Title
The grease trap: Uncovering the mechanism of the hydrophobic lid in Cutibacterium acnes lipase

Authors
Hyo Jung Kim1,2, Bong-Jin Lee2 and Ae-Ran Kwon3

1College of Pharmacy, Woosuk University, Wanju 55338, Republic of Korea
2Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Gwanak-gu, Seoul 151-742, Korea
3Department of Beauty Care, College of Medicine and Science, Deagu Haany University, Gyeongsan 38610, Republic of Korea

Corresponding author:
arkwon@dhu.ac.kr Phone +82-53-819-1585

Running title
Structural and functional study of C. acnes lipase

Abbreviations
BMK Benzyl Methyl Ketone
c4l lipase C.acnes lipase
LPC Lysophosphatidylcholine
Abstract

Acne is one of the most common dermatological conditions, but the details of its pathology are unclear, and current management regimens often have adverse effects. *Cutibacterium acnes* is known as a major acne-associated bacterium that derives energy from lipase-mediated sebum lipid degradation. *C. acnes* is commensal, but lipase activity has been observed to differ among *C. acnes* types. For example, higher populations of the type IA strains are present in acne lesions with higher lipase activity. In the present study, we examined a conserved lipase in type IB and II, but truncated in type IA *C. acnes* strains. Closed, blocked, and open structures of *C. acnes* ATCC11828 lipases were elucidated by X-ray crystallography at 1.6-2.4 Å. The closed crystal structure, which is the most common form in aqueous solution, revealed that hydrophobic lid domain shields the active site. By comparing closed, blocked, and open structures, we found that the lid domain-opening mechanisms of *C. acnes* lipases involve the lid-opening residues, Phe-179 and Phe-211. To the best of our knowledge, this is the first structure-function study of *C. acnes* lipases, which may help shed light on the mechanisms involved in acne development and may aid in future drug design.

Key Words

**Listed** phospholipases, lysophospholipid, lipase, skin lipid metabolism, protein structure

**Not-listed** *C. acnes*, acne, lysophosphatidylcholine, lysophospholipase, X-ray crystallography
Introduction

Acne is a common skin condition widespread across all cultures and ages. The prevalence rates are up to 85% in adolescents and 40% in adults (1-3). Acne leaves scarring, symptomatic pain, emotional and psychological disturbances. While neither life threatening nor physically debilitating, acne can cause social and psychological discomfort. Even mild facial acne is more commonly associated with significant depression compared to other skin conditions such as alopecia or atopic dermatitis (4). Current studies focus on the *Cutibacterium acnes* colonization of sebaceous follicles as a major cause of acne (5). The pathogenicity is thought to be due to substances generated by *C. acnes*, such as free fatty acids, extracellular enzymes, or virulence factors (6, 7). However, it is one of the most common bacteria on human skin that metabolizes sebum and releases fatty acids by secreting lipases in healthy skin. In this process, skin acidity is maintained and this functions as a natural barrier from harmful pathogens, providing innate skin immunity (8). Recently, studies have shown that the proliferation of *C. acnes* is not the trigger of acne but disturbances to a tight equilibrium between phylotypes might perform a key role (5, 9).

To date three phylogenetic groups of *C. acnes* have been described, type I, II and III according to genome sequences or biological characteristics (lipase activity). Type I is further divided into IA1, IA2, IB, and IC (10-12). Recent evidence suggests type IA is strongly associated with acne while IB, II, and III are less involved (11-13). Type IA was predominant in severe, moderate, and mild acne but type II was preferentially present in healthy skin (14, 15). The notable characteristics of type IA strains are increased lipase activity and secretion. Contrastingly, the distinctive features of type II strains are decreased secretion of virulence factors including lipase (16, 17). To understand different roles depending on *C. acnes* types, genetic studies based on sequence data are being conducted (18, 19).

Since lipolytic activity is an essential metabolic pathway for *C. acnes*, we focused on lipase from the type II *C. acnes* ATCC11828 (CALipase hereafter). This gene is absolutely conserved across type II strains, while shows 85-95% sequence homology with type IB, IC, or III strains. The known pathogenic type IA strains possess the corresponding gene but 13 nucleotides deletion was detected. The deletion causes
premature stop codon, resulting in truncated fragments of \( C_{a} \)lipase in type IA strains. Consequently, the catalytic triad is dissociated into two different fragments. This implies \( C_{a} \)lipase is originally conserved across all types \( C. \ acnes \) but selectively mutated in genetic levels during evolution into new strains. In this regard, we could expect that conserved \( C_{a} \)lipase performs well-controlled lipolysis in healthy skin but the mutation causes deficiency of controlled lipase in acne lesion. By revealing how the conserved \( C_{a} \)lipase regulates their activities, we could surmise different behavior of type I \( C. \ acnes \) as an opportunistic pathogen. In the present study, the crystal structures of closed, blocked, and open states of \( C_{a} \)lipase were identified. We elucidated the protein shields and reveals active site with bulky side chains. Our study will promote a better understanding the \( C. \ acnes \) lipase mechanism on a molecular basis.
Materials and Methods

Cloning, expression, and purification

The predicted ORF of C. acnes ATCC 11828 genomic DNA using by PCR. The Ndel and Xhol restriction sites were used for cloning into the pET-21a(+) vector (Novagen, U.S.A.). The resulting construct has 8 additional residues (LEHHHHHHH) that encode a C-terminal hexa-histidine tag. The sequences of the cloned genes were confirmed by DNA sequencing (results not shown). To prepare mutants, QuickChange Site-Directed Mutagenesis kit (Agilent, U.S.A.) was used to generate point mutations in the recombinant pET-21a(+) plasmid. The point mutations resulted in multiple recombinant plasmids, specifically F176F179W192F211A (active site shielding residues mutant), F179W192A (lipophilic path forming residues mutant), and E5D54D202K205D206A (dimer mutant).

Each recombinant plasmid was transformed into Escherichia coli BL21 (DE3). Cells were grown at 37°C in LB medium supplemented with ampicillin (50 μg/ml). For the selenoproteins, cells were grown in minimal medium supplemented with selenomethionine. Recombinant protein expression was induced by the addition of IPTG to 0.5 mM when the OD600 reached to 0.5. After an additional 4 h of growth, cells were harvested by centrifugation at 4,500 g at 4°C. Cell pellet was resuspended in 50 mM Tris (pH 7.5), 500 mM NaCl, and 20 mM imidazole buffer and disrupted using an Ultrasonic processor (Cole-Parmer, U.S.A.). The cell lysate was centrifuged at 20,000 g for 1 h at 4°C. The cleared supernatant was purified by binding to a Ni-NTA (Ni2+-nitritotriacetate) affinity column (Qiagen, Germany; 3ml of resin per litre of cell culture) previously equilibrated with the same buffer. The Ni2+ bound protein was eluted with elution buffer (50 mM Tris (pH 7.5), 500 mM NaCl, and 200 mM imidazole) until there was no detectable absorbance at 280 nm. Further purification and buffer exchange were achieved by size-exclusion chromatography using a Superdex 75 (10/300 GL) column (GE Healthcare Life Sciences, U.S.A.) that was previously equilibrated with 50 mM Tris (pH 7.5), and 150 mM NaCl. The purity of each recombinant protein was estimated to be over 95% by SDS-PAGE. The purified protein was concentrated to 10 mg/ml by ultrafiltration in 10,000 Da molecular-mass cut-off spin columns (Millipore, U.S.A.).
Crystallisation, data collection, and structure determination

Crystallisation was performed at 293 K using 24-well VDX plates (Hampton Research, U.S.A.). Initial crystallisation conditions were established using screening kits from Hampton Research (Crystal Screen I and II, Index, PEG/Ion, and MembFac) and from Emerald Biosystems (Wizard I, II, III, and IV). For the optimal growth of \( \text{Calipase} \) crystals, each hanging drop was prepared on a siliconised cover slip by mixing 1 μl of precipitant solution (30% (w/v) PEG 8K and 0.1 M Tris (pH 8.0)) and 1μl of protein solution (10 mg/ml). This drop was equilibrated against 1 ml reservoir of precipitant solution. This condition yielded rod-shaped crystals in three days. Selenoprotein was crystallised in the same procedure. The co-crystal with LPC was prepared by addition of 20 mM LPC to the drop. The best crystals were grown from 2M (NH₄)₂SO₄, 0.2M MgCl₂, and 0.1 M Bis-Tris (pH 6.2) as a diamond shape.

\( \text{Calipase} \) crystals were transferred to a cryoprotectant solution with 30% (v/v) glycerol in the crystallisation condition for several minutes before being flash-frozen in a stream of nitrogen gas at 100 K. For the comparison, the same crystal was transferred to a different cryoprotectant solution, Al’s oil (Hampton Research, U.S.A.). \( \text{Calipase} \) crystals and \( \text{Calipase}:\text{LPC} \) co-crystals were transferred to an Al’s oil before freezing for several minutes. Diffraction data were collected on beamlines 5C and 7A at the Pohang Light Source, Korea. The raw data were processed and scaled using the HKL2000 program suite (20). Further data analysis was carried out using CCP4 suite (21). The \( \text{Calipase} \) crystals belong to space group P2₁2₁2 and contained two molecules per asymmetric unit. The \( \text{Calipase}:\text{LPC} \) co-crystals were determined as P3₁ with 26 molecules per asymmetric unit.

To determine the \( \text{Calipase} \) structure, MAD dataset was used for solving the structure using the Autosol and Autobuild Wizards in the Phenix package (22). The selenium sites were identified and the initial phases calculated from these sites were further improved by density modification. The structures for Al’s oil soaked and \( \text{Calipase}:\text{LPC} \) co-crystal were determined using molecular replacement with the program MolRep within CCP4 suite (23). Iterative cycles of model building were performed using COOT, followed by refinement with Refmac5 (24). A portion of data (5%) was set aside for the refinement calculations of \( R_{\text{free}} \). Data collection and final crystallographic statistics are summarized in Table 1.
SDS-PAGE was conducted according to the Laemmli method using a 12% (w/v) polyacrylamide gel (25). The samples were treated with 1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol at 100˚C for 5 min before electrophoresis in a vertical Mini Gel system (Bio-Rad Laboratories, U.S.A.). The proteins were stained with Coomassie Brilliant Blue R250 (Thermo Scientific, U.S.A.). Additionally, for the separation of CALipase depending on the solvent condition, native PAGE was performed and analysis was conducted using a 10% (w/v) polyacrylamide gel without either SDS or 2-mercaptoethanol. Native PAGE was performed in a buffer (25 mM Tris (pH 8.3) and 192 mM glycine). The staining was performed as described above for SDS-PAGE.

Lipase/phospholipase activity assay
The colorimetric assay was performed to detect hydrolysis of thioester bond (26, 27). To monitor the lipase activity, 3 mM of 2,3-dimercapto-1-propanol tributyrate (DMPTB) was used (Sigma, U.S.A.). For lysophospholipase activity, the same amount of palmitoyl thio-phosphocholine (Cayman, U.S.A.) was used. The released free thiols are subsequently reacted with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma, U.S.A.). 10 μg of CALipase, F176F179W192F211A (active site shielding residues mutant), or F179W192A (lipophilic path forming residues mutant) was incubated for 30 min at 37˚C with 10 μl of DTNB in a buffer containing 25 mM Tris (pH 7.5), 10 mM CaCl2, and 100 mM KCl. The free thiol groups reacted with DTNB to produce a yellow precipitate and absorbance was measured at 412 nm using TECAN Microplate Reader Spark (TECAN, Switzerland).

Isothermal Titration Calorimetry (ITC)
ITC was performed using a MicroCal PEAQ ITC (Malvern Instruments, UK). 300 μl of 40 μM CALipase in 50 mM Tris (pH 7.5) and 150 mM NaCl was injected into the sample cell at 25˚C and buffer only into the control cell. Repeated 0.4-2 μl injections of 400 μM LPC in the same buffer were applied to both cells every 150 s with 500 rpm mixing. Thermograms were obtained and fitted via nonlinear least squares
minimization method to determine the $K_D$ and change in enthalpy of binding ($\Delta H$). The Gibbs free energy of binding, $\Delta G$, was calculated from Ka values and entropic term, $T\Delta S$, was derived from the Gibbs-Helmholtz equation using the recorded $\Delta H$ values. The data presented are the mean ±SEM of four separate experiments.

**Data Availability Statement**

Protein coordinates and structure factors have been deposited in the RCSB PDB under code 6KHK for closed, 6KHL for blocked, and 6KHM for open state structures.
Results

Conserved lipases in different types of C. acnes

C. acnes releases lipases to degrade sebum lipids, providing energy to the bacteria and skin with hydration. While the lipase serves in the nutrition of C. acnes and maintenance of a healthy skin, excessive products (fatty acids) are known as major factors of inflammation. In inflammatory lesions, the quantities of C. acnes were similar as in healthy skin, but preferential proliferation of type IA C. acnes is detected while type II C. acnes is major in healthy skin. Although there are only limited genetic studies to reveal the phenotypic and functional differences, virulent and lipases genes showed apparent differences between type IA and II C. acnes. Indeed, the degree of lipase activity was elevated in type IA C. acnes (16, 28, 29). Each strain produces ~15 lipases, and many of them are well conserved within types. However, these lipases are not conserved across types. The conserved lipases in type IA are not observed in type IB, IC, or II and type II conserved lipases are not observed in type IA. The examples of conserved lipases in type IA are shown in Supplemental Table S1.

Bioinformatics showed that two of conserved hydrolases from type IA C. acnes are relatively short in size. Most lipases consist of an enzymatically active core domain controlled by a lid domain. Therefore, lipases are typically >30 kDa in size which makes these short hydrolases unique. As genes for two short hydrolases are adjacently positioned in type IA, nucleotides in the region are together aligned with type IB, IC and type II corresponding lipase genes. The alignment shows 97%, 96%, and 83% homologies with type IB, IC, and II, respectively. Notably, the 13 nucleotides deletion in type IA genes is observed and this causes a frameshift, resulting in a premature stop codon (TGA). This mutation yields two short hydrolases, sequence ID: WP_002515192.1 and sequence ID: WP_002519208.1. To gain further insight into truncated in type IA but intact lipase in other types (IB, IC and II), we investigated structural and functional study of type II C. acnes lipase (WP_002514103.1, named as CALipase) (Supplemental Figure S1A).

C. acnes lipase dimer shows core and lid domain
The crystal structure of Calipase was determined to a 1.75 Å resolution. The asymmetric unit contains two subunits and this dimeric state was supported by size-exclusion chromatography (Figure 1A). A single sharp peak eluted with an apparent molecular weight of 66 kDa, suggesting the formation of dimer. Six salt bridges (Ala2-Asp202, Arg69-Glu5, Glu5-Arg43, Glu23-Lys205, His47-Asp54, and Asp54-Arg294) and a hydrogen bond at Leu3-Asp206 form a stable dimerization interface. This dimerization mode is unique and these residues show poor conservation across similar structures. Most structurally known lipases are monomers and function as such. Moreover, when they form crystallographic dimers, these interfaces are highly different from the Calipase dimer (Figure 1B and 2) (30-33). The dimeric state of Calipase contributes solubility and stability. The dimerization interface mutant, E5D54D202K206D206A was expressed in inclusion bodies, although wild type is highly soluble (Supplemental Figure S2).

Each monomer consists of two domains: a core domain that possesses a catalytic triad and a lid domain (residues 144-231). The core domain shares a classic α/β hydrolase fold: (1) mostly parallel eight-stranded β-sheet surrounded on both sides by α-helices. (2) A catalytic triad serine (Ser114) is coordinated after β5-strand, aspartate (Asp252) after β7-strand, and histidine (His285) after β8-strand (Figure 1C) (34). The Calipase shows typical characteristics in the core domain but the distinctive features are in the lid domain. In our structure, four α-helices between β6-and β7-strand form the lid domain and widely cover one side of the core domain. The lid domain shields the active site by bulky hydrophobic residues, preventing approaches from other substances. Especially, as shown in Figure 1B, Phe176, Phe179, Trp192, and Phe211 coordinated around the active site pocket with side chains being headed toward pocket. The two short hydrolases in type IA C. acnes, WP_002515192.1 and WP_002519208.1, lack β5-strand including active serine, therefore hydrolysis is not expected. When the structures are modeled based on Calipase crystal structure using SWISS-MODEL workspace, both are considered unstable (35) (Supplemental Figure S1B). The hydrophobic residues are exposed to solvent areas. While lid and core domains are tightly interacting in Calipase, protein WP_002519208.1 loops are exposed freely to solvent areas. Protein WP_002515192.1 shows two folded domains separated by a single loop, which is physically unreasonable.
Comparison of *C. acnes* lipase with related proteins

Using the DALI server, the two strongest matches (Z-scores over 30), *Bacillus coagulans* carboxyesterase (PDB ID 5O7G, 1.5 r.m.s.d., 39% sequence identity) and Human monoglyceride lipase (PDB ID 3HJU, 2.2 r.m.s.d., 22% sequence identity) were found (30, 33) (Figure 2). When these structures are aligned, clear differences are apparent in the lid domain. In the crystal structure of Human monoglyceride lipase, one α-helix that corresponds α6-helix of *C. a.* lipase is absent and this causes different arrangement in the loop between α6 and α7-helix. Comparing *C. a.* lipase with the highest Z-scored *B. coagulans* carboxyesterase, although both structures show four α-helices in the lid domain, longer loop between α6 and α7-helix and two 3_10 helical turns are distinct to *C. a.* lipase. Notably, loop between α7 and α8-helix shows major differences while α7-helix coordination is well conserved (Figure 3A). Another notable feature can be found in surface structure. Although the lid domains cover active sites in *B. coagulans* carboxyesterase and Human monoglyceride lipase, the nucleophile serine residue is solvent accessible. Contrastingly, *C. a.* lipase lid domain completely shields the catalytic triad (Figure 3B). The structural differences in the lid domain are important because lipolysis activity is controlled by rearrangement of lid domain structure generally. However, the open-close mechanisms are not predictable due to the poor structural conservation (36). Judging from the crystal structure of *C. a.* lipase, the lid domain should be further open and expose the hidden active site for the lipolysis activity.

Lipase and lysophospholipase activity

The lipolysis activity was tested using two different sebum lipids, triglyceride and lysophosphatidyl choline. Triglyceride and lysophosphatidyl choline are natural modulators that emulsify and moisturize skin or hair to prevent dryness. However, free fatty acids digested by *C. acnes* lipases from triglyceride and lysophosphatidyl choline induce marked inflammation (37). In this regard, understanding the regulatory mechanisms underlying activation of *C. acnes* lipases will provide new strategies for relieving inflammation in acne patients. To understand hydrolysis activities depending on structural characteristics, wild type, active site shielding residues mutant (F176F179W192F211A), and lipophilic path forming residues mutant (F179W192A) were used. Both substrates were degraded actively by protein, showing
higher affinity to triglyceride. Since triglyceride occupies the predominant proportion of sebum, the substrate selectivity toward triglyceride is convincing (38). Interestingly, the active site shielding residues mutant showed higher activity than wild type or lipophilic path forming residues mutant (F179W192A). This implies CAlipase activity is closely related with bulky side chain residues, especially Phe176 and Phe211, which shield the active site in the wild type (Figure 4).

**Interaction study with lysophosphatidyl choline by ITC**

Lipase and lysophospholipase activities were identified, but our crystal structure showed a completely closed conformation. Lipolysis in lipases requires the catalytic triad to be accessible by solvents but most of lipases possess lid domain adjacent to active site. Accordingly, some lid opening mechanisms of lipases are known: temperature-dependent, oil-water interfacial, and polarity-dependent activation (36). These activation mechanisms are markedly different between species, lid conformations, and even specific residues (39). To open the lid domain of CAlipase, we changed buffer conditions by adding surfactant or oil in the presence of LPC and checked changes of charge states (Figure 5A). The final concentration of 0.1% Triton X-100 was used as surfactant or Al’s oil (combination of paraffin and silicon oil) was suspended to buffers. A single intense peak in size-exclusion chromatography indicates homogenous size in aqueous solution, but native PAGE result shows various surface charges. With the addition of 0.1% Triton X-100, bands are reduced as a single band. When excess oil was mixed to CAlipase protein, multiple bands are condensed. Native PAGE bands are further reduced by the concurrent addition of oil and LPC.

To characterize the interaction between LPC and CAlipase, ITC experiments were performed. Under Tris buffer, the result clearly confirmed interaction and binding of LPC and CAlipase. ITC data also indicates a stoichiometry of 0.07, implying most of CAlipase remain in the closed state as determined from crystal structure (Figure 5B). However, ITC data did not indicate any binding under 0.1% Triton X-100 (data not shown). For the Al’s oil, ITC experiments were not able to be conducted due to the buffer phase separation. From this experiment, the closed form of CAlipase is expected as the major form in aqueous
solution and it is shown that changing the characteristics of the reaction buffer will cause structural differences in cAlipase.

**Lid opening mechanism for substrate entry**

Since Al’s oil and LPC addition showed condensed bands in native PAGE, we tried co-crystallisation of cAlipase with LPC. The co-crystals were soaked in Al’s oil before freezing. cAlipase:LPC complexes have been found with different space group (P31) from wild type crystal structure (P21212). cAlipase:LPC has 13 dimers (26 monomers) in asymmetric unit. For the comparison, crystals prepared in the absence of LPC were also soaked by Al’s oil. Two different structures (blocked and open) were identified in addition to the previous closed conformation. In the absence of LPC, benzyl methyl ketone (BMK hereafter), one of the component of Al’ oil, was covalently bound to Ser114 (blocked structure).

Structural differences of the closed, blocked, and open states of cAlipase are found in the lid domain (Figure 6A). While α7-helix (which is responsible for dimerization) is fixed, 3 other helices are shifted slightly towards the active site pocket. The notable movement is in the loop between α7 and α8-helix that includes Phe211, which is located right above the active serine. In the closed state structure, Phe211 occludes the active site pocket with Phe176. The distance between C\textsubscript{\varepsilon} atoms of both phenylalanine is 5.2 Å, not allowing ligands to pass through. In the blocked structure with BMK binding, this obstruction was relieved by a changed coordination of Phe176. Furthermore, after introduction of LPC in the open structure, Phe211 moves with the loops and the distance between C\textsubscript{\varepsilon} atoms of Phe211 and Phe176 becomes 11.6 Å, providing enough space for the ligand LPC. After the carbonyl carbon of LPC is attacked, products, fatty acids, remain bound to serine. Many hydrophobic residues, Leu38, Val140, Phe146, Phe176, Phe179, Trp192, Phe211, and Leu223, form a stable hydrophobic path for fatty acids (Figure 6B). These hydrophobic residues including key lid opening residues (Phe176 and Phe211) are not conserved across similar structures, indicating that this lid opening mechanism is unique in cAlipase (Figure 2).

Although crystallography only provides a proteins’ static structure, crystallographic temperature factors, known as B-factors, constitute a measurement of structure’s flexibility. Moreover, open structure of
CAlipase:LPC complex has 26 chains in an asymmetric unit, showing slightly different structural state. Relatively large mean B-factors are monitored around lid domain (residue 144-231). Considering B-factors, closed structure is rigid showing constant values under 40 throughout the whole region and we could check the flexibility of lid domain region after the introduction of substrate (Figure 6C). From three different crystal structures, we identified CAlipase moves its lid domain and two phenylalanine residues are responsible for the exposure of core domain active site to the solvent area.

The modified serine residue after hydrolysis

The structure of BMK is similar to PMSF, well-known serine protease inhibitor which resulting in sulfonate ester after binding to active serine residue (Supplemental Figure S3). There are lipase crystal structures with PMSF-bound to active serine (PDB ID 5MII, 3RLI, and 3H17), but PMSF molecule was not added in any step of purification or crystallization in our experiment (40-42). In the blocked crystal structure, Ser114 bound BMK forms stable tetrahedral intermediate coordination. When His285 acquires positive charge, Asp252 is stabilized. The BMK carbonyl oxygen forms hydrogen bonds with the main chain amides of Leu38 and Trp115 (oxyanion hole). The remaining moieties interact dominantly with hydrophobic residues: methyl group with Val140 and benzyl group with Phe176, Phe179, Trp192, and Phe211 (Figure 7A).

In the CAlipase:LPC crystal structure (open structure), the flexibility of 16 carbon chains led to poor electron density and they could not be resolved. However, 2Fo-Fc map indicates covalently bound ester moiety to Ser114 and about 5 carbon chains are resolved in each domain. The carbon chains are extended through hydrophobic path of the lid domain. Compared to blocked structure, although hydrogen bonds between one oxygen atom from LPC is maintained, missing hydrophobic interactions with Val140, Phe179, and Phe211 are detected. Since LPC does not have methyl group and sterically bulky benzyl group, Val140 and Phe179 lost their hydrophobic interaction. Phe211 is relocated by ~5 Å away from active site in open structure (Figure 7B).
Discussion

Acne is the most common skin problem with a prevalence rate of nearly 100%. Most people are affected at some point during their lifetime. Although acne is not a fatal threat, severe acne can lead to disfigurement and permanent scarring. The psychosocial aspects of acne are of great importance for the patients in dealing with interpersonal relationships or study/work (43). Production of excessive oily substances, known as sebum can be caused by a number of factors, including hormones, foods, stresses, and hereditary conditions. When the excess sebum forms a plug in the follicle, *C. acnes* in the follicle digests sebum, creating inflammation and lead to papules, pustules, nodules or cysts. *C. acnes* is normally harmless bacteria but recent studies have revealed that the proportion of *C. acnes* strains, not the population of bacteria, changes between healthy and acne patients (5, 9). Type IA strains are common in patients with inflammatory lesions while type IB/IC and II are not. This is due to the different lipases expressed from diverse typed strains (11-13). In the present study, we focused a highly conserved lipase across type IA, IB, IC and II but truncated only in virulence type IA *C. acnes*.

At present, the available remedies, such as benzoyl peroxide, retinoid and antibiotics focus on reducing symptom. However, there is no direct drug treatment for acne. Because of limited studies about the pathology, the aim for the therapy is relieving symptom or killing bacteria using antibiotics. To facilitate intelligent drug design, high resolution structures provide both starting point for structure-based drug design, and enabling comparisons to find successful approaches in similar species. However, in the case of lipases, the activity is hard to predict because of their poor conservation in lid domains. The crystal structures of closed, blocked and open *C. aliphases*, which are the first structures of *C. acnes* lipase, revealed the novel lid opening mechanism (Figure 8 and Supplemental Movie 1). In healthy skin, lipase controls its activity depending on environmental lipid level. When the lipase is exposed to a certain lipid level, the loop between α7 and α8-helix moves by ~2.5 Å, providing enough space for Phe211 side chain flipping. Additionally, Phe176 changes its position to open up the blockage and make the active site ligand-accessible. The hydrophobic residues such as Phe179 and Trp192 form lipophilic path for carbon chains of substrate and amides from Leu38 and Trp115 constitute oxyanion hole for the catalytic reaction. This
pattern is unique compared to previously known lid-opening mechanisms. *Candida antarctica* lipase B (CALB) structure opens up by releasing salt bridges between aspartate and lysine residues. A whole α-helix movement make a path for the fatty acids in *Saccharomyces cerevisiae* monoglyceride lipase Yju3 (PDB ID 4ZXF) (31, 44). To date, genome sequencings for various types of *C. acnes* strains are being studied and the biological behavior of type IA are now being revealed. From our study, we could gain insight into the property of conserved *C. acnes* lipase. Further researches on various *C. acnes* lipases, exposed to different environments, will aid novel inhibitor development which has been neglected for over a decade.

**Acknowledgements**

We are grateful to the staff of Pohang Light Source (beamlines 5C and 7A) in Korea for their help with the X-ray experiments. We also thank to Dr. Weng Chan from University of Nottingham and Dr. Sarah Kuehne from University of Birmingham for informative discussions. This work was supported through the National Research Foundation of Korea (NRF) funded by the Ministry of Education of the Korean government [grant number 2017R1D1A1B03033857] and [grant number 2018R1A5A2024425].
References

1. Poli, F., B. Dreno, and M. Verschoore. 2001. An epidemiological study of acne in female adults: results of a survey conducted in France. *Journal of the European Academy of Dermatology and Venereology : JEADV* **15**: 541-545.

2. Tasoula, E., S. Gregoriou, J. Chalikias, D. Lazarou, I. Danopoulou, A. Katsambas, and D. Rigopoulos. 2012. The impact of acne vulgaris on quality of life and psychic health in young adolescents in Greece. Results of a population survey. *Anais brasileiros de dermatologia* **87**: 862-869.

3. Li, D., Q. Chen, Y. Liu, T. Liu, W. Tang, and S. Li. 2017. The prevalence of acne in Mainland China: a systematic review and meta-analysis. *BMJ open* **7**: e015354.

4. Gupta, M. A., and A. K. Gupta. 1998. Depression and suicidal ideation in dermatology patients with acne, alopecia areata, atopic dermatitis and psoriasis. *The British journal of dermatology* **139**: 846-850.

5. Dreno, B., S. Pecastaings, S. Corvec, S. Veraldi, A. Khammari, and C. Roques. 2018. *Cutibacterium acnes* (*Propionibacterium acnes*) and acne vulgaris: a brief look at the latest updates. *Journal of the European Academy of Dermatology and Venereology : JEADV* **32 Suppl 2**: 5-14.

6. Institute of Medicines (US) Forum on Microbial Treats. 2004. *In The Infectious Etiology of Chronic Diseases: Defining the Relationship, Enhancing the Research, and Mitigating the Effects: Workshop Summary*. S. L. Knobler, S. O'Connor, S. M. Lemon, and M. Najafi, editors, Washington (DC).

7. Mollerup, S., J. Friis-Nielsen, L. Vinner, T. A. Hansen, S. R. Richter, H. Fridholm, J. A. Herrera, O. Lund, S. Brunak, J. M. Izarzugaza, T. Mourier, L. P. Nielsen, and A. J. Hansen. 2016. *Propionibacterium acnes*: Disease-Causing Agent or Common Contaminant? Detection in Diverse Patient Samples by Next-Generation Sequencing. *Journal of clinical microbiology* **54**: 980-987.

8. Cogen, A. L., V. Nizet, and R. L. Gallo. 2008. Skin microbiota: a source of disease or defence? *The British journal of dermatology* **158**: 442-455.

9. Holland, C., T. N. Mak, U. Zimny-Arndt, M. Schmid, T. F. Meyer, P. R. Jungblut, and H. Bruggemann. 2010. Proteomic identification of secreted proteins of *Propionibacterium acnes*. *BMC microbiology* **10**: 230.
10. McDowell, A., E. Barnard, I. Nagy, A. Gao, S. Tomida, H. Li, A. Eady, J. Cove, C. E. Nord, and S. Patrick. 2012. An expanded multilocus sequence typing scheme for Propionibacterium acnes: investigation of 'pathogenic', 'commensal' and antibiotic resistant strains. PloS one 7: e41480.

11. Kwon, H. H., J. Y. Yoon, S. Y. Park, and D. H. Suh. 2013. Analysis of distribution patterns of Propionibacterium acnes phylotypes and Peptostreptococcus species from acne lesions. The British journal of dermatology 169: 1152-1155.

12. Yu, Y., J. Champer, G. W. Agak, S. Kao, R. L. Modlin, and J. Kim. 2016. Different Propionibacterium acnes Phylotypes Induce Distinct Immune Responses and Express Unique Surface and Secreted Proteomes. The Journal of investigative dermatology 136: 2221-2228.

13. McDowell, A., A. L. Perry, P. A. Lambert, and S. Patrick. 2008. A new phylogenetic group of Propionibacterium acnes. Journal of medical microbiology 57: 218-224.

14. Lomholt, H. B., and M. Kilian. 2010. Population genetic analysis of Propionibacterium acnes identifies a subpopulation and epidemic clones associated with acne. PloS one 5: e12277.

15. Nakase, K., N. Hayashi, Y. Akiyama, S. Aoki, and N. Noguchi. 2017. Antimicrobial susceptibility and phylogenetic analysis of Propionibacterium acnes isolated from acne patients in Japan between 2013 and 2015. The Journal of dermatology 44: 1248-1254.

16. Tomida, S., L. Nguyen, B. H. Chiu, J. Liu, E. Sodergren, G. M. Weinstock, and H. Li. 2013. Pan-genome and comparative genome analyses of Propionibacterium acnes reveal its genomic diversity in the healthy and diseased human skin microbiome. mBio 4: e00003-00013.

17. Achermann, Y., E. J. Goldstein, T. Coenye, and M. E. Shirtliff. 2014. Propionibacterium acnes: from commensal to opportunistic biofilm-associated implant pathogen. Clinical microbiology reviews 27: 419-440.

18. Horvath, B., J. Hunyadkurti, A. Voros, C. Fekete, E. Urban, L. Kemeny, and I. Nagy. 2012. Genome sequence of Propionibacterium acnes type II strain ATCC 11828. Journal of bacteriology 194: 202-203.

19. Kasimatis, G., S. Fitz-Gibbon, S. Tomida, M. Wong, and H. Li. 2013. Analysis of complete genomes of Propionibacterium acnes reveals a novel plasmid and increased pseudogenes in an acne associated strain. BioMed research international 2013: 918320.
20. Otwinowski, Z., and W. Minor. 1997. [20] Processing of X-ray diffraction data collected in oscillation mode. *Methods in enzymology* **276**: 307-326.

21. Winn, M. D., C. C. Ballard, K. D. Cowtan, E. J. Dodson, P. Emsley, P. R. Evans, R. M. Keegan, E. B. Krissinel, A. G. Leslie, A. McCoy, S. J. McNicholas, G. N. Murshudov, N. S. Pannu, E. A. Potterton, H. R. Powell, R. J. Read, A. Vagin, and K. S. Wilson. 2011. Overview of the CCP4 suite and current developments. *Acta crystallographica. Section D, Biological crystallography* **67**: 235-242.

22. Terwilliger, T. C., P. D. Adams, R. J. Read, A. J. McCoy, N. W. Moriarty, R. W. Grosse-Kunstleve, P. V. Afonine, P. H. Zwart, and L. W. Hung. 2009. Decision-making in structure solution using Bayesian estimates of map quality: the PHENIX AutoSol wizard. *Acta crystallographica. Section D, Biological crystallography* **65**: 582-601.

23. Vagin, A., and A. Teplyakov. 2010. Molecular replacement with MOLREP. *Acta crystallographica. Section D, Biological crystallography* **66**: 22-25.

24. Emsley, P., B. Lohkamp, W. G. Scott, and K. Cowtan. 2010. Features and development of Coot. *Acta crystallographica. Section D, Biological crystallography* **66**: 486-501.

25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

26. Petrovic, N., C. Grove, P. E. Langton, N. L. Misso, and P. J. Thompson. 2001. A simple assay for a human serum phospholipase A2 that is associated with high-density lipoproteins. *Journal of lipid research* **42**: 1706-1713.

27. Santana, C. C., L. A. Barbosa, I. D. B. Junior, T. G. D. Nascimento, C. B. Dornelas, and L. A. M. Grillo. 2017. Lipase Activity in the Larval Midgut of *Rhynchophorus palmarum*: Biochemical Characterization and the Effects of Reducing Agents. *Insects* **8**.

28. Higaki, S., T. Kitagawa, M. Kagoura, M. Morohashi, and T. Yamagishi. 2000. Correlation between *Propionibacterium acnes* biotypes, lipase activity and rash degree in acne patients. *J Dermatol* **27**: 519-522.

29. Bruggemann, H., H. B. Lomholt, H. Tettelin, and M. Kilian. 2012. CRISPR/cas loci of type II *Propionibacterium acnes* confer immunity against acquisition of mobile elements present in type I *P. acnes*. *PloS one* **7**: e34171.
30. Labar, G., C. Bauvois, F. Borel, J. L. Ferrer, J. Wouters, and D. M. Lambert. 2010. Crystal structure of the human monoacylglycerol lipase, a key actor in endocannabinoid signaling. *Chembiochem: a European journal of chemical biology* **11**: 218-227.

31. Stauch, B., S. J. Fisher, and M. Cianci. 2015. Open and closed states of *Candida antarctica* lipase B: protonation and the mechanism of interfacial activation. *Journal of lipid research* **56**: 2348-2358.

32. Kaschner, M., O. Schillinger, T. Fettweiss, C. Nutschel, F. Krause, A. Fulton, B. Strodel, A. Stadler, K. E. Jaeger, and U. Krauss. 2017. A combination of mutational and computational scanning guides the design of an artificial ligand-binding controlled lipase. *Scientific reports* **7**: 42592.

33. De Vitis, V., C. Nakhnoukh, A. Pinto, M. L. Contente, A. Barbiroli, M. Milani, M. Bolognesi, F. Molinari, L. J. Gourlay, and D. Romano. 2018. A stereospecific carboxyl esterase from *Bacillus coagulans* hosting nonlipase activity within a lipase-like fold. *The FEBS journal* **285**: 903-914.

34. Nardini, M., and B. W. Dijkstra. 1999. Alpha/beta hydrolase fold enzymes: the family keeps growing. *Current opinion in structural biology* **9**: 732-737.

35. Waterhouse, A., M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T. A. P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, and T. Schwede. 2018. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic acids research* **46**: W296-W303.

36. Khan, F. I., D. Lan, R. Durrani, W. Huan, Z. Zhao, and Y. Wang. 2017. The Lid Domain in Lipases: Structural and Functional Determinant of Enzymatic Properties. *Frontiers in bioengineering and biotechnology* **5**: 16.

37. Marples, R. R., D. T. Downing, and A. M. Kligman. 1971. Control of free fatty acids in human surface lipids by *Corynebacterium acnes*. *The Journal of investigative dermatology* **56**: 127-131.

38. Greene, R. S., D. T. Downing, P. E. Pochi, and J. S. Strauss. 1970. Anatomical variation in the amount and composition of human skin surface lipid. *The Journal of investigative dermatology* **54**: 240-247.

39. Skjold-Jorgensen, J., V. K. Bhatia, J. Vind, A. Svendsen, M. J. Bjerrum, and D. Farrens. 2015. The Enzymatic Activity of Lipases Correlates with Polarity-Induced Conformational Changes: A Trp-Induced Quenching Fluorescence Study. *Biochemistry* **54**: 4186-4196.
40. Nam, K. H., S. J. Kim, A. Priyadarshi, H. S. Kim, and K. Y. Hwang. 2009. The crystal structure of an HSL-homolog EstE5 complex with PMSF reveals a unique configuration that inhibits the nucleophile Ser144 in catalytic triads. Biochemical and biophysical research communications 389: 247-250.

41. Rengachari, S., G. A. Bezerra, L. Riegler-Berkt, C. C. Gruber, C. Sturm, U. Taschler, A. Boeszoermenyi, I. Dreveny, R. Zimmermann, K. Gruber, and M. Oberer. 2012. The structure of monoacylglycerol lipase from Bacillus sp. H257 reveals unexpected conservation of the cap architecture between bacterial and human enzymes. Biochimica et biophysica acta 1821: 1012-1021.

42. Cavazzini, D., G. Grossi, E. Levati, F. Vallese, B. Montanini, A. Bolchi, G. Zanotti, and S. Ottonello. 2017. A family of archaea-like carboxylesterases preferentially expressed in the symbiotic phase of the mychorrizal fungus Tuber melanosporum. Scientific reports 7: 7628.

43. Hazarika, N., and M. Archana. 2016. The Psychosocial Impact of Acne Vulgaris. Indian journal of dermatology 61: 515-520.

44. Aschauer, P., S. Rengachari, J. Lichtenegger, M. Schittmayer, K. M. Das, N. Mayer, R. Breinbauer, R. Birner-Gruenberger, C. C. Gruber, R. Zimmermann, K. Gruber, and M. Oberer. 2016. Crystal structure of the Saccharomyces cerevisiae monoglyceride lipase Yju3p. Biochimica et biophysica acta 1861: 462-470.
Table 1.

|                              | Closed Calipase (wild type) | Blocked Calipase (BMK-bound) | Open Calipase (LPC-bound) |
|------------------------------|-----------------------------|------------------------------|---------------------------|
| **Data collection**          |                             |                              |                           |
| Beamline                     | PAL-5C                      | PAL-7A                       | PAL-7A                    |
| Wavelength (Å)               | 0.98                        | 0.98                         | 0.98                      |
| Resolution range (Å)         | 38.36-1.75                  | 31.89-1.60                   | 35.88-2.40                |
| Space Group                  | P2;2;2                      | P2;2;2                      | P3;1                      |
| Unit cell parameters (Å)     | a=55.3                      | a=94.5                      | a=185.9                   |
|                              | b=93.3                      | b=129.7                     | b=185.9                   |
|                              | c=129.7                     | c=55.5                      | c=205.1                   |
| Observations (total/unique)  | 68,476/63,249               | 91,178/89,860               | 311,475/68,958            |
| ComPLEteness (%)             | 92.8 (87.6)                 | 98.7 (84.0)                 | 97.6 (83.3)               |
| R<sub>sym</sub>              | 7.7 (29.5)                  | 6.1 (23.3)                  | 10.7 (34.1)               |
| CC<sub>1/2</sub>             | 0.95 (0.82)                 | 0.99 (0.96)                 | 0.99 (0.83)               |
| Redundancy                   | 3.7 (3.7)                   | 12.4 (5.9)                  | 3.5 (2.5)                 |
| I/sigma                      | 21.0 (10.1)                 | 25.7 (7.8)                  | 19.0 (5.8)                |
| **Refinement**               |                             |                              |                           |
| R<sub>work</sub> / R<sub>free</sub> (%) | 17.9/22.2                  | 14.5/16.7                   | 18.5/25.6                 |
| Protein/ligand atoms         | 4712/60                     | 4712/114                    | 61283/204                 |
| Water molecules              | 521                         | 705                         | 2515                      |
| Average B value (Å<sup>2</sup>) | 18.0                        | 14.0                        | 32.0                      |
| r.m.s.d. bond (Å)            | 0.013                       | 0.014                       | 0.010                     |
| r.m.s.d. angle (°)           | 1.728                       | 1.638                       | 1.686                     |
| **Ramachandran plot (%)**    |                             |                              |                           |
| Favoured                     | 93.97                       | 94.95                       | 93.05                     |
| Allowed                      | 5.70                        | 5.05                        | 6.79                      |
| Disallowed                   | 0.33                        | 0.00                        | 0.16                      |
Table 1. Crystallographic data collection and refinement statistics.

a Numbers in parentheses indicate the statistics for the last resolution shell.

b $R_{sym} = \frac{\sum |I_{hkl} - <I_{hkl}>|}{\sum <I_{hkl}>}$, where $I_{hkl}$ = single value of measured intensity of $hkl$ reflection, and $<I_{hkl}>$ = mean of all measured value intensity of $hkl$ reflection.

c $R_{work} = \frac{\sum |F_{obs} - F_{calc}|}{\sum F_{obs}}$, where $F_{obs}$ = observed structure factor amplitude, and $F_{calc}$ = structure factor calculated from model. $R_{free}$ is computed in the same manner as $R_{work}$, but from a test set containing 5% of data excluded from the refinement calculation.
Figure 1.

A

B

C
**Figure 1. Crystal structure of calipase**

A) The calipase dimer is shown in ribbon representation. Core domain is coloured in light cyan and teal while lid domain is coloured as pink and magenta.

B) Detailed view of dimerization interface. *Left* Hydrogen bonds and salt bridges are indicated. *Right* The catalytic triad (coloured as red) is covered by bulky hydrophobic residues (coloured as grey).

C) Secondary structure diagram of calipase monomer. The locations of catalytic triad are indicated.
Figure 2.
Figure 2. Sequence comparison of CAlipase with related proteins

Sequence alignment of CAlipase with type IB/IC lipases, type IA C. acnes short hydrolases (separated by dotted line), and two highest structural matches from DALI algorithm. Identical residues are coloured white on a red background and similar residues are red on white background. Additionally, identical residues across type IA/IB/IC/II C. acnes are coloured as light blue background. The CAlipase shows 83%, 97%, 96%, 39%, and 22% sequence identities with type IA, IB, IC C. acnes lipase, B. coagulans carboxyl esterase (5O7G), and human monoglyceride lipase (3HJU), respectively. Secondary-structure elements (springs: α-helices and arrows: β-strands) are shown above. The residues responsible for dimeric formation are marked as blue circles. Catalytic triad residues are marked as red triangles. Two phenylalanine residues that open up closed structure are indicated as black stars. Hydrophobic residues around fatty acid are shown by yellow drops. The lid domain region including α5, α6, α7, and α8 is indicated as pale yellow background.
Figure 3. Structural comparison of Cₐlipase with related proteins

A) The superposition of Cₐlipase with carboxyl esterase from B. coagulans (core and lid domains are coloured in salmon and brown) and monoglyceride lipase from human (core and lid domains are coloured in lime and green). Core domain is coloured in light colour. The conserved catalytic triads (serine, aspartate, and histidine) are indicated as sticks.

B) Surface structures of superposed structures in A). The catalytic triad is coloured in red and detected under the lid region, but completely hidden in Cₐlipase.
Figure 4.

A

Absorbance (412 nm)

Time (min)

B

Activity (arbitrary unit)

Lysophospholipase WT
Lipase WT
Lipase Mutant
Figure 4. Lipase and lysophospholipase activity

A) *Left* wild type CAlipase (▲) and lysophospholipase (■) activity were measured by colorimetry. The wild type CAlipase hydrolyse both substrates but preferred activity toward lipase substrate is observed. The active site shielding residues mutant (F176F179W192F211A, ◆) indicates higher activity. *Right* Relative initial rate of hydrolysis. Lipolytic activity is normalised to the active site shielding residues mutant.

B) To compare the mutants’ activity, the lipase activity of active site shielding residues mutant (F176F179W192F211A, ●) was compared to wild type CAlipase (■) and lipophilic path forming residues mutant (F179W192A, ◆). The active site shielding residues mutant showed higher activity. Values are the mean of three separate determinations.
Figure 5. Charge states of Ca lipase and binding assay

A) The size-exclusion chromatography of Ca lipase and corresponding SDS-PAGE and native PAGE. The native PAGE shows broadened bands. Bands reduced upon addition of Al’s oil and Triton X-100. Addition of LPC in Al’s oil induced further band reduction.

B) ITC experiment of raw data and binding isotherms fitted to a one site binding model. The dissociation constant, ΔG, ΔH, and -TΔS are indicated in the figure.
Figure 6. Structural comparison of closed, blocked, and open CaLipase

A) Cross-section of CaLipase structures. A path to the catalytic triad (coloured as red) is closed, blocked, and open in each crystal structure.

B) *Left* Superposition of crystal structures of closed (core domain: cyan, lid domain: pink), blocked (core domain: yellow, lid domain: gold), and open (core domain: palegreen, lid domain: green) state CaLipase. Two phenylalanine residues that show rearrangement are represented as sticks. Distances are given in Å.

*Right* Hydrophobic residues around modified serine in open state crystal structure are indicated as sticks.

C) B-factor difference plots of open state CaLipase crystal structure. B-factor of all 26 chains are showed (*left*: main chain, *right*: side chain). Lid domain region is highlighted.
Figure 7.
Figure 7. Modified serine at residue 114

A) The crystal structure of blocked Ser114 with BMK (blocked structure). The catalytic triad is shown as red sticks. Ligplot showing residues involved in interactions between BMK and CALipase is shown. The covalently bound BMK forms hydrogen bonds with Leu38 and Trp115. The methyl and benzene moieties are stabilized predominately by hydrophobic interactions with Val140, Phe176, Phe179, Trp192 and Phe211.

B) Ser114 is modified as fatty acid bound form in open structure by introduction of LPC. Ligplot analysis for bound LPC and CALipase is shown. The hydrophobic interactions between Val140, Phe179, and Phe211 are absent due to the lack of methyl and benzyl groups.
Figure 8. Mode of action of *C. acnes* lipase

In aqueous solution, the major form of lipase is closed form, shielding the active site (coloured in red). However, when the lipase is exposed to a high level of lipid, the lid domain moves to reveal the active site. Two bulky side chain residues, Phe176 and Phe211, are responsible for the lid opening. The entry of the substrate is helped by lipophilic path, followed by the catalytic reaction through active site and oxyanion hole (coloured in red and purple, respectively).