Open and closed states of *Candida antarctica* Lipase B: protonation and the mechanism of interfacial activation

Benjamin Staucha, Stuart J. Fishec and Michele Cianci*d*

*aEuropean Molecular Biology Laboratory - European Bioinformatics Institute (EMBL-EBI), Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SD, United Kingdom, bRobinson College, University of Cambridge, Grange Road, Cambridge CB3 9AN, United Kingdom, cDiamond Light Source, Didcot, Oxfordshire OX11 0DE, United Kingdom, dEuropean Molecular Biology Laboratory, c/o DESY, Building 25A, Notkestrasse 85, Hamburg, 22607, Germany

*Corresponding e-mail: michele.cianci@embl-hamburg.de

1 Present address: Department of Chemistry, Bridge Institute, University of Southern California, Los Angeles, CA 90089 USA
ABSTRACT Lipases (E.C. 3.1.1.3) are ubiquitous hydrolases for the carboxyl ester bond of water-insoluble substrates such as triacylglycerols, phospholipids, and other insoluble substrates, acting in aqueous as well as in low-water media, thus being of considerable physiological significance with high interest also for their industrial applications. The hydrolysis reaction follows a two-step mechanism, or ‘interfacial activation’, with adsorption of the enzyme to an heterogeneous interface and subsequent enhancement of the lipolytic activity. Among lipases, *Candida antarctica* Lipase B (CALB) has never shown any significant interfacial activation, and a closed conformation of CALB has never been reported leading to the conclusion that its behaviour was due to the absence of a lid regulating the access to the active site. The lid open and closed conformations and their protonation states are observed in the crystal structure of CALB at 0.91 Å resolution. Having the open and closed states at atomic resolution allows relating protonation to the conformation, indicating the role of Asp145 and Lys290 in the conformation alteration. The findings explain the lack of ‘interfacial activation’ of CALB and offer new elements to elucidate this mechanism, with the consequent implications for the catalytic properties and classification of lipases.

KEYWORDS: Lipase; Fatty acid/metabolism; Lipids/chemistry; Enzymology/Enzyme regulation; X-ray crystallography.
INTRODUCTION

Lipids are, together with proteins and carbohydrates, the basic constituents of the architecture of a living organism. Lipids form membrane systems, which function as physical and chemical barriers separating aqueous compartments, regulating processes such as secretion, endocytosis, and signal transduction. Lipids are one of the major sources of energy, or serve as biological modulators and signal transducers (e.g., steroids, phosphatidylinositol derivates, etc.) and as vehicles for carrying fat-soluble vitamins. The functional interaction between lipids and proteins is essential for such activities.

Lipases (E.C. 3.1.1.3) are ubiquitous hydrolases for the carboxyl ester bond of water-insoluble substrates as triacylglycerols, phospholipids, and other insoluble substrates, acting in aqueous as well as in low-water media. For these reasons lipases are enzymes of considerable physiological significance (1) with high interest also for their industrial applications (2).

The hydrolysis reaction, catalysed by lipases, does not follow Michaelis-Menten kinetics, and presents a two-step mechanism, defined as ‘interfacial activation’, with adsorption of the enzyme to an heterogeneous interface and subsequent enhancement of the lipolytic activity (3). The crystal structures of human pancreatic lipase (HPL) (4) and of lipase-procolipase complex (5,6), a fungal enzyme from Geontrichum candium (7), and a lipase from Pseudomonas glumae (8) showed a lid domain, either a loop or a helix, shielding the active site from the solvent and thus regulating the exposition of the substrate to the catalytic triad. It was postulated that a conformational change would occur in the presence of a lipid-water interface inducing interfacial activation. Thus the presence of a lid domain and interfacial activation have long been used to distinguish lipases from esterases, where the interfacial activation phenomenon was not observed (9). More recently the observation of a novel lid domain allowed the formulation of hypothesis for a likely mode of interfacial activation of Candida antarctica Lipase A (10), while a cutinase
from *Trichoderma reesei*, showing interfacial activation behaviour and the presence of the lid domain, has been reported as having the kinetic and structural features of a ‘true lipase’ (11).

Among lipases, *Candida antarctica* Lipase B (CALB) is the one that found the widest application in many industrial processes because of its high enantioselectivity, wide range of substrates, thermal stability, and stability in organic solvents (12,13). CALB belongs to the α/β hydrolase fold family with a conserved catalytic triad consisting of Ser, His, and Asp/Glu within an acyl binding pocket and binding pocket for the moiety of secondary alcohols (14,15). However a closed conformation of CALB has never been reported and CALB has never shown any significant interfacial activation (16). Taken together, these observations have lead to the conclusion that this behaviour was due to the absence of a lid regulating the access to the active site (16).

Whether or not CALB lacks a lid structure is matter of debate according to several molecular dynamics simulation studies (17-20). CALB has two α-helixes surrounding the active site, namely α5 and α10 (14,15) which have been shown to be very flexible regions and could work as a lipase lid by a relative motion between them either by increasing the working temperature (19) or by working in organic solvents (17). Such helix motions, in aqueous media, could not be large enough to produce variation in the hydrophobic surface and in the solvent accessible area and to close the active site (20). However, the CALB sequence stretch around the α-helix 5 has been shown to significantly influence the catalytic properties of the enzyme, including the enantioselectivity, which, consequently, seems to be not exclusively directed by the fit of the substrate into the enzyme’s active site pocket (18). The issue of the open and closed conformations of CALB is then not only related to the ‘interfacial activation’ but it has implications for the catalytic properties of the enzyme (18).

We report here the crystal structure of *Candida antarctica* Lipase B (CALB) in open and closed conformation refined at 0.91 Å resolution, representing the highest resolution structure of CALB.
to date, and of all currently available triacylglycerol lipase X-ray structures (EC 3.1.1.3). Probing
the crystals using xenon validated the open and closed conformations. Having available the open
and closed conformations, in the same unit cell at atomic resolution allows relating the
protonation states (21,22) of each monomer to their conformation. Resolving the open and closed
conformations offers new explanations for the lack of interfacial activation mechanism of CALB
pointing to a role of Asp145 and Lys290 in the conformation alteration. A search for homologues
with conserved Asp145 and Lys290 residues returns five hits potentially leading to a new sub-
classification of lipases.
MATERIALS AND METHODS

Crystallization conditions and xenon derivativation

*Candida antarctica* Lipase B (CALB) was purchased from Hampton Research and crystallized without further purification. Crystallization trials were performed at 293 K using the hanging-drop method using a Quiagen™ EasyXtal 15-well plate. 1 µL of a 15 mg/mL CALB solution in 20mM Na(CH₃COO) pH = 4.8 was diluted with 1 µL of the precipitant solution, made of 200mM Na(CH₃COO) pH = 4.8, 20% (w/v) PEG4000, and 10-13% (v/v) 2-propanol. The drop was equilibrated by vapor diffusion against 500 mL of the precipitant solution. Protein crystals of native CALB appeared within one week and grew to a size of 0.2 x 0.4 x 0.5 mm³ in three weeks.

The xenon complex of native CALB was prepared by exposing the crystals to xenon pressure (10 atm) by using the Hampton Research xenon chamber available at the EMBL laboratory. Xenon incubation times were kept to around 15-20 minutes. After pressure release the loops with the crystal was quickly plunged into liquid nitrogen for flash cooling and then transferred to the beam line. The crystal loop was maintained at 100 K with a cold nitrogen stream.

X-ray diffraction data collection and structure analysis

Diffraction data were collected at 100 K using synchrotron radiation at the EMBL P13 beamline at the Petra III storage ring, c/o DESY, Hamburg (Germany). The beam line was equipped with a Dectris Pilatus 6M detector and a MD2 goniometer (MAATEL-EMBL) with horizontal spindle axis. Crystals were cooled at 100 K with a cold nitrogen stream. The wavelength was set to 0.826 Å, using a Si(III) crystal monochromator (FMB-Oxford). Data were collected from four crystals oriented with different kappa angles while performing helical scans. The data were integrated using the program XDS and scaled with XSCALE (23). Crystals of the native enzyme diffracted to 0.91 Å resolution with unit cell dimensions of a = 39.67 Å, b = 48.94 Å, and c = 71.61 Å and space group P1.
For the xenon complex of native CALB, data collection was carried on at a wavelength of 2.25 Å, chosen to enhance the anomalous signal from the expected xenon atoms and warrant the necessary resolution for the experiments. At λ = 2.25 Å, close to the L₁ edge of Xe, the f’ is of 13.14 e⁻. Crystals of the xenon complex diffracted to 2.27 Å resolution with unit cell dimensions of a = 39.6 Å, b = 48.8 Å, and c = 71.6 Å and space group P1. The resolution of data for the xenon complex was limited by the geometry of the camera.

The structures of native CALB and of the xenon complex were solved by molecular replacement using MOLREP (24) starting from the deposited structure of CALB as search model (Protein Data Bank (PDB): 1TCA (14)). The models were subjected to rigid-body minimization and subsequently to refinement steps with REFMAC (25). For the native structure, anisotropic temperature factors refinement was used and all the hydrogen generated, with automatic weighting scheme, given the value of 53 for ratio reflection/atom. For the xenon complex isotropic temperature factors refinement was used. Manual rebuilding of the models were performed using the COOT graphic interface (26) by inspecting the electron density map, calculated with 2F_{obs}-F_{calc} or F_{obs}-F_{calc} coefficients and calculated phases from the model. Models were validated using the PDB_REDO web server (27). The native structure and its xenon complex were refined to an R_{factor} and R_{free} (5% of data) of 11.1% and 13.2%, and of 13.9% and 19.4% respectively. Data collection and refinement statistics are reported in Table 1. The protonation-state determination of the amino acid side chains followed the protocol described by Fisher et al. (22).
RESULTS

Overall structure

In the asymmetric unit there are two monomers (Figure 1a), which show the α/β type fold described by Uppenberg et al. (14) (Figure 1b). These two monomers interact via a network of non-bonded contacts between residues Pro143, Leu144, Leu147 (α-helix 5), Thr186, Asp187, Glu188 (loop between sheet β6-β7), Ala282 and Val286 (α-helix 10) in monomer A and Thr186, Asp187, Gln191 (loop between sheet β6-β7), Pro218, Leu219, Asp223 (α-helix 5 and subsequent loop), Pro260 and Leu261 (loop between α-helix 9 and 10) in monomer B, representing interface areas of 463 and 442 Å² in monomer A and B, respectively (Figure 1c). Two N-acetylglucosamine molecules are observed in both monomer A and monomer B. In monomer A, the triad Asn74-NAG-NAG show two alternative conformations while in monomer B only a single conformation is observed. Several multiple amino acid side chain conformations are observed throughout the structure (Figure 1b).

Open and closed conformations

The rms deviations of the Cα atoms of CALB monomers A and B, reported here, against the monomer A of the structure reported by Uppenberg et al. (14) (PDB code 1TCA) are 0.34 Å and 0.54 Å respectively, as calculated using SUPERPOSE (28) over 310 residues (Figure 2a). The rms deviation values of Cα atoms, of monomer A against monomer B, presented here, is 0.53 Å. The rms deviation values (Figure 2a) indicate that the secondary structure of monomer A is maintained and consistent with the classical open conformation previously observed (14,15,29) while the rms deviation values of Cα atoms for monomer B show a dramatic change between residues 140 and 147. The residue range Leu140-Ala141-Gly142-Pro143-Leu144-Asp145-Ala146-Leu147, corresponding to α-helix 5, undergoes a conformational change from an α-helix structure to a loop as clearly shown by the assignment of the secondary structure computed with PROCHECK (30) (Figure 2b). The electron density maps at 0.91 Å resolution clearly describe the
residue range 140-147 of monomer A as an $\alpha$-helix, corresponding to an open conformation, and of monomer B as an unfolded loop, corresponding to a closed conformation (Figure 2c). The residue range 140-147 presents Asp145 as the only amino acid with a charged side chain.

The final model of Candida antarctica Lipase B (CALB) has a standard uncertainty for the positional parameters of all atoms of 0.015 Å, as calculated using the Cruickshank’s DPI factor (31) (Table 1), which is six times lower than the values of the best currently reported Candida antarctica Lipase B (CALB) structures. Determination of the protonation state by bond length analysis (21,22) of the Asp and Glu amino acid side chains (Figure 3) resulted in a final computed average standard deviation of 0.005 Å. The analysis reveals that, in chain A, the carboxylic group of Asp145 is protonated with bond lengths of C-O$_{\gamma2}$ $\approx$ 1.3 Å and of C-O$_{\gamma1}$ $\approx$ 1.2 Å, while in chain B it is deprotonated due to salt bridge formation, with bond lengths of the Asp145 carboxylic group of C-O$_{\gamma2}$ $<$1.2 Å and of C-O$_{\gamma1}$ $>$1.4 Å outside standard values. In the open conformation, Asp145 is placed at a distance of 3.8 Å above the indole group of Trp155, within hydrogen bond distance from the carboxylic group of the main chain of Ala141, the hydroxyl group of Ser150 and Thr158 side chains (Figure 4a). The side chain of Asp145 shifts by 7.4 Å, thus reaching the side chain of Ly290 at a distance between NZ atom Ly290 – O$_{\gamma1}$ Asp145 of 2.7 Å and generating a salt bridge (Figure 4b,c). The consequent large conformational change of the Leu140-Leu147 region results in the closing of CALB active site as shown by the space-fill representation (Figure 4d,e).

Pressurizing the crystal with xenon probed the tightness of the closed lipase conformation. Three xenon atoms, with anomalous signal bigger than 5$\sigma$, were located in monomer A in the catalytic site in close proximity to the catalytic triad. In particular, the three xenon atoms (8.3$\sigma$, 6.3$\sigma$ and 6$\sigma$ map level, respectively, refined with fractional occupancies) in monomer A are located above His224, in the hydrophobic region of the catalytic site defined by Leu144, Val154, Ile189, Leu219, Val221, Ala281, Ala282, Ile285, Leu287 (Figure 4f). No xenon atoms were observed in
the active site of monomer B, thus confirming that the conformational rearrangement of the residue range 140-147 prevents the access of any substrate to the active site (Figure 4g). Xenon is also found in the hydrophobic cavity defined by Met83, Val101, Leu115, Ile121, Val125, Val37 and Ile187 in both monomer A (10.2σ) and B (10.5σ). A further site is found next to Trp155 in monomer B (7.2σ).

The rms deviation values of Cα atoms (Figure 2a) confirm a reorientation of the loop residues between α-helixes 8 and 9, localized on Leu219 and around α-helix 10. These shifts are compatible with the protein-protein interaction and with the crystal packing. In particular, Leu219 of monomer A, in open conformation, is pointing towards α-helix 10 of the symmetry related molecule in closed conformation, in a proximal position to Lys290. Leu219 of monomer B is found above the active site of monomer A.

Active sites

In the active sites of both monomer A and monomer B, Ser105 is refined with multiple conformations, with the hydroxyl group swinging towards and away from NE2 of His224 and one water molecule. In both active sites molecules of isopropanol are found tethered by the hydrogen bonding with the hydroxyl group of the side chain of Thr40, with the alkane chain positioned in the void between Asp134, Gln157, and Ile189. A network of waters is found within the active site and extending from the catalytic triad towards α-helix 5 and α-helix 10.

The bond length analysis (22) shows that, in monomer A (Figure 5a), the side chains of Asp187 and Glu188 are deprotonated, with bond lengths of C-Oγ1=1.225(9) Å and C-Oγ2=1.246(8) Å for the Asp187 residue and of C-Oδ1=1.233(12) and C-Oδ2 =1.225(14) Å for the Glu188. The protonation state in His224 can be rationalised by analysing the chemical environment as suggested by Fisher et al. (22). In monomer A Asp187 forms hydrogen bond via Oγ2 with the His224 ND1 proton (2.79 Å), while a water molecule donates protons to NE2 (2.73 Å). In
monomer B (Figure 5b), the side chain of Asp187 is also deprotonated with bond lengths of C-Oγ1=1.277(9) and C-Oγ2=1.254(8) Å. The side chain of Glu188 appears to be protonated or partially protonated as evident from the different C-Oδ1 and C-Oδ2 distances, where the C-Oδ1 bond, hydrogen bonded to the N-H of the backbone of His224, is 0.05 Å shorter than the C-Oδ2 facing towards a water molecule. When looking at the maps and the structures of His224, ND1 proton makes a hydrogen bond to the deprotonated Asp187. Ser105 proton hydrogen bonds to His224 NE2. In both monomer A and monomer B a water molecule is shared between His224 and Ser105 (Figure 5a,b).
DISCUSSION

The presence of a lid covering the active site in *Candida antarctica* Lipase B has long been matter of debate. The lack of structural evidence (14,15) and the lack of obvious interfacial activation during catalysis (16) have been the main argument against the existence of a lid in CALB. The \(\alpha\)-helix 5 and \(\alpha\)-helix 10 are shown here to be responsible for the closed conformation of CALB, and describe a completely unforeseen closing mechanism (Figure 6) at atomic level. In the open conformation \(\alpha\)-helix 5 shows a series of aliphatic residues that lines the channel leading to the active site (14) with the only polar residue Asp145 making hydrogen bonds with the side chains of Ser150 and Thr158. When closing the active site, the \(\alpha\)-helix 5 undergoes a dramatic conformational change to an unstructured loop while bringing the carboxylic group of Asp145 close to the side chain of Lys290 thus forming a salt bridge. The spatial rearrangement of Lys290 brings \(\alpha\)-helix 10 closer to new lid region completing the closure of the catalytic cavity. The unfolding of \(\alpha\)-helix 5 results in the closing of the lid over the cavity, preventing the access of any substrate to the catalytic site as shown by probing binding sites with xenon.

Xenon is able to bind to a protein in closed intramolecular hydrophobic cavities, accessible active sites, intermolecular cavities and channel pores, as the result of non-covalent weak-energy van der Waals forces (32) by diffusing rapidly towards potential interaction sites through the solvent channels of a protein crystal (32). The relatively high atomic number and its anomalous dispersion of xenon together with data collection at long wavelengths make it possible to locate it within protein structures in crystallography (33).

The location of three xenon atoms in monomer A, in close proximity to the catalytic triad, and none in monomer B, confirms that the conformational rearrangement of \(\alpha\)-helix 5 seals the active site. In our structure the lid region in monomer B is held in place by a close contact with the \(\alpha\) helix 9’ region of the symmetry related of monomer A. No evident hydrogen bond pattern is present, and the interaction happens via hydrophobic surface patches from each monomer.
Comparison with previously reported CALB structures reveals reasons for the absence, so far, of structural evidence for this closed lid conformation. In the structures reported by Uppenberg et al. (14) (PDB identifiers 1TCA, 1TCB and 1TCC), the range Asp145-Pro143, would clash with residue Leu199 of a symmetry related CALB monomer that is part of a face-to-face, i.e. binding site to binding site, dimer, if the lid segment would adopt the more extended, closed conformation (Figure 2d). Instead, in these structures, the lid segment adopts the more compact, open, α-helical conformation. In our structure, however, the monomers are shifted with respect to each other such that for the monomer in closed conformation this clash does not occur (Figure 2c). A similar clash between residues Asp145-Pro143 in one and Leu199 in the other monomer would occur for a closed lid conformation in an additional CALB structure (PDB identifier 1LBS; (15)) where the open conformation of α-helix 5 has been modelled (15). Interestingly, in the same study, the authors obtain an alternative crystal form of CALB in the presence of Tween 80 at pH 4.0 that is unusually loosely packed and has a solvent content of over 60% (15). The hydrophobic surface around the active site is fully exposed to the solvent, and the lid segment is found in the open conformation. The absence of the closed conformation in this case cannot be attributed to steric reasons alone as the crystal packing would allow for the lid to adopt the more extended, closed conformation. However, it is tempting to speculate that the predominantly hydrophobic lid segment α-helix 5 upon exposure to polar solvents and/or high pH prefers a more compact, helical conformation, minimizing unfavourable solvent contacts. In our structure, however, the lid segment is better protected against solvent exposure by tighter crystal packing (solvent content 38%).

CALB was crystallized in conditions very similar to the reported by Uppenberg et al. (29). We buffered the precipitation solution to pH 4.8, a value that is just above the pKa of the side chains Asp (pKa = 3.86) and Glu (pKa = 4.07) residues. At pH =4.8 it is reasonable to expect that most
of the carboxylic groups would be in the deprotonated state, subject to the local protein environments.

Figure 3, reporting the bond lengths of C-O<sub>γ1</sub> and C-O<sub>γ2</sub> of the carboxylic group of aspartic residues, and of C-O<sub>δ1</sub> and of C-O<sub>δ2</sub> of the carboxylic group of glutamic residues, clearly show that most Glu and Asp residues are in fact in a deprotonated state, but exceptions in a protonated state are also present. However, the protonation state of the charged residues in the active site of the two monomers (Table 2) indicates that the charge inside the cavity is conserved. Residue Asp187 maintains its deprotonated state, Glu188 is deprotonated in monomer A and protonated in monomer B, while Asp145 is protonated in monomer A and deprotonated in monomer B (Table 2) as it forms a salt bridge.

The bond lengths of Asp187 within the catalytic triad in open and closed conformations are consistent with the bond lengths of Asp102 in trypsin and of Asp32 in subtilisin reported by Fisher et al. (22) and further confirm previous NMR (34) and crystallographic evidence (35) that the key catalytic proton is positioned between the two key catalytic residues His and Asp, rather than being fixed on the histidine, thus supporting the theory that the proton may in fact be involved in a low-barrier hydrogen bond (34).

Overall there is no change of the net charge on the protein in the open and closed conformations (Table 2) following the destructuring of α-helix 5 and the subsequent ion pairing of Asp145 with Lys290 from α-helix 10.

The optimal pH for catalysis is 7, and the enzyme is stable in aqueous media in the range of pH 3.5-9.5 (12), values close to the pKa of the side chains of Asp145 and Lys290 residues discussed before. It was already noted that, in the monoclinic form P<sub>2</sub>1, raising the pH from 3.6 to 5.5 would cause dramatic changes to α-helix 10, whose residues would change from an ordered structure to disordered structure, manifested by a lack of continuous electron density (14). The lid region itself, with its primary (amino acid composition and number of residues) and secondary
structure can modulate the ‘interfacial activation behaviour’ of CALB, but also of lipases in general. Chimeras of *Candida antarctica* lipase B, generated by lid swapping, displayed large variations in kinetic constants and enantiomeric ratio for hydrolysis of p-nitrophenyl esters (18). So while the previous studies pointed towards an activation of lipases based on interfacial oil-water mechanism, in the case of CALB the environmental pH could also play a role. At pH 7 CALB in solution is likely to oscillate between the open and the close conformation, hence the reason for the lack of interfacial activation of CALB must be searched for elsewhere rather than in the lack of a lid region in the structure. It is noteworthy that the removal of the Asp145 from α-helix 5 region in the chimeras of *Candida antarctica* lipase B, removed the activity pH dependence (18), clearly indicating a relationship between optimal activity pH and primary sequence of the lid region. The two variants, where the lid region was cloned from *N. crassa* and from *G. zeae*, on the other hand, yielded surprisingly low specific activities in the assay with no clear pH dependency (18). In these two variants, the Asp145 is missing from α-helix 5 sequence. A BlastP search (36) using CALB sequence as search motif, returns more than 50 results with sequence identity higher than 20% (results not shown). However, among these, only five hits are found, which conserve Asp145 in the α-helix 5 region and Lys290 after the α-helix 10 region (Figure 7). *Candida Antarctica* (LIPB_CANAR), *Pseudozyma antarctica* (M9MDK9_PSEA3) and *Pseudozyma aphidis* (W3VNS6_9BASI) share a sequence identity of more than 99%, while *Ophiocordyceps sinensis* (T5ACW5_OPHSC), *Gibberella moniliformis* (W7N1B5_GIBM7), *Fusarium oxysporum* (W9LLN1_FUSOX) have a sequence identity of about 25% with *Candida Antarctica* (LIPB_CANAR). The fact Asp145 is rarely conserved in the sequence of lipases, would explain why, among lipases, CALB is the only lipase reported so far, which does not show the ‘interfacial activation’ behaviour typical of other lipases, but rather shows a ‘pH activation’ behaviour as shown by Skjot et al. (18). As our structure has shown a salt bridge is formed between Asp145 and Lys290 upon lid closure. In absence of Asp145, such a salt bridge cannot
form, thus a pH dependent behaviour is not possible. Because of the presence of Asp145 in the α-helix 5 region, *Candida Antarctica* lipase B does represent a class of its own among lipases on the basis of the sequence homology and its characteristic behaviour as lipase.

Several molecular dynamics simulation studies (17-20) have shown that α-helix 5 is a highly dynamic region and works as a lipase lid depending on the working temperature (19) or the working organic solvents (17). Derewenda et al. (37) suggested that the interfacial activation has both enzyme and substrate components, and that conformational changes in the enzyme are essential but not sufficient for its maximum activity. Schrag et al. (38) proposed that the open or closed conformation of lipases was determined by the solution conditions, and perhaps the dielectric constants, on the basis of the crystallization conditions of various microbial lipases and their observed conformations. Viceversa, in non-aqueous media the lack of interfacial activation suggested that the lid was predominantly closed, thus resulting in a reduced enzymatic activity (39).

Here we have shown that the basis of the ‘interfacial activation’ for lipases should therefore not be related to the presence or the absence of a lid region in an enzyme structure, which went so far unobserved in the case of CALB for instance, but rather related to both the amino acid composition of the lid regions and how and why these regions react to changes in the dielectric value or ionic strength of the media. The ‘interfacial activation’ phenomenon is then the convolution of the properties of the substrate, the media and the enzyme with its fold and with its amino acid sequence.

**Accession Numbers:** The refined coordinate and structure factor files for the atomic resolution structure and the xenon derivative have been deposited in the Protein Data Bank with accession number 5A71 and 5A6V, respectively.

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References


Figure 1. Graphical representation of Candida antarctica Lipase B (CALB). a) asymmetric unit; b) general secondary structure topology with the position of residues showing multiple conformations highlighted; c) non-bonded contacts between residues, the width of the striped line is proportional to the number of atomic contacts. Figures were generated using PDBsum web server (40).

Figure 2. Structural evidence of open and closed conformation in CALB. a) The rms deviation values of Cα atoms for monomer A and B computed against the currently deposited CALB structure (PDB code 1TCA(14)), versus residue number; b) PROCHECK (30) analysis of the secondary structure region surrounding α-helix 5, showing the unfolding of residues 140-147; c) ribbon representation of the residue range 140-147 (momomer A, folded in light blue colour; momomer B, defolded in gold colour) superimposed to the Fo-Fc difference Fourier OMIT map (blue mesh; contour level 3.0σ, calculated without the residues) at 0.91 Å resolution; d) Steric clash between extended, closed lid segment/α-helix and neighbouring monomers in other crystal forms of CALB. The structure of CALB assuming the closed lid conformation described in this study (magenta, shown as backbone trace/ribbon) was superimposed to the structure 1TCA (blue ribbon and surface) present in an open conformation. Residue Pro143 (magenta) in the closed conformation would sterically clash with residue Leu199 of a neighbouring CALB monomer (cyan) within the same crystallographic unit cell. The superimposition was carried out and images were generated using PyMol (41).

Figure 3. Bond length and bond angles analysis. a) histidine (bond angles); b) glutamic acid (bond lengths); c) aspartic acid residues (bond lengths). Values are indicated with standard deviations.

Figure 4. Open and closed conformations of CALB. The two monomer A (open conformation) and B (close conformation) that constitute the unit cell are depicted in light blue and gold,
respectively. Amino acid residues are depicted as ball and stick with carbon = green; oxygen = red; blue = nitrogen: a) stereoview of the Asp145 region in the open conformation with 2Fo-Fc electron density map shown contoured at 1.5σ level; b) the amino acid residues Asp145 and Lys290 in monomer A (light blue), open conformation, superimposed to the Fo-Fc difference Fourier OMIT map (blue mesh; contour level 3.0σ, calculated without the residues); c) in monomer B (gold), close conformation; d,e) space fill representation of CALB surface in open conformation (d) and closed conformation (e) with the Leu140-Ile147 region highlighted. Hydrophobic region are depicted in blue, hydrophobic region are depicted in red; f) xenon sites (represented as spheres in grey) superimposed to the anomalous difference Fourier map (green mesh; contour level 4.0σ, calculated phases without the residues) present into the active site in open conformation; g) absent in the cavity in closed conformation. Images were generated using CCP4MG (42).

Figure 5 Active site of CALB. 2Fo-Fc electron density map shown contoured at 1.5σ level: a) open conformation; b) closed conformation. Bond lengths in red and hydrogen bond distances in black reported in Å.

Figure 6 diagrammatic summary of the overall mechanism of CALB catalysis. A triacylglycerol molecule (TAG) is hydrolyzed to diacylglycerol (DAG) and monoacylglycerol (MAG) when CALB is present in open state.

Figure 7 Alignment of CALB homologues with conserved Asp145 and Lys290 residues. UniProt gene codes, organism and identity (%) are reported from the left, respectively. Yellow boxes mark the position of Asp145 and Lys290 residues. Amino acid coloring scheme is according to the amino acid family.
Table 1  Data collection, processing and refinement statistics for the native *Candida antarctica* and xenon complex.

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<td>Resolution range (Å)†</td>
<td>71.04 – 0.91 (0.93 - 0.91)</td>
<td>70.96 – 2.27 (2.36 - 2.27)</td>
</tr>
<tr>
<td>Total number of reflections†</td>
<td>5925539 (74630)</td>
<td>133177 (7287)</td>
</tr>
<tr>
<td>Unique reflections†</td>
<td>343684 (20871)</td>
<td>21330 (1559)</td>
</tr>
<tr>
<td>Multiplicity†</td>
<td>17.2 (3.5)</td>
<td>6.2 (4.6)</td>
</tr>
<tr>
<td>Completeness† (%)</td>
<td>94.5 (77.4)</td>
<td>92.7 (70.5)</td>
</tr>
<tr>
<td>R_{sym}† (%</td>
<td>10.9 (56.7)</td>
<td>5.7 (34.0)</td>
</tr>
<tr>
<td>Mean (I) half-set correlation CC(1/2)</td>
<td>99.9 (80.2)</td>
<td>99.9 (95.0)</td>
</tr>
<tr>
<td>Mean I/σ(I)†</td>
<td>13.65 (1.59)</td>
<td>29.5 (7.2)</td>
</tr>
</tbody>
</table>

**Refinement statistics**

<p>| Number of monomers in the asymmetric unit | 2 | 2 |
| R_{factor}† (%)                          | 11.1 | 13.9 |
| R_{free}† (%)                            | 13.2 | 19.4 |</p>
<table>
<thead>
<tr>
<th></th>
<th>0.01</th>
<th>0.23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cruickshanks DPI for coordinate error(^1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>based on R(_{\text{factor}}) (Å)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wilson plot B-factor</td>
<td>8.69</td>
<td>13.78</td>
</tr>
<tr>
<td>Average all atom B-factor(^3)</td>
<td>13.0</td>
<td>14.4</td>
</tr>
<tr>
<td>RMS(bonds)</td>
<td>0.02</td>
<td>0.007</td>
</tr>
<tr>
<td>RMS(angles)</td>
<td>2.23</td>
<td>1.12</td>
</tr>
<tr>
<td>Total number of non-hydrogen atoms(^3)</td>
<td>5955</td>
<td>5193</td>
</tr>
<tr>
<td>Total number of water molecules</td>
<td>1019</td>
<td>484</td>
</tr>
<tr>
<td>Solvent content (%)</td>
<td>38.0</td>
<td></td>
</tr>
<tr>
<td>Matthews Coefficient (Å(^3)/Da)</td>
<td>1.98</td>
<td></td>
</tr>
</tbody>
</table>

**Ramachandran plot\(^6\)**

<table>
<thead>
<tr>
<th>Region</th>
<th>90.2</th>
<th>90.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most favored region (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additionally allowed region (%)</td>
<td>9.1</td>
<td>9.1</td>
</tr>
<tr>
<td>Generously allowed region (%)</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Disallowed region (%)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^1\)highest resolution bin in parenthesis

\(^2\)R\(_{\text{sym}}\) = \(\frac{\sum_{hkl} \sum_{j} | I_j - <I> |}{\sum_{hkl} \sum_{j} I_j}\) where \(I\) is the intensity of a reflection, and \(<I>\) is the mean intensity of all symmetry related reflections \(j\).

\(^3\)taken from REFMAC5 (25) (R\(_{\text{free}}\) is calculated using 5% of the total reflections that were randomly selected and excluded from refinement)

\(^4\)DPI = \(\sqrt{\frac{N_{\text{atoms}}}{(N_{\text{refl}} - N_{\text{params}})} \cdot R_{\text{factor}} \cdot D_{\text{max}}^{0.16}}\), where \(N_{\text{atoms}}\) is the number of the atoms included in the refinement, \(N_{\text{refl}}\) is the number of the reflections included in the refinement, \(D_{\text{max}}\)
is the maximum resolution of reflections included in the refinement, compl is the completeness of the observed data, and for isotropic refinement, \( N_{\text{params}} \approx 4N_{\text{atoms}} \) (31).

\(^5\)taken from BVERAGE (28)

\(^6\)taken from PROCHECK (28)
Table 2  Protonation states of the charged residues in the active site of the two monomers.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Conformation</th>
<th>Protonation State</th>
<th>Charge</th>
<th>Protonation State</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Open</td>
<td>protonated</td>
<td>0</td>
<td>protonated</td>
<td>0</td>
</tr>
<tr>
<td>Asp134</td>
<td></td>
<td>protonated</td>
<td>0</td>
<td>protonated</td>
<td>0</td>
</tr>
<tr>
<td>Asp145</td>
<td></td>
<td>protonated</td>
<td>0</td>
<td>deprotonated*</td>
<td>-1</td>
</tr>
<tr>
<td>Asp187</td>
<td></td>
<td>deprotonated</td>
<td>-1</td>
<td>deprotonated</td>
<td>-1</td>
</tr>
<tr>
<td>Glu188</td>
<td></td>
<td>deprotonated</td>
<td>-1</td>
<td>protonated</td>
<td>0</td>
</tr>
<tr>
<td>His224</td>
<td></td>
<td>single protonated</td>
<td>0</td>
<td>single protonated</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Closed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall Charge</td>
<td></td>
<td>-2</td>
<td></td>
<td>Overall Charge</td>
<td>-2</td>
</tr>
</tbody>
</table>

* in ionic pair with the side chain of Lys290
Figure 1
Figure 2
Figure 3

(a)

(b)

(c)
Figure 4
Figure 5
Figure 6
Figure 7

1. LIP8_CANAR(C. antarctica) 100.0%
2. WYGRK_PSEAD(P. antarctica) 99.4%
3. WYGRK_PSEAD(P. aphidis) 99.4%
4. WYGRK_PSEAD(P. sinensis) 99.4%
5. WYGRK_PSEAD(P. oxyporum) 99.4%
6. WYGRK_PSEAD(P. oxyporum) consensus/100%

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