CHARACTERIZATION OF A KEY *MYCOBACTERIUM TUBERCULOSIS* LIPASE, LIPY

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ABSTRACT

Christopher K. Garrett: Characterization of a Key *Mycobacterium tuberculosis* Lipase, LipY
(Under the direction of Saskia Neher)

The causative agent of tuberculosis, Mycobacterium tuberculosis is estimated to be present in roughly a third of the world's population. One of the hallmarks of the pathogen is its reliance on lipids as a source of energy in order to survive in a non-replicating state and ultimately cause infection. A key player in lipid utilization by M. tuberculosis is the triglyceride lipase, LipY. LipY is the rate-limiting catalyst in the mobilization of free fatty acids from triglycerides acting on lipid bodies both within the tubercule bacillus and outside the bacterium. LipY also belongs to the PE/PPE family of proteins, a group of approximately 169 virulence-associated proteins characterized by a proline-glutamate or proline-proline-glutamate motif at their N-termini.

It has been suggested that the PE domain of LipY may act as a regulator of enzymatic activity. Using several biochemical and activity-based approaches, we established a method for purifying LipY with improved resolution. We also introduced a rapid and reliable method for quantification of active sites for serine hydrolases using activity-based protein profiling (ABPP). Finally, by imploring these methodologies, we discovered that the PE domain of LipY acts as a non-competitive inhibitor of LipY enzymatic activity when it is attached to the mature lipase. The methodologies and evidence presented here may lead to discovery of additional PE/PPE family enzymes

with roles in *M. tuberculosis* lipid metabolism and/or virulence. This work may also lead to expansion of our understanding of the roles of PE/PPE proteins and domains.

To every Black boy and Black girl who has been told, “no, you can’t,” you can. And to the Trayvons, Mikes, Tamirs, Jordans and Sandras of the world, I see you. BLM.

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LIST OF SYMBOLS AND ABBREVIATIONS

Å	angstrom
ABP	activity-based probe
ABPP	activity-based protein profiling
ACS	acetyl-CoA synthetase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CD	circular dichroism
CMC	critical micelle concentration
CoA	coenzyme A
°C	degrees Celsius
Δ	deletion/deleted
DGGR	1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester)
FAD	flavin adenine dinucleotide
FFA	free fatty acid
IPTG	isopropyl β-D-1 thiogalactopyranoside
ITC	isothermal titration calorimetry
kDa	kilodalton
K_m	Michaelis-Menten constant
M. bovis BCG	*Mycobacterium* bovis Callmete-Guerin
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
MDR-TB	multidrug-resistant tuberculosis

MRE	mean residue ellipticity
POP	peroxidase
pNPB	*p*-Nitrophenyl butyrate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG	triglyceride
THL	tetrahydrolipstatin
T_m	melting temperature
TOOS	N-ethyl-N-sulphohydroxy propyl-m Toluidine
TRL	triglyceride rich lipoprotein
V_{max}	maximum reaction velocity
XDR-TB	extensively drug-resistant tuberculosis

CHAPTER 1: INTRODUCTION

Tuberculosis Overview

Mycobacterium Tuberculosis

The tuberculosis (TB) disease is caused by a single deadly pathogen, *Mycobacterium tuberculosis* (*M. tuberculosis*), resulting in upwards of 9 million new infections and roughly 1.5 million deaths every year[1]. *M. tuberculosis* was first described as the causative agent of tuberculosis in 1882[2]. Although *M. tuberculosis* can affect most tissues and organs (extrapulmonary TB), the majority of infections begin in the lungs (pulmonary TB) where alveolar macrophages phagocytose the bacteria[1,3,4]. Subsequently, most bacteria are destroyed although in a number of cases, the opportunistic pathogen is able to modulate the host's response such that it avoids destruction and proliferates, ultimately leading to cell death and infection[5-7].

Ninety-five percent of all TB deaths occurred in developing countries around the world with 22 of those countries accounting for 80% of all TB cases[1]. *M. tuberculosis* is the second deadliest single causative agent in the world with HIV leading the way[1]. A vaccine widely used in countries where TB is most prevalent, bacillus Calmette-Guérin (BCG), is a strain derived from attenuation of the *Mycobacterium bovis* species[8]. However, the BCG vaccine is limited in its ability to protect individuals from certain forms of *M. tuberculosis* transmission[9]. When individuals with active TB infections cough, bacilli-containing sputum is released and can be inhaled resulting in TB infection. Those most susceptible to TB infection generally have weakened immune systems and

include children, the elderly and individuals with HIV as TB-HIV co-infection can make up more than two thirds of all TB cases in certain developing countries[10].

One of the major breakthroughs towards a more comprehensive understanding *M. tuberculosis* was the sequencing of its genome in 1998[11]. *M. tuberculosis* is made up of roughly 4,000 genes spanning an approximately 4.4 million base pair genome[11,12]. One of the defining characteristics of the bacteria and sources of its name is a thick, waxy cell wall made up primarily of mycolic acids and other lipids that aid the pathogen in virulence and resistance to drugs[13,14]. *M. tuberculosis* is also considered a slow-growing pathogen, requiring up to 16 hours to double in cultured medium[15]. This is also one of the major features that allows infection to go undetected for extended periods of time.

Treatment

One of the biggest impediments to drug effectiveness is the *M. tuberculosis* cell wall, an extremely hydrophobic layer with a variety of lipids, glycolipids and polysaccharides and other molecules that limit cell entrance by foreign bodies[16,17]. Not surprisingly, one of the major first-line anti-tuberculosis drugs, isoniazid, specifically targets synthesis of cell wall mycolic acids by targeting the *M. tuberculosis* enoyl reductase InhA[18-20]. Targeting the cell wall via an alternative mechanism, ethambutol is a first-line anti-tuberculosis drug with effects similar to those of isoniazid[21]. The remaining first-line anti-tuberculosis drugs are rifampicin and pyrazinamide which target the activity of RNA polymerase and, purportedly, fatty acid synthetase, respectively[22-24].

Drug regimens for individuals infected with *M. tuberculosis* typically require a treatment period of approximately 6 months[25]. Fluoroqunolones targeting *M. tuberculosis* DNA gyrase and injectable aminoglycosidases such as kanamycin and amikacin which

inhibit ribosome activity, are the major second-line anti-tuberculosis drugs currently used for treatment[26,27]. It is important to note that current anti-mycobacterial drugs almost exclusively target replicating mycobacteria resulting in extended periods of treatment in order to eradicate slower, non-replicating bacilli.

Drug resistance

One of the major contributors to intrinsic *M. tuberculosis* antibiotic resistance is the activity of β-lactamases[28]. However, instances of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) can often times be attributed to case mismanagement and transmission within a particular community[29]. MDR-TB is defined by resistance to either of the common first-line drugs isoniazid or rifampicin while XDR-TB refers to resistance to any fluoroquinolone and at least one injectable drug in addition to resistance to isoniazid or rifampicin[1]. In 2013 alone, MDR-TB accounted for an estimated 5% (480,000) of all TB cases around the world with almost half of those cases resulting in death[1]. Therefore, identification and characterization of novel therapeutic targets, and development of novel drugs and therapeutic strategies must be at the forefront of research endeavors seeking to reduce the global burden of the tuberculosis disease.

PE/PPE Protein Family

PE/PPE family overview

The PE/PPE family of proteins, named after a proline-glutamate (PE) or proline-proline-glutamate (PPE) motif beginning at the ninth amino acid position, was discovered in the 1998 sequencing of the *M. tuberculosis* genome[11]. The N-termini of these protein families were highly conserved and the newly denoted proteins had previously been

implicated in antigenic variation prior to their reclassification[30,31]. PE and PPE proteins are made up of a 110 or 180 amino acid N-terminus, respectively, and, in some cases contain C-terminal extensions with a variety of functions[11,32]. The PE and PPE genes account for ~10% of the coding capacity of the *M. tuberculosis* genome, adding to the curiosity surrounding this relatively new family of proteins[11]. The number of identified PE and PPE genes varies slightly depending on the report with roughly 99 PE genes and 68 PPE genes being reported[11].

PE and PPE proteins are found in gene clusters throughout the *M. tuberculosis* genome. Several pieces of genomic, biochemical and structural data point to an important relationship between proteins of the two families. In some cases, PE and PPE proteins are present in operons where they are co-transcribed with their specific protein partner[33]. For example, PE25 (Rv2431c) and PPE41 (Rv2430c) can only be purified when co-transcribed and co-purified in *E. coli* cells[33]. Until Strong et al. used this approach, the two proteins were only purified as aggregates suggesting that some PE and PPE protein pairs stabilize each other.

Structural data confirmed that some PE and PPE protein pairs interact. The crystal structure of the PE25-PPE41 complex revealed a ~100 Å extended heterodimeric alpha-helical bundle[33-35]. EspG, a protein involved in secretion, was also crystallized with the PE25-PPE41 complex and shown to interact exclusively with PPE41[34]. The predicted corresponding EspG-binding sites of several other PPE proteins were used to create chimeras and test binding to EspG, revealing nanomolar affinity and implicating PPE proteins as containing the signal for secretion of the PE/PPE pair[34].

Some PE and PPE genes are found adjacent to one another throughout the *M. tuberculosis* genome and sometimes interact with one another after expression for proper cellular localization and function[33]. However, many PE proteins can either be found clustered or dispersed throughout the genome[33]. Therefore, much evidence indicates that not all PE proteins have a cognate PPE partner and often times function alone.

PE proteins and PE_PGRS (polymorphic GC-rich repetitive sequences) proteins

The polymorphic GC-rich repetitive sequence (PGRS) family was first described in 1995 with the consensus nucleotide sequence CGGCGGCAA at 26 *M. tuberculosis* loci[30]. However, it wasn't until later when Cole et al. published the sequence of the *M. tuberculosis* H37Rv genome that the PE family of proteins was described and later separated into 3 subcategories that included the PE_PGRS subfamily[11]. The first type of PE protein was described as being comprised of the ~110 amino acid PE domain alone. A second subcategory of PE proteins is made up of the PE domain with an extended C-terminal domain with a unique sequence and unknown function. For example, using an *in silico* sequence analysis, it was discovered that five proteins from this subcategory of PE proteins possess an α/β hydrolase fold indicating some sort of enzyme function[36]. It was later confirmed that one of those proteins does in fact possess esterase activity at its C-terminus[37].

The majority of PE proteins, however, fall under the third subfamily of PE proteins called PE_PGRS proteins, which are characterized by an N-terminal PE domain and C-terminal PGRS region. The tandem repeats that make up the PGRS domain are Gly-Gly-Ala or Gly-Gly-Asn[38]. One piece of evidence points to the repeat sequences as acting as protectors of the PE_PGRS protein from proteosomal degradation and thus,

antigen processing by the host[39]. However, it does not appear that the PE_PGRS protein family adheres to the classical model of antigenic variation[40]. PE_PGRS proteins are mostly cell membrane and cell wall associated where they can be present on the cell surface at the host-pathogen interface[38,41,42]. The PGRS domain was also determined to be necessary for PE_PGRS33 localization to the mitochondria of eukaryotic cells and to induce apoptosis signifying a role for PE_PGRS proteins in pathogenesis[43].

PPE and PPE_MPTR (major polymorphic tandem repeats) proteins

As with the PE_PGRS and the PPE families of proteins, the PPE_MPTR subgroup wasn't discovered until 1998 with the sequencing of the *M. tuberculosis* genome[11]. Much like the PGRS protein family, the major polymorphic tandem repeats (MPTR) family of proteins was discovered several years before the discovery of the PPE protein family[31]. MPTRs, believed to be in approximately 80 regions of the *M. tuberculosis* genome, are made up of a consensus 10-nucleotide sequence (GCCGGTGTTG) separated by 5-nucleotide segments.

Similar to the PE family, the PPE family can be broken down into several subgroups including the ~180 amino acid PPE domain alone, PPE_PPW which contains a PXXPXXW motif, PPE_SVP which contains a conserved GXXSVPXXW motif around the 350 amino acid position and the most abundant and well-studied PPE subgroup, PPE_MPTR[11,44]. Of the 68 or so PPE proteins, 23 belong to the PPE_MPTR subgroup[40]. PPE_MPTR proteins contain tandem copies of NXGXGNXG at their C-termini and similar to PE_PGRS proteins, it is generally accepted the PPE_MPTR proteins play a role in *M. tuberculosis* pathogenesis and, potentially, antigenic variation[11].

M. tuberculosis secretion overview

M. tuberculosis possesses several export systems utilized for secreting numerous extracellular and cell wall-associated virulence factors from the cytosol to their final subcellular locations. In this section I discuss the Seca1 and Seca2 export pathways, the twin arginine translocation (TAT) pathway and the mycobacteria-specific type VII secretion pathways. Discussed are the components of each export pathway, the type of cargo that is exported by each system and the relevance of each system to mycobacterial pathogenesis.

General Secretion Pathway (Seca1/Seca2)

One of the keys to the functions of PE and PPE proteins and their subgroups is their ability to be exported to the cell surface or extracellular space where they often times come into contact with the host cell. The general secretion pathway, also called the Sec export pathway is the most common means of exporting proteins across the cell membrane. The Sec pathway is conserved in all bacteria although it has been most thoroughly studied in *E. coli*. Twelve different proteins of the Sec export pathway are required for *M. tuberculosis* growth[45]. The preproteins exported by the general Sec pathway are recognized in an unfolded state[45]. They are recognized by the Sec pathway secretion machinery by an N-terminal signal peptide that is positively charged at the N-terminus, possesses an uncharged polar C-terminus and 10-15 amino acid hydrophobic center[46]. After being exported across the cell membrane the signal peptide is cleaved at the uncharged, polar region, thus liberating a mature protein[46].

The primary housekeeping SecA protein, SecA1, which acts as an ATPase that binds to Sec pathway cargo and pushes the preproteins through the Sec export machinery, is essential in *M. tuberculosis*[45,47]. The transmembrane pore through which Sec export

pathway machinery, SecYEG, is made up of 3 proteins: a large transmembrane protein (SecY), a clamp-like protein that stabilizes SecY (SecE) and a protein that improves export efficiency (SecG)[45]. The signal peptidase responsible for cleavage of most Sec export pathway substrates is LepB although a second signal peptidase exists.

Not surprisingly, the Sec export pathway is important for *M. tuberculosis* virulence. For example, important virulence-related lipoproteins that are exported by *M. tuberculosis* such as LpqH, LppX and LprG are dependent on the Sec export pathway. In addition to exporting virulence factors, the Sec export pathway is also responsible for co-translational insertion of integral membrane proteins into the mycobacterial cell membrane. The FtsY receptor, required for all bacterial growth including *M. tuberculosis*, recognizes the signal recognition particle, SRP, of nascent integral membrane proteins. SecA assists in the export process and the SecYEG channel delivers the membrane protein to the membrane. This along with the other secretory functions is a clear indication of the significance and breadth of Sec export in *M. tuberculosis*.

A second accessory export pathway protein, SecA2, also exists in mycobacteria. Although its function is similar to that of SecA1, there are some key differences between the two proteins and, thus, the pathways. SecA2 is smaller than SecA1 by approximately 21 kDa and only shares 50% sequence similarity to SecA1[47,48]. Unlike the Sec export pathway, the accessory SecA2 export pathway exports proteins with and without the Sec signal peptide. For example, KatG, which activates the antimycobacterial pro-drug isoniazid and the superoxide dismutase, SodA both lack the Sec signal peptide[49,50]. Both proteins, however, are exported in a SecA2-dependent manner. Nevertheless, SecA1 and SecA2 also share some similarities. Both SecA1 and SecA2 use ATPase activity to

function. As it is understood today, the accessory SecA2 export pathway uses the same export components of the Sec export pathway, namely SecYEG, in order to transport its cargo outside the cell[51].

Twin Arginine Translocation Pathway

The twin arginine translocation pathway (TAT) is named after a motif found in an N-terminal signal peptide similar to that of the Sec export pathway. However, there are several distinctions between the Sec export and TAT pathways. One of the major distinctions between the signal peptide of TAT pathway preproteins and Sec export pathway preproteins is a S/TRRXFLK motif containing 2 consecutive arginine residues (twin-arginines) although evidence indicates that one arginine can be sufficient for TAT export of a small number of proteins[52-54]. In addition to the presence of the twin-arginine motif in the signal peptide, preproteins must be properly folded for recognition and export by the TAT export system, a clear deviation from the requirement of unfolded preproteins in the Sec export pathway[55,56]. In addition, the TAT system, although present in Gram-negative and Gram-positive bacteria is not present in all bacteria like its protein export pathway companion SecA[57].

The TAT export system, because it exports folded preproteins, is made up of slightly different components from the Sec export pathway that can accommodate the structurally diverse cargo. Three transmembrane proteins, TatA, TatB and TatC, constitute the bulk of the TAT system machinery with TatB and TatC forming a preprotein-binding complex. A TatB-TatC complex is formed after both proteins recognize and bind the twin-arginine motif of the preprotein's signal peptide with TatC being the primary binding protein[58]. TatA has been shown to forma a variety of

oligomers (130 kDa- 390 kDa) that are believed to make up the pore through which TAT cargo traverses the cell membrane[59,60]. It is recruited to the TatB-TatC-preprotein complex, interacting primarily with TatB, and the cargo is exported by means of the proton motive force[58]. In E. coli, LepB, the same signal peptidase used to cleave Sec export pathway peptide signals, liberates TAT preporteins from their signal peptides[61]. This function has been extrapolated to be present in the homologous LepB protein in *M. tuberculosis*.

The *M. tuberculosis* TAT export system is essential and, to date, only 18 TAT export proteins have been identified in *M. tuberculosis* although several more likely exist[45,62,63]. Of these proteins with an experimentally determined TAT signal peptide, five (~28%) have been identified as required for virulence in *M. tuberculosis*[45]. Therefore, the TAT export system has been implicated in *M. tuberculosis* virulence. Also, a key β-lactamase that helps protect the cell from damage by β-lactam antibiotics, BlaC, is exported via the TAT system[64]. Therefore, in addition to pathogenesis, the TAT export system plays a role in *M. tuberculosis* drug resistance.

ESX-1

ESX-1 is the best characterized Type VII secretion systems in *M. tuberculosis*. The ESX system derived its name from one of its substrates, ESAT-6, which, along with its heterodimeric partner CFP-10, are required for full *M. tuberculosis* virulence[65]. In cultured bacteria, the two proteins are found in the culture filtrate and have also been found to associate with the cell wall[66,67]. A general secretion motif, YxxxD/E, was discovered to be necessary for export of ESX-1-specific proteins including one of the

hallmark substrates of ESX-1, CFP-10, the virulence-associated protein EspB, and some PE proteins[68]. This motif is absent in PPE proteins and ESAT-6-like proteins.

ESX-1 is also the best-characterized export system in terms of its architecture. The genes comprising the ESX-1 export system are found in the *esx-1* gene locus of *M. tuberculosis*. Interestingly, the *esx-1* gene locus is absent in the attenuated *M. bovis* BCG vaccine strain as it is part of the region of difference (RD1), which is missing in *M. bovis* BCG[69]. This is the earliest and, perhaps, strongest piece of evidence indicating an essential role of the ESX-1 system in *M. tuberculosis* virulence. The proteins located in the esx-1 gene locus that constitute the ESX-1 secretion machinery include one membrane-bound serine protease (MycP1), two cytosolic ATPases (EccA1 and EccCb1) and four membrane associated proteins (EccB1, EccCa1, EccD1, and EccE1)[45,70].

MycP1, a subtilisin-like protease, cleaves ESX-1 substrates after export to the cell wall whereas EccA1 and EccCb1 interact with the C-terminus of ESX-1 substrates on the cytosolic side of the cell membrane and use their ATPase activity to assist translocation[45,70-72]. It is generally accepted that EccD1, a protein with 11 membrane-spanning helices, forms the channel through which ESX-1 substrates pass whereas the remaining membrane proteins play a more peripheral role interacting with ESX-1 machinery[45,70,73].

ESX-3

To date, there is limited information regarding the ESX-3 secretion system. Using an *esx-3* conditional knockout, Serafini et al. discovered that growth of cultured *M. tuberculosis* could be restored when the media was supplemented with iron and zinc[74]. It was also discovered that ESX-3 is essential for cultured *M. tuberculosis* growth[74].

Although many of the genes encoded in the *esx-3* gene locus remain to be identified, it has been suggested that the ESX-3 system secretes factors such as the EsxG-EsxH complex that assists in regulating metal uptake by *M. tuberculosis*[74-76]. One group demonstrated that scavenging of iron via the *M. tuberculosis* mycobactin pathway requires the presence of ESX-3, further highlighting the importance of the export system in *M. tuberculosis* growth[77]. Although there has been some success with regards to elucidating the role of ESX-3 export in *M. tuberculosis*, the genes comprising specific components of the ESX-3 export machinery remain to be identified.

ESX-5

The ESX-5 secretion system is specific to slow growing mycobacteria including members of the *M. tuberculosis* complex (MTC), *M. tuberculosis*, *M. africanum*, and *M. bovis*[78]. Most research centered on ESX-5 has used *M. marinum* as a model. However, Bottai et al. were able to directly study the role of *M. tuberculosis* ESX-5 in protein export and virulence by creating mutants lacking proteins from the ESX-5 locus[79]. These studies showed that ESX-5 in *M. tuberculosis* is a functional secretion system that is important for maintenance of cell wall stability and virulence. ESX-5 is responsible for exporting a number of PE and PPE proteins to the cell surface and extracellular space including all of the PE_PGRS proteins in *M. marinum* (not confirmed in *M. tuberculosis*)[80]. Many PE proteins with roles in virulence are exported by ESX-5. The PE/PPE heterodimer, PE25-PPE41, is one example of a PE/PPE pair with a role in virulence exported by ESX-5[33,68,81]. Other PE_PGRS and PPE_MPTR proteins identified in *M. marinum* are also exported by the ESX-5 system[80].

There is no crystal structure of the ESX-5 export machinery. However, there have been some efforts to characterize the architecture of the ESX-5 membrane complex of *M. tuberculosis* using *M. marinum* and *M. bovis* BCG as model organisms. The five genes comprising the ESX-5 export system are located in the *esx-5* gene locus and named $EccA_5$, $EccB_5$, $EccC_5$, $EccD_5$, $EccE_5$ for ESX-conserved component[82,83]. The subscript '5' refers to these proteins comprising the machinery of the ESX-5 system as opposed to the four remaining ESX systems. $EccC_5$ and $EccD_5$ are both believed to make up the membrane channel for translocation of substrates because of their essentiality in ESX-5 dependent secretion[82]. In addition, $EccC_5$ has three predicted nucleotide-binding domains and shares homology with the FtsK/SpoIIIE ATPase family[82]. Therefore, it is likely that $EccC_5$ uses rounds of ATP hydrolysis to help translocate ESX-5 substrates across the cell wall[82]. $EccB_5$, $EccC_5$, $EccD_5$ and $EccE_5$ all make up a 1,500 kDa protein complex that localizes to the cell wall while $EccA_5$ is not associated with the cell wall or insoluble fractions in experiments exploring ESX-5 architecture[82]. It is likely that EccA5 interacts with ESX-5 substrates and/or the cytosolic portion of the ESX-5 membrane complex. $EccB_5$ and $EccE_5$ likely play a peripheral role helping to stabilize the remainder of the EccBCDE complex for efficient translocation of ESX-5 substrates.

Although ESX-5 substrates lack Sec and TAT signal peptides, a general motif at the N-terminus of PE proteins, YxxxD/E, is required for ESX-5 dependent secretion[68]. It has also been demonstrated that some ESX-5 substrates are proteolytically cleaved upon export[68,84,85]. The PE_PGRS protein, LipY, was the first PE protein with an experimentally determined enzymatic function and its export to the cell wall has been thoroughly characterized. LipY is composed of an N-terminal PE domain containing a

YAAAE secretion motif and a C-terminal lipase domain capable of hydrolyzing long chain triglycerides[68,86]. LipY's PE domain is cleaved between a glycine and alanine upon export indicating that the PE domain acts a pseudo-secretion signal for export via ESX-5[85]. However, it does not appear all ESX-5 substrates are proteolytically processed although this cleavage feature does appear to be one of the many putative functions of the ESX-5 system. In addition, some PE and PPE proteins such as PE25 and PPE41 are exported as obligate-heterodimers by ESX-5 and depend on a YATAE secretion motif in PE25[33,68]. Some structural data suggest that the YxxxD/E motif of the PE domain of PE proteins may interact with the PPE domain of its associated PPE protein although PE/PPE complex formation does not appear to be a prerequisite for all ESX-5 substrates[68,70]. Taken together, biochemical, structural, and functional studies indicate that PE proteins contain an export motif that can, in some cases, direct the export of a partner protein from the *M. tuberculosis* cytosol.

Role of Lipids and Mycobacterial Lipases

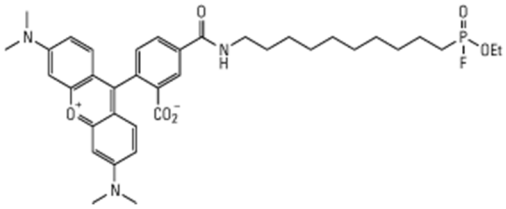
Mycobacterium Tuberculosis Lipid Metabolism

The glycoxylate cycle is a key metabolic process for providing *M. tuberculosis* with energy during pathogenesis[87]. Isocitrate lyase, an essential enzyme in the glyoxylate cycle that converts isocitrate to glyoxylate and succinate which is converted into energy in the tricarboxylic acid cycle, is upregulated under minimal growth conditions such as acetate and low oxygen[88,89]. The glyoxylate cycle, however, begins with acetyl coenzyme A (acetyl-CoA), which is a product of β-oxidation. Beta-oxidation involves the catabolic processing of fatty acids derived from lipids such as triacylglycerols (TAGs). Therefore

processing of lipids into free fatty acids represents the initial step for β-oxidation and ultimately energy production for *M. tuberculosis*, especially during dormancy.

Based primarily on sequence identity, *M. tuberculosis* H37Rv is believed to have approximately 250 genes involved in lipid metabolism[11]. Some of these genes encode enzymes such as TAG synthetases, fatty acid synthases and numerous serine hydrolases including lipases (Fig 1). One of the critical reasons why *M. tuberculosis* possesses such a large number of lipid metabolism-related genes, many of which are redundant, is because of its complex, lipid-rich cell wall[11]. Synthesis of the major cell wall component, mycolic acids, requires a number of biosynthetic steps. The *M. tuberculosis* cell wall is key to its drug resistance and is the target of the first-line tuberculosis pro-drug isoniazid which specifically targets the synthesis of cell wall mycolic acids after being activated by the katG gene[19,50,90]. Unfortunately, isoniazid only targets actively replicating *M. tuberculosis*, not dormant bacilli.

Unlike the lipids that make up the cell wall, intracellular lipids, specifically TAGs, are key to fueling *M. tuberculosis* metabolic processes. Lipid droplets containing mostly cholesterol and TAGs can be found in *M. tuberculosis* grown *in vitro* as well as in samples of sputum from patients classified as having clinical tuberculosis[91]. Host foamy macrophages loaded with lipid droplets contain *M. tubercul*osis bacilli and provide a means for the bacteria to accumulate TAGs. It is known that *M. tuberculosis* accumulates TAGs during dormancy; several lipid metabolism genes are upregulated during dormancy and that the lipids within the bacilli are derived from host cell lipids[86,92,93]. This evidence points to TAG lipases having a direct role in the accumulation and utilization of lipids for *M. tuberculosis* energy.



TAMRA FP-Serine hydrolase probe

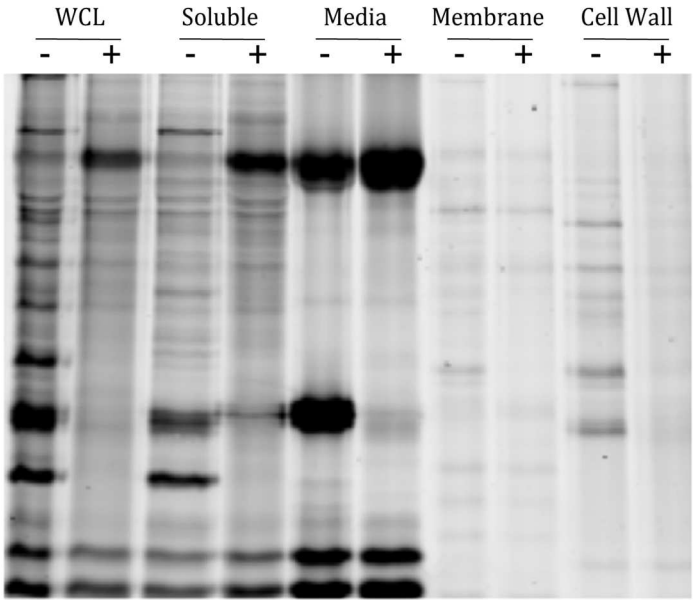


Figure 1. Activity-Based Protein Profiling of Several Fractions of *M. tuberculosis.* Serine hydrolases present in the indicated fractions of *M. tuberculosis* were probed using the fluorescent TAMRA-FP Serine Hydrolase probe for 30 minutes at room temperature, Reactions were quenched with SDS-loading dye and samples were boiled for 10 minutes at 100°C. Samples were resolved using SDS-PAGE and imaged using a Typhoon imager. (+ and – indicate boiled and unboiled samples, respectively, prior to reaction with the activity-based probe)

Lipases

Lipases are enzymes that catalyze the hydrolysis of lipids to free fatty acids. TAGs, which are made up of a glycerol backbone and three fatty acids, are hydrophobic energy-possessing molecules that can be mobilized by TAG lipases. TAG lipases specifically hydrolyze the fatty acid of TAGs at the *sn*1 or *sn*3 (terminal) positions yielding diacylglycerol and one free fatty acid although some reactions result in cleavage of two free fatty acids, one from both the *sn1* and *sn2* positions. Therefore, TAG lipases represent the rate-limiting enzymes in mobilizing free fatty acids for energy use.

Lipases belong to the superfamily of enzymes called serine hydrolases, which are characterized by a nucleophilic serine residue in a loop region flanked by glycine residues[94]. A histidine residue and either aspartate or glutamate residue make up the remainder of the catalytic triad which behaves as a charge relay system to activate the serine residue[95]. Structurally, TAG lipases have an α/β hydrolase fold characterized by alternating beta strands and alpha helices forming a unique α/β sheet[95]. A hydrophobic pore leading to the active site accommodates the lipid substrate. A lid domain, usually made up of one or two α-helices controlled by a hinge-like function, helps direct substrate specificity by presenting a hydrophobic surface for contact with the lipid[96]. Exchanging of lids from different lipases can result in a difference in relative specific activity, implicating lipase lids as crucial structural components for enzymatic activity[97].

Bacterial Lipases

Lipases have been implicated in the virulence and biofilm formation of bacteria. In *Stapylococcus aureus*, deletion of the lipase-encoding gene led to a reduction in biofilm formation *in vitro* and a decreased bacterial load in the liver, kidney and spleen of

infected mice[98]. It has also been more directly demonstrated that bacterial lipases have play a role in altering the host immune system in order to more effectively cause infection. Independent of enzymatic activity, *Staphylococcal* lipase had a protective effect on bacterial phagocytosis by granulocytes[99]. The presence of *Staphylococcal* lipase also immobilized granulocytes and drastically altered the morphology of granulocytes proving bacterial lipases have a direct role in the infection process[99]. Therefore, expanding the biochemical and structural information concerning bacterial lipases is of utmost importance to targeting such pathogens for therapeutic discovery and development.

LipY

Regulation during dormancy and regrowth

LipY, like many genes believed to be involved in lipid metabolism, is upregulated during the *M. tuberculosis* dormancy phase[86]. Deb et al. demonstrated this by measuring expression of the 24 putative lipase genes including LipY in *M. tuberculosis* grown under dormancy-like conditions[86]. Deb et al. also demonstrated that LipY is the major contributor to *M. tuberculosis* lipase activity in vitro and that its enzymatic activity is upregulated during dormancy[86]. It also appears that LipY is important for breaking down stored TAGs within *M. tuberculosis* during regrowth from dormancy[100]. A knockout mutant of the *M. bovis* BCG LipY ortholog had a negative effect on the amount of stored TAGs, rate of bacterial regrowth and lipase activity during the first day of regrowth[100]. Although LipY appears to be upregulated during the dormancy phase and a key player in regrowth of *M. tuberculosis* during the regrowth phase, the specific factors that regulate expression of LipY are not yet known.

Subcellular location

Several groups have attempted to elucidate the precise subcellular location of LipY. The initial report describing LipY's biochemical and enzymatic properties demonstrated that LipY hydrolyzes TAGs intracellularly[86]. A later study used immunoblot analysis to demonstrate that *M. tuberculosis*-LipY overexpressed in *M. bovis* BCG could be detected both in the cytosol and cell wall[101]. Mishra et al. also demonstrated that the absence of LipY's PE domain (LipYΔPE) did not impact its subcellular localization. Furthermore, it was demonstrated using electron microscopy that LipY and LipYΔPE are located on the outer most surface of the *M. bovis* BCG cell wall. Similarly, it was demonstrated by Daleke et al. that LipY is exported to the cell wall of *M. marinum* by immunoblot analysis. Daleke et al. also demonstrated that LipY is responsible for the extracellular hydrolysis of a lipid-like substrate when incubated with intact *M. marinum* cells expressing the enzyme[85]. Although Daleke et al. provided evidence that supported the idea of LipY being exported to and localized on the cell wall, the group also demonstrated that LipY's PE domain is necessary for export via the type VII secretion system, ESX-5[85]. This contrasts with evidence from Mishra et al. that demonstrated that LipY is localized to the cell wall in the presence or absence of its PE domain[101]. However, it wasn't until 2012 that Daleke et al. showed that a general secretion motif (YxxxD/E) located in the PE domain of LipY and other ESX-5 substrates is required for export to the cell wall and extracellular space[68].

Purification of LipY

Purification of the 45 kDa lipase, LipY, has proven difficult over the last decade. In all attempts to purify the enzyme, denaturants such as 8 molar urea, detergents such as

N-lauroylsarcosine or both have been used to solubilize the protein because of its lipophilic nature and tendency to be found in inclusion bodies[86,101-103]. LipY has been expressed in both *Escherichia coli* and *Mycobacterium smegmatis*[86,101-104]. In some cases, a histidine tag is placed at the N-terminus of the protein and used for nickel affinity chromatography as a purification step[86,101-104]. However, LipY was subjected to further purification by gel filtration chromatography in only 2 cases[86,102]. In one case, LipY eluted near the void volume of the gel filtration column where it likely existed as a soluble aggregate while the elution profile of LipY was not presented in the other study[86,102].

Role of LipY's PE Domain

The main function of LipY's PE domain is likely as a non-typical secretion signal that is recognized by the ESX-5 secretion system[68,85]. Generally, the PE domain is described as being ~110 amino acids[11]. However, LipY's PE domain has been described as being 99 amino acids[101]. This distinction agrees with evidence that the position of the YxxxD/E motif (YAAAE) responsible for export to the cell wall begins at position 88 and ends at position 92 of the N-terminus[68]. It is unclear how the YxxxD/E motif targets proteins to the cell wall but it has been suggested that, based on the crystal structure of a different PE protein, the secretion motif may be recognized by the ESX-5 membrane complex[35]. Once exported to the cell wall, the LipY's PE domain is proteolytically cleaved by an unknown mechanism between a glycine and alanine at positions 149 and 150, respectively[85]. Therefore, although the PE domain has been described as being made up of the first 99 amino acids of LipY, the physiological cleavage site of the PE domain is much further away. In addition to acting as a non-traditional secretion signal, it has

also been implied that LipY's PE domain is capable of regulating its lipase activity[101]. This result was demonstrated when Mishra et al. expressed full length LipY and a LipY mutant devoid of the PE domain (first 99 amino acids) in *M. smegmatis* and compared the hydrolase activity of increasing amounts of enzyme-containing cell wall fragments[101]. In the absence of the PE domain, there was a greater increase in LipY hydrolase activity compared to the full-length enzyme, which suggests that the PE domain is a downregulator of LipY activity.

Substrate specificity and activity

In 2006, Deb et al. tested the effects of several different conditions on recombinantly expressed and purified LipY activity[86]. First, it was discovered that purified LipY has a preference for hydrolyzing long-chain TAGs (12-22 carbon fatty acid chain) *in vitro* by using a radiometric assay with triolein as a substrate. Triolein is a triglyceride composed of glycerol and oleic acid, an unsaturated 18-carbon fatty acid. LipY demonstrated K_m and V_{max} values of 7.57 mM and 653.3 nmol/mg/min, respectively. The optimal pH for LipY activity is between 8 and 9 while LipY appears to retain activity at temperatures near 60°C. The effects of sodium dodecyl sulfate (SDS) and non-ionic detergents Triton X-100 and Tween-20 on LipY were also explored. 4 mM SDS completely inactivates LipY activity while a slight increase in activity is observed when LipY is incubated with 0.1% Triton X-100 (v/v). Interestingly, Tween-20 abolishes LipY activity above 0.5% (v/v). The active site serine residue of LipY is present in a GDSAG motif at the C-terminus of the protein, a conserved motif characteristic of the hormone-sensitive family of lipase[86]. The residues comprising the catalytic triad of LipY include serine 309, aspartate 383, and histidine 413[104].

LipY's role in virulence

LipY has also been implicated in *M. tuberculosis* virulence. The only available vaccine for protection against tuberculosis, the *M. bovis* BCG strain, has been used to study the effect of overexpression of LipY on host protection[105]. Balb/c mice immunized with recombinant *M. bovis* BCG overexpressing LipY challenged with the virulent *M. tuberculosis* H37Rv strain leads to decreased survival compared to mice immunized with the wild type vaccine strain[105]. In addition, mice immunized with purified recombinant LipY and challenged with *M. tuberculosis* H37Rv experienced increased survival compared to non-immunized mice suggesting LipY can be used as an antigen to provide protection against *M. tuberculosis* infection[105]. These mice also experienced an enhanced IL-2, IL-6, IFN-gamma and TNF cytokine response compared to non-immunized mice[105]. When mice were immunized with *M. bovis* BCG overexpressing LipY, the same cytokines were enhanced but to a lesser extent than the cytokines of mice immunized with the wild type *M. bovis* BCG strain[105]. Therefore, LipY appears to play a role in the virulence of *M. tuberculosis*.

A second study on the role of LipY in *M. tuberculosis* virulence demonstrated similar results. Singh et al. discovered that overexpression of LipY in *M. tuberculosis* H37Rv results in weight loss, a significant increase in bacterial load in the lungs, damage to the lungs and spleen and decreased survival of approximately one week of infected mice compared to mice infected with wildtype *M. tuberculosis* H37Rv[106]. Immunization of mice with recombinant purified LipY prior to challenge with *M. tuberculosis* H37Rv overexpressing LipY led to an increased median survival of approximately 2 weeks compared to non-immunized mice demonstrating the ability of LipY to have a protective

effect against infection[105]. The ability of LipY overexpression in *M. tuberculosis* H37Rv to cause increased mortality in challenged mice is likely a result of the decreased cytokine response compared to uninfected mice and mice challenged with wildtype *M. tuberculosis* H37Rv as evidenced by decreases in IL-2, Il-6, IL-17, TNF, and IFN-gamma[105].

Inhibitors against LipY

LipY has also been researched as a potential therapeutic target. The well-characterized lipase inhibitor, tetrahydrolipstatin (THL), has been shown to be a potent inhibitor of *M. tuberculosis* growth and disrupt *M. tuberculosis* morphology[102,107]. A derivative of the compound oxadiazolone called M*m*PPOX was also tested for its inhibitory function against recombinant purified LipY as well as 8 additional putative *M. tuberculosis* lipases and *M. tuberculosis* and *M. bovis* BCG cells[102]. M*m*PPOX inhibited LipY with an apparent inhibitory constant (K_i) of 7.7 μM[102]. It was also demonstrated that M*m*PPOX inhibited *M. tuberculosis* and *M. bovis* BCG growth with potency comparable to that of THL and it was inferred that this effect comes as a result of inhibition of LipY and related lipases[102]. Screening of different inhibitors against LipY activity lead to synthesis of several compounds, one of which displayed a half maximal inhibitory concentration (IC_{50}) value of 5.13 μM and very little cytotoxicity at high concentrations when tested against *M. tuberculosis* H37Rv suggesting their activities are specific to LipY[104].

The work presented here lays a foundation for studying and better understanding the enzyme kinetics of established bacterial lipases as well as the activities of putative enzymes from the *M. tuberculosis* PE/PPE family of proteins. The need for an improved

purification method and more thorough characterization of LipY and its PE domain is also addressed in this work. Not only does this work improve our overall understanding of LipY and the function of its PE domain but it also gives us insight into how additional PE and PPE proteins may behave differently in the absence of their N-terminal domains. The difference in the activities of these enzymes with and without their associated domains may ultimately impact the mechanism in which they are inhibited and, subsequently, the manner in which they must be investigated.

CHAPTER 2: MODULATION OF THE ACTIVITY OF MYCOBACTERIUM TUBERCULOSIS LIPY BY ITS PE DOMAIN[1]

Summary

Mycobacterium tuberculosis harbors over 160 genes encoding PE/PPE proteins, several of which have roles in the pathogen's virulence. A number of PE/PPE proteins are secreted via Type VII secretion systems known as the ESX secretion systems. One PE protein, LipY, has a triglyceride lipase domain in addition to its PE domain. LipY can regulate intracellular triglyceride levels and is also exported to the cell wall by one of the ESX family members, ESX-5. Upon export, LipY's PE domain is removed by proteolytic cleavage. Studies using cells and crude extracts suggest that LipY's PE domain not only directs its secretion by ESX-5, but also functions to inhibit its enzymatic activity. Here, we attempt to further elucidate the role of LipY's PE domain in the regulation of its enzymatic activity. First, we established an improved purification method for several LipY variants using detergent micelles. We then used enzymatic assays to confirm that the PE domain down-regulates LipY activity. The PE domain must be attached to LipY in order to effectively inhibit it. Finally, we determined that full length LipY and the

[1]A significant portion of Chapter 2 appeared in an article in PLOS One. The original citation is as follows:
Garrett CK, Broadwell LJ, Hayne CK, Neher SB (2015) Modulation of the Activity of *Mycobacterium tuberculosis* LipY by Its PE Domain. PLoS ONE 10(8): e0135447. doi:10.1371/journal.pone.0135447

mature lipase lacking the PE domain (LipYΔPE) have similar melting temperatures. Based on our improved purification strategy and activity-based approach, we concluded that LipY's PE domain down-regulates its enzymatic activity but does not impact the thermal stability of the enzyme.

Introduction

Mycobacterium tuberculosis (Mtb) is remarkably adept at interfering with host cellular processes in order to evade destruction. This ability depends on the secretion of virulence factors that modify the host environment. One family of proteins, known as PE and PPE proteins, are involved in immune evasion and virulence[43,108,109]. PE/PPE proteins are unique to mycobacteria; they were initially discovered when sequencing of the *Mtb* genome revealed approximately 160 genes encoding proteins with Pro–Glu (PE) or Pro–Pro–Glu (PPE) motifs near their N-termini[11]. Subsequent analysis revealed that PE/PPE proteins comprise about 7% of the coding capacity of the *Mtb* genome[110]. Although PE/PPE domains have been identified in both pathogenic and saprophytic mycobacteria, pathogenic mycobacteria maintain the highest number of PE/PPE proteins[111].

The PE motif is a moderately conserved, 110-residue domain found at the N-terminus of PE proteins[11]. The PPE motif is a distinct, but also conserved, domain of about 180 residues found at the N-terminus of PPE proteins[11]. The C-terminal domains of both PE and PPE proteins are highly variable and can encode enzymatic domains, conserved sequence motifs or large, repeated arrays of peptide motifs[11,110]. Genes encoding PE and PPE proteins are often proximal on the *Mtb* genome and functionally linked[112]. In fact, structural studies show that in some cases, PE and PPE proteins form

heterodimeric complexes[33]. PE/PPE gene families co-evolved with specialized, type VII secretion systems important to *Mtb* virulence known as the ESX secretion systems[113]. The *Mtb* genome encodes five type VII secretion systems, named ESX-1 to ESX-5[51]. Studies using both *Mtb* and *Mycobacterium marinum* revealed that several PE and PPE proteins depend on ESX-5 for export[79,84].

LipY is a PE protein with a C-terminal triglyceride (TG) lipase domain[86]. LipY is proposed to have a dual role in *Mtb* pathogenesis[106]. First, *Mtb* is known to store host-derived TGs in lipid droplets that provide fuel during reactivation from dormancy[93,100,114]. LipY is the primary contributor to the break down of these stored TGs[86]. Next, overexpression of LipY has been implicated in increased *Mtb* virulence as shown by the enhanced mortality of TB-infected mice[106]. The increased mortality associated with LipY overproduction is attributed to down-regulation of host immunity by the products of LipY TG hydrolysis[99,106]. These two roles for LipY are consistent with the observation that LipY is found both intracellularly and on the cell exterior[85].

LipY lacks a classic secretion signal but contains an YxxxD/E motif (Y-A-A-A-E) beginning at position 88 of its PE domain. The YxxxD/E motif is found in several other PE proteins and appears to be a general secretion signal required for recognition by the ESX-1 and ESX-5 secretion systems[68]. In LipY the motif is essential for secretion by ESX-5[68]. In some ESX and PE/PPE protein pairs, the YxxxD/E motif in one protein forms a joint motif with the sequence WxG present in its partner[70]. However, there is little evidence to suggest LipY has a PPE binding partner necessary for secretion[33,115].

Upon export to the cell wall, LipY's PE domain is removed by proteolytic cleavage[85]. One study using the cell wall fraction of *Mycobacterium smegmatis*

containing LipY hinted that LipY's PE domain could down-regulate its enzymatic activity[101]. This study also showed that mycobacteria expressing LipY lacking its PE domain exhibited a greater reduction in intracellular TG pools than mycobacteria expressing LipY. Therefore, it appears that although the Y-A-A-A-E motif in the N-terminus of LipY is necessary for its export to the cell wall, the PE domain likely has additional, unexplored functions. Here, we utilize biochemical assays with purified proteins and determined that LipY's PE domain regulates its enzymatic activity.

Materials and Methods

LipY, LipYΔPE and PE Domain Purification

LipY, LipYΔPE (amino acids 150-437) and the PE Domain (1-149) were cloned into pET16b expression vectors (Novagen) with a C-terminal His tag. The proteins were expressed in BL21 (DE3) *E. coli* cells and grown to an OD_{600} of ~0.7 at 37°C. Protein expression was induced with 0.7 mM isopropyl-beta-D-thiogalactopyranoside and cells were shaken at 18°C for 16 h. Cells were then pelleted at 5,900 x g for 25 min and the pellet suspended in buffer A (20 mM HEPES pH 7, 300 mM NaCl, 10 mM imidazole, 10% glycerol) before being lysed in a Nano DeBEE Laboratory homogenizer (BEE International) at 20,000 psi. The lysates were cleared by centrifugation at 23,000 x g for 40 minutes. The resulting pellet was resuspended in buffer B (20 mM HEPES pH 7, 300 mM NaCl, 10 mM imdazole, 10% glycerol, 0.5% w/v N-laurylsarcosine) with agitation at 4°C for 1 hour (incubation time is critical) followed by centrifugation at 23,000 x g for 40 minutes. The supernatant was then incubated with pre-equilibrated nickel-nitrilotriacetic acid resin (Qiagen) with gentle agitation for 1 hour. The resin was washed with 4 column volumes of buffer A followed by elution with buffer C (20 mM HEPES pH 7, 300 mM

NaCl, 350 mM imidazole, 10% glycerol). Eluted protein was incubated with Tween 20 with gentle agitation for 1 hour at 4°C and then loaded onto a pre-equilibrated Superdex 200 10/300 HR gel filtration column (GE Healthcare) with Buffer D (20 mM HEPES pH 7, 100 mM NaCl, 10% glycerol). Protein was eluted over 1 column volume with Buffer D. Protein that eluted between 8 and 10 mLs was collected and labeled 'Peak 1' while protein that eluted between 12 and 14 mL was labeled 'Peak 2'. Protein was flash frozen in liquid nitrogen and stored at -80°C. A final purity of >95% was achieved for both LipY and LipYΔPE (S1 Fig). Because LipY and LipYΔPE were purified with detergent, we tested their ability to hydrolyze a natural triglyceride substrate in buffer that did not contain detergent and found that both were active (S2 Fig).

Quantification of active LipY and LipYΔPE by activity-based protein profiling (ABPP)

LipY and LipYΔPE were diluted to the same concentration using ABPP buffer (20 mM Tris pH 8, 100 mM NaCl). A 1.5 molar excess of ActivX TAMRA-FP serine hydrolase probe (Thermo Scientific) was added to each sample and incubated for 30 minutes. The reactions were quenched with addition of SDS loading dye and boiling for 10 minutes. To quantitate the amount of active protein, a standard of the probe alone was prepared by serial dilution with ABPP buffer. All samples were resolved using SDS-PAGE and imaged using a Typhoon Trio+ imager (GE Healthcare Life Sciences). ImageQuant TL software was used for quantification of bands corresponding to the TAMRA-FP serine hydrolase probe alone, probe-labeled LipY and probe-labeled LipYΔPE.

Enzymatic Assays

Michaelis-Menten kinetics were measured as previously described[116]. Briefly, LipY or LipYΔPE was diluted in assay buffer (20 mM Tris pH 8, 150 mM NaCl, 0.2% Fatty acid free BSA, and 0.4% (v/v) Triton X-100) to a set concentration according to quantification by ABPP. Next, the fluorogenic substrate 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) known as DGGR (Sigma Aldrich) was added. Substrate hydrolysis was measured by monitoring fluorescence with a 529 nm excitation wavelength and reading emission at 600 nm with a 590 nm cutoff filter over 30 minutes at 37°C. Non-enzymatic hydrolysis was subtracted and initial rates of hydrolysis were calculated using the first 10% of data. Initial rates were plotted against substrate concentration and fitted to the Michaelis-Menten equation using KaleidaGraph software.

Circular Dichroism Measurements

Thermal stability assays were measured as previously described with some modifications[117]. Briefly, LipY or LipYΔPE was diluted to 10 μM in Buffer D and data were collected at 20°C. Molar ellipticity of thermal denaturation was monitored at 222 nm from 4-90°C. Buffer D was used as a blank and all data were converted to mean residue ellipticity (MRE) using eq 1, where Θ is MRE, signal is the CD signal, C is the protein concentration (mM), n is the number of amino acids and l is the cell path length (cm)[118].

$$\Theta = (100 \times signal) \div (C \times n \times l) \qquad (1)$$

Results

Detergent Separates Lipy into 2 Distinct Peaks

Most PE/PPE proteins do not have well-characterized functions, but LipY is a well-characterized lipase. We therefore used LipY to determine how the PE domain affects its enzymatic activity. To carry out these studies we first needed to resolve the oligomeric state of active LipY. Although LipY is a 45 kDa protein, previous reports of its purification show that it elutes from a gel filtration column at or near the void volume, indicating a protein of over 600 kDa[86]. LipY purified from this void fraction exhibited TG hydrolysis activity and in a previous study was used for its biochemical characterization[86]. We developed an improved purification protocol in which LipY migrates in the included volume of a Superdex 200 gel filtration column (Fig 2A, gray trace). To achieve this, we utilized detergent micelles to help solubilize nickel-purified LipY. Lipases function at the lipid-water interface, and they are frequently purified and crystallized in the presence of detergent micelles[119,120]. After addition of Tween 20 (CMC= 0.6 mM) at a 270-fold molar excess over LipY, we observed that LipY elutes from a size exclusion column in 2 distinct peaks: one is in the void volume (Peak 1) and one in the included volume (Peak 2). By contrast, when LipY was not supplemented with detergent, only Peak 1 was observed (Fig 2A, black trace). We next tested the contents of both peaks by Western blot. These experiments confirmed that LipY migrates mainly in Peak 1 in the absence of Tween 20 but migrates in Peak 2 when Tween 20 is added (Fig 2, inset).

This result raised the question of whether LipY was migrating independently or in/on the detergent micelles. We next ran a sample containing only Tween 20 micelles on

the size exclusion column (Fig 2B). This trace revealed that LipY from Peak 2 matched the elution profile of the Tween 20 micelles. Next, we wanted to determine if the protein in Peak 1 was refractory to solubilization by detergent. We supplemented LipY isolated from Peak 1 with Tween 20, repeated the purification strategy using gel filtration and observed both Peak 1 and Peak 2 in the elution profile (Fig 2B, black trace). The appearance of LipY in both peaks was confirmed via immunoblot analysis. Taken together, these results indicate that Tween 20 micelles can extract LipY from a large, soluble aggregate.

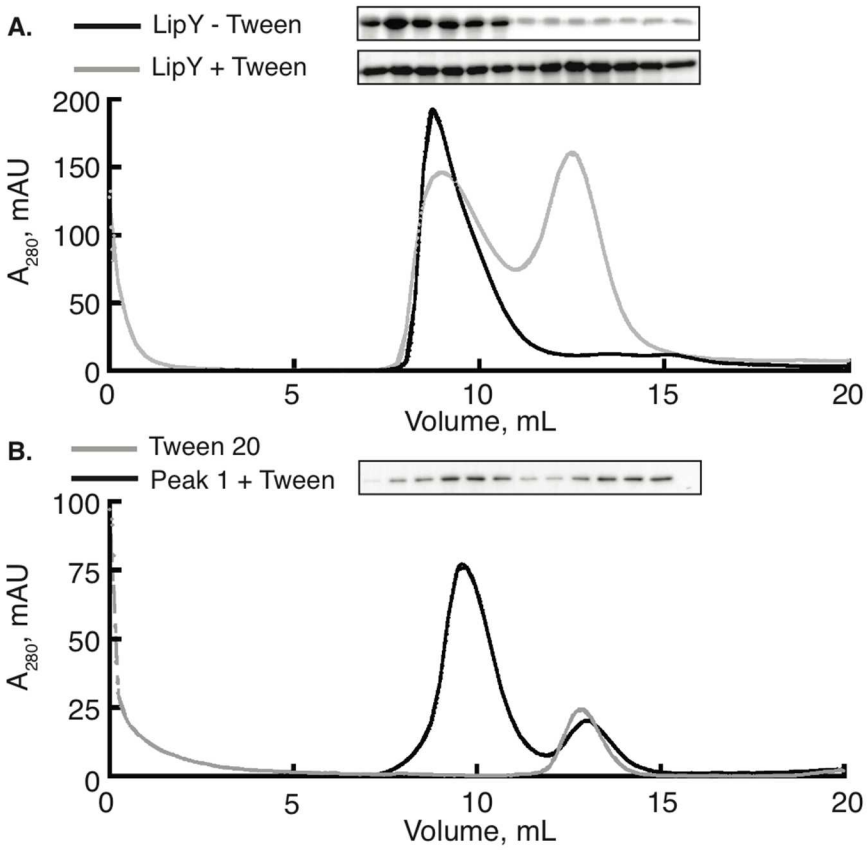


Figure 2. Size Exclusion Chromatography of Lipy With or Without Tween 20.
(A) LipY purified via Nickel affinity chromatography was resolved on a Superdex 200 gel filtration column with (gray trace) or without (black trace) pre-incubation with a 270 fold molar excess of Tween 20. In the presence of detergent a second peak in the included volume appears whereas a single peak in the included volume is observed when no detergent is present. (B) LipY purified in the void volume (Peak 1) was pre-incubated with a 270 fold molar excess of Tween 20 and subjected to size exclusion chromatography (black trace). This trace is overlayed with a trace of 6 mM Tween 20 alone (gray trace). Western blots against the His tag of LipY were used to detect the presence of protein eluted from the gel filtration column in each experiment.

The PE Domain of Lipy Contributes to Formation of Lipy Aggregates in Peak 1

We next sought to determine whether the PE domain of LipY or the mature lipase (LipYΔPE) is responsible for LipY aggregation. On one hand, PE proteins are known to form dimers with their cognate PPE partner for recognition and exportation by unique type VII secretion systems[33,121]. Conversely, there are many examples of bacterial lipases that are prone to aggregation upon purification[120,122]. To answer this question, we first expressed and purified recombinant LipYΔPE (residues 150-437) and recombinant PE domain (residues 1-149) using nickel affinity chromatography. Consistent with the purification of LipY, we added Tween 20 at a 270 fold molar excess over protein to help solubilize the purified proteins prior to size exclusion chromotography. The elution profile for the PE domain revealed the formation of two distinct peaks as we previously observed with full length LipY (Fig 3). However, the elution profile of LipYΔPE featured a distinct peak in the included volume with a smaller, broad peak in the void volume. Immunoblot analysis of the size exclusion column fractions of LipYΔPE and the PE domain revealed that the majority of purified PE domain is in the void volume (Peak 1) whereas the majority of purified LipYΔPE is in the included volume (Peak 2). The data show that removal of the PE domain from purified LipY dramatically reduces its aggregation into Peak 1. Taken together with data from Figure 2, these results demonstrate that the PE domain drives the aggregation of LipY.

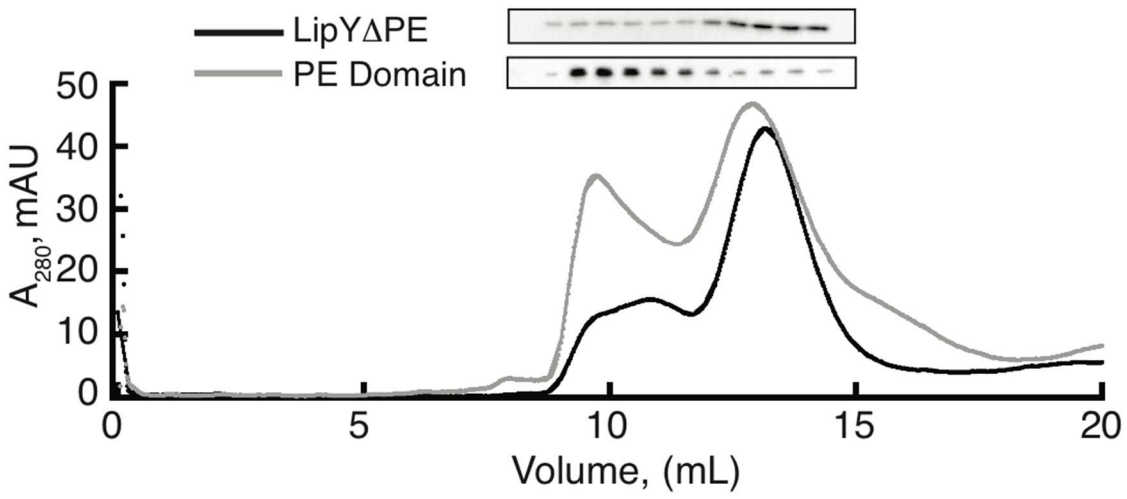


Figure 3. The PE Domain Contributes to Lipy Aggregation *in vitro*.
LipYΔPE and the PE domain were resolved on a Superdex 200 gel filtration column. Both proteins were pre-incubated with a 270 fold molar excess of Tween 20 for 1 hour prior to being resolved on the column. In the gray PE domain trace, Peak 1 (~9.5 mL) elutes near the void volume and Peak 2 (~13 mL) elutes in the included volume. In the black LipYΔPE trace, a weak A_{280} signal was observed between 9 and 12 mL and a single distinct peak (Peak 2) elutes in the included volume. Western blots against the His tag of each construct show that the majority of the PE domain elutes in the void volume while the majority of LipYΔPE elutes in the included volume.

Lipy from Peak 2 is More Active Than Lipy from Peak 1

Although LipY from Peak 1 elutes in the void as a high molecular weight aggregate, our lab and others have observed that the LipY purified from Peak 1 maintains lipase activity[86]. We therefore set out to compare the fraction of active LipY purified in Peak 1 and Peak 2. First, we used activity-based protein profiling (ABPP) to measure the amount of active enzyme in both Peak 1 and Peak 2. In this method, the reactive fluorophosphonate group of the activity-based probe (ABP) forms a covalent bond with the active site serine residue of *active* serine hydrolases, including lipases with a 1:1 ratio of ABP:active site[123]. We calculated the amount of active enzyme in each peak (plotted in Fig 4A). LipY in Peak 2 contains more active enzymes (~45% active) than Peak 1 (~32% active). We then compared the Michaelis-Menten kinetics of LipY from Peak 1 and Peak 2 using the long-chain lipase substrate DGGR (Fig 4B). These experiments confirmed that LipY in Peak 2 (V_{max}= 3.2 ± 0.3 RFU s^{-1} $pmol^{-1}$ *total* LipY) contains more active enzyme molecules than LipY in Peak 1 (V_{max}= 2.5 ± 0.2 RFU s^{-1} $pmol^{-1}$ *total* LipY). The K_m values for Peak 1 and Peak 2 are within the experimental error of each other (5.0 ± 1.5 and 4.8 ± 1.1 μM, respectively). However, when comparing the enzyme kinetics of equal amounts of *active* LipY from Peak 1 and Peak 2 based on our ABPP measurement, we observed almost no difference in the V_{max} (S3 Fig). As a result, we concluded that although LipY from Peak 2 to contains more active enzymes than LipY from Peak 1, the enzymes from both fractions are catalytically equivalent.

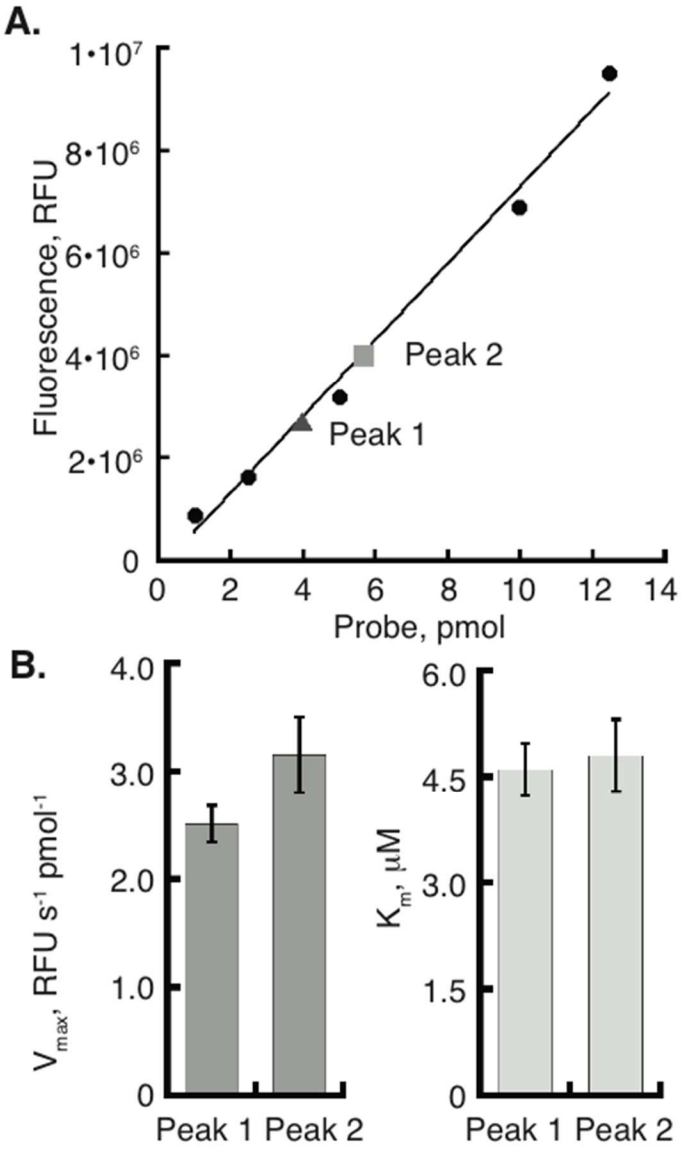


Figure 4. There are More Active Lipy Molecules in Peak 1 Compared to Peak 2. (A) Equal concentrations of *total* LipY Peak 1 and Peak 2 were incubated with a 1.5 molar excess of TAMRA-FP serine hydrolase probe for 30 minutes at room temperature. A standard was created using the probe alone. The amount of active Peak 1 and Peak 2 were calculated based on the standard curve. (B) Bar graph showing the V_{max} and K_m from Michaelis-Menten curves comparing equal amounts of *total* LipY from Peak 1 and Peak 2 using the DGGR substrate. Error bars represent the standard error of the mean of 6 independent measurements[124].

To confirm that Peak 2 is more active than Peak 1 we used an established method to compare the activities of the two peaks[125]. We used a separate protein purification as we observed that the exact fraction of active protein in each peak varied from purification to purification but Peak 2 was always more active than Peak 1. Tetrahydrolipstatin (THL) is a lipase inhibitor that covalently modifies the active site of lipases[126,127]. LipY (5 nM) was titrated with increasing concentrations of THL, and the residual lipase activity was compared to untreated LipY. Residual LipY activity was measured using the small, soluble substrate *p*-Nitrophenyl butyrate (pNPB) at a concentration of 200 μM, which is 2 fold over the K_m for LipY hydrolysis of pNPB. Consistent with data shown in Figure 4, LipY in Peak 2 is more active than LipY in Peak 1 (S4 Fig). Protein purified from Peak 2 was used in all subsequent experiments as our studies show that it is the disaggregated, more active form of the enzyme.

Lipyδpe is More Active Than Lipy

A study comparing cells expressing LipY vs. LipYΔPE reported that the crude cell wall fraction from the LipYΔPE-expressing cells had greater enzymatic activity[101]. These results suggest that the PE domain may down-regulate LipY enzymatic activity. However, because this result was obtained using a crude cell wall fraction, it could also be explained by loss of an inhibitor-binding site as a result of the deletion of the PE domain. Additionally, the authors created the initial LipYΔPE construct by deleting the first 99 residues from LipY's N-terminus. Subsequently, experimental evidence showing the precise cleavage site (after glycine 149) for removal of the PE domain emerged[85]. We tested the role of the PE domain in LipY activity by comparing full length LipY and LipYΔPE generated using the physiological cleavage site. To compare the activities of

LipY and LipYΔPE, we first employed the method used in Figure 4A to calculate the amount of active protein. After determining the amounts of active LipY and LipYΔPE using the ABPP method, we compared Michaelis-Menten kinetics of equal amounts of active protein using DGGR as a substrate. These data show that LipYΔPE is more active than LipY, as shown by the representative plot in Figure 5A. Analysis of multiple data sets revealed V_{max} values of 6.3 ± 0.8 and 11.3 ± 1.3 RFU s^{-1} $pmol^{-1}$ for LipY and LipYΔPE, respectively, indicating a significant difference in activity ($p<0.015$) between the two LipY variants (Fig 5B, left graph). Loss of the PE domain does not affect substrate binding, as the K_m values are equivalent for LipY and LipYΔPE (Fig 5B, right graph).

As shown in Figure 3, we successfully expressed and purified the 149-amino acid PE domain of LipY. We tested the ability of the purified PE domain to act as an inhibitor of LipYΔPE *in trans*. Increasing amounts of PE domain were incubated with a constant amount of LipYΔPE for 10 minutes prior to testing lipase activity. The initial velocity was plotted against substrate concentration and data for each concentration of PE domain tested were fitted to the Michaelis-Menten equation. We observed negligible differences in activity of LipYΔPE alone and LipYΔPE pre-incubated with PE domain (Fig 5C). This lack of inhibition indicates that the PE domain only inhibits LipY when it is part of the same molecule.

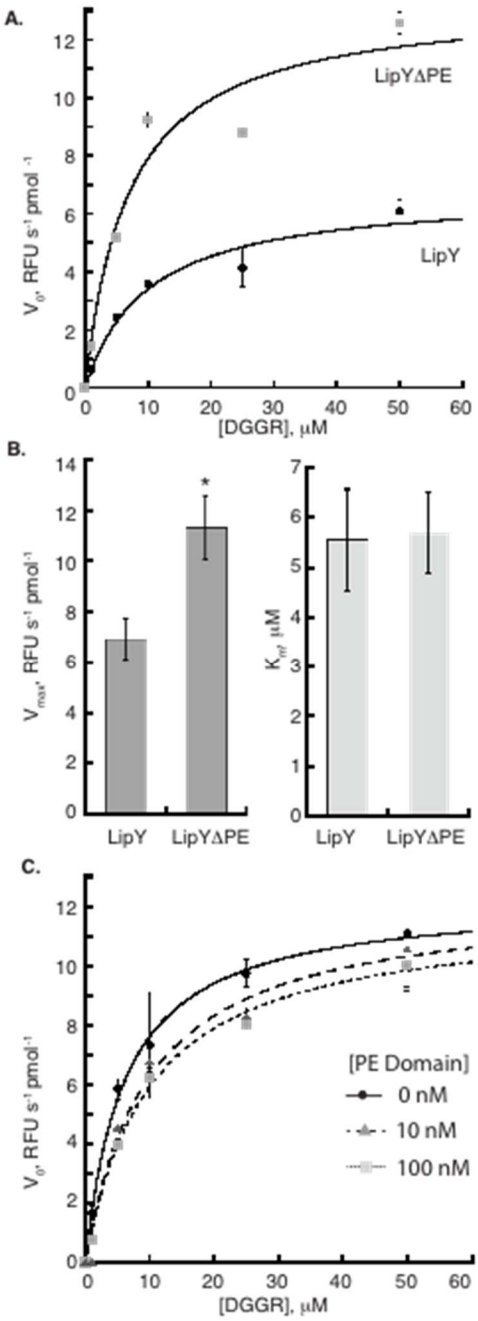


Figure 5. Lipy's PE Domain Downregulates its Enzymatic Activity.
(A) Equal amounts of *active* LipY and LipYΔPE as determined by ABPP were used to compare the Michaelis-Menten kinetics of the two constructs. Hydrolysis of the lipase substrate, DGGR was monitored at 37°C. Initial velocities for a representative data set were fit to the Michaelis-Menten equation and plotted against substrate concentration. (B) Analysis of multiple data sets reveals that LipYΔPE has a V_{max} that is approximately two fold higher than the V_{max} for LipY (left graph). However, the K_m is not affected (right graph). Error bars represent the standard error of the mean of 6 independent measurements[124]. "*" corresponds to $p<0.015$ of the V_{max} of LipY compared to LipYΔPE. (C) LipYΔPE was titrated with 0, 10 or 100 nM PE domain and incubated for 10 minutes before testing its residual lipase activity as described in (A).

The PE Domain Does Not Affect Thermal Stability of Lipy

Finally, we asked if the PE domain might affect the thermal stability of full length LipY. We probed the thermal stability of both LipY and LipYΔPE by measuring their thermal denaturation using circular dichroism (CD) spectroscopy. Based on far-UV CD scans of both proteins, we elected to measure unfolding at 222 nm. Both constructs showed a single thermal unfolding transition. Furthermore, the melting temperatures (T_m) of the two constructs were very similar (Fig 6). Both constructs had T_ms near 75°C, which suggests that even in the absence of the PE domain LipY is highly stable. This is in good agreement with a previous study measuring the activity of LipY as a function of temperature. In this study LipY retained partial activity even after incubation at 60°C for 15 minutes prior to an activity assay[86].

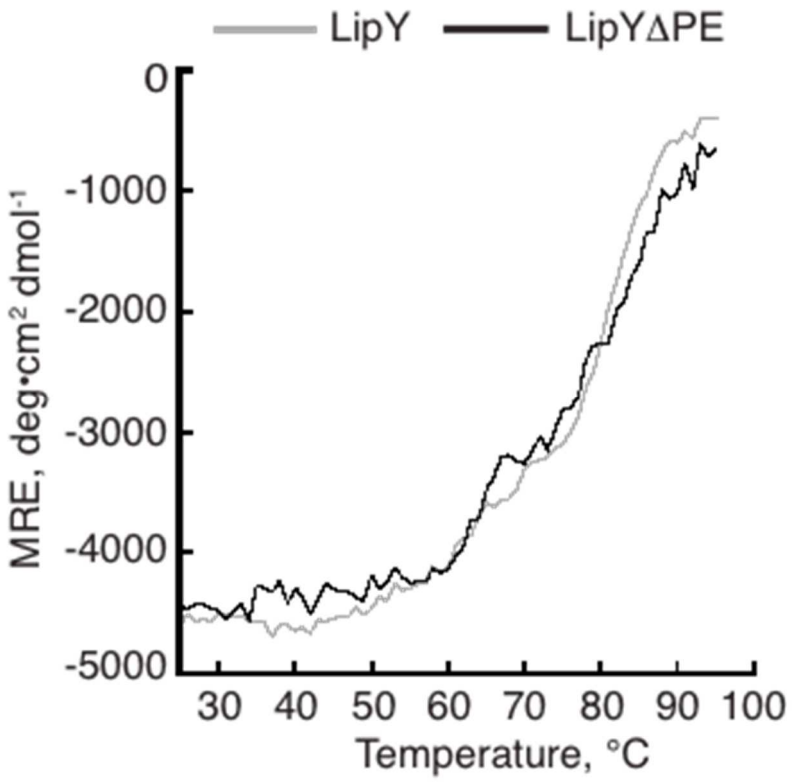


Figure 6. LipY and LipYΔPE Share Similar Thermal Unfolding Profiles.
CD temperature scans of LipY (black) and LipYΔPE (gray) monitored at 222 nm between 25 and 95°C. Data points were collected every 1°C.

Discussion

PE and PPE proteins of *Mtb* were first described almost 17 years ago, and their molecular functions are still not fully understood. Studies with individual PE/PPE proteins suggest that they serve to target their C-terminal cargo to the type VII secretion systems[85,128,129]. However, the physiological utility of employing a large, aggregation prone domain to target proteins for secretion is not clear, and it is tempting to speculate that the PE domain may have additional roles. LipY represents one of the best candidates for studying the PE domain because its enzymatic activity is well established and the cleavage site for the PE domain is known. In the case of LipY, the PE domain has at least two distinct functions. First, it is clear that the YxxxD/E motif acts as a secretion signal for export of the mature lipase to the cell wall[68]. Second, the PE domain has been implicated as a regulator of enzyme activity[101]. Here, we utilize biochemical assays to investigate this observation because experiments initially describing this regulatory function were carried out using crude cell wall fraction. Thus, it is possible that other factors interacted with the PE domain to regulate LipY activity. Furthermore, only the first 99 amino acids were removed from LipY, as it was only later demonstrated that the PE domain precedes a linker region and the physiological cleavage site is after amino acid position 149[85].

We therefore compared the lipase activity of purified LipY and LipYΔPE generated using the physiological cleavage site. We first needed to de-aggregate LipY and did so by utilizing Tween 20 detergent micelles. These experiments led to the finding that the PE domain of LipY provides an interface for its aggregation, as the isolated PE domain aggregated whereas LipYΔPE was less susceptible to aggregation. Our data

imply that PE proteins, especially those associated with the cell wall of *Mtb,* are prone to aggregation via their PE domain. This should be taken into consideration when purifying proteins from this family. We next quantified the amount of active enzyme molecules using an activity-based method to compare equal amounts of active LipYΔPE and LipY. We observed a clear increase in the maximum reaction velocity of purified LipYΔPE compared to LipY thus confirming that the PE domain does indeed down-regulate LipY activity. Finally, we added the isolated PE domain back to LipYΔPE and found that it did not inhibit *in trans.*

Thus, the PE domain must be attached to the mature lipase in order to inhibit lipase activity. It is likely that this connection is necessary because it increases the local concentration of the PE domain at the site of inhibition. In data not shown, we tested PE domain concentrations up to 0.5 μM but were not able to test higher concentrations due to the risk of PE domain aggregation. Our kinetic analysis shows that LipY has a lower V_{max} and an equivalent K_m as compared to LipYΔPE. This is characteristic of noncompetitive inhibition, in which an inhibitor affects enzyme activity by binding to a site other than the substrate-binding site (for example an allosteric site)[130]. However, understanding the precise molecular mechanism by which the PE domain reduces LipY activity will require further mechanistic studies.

Our study includes a useful technical advance. We used three different methods to compare the activities of LipY from Peak 1 and Peak 2: ABPP, active site titration using THL, and Michaelis-Menten kinetics using a model triglyceride substrate. All methods demonstrated that LipY from Peak 2 was more active than LipY from Peak 1. Generally, ABPP has been used as a proteomics tool for screening inhibitors and identifying

enzymes of interest in cell fractions[131]. Our study shows that the TAMRA-FP serine hydrolase activity based probe is also useful to quantify enzyme active sites. Because the ABPs only label active protein, this approach offers a quicker, easier way to quantitate enzyme active sites than using pre-steady state kinetics or active site titration.

LipY functions both intracellularly and extracellularly, and the PE domain is removed upon secretion[85]. Our data suggest that the absence of the PE domain allows the mature lipase to better hydrolyze TGs outside the cell, thus tuning LipY activity to its environment. The observations that the PE domain reduces LipY activity leads to the question of whether this is a LipY-specific phenomenon or a common occurrence. Currently, few other PE/PPE proteins have known enzymatic functions. It is also not well established if other PE/PPE domains are cleaved upon secretion. However, some studies do hint that this may be true for certain PE/PPE proteins. For example, a 2-D gel analysis showed that a number of proteins dependent on ESX-5 for secretion into the culture were present at lower than expected molecular weights[84]. Several PE/PPE proteins were among this group. As the biological functions of additional PE/PPE proteins are uncovered, careful biochemical analysis will provide a better understanding of the full significance and functionality of PE/PPE domains.

CHAPTER 3: DISCUSSION

Conclusions

Until now, LipY has only been purified as a soluble aggregate, eluting only in the void volume from a gel filtration column[86]. Problems with the purification of LipY have mostly arisen as a result of the enzyme's lipophylic properties. Denaturants such as urea, and detergents have been used to solubilize LipY when it has been expressed in *E. coli*. However, it has still proven difficult to purify LipY in a non-aggregate state. Here, I present a new a purification method for LipY that utilizes Tween 20 micelles to disaggregate aggregated LipY. Presumably, this method can be used to purify additional mycobacterial lipases and lipohilic proteins. Importantly, LipY variants (LipYΔPE and the PE domain) share similar properties as LipY when purified using this method.

ABPP Method for Active Site Quantitation

In addition to establishing a purification protocol for LipY, I also introduced a novel method for active site quantitation using activity-based protein profiling (ABPP). Generally, there are 2 methods used to quantify active enzyme molecules in vitro: 1) burst phase kinetics and 2) active site titration. With both approaches, either a well-defined, active site-specific substrate or inhibitor is required. With regards to burst phase kinetics, usually either a chromogenic or fluorophore-containing substrate must be used in order to monitor enzyme activity. In addition, the rate of the reaction must also be considered in order to obtain data that reflects the rate of the enzymatic reaction as opposed to autohydrolysis (non-enzymatic activity). Active site titration methods also

require a tight-binding or covalent inhibitor specific to the enzyme's active site. Residual enzyme activity can be measured and in some cases isothermal titration calorimetry (ITC) can be used to measure enzyme-inhibitor stoichiometry.

Using ABPP, I have introduced a method of active site quantification that requires minimal reagents and is reliable. Generally, fluorescent ABPP is used to identify enzymes in a particular proteome by labeling cell lysates or subcellular fractions with a probe, separating the proteins using SDS-PAGE and performing an in-gel fluorescence analysis[131]. The cell fractions or lysates can also be manipulated by adding inhibitors or potential inhibitors to test their activities against enzymes within a particular proteome. In this work, I describe a method in which the commercially available serine hydrolase-specific activity-based probe (ABP) is used to create a standard with as little as 2.5 pmols of the probe. Equal amounts of the serine hydrolase or serine hydrolases with an unknown active site concentration can then be labeled with a 1.5 fold molar excess of the ABP and reacted for 30 minutes. After labeling, the reaction is quenched with an excess of loading dye, boiled for 5-10 minutes, and separated using SDS-PAGE before performing in-gel fluorescence. Quantitation software is then used to quantify the ABP standard and the amount of labeled enzyme. This method produces a reliable value of the moles of active enzyme in a particular sample and, upon further testing, can be applied to quantifying lipases, serine proteases, esterases, cutinases and several other serine hydrolase family proteins.

Testing Inhibitory Function of Candidate PE/PPE Domains

It is still unclear whether or not the inhibitory role of the PE domain is a common function or is unique to LipY. In order to test the potentially ubiquitous inhibitory

property of PE and PPE domains, enzymatic assays similar to what have been described in this work could be carried out. There are 5 PE (Rv0151c, Rv0152c, Rv0159c, Rv0160c, Rv1430) and 3 PPE (Rv1800, Rv2608, Rv3539) proteins with predicted C-terminal α/β hydrolase folds based on *in silico* data[36]. It is therefore likely that these enzymes possess some sort of serine hydrolase activity (lipase, cutinase, esterase, protease, etc.). For example, Rv1430 was subsequently discovered to possess esterase activity based on experimental data[37]. Therefore, it is plausible that, if PE/PPE domains act in a regulatory capacity in addition to their other functions such as targeting the protein to the cell wall, these 8 candidate PE/PPE domains may also possess that regulatory function. This hypothesis could be tested using chimeras with the candidate PE/PPE domains at the N-terminus and the linker and lipase domains of LipY at the C-terminus.

A similar enzymatic assay as described in the previous chapter can be used to test the putative inhibitor ability of candidate PE/PPE domains in the LipY-PE/PPE chimeras using the fluorogenic lipase substrate, DGGR. Based on the inhibitory function of LipY's PE domain when attached to LipY, I anticipate that some, if not all, of the LipY-PE/PPE chimeras tested will possess some inhibitory function evidenced by a decreased maximum reaction velocity (V_{max}). This result would suggest that a common inhibitory motif exists in some PE/PPE proteins. Another characteristic that would need to be considered when testing inhibition of LipY by candidate PE/PPE domains is whether or not those PE/PPE proteins are exported and whether or not the domains themselves are cleaved. This impacts how the inhibitory function of such domains can be exploited for therapeutic development. Only the LipY's PE domain has been experimentally determined to be proteolytically cleaved after export to the cell wall although it has been

suggested that other PE/PPE domains may also be cleaved after export via the ESX-5 secretion systems[80,85]. It is unclear whether or not the candidate PE/PPE proteins are exported and whether or not their respective PE or PPE domains are cleaved.

Probing Enzymatic Functions of Candidate PE/PPE Proteins

Only one of the 8 candidate PE/PPE proteins previously discussed has been successfully expressed, purified and had its enzymatic activity tested[37]. A likely reason for the lack of data concerning the 7 remaining PE/PPE proteins can likely be attributed to difficulties in purifying the proteins, as we know many PE/PPE proteins localize to the *M. tuberculosis* lipid-rich cell wall. The experiments described in the previous chapter provide a methodology for expressing, purifying and testing the activity of these putative enzymes. Here, I propose an approach that could provide at least two important pieces of information regarding PE/PPE proteins: 1) whether or not the 7 candidate PE/PPE proteins possess some hydrolase activity and 2) whether or not their PE/PPE domains possess some inhibitor function. We could clone, express and purify the previously mentioned PE/PPE candidates in the presence and absence of their PE or PPE domain using a similar approach used to purify LipY. Then, using p-Nitrophenly butyrate (pNPB), a general substrate with a short 4-carbon chain to test esterase activity, our lab could test the activities of the different variants. pNPB is a suitable substrate because it is unclear whether the candidates are lipase, cutinases, proteases, etc.

The expected outcome from these experiments would be the discovery of additional PE/PPE proteins with C-terminal enzymatic domains. This would be a major discovery in defining the functions of PE/PPE proteins. The other potential outcome of these experiments would be the discovery of PE/PPE domains with inhibitory functions.

If we compared the activities of the full-length PPE candidate Rv1800 and its PPE domain mutant, for example, the mutant protein may have a higher V_{max}, indicating it has an inhibitory role. The work presented here indicates that these possibilities are worth testing, are feasible and could be beneficial to researchers interested in PE/PPE proteins as therapeutic targets.

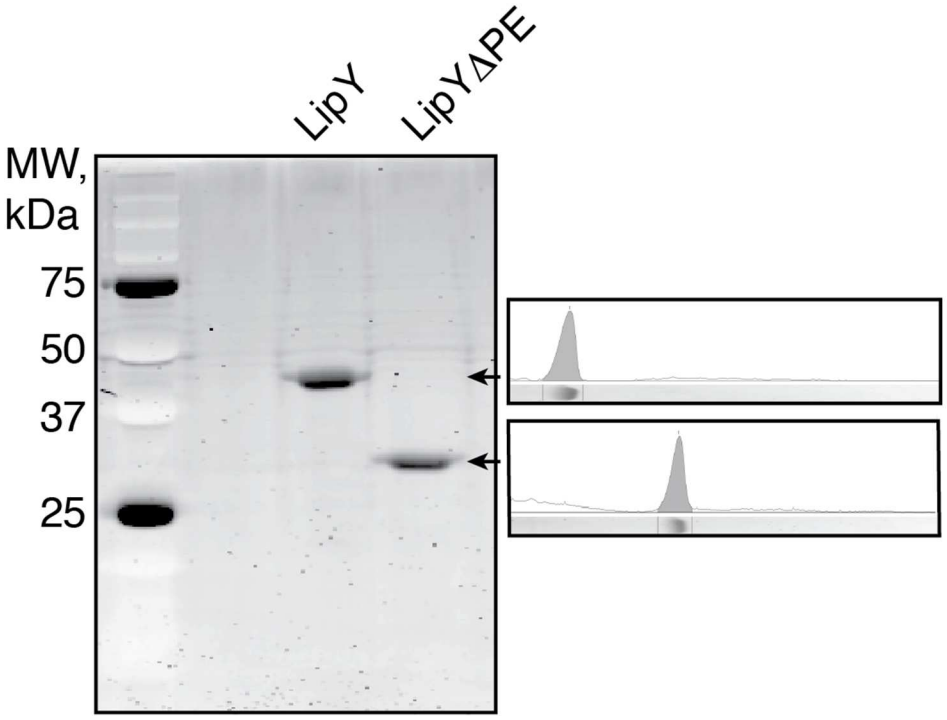
Broader Implications

The work presented in this body provides a template of sorts for recombinantly expressing, purifying, quantifying and measuring the enzymatic activity of potential PE and PPE proteins. This work also provides a framework for testing the inhibitory properties of PE and PPE domains against enzymatic activity. Future experiments, as proposed in this chapter, will likely result in a discovery of additional PE/PPE proteins with C-terminal hydrolase domains as well as the discovery of additional PE/PPE domains with regulatory properties. The discovery of previously unidentified PE/PPE enzymes could provide researchers with more information on how to best target these PE/PPE proteins with therapeutics.

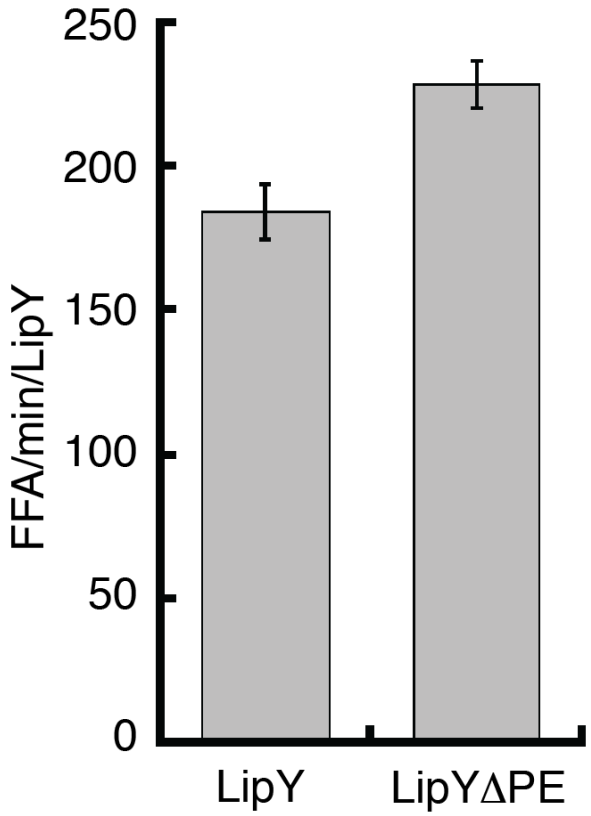
It could also be the case that the outcomes of the proposed experiments are negative, indicating that LipY and Rv1430 are the only PE/PPE proteins with enzymatic functions. This outcome would also be beneficial to the scientific community as it would focus attention away from discovery of novel PE/PPE enzymes and towards better characterizing and targeting the current PE/PPE enzymes (LipY and Rv1430) for therapeutic discovery. In this scenario, which is the current state with respect to discovery of PE/PPE enzymes, researchers should consider the different catalytic properties of full length LipY (intracellular) and mature LipY (cell wall-associated) when assessing

accessibility of the target by inhibitors and screening inhibitors as this could affect the efficacy of the prospective therapeutic. Inhibitors targeting mature LipY located on the cell wall of *M. tuberculosis* would likely have a greater affect on *M. tuberculosis* growth compared to full length LipY as they would not have to cross the cell wall.

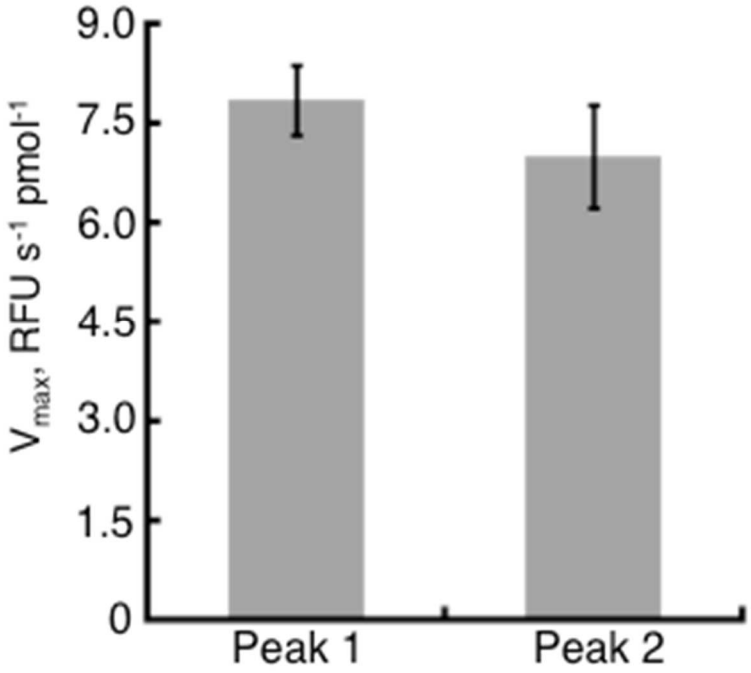
APPENDIX: SUPPORTING INFORMATION



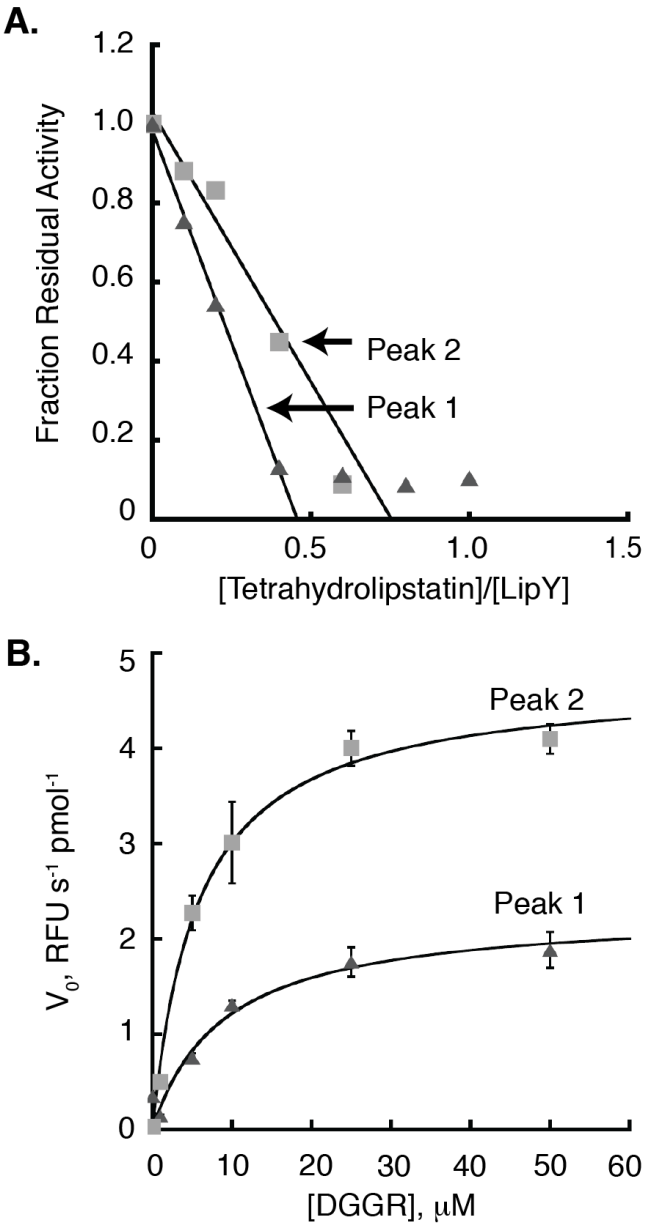
S Figure 1. LipY and LipYΔPE after Purification.
(A) 50 pmoles each of LipY and LipYΔPE were loaded on a 12% SDS-PAGE gel, which was stained with Sypro Orange and imaged using a BioRad ChemiDoc™ Imaging System. The purity of the protein is demonstrated in the lane profiles to the right, which were analyzed using Image Lab 4.1.



S Figure 2. LipY and LipYΔPE are Active on a Natural Triglyceride Substrate. LipY and LipYΔPE from Peak 2 (50 nM active protein) were incubated with triglyceride-rich lipoprotein particles. Free fatty acids were released upon substrate hydrolysis; these were measured using a colorimetric assay as described in the supplemental methods. Three independent measurements were taken and error bars represent the standard deviation.



S Figure 3. Comparison of Equal Amounts of Active LipY from Peak 1 and Peak 2. The V_{max} values for Peak 1 and 2 were adjusted using the active site quantitation shown in figure 4A. Values represent the V_{max} for LipY in Peaks 1 and 2 per amount of active enzyme molecule. Error bars represent the standard error of the mean of 6 independent measurements.



S Figure 4. Active Site Titration and Kinetics of Lipy in Peak 1 and Peak 2.
(A) Titration curve of LipY Peak 1 and Peak 2 inhibited with THL. Residual enzyme activity was measured using *p*-Nitrophenyl butyrate and normalized to uninhibited LipY. Activity versus ratio of THL to enzyme was plotted and the linear portions of each set of data were fit linearly. (B) Michaelis-Menten curves comparing equal amounts of *total* LipY from Peak 1 and Peak 2 using the DGGR substrate.

Supplemental Methods

Isolation of Plasma Derived Triglycerides

Plasma derived triglyceride rich lipoprotein (TRL) particles were isolated as previously described[132].

Quantification of Substrate Triglyceride Levels

The triglyceride content of the sample was quantified as previously described with some modifications[133]. Briefly, samples were diluted in PBS and incubated with 7.25 μM Candida Rugosa Lipase (Sigma Aldrich) in hydrolysis buffer (133 mM KPO_4 and 0.25% FFA BSA) for 30 minutes at 37 °C. Next, quantification buffer (133 mM KPO4, 3.3 mM McCl2, 4.4mM ATP, 0.6 mM TOOS, 6 U/mL POD, 1.73 mM bromophenol, 1.73 mM aminoantopyrine, 0.1 mM FAD, 0.5% Triton X-100, 0.25 U/mL Glycerol Kinase (Fisher Scientific), 4 U/mL Glycerolphosphate oxidase (Fisher Scientific)) was added each sample and allowed to incubate for 15 minutes at 37 °C. A sample containing no lipase was used for background subtraction for the hydrolyzed sample. Samples were read on a Spectromax M5 plate reader at 555 nm. Samples were fit to the standard curve to give the final triglyceride concentration.

Triglyceride Hydrolysis Assays

Reaction samples were loaded into wells of a clear 96-well plate (Greiner). 50 nM LipY was incubated with TRLs (at a concentration of approximately 2.6 mM triglycerides). Individual reactions were quenched by the addition of Orlistat (Cayman Chemical Company, Ann Arbor, Michigan, USA) to a final concentration of 80 μM. Released free fatty acids (FFA) were quantified using a slight adaptation of a previously reported assay[134]. Briefly, reagent A (final concentrations: 133 mM KPO_4 pH 7.5, 3.3

mM $MgCl_2$, 4.4 mM ATP, 1 mM CoA, 0.055 U/mL ACS, 0.5% Triton X-100) was added to each sample (including palmitate standards) and the mixtures were incubated for 15 minutes at 37°C. Reagent B (final concentrations: 133 mM KPO_4 pH 7.5, 0.6mM TOOS, 6 U/mL Horse POD, 1.73 mM dibromophenol, 1.73 mM 4-aminoantipyrine, 0.1 mM FAD, 10 mM NEM, 5 U/mL ACO, 0.5% Triton X-100) was then added to each sample and further incubated at 37°C for 10 minutes. Samples were assayed in triplicate. Samples were read on a Spectromax M5 plate reader at 555 nm. FFA release for each sample was quantified using a standard curve of palmitate.

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