review by Belfrage *et al.*, 1984). Binding of bile salts stimulates pancreatic and milk cholesterol esterase, but inhibits lipoprotein lipase. Gastric lipase has an acidic pH optimum, whereas pancreatic lipase activity is increased at neutral pH. Interactions with macromolecular cofactors such as colipase or apolipoproteins regulate pancreatic and lipoprotein lipases, respectively.

Specificities of lipases

Because lipases function at interfaces, assessment of substrate specificity is influenced by many factors (Jensen, Gazzullo and Bush, 1990). Few lipases exhibit strict substrate specificity, but instead, they show preferences, or selectivity, for some substrates. The mode of presentation of the substrate is also very important (Brockman, 1984). The same enzyme may exhibit differing selectivities when the substrate is added in the form of mixed micelles, monolayer films, or dissolved in organic solvents. Immobilization of the enzyme on inert supports may also affect the selectivity of the enzyme.

Classically, lipase selectivity for triacylglycerols is viewed from two per-

spectives. One can discuss the selectivity either in terms of the position of the hydrolysed ester bond on the glycerol skeleton (regioselectivity), or in terms of the fatty acid type. This subject has been well described by Macrae and Hammond (1985) and in previous reviews (Desnuelle, 1972; Macrae, 1983).

In terms of regioselectivity, lipases are usually considered either as 1,3-specific or non-specific. The selectivities reported in the early literature are somewhat in doubt, because crude or only partially purified enzyme preparations containing multiple lipase forms were often used. For example, *Geotrichum candidum* and *Candida rugosa* were considered non-regiospecific (Macrae, 1983). Recently, however, after careful purification of the lipases produced by *C. rugosa*, we have separated multiple fractions which generate 1,2- and 1,3-dioleins at different rates under the same reaction conditions (*Figure 1*), indicating different positional specificities for these fractions (A. Bernier, F. Ergan and M. Trani, unpublished data). Also, one of the extracellular lipases isolated recently from *G. candidum* was reported to show a preference for the 2-position of the triacylglycerol (Sugihara, Shimada and Tominaga, 1991). A few lipases are known to exhibit an almost absolute specificity regarding chain position of the fatty acid. The *Rhizomucor miehei* and pancreatic lipases specifically hydrolyse the 1,3-ester bond positions (Macrae and Hammond, 1985; Huge-Jensen, Galluzzo and Jensen, 1987).

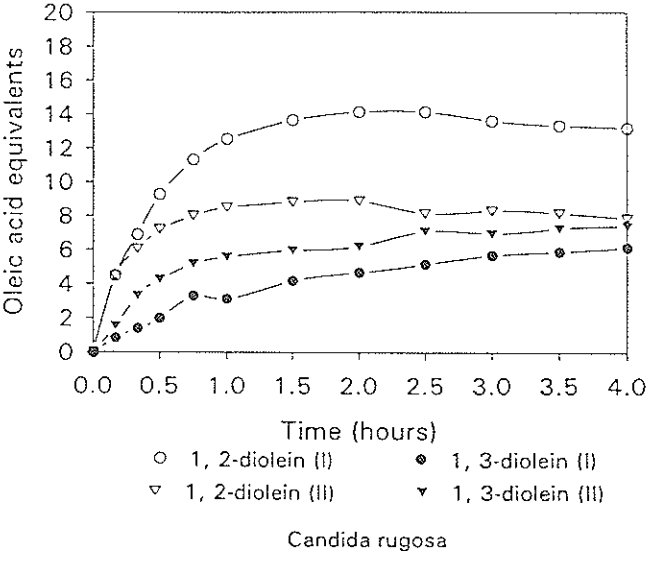

Figure 1. Appearance, as a function of time, of 1,2-diolein and 1,3-diolein obtained from triolein hydrolysis catalysed by two lipase fractions (I and II) prepared from crude *Candida rugosa* lipase. The results are expressed in oleic acid equivalents. The reaction medium was composed of 0·522 g of triolein mixed with 0·5 ml of water and lipase sample.

Lipase from *G. candidum* is probably the best known example of a fatty-acid-specific lipase. The enzyme from one strain was reported long ago to be specific for *cis*-Δ9 unsaturated fatty acids (Alford, Pierce and Suggs, 1964) and, more recently, an enzyme with this specificity was purified to

homogeneity from this organism (Sidebottom *et al.*, 1991; Charton and Macrae, 1992). This lipase also shows a preference for long chain fatty acids, particularly C_{18} chains (Macrae, 1983). As another example, lipase from *Candida deformans* hydrolyses triacylglycerols with mono-unsaturated fatty acids faster than ones with saturated fatty acids, and the presence of two or three double bonds slows down the rate of hydrolysis (Muderwha *et al.*, 1985).

In some cases, lipases only slowly hydrolyse certain fatty esters. The lipase from *Brassica napus* L. was recently shown to discriminate against *cis*-4 and *cis*-6 unsaturated fatty acids (Hills, Kiewitt and Mukherjee, 1990). We have shown that *C. rugosa* lipase releases erucic acid from high erucic acid rapeseed (HEAR) oil or γ-linolenic acid from borage and evening primrose oils only very slowly (Ergan, Lamare and Trani, 1992). Similar behaviour was observed for lipases from *G. candidum* (F. Ergan and M. Trani, unpublished data).

Identification of catalytic residues

For many years, the reaction mechanism of lipases has been thought to be similar to that of the serine proteases. Pancreatic lipase was the subject of many of the first chemical modification studies. These early results have been reviewed (Verger, 1984; Chapus *et al.*, 1988). To summarize these findings, lipolytic activity was shown to be inhibited by modification of serine, histidine, and carboxyl groups and acyl–enzyme intermediates were identified. These data all suggest the presence of a catalytic triad and a reaction mechanism involving acylation and deacylation which is similar to that of the serine proteases. Attempts to identify the acylated residue led to some confusion. The serine labelled by the inhibitor diethyl-*p*-nitrophenyl phosphate (E_{600}) was identified as serine 152. However, the E_{600}-modified enzyme was still able to hydrolyse small soluble substrates like *p*-nitrophenylacetate (PNPA), but activation by inert interfaces was impaired (Chapus and Sémériva, 1976). As a result, the Ser-152 residue was suggested to be involved in interface binding rather than catalysis. The inhibition of lipolytic activity by boronic acids further suggested that a serine is susceptible to acylation (Garner, 1980). Other experiments showed that the C-terminal fragment of pancreatic lipase is able to hydrolyse PNPA and Lys-373 was shown to be stoichiometrically acetylated (DeCaro, Rouimi and Rovery, 1986; DeCaro *et al.*, 1988). An alternative model for catalysis with lysine as the nucleophile was developed (Kaimal and Saroja, 1989). The discrepancy regarding the identity of the catalytic nucleophile began to unravel with the observation of a Ser–His–Asp triad involving Ser-152 in the three-dimensional structure of human pancreatic lipase (Winkler, D'Arcy and Hunziker, 1990). The labelling of this residue by butyl boronic acid provided strong evidence that this is the catalytic residue (Winkler, D'Arcy and Hunziker, 1990; Gubernator, Müller and Winkler, 1991). Specific labelling of Ser-152 by the lipase inhibitor tetrahydrolipstatin lent further support for the assignment of this serine as the catalytic nucleophile (Hadváry *et al.*, 1991;

Lüthi-Peng, Märki and Hadváry, 1992). Ser-132 of lipoprotein lipase (Faustinella *et al.*, 1991; Emmerich *et al.*, 1992) and Ser-147 of hepatic lipase (Davis *et al.*, 1990), the residues corresponding to Ser-152 of pancreatic lipase in sequence alignments, were shown to be essential for activity in these enzymes.

Further support for the presence of a catalytic triad in lipoprotein lipase was provided when the catalytic acidic group of this enzyme was identified by analysis of naturally occurring mutations and comparison with the pancreatic lipase structure (Ma *et al.*, 1992) and by site-directed mutagenesis (Emmerich *et al.*, 1992). Sequence alignment shows that this acid is the equivalent of Asp-176 of pancreatic lipase, the acid observed in the triad. The site-directed mutagenesis study also identified His-241 as the catalytic histidine in lipoprotein lipase (Emmerich *et al.*, 1992). These results establish that pancreatic, hepatic, and lipoprotein lipases are indeed serine esterases, and that the Ser–His–Asp triad observed in pancreatic lipase is indeed the catalytic site.

Other lipases have also been established to be serine esterases, and a Ser–His–Acid triad is presumed to be present in each case. Inactivation of *Rhizomucor miehei* lipase with phenylmethanesulphonyl fluoride (PMSF) labels Ser-144, which takes part in a Ser–His–Asp triad in the crystal structure (Brady *et al.*, 1990). Hormone-sensitive lipase from adipose tissue is readily inhibited by diisopropyl fluorophosphate (DFP) (Belfrage *et al.*, 1984). *Pseudomonas* lipases are inhibited by E_{600} and by DFP (Nishioka *et al.*, 1991; Kordel *et al.*, 1991). Pancreatic cholesterol esterase is inhibited by both DFP and PMSF as well as carboxyl-modifying reagents (*see* review by Rudd and Brockman, 1984). Kinetic analysis of this enzyme also suggests a serine protease-like reaction mechanism (Stout, Sutton and Quinn, 1985). The essential serine residue was identified by a combination of chemical modification with DFP and site-directed mutagenesis (DiPersio, Fontaine and Hui, 1990; Christie, Cleverly and O'Connor, 1991). The essential histidine was also identified by site-directed mutagenesis (DiPersio, Fontaine and Hui, 1991). The *Geotrichum candidum* lipase is inhibited by PMSF and is sensitive to modification of carboxyl moieties by carbodiimides (Sugihara, Iwai and Tsujisaka, 1982; Spener *et al.*, 1991). A catalytic triad has been confirmed in the three-dimensional structure of a *G. candidum* lipase (GCL) (Schrag *et al.*, 1991b). The three-dimensional structure of GCL revealed for the first time a catalytic triad containing glutamate rather than aspartate. Although there is no chemical reason for favouring Asp over Glu, only Asp was found in the triads of serine proteases, other lipases and some other hydrolytic enzymes (Ollis *et al.*, 1992). Based on sequence comparison, acetylcholinesterase was also predicted to utilize glutamate as the third partner in the catalytic triad. Subsequently, the structure determination of acetylcholinesterase (AChE) from *Torpedo californica* (Sussman *et al.*, 1991) confirmed this prediction. The fact that many other esterases belonging to the same family as GCL and AChE also utilize glutamate in this role is now recognized (Cygler *et al.*, 1992).

Gastric lipases are inhibited by micromolar concentrations of mercurials, indicating that a sulphydryl group is essential for activity (Gargouri *et al.*,

1988). However, human and rabbit gastric lipases are also inhibited by boronic acids, tetrahydrolipstatin and E_{600} (Sando and Rosenbaum, 1985; Anderson and Sando, 1991; Ransac *et al.*, 1991). Lysosomal acid lipase, an enzyme related to the gastric lipases, is also inhibited by boronic acids (Sando and Rosenbaum, 1985). These data suggest that, despite the importance of a sulphydryl group, these enzymes are probably serine hydrolases. This is supported by the results of Moreau *et al.* (1991), who showed that the essential sulphydryl group and the E_{600}-labelled serine function independently.

The cutinases are also inhibited by DFP, phenylboronate, the histidine-modifying reagent diethyl pyrocarbonate, and by carboxyl-modifying carbodiimides (Kolattukudy, 1984). Loss of activity is correlated with modification of a single carboxyl group. Identification of the DFP-labelled serine by amino acid sequencing has also been accomplished (Soliday and Kolattukudy, 1983) and the catalytic histidine is the lone histidine residue in the polypeptide chain (Ettinger, Thukral and Kolattukudy, 1987). The recent determination of the three-dimensional structure of a cutinase from *Fusarium solani* confirms the presence of this triad (Martinez *et al.*, 1991).

For serine proteases, the enzyme family from which much of our knowledge about catalytic triads has derived, the active site serine is always found in a G–X–S–X–G(A) consensus sequence. Lipases for which the active site serine has been identified follow the same pattern. This is also true for other serine hydrolases (e.g. esterases) and some other hydrolytic enzymes (Brenner, 1988). This motif has become a paradigm, and for many serine hydrolases, the identity of the catalytic serine has been predicted solely from the presence of this pentapeptide consensus.

Sequence homology among lipases

The names given to lipases have contributed to some confusion regarding relationships between the various enzymes. Frequently the name reflected either the particular tissue from which the enzyme was purified or a substrate preference. However, many tissues, including pancreas, liver and adipose, produce multiple lipases, and many of these lipases hydrolyse a wide variety of substrates. For example, colipase-dependent pancreatic lipase and cholesterol esterase can both be purified from pancreas, and adipose tissue produces both lipoprotein lipase and hormone-sensitive lipase which hydrolyse both cholesterol esters and triacylglycerols. Only the appearance of amino acid sequences has begun to delineate the relationships between the various lipases. In the last few years, the primary structures of many lipases have been reported. The polypeptide chain length of lipases varies over a wide range. Amino acid sequence similarity among the lipases is often limited to the five residue consensus sequence G–X–S–X–G, but families of related enzymes can clearly be recognized by sequence similarity (*Table 1*).

Table 1. Classification of lipases according to sequence homology

Mammalian lipases			
I	II	III	IV
Pancreatic	Gastric	Hormone-sensitive	Bile-salt stimulated
Lipoprotein	Lingual		
Hepatic	Lysomal acid		
Microbial lipases			
V	VI	VII	
Rhizomucor miehei	*Pseudomonas cepacia*[*]	*Moraxella* TA-144[‡]	*Geotrichum candidum*[§]
Rhizopus delemar	*Pseudomonas fragi*		*Candida rugosa*
Humicola lanuginosa	*Pseudomonas aeruginosa*		
Penicillium camembertii	*Pseudomonas* sp.		
	Staphylococcus aureus		
	Staphylococcus hyicus		
	Chromobacterium viscosum		
Cutinases			
VIII			
Fusarium solani			
Colletotrichum capsici			
Colletotrichum gloeosporoides			

[*] This classification has been devised so as to minimize the number of families. The grouping of *Pseudomonas* and *Staphylococcus* lipases is based on the homology shown over five separate short spans of the sequence.
[‡] Three different lipase genes which show little homology to each other are included in this grouping.
[§] The *Geotrichum* and *Candida* lipases are members of the family of enzymes which includes the bile-salt stimulated lipase.

MAMMALIAN LIPASES

Based on sequence comparisons, the mammalian lipases which have been studied can be divided into several different enzyme families. The first family includes colipase-activated pancreatic lipase, hepatic lipase and lipoprotein lipase. These enzymes have long been recognized as being related, and numerous sequence alignments have appeared in the literature (Komaromy and Schotz, 1987; Datta *et al.*, 1988; Cooper *et al.*, 1989; Kirchgessner *et al.*, 1989; Mickel *et al.*, 1989). The sequence homology among these enzymes extends throughout the entire length of the polypeptide chain, and alignment of the sequences requires few insertions or deletions. The overall structures of lipoprotein and hepatic lipases must be very similar to the pancreatic lipase, whose structure has been reported. The gene structures of these enzymes are also similar and these enzymes form a multigene family (Komaromy and Schotz, 1987; Cai *et al.*, 1989; Kirchgessner *et al.*, 1989).

A second family of mammalian lipases is characterized by an acidic pH optimum. This lipase family includes the gastric lipases, lingual lipase and lysosomal acid lipase. Some of the properties of these enzymes have been reviewed previously (Fowler and Brown, 1984; Hamosh, 1984). As mentioned above, there is strong evidence to suggest that these enzymes are serine esterases despite their sensitivity to sulphydryl modifying reagents. The amino acid sequences of members of this lipase family have two G–X–S–X–G sequences (Docherty *et al.*, 1985; Bodmer *et al.*, 1987; Anderson and Sando, 1991). No conclusive evidence as to which of these motifs contains the

catalytic serine has been presented, but the sequence around Ser-153 (numbering according to Anderson and Sando, 1991), G–H–S–Q–G, shows more similarity to other lipase consensus sequences and is more likely to be the catalytic site. Sequence similarity between this family and other lipase families is very limited. The sequence around histidine 353 shows some similarity to some of the microbial lipases, including the sequence surrounding the catalytic histidine in the *Rhizomucor miehei* lipase. This suggests that His-353 may be part of a catalytic triad in the gastric lipase family. The acid group of a supposed triad cannot be clearly identified from the sequence either. If one assumes that the linear sequence of catalytic residues is Ser–Asp/Glu–His, as in other lipases (discussed in more detail below), the identity of the catalytic acid can be narrowed to one of four aspartates which are located between the G–H–S–Q–G motif and His-353. These are the only acidic residues in this region which are conserved in the three known lipase sequences of this family.

The lysosomal acid lipase has also been referred to as cholesterol ester hydrolase. Comparison of amino acid sequences shows that enzymes which have been called cholesterol esterases represent at least three different families of lipases. The hormone-sensitive lipase (HSL) from adipose tissue hydrolyses both cholesterol esters and triacylglycerols. Like the gastric lipases, this enzyme is inhibited by micromolar concentrations of $HgCl_2$ (*see* review by Belfrage *et al.*, 1984). However, the amino acid sequence of HSL (Holm *et al.*, 1988) shows little similarity to gastric lipases or any other lipases beyond the G–X–S–X–G motif. HSL is regulated by phosphorylation/dephosphorylation of two serine residues near the C-terminus of the protein (Strålfors, Björgell and Belfrage, 1984; Garton and Yeaman, 1990 and references cited therein). Lipases which may be identical or closely related to HSL have been purified from adrenal cortex (Belfrage *et al.*, 1984 and references cited therein) and from liver and mammary gland (West and Shand, 1991; Ghosh and Grogan, 1991). The sequences of these enzymes have not been reported, but their regulation by phosphorylation suggests that they may be closely related to HSL (Martinez and Botham, 1990; Ghosh and Grogan, 1991). HSL from chicken adipose tissue has a molecular weight of 42 000 (Berglund *et al.*, 1980), approximately half the size of the mammalian HSL. No amino acid sequence has been reported, but its regulation by phosphorylation/dephosphorylation (Khoo and Steinberg, 1974) suggests that it, too, belongs to the same family as the mammalian HSL.

Bile-salt-stimulated lipase, also known as cholesterol esterase, represents yet another enzyme family. This lipase has been cloned from pancreas and from milk (Han, Stratowa and Rutter, 1987; Kissel *et al.*, 1989; Kyger *et al.*, 1989; Hui and Kissel, 1990; Nilsson *et al.*, 1990; Baba *et al.*, 1991; Christie, Cleverly and O'Connor, 1991). These enzymes show strong sequence homology to the carboxylesterases, cholinesterases (Kissel *et al.*, 1989; Nilsson *et al.*, 1990; Krejci *et al.*, 1991), and the lipases from *Geotrichum candidum* (Krejci *et al.*, 1991) and *Candida rugosa* (Schrag *et al.*, 1991b; Cygler *et al.*, 1992). This enzyme family is interesting in that it includes both microbial and

mammalian enzymes. The homologies among the enzymes in this family are discussed in more detail below.

MICROBIAL LIPASES

Lipases have been purified from many microbes. Multiple forms of lipase are commonly produced by these organisms. *Aspergillus niger* and *Penicillium cyclopium* have been shown to produce multiple lipase forms (Iwai and Tsujisaka, 1984). *Penicillium* lipases differ greatly in molecular weight (Iwai and Tsujisaka, 1984; Isobe, Akiba and Yamaguchi, 1988). *Rhizomucor miehei* produces lipases A and B, which differ only in their carbohydrate content (Boel *et al.*, 1988). The lipase from *Geotrichum candidum* has received a great deal of attention because of its reported specificity for *cis*-$\triangle$9 oleic acid. Lipases have been purified from many different strains of *G. candidum* (Baillargeon, Bistline and Sonnet, 1989; Jacobsen *et al.*, 1989; Baillargeon, 1990; Veeraragavan, Colpitts and Gibbs, 1990; Baillargeon and McCarthy, 1991; Hedrich *et al.*, 1991; Sidebottom *et al.*, 1991) and microheterogeneity has often been reported. Treatment of the enzyme preparations with endoglycosidases reduces the heterogeneity observed on isoelectric focusing gels, indicating that some of the heterogeneity results from differences in glycosylation (Baillargeon and McCarthy, 1991; Hedrich *et al.*, 1991; Menge *et al.*, 1991; M. Cygler *et al.*, unpublished data). A similar situation was observed for the mono-, diacylglycerol lipase from *Penicillium camembertii* (Isobe and Nokihara, 1991; Isobe *et al.*, 1992). Heterogeneity also results from the expression of multiple lipase genes. Two cDNAs have been identified and cloned from *G. candidum* strain ATCC 34614, and purification of both lipases has been accomplished and confirmed by partial amino acid sequencing (Shimada *et al.*, 1989, 1990; Sugihara *et al.*, 1990). Purification of two lipases with different specificites has also been reported for another strain of *G. candidum* (Sidebottom *et al.*, 1991; Charton and Macrae, 1992). Partial sequencing of these two lipases shows that they are different enzymes (Charton, 1991). The presence of multiple genes in *Candida rugosa* (formerly *C. cylindracea*) has also been demonstrated (Alberghina *et al.*, 1991; Longhi *et al.*, 1992; L. Alberghina and R. Grandori, personal communication). In most cases, the different gene products or lipases from different strains of an organism are very similar.

Sequence comparisons indicate that microbial enzymes represent several distinct lipase families. The *Rhizomucor miehei, Humicola lanuginosa* triacylglycerol lipases and the *Penicillium camembertii* U-150 mono-, diacylglycerol hydrolase are homologous enzymes (Yamaguchi, Mase and Takeuchi, 1991). Alignment of the *Rhizopus delemar* triacylglycerol lipase sequence to the other three, and in particular to that of *Rhizomucor miehei*, requires few insertions or deletions (Haas, Allen and Berka, 1991). These enzymes clearly form one family. Catalytic residues can be readily identified by comparison of the aligned sequences with the triad identified from the *R. miehei* three-dimensional structure.

Lipases have been purified and characterized from a number of strains of

Pseudomonas. There is some confusion regarding the naming of the *Pseudomonas* species and strains. Some lipases which were thought to have been derived from different species and strains have subsequently been shown to be identical in sequence. It is possible that the organisms were, in reality, the same, and future work may further revise the identifications of the *Pseudomonas* strains used in lipase production. Complete amino acid sequences have been reported for *P. cepacia* DSM 3959 (Jorgensen, Skov and Diderichsen, 1991), *P. fragi* IFO-12049 (Aoyama, Yoshida and Inouye, 1988), *P. sp.* KWI-56 (Iizumi *et al.*, 1991) and *P. aeruginosa* (Nishioka *et al.*, 1991; formerly called *P. fluorescens*, but renamed *P. aeruginosa* as cited in Iizumi *et al.*, 1991). Another sequence from a clone derived from *P. fragi* was reported by Kugimiya *et al.* (1986). This cDNA-derived sequence is considerably shorter than all of the other *Pseudomonas* lipases and is identical to the N-terminal portion of the *P. fragi* IFO-12049 lipase sequence, with the exception of three positions. This suggests that the sequence reported by Kugimiya *et al.* (1986) may be a truncated form of the lipase. The sequence homologies of these lipases are readily apparent and alignment requires few insertions or deletions. Comparison of amino terminal sequences suggests that several additional *Pseudomonas* species and strains are also homologous (Gilbert, Cornish and Jones, 1991). Lipases cloned from *Pseudomonas* sp. ATCC 21808 and *P. aeruginosa* also show more than 60% identity with lipase from *P. fragi* (Hom *et al.*, 1991; Jaeger, Wohlfarth and Winkler, 1991). These data indicate that the homology among *Pseudomonas* enzymes is very high. Homology of *P. cepacia* M-12-33 with lipase from *Chromobacterium viscosum* lipase has also been reported (Nakanishi *et al.*, 1991).

Sequence similarity of *Pseudomonas* lipases to other lipases, however, is limited to only short spans. Five regions of similarity with lipases from *Staphylococcus aureus* and *S. hyicus* were identified (Iizumi *et al.*, 1991). This suggests that *Pseudomonas* and *Staphylococcus* lipases may be distantly related and for that reason we have grouped them into the same family. One of the spans which shows some similarity is the region surrounding the G–X–S–X–G motif. This is, in all likelihood, the catalytic serine. Near the C-terminus is another short stretch of 10 residues which contains a conserved histidine residue (His-260 in *P. fragi*). This sequence shows some similarity to the *Rhizomucor* sequence which contains the catalytic histidine and to the His-353-containing segment of gastric lipases, suggesting participation of these histidines in their respective catalytic triads. Two regions of sequence similarity between *Pseudomonas* and *Staphylococcus* lipases are located between the G–X–S–X–G and the histidine-containing regions. Both of these contain a conserved aspartate residue. If one assumes that the linear sequence of a catalytic triad is Ser–Asp–His, then one of these may be the catalytic acid. Since the number of absolutely conserved residues immediately surrounding the aspartate is higher in the second of the two spans, this N–D–G sequence around Asp-238 (*P. fragi* numbering) is the more likely to contain the catalytic acid.

Three lipase genes have been cloned from the antarctic psychrotroph *Moraxella* (Feller, Thiry and Gerday, 1990, 1991a, b). These lipases are not

only different from other lipases, but also show little similarity among themselves. All contain the G–X–S–X–G consensus sequence. Two of the lipases contain a conserved His–Gly (HG) dipeptide which is similar to HG dipeptides found in pancreatic lipases, lipoprotein lipases, hepatic lipases, gastric lipases, and the lipases of *Pseudomonas* and *Staphylococcus* lipases (Feller, Thiry and Gerday, 1991b). Based on this conservation, some investigators suggest that this dipeptide marks the catalytic histidine (van Oort *et al.*, 1989; Götz, 1991). This is contradicted by the evidence from the three-dimensional structure of the pancreatic lipase and by the absence of similar HG sequences in the *Rhizomucor*, *Geotrichum* and related lipases and esterases. The location of this HG dipeptide prior to the G–X–S–X–G pentapeptide in the sequence is at variance with the linear sequence of catalytic residues observed in all other lipases to date.

The lipases from *Geotrichum candidum* and *Candida rugosa* are members of a large family of esterases and lipases. This family includes carboxylesterases, cholinesterases, and both mammalian and microbial lipases. The sequence homology between the *G. candidum* lipase and acetylcholinesterase was recognized before either of the structures was reported (Shimada *et al.*, 1990; Slabas, Windust and Sidebottom, 1990). Determination of the GCL structure permitted prediction of the catalytic residues of the *Candida* lipase and cholinesterases based on sequence comparisons (Schrag *et al.*, 1991b). Comparison of the three-dimensional structures of the GCL (Schrag *et al.*, 1991b) and AChE (Sussman *et al.*, 1991), and alignment of the sequences of 25 related enzymes, shows that the strongest sequence conservation is in the core of the molecule, particularly in the β-sheet (Schrag *et al.*, 1991b; Cygler *et al.*, 1992). Approximately 4% of the residues are strictly conserved in all of the sequences and another ~20% of the residues show only slight variability. Among the absolutely conserved residues are the catalytic serine and histidine residues. The catalytic acid in most of the members of this enzyme family is glutamate, but aspartate has been identified in some, including cholesterol esterases. The three-dimensional structures of GCL and AChE show that most of the strictly conserved residues are located at the ends of the β-strands and are in positions which are important in maintaining the fold. Most of the residues with low variability are located in the β-sheet. The residues with high variability are also clustered and are on the protein surface, where some may be involved in quaternary associations, or in positions which are likely to relate to substrate binding and specificity.

The cutinases represent yet another family of enzymes. The amino acid sequences of three cutinases have been reported (Soliday and Kolattukudy, 1983; Ettinger, Thukral and Kolattukudy, 1987). The sequences are very similar and all can be expected to have structures similar to that of the *Fusarium solani* cutinase, which was recently determined (Martinez *et al.*, 1992). Catalytic triad residues can be identified by homology with the *Fusarium* cutinase.

Lipases have also been purified from flowering plants. Some characterization of these enzymes has been done and for details of this work the reader is referred to a review by Huang (1990). These enzymes are, generally,

membrane or lipid associated and are difficult to purify. No plant lipase amino acid sequences have been reported to date. Since little is known about the struetures of these lipases, they will not be mentioned further in this review.

Structural studies

Many of the questions regarding the mechanism of catalysis and the molecular nature of the interfacial activation process remained unanswered until the first three-dimensional structures of lipases were determined. Attempts at crystallization of lipases date back some 30 years. The first efforts were directed toward fungal lipases. *Aspergillus niger* lipase (Fukumoto, Iwai and Tsujisaka, 1963) and *Geotrichum candidum* lipase (Tsujisaka, Iwai and Tominaga, 1973) were the first ones to be crystallized. These crystals were not very stable and were of rather poor quality, which probably was a result of heterogeneity in the enzyme preparations. Nevertheless, Hata *et al.* (1979) succeeded in obtaining a low resolution (6 Å) model of the lipase from *Geotrichum candidum*, ATCC 34614 strain. This resolution was, however, insufficient to identify the secondary structural elements, determine the chain tracing or to locate the active site. Since then many lipases have been crystallized in a form suitable for high resolution X-ray diffraction studies and their crystallographic analysis is in progress. Data for lipases that have been crystallized to date are shown in *Table 2*. These lipases span the spectrum of molecular weights and organisms. Along with fungal lipases, much attention has been given to pancreatic lipase from various sources. This enzyme functions in the environment where triacylglycerols are solubilized by bile salts and, under those conditions, it requires a small protein helper, a colipase, for efficient hydrolysis. Pancreatic lipases from human, horse and pig have been crystallized with and without a colipase (Lombardo *et al.*, 1989; Cambillau *et al.*, 1991; Chapus *et al.*, 1991). A number of extracellular lipases from various strains of *Geotrichum candidum* have been purified to homogeneity (as far as protein content is concerned), and well diffracting, stable crystals have been obtained (Hedrich *et al.*, 1991; Schrag *et al.*, 1991a). Also, a lipase from the yeast *Candida rugosa* has been crystallized by different groups (Ghosh, Erman and Duax, 1991; Rubin, Jamison and Harrison, 1991; M. Cygler *et al.*, unpublished data). Despite the fact that most of the lipases are highly glycosylated, the crystals obtained are usually of very good quality, diffracting to high resolution. In particular, the crystals of *G. candidum* lipase diffract to 1.5 Å resolution (M. Cygler and J. Schrag, unpublished data) and those of cutinase to 1·3 Å (Martinez *et al.*, 1992).

Although crystals of some of the lipases have been available for many years (Fukumoto, Iwai and Tsujisaka, 1963), only within the last two years have the first high-resolution three-dimensional structures of lipases been determined and reported in the literature. They are from: *Rhizomucor miehei* (RML; Brady *et al.*, 1990), human pancreas (HPL; Winkler D'Arcy and Hunziker, 1990) and *Geotrichum candidum* (GCL; Schrag *et al.*, 1991b). Recently, the structure of a cutinase has also been determined at the molecular level

Table 2. Pertinent crystallographic data for lipases that have been crystallized to date

Source	Space group	Cell dimensions						Resolution (Å)	Reference
		a(Å)	*b*(Å)	*c*(Å)	α	β	γ		
Human pancreas	$P2_1$	47·8	112·8	91·0		99·3		2.3	Winkler, D'Arcy and Hunziker (1990)
	$P2_1$	68·1	88·6	92·1		94·3		?	Chapus *et al.* (1991)
	$P1$	65·6	97·7	91·	93·3	94·3	109·5	?	Chapus *et al.* (1991)
	$P1$	61·7	89·3	90·6	98·8	109·2	91·0	3·0	Cambillau *et al.* (1991)
Horse pancreas	$P2_12_12_1$	79·8	97·2	145·3				1·8	Lombardo *et al.* (1990)
Human pancreas lipase/ porcine procolipase	$P3_221$	80	80	251				3·0	van Tilbeurgh *et al.* (1992)
Rabbit gastric	trigonal	164·0	164·0	510·0				8·0	Abergel, Fonticella and Cambillau (1991)
Human gastric	?	80·0	190·0	290·0				8·0	Abergel, Fonticella and Cambillau (1991)
Dog gastric	$P2_12_12_1$	182·0	211·0	98·0				4·0	Abergel, Fonticella and Cambillau (1991)
Rizomucor miehei	$P2_12_12_1$	71·6	75·0	55·0				1·9	Brady *et al.* (1990)
Rizomucor miehei +NHPEE*	$C222_1$	48·3	93·9	121·1				3·0	Brzozowski *et al.* (1991)
Rizomucor miehei E_{600}†	$C222_1$	48·6	93·9	121·1				3·0	Derewenda *et al.* (1992)
Candida rugosa	$C222_1$	64·9	97·2	175·8				2·2	Rubin, Jamison and Harrison (1991)
	$P2_1$	122·9	101·0	95·2		108·3		3·0	Ghosh, Erman and Duax (1991)
	$P1$	58·6	88·7	58·6	93·3	113·8	96·0	3·0	Ghosh, Erman and Duax (1991)
Pseudomonas fluorescens	?							?	Sugiura *et al.* (1977)
Pseudomonas fluorescens‡	$C2$	91·0	47·1	85·2		121·4		1·6	Larson *et al.* (1991)
Pseudomonas sp. §	$C2$	92·7	47·4	86·5		122·3		2·5	Kordel *et al.* (1991)
Pseudomonas putida	$P4_32_12$	58·5	58·5	144·8				2·5	Sarma *et al.* (1991)
Pseudomonas Arnano	?	?	?	?				3·0	Cleasby (1991)
Pseudomonas glumae	$P4_12_12$	89·3	89·3	180·4				3·0	Cleasby (1991)
Penicillium cyclopium	?							?	Isobe, Akiba and Yamaguchi (1988)
Aspergillus niger	?							?	Fukumoto, Iwai and Tsujisaka (1963)
Candida antarctica	$P2_1$	95·2	50·2	99·5		90·6		2·0	J. Uppenberg (pers.comm.)
Geotrichum candidum ATCC 34614	$P2_1$	59·5	83·6	56·1		99·9		?	Hata *et al.* (1979)
	$P2_1$	59·4	84·4	56·0		100·1		1·5	Schrag *et al.* (1991a)
	$P2_1$	59·4	92·1	54·4		101·1		2·2	Schrag *et al.* (1991a)
	$P1$	59·4	92·0	55·7	98·1	102·3	90·0	2·0	Schrag *et al.* (1991a)
G. candidum Amano GC-4	$P2_1$	53·1	83·5	57·8		100·0		2·5	Hedrich *et al.* (1991)
Fusarium solani cutinase	$P2_1$	35·1	67·4	37·1		94·0		1·3	Martinez *et al.* (1992)

* NHPEE, *n*-hexylphosphonate ethyl ester.
† E_{600}, diethyl *p*-nitrophenyl phosphate.
‡ Sequence of proteolytic fragments of this lipase and its molecular weight correspond to the published sequence of *Pseudomonas cepacia* (J. Oliver, personal communication).
§ The sequence of N-terminal 24 amino acids corresponds to the published sequence of *Pseudomonas cepacia* (Kordel *et al.*, 1991).

Table 3. Characteristics of the lipolytic enzymes with known three-dimensional structures

Name	Molecular Weight (Da)	Glycosylation	Source	Selectivity	References
RML	29 472	Yes	Recombinant	1,3 position	Brady *et al.* (1990)
HPL	49 522	Yes	Purified	1,3 position	Winkler, D'Arcy and Hunziker (1990)
GCL	59 671	2-N, 1-O sites	Purified	*cis*-Δ9	Schrag *et al.* (1991b)
Cutinase	20 697	Yes	Recombinant		Martinez *et al.* (1992)

(Martinez *et al.*, 1992). The latter enzyme is able to hydrolyse triacylglycerols, but, unlike lipases, it does not undergo interfacial activation and is able to hydrolyse soluble substrates (below their critical micellar concentration). The pertinent information about these proteins is provided in *Table 3*. The structure determinations of lipases from *Pseudomonas fluorescens* (J. Oliver, personal communication), *Pseudomonas putida* (Sarma *et al.*, 1991; R. Bott, personal communication), *Candida antarctica* (M.J. Uppenberg, T.E. Bergfors and T.A. Jones, personal communication), horse pancreatic lipase (C. Cambillau, personal communication) are all well advanced and should provide a wealth of new data in the near future.

One of the important questions about the catalytic mechanism answered by the structural data was the existence in all of them of a Ser–His–Asp acid catalytic triad analogous to the active site of serine proteases. Although a mechanism similar to proteases has been suggested for lipases for a long time, the three-dimensional structures provided the strongest experimental support for this hypothesis and identified all residues forming catalytic triads.

The lipases for which the three-dimensional structures have been determined show very little similarity in their sequences and represent three different families of proteins (*Table 3*). Cutinase comes from yet another family. Although at the sequence level there seems to be little similarity between the lipases, their three-dimensional structures have a number of features in common.

DESCRIPTION OF THE STRUCTURES

The schematic representation of the structures of the three lipases are shown in *Figure 2*. All of them are α/β type proteins and the central part of each of them is formed by a mixed β-pleated sheet, which provides the scaffold for additional α-helices and loops.

Rhizomucor michei *lipase*

Rhizomucor miehei lipase is the smallest of the three. It contains 269 residues, the first five of which are disordered and are not included in the model (Brady *et al.*, 1990). The dimensions of the molecule are approximately $35 \times 40 \times 45$ Å^3. The β-sheet contains nine strands (Derewenda, Derewenda and Dodson, 1992), most of them parallel (*Figure 3*). The sheet is twisted, folding around a long, amphipathic, N-terminal α-helix which runs along the diagonal of the sheet (*Figure 2*). The convex side of the β-sheet is covered by two α-helices running nearly parallel to the strands. Three disulphide bridges are formed between Cys residues. One of them, between residues 29 and 268, seems to be important for the stabilization of the global fold, while the two others encompass short loops. Importantly, the three-dimensional structure of RML revealed an arrangement of three residues, Ser-144–His-257–Asp-203, which was reminiscent of the catalytic triad in serine proteases. Ser-144 has been shown, by chemical inactivation with PMSF followed by sequence analysis, to be the active site nucleophile. This

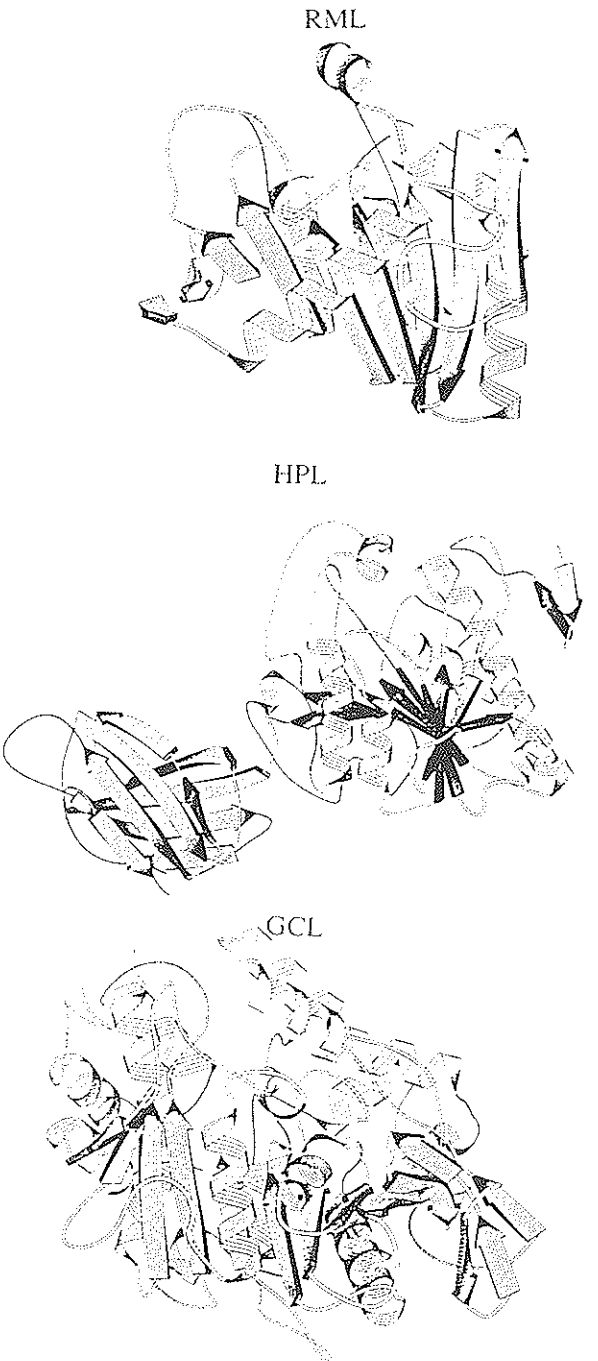

Figure 2. Ribbon diagrams of the structures of three known lipases. (A, upper) *Rhizomucor miehei* lipase (coordinates obtained from Brookhaven Protein Databank, 1tgl; Bernstein *et al.*, 1977). (B, middle) Human pancreatic lipase (coordinates provided by F. Winkler). (C, lower) *Geotrichum candidum* lipase.

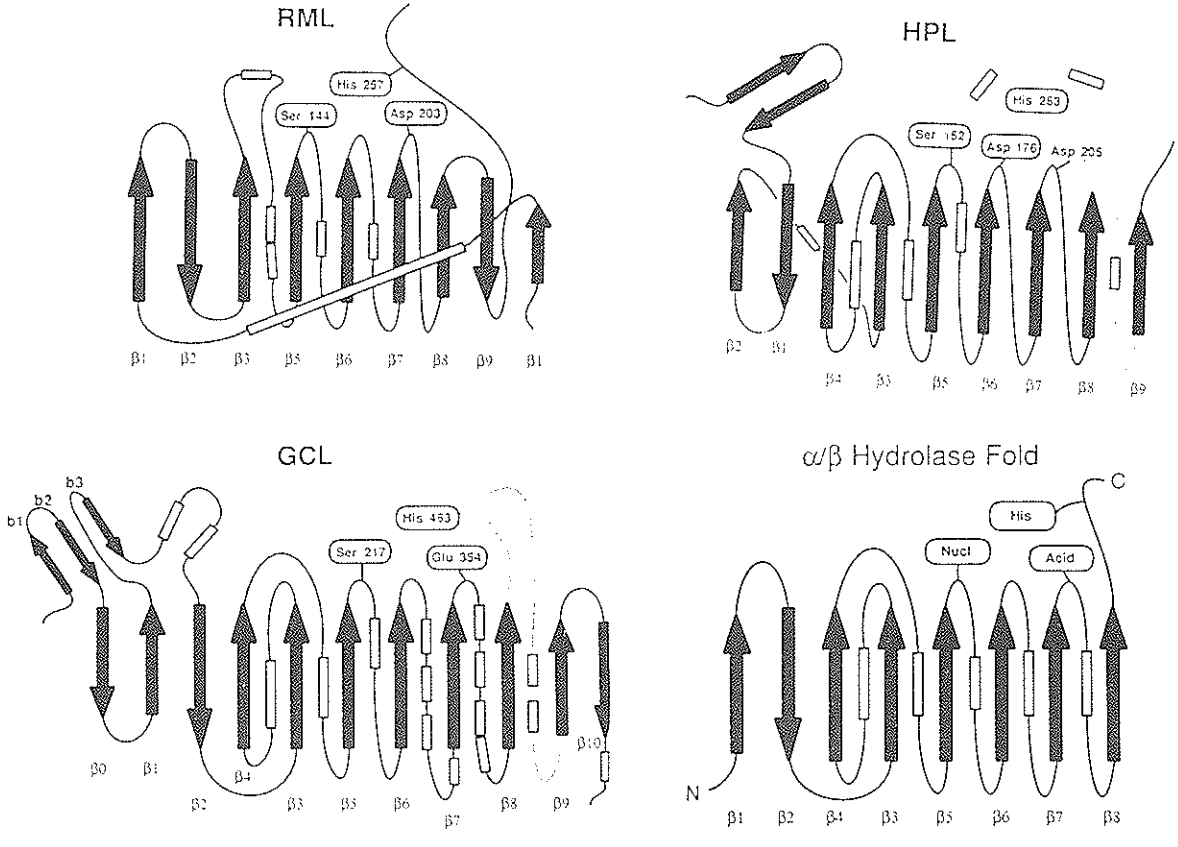

Figure 3. Topology diagrams describing strands connectivity. In order to make the numbering of the strands consistent with Ollis *et al.* (1992) for the α/β hydrolase fold, the first strand of GCL is numbered β_0 and the symbol β_4 is omitted in RML. (A, upper left) *Rhizomucor miehei* lipase. (B, upper right) N-terminal domain of human pancreatic lipase (residues 1–335). (C, lower left) *Geotrichum candidum* lipase. (D, lower right) α/β hydrolase fold (Ollis *et al.*, 1992).

serine is located at the end of the middle strand of the β-sheet (β_5; *Figures 2* and *3*), in a tight turn between the strand and an α-helix (Derewenda and Derewenda, 1991). Asp-203 comes from a loop following strand β_7, two strands after the serine. Finally, His-257 is embedded in the C-terminal loop following strand β_9. Within the three-dimensional structure of this enzyme, the triad residues are located at the bottom of a large cleft which is covered from the top by a short α-helix (*Figure 4*). In the absence of substrate the serine side-chain is totally buried and inaccessible from the solvent. The distance from the Ser O_γ to the surface is approximately 10 Å. The α-helix forming the flap over the active site comes from the segment connecting parallel strands β_3 and β_4 and is located between strand β_3 and a long, kinked α-helix (*Figure 3*). In analogy to the serine proteases, an oxyanion hole has been suggested to exist in RML. The backbone nitrogen atoms of residues 145 and 146 have been proposed to play this role.

Human pancreatic lipase

Human pancreatic lipase is a larger protein, containing 449 residues and is made up of two domains (Winkler, D'Arcy and Hunziker, 1990). It is elongated in one direction, with overall dimensions of approximately $35 \times 55 \times 90$ Å^3 (*Figure 2*). The crystal contains two molecules in the asymmetric unit. They form a dimer and are arranged head-to-tail, with the pseudo-two-fold symmetry axis being perpendicular to the long dimension of the molecule. The N-terminal domain comprising the first 335 residues and having the dimensions $35 \times 50 \times 55$ Å^3, contains the active site, whereas the C-terminal domain is involved in the interactions with colipase (Abousalham *et al.*, 1992). The main feature of the N-terminal domain is a nine-stranded mixed β-sheet (*Figure 3*). The first three strands are anti-parallel, while the last seven run parallel to each other. There is also a small two-stranded β-sheet formed by the N-terminal segment of the polypeptide chain. On each side of the large β-sheet there are two α-helices packed against central strands and running approximately parallel to them. The connections between parallel strands are of the common right-handed type, except for the connection between the last two strands. It makes a left-handed cross-over, rarely found in proteins. There are four disulphide bridges in the N-terminal domain, all of them encompassing rather short segments. The active site is composed of a triad: Ser-152–His-263–Asp-176. Ser-152 is found in a similar motif as in RML, in a tight bend between strand β_5 and an α-helix (*Figure 3*). The aspartate comes from a loop following strand β_6 and the histidine from a long loop after strand β_8. As is the case with RML, an α-helix restricts access to the active site Ser-152 (*Figure 4*). This helix is embedded in the left-handed cross-over between the strands β_8 and β_9, and precedes His-263, which comes from the same connection. One of the disulphide bridges closes the bottom of this loop (Cys-237–Cys-261). The loops surrounding the triad are longer than in RML and, as a result, the active site is further away from the surface (approximately 14 Å; *Figure 4*). The oxyanion hole in this enzyme has been

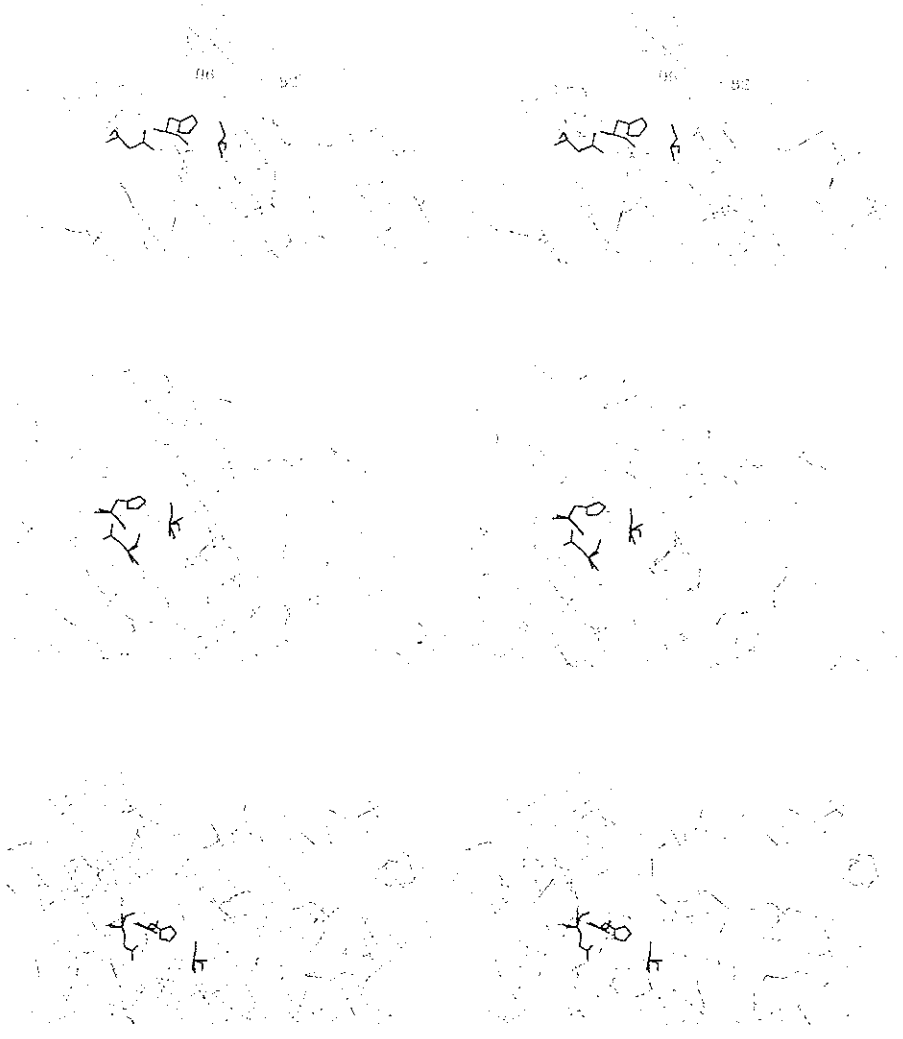

Figure 4. Close-up of the active site of the three lipases. C_α tracing of the polypeptide chain (thin lines) with triad residues shown in full (thick lines). (A, upper row) *Rhizomucor miehei* lipase. (B, middle row) Human pancreatic lipase. (C, lower row) *Geotrichum candidum* lipase.

predicted to be formed by the backbone NH groups of Leu-153 and Phe-77 (Winkler, D'Arcy and Hunziker, 1990).

The C-terminal domain is well separated from the N-terminal domain (*Figure 1*). It forms a β-sandwich made of two anti-parallel four-stranded β-sheets. The C-terminal cysteine forms a disulphide bridge with the cysteine in the edge strand of the other β-sheet in this domain.

Geotrichum candidum *lipase*

Geotrichum candidum lipase is the largest of the three lipases. It has 544 amino acids folded into a single domain with dimensions $45 \times 60 \times 65$ Å^3. As in the other two lipases, the scaffold of this protein is made of a mixed β-sheet. The sheet is somewhat larger than in the other two proteins and contains eleven strands. The central strands are all parallel, whereas the first four and the last two strands run antiparallel to each other (*Figure 3*). In addition to this large β-sheet there is also a smaller, three-stranded β-sheet formed by the residues from the N-terminus. The large β-sheet is strongly twisted. Two α-helices line the concave side of the sheet running along the strands and three helices cover the convex side, with their axes also nearly parallel to the direction of the strands they cover. The connections between parallel strands are right-handed except for the connection between strands β_8 and β_9, which forms a long left-handed cross-over. Based on the fact that, in all documented cases (see above), the active site serine in lipases is part of the G–X–S–X–G motif, the catalytic role in GCL was ascribed to Ser-217 (Shimada *et al.*, 1989). The three-dimensional structure of this enzyme confirmed this prediction. Ser-217 forms a triad arrangement with His-463 and Glu-354 in a similar way to that observed in the other two lipases (Schrag *et al.*, 1991b). It is also found in a tight bend between a β-strand (β_5) and an α-helix (*Figure 3*). When this serine was mutated to alanine, the protein was produced at the same level as wild-type, but showed no lipase activity, consistent with a role for this serine in catalysis (T. Vernet, unpublished results).

The strands of the β-sheet are connected by long loops which form excursions on the C-terminal side of the large β-sheet, whereas the loops on the N-terminal side of the sheet are rather short. The active site is located on the C-terminal side of the β-sheet and is covered by two extensive loops, 61–99 and 262–311 (*Figure 4*), in a more complex way than in the other two lipases. Access to Ser-217 is blocked by two α-helices, 66–76 and 294–310, coming from these two loops. The distance from this serine to the surface of the molecule is approximately 18 Å. There are only two disulphide bridges in GCL and both of them are involved in the stabilization of the two loops covering the active site. The first disulphide bridge is formed at the bottom of loop 61–99. The second one is formed near the tip of loop 262–311. The tertiary fold of GCL is additionally stabilized by a number of salt bridges, which are formed between charged residues embedded in nearby loops. Three of these bridges are located in the same region of the structure.

The oxyanion hole is also presumed to exist in this enzyme. The backbone nitrogens of residues Ala-218 and Ala-132 are in a likely position to form hydrogen bonds with the transition state, although some rearrangement of the loop containing Ala-132 upon substrate binding seems highly probable.

Other lipases

The three-dimensional structure of the lipase from *Candida rugosa* (CRL) has just been determined and the major structural features of this enzyme are nearly identical to those of GCL (Grochulski *et al.*, 1992). As predicted from sequence alignment with GCL (Schrag *et al.*, 1991b), a Ser–His–Glu catalytic triad is observed and involves Ser-209, Glu-341 and His-449. Over 80% of the C_α atoms of CRL can be superimposed with the corresponding atoms in GCL with a root mean square deviation in atom position of ~1 Å. Unlike GCL, however, CRL is in an 'open' conformation with Ser-209 exposed to solvent. This is the first observation of a 'native' lipase crystallized in an 'open' conformation. The 'open' conformation results from differences in the positions of the residues comprising the flap and adjacent loops. The only other differences between GCL and CRL are in the region of the C-terminal helix and do not appear to be related to the interfacial activation or catalysis.

The structure of cutinase shows many of the same features as the other lipases (Martinez *et al.*, 1992). It is also built around a mixed β-sheet with the active site triad occupying a position equivalent to that in the other lipases. The important difference between this structure and the true lipases is the fact that the cutinase active site is accessible from the solvent and is not occluded by parts of the polypeptide chain.

COMMON STRUCTURAL FEATURES

These lipases represent four different families of enzymes which hydrolyse triacylglycerols (*Table 1*). Sequence alignment between lipases of different families is difficult and, except for the region around the active site serine, no significant sequence similarities are apparent. Despite the lack of sequence similarity, their three-dimensional structures show significant similarities.

All of them are α/β proteins, with a β-sheet forming the scaffold of the structure. The central part of the β-sheet is made of parallel strands, and the topology (connectivity) of the strands around the active site serine (β_5 to β_8) is maintained. A closer look at RML shows that, as compared to the other two lipases, it lacks strand β_4, which became part of a long loop between strands β_3 and β_5 (*Figure 3*). Taking this into account, the topology of strands β_1–β_8 of RML is the same as corresponding strands in GCL. Comparison of HPL with GCL shows that the two share the same topology for strands β_3–β_9, including the left-handed cross-over between strands β_8 and β_9. Strands β_1 and β_2 have the same directions relative to the sheet in both proteins, but their connectivity is different (*Figure 3*).

These lipases share the same linear sequence of the triad residues: Ser–Acid–His. Garavito *et al.* (1977) recognized that the triad arrangement has a handedness, which is reflected by the side from which the serine (nucleophile) approaches the plane of the histidine ring. The structures show that the triads of all four lipases have the same handedness (*Figure 5*). The oxyanion hole of each is proposed to involve the backbone NH group of the residue immediately C-terminal to the serine. In all of these proteins, the

active site serine is embedded in a similar supersecondary structural element: β-strand–turn–α-helix. This common element was recognized early on (Derewenda and Derewenda, 1991; Schrag *et al.*, 1991b) and was found not only in lipases, but also in a number of other hydrolytic enzymes (Schrag *et al.*, 1991b; Ollis *et al.*, 1992). They include enzymes belonging to a family of esterases (GCL and AChE) and, on the basis of sequence homology, it appears that all members of this family will also have the serine embedded in the same supersecondary motif (Derewenda and Derewenda, 1991; Cygler *et al.*, 1992). The serine located in the bend between the strand and the helix adopts a conformation with (ϕ, ψ) torsion angles around ~(60°, −110°). On a Ramachandran plot (Ramachandran and Sasisekharan, 1968), this is a high energy area that involves some internal strain, yet it is consistently observed in all of these structures. It has been suggested by Ollis *et al.* (1992) that this conformation of the serine (or, in general, a nucleophilic group) provides very good exposure for the short side-chain of the nucleophile, projecting it away from the rest of the structure, and makes it readily accessible to both the histidine of the triad and to the substrate. It is also of importance that the axis of the α-helix following the nucleophile is directed along the line of access of the substrate. The helix dipole moment may then play a role in the stabilization of the tetrahedral intermediate during the catalytic reaction (Hol, van Duijnen and Berendsen, 1978), in addition to the stabilization provided by the oxyanion hole.

The two glycine residues of the G–X–S–X–G consensus sequence are very important for the maintenance of the tight bend between the β-strand and the α-helix (*Figure 6*). These two residues face each other and the distance between their C_α atoms is only ~4·5 Å, while they are even closer to other atoms from the backbone opposite to them (~4·0 Å). If either of them was replaced by another amino acid, even an alanine, the disposition of the strand and the helix would have to change. For similar steric reasons the residue after the second Gly is always one with a small side-chain, usually Gly or Ala. There is a second pair of residues that come into close contact in this supersecondary element: position –4 and +6 relative to the serine (*Figure 6*). The residue in position +6 packs against the side-chain of residue –4 and the backbone of residue –3 and always has a small side-chain. The residue in position –4 is either hydrophobic, with a branched side-chain, or aromatic.

The His of the triad comes from the C-terminal end of the molecules. In HPL and GCL it is located in a long loop after strand β_8 (*Figure 3*), which forms the left-handed cross-over connection to strand β_9. There, however, the similarity ends. In HPL the His-263 comes from the C-terminal end of this connection, while in GCL the His-463 comes from the N-terminal part of it. In RML, the polypeptide chain meanders after strand β_8, forms an anti-parallel strand β_9 and then comes back toward the active site. His-257 is located near the end of the chain (*Figure 3*). Cutinase (Martinez *et al.*, 1992) is shorter at the C-terminus than the above-mentioned lipases and it does not have strand β_8. His-188 is located in the same loop as its triad partner Asp-175, separated from it only by a short polypeptide segment.

The acid member of the triad is usually located in the loop after strand β_7,

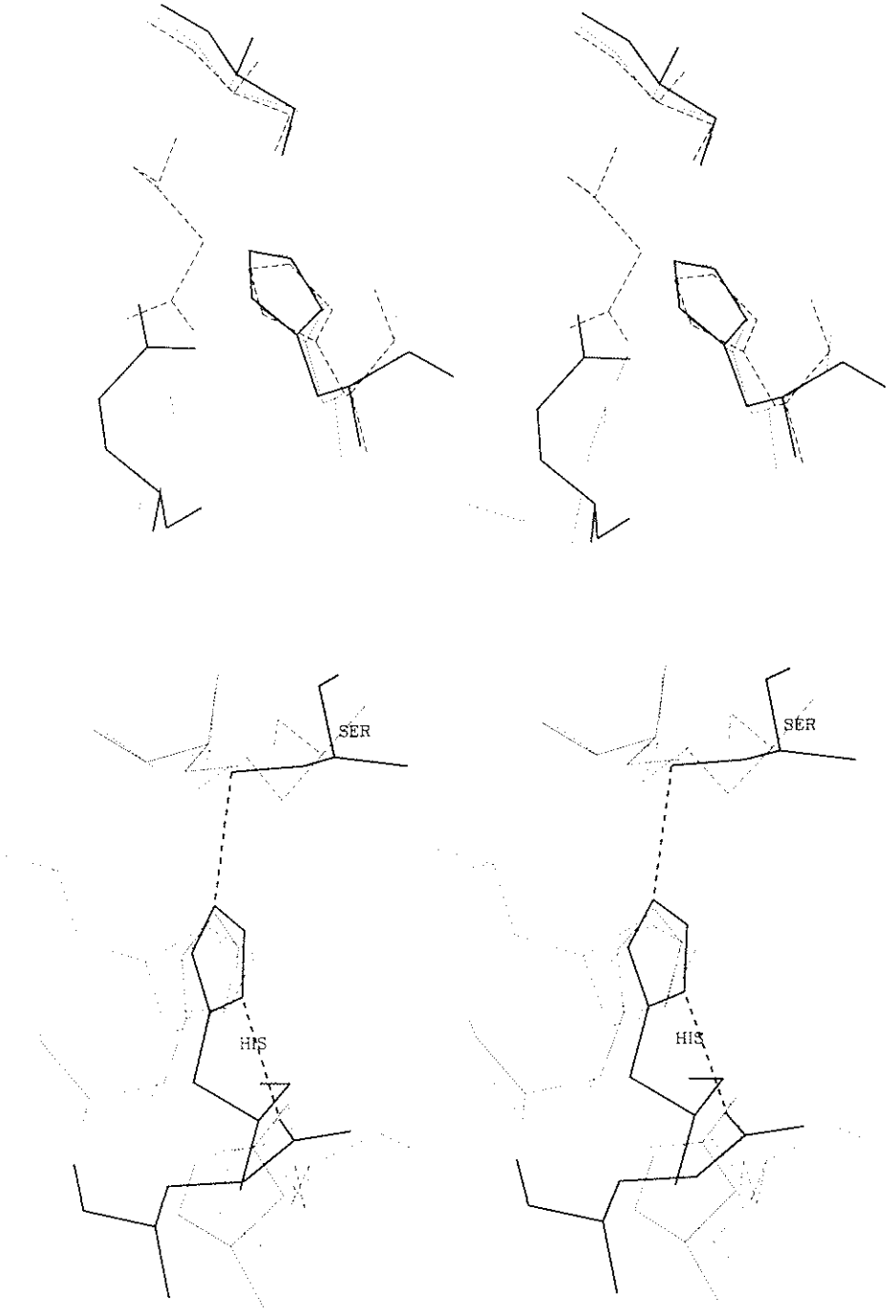

Figure 5. Superposition of the catalytic triad residues. (A, upper) RML (thin line), HPL (dashed line), GCL (thick line). (B, lower) GCL (thick line), chymotrypsin (thin line, PDB code 2gch), papain (dashed line, PDB code 9pap), trypsin (dotted line, PDB code 1sgt).

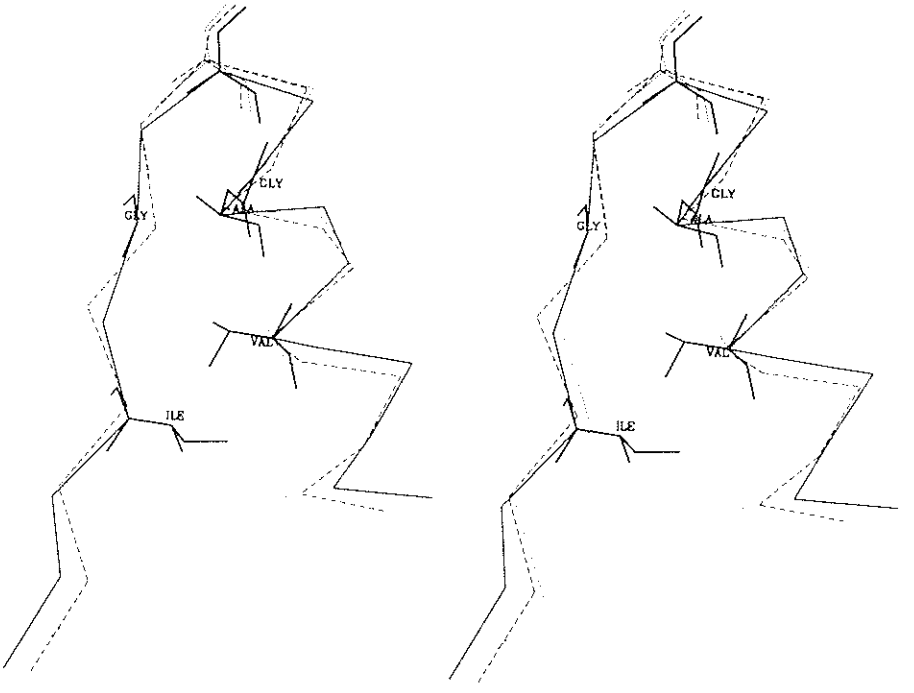

Figure 6. Superposition of strand-serine-helix supersecondary motif. RML (thin line), HPL (dashed line), GCL (thick line).

two strands after the active site serine. This holds for RML, GCL, CRL, cutinase and for other enzymes with the α/β hydrolase fold (Ollis *et al.*, 1992). The lipase HPL is an exception to this rather general observation. In HPL, Asp-176 is embedded in the loop following strand β_6 (*Figure 3*), resulting in a different direction of approach to the histidine (*Figure 5A*). Sequence alignment of pancreatic, hepatic and lipoprotein lipases clearly identified this position as absolutely conserved.

The three lipases and the cutinase vary greatly in size, ranging from 196 amino acids (cutinase) to 544 (GCL), yet the core of the structure is very much the same in all of them. The differences are in the N- and C-terminal extensions (additional strands, small β-sheet in HPL and GCL) and in the size of the loops between the strands of the conserved β-sheet. These loops extend mainly on the C-terminal side of the β-sheet, forming a cap over the active site. The extensions on the N-terminal side of the β-sheet are much shorter. Although the roles of these 'extra' features are not clear, it seems reasonable to expect that they are important for the substrate binding and for the interactions at the lipid–water interface. A more detailed understanding of their role requires further structural studies.

Conformational changes during catalysis

The first evidence of conformational changes associated with interfacial activation came from chemical modification studies. The inactivation of pancreatic lipase by organophosphates was shown to require the presence of mixed micelles and colipase (Maylié, Charles and Desnuelle, 1972; Rouard *et al.*, 1978). The sensitivity of *Geotrichum candidum* and *Pseudomonas* lipases to organophosphates was increased in the presence of lipids or in organic solvents, suggesting that a conformational change was required for the inhibitor to reach the active site (Kordel and Schmid, 1991; Nishioka *et al.*, 1991; Spener *et al.*, 1991). Limited tryptic or chymotryptic digestion of lipoprotein lipase was shown to alter the lipase activity toward triacylglycerols, but not to soluble substrates (Bengtsson and Olivecrona, 1981). Digested enzyme was only active toward emulsified substrate in the presence of the apolipopotein cofactors. This suggested that the modified enzyme was incapable of undergoing the conformational change required for activation by the interface. Amino-terminal sequencing of the fragment identified the cleavage site (Bengtsson-Olivecrona, Olivecrona and Jörnvall, 1986), and comparison of this site with the equivalent site in pancreatic lipase (Winkler, D'Arcy and Hunziker, 1990) reveals that the cleavage occurs in the flap which covers the active site.

The results of the chemical modification studies are corroborated by the crystallographic data, which show that a common feature of all three lipases is the inaccessibility of the active site from the bulk solvent. Substrates of the size of triacylglycerols cannot be accommodated in their active sites without some conformational changes to parts of the structures. Even small, water-soluble esters like PNPA, which are hydrolysed by lipases in solution, could not be modelled to access the active site of GCL without steric hindrance (M. Cygler and J. Schrag, unpublished data). That implies that the structures observed in the crystals grown in the absence of sustrates or inhibitors do not represent the interfacially active conformations of these enzyme.

Although the scaffolds and the active sites of all three lipases and the cutinase share many common features, accessibility to the active sites in each of the lipases is restricted in a different way. The extent of protection increases with the protein size. In RML, the active site is covered by a short α-helix (*Figures 1* and *3*) and is about 10 Å from the protein surface. The active site of HPL is also covered by an α-helix but the loops around the active site are longer and, as a result, the distance to the surface is about 14 Å. The loop containing the α-helical flap comes from a very different region than in RML, but its final position is very close to that in RML. GCL is the most complex of the three. Its active site is covered by segments from two large loops and is nearly 18 Å from the surface (*see* above and *Figure 3*). Cutinase is fully active in solution and its active site is on the surface of the molecule (Martinez *et al.*, 1992).

Understanding the triggering mechanism and the nature of the conformational changes at the lipid–water interface is, at the present time, one of the most fascinating and challenging problems with regard to lipase function. The

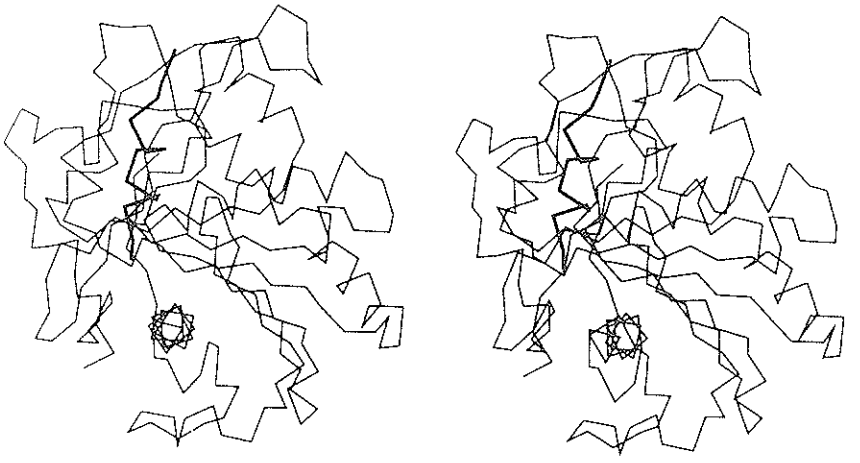

Figure 7. C_α tracing of RML showing the residues of the loop above the active site in the 'closed' (thin line) and 'open' (thick line) conformations. Reprinted from Derewenda *et al.* (1992), with permission from the publisher.

first insight was provided by crystallographic studies of RML with covalently bound inhibitors. Recently, Brzozowski *et al.* (1991) succeeded in crystallizing RML inhibited by the *n*-hexyl chlorophosphonate ethyl ester. This ester reacted stoichiometrically with the lipase, inhibiting it irreversibly. The crystals obtained were of relatively poor quality and diffracted only to 3.0 Å resolution. Nevertheless, they provided the first evidence of the conformational change in the lipase. Continuing the search for better quality crystals, Derewenda *et al.* (1992) crystallized a complex of RML with the known serine protease inhibitor, E_{600}. These crystals were isomorphous to the previously obtained complex, but diffracted to 2·65 Å resolution. They confirmed the previous results and allowed for a more detailed description of the conformational change from a 'closed' to an 'open' form in which the active site is accessible to the substrate. Superposition of the native lipase structure and the inhibited complex identified four regions displaying different positions of the backbone. Three short segments show rather small displacements and correspond to the areas involved in intermolecular crystal contacts, either in the native or complexed crystals. These changes are related to differences in the environments (arrangement) of the molecules in the two different crystal lattices. A major change was observed in the position of residues 82–96, which constitute the loop containing the α-helix covering the active site (*Figures 4* and *7*). This large conformational change can be described as a rigid body movement of the α-helix around two hinge regions (Brzozowski *et al.*, 1991). One of the hinge regions consists of Ser-83–Ser-84, while the other includes Asp-91–Phe-94. The middle, α-helical part of this loop, comprising residues 85 to 92, has the same local backbone conformation in the native and complexed molecules (*Figure 8*). There is, however a change in the conformation of some of the side-chains in this region, notably those of Trp-88 and

Asn-91. As a result of this rigid body movement, the centre of the α-helix moves by about 8 Å and the helix rotates by approximately 167°, allowing access to the active site (Brzozowski *et al.*, 1991). This is accompanied by a change in the character of the protein surface. Some of the previously exposed hydrophilic residues of the flap (Ser-84, Asn-87, Asp-91 and Thr-93) become partially buried, interacting with the end of the loop 58–61. This corresponds to a total loss of hydrophilic surface area on the order of 550 $Å^2$. At the same time, there is an increase in the hydrophobic surface area, due mainly to increased exposure of hydrophobic side chains which are covered in the native structure by the α-helix of the flap. The increase amounts to more than 700 $Å^2$, ~7% of the total solvent accessible surface of RML (Derewenda *et al.*, 1992).

To what extent the conformation observed in the complex represents the active conformation of RML at the interface is somewhat uncertain. Although there is no direct evidence, the 'open' conformation observed in the complex seems likely to represent the active state achieved by the lipid–water interface. The new position of the flap in the 'open' conformation is stabilized by many hydrogen bonds, and the polar residues that became buried in the new conformation participate in this new network. The 'open' position of the flap appears to form an alternative stable conformation of loop 82–96 (Derewenda *et al.*, 1992). This is supported by the fact that two inhibitors, although different in size, induce the same 'open' conformation. The newly created hydrophobic surface, part of which comes from the bound inhibitor, is involved, in the crystal environment, in hydrophobic interactions with the equivalent surface of a molecule related by a two-fold symmetry. Thus, similar interactions of this surface with the lipids at the interface seem plausible. Another possibility, of course, is that the observed 'open' conformation is to some extent influenced by crystal packing, but on the other hand, such packing provides a very good simulation of the hydrophobic interface. To what extent the observed 'open' form represents a fully open conformation characterizing interactions with real substrates (triacylglycerols) will, hopefully, be answered either by structural studies of complexes with larger inhibitors or by observing different conformations of the enzyme in crystals which are grown under different conditions.

For the other two lipases the question of a conformational change was addressed only in a more speculative fashion. Neither of them appears to have been observed in its lipolytic conformation. The blocking of the entrance to the active site in HPL by an α-helix suggests that there is some similarity in the activation mechanism to the change observed in RML. However, the movement of only the loop containing this helix is not sufficient to provide access to the active site. Winkler, D'Arcy and Hunziker (1990) suggest that additional changes, in the regions 75–79, 212–216 and in the position of Trp-252, are also likely to occur upon binding triacylglycerols. Small substrates might be able to enter the active site without its full opening. In order to identify unambiguously the active site serine, these authors soaked the HPL crystals in a solution containing butylboronic acid, a small, reversible inhibitor, collected 3·0 Å resolution data and calculated a differ-

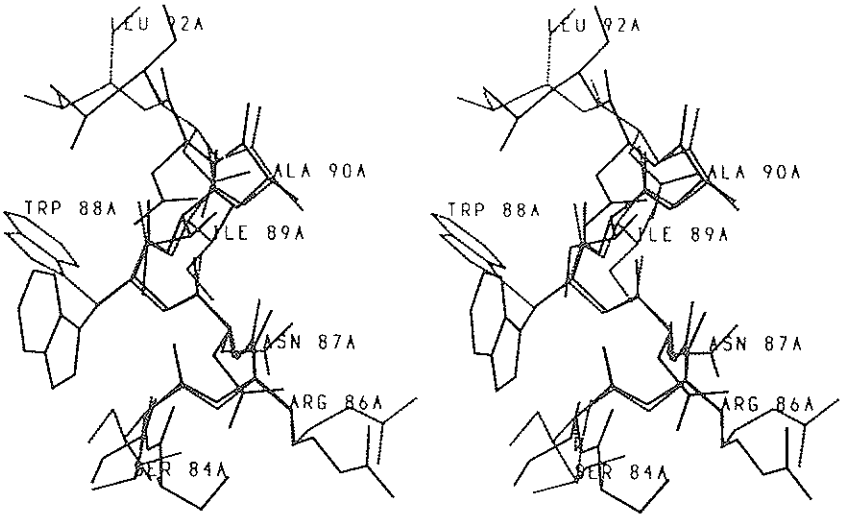

Figure 8. Superpositionof the segment 85–92 of RML in the 'open' and 'closed' conformation. Reprinted from Derewenda *et al.* (1992), with permission from the publisher.

ence electron density map. The highest peak in the map occurred near the position of Ser-152. There was no indication of a conformational change, but the level of formation of the boronate adduct was quite low. Apparently, this small inhibitor was able to diffuse, although inefficiently, into the active site without causing a major conformational change. This is consistent with the low level of activity displayed by lipases toward small, soluble substrates.

Comparison of the GCL and CRL structures provides strong evidence for the types of conformational changes which accompany activation of these enzymes at the lipid–water interface. The greatest differences in the crystal structures of these enzymes are seen in the region of the flap and loops adjacent to the active site (Grochulski *et al.*, 1992). One may reasonably presume, then, that the conformation displayed by CRL is a good representation of the 'open', lipolytic conformation adopted by GCL at the interface, and that, in aqueous media, CRL adopts a 'closed' conformation similar to that seen in GCL. The movement of the flap (residues 61–99 in GCL) associated with interfacial activation most closely resembles the unwinding of the twist in the polypeptide chain at the base of the flap suggested and discounted by Schrag *et al.* (1991b). This movement shifts the flap from an orientation which is nearly parallel to the protein surface to one which is nearly perpendicular to the protein surface. The second helix of the flap (helix 85–99) in GCL, rather than helix 66–76, would undergo the largest displacement. This idea was discounted by Schrag *et al.* (1991b) because the position of an internal cavity in GCL suggested a direction of approach of substrate which required movement of helix 66–76. Opening of the flap in CRL and GCL exposes hydrophobic residues and forms a broad, mostly hydrophobic depression which leads to the catalytic serine.

The second loop which differs in position in CRL and GCL is the loop

containing Ala-132 in GCL, which is proposed to contribute to the oxyanion hole. In the 'closed' conformation observed in GCL, this loop is directed toward the surface of the protein and makes hydrophobic contacts with the underside of the flap. In the 'open' conformation of CRL this loop folds downward and is directed more toward the centre of the molecule. The conformation of this loop in CRL is nearly identical to that of the corresponding loop in AChE. In this conformation, Ala-132 in GCL would be in a better position to form part of the oxyanion hole and the catalytic serine would be more fully exposed. The shift to this conformation must be accompanied by adjustments in the positions of some adjacent residues, which is also observed in comparing GCL and CRL.

Phospholipases are another family of proteins working at the lipid–water interface and are also known to be activated by the presence of the interface. The mechanisms of activation of lipases and phospholipases A_2 appear to be different. Crystal structures of complexes of phospholipases A_2 with substrate-like inhibitors indicated that the protein does not undergo a major conformational change upon substrate binding (Scott *et al.*, 1990). Instead, these authors speculate that the interface plays a role through pre-forming the flexible substrate — phospholipid molecule — into a conformation easily accepted by the enzyme. To what extent this function of the interface plays a role in lipases remains to be seen.

Modelling of other lipases by homology

The three-dimensional structures of the four lipases will provide a basis for systematic investigation of similarities and differences in related enzymes. The structure of the pancreatic lipase has provided the basis of several modelling studies of lipoprotein lipase and the non-hydrolytic vitellogenin relatives. Derewenda and Cambillau (1991) modelled the structures of hepatic and lipoprotein lipases from HPL and examined the positions of naturally occurring mutations in the hepatic and lipoprotein lipase genes and the consequences of the mutations on the lipolytic function of the enzyme. Many of the mutations are in positions which appear to compromise proper folding of the enzyme rather than altering the catalytic or cofactor binding sites directly. Another study of naturally occurring mutants in lipoprotein lipase identified two mutations affecting Asp-156, presumably a member of the catalytic triad (Ma *et al.*, 1992). These mutations result in inactive enzyme, confirming a role for Asp-156 in catalysis. This study also identified a Cys to Ser mutation disrupting a disulphide bond at the base of the loop which covers the active site in the model of lipoprotein lipase. This mutation compromises the interfacial activation of the enzyme.

Sequence similarity of the pancreatic lipase family with vitellogenins has been recognized (Bownes *et al.*, 1988; Persson *et al.*, 1989). A recent study shows that the vitellogenins are very similar to the lipases in five localized segments (Persson *et al.*, 1991). Four of these segments form consecutive β-strands in pancreatic lipase, and all are located in the core of the HPL molecule. Secondary structure predictions for these regions of vitellogenin

suggest a conformation similar to that observed in HPL. The fifth segment which shows strong homology is, in HPL, a helix which packs against two of the β-strands. This region forms a largely hydrophobic pocket, which these authors suggest acts as a lipid binding site in the vitellogenins. The vitellogenins and pancreatic lipases are suggested to have diverged from a common ancestor.

Evolutionary relationships

The similarities in the three-dimensional structures of the lipases despite limited sequence homology raises the question of their evolutionary relationship. The amino acid sequence homology among the various lipase families is largely limited to the G–X–S–X–G motif which, in all known cases, contains the catalytic serine. In all of the lipase structures, this motif is found in a β-strand–turn–α-helix supersecondary structure (Derewenda and Derewenda, 1991; Schrag *et al.*, 1991b). Derewenda and Derewenda (1991) have analysed the stereochemistry of this element and have compared the sequences of a number of lipases and esterases in this region. They conclude that all of the lipases which they studied will have the same structure around the catalytic serine. In addition to the families represented by the four three-dimensional structures described in this review, this includes the gastric lipase family and the *Staphylococcus* lipases. The essential elements described for this supersecondary structure are also present in the hormone-sensitive lipase and in the *Moraxella* lipases (J. Schrag, unpublished observations). The identical β-strand–turn–α-helix supersecondary structure was found not only in serine hydrolases, but also in hydrolases which contain catalytic nucleophiles other than serine (Ollis *et al.*, 1992).

The core of the *Geotrichum* lipase is identical in topology to four other hydrolases. One of these hydrolases is *Torpedo californica* acetylcholinesterase, whose homology and structural similarity (Sussman *et al.*, 1991) to the *Geotrichum candidum* lipase has been mentioned previously in this review. The remaining three hydrolases, namely: dienelactone hydrolase from *Pseudomonas* (Pathak and Ollis, 1990), wheat serine carboxypeptidase II (Liao and Remington, 1990), and haloalkane dehalogenase from *Xanthobacter autotrophicus* (Franken *et al.*, 1991), show little sequence homology, either to each other or to GCL or AChE. This conserved topology has been named the α/β hydrolase fold (*Figure 2*) and is described in detail by Ollis *et al.* (1992). The topology of the β-sheet of these enzymes is strictly conserved, including the location of the active site residues. The identities of the essential residues, however, are not conserved. The nucleophile can be either Ser, Cys, or Asp and the acid can be either Asp or Glu. The conservation of the topology and location of the catalytic residues, despite the nature of the nucleophile or acid, suggests that these enzymes have diverged from a common ancestor.

The topologies of RML and HPL show strong similarities to the α/β hydrolase fold, but the identical portions are somewhat shorter and encompass fewer strands. Although the strand directions are generally conserved,

the connectivities between some of the strands are different (see above). In HPL the location of the catalytic acid is different, coming after strand β_6 rather than β_7. The question of whether this arrangement evolved independently or these two assemblies of the triad are evolutionarily related has been addressed by Schrag, Winkler and Cygler (1992). These authors pointed out that in the HPL structure there is an aspartate (Asp-205) in a position equivalent to that of the catalytic acid in the α/β hydrolase fold enzymes. The side-chain of Asp-205 is directed away from His-263 and makes no close contact with it. Instead, it is surrounded by a few water molecules to which it makes hydrogen bonds. Model building showed that, by a simple rotation of this side-chain (χ_1, χ_2 torsion angles), its carboxylate group can be brought into hydrogen bonding distance to His-263 in a fashion analogous to the other lipases. A parallel modelling experiment showed that if aspartate is placed in position 249 of GCL, corresponding to catalytic Asp-176 in HPL, its side-chain can easily be positioned to make a hydrogen bond with His-463 (Schrag, Winkler and Cygler, 1992). Asp-205 of HPL is conserved in all pancreatic lipases for which sequences are available, but not in the closely related hepatic or lipoprotein lipases. These authors suggest that, at some point during the evolution of pancreatic lipase, the position of the acid member of the triad switched from one position (after strand β_7) to another (after β_6). They further suggest that pancreatic lipases represent an evolutionary link between enzymes whose catalytic triads are arranged differently within the same basic protein fold. Comparison of the gene structures of these enzymes suggests that the pancreatic lipase gene most closely resembles the primordial lipase gene, and that the hepatic and lipoprotein lipase genes are more similar to each other than to the pancreatic lipase gene (Kirchgessner *et al.*, 1989). Analysis of the variability in the gene sequences further suggests that pancreatic lipase is evolving at twice the rate of the hepatic lipase, whereas lipoprotein lipase appears to be changing at a slower rate (Datta *et al.*, 1988). The arguments based on the three-dimensional structures and model building (Schrag, Winkler and Cygler, 1992), together with the analysis of gene structures of the pancreatic, hepatic and lipoprotein lipases (Kirchgessner *et al.*, 1989), suggest that the hepatic and lipoprotein lipases diverged from pancreatic lipase after the switch in acid position had already occurred. Schrag, Winkler and Cygler (1992) speculate that the differences in topology and locations of catalytic residues between HPL, RML and α/β hydrolase fold enzymes are a result of divergence from a common ancestor.

Active site serine residues of lipases have always been found in the G–X–S–X–G consensus sequence. This consensus is common not only to lipases, but also has been observed at the active site serines in serine proteases, esterases, and many other hydrolytic enzymes. Cysteine proteases have the same sequence at the active site, except that cysteine replaces serine as the nucleophile. The presence of this pentapeptide consensus in a polypeptide sequence does not necessarily indicate a catalytic role for this serine. In 4085 protein sequences in the SwisProt databank, this pentatpeptide was found 5770 times (R. Brousseau, unpublished results), and there were over 1400 occurrences in the GenBank (Derewenda and Derewenda,

1991), many of which were in proteins with no hydrolytic activity. From an analysis of the codon representation of the catalytic serine residues, which fall into two classes, TCN codon and AGN codon, Brenner (1988) hypothesizes that all of the catalytic serines have derived from the cysteine TGN codon, by single mutations. He argues that the ancestral enzyme used cysteine rather than serine as the catalytic nucleophile. The three-dimensional structures of serine proteases show that, in these enzymes, the conformation of the consensus pentapeptide around the catalytic serine is different from that in lipases. Although the two glycines are conserved, their apparent structural role in proteases is not the same as in lipases (for details see Derewenda and Derewenda, 1991). In fact, proteases of the trypsin family differ in this respect from the subtilisin family. In the latter, the second Gly of the consensus sequence is replaced by Ala. Additionally, the linear order of catalytic residues in serine proteases is different from that found in lipases. For example, in the chymotrypsin-like family the triad order is: His–Asp–Ser, whereas in the subtilisin family the order is Asp–His–Ser and in the cysteine protease papain-like family it is Cys–His–Asn. Consequently, the triad handedness also may differ. As shown in *Figure 5*, the handedness of the triads in lipases is opposite to those in the trypsin, chymotrypsin, and subtilisin families of proteases (Brady *et al.*, 1990; Winkler, D'Arcy and Hunziker, 1990; Schrag *et al.*, 1991b), but similar to that observed in cysteine proteases of the papain family (Schrag *et al.*, 1991b). Due to this difference, the postulated position of the oxyanion hole in lipases is also different from serine proteases. In serine proteases it is the NH group of the active site serine that contributes to the oxyanion hole, whereas, in lipases, the backbone NH of the residue immediately C-terminal to the catalytic serine is postulated to contribute to transition state stabilization. The change in handedness results in the migration of the oxyanion hole from the N-terminal side to the C-terminal side of the serine. The triad arrangements observed in the α/β hydrolases and the subtilisin, trypsin and papain families seemingly represent an example of convergent evolution. In this context, the strict conservation of the G–X–Nuc–X–G(A) sequence around the active site nucleophile is intriguing.

Concluding remarks

The three-dimensional structures reported to date represent four of the enzyme families described in *Table 1* and provide insights into the structures of homologous enzymes. The similarities between the four structures also suggest that other lipase families will have similar features. While the similarities are interesting and informative, there is much to be learned by looking at the differences as well. For example, pancreatic lipase and lipoprotein lipase undoubtedly have similar structures, and yet their activities are modulated by different cofactors. The *Rhizomucor, Rhizopus* and *Humicola* lipases are homologous to the *Penicillium* mono-, diacylglycerol lipase, but their substrate specificities are quite different. The bile salt-stimulated lipase and those of *Geotrichum* and *Candida* are homologous, but

also show different substrate specifities and different cofactor dependence. The structures solved to date do not provide the information necessary to understand the structural basis of selectivity differences. Clearly we are only beginning to understand the structure and function relationship in lipases. We anticipate that developments in this field will be both rapid and exciting.

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Note added in proof

An important paper describing the structure of the complex between human pancreatic lipase and porcine procolipase has been published (van Tilbeurgh *et al., Nature* **359**, 159–162, 1992) since this review was submitted and for that reason is not discussed in this review.

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