Published in final edited form as:

Biochemistry. 2019 September 24; 58(38): 3943–3954. doi:10.1021/acs.biochem.9b00462.

The Structural Basis by Which the N-terminal Polypeptide Segment of *Rhizopus chinensis* Lipase Regulates Its Substrate Binding Affinity

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Abstract

An important group of industrial enzymes, *Rhizopus* lipases exhibit valuable hydrolytic features that underlie their biological functions. Particularly important is their N-terminal polypeptide segment (NTPS), which is required for secretion and proper folding, but is removed in the process of enzyme maturation. A second common feature of this class of lipases is the α -helical “lid”, which regulates accessibility of substrate to the enzyme active site. Some *Rhizopus* lipases also exhibit “interfacial activation” by micelle and/or aggregate surfaces. While it has long been recognized that the NTPS is critical for function, its dynamic features have frustrated efforts to characterize its structure by X-ray crystallography. Here, we combine nuclear magnetic resonance spectroscopy and X-ray crystallography to determine the structure and dynamics of *R. chinensis*

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Author Contributions

M.Z., X.W., Y.X., R.G., G.V.T.S., T.S., J.F.H. and G.T.M. designed experiments and interpreted data. M.Z. carried out protein biochemistry and enzymology studies. M.Z. and G.V.T.S. carried out NMR experiments. M.Z. and R.G. carried out crystallography experiments. All authors contributed in writing the manuscript.

J. F. H. and G. T. M. are scientific advisors to Nexomics, Biosciences.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Critical Micelle Concentration (CMC) determination of pNPB, table of ¹⁵N nuclear relaxation data, ¹⁵N R₂/R₁ relaxation rates, key long range NOEs involving the NTPS, lid domain and binding pocket residues, representative electron density of core sidechains and NTPS, crystallization, data collection and refinement statistics for r27RCL, and multiple sequence alignment.

lipase (RCL) with its 27-residue NTPS prosequence (r27RCL). Both r27RCL and the truncated mature form of RCL (mRCL) exhibit bi-phasic interfacial activation kinetics with *p*-nitrophenyl butyrate (*p*NPB). r27RCL exhibits a significantly lower substrate binding affinity than mRCL due to stabilization of the closed lid conformation by the NTPS. In contrast to previous predictions, the NTPS does not enhance lipase activity by increasing surface hydrophobicity, but rather inhibits activity by forming conserved interactions with both the closed lid and the core protein structure. Single site-mutations and kinetic studies were used to confirm that the NTPS serves as internal competitive inhibitor, and to develop a model of the associated process of interfacial activation. These structure-function studies provide the basis for engineering RCL lipases with enhanced catalytic activities.

Graphical Abstract

Keywords

Rhizopus chinensis lipase; X-ray structure; NMR nuclear spin relaxation; protein engineering

Introduction

Lipases (triacylglycerol ester hydrolases EC 3.1.1.3) catalyze the hydrolysis and synthesis of ester bonds of lipids and triglycerides. In addition to their natural roles in catalyzing triglyceride hydrolysis, lipases can catalyze interesterification, alcoholysis, acidolysis, and esterification in non-aqueous media¹. They are an important group of biotechnologically relevant enzymes, with extensive applications in the food, dairy, detergent, leather, paper, fine chemical synthesis, and pharmaceutical industries^{2, 3}. Lipases exhibit regioselectivity, stereoselectivity, and fatty acid selectivity in hydrolyzing triglycerides, which can be exploited for production of valuable lipids⁴ including cocoa butter substitutes⁵, infant formulas⁶, and triacylglycerols rich in omega-3 polyunsaturated fatty acids^{7, 8}. In the pharmaceutical industry, lipases also can provide kinetic resolution of racemic mixtures, and are utilized for synthesis of fine chemicals and drugs, including the anticancer drug paclitaxel (taxol), and its derivatives⁹.

Fungal lipases are particularly important industrial enzymes because of their high stability and wide substrate specificity¹⁰. In particular, *Rhizopus* fungi lipases are an important group of industrial microbial lipases, widely used in food industries^{11, 12}, as well as for production of biodiesel and chiral compounds¹³. *Rhizopus* species of particular interest include *R. microsporus*, *R. oryzae*, and *R. stolonifer*¹⁴. The corresponding *Rhizopus* lipase genes code for open reading frames that include (i) a signal peptide, (ii) a prosequence, and (iii) a mature lipase sequence. The prosequence, or N-Terminal Polypeptide Segment (NTPS), is important for the secretion, folding and catalytic properties of *Rhizopus* lipases^{15–19}.

The primary lipase, RCL, of *Rhizopus chinensis* (*Rhizopus microsporus* var. *chinensis*) a fungal strain isolated from leaven (mouldy grain), is found in several forms, including the pre-proRCL (42 kDa), proRCL (39 kDa), r27RCL (33 kDa), and truncated “mature” (30 kDa) mRCL forms²⁰. When produced in *Pichia pastoris*, the 324-residue proRCL protein is cleaved by an endogenous Kex2-like protease at a Lys-Arg-Asp sequence motif, between residue positions –29 and –28 of the prosequence, resulting in a 296-residue r27RCL enzyme, the dominant form of the protein when expressed in *P. pastoris*. This r27RCL form is further proteolytically truncated to 269-residue mRCL by removal of its 27-residue NTPS²⁰. *R. oryzae* fungal lipase (ROL32) and ostrich pancreatic lipase (OPL) also have NTPS regions, that have been reported to function to enhance lipase activity^{21, 22}.

Crystal structures of *Rhizopus* lipases have been available since the 1990s, including lipases from *R. niveus* (RNL, PDB i.d. 1LGY, 2.20 Å²³) and *R. oryzae* (ROL, PDB i.d.'s 1TIC, 2.60 Å and 1TIB 1.84 Å²⁴). Like many other lipases and esterases, as well as many serine proteases, these enzymes have α/β hydrolase folds, and a Ser- His- Asp/Glu active site²⁵. The structures available for *Rhizopus* lipases are for the mature (mRCL) forms, and the structures of the NTPSs were not reported in these crystal structures. For RNL, the 28-residue N-terminal segment was apparently hydrolyzed during crystallization, providing a structure only for the mature, truncated lipase²³. For ROL, both its proenzyme and mature forms have been crystallized²⁶, but the X-ray crystal structure could only be determined for the “mature” truncated form²⁴.

As crystal structures have not been available to use as templates, the 3D structure of the NTPS and its interactions with the core ROL lipase structure has been modeled using secondary structure prediction and conformational energy minimization^{21, 22}. Using these predicted structures, it was proposed that the function of the NTPS is to extend the hydrophobic surface of the lipase adjacent to the active site in its open form, providing crucial hydrophobic interactions with lipid substrates^{21, 22}, enhancing the specific activity of ROL32, and modulating its substrate affinity relative to the truncated form lacking the NTPS. A similar mechanism, based on a predicted NTPS structure, has also been proposed for the enhanced specific activity of full-length over truncated OPL lipase²².

A common functional feature of most (but not all) lipases, called “interfacial activation”, is characterized by enhanced enzymatic activity in the presence of membranes or micelles, or when the lipid substrate solubility is exceeded, resulting in a lipid-water interface²⁷. Under these conditions, lipases may form activated “open” conformational states^{24, 27–30}. In the case of *Rhizopus* lipases, this “interfacial activation” has been proposed to arise, at least in part, from the structural displacement of a small, largely α -helical “lid” polypeptide segment, providing better access of substrate into the active site^{24, 27}.

The structural basis for interfacial activation has been studied by various biophysical methods, including X-ray crystallography^{27, 30}. For example, the asymmetric unit of the crystal structure of ROL lipase (PDB i.d. 1TIC) includes two protein molecules, one with a “closed lid” conformation, blocking the active site, and the other with a partially-open conformation stabilized by a detergent molecule²⁴. Stabilization of the “open lid” conformation by hydrophobic interfaces is proposed to provide a basis for its interfacial activation.

Here, we describe the 2.0 Å X-ray crystal structure of *Rhizopus chinensis* r27RCL lipase produced in *P. pastoris*. Guided by NMR data, the conformation of most of the NTPS in the r27RCL structure was also traced in the electron density, allowing identification of specific interactions between the NTPS and both the core and the lid regions of the α/β hydrolase fold. Significantly, this experimental structure provides novel structure-function insights which are not evident from previously published predicted models^{21, 22}. Site-specific mutations of residues of the NTPS demonstrate the importance of specific atomic interactions between the NTPS and the lid region in inhibiting, rather than activating, the enzymatic activity of r27RCL, and provide novel r27RCL analogs with enhanced lipase activities relative to wt r27RCL.

Results

Enzyme kinetic studies demonstrate that the NTPS of RCL inhibits its enzymatic activity at low substrate concentrations

Rates of *p*-nitrophenyl butyrate (*p*NPB) substrate hydrolysis as a function of substrate concentration were compared for *P. pastoris* r27RCL and mRCL enzymes (Figure 1). In these plots, r27RCL exhibits bi-phasic kinetic behavior, suggesting an induced activation process at substrate concentrations above ~ 4 mM at 40 °C. In the first phase, the rate of *p*NPB hydrolysis rises from 0 to about 0.31 s⁻¹ over the concentration range of 0 to 4 mM.

However, at *p*NBP substrate concentrations greater than ~ 4 mM, there is a substantial activation of activity. Notably, *p*NBP has a critical micelle concentration (CMC) of ~ 4.1 mM at 40 °C, measured by fluorescence analysis (Figure S1), suggesting that this activation is mediated by interactions with substrate micelles through a modest (~ 1.5-fold) interfacial activation process. In *P. pastoris* mRCL (Figure 1B), a form lacking the 27-residue NTPS, the kinetics reaches the same k_{cat} value (~ 0.35 s⁻¹) at much lower *p*NBP concentration (< 1 mM), while the second substrate-induced activation phase is unaffected. The K_{M} values measured for the first phase of r27RCL and mRCL kinetics are 1.19 ± 0.15 and 0.12 ± 0.02 mM, respectively, suggesting that the interactions between NTPS and the rest of the enzyme inhibit substrate binding at low substrate concentration. Based on these data (Figure 1), the NTPS is not the cause of the interfacial activation. Rather, contrary to what has been reported for ROL and OPL lipases^{21, 22}, the presence of the NTPS inhibits the hydrolysis activity of r27RCL at low substrate concentrations, but has little effect on the lipase activity at high substrate concentrations.

¹⁵N relaxation measurements reveal a partially-ordered NTPS of RCL

r27RCL can be produced in either *P. pastoris* or *E. coli* expression hosts with uniform ¹³C, ¹⁵N- or ²H, ¹³C, ¹⁵N-enrichment for NMR studies³¹. Using r27RCL samples produced in *E. coli*, in which the Ile (δ1), Leu, and Val methyl groups were also ¹³CH₃ labeled (i.e. [²H, ¹³C, ¹⁵N; ¹H-¹³C Ile δ1, Leu δ, Val γ methyl labeled]-r27RCL, or more concisely ILV-r27RCL), we have previously published extensive backbone and sidechain methyl resonance assignments³². These backbone resonance assignments provide the basis for ¹⁵N nuclear spin relaxation measurements using uniformly ¹⁵N-enriched r27RCL, including longitudinal (R_1) and transverse (R_2) relaxation rate, and ¹⁵N-¹H heteronuclear NOE (hetNOE), measurements for ~ 77% of the backbone amide sites (Table S1). These data are plotted (Figure 2) along with secondary structure elements, and the locations of key structural elements, including the NTPS, the core hydrolase structure, and the lid α-helical segment, which were determined from the X-ray crystal structure described below. Using relaxation delays longer than 3 s, most residues in the folded core have the same hetNOE intensities within experimental error. Overall, these relaxation data are consistent with a largely well-ordered protein structure, with a rotational correlation time τ_c determined from the ¹⁵N R_2/R_1 ratios^{33–36} of 10.6 ± 1.3 ns at 35 °C. Most notable in these data are relaxation rates and heteronuclear NOEs in the NTPS of r27RCL (designated with a magenta horizontal bar in Figure 2). Some amide sites in the NTPS have much lower hetNOE and R_2 values, and higher R_1 values, than amide sites in the core protein structure. While residues Ala21-Ser27 appear to be largely disordered on the timescale of the nuclear relaxation rate measurements, which are sensitive to internal motions faster than the overall tumbling time τ_c , residues within the N-terminal polypeptide segment Thr4-Ile19 exhibit ¹⁵N nuclear relaxation rates and hetNOE values similar to the rest of the r27RCL protein structure (Figure S2). These observations are consistent with a r27RCL structure in which the polypeptide segment Thr4-Ile19 is packed back onto the r27RCL core structure, while the intervening polypeptide linker, Ala21-Ser27, forms a disordered loop within the protein structure. Significantly, amide sites within the α-helical lid structural element (indicated in blue in Figure 2), and at the interface of the lid and core hydrolase structure, are not especially remarkable compared with amide sites in the rest of the core hydrolase structure, indicating that these nuclear

relaxation measurements are not detecting any lid opening/closing dynamics that may occur under the conditions studied here. ^{15}N CPMG NMR experiments (Figure S3) also provide no evidence for relaxation dispersion at the lid/core interface or other indications of lid open/close dynamics on the CPMG timescale (100 μs to tens of milliseconds). These dynamic features are addressed further in the Discussion section, below.

X-ray crystal structure of RCL

Single crystals (dimensions of about $0.1 \times 0.1 \times 0.3$ mm) of r27RCL were obtained from 0.25 M ammonium sulfate and 25% (w/v) PEG 4000 after 3–4 days. In the initial model of r27RCL (R -factor = 26.4, R_{free} = 30.1) weak electron density adjacent to the lid was interpreted as bound PEG, and the conformation of the NTPS, specifically residues Asp1-Ser27, could not be traced. However, as ^{15}N nuclear relaxation and hetNOE data, outlined above, indicated that residues Val5-Ile19 of the NTPS are well-ordered, efforts were made to use NMR data to identify how the NTPS packs against the rest of the r27RCL structure. Using the perdeuterated ILV-r27RCL sample, many ^1H - ^1H NOEs were identified throughout the protein structure that are consistent with the initial X-ray crystal model. In particular, 53 H^{N} - H^{N} , H^{N} -Me, and Me-Me NOEs were observed between the NTPS, lid, and core regions of the structure (Table S2). Overall, these NOEs and relaxation data confirmed that the “closed” conformation of r27RCL determined by X-ray crystallography is highly populated under these solution conditions. However, in addition to these data supporting the initial crystal structure, many NOEs were identified and assigned to contacts between the ordered region of the NTPS (Val5-Ile19) and both the lid and core regions of r27RCL (Table S2). Based on these NMR data, we reinvestigated the electron density map of r27RCL. Representative electron density for the core region and the NTPS are shown in Figure S4. Guided by these data, we identified the NTPS segment in the electron density, which had been initially misassigned as bound PEG. This allowed us to trace the structure of NTPS residues Thr2-Ser23 in the electron density, allowing further refinement of the structure to an R -factor of 0.167 (R_{free} = 0.208). Consistent with the ^{15}N relaxation measurements, no electron density could be observed for the disordered NTPS region residues Thr24-Gly31.

Crystal structure quality statistics for this final r27RCL structure are summarized in Table S3, and the resulting model is illustrated in Figure 3. Preliminary structural analysis showed that the catalytic triad residues Ser172, Asp231, and His284 are located at their canonical positions in the α/β hydrolase fold, which is a characteristic of lipase family members. The “lid” region, residues Gly109 to Thr123, includes a short α -helix (Phe113-Asp119) linked to the “core” of the protein structure by polypeptide hinge residues (Gly109-Ser112 and Met120-Thr123). In the crystal lattice, r27RCL is in a “closed” conformational state in which the active site is covered by the “lid” helix. The backbone atoms of the NTPS have higher B-factors compared with core residues of r27RCL (Figure S5), which further indicates that the NTPS has higher flexibility. Notably, within the NTPS segment, residues Thr2, Pro13, Gln14, Asn15, and Ser23 have the largest B-factors, while the remaining residues that interact with the lid and core regions, and for which electron density is observed, exhibit smaller B-factor values, suggesting less flexibility.

The 3D structure of r27RCL reveals the structural basis by which the NTPS modulates its lipase function

Having characterized the structure and dynamics of r27RCL, we next set out to understand the role of the NTPS in modulating its hydrolytic activity. We first examined whether the NTPS of r27RCL extends the hydrophobic surface near the active site, as has been proposed for the ROL lipase based on a different predicted binding mode for the NTPS²¹. The open form of r27RCL was homology modeled based on the open conformation reported for the homologous RML (*Rhizomucor miehei* lipase)³⁷, and the hydrophobic surface of RCL with (r27RCL) and without (mRCL) the NTPS region were compared for both the open and closed conformational states. This analysis (shown in Figure S6) demonstrates that, contrary to conclusions reached using a predicted binding model of the NTPS region for ROL^{21, 22}, in its open state r27RCL does not have an especially more extensive hydrophobic surface than mRCL in the vicinity of the active site. While this surface itself is likely to have some plasticity, such structural variations are not likely to be a broad distribution, and hence potential dynamics of the surface are not expected to significantly change the hydrophobic / hydrophilic character shown in these static models. Hence, the suggested role of the NTPS in significantly extending the hydrophobic surface in order to enhance substrate binding and/or interfacial activation, is not supported by our X-ray crystal structure of r27RCL which includes the bound-state structure of the NTPS.

We next examined the hypothesis that the NTPS may modulate lipase activity in r27RCL by interacting with the lid and/or active site of the enzyme. Examination of the crystal structure of r27RCL reveals several interactions between residues of the NTPS with residues in the lid and/or residues in close proximity to the lipase active site (Table S4). These interactions are also supported by specific NOEs between atoms in the NTPS and atoms of the lid and/or core regions (Table S2). For example, residues Glu3 and Asp11 of the NTPS form salt bridges with residue Arg114 in the lid, and the δ 1 methyl group of Leu10 in the NTPS forms a well-packed hydrophobic interaction with the δ 1 methyl group of Ile117 in the lid (Figure 3C). NTPS residues in polypeptide segment Met8-Leu12 and residue Pro17 also interact with residues that are in close proximity to the lipase active site, particularly in the polypeptide segment Pro277-Ser280, adjacent to active-site triad residue His284, and residue Arg230 which is adjacent to active-site triad residue Asp231 (Figure 3D). These structural features of r27RCL suggest that the NTPS could modulate lipase activity either by influencing the lid open/close dynamic equilibrium, and/or by directly affecting the local environment and conformation of the active-site triad.

The key residues mediating NTPS interactions with the lid and active site are conserved

Multiple sequence alignment of homologous lipases (Figure S7) reveals conservation of residues in the NTPS, most of which are residues interacting with the lid and active site. Residues at the NTPS / lid / binding pocket interface that are most conserved include Val5, Gly7, Thr9, Pro13, and Pro17 in the NTPS, Gly109 and Thr118 in the lid domain, and Arg230, Thr279 in binding pocket. Residue Asp11 was observed to co-evolve with Arg114; when residue 11 mutates to Glu, residue 114 mutates to Ser, perhaps conserving hydrogen-bonded interactions. Residues 5, 10, and 117 form a conserved hydrophobic cluster; residue 10 is either Leu or Met, while residue 117 is either Ile or Val. This analysis suggests that

these homologous lipases may also form similar interactions between their NTPS regions and the lid and core protein structure.

Site-directed mutagenesis of key residues in the NTPS demonstrate its role in inhibiting RCL lipase activity

In order to test the hypothesis derived from our crystallography and NMR studies that specific NTPS interactions modulate lipase activity, site-specific mutations were made for several residues in the NTPS involved in interactions with the lid (i.e., residues Glu3, Val5, Leu10, and Asp11) and/or lipase active site (i.e., Met8, Thr9, Leu10, Leu12, Pro17). As a control, residue Thr22, which does not contact the lid or active site region, was also mutated to Asp. These mutant r27RCL proteins were expressed in *E. coli*, purified, initial rate measurements were made, and steady-state Michaelis-Menten kinetic parameters K_M and k_{cat}^1 and k_{cat}^2 , and were determined for the first phase of the bi-phasic kinetics and for the second interfacial-activated phase, respectively using *p*NPB substrate (Figure 4 and Figure S8). These values are summarized in Figure 5 and Table S5. As the second phase appears to involve a micelle-induced structural transition, no attempt was made to fit a K_M to this phase. The K_M and k_{cat}^1 values of wild-type r27RCL expressed in *E. coli*, determined from these steady-state kinetic measurements made at *p*NPB concentrations lower than its solubility limit, were 0.81 ± 0.14 mM and 0.41 ± 0.03 s⁻¹, similar to the corresponding values for r27RCL expressed in *P. pastoris*. Significantly, all of the mutations have similar k_{cat}^1 and k_{cat}^2 values. The T22D control mutation, and mutants that interact with, or are spatially close to, active site residues (M8A, T9A, L12A, P17G) had no significant (< 2-fold) effect on the K_M values for hydrolysis. However, most of the mutants of lid-interacting residues have reduced K_M values (0.21 – 0.42 mM), except for the E3A mutation. Particularly notable are the L10A and D11A mutants, which disrupt hydrophobic, electrostatic, and hydrogen bonding interactions between the NTPS and the lid helix (Figure 3C and 3D), with K_M more than 3-fold lower than the wt enzyme. The K_M of the V5A mutant is also half that of wt r27RCL. Note that residue E3 has relatively high B factors in the crystal structure. These mutagenesis and enzyme kinetics data demonstrate that specific interactions between the NTPS and the lid domain of r27RCL modulate the activity of the lipase at low substrate concentrations by affecting K_M , but not k_{cat}^1 . Significantly, however, none of these mutations affect the k_{cat}^2 values associated the modest interfacial activation of r27RCL observed at substrate concentrations above its CMC. Taken together, these data demonstrate a key, previously unrecognized, role for the NTPS in modulating the lipase activity of r27RCL by specific interactions that stabilize the lid-closed state, independent of the interfacial activation process.

Discussion

Using *p*NPB as substrate, we have characterized the role of the NTPS in modulating RCL lipase activity. From ¹⁵N relaxation measurements, we determined that residues in the N-terminal portion of the NTPS have backbone rotational correlation times that are similar to those of residues in the protein core. This observation prompted us to reexamine the initial electron density map generated for the crystal structure of r27RCL, and allowed us to identify the lipase-bound structure of the NTPS and its interactions with the rest of the

enzyme. The refined enzyme structure has much improved crystallography R factors. These structural observations then drove design of enzyme kinetic studies, using site-specific mutations, that elucidate a previously unrecognized role for the NTPS in modulating RCL activity by stabilizing the lid-closed conformation of the enzyme. Significantly, in the system studied here, the NTPS does not modulate the modest interfacial activation provided by the *p*NPB substrate at concentrations above its CMC. However, it could still play a role under conditions in which interfacial activation is stimulated by different hydrophobic interfaces.

Our kinetic data indicate that the function of the NTPS is to modulate substrate binding to the active site, rather than in product release or catalytic turnover. In the classical Michaelis-Menten model of steady-state kinetics³⁸, if $k_{\text{cat}} \ll k_{-1}$, then $K_M \approx K_S = [E][S]/[ES]$, and changes in K_M do not reflect changes in the rate of product release. It is well established for many lipases that the rate limiting step in catalysis is hydrolysis of the acyl intermediate^{39,40}. Hence, even if the NTPS affects product release, it should not affect catalytic efficiency, and differences in K_M between r27RCL and mRCL resulting from removing the NTPS thus largely reflect changes in the substrate binding equilibrium. This interpretation is supported by our kinetic data. Turn-over rates k_{cat} , which include the processes of both acyl-intermediate hydrolysis and product release, are essentially the same for r27RCL (with the NTPS) and mRCL (without the NTPS). While we cannot entirely rule out the possibility of the NTPS having some mutually compensating effects on acyl intermediate hydrolysis and product release, there is no basis to consider such compensation for the interpretation of our data. Hence, we conclude that the primary function of the NTPS of r27RCL (and in larger precursor forms of RCL) is to modulate lid opening and substrate binding affinity.

There is no significant effect on the second-phase of the kinetic data due to removal of the NTPS. Similar results were observed for mutations of the NTPS / lid interacting residues. Hence, we also conclude that the NTPS does not contribute to modulating K_M (or substrate binding affinity) in the second, micelle-activated phase of the kinetics. While this conclusion is consistent with both our structural and kinetic data, it is always possible that kinetic data may arise from more complex models than the simplest model that fits the data. Hence we cannot absolutely rule out some non-innocent (and non-observed) role of the NTPS in the second phase of the bi-phasic kinetics. This is a general shortcoming when fitting “the simplest model that adequately explains the data.”

Based on the combined structural and kinetic data provided in this study, we propose a bi-phasic interfacial activation model for *Rhizopus chinensis* lipase (Figure 6). At low substrate concentration, when the amphiphilic lipid substrate exists predominately as a monomer, specific interactions between the NTPS and the lid stabilize the lid-closed form of r27RCL (Figure 6A). Under the same conditions mRCL, which lacks the NTPS, or in mutants of r27RCL, in which key interactions between the lid and NTPS are disrupted, the lid closed $\leftrightarrow$ open equilibrium is shifted toward the lid open form in (Figure 6C), and the enzyme has higher activity. As the substrate concentration reaches the CMC, the system undergoes a structural transition in which both the NTPS and the α -helical lid are dissociated from the enzyme, exposing the active site (Figure 6B). Both mRCL and r27RCL are activated by

micelle-stabilization of this lid-open conformation(s) (Figure 6B, D). This stabilization of the lid-open conformation(s) is attributable to interactions between otherwise buried hydrophobic residues of the α -helical lid with substrate micelle and/or aggregate surfaces, as proposed for other one-lid lipases^{24, 27}.

Our studies demonstrate that r27RCL exhibits only modest (~ 1.5-fold) interfacial activation by *p*NPB substrate, at concentrations above the CMC. Removal of the N-terminal 27-residue NTPS does not affect this interfacial activation process, and both r27RCL and mRCL have similar apparent k_{cat} parameters under assay conditions above the substrate CMC. This is consistent with previous studies demonstrating similar (or slightly reduced) activity for mRCL compared with r27RCL using lipase assay conditions under which substrates form (or may form) micelles and/or aggregates, and/or which include sodium deoxycholate or Triton X-100²⁰. However, at lower substrate concentration conditions (below the CMC) and in the absence of emulsifiers, K_M (but not k_{cat}) is significantly higher for r27RCL than for mRCL. This same relief of enzyme inhibition by truncation of the N-terminal 27 residues is observed in mutants designed to destabilize specific interactions between the NTPS and the lid α -helix. This inhibition of lipase activity by the NTPS is masked when the enzyme is activated by micellar substrates or emulsifiers used in some lipase assay conditions, and has therefore been overlooked in previous studies of RCL function.

Like RCL, RAL has a pro-enzyme form, rPro28RAL, which has 28 N-terminal residues compared to its mature form, mRAL. The specific activity of rPro28RAL (1543 U/mg) is reported to be significantly lower than mRAL (2437 U/mg) using an olive oil emulsion substrate. For ROL, a less-active truncated form, ROL29, was produced from the pro-enzyme form, ROL32, by incubation for days or months²¹, but other changes may occur to the enzyme in this process.

In our model of the interfacial activation process for r27RCL (Figure 6), the NTPS plays an inhibitory functional role, which is overcome by micellar or other lipid interfacial interactions. For *Gibberella zeae* lipase (GZEL), there is no NTPS. Rather, its C-terminal helix (CTH) provides an inhibitory function⁴¹ analogous to the NTPS of r27RCL. Despite their sharing less than 23% sequence identity, structural alignment of r27RCL with GZEL (excluding the NTPS and CTH regions, respectively) provides an excellent superimposition, with a backbone r.m.s deviation of ~ 1.0 Å. In this superimposition (Figure 7) the NTPS of r27RCL and the CTH of GZEL occupy similar spatial locations relative to the active-site and core structure, and each interacts extensively with their corresponding α -helical lid regions. In the 3D structure of GZEL, residue Arg86 on the lid has been proposed to act as a “lockpin”, which fits into a structural “lockhole” formed by polypeptide segment Met291-Asp293 from the CTH⁴¹. In the lid-open conformation, two hydrogen bonds formed by Arg86 and Asp292 with Met291 are broken, and the Arg86 lockpin is released. In the lid opening process, Arg86 is proposed to turn toward the solution, as the lid assumes a fully open conformation⁴¹. The NTPS of RCL, like the CTH of GZEL, forms an analogous “lockhole-hinge-lockpin” structure, which stabilizes the lid-closed conformation, but is relieved by micellar or alternative interfacial activation interactions.

While we have not been able to measure the time scale of the opening/closing of the lid, we can put bounds on the timescale of these dynamics. First, if such dynamics are present they do not impact the transverse (R_2) and longitudinal (R_1) ^{15}N relaxation rates (or their ratio) for amide groups in residues in the lid, or at the lid/core interface (Figure 2). Hence any dynamics involving a significantly populated “open” state are slower than the timescale for such measurements, which are bounded by the rotational correlation time of the protein, ~ 10 ns. There are many NOEs to the lid domain which are consistent with a significant population of “closed state” with lifetime longer than this timescale. This observation, however, does not rule out a population of open lid states. We also have carried out ^{15}N CPMG NMR experiments (Figure S3), to probe for lid open/close dynamics. These data also provide no evidence for lid open/close dynamics on this CPMG timescale. Since R_2 CPMG is not affected for *any* of the monitored lid residues even with the 180° pulse spacing being only 20 ms, our rotating-frame ^{15}N spin relaxation data reveal no evidence for fast conformational exchange between a major closed and minor open conformation(s) with sizable populations on the approximate time scale ranging from 100 μs to 10 ms. In addition, there is no evidence for resonances of lid residues in slow exchange. These combined data, however, do not exclude a significant population of open conformers converting on the 100 ns to 100 μs timescale.

These combined structural and kinetic studies reveal that the pro-enzyme NTPS regions of *Rhizopus* lipases, or C-terminal regions of other lipases, function, at least in part, to inhibit enzyme activity. This inhibition is fully overcome by interfacial activation by micelles or aggregate surfaces. This inhibition may be masked when carrying out lipase assays in the presence of emulsifiers.

Inhibition of enzyme activity in zymogen forms is common feature of the pro-enzymes of many hydrolytic enzymes^{42, 43}. Biologically, such inhibition can provide for stabilization of lipid components of the cell during storage and secretion of a lipase. This proposed biological function of the NTPS is supported by our observations that the yield of mRCL produced in *P. pastoris* is very low, unless it is first produced with an intact N-terminal segment (i.e. as proRCL), and then enzymatically processed after purification to remove the NTPS. While such regulation of hydrolase function is biologically important, in the context of industrial biotechnology applications where enhanced lipase activity is valued, these structural and functional studies suggest the future design and development of enzymatically-enhanced RCL molecules in which the NTPS still functions to suppress enzyme activity during storage, transport, and secretion, but is subsequently removed and the lid locked in an open conformation by appropriate photo- or redox-activated chemical crosslinks.

Experimental section

Protein preparation for crystallization

Protein expression followed the previous procedures⁴⁴. Briefly, the PCR-amplified gene products were inserted into pPIC9K expression vector. The plasmids were electroporated into *P. pastoris* GS115 and expressed in BMGY and BMMY medium at 30 °C. After 84 h of induction at 28 °C, culture was centrifuged at $6000 \times g$ for 30 min. The supernatant was

filtered and purified on an AKTAPurifier 10 (GE Healthcare Life Sciences) using a Ni-NTA column, equilibrated in binding buffer containing 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 20 mM imidazole. The target protein was eluted at about 250 mM imidazole when using a 20–500 mM imidazole gradient. The fractions contain target protein were dialyzed twice against 25 mM Tris-HCl buffer (pH 8.0) and concentrated to 50 mg mL⁻¹ using an Amicon Ultra-15 Centrifuge (Millipore). The final protein purity was estimated by SDS-PAGE analysis.

Crystallization and X-ray diffraction

Crystallization screening was performed manually using 768 reservoir conditions from Hampton Research (Laguna Niguel, CA, USA) including Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen Lite, MembFac, Natrix, Index, SaltRx, SaltRx 2, PEG/Ion Screen, PEG/Ion 2 Screen, Quick Screen and Grid Screen (ammonium sulfate, MPD, sodium chloride, sodium malonate, PEG 6000, PEG/LiCl). Initial assays were carried out by the sitting-drop vapor-diffusion method at 22 °C by mixing 1 µL protein solution (dissolved in 25 mM Tris-HCl, 150 mM NaCl, pH 8.0; 25 mg mL⁻¹) with 1 µL reservoir solution in 24-well Cryschem plates (Hampton Research).

Prior to flash-cooling to -173 °C using a cryogenic system, all crystals were soaked for 3 s in a cryoprotectant solution consisting of 0.25 M ammonium sulfate, 25% (w/v) PEG 4000 and 10% (v/v) glycerol. An X-ray diffraction data set was collected to 2.0 Å resolution on beamline BL13C1 of the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan). The diffraction images were processed using the program HKL-2000⁴⁵. The crystal structure of RCL was solved by the molecular-replacement (MR) method with the Phaser program⁴⁶ from the CCP4 suite⁴⁷ using a homology model based on the X-ray crystal structure of *Rhizopus niveus* lipase (PDB i.d. 1LGY²³; 82% sequence identity), produced using the SWISS-MODEL website, as the search model. Structural refinement was done using REFMAC⁵⁴⁸ and CNS⁴⁹. Coordinates and structure factors have been deposited in the Protein Data Bank (pdb id 6a0w).

Nuclear relaxation measurement

Extensive backbone and methyl sidechain resonances assignments for r27RCL have been published separately³². ¹⁵N-enriched r27RCL was prepared at 0.5 mM protein concentration in 0.1 M sodium phosphate buffer pH 6.0 containing 50 µM 4,4-dimethyl-4-silapentane-1-sulfonate sodium salt (DSS) and 10 % ²H₂O. All NMR data were collected at 35 °C on Bruker AVANCE 800 MHz spectrometer. Heteronuclear ¹⁵N-¹H NOE, ¹⁵N longitudinal relaxation rates (R₁) and ¹⁵N transverse relaxation rate (R₂)^{50–52} measurements were acquired using interleaved pulse sequences. The recycle delay time for hetNOE experiment was 3 s. Relaxation delays were 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5 and 2.5 s for R₁ and 0.016, 0.032, 0.048, 0.064, 0.080, 0.096, 0.128, 0.16, 0.192 and 0.24 s for R₂ measurements.

Topspin (Bruker Biospin, Karlsruhe, Germany), NMRPipe, nmrDraw⁵³, and SPARKY (Goddard and Kneller, University of California, San Francisco) software were used for data

processing and spectral analysis. Equations $I(t)=I_{\infty}-(I_{\infty}-I_0)\cdot e^{-t\cdot R_1}$ and $I(t)=I_0\cdot e^{-t\cdot R_2}$ were used for R_1 and R_2 data fitting in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Site directed mutagenesis, expression and purification

Nine site-specific mutations in the NTPS were designed based on the X-ray crystal structure of r27RCL: E3A, V5A, M8A, T9A, L10A, D11A, L12A, P17G, T22D. The mutations were constructed using QuikChange® Lightning Site Directed Mutagenesis Kit (Agilent technologies, La Jolla, CA, USA) with MBP-proRCL plasmid as template and transformed into *E. coli* BL21 *trxB* (DE3) (Novagen). The mutations were expressed and purified as described previously³¹. Briefly, incubation was started by inoculation of a single colony into 500 μ L LB media. Cultivation was carried on for 4–6 h at 37 °C and 200 rpm before inoculation into 50 mL of LB media, and incubated overnight. This overnight culture was then transferred into a 2 L flask, and incubated at 37 °C and 200 rpm until the OD₆₀₀ reached 0.6–0.8 units. Protein expression was induced by adding 1 mM IPTG at 17 °C. Cells were harvested by centrifugation after overnight induction, resuspended in Ni-NTA Binding Buffer (50 mM Tris–HCl, 500 mM NaCl, 40 mM imidazole, 1 mM TCEP and 0.02% NaN₃, pH 7.5), and sonicated (Ninbo Scientz Biotechnology Co., LTD) for 10 min using a 30 s on / 30 s off program in an ice water bath. The supernatant was loaded onto the AKTAPurifier 10 (GE Healthcare Life Sciences) system after centrifugation at 15,000 $\times$ g for 45 min and filtration (0.22 μ m). The 8-His-tagged mutants were eluted from the HisTrap HP column (5 mL) at about 300 mM imidazole when using a 20–500 mM gradient of imidazole Elution Buffer (50 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, 1 mM TCEP and 0.02% NaN₃ at pH 7.5). The elution fractions were collected and buffer exchanged to Binding Buffer 2 (50 mM Tris–HCl, 500 mM NaCl, 1 mM TCEP and 0.02% NaN₃, pH 7.5) using Amicon ultrafiltration concentrators (Millipore). MBP-proRCL and mutants were digested by Kex2 protease (PeproTech Inc.) with a ratio of 1:100 (protease: protein) at 30 °C for 3 h. Finally, r27RCL and mutants were collected from Ni-NTA column flow through equilibrated in Binding Buffer 2.

Biochemical assay

Protein concentration was estimated by SDS-PAGE and determined by BCA Protein Assay Kit (Thermo Scientific) using Bovine Serum Albumin (BSA) as a standard. For interfacial activation assay, 1% (v/v) *p*NPB dissolved in acetonitrile was added into 50 mM sodium phosphate buffer, pH 8.0 and sonicated for 2 min as substrate. The reaction was started by the addition of 10 μ L enzyme solution into 190 μ L substrate. The rate of hydrolysis was monitored at 410 nm for 2 min at 40 °C with a Cytation 3 imaging reader (BioTek Instruments, Inc.). The solubility limit of *p*NPB was determined under same conditions using fluorescence spectrophotometer F-7000 (Hitachi High-Technologies, Japan) at 500 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank Dr. Chun-Hsiang Huang for helpful scientific discussions.

Funding Sources

Financial support from the National Natural Science Foundation of China (NSFC) (31671799), Six Talent Peaks Project in Jiangsu Province (NY-010), the High-end Foreign Experts Recruitment Program (GDT20153200044), the National First-Class Discipline Program of Light Industry Technology and Engineering (LITE2018-09), and the 111 Project (111-2-06) are greatly appreciated. G.T.M.'s contributions to this work were also supported in part by NIH NIGMS grant 1R01-GM120574.

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Figure 1.

Initial substrate hydrolysis rates vs. substrate concentration reveal a bi-phasic substrate-induced enzyme activation profile for *R. chinensis* variants (A) r27RCL and (B) mRCL, produced in *P. pastoris*. Initial hydrolysis rates were determined with pNPB substrate at different concentrations.

Figure 2. ^{15}N nuclear spin relaxation measurements for r27RCL. (A) ^{15}N - ^1H hetNOE measurements. (B) ^{15}N longitudinal relaxation rates, R_1 . (C) ^{15}N transverse relaxation rates, R_2 . The locations in the sequence of regular secondary structure elements, along with the residue ranges of the NTPS (magenta bar), core (orange), and lid helix (blue) regions of the protein structure, are illustrated at the top of the figure. All measurements were made using a sample prepared at protein concentration of 0.5 mM in 0.1 M sodium phosphate buffer at pH 6.0, using a spectrometer operating at 800 MHz ^1H resonance frequency.

Figure 3.

The NTPS of r27RCL interacts with residues in the lid region and nearby to the active-site triad, to modulate lipase activity. (A) Top view of r27RCL X-ray crystal structure. (B) Side view of r27RCL structure. Residues in the NTPS, core, and lid regions are colored magenta, orange, and blue, respectively. Atoms of active site triad Ser172, His284, Asp231 atoms are colored green. (C) Key salt-bridge and hydrophobic interactions between the NTPS and the lid α -helix, with electrostatic interactions shown as dashed red lines. (D) Key interactions between the NTPS (magenta space-filling representation) and the polypeptide chains (orange) adjacent to active-site residues His284 and Asp231 (green), respectively, with key

hydrogen-bonded interactions shown as dashed green lines. The figure was made using PyMOL Graphics System, Version 2.0 (Schrödinger, Inc.).

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Figure 4. Initial substrate hydrolysis rates vs. substrate concentration of r27RCL and mutants expressed in *E. coli*. (A) Wild-type r27RCL, (B) E3A, (C) V5A, (D) L10A, (E) D11A, (F) T22D.

Figure 5.

Kinetic parameters of RCL and its mutants. k_{cat}^1 and k_{cat}^2 correspond to the k_{cat} values of the first and second phase of the kinetic curves. K_M represents the K_M value of the first phase. PPMRCL and PPr27RCL were produced in *P. pastoris* while ECr27RCL was produced in *E. coli*.

Figure 6.

Schematic diagram of r27RCL and mRCL interfacial activation mechanism. (A) Inactive form of r27RCL at low substrate concentration. (B) Active form of r27RCL when substrate forms micelle or aggregate surfaces. Interactions between the NTPS and the lid stabilize the closed form of r27RCL when substrate concentration is very low. When substrate concentration is sufficiently high to form micelles, the NTPS is fully dissociated from its binding site on the enzyme, the lid opens, and r27RCL is activated. (C) mRCL, lacking the NTPS, interconverts between inactive and partly active forms at low substrate

concentrations. (D) Active form of mRCL when substrate forms micelles. When substrate concentration is low (less than CMC), mRCL exists in a dynamic equilibrium between lid-closed and more active lid-open conformations. When substrate molecules form micelle or aggregate surfaces, mRCL behaves similarly to r27RCL, with similar activated k_{cat} . For both r27RCL and mRCL, substrate micelle and/or aggregate stabilizes the lid-open conformation(s).

Figure 7.

Structural superimpositions of r27RCL and GZEL (PDB i.d. 3NGM) lipases, revealing a very similar core and lid structures (A), and analogous “terminal binding” interactions with the lid (B). The structures and structural elements are labeled: gray: GZEL, forest: C-terminal α -helix of GZEL, green: interactions between M291, T292, D293 with lid residue R86 of GZEL, orange: r27RCL, blue: lid of r27RCL, magenta: NTPS of r27RCL, red: interactions between E3, D11 with lid residue R114 of r27RCL