Engineering Lipases: Walking the Fine Line Between Activity and Stability

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Engineering Lipases: Walking the Fine Line Between Activity and Stability

Siva Dasetty, Mark A. Blenner, and Sapna Sarupria

Department of Chemical & Biomolecular Engineering
Clemson University, Clemson, SC 29634
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Abstract

Lipases are enzymes that hydrolyze lipids and have several industrial applications. There is a tremendous effort in engineering the activity, specificity and stability of lipases to render them functional in a variety of environmental conditions. In this review, we discuss the recent experimental and simulation studies focused on engineering lipases. Experimentally, mutagenesis studies have demonstrated that the activity, stability, and specificity of lipases can be modulated by mutations. It has been particularly challenging however, to elucidate the underlying mechanisms through which these mutations affect the lipase properties. We summarize results from experiments and molecular simulations highlighting the emerging picture to this end. We end the review with suggestions for future research which underscores the delicate balance of various facets in the lipase that affect their activity and stability necessitating the consideration of the enzyme as a network of interactions.

Keywords: Lipase; Enzyme; Stability; Activity; Flexibility
I. INTRODUCTION

Enzymes are proteins that serve as biological catalysts and play a critical role in regulating metabolic pathways in organisms. Enzymes possess high specificity, selectivity and environmental benignity in contrast to the traditionally used non-biological catalysts. Consequently, there is a high demand for using enzymes for industrial applications. The limitations to this end are the narrow range of environmental conditions in which the enzymes are active. Enzymes have evolved to perform optimally near the physiological conditions of the source organism, and often lose their functionality in industry-relevant conditions that deviate from their normal environment, such as high temperatures and acidic conditions. Therefore, there has been considerable interest in engineering enzymes to function in a broad range of conditions.

Protein engineering, and specifically enzyme engineering, is an active area of research. The goal of enzyme engineering is to design enzymes for specific applications through an ability to tune its window of functionality (e.g., temperature range, pH conditions, substrate specificity, enantioselectivity, etc). Enzyme structure plays the key role in determining the activity and stability of an enzyme. Therefore, understanding the relationship between enzyme structure, activity and stability is the holy grail of enzyme engineering. This relationship is often investigated through studies of enzyme evolution in related organisms from different environments and through more systematic studies comparing wild type (WT) and mutant enzymes. For example, comparative studies of similar enzymes from psychrophilic and thermophilic organisms have long suggested that enzymes adapt to different temperatures by modulating their flexibility. Psychrophilic enzymes seem to function at low temperatures through evolution of high active site flexibility, while thermophilic enzymes adapt high temperatures by reducing their overall flexibility. To further elucidate the relation between specific residues and flexibility, various experimental techniques such as directed evolution and rational engineering have been used. These methods enable systematic studies of mutant enzymes and their corresponding effects on stability and activity. Directed evolution imitates natural adaptation of enzymes in a controlled environment by selection for enzymes with specific properties (e.g., activity at higher temperature) through iterative generation of mutagenesis. The effectiveness of directed evolution can be improved by evolving the enzyme semi-rationally. With
increasing experimental data on enzyme activity, stability and structure, there has been a concurrent increase in the computational studies geared towards elucidating the enzyme structure-activity-stability relationship.

In this review, we focus on the recent advances in lipase engineering. Lipases catalyze the hydrolysis of lipid molecules and therefore, have several industrial applications. They have been used in pharmaceuticals, detergents, paper and pulp industry, and food and beverages. These applications require lipases to function in various conditions and therefore, in recent years several studies focused on developing strategies to engineer lipases have been reported. While these studies have established promising guidelines, a clear strategy to manipulate the activity, specificity and stability for given conditions remains elusive. We summarize the emerging understanding of the relationship between lipase structure, flexibility, activity and stability. In doing so, we also highlight the complexity in establishing these relationships. Finally, we provide our perspective on the possible future directions of research to achieve an ability to engineer lipases as desired.

The outline of the review is as follows: We begin with describing the terminology used to characterize enzyme activity and stability (Section II). In Section III we introduce the structure and reaction mechanism of lipases. In the next two Sections (IV and V) we summarize the recent experimental and simulation studies performed on *Bacillus subtilis* lipase A and *Candida antartica* lipase B, which are the two most widely studied lipases. Studies focused on other lipases are discussed in Section VI. We end with our conclusions and perspectives in Section VII.

II. ENZYME ACTIVITY AND STABILITY

Activity measures the potential of an enzyme to catalyze a given reaction. It is commonly quantified as the amount of product produced per unit time under specified conditions. Activity is obtained by monitoring the change in the concentration of the substrate or product with time using enzyme assays. The results are reported in terms of specific activity, which refers to activity normalized by the total utilized mass of enzyme in the reaction. Fig. 1 shows an example of such results obtained for *Bacillus subtilis* lipase A (BSLA) and its mutants at different temperatures. The specific activity-temperature plot shows a skewed parabolic behavior. The parabolic shape is typically
ascribed to Arrhenius-like increase in reaction rate with temperature competing against thermal unfolding of enzyme structure. The point of maximum activity is referred to as the optimal activity, and the corresponding temperature is called optimal temperature ($T_{opt}$, see Fig. 1). The shift of the curve towards higher temperatures for the mutants in Fig. 1 indicates that the mutants are active at higher temperatures than the WT.

**FIG. 1.** An illustration of the typical relationship between specific activity and temperature. The specific activities as a function of temperature are shown for WT and mutant (TM, 2D9, 4D3) BSLA. The point of maximum activity is shown by arrows. Reprinted from Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics, 381, Ahmad et al., Thermostable Bacillus subtilis lipases: in vitro evolution and structural insight, 324-330, Copyright (2008), with permission from Elsevier.

In addition to specific activity, kinetic and stability parameters of enzymes are also evaluated. These parameters are used to compare the specificity of enzymes and their corresponding capacity to catalyze reactions in different conditions. The kinetics are quantified by describing the reaction using the Michaelis-Menten model. This model relates the reaction rate to the substrate concentration using two parameters, $V_{max}$ and $K_m$. These parameters describe the maximum reaction rate and the substrate concentration giving rise to half the maximum reaction rate ($V_{max}$), respectively. $V_{max}$ is the product of the catalytic rate constant ($k_{cat}$) and the total enzyme concentration. $k_{cat}$ describes the frequency of conversion of enzyme-substrate complex to product per time. The ratio of $k_{cat}$ to $K_m$ ($k_{cat}/K_m$) is called the overall catalytic efficiency or specificity constant of
enzymes and is often used to compare the activity changes with mutations. These parameters are specific to substrate and reaction conditions as shown in Table II. An enzyme with lower \( K_m \) and higher \( k_{cat} \) values indicates that it has both high affinity with the substrate and faster catalytic rate under the specified reaction conditions.

The stability of an enzyme refers to its ability to tolerate environmental conditions, such as temperature, pressure, pH, and chemicals. Temperature-based stability of an enzyme is expressed by either its ability to function after thermal treatment or tendency to undergo unfolding. The former type of stability is referred as the kinetic stability and the latter as thermal stability in this manuscript. Kinetic stability is determined by incubating the enzyme for specified duration at higher temperature and then measuring the residual activity when cooled back to the lower (predetermined) temperature. It therefore characterizes the resilience of the enzyme structure to higher temperatures and strongly depends on the unfolding aggregation propensity of enzymes. Kinetic stability is reported as the change in residual activity with temperature and is described by \( T_{50} \) and half-life values \( (t_{1/2}) \) as shown in Fig. 2a and 2b. \( T_{50} \) corresponds to the temperature at which the enzyme loses 50% of its activity. \( t_{1/2} \) is defined as the time required for the enzyme to attain 50% of its residual activity when incubated at a predetermined higher temperature. An increase in these values indicates an increase in the corresponding kinetic stabilities. Thermal stability is measured by melting temperature \( (T_m) \) and stability curves as illustrated in Fig. 2c and 2d, respectively. It is measured by monitoring the fraction of folded protein as a function of temperature using experimental techniques such as nuclear magnetic resonance (NMR), and circular dichroism (CD) spectroscopy. The temperature at which the fraction equals 0.5 is called the \( T_m \). The stability curves represent the variation in the free energy of unfolding \( (\Delta G) \) with temperature. They are parabolic in shape and intersect the \( \Delta G = 0 \) line at both low and high temperatures. The unfolded state of the protein is more favored at temperatures lower and higher than these limits. Broadening and/or shifting of these curves is associated with changes in the thermal stability. For example, as seen in Fig. 2d, the stability curves of the mutants were broader and shifted towards higher \( \Delta G \) values indicating improved thermal stability of the BSLA mutants.

Fig. 3 illustrates the typical behavior of specific activity versus temperature for enzymes adapted to different temperatures. The decrease in the specific activity at higher...
FIG. 2. (a) Residual activities of WT BSLA and mutants determined at 25 °C after incubating at various temperatures for 20 min. $T_{50}$ corresponds to the temperature at which the residual activity is 50%. (b) Change in residual activities with time of WT BSLA and its mutants after incubating at 55 °C. $t_{1/2}$ is the time taken to reach half the initial residual activity. (c) Unfolding transitions of WT BSLA and its mutants obtained by CD spectroscopy. $T_m$ is the temperature at which the fraction of folded (or native) states is 0.5. (d) Stability curves showing the change in free energy of unfolding of the WT BSLA and its mutants determined by differential scanning calorimetry at pH 4.6. (a) and (c) are Reprinted from Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics, 381, Ahmad et al., Thermostable Bacillus subtilis lipases: in vitro evolution and structural insight, 324-330, Copyright (2008), with permission from Elsevier. (b) is Reprinted from Journal of Molecular Biology, 341, Acharya et al., Structural Basis of Selection and Thermostability of Laboratory Evolved Bacillus subtilis Lipase, 1271-1281, Copyright (2004), with permission from Elsevier. [31] (d) is Reprinted from Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics, 1804, Kamal et al., Stability curves of laboratory evolved thermostable mutants of a Bacillus subtilis lipase, 1850-1856, Copyright (2010), with permission from Elsevier.[86, 87]

temperatures ($T>T_{opt}$) has generally been attributed to the loss of the enzyme’s folded structure. Alternative theories have also been proposed to explain the loss of activity.
in the absence of unfolding. However, in all these theories the structural stability and
dynamics (i.e. conformations sampled) of the enzyme play a central role. Therefore, in or-
der to improve activity at higher temperatures, several studies have focused on increasing
either kinetic or thermal stability of the enzyme. Previous work comparing homologous
proteins from psychrophiles, mesophiles and thermophiles concluded that the stability
of these proteins was regulated by changing the flexibility (or rigidity) of the protein
structure. It has been suggested that thermophilic enzymes evolve rigid structures
to remain stable and the thermal energy at high temperatures provide the flexibility to
the active site. On the other hand, the psychrophilic enzymes have not evolved under
thermal selection pressure and generally have lower thermostability but higher flexibility
especially in the active site region. The increased flexibility contributes to the lower
thermal stability of psychrophiles. Consequently, flexibility has become a key parameter
to bridge the mutations (therefore, effect of a residue) to the observed changes in activity
and stability when comparing enzymes.

Flexibility has been used in the literature to describe changes in protein confor-
station over a broad range of spatial and temporal scales. For example, flexibility has
been used to describe the thermal fluctuations in the positions of the protein atoms, as
well as conformational changes involving rearrangement of secondary structure elements.
Flexibility is often measured by B-factors in crystallographic experiments and root mean
square fluctuations (RMSF) per residue in molecular simulations. B-factors are associ-
ated with correcting the scattering power of X-rays related to the mean square atomic
vibrations. Protein flexibility has also been probed by H/D exchange and dynamic flu-
orescence quenching techniques. In molecular simulations, RMSF is calculated from
the fluctuations in the atomic positions of the protein atoms. Usually RMSF is calculated
on a per residue basis and the residues with higher RMSF values are inferred to be more
flexible. Other properties such as changes in number of salt-bridges or hydrogen bonds
and side chain orientations have also been used to quantify flexibility.

Several excellent reviews have discussed the interplay between activity and stability.
We highlight the key points relevant to the manuscript and direct the readers to these
reviews for more detailed discussions. Teilum et al. focused on the relationship between
enzyme stability and substrate/ligand affinity. They presented examples where mutations
in enzymes resulted in positive, negative, and low correlations between their stability and
FIG. 3. Relative activities of enzymes adapted to different temperatures. Psychrophilic (blue), mesophilic (black), and thermophilic (red) enzymes are adapted to low, moderate, and high temperatures, respectively. Hypothetical curve demonstrating the desired activity–temperature relationship in enzymes is shown in green.

substrate affinity. These diverse outcomes were attributed to flexibility changes in the enzyme caused by mutations. It was suggested that the location of the flexibility change within the enzyme structure governed the negative versus positive correlation between stability and affinity. A discussion of the balance between activity and stability in psychrophilic enzymes was presented by Feller. It appears that these enzymes are more flexible to remain active in low thermal energy conditions. In doing so, they sacrifice their substrate affinity and stability. This was supported by the studies that incorporated mesophilic activity in psychrophilic enzymes by targeting residues with potential role in stabilizing local interactions and hence, decreasing the local flexibility. The result was a negative correlation between catalytic rate and affinity. Siddiqui presented the activity-stability trade-off in terms of enthalpy-entropy compensation. The idea is that activity can be increased if the enthalpic barrier decreases and/or the entropic barrier increases. Similarly, the stability can be increased by increasing the associated enthalpic barrier and decreasing the entropic barrier. Enthalpic activation barrier can be achieved by eliminating some enthalpy-related interactions within the active site that will be broken in the transition state. This will result in higher flexibility of the active site. Similarly, mutations on the surface of the protein to enhance electrostatic interactions (and hence rigidifying the protein) for thermostabilization and including solvent molecules in the
active site that are expelled during substrate binding (and hence decreasing the entropic barrier) can be used to balance activity and stability. In most cases, the structural origins for the changes in activity and stability were mapped to the changes in flexibility caused by the mutations. Comprehensively, these investigations demonstrate the possibility of changing activity and stability as well as in the potential of escaping the trade-off between activity, stability and affinity (specificity). However, what remains challenging is the capability to map the origins of these changes to the protein structure and dynamics, which is a prerequisite to develop predictive abilities for engineering enzymes.

In the following sections, we outline the studies probing the relationship of activity and stability in lipases. We primarily focus on *bacillus subtilis* lipase A (BSLA) and *candida antartica* lipase B (CALB) which are two of the most studied lipases. We also briefly discuss relatively less studied lipases such as geobacillus thermocatenulatus (GTL) lipase. In doing so, we span lipases of different sizes as well as broad range of optimal and melting temperature. BSLA, CALB, and GTL are considered mesophilic, psychrophilic, and thermophilic lipases, respectively.

III. LIPASES: STRUCTURE AND REACTION MECHANISM

A. Lipases: Structure

Lipases are enzymes that catalyze the hydrolysis of lipids and belong to the enzyme class of esterases. Over 150 structures of wild type lipases from various organisms and their mutants have been deposited in the protein databank. Lipases from different source organisms and with different substrate preference, have common protein structural features. Representative crystal structures of lipases from three different organisms are shown in Fig. 4a-d. Fig. 4f shows the three structures superimposed on each other. The lipases have a similar core formed by a $\alpha/\beta$ hydrolase fold which comprises of $\beta$-strands enclosed by $\alpha$-helices and loops. The hydrolysis of ester bonds is assisted by a catalytic triad formed by Glu/Asp-His-Ser residues. The nucleophilic Ser is located near a loop between a central $\beta$-strand and an $\alpha$-helix (Fig. 4f). Asp/Glu acid and His, which form the acid-base pair that generate the nucleophile are located in the surrounding loops.

Lipases vary in their molecular weights and amino acid sequence homology. This
FIG. 4. Wild type crystal structures of (a) *Bacillus subtilis* lipase A (BSLA, PDB: 1ISP), (b) *Candida antarctica* lipase B (CALB, PDB: 1TCA), and (c) *Geobacillus thermocatenulatus* lipase (GTL, PDB: 2W22). (d) Superimposed structures of BSLA (grey), CALB (blue), GTL (red) obtained after aligning and minimizing the root mean square deviation between the catalytic triad residues. (e) Organization of the catalytic triad. The enzyme is illustrated by cartoon representation with helices in green, sheets in yellow, and loops in grey (a, b, and c). The catalytic triad is colored by element type and is shown by sticks.

Variance comes from the presence of additional structural elements in addition to the α/β core and include the lid domain, zinc domain and calcium binding domain (Fig. 5). These domains play important functional roles in the activity and stability of lipases. The lid domain, also referred to as extra cap or flap, is made of amphipathic loops or α-helices and protects the lipase binding site. This domain is activated in the presence of a hydrophobic phase (e.g., oil phase) such that the hydrophobic side of the lid is exposed to the hydrophobic phase. This has led to the suggestion that lipases with lid domains have
two conformations – open and closed – which essentially refers to the lid conformations that allow or restrict the access to the binding site, respectively (see Fig. 5). The zinc binding domain has been found to play an important role in the opening and closing of the lid domain as well as in stabilizing thermophilic lipases. Lipases also differ in their substrate specificity. Specificity implies the selectivity of the enzyme towards particular substrates based on the substrate chemistry, chirality and/or chain length. The various structural entities of lipases – active site, substrate binding site, lid domain, zinc domain, calcium binding site – therefore, represent various knobs that can be tuned to engineer the activity and stability of lipases.

FIG. 5. (a) A cartoon representation of the structure of *Geobacillus stearothermophilus* (GSL, PDB: 1JI3). The zinc and lid domains are shown in blue and orange, respectively. The cofactor zinc (blue) and calcium (magenta) ions are illustrated by spheres with their corresponding interacting residues shown by sticks. The core fold containing $\alpha$-helices and $\beta$-sheets are shown in green and yellow, respectively. (b) A demonstration of the difference between the open and closed states of a lipase with lid domain. GSL and *Geobacillus thermocatenulatus* (GTL, PDB: 2W22) were used as models for the closed and open conformations, respectively. Pymol was used to align the structures. Only the lid domains in the open (red) and closed (blue) conformations are colored differently to highlight the differences. (c) A magnified view of the substrate analog (sticks) in the binding site of GTL (surface). The residues constituting the wall of the binding site are colored in green. The catalytic triad is shown by sticks in (a, b) and by spheres in (c).
B. Lipases: Reaction mechanism

The reaction mechanism of ester hydrolysis in lipases is shown in Fig. 6. The hydrolysis involves three residues – Asp/Glu, His, and Ser – referred to as the catalytic triad. The mechanism involves two steps – acylation and deacylation. In the acylation step, the hydroxyl group of Ser is deprotonated by the His residue. This is driven by the interaction of Asp/Glu with His which allows the His to act as a base and deprotonate Ser. The deprotonated Ser is nucleophilic and binds to the carbonyl carbon of the substrate (triglycerides). A negatively charged tetrahedral intermediate is formed and is stabilized by the neighboring oxyanion hole. Hydrogen from His is transferred to this intermediate producing the first product of ester hydrolysis (diglyceride) and acylated lipase intermediate. This is followed by the deacylation step, which begins with the donation of a proton from a water molecule to His. The resulting hydroxyl ion from the water molecule binds with acyl group of the acylated lipase. The negatively charged tetrahedral intermediate thus formed is stabilized in the oxyanion hole. Finally, the protonated His donates its hydrogen to the intermediate producing the second product of the ester hydrolysis (fatty acid) and substrate free lipase.

IV. BACILLUS SUBTILIS LIPASE A

BSLA is obtained from bacillus subtilis, which is a gram-positive, aerobic and endospore forming bacterium. The small size of BSLA (∼19.4 kDa) compared to other lipases, and large biotechnological applications has made BSLA a choice lipase system for several studies probing activity and stability. The crystal structures were resolved at various pH values. BSLA was found to contain only the core α/β hydrolase fold of lipases (Fig.4a) and structure lacks the lid domain. Thus, the catalytic site is exposed to the solvent. It is tolerant to extreme alkaline environments with an optimal activity at pH 10, $T_{opt} = 35^\circ C$ and $T_m = 56^\circ C$.

Several rational and semi-rational mutagenesis strategies to generate variants of BSLA that are more stable, or more selective, or more active, or any combination thereof, have been reported. It seems that while it may be relatively straightforward to enhance one of these properties, being able to achieve two or more with minimal mutations is challenging.
FIG. 6. Schematic of the reaction mechanism of the breakdown of a triglyceride into a fatty acid and diglyceride facilitated by a lipase. O, N, H, and R indicate oxygen (red), nitrogen (blue), hydrogen (black), and side chains (black), respectively. R1, R2, and R3 represent the fatty acid chains in the triglyceride. Marvin sketch was used to draw the reaction mechanism.

Funke et al. performed directed evolution experiments to improve selectivity of BSLA by checking for variants with higher enantioselectivity in each generation. They were able to obtain more selective mutants in this manner. However, it was observed that the more selective mutants had lower kinetic stability. The authors speculated that while some mutations were enhancing the selectivity, others were negatively affecting the stability.

In a series of experiments by Rao’s group, error-prone polymerase chain reaction was combined with saturation mutagenesis to obtain more stable variants of BSLA (Table I Rows 1-23). From the first generation, they identified a single-site mutant (N166Y) for further evolution. In the later generations, mutants with potential destabilizing local contacts, estimated based on their physicochemical properties and location within the BSLA structure, were eliminated. Consequently, variants with single and multiple site
mutations distributed throughout the structure were obtained. A variant with 12 mutations (Table 1 Row 21, 22, and 23) was attained at the end of the fourth generation (referred to as 6B), which showed improved thermodynamic stability, higher specific activity over a wide range of temperatures, higher catalytic efficiency, tolerance to chemical denaturants, and lower aggregating tendency. The success of this method indicates the possibility of modulating activity-temperature relationship using mutagenesis. Various experiments probing the structural and thermodynamic properties of the variants have been performed. Thermodynamic analysis indicated that the BSLA variants obtained in Rao’s group have broader stability curves with a higher maximum than the mesophilic wild type BSLA (Fig. 2b). This suggests that the observed activity at higher temperatures in these variants could be a result of increased population of active conformations with higher thermodynamic stability.

The challenge has been in elaborating the contribution of each mutation on the activity and stability of the variants. Simulations and experimental studies have been performed to probe this and in some cases, have resulted in contradictory conclusions. The three-dimensional crystal structures of the different mutants are similar with the largest RMSD of ~0.4Å between the structures. Based on the premise of the flexibility-activity-stability relation, the active site flexibility of BSLA and its variants has been probed. The crystallographic B-factors were determined to relate the differences in flexibility to the possible origins of the enhanced activity and stability in the BSLA mutants. This indicated that the active site flexibility decreased for the mutants and was suggested to be the reason for enhanced thermostability. A subsequent study used molecular dynamics (MD) simulations and time-resolved fluorescence anisotropy measurements to further probe this in WT and 6B BSLA mutant. They also found diminished active site fluctuations. In addition, it was reported that the organization of residues in the active site became more favorable in the mutants. Both these factors were surmised to contribute to the enhanced activity. In contrast, Singh et al concluded that the active site flexibility increased for the mutants based on their MD simulations. It is worth noting that the conditions (temperature) were different between the two simulations – higher temperature was used in Singh et al. study. This also suggests that perhaps the change in flexibility with temperature needs consideration. This could affect the flexibility and hence, the conclusions drawn.
In addition to flexibility, other potential factors affecting the stability and activity interplay have been proposed. Iterative saturation mutagenesis was performed on residues with high B-factors obtained from crystallographic structure of BSLA to enhance stability. This method was developed to accelerate the search for better variants using directed evolution. The mutants (Table I Rows 24-26) generated had high residual activities following incubation at different temperatures. However, their thermal stability was found to be lower than the WT. Later experiments indicated lower aggregation propensity of the variants as the likely reason for the improved residual activities at high temperatures. This role of aggregation in improving residual activity was also indicated as a factor in enhancing stability of the 6B mutant from Rao’s group. Yoo’s group used an alternative approach focused on enhancing local packing by replacing smaller residues with larger residues (e.g. Gly to Ala or Ala to Val) in the core of the lipase. This method was successful in producing mutants (Table I Rows 26-37) with high stability but resulted in compromised activity. The authors hypothesized that the stability could be improved while retaining activity by increasing local packing with minimal perturbations to the local network of interactions. Accordingly, a later study resolved this activity-stability trade-off by using an additional criteria related to the local interactions of the residue for mutating sites.

Computational studies provide a parallel tool to probe the structural origins underlying the changes in the properties of the mutants. Several MD simulation studies of the WT BSLA and its mutants have been reported. Differences in various parameters such as RMSD, changes in number of salt-bridges, active site residue conformations, change in hydrogen bonding patterns etc have been reported. Collectively, these studies have found several different interactions/phenomena that could play a role in affecting stability and activity. Motivated by this network analysis based methods have been applied to study lipase structures. In these methods, lipase is represented in terms of networks of interactions and insights into the role of different amino acids is assessed based on its connections to the other residues in the lipase.

Rathi et. al used constraint network analysis (CNA) to relate structure and stability of BSLA. CNA is based on the rigidity theory and identifies residues that are structural weak spots based on their interaction networks. Using this methodology, Gohlke and coworkers identified that the more thermostable mutants of BSLA obtained
FIG. 7. Panels (a) and (b): The crystal structures of WT BSLA and its mutant (6B), respectively. (c) and (d) represent the corresponding network communities in each structure. Each community of WT (c) is represented by different color. The same color scheme was used in (d) to illustrate the reorganization of the network in the mutant. Each number indicates the node (C\text{\alpha} of each residue) in the network. Additional contacts established in mutant are represented by black lines in (b). Figure is reproduced from Srivastava and Sinha.\cite{90}

in Rao’s group were more rigid than the WT and when applied to the less thermostable mutants obtained by Reetz et al.\cite{32}, they found that the mutants were more flexible. While this indicated some correlation between the flexibility and stability, further decomposing this into the effects of the individual mutated residues became difficult. For example, the observed increase in rigidity was suggested to arise from stronger contacts between the \beta-strands and \alpha-helices buried in the core even though the mutations were at the lipase surface. This indicates that mutations can have long-range effects in the protein and therefore, while no large change may be obvious, collective subtle changes across the lipase structure can lead to differences in the activity and stability. This aspect was fur-
ther highlighted by the work of Srivastava and Sinha. They performed protein contact network analysis on the six BSLA mutants including 6B reported by Rao and coworkers. Based on the network, the authors identified communities – residues within the network that are more connected among themselves compared to other residues. It can be loosely thought of as cluster of interacting residues. Fig. illustrates the crystal structure and communities of WT BSLA and 6B mutant. While no major differences are seen in the crystal structure, the changes in the communities of the WT and 6B mutant are clearly seen. For example, the first mutation of the 6B generation (N166Y) splits the red community of WT into two communities. This can have potential impact on the contacts between different helices in the lipase structure. The network based approaches highlight the intricate interconnection of the residues within the protein structure. In a recent study, Tian et al. were able to develop a relation between residue-residue contact energy matrix to change in melting temperature upon mutation. They obtained encouraging results when the strategy was applied to BSLA. Collectively, these studies indicate that proteins are networks of contacts and therefore, to successfully develop predictive tools to ascertain the activity and stability of a mutant we need to begin with considering the protein as a whole and probe the interplay of local and global changes.

**TABLE I. Thermodynamic and kinetic properties of BSLA**

<table>
<thead>
<tr>
<th>ID</th>
<th>Lipase</th>
<th>Substrate</th>
<th>(T_{\text{opt}}) (°C)</th>
<th>(T_{\text{m}}) (°C)</th>
<th>(t_{1/2}/T) (min)/(°C)</th>
<th>(K_m) (mM)</th>
<th>(k_{\text{cat}}) (min(^{-1}))</th>
<th>(k_{\text{cat}}/K_m) (min.mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WT(^{\text{ES}})</td>
<td>PNPA</td>
<td>31</td>
<td>–</td>
<td>2.5/55.0</td>
<td>0.97</td>
<td>520</td>
<td>540</td>
</tr>
<tr>
<td>2</td>
<td>WT(^{\text{EO}})</td>
<td>PNPA</td>
<td>35.0</td>
<td>55.3</td>
<td>2.8/55.0</td>
<td>0.98</td>
<td>220</td>
<td>220</td>
</tr>
<tr>
<td>3</td>
<td>WT(^{\text{ES}})</td>
<td>PNPA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.98</td>
<td>220</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>WT(^{\text{ES}})</td>
<td>PNPA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.29</td>
<td>261</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>1-1E11 (\text{N166Y})</td>
<td>PNPA</td>
<td>–</td>
<td>–</td>
<td>25.4/55.0</td>
<td>1.03</td>
<td>500</td>
<td>480</td>
</tr>
<tr>
<td>6</td>
<td>DM(^{\text{ES}})</td>
<td>PNPA</td>
<td>–</td>
<td>–</td>
<td>228.0/55.0</td>
<td>1.22</td>
<td>680</td>
<td>560</td>
</tr>
<tr>
<td>7</td>
<td>TM(^{\text{ES}})</td>
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<td>61.2</td>
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<td>61.2</td>
<td>530.0/55.0</td>
<td>0.93</td>
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<td>61.2</td>
<td>530.0/55.0</td>
<td>0.93</td>
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<td>0.79</td>
<td>290</td>
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<td>101.2/75.0</td>
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<td>430.5/75.0</td>
<td>0.51</td>
<td>414</td>
<td>811</td>
</tr>
<tr>
<td>22</td>
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<td>–</td>
<td>93.0</td>
<td>78.2</td>
<td>430.5/75.0</td>
<td>0.51</td>
<td>414</td>
<td>811</td>
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<td>78.2</td>
<td>430.5/75.0</td>
<td>0.51</td>
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<td>0.51</td>
<td>414</td>
<td>811</td>
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<td>25</td>
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<td>–</td>
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<td>26</td>
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<td>78.2</td>
<td>430.5/75.0</td>
<td>0.51</td>
<td>414</td>
<td>811</td>
</tr>
</tbody>
</table>

* Reported units of kcat are mM in Ahmad et al.85
* This is the the unfolding initiation temperature, which indicates the first point of folded to unfolded transition detected in CD spectroscopy.
* These are specific activities in U/mg at 50°C.
* PNPA: p-nitrophenylacetate, PNPB: p-nitrophenyl butyrate, PNPC: p-nitrophenyl caprylate.
V. *CANDIDA ANTARCTICA* LIPASE B

*Candida antarctica* lipase B (CALB) was widely used psychrophilic lipase with an optimal activity at pH 7 and $T_{opt} = 45^\circ$C. CALB is larger than BSLA with a molecular weight of $\sim 33$ kDa (Fig. [1]). It has also been suggested that CALB has a lid domain shielding its catalytic triad. However, the presence of the lid domain is debated. Interfacial activation, which is considered the signature for lid mechanism, is reported to be absent for CALB. On the other hand, Stauch et al. determined open and closed conformations of CALB. The open and closed conformations showed displacement of, and differences in the secondary structure elements of the presumed lid region. The resulting narrow opening to the active site results in the selectivity of CALB towards smaller substrates such as secondary alcohol with one substituent being an ethyl group.

Based on the idea of the narrow opening to the active site, Magnusson et al. used site-directed mutagenesis to engineer CALB for larger substrates. They mutated the tryptophan near the catalytic Ser to smaller residues (His, Gln and Ala), and were able to improve the specificity towards larger substrates. The corresponding structural and stability changes were however, not investigated. It is possible that because the mutated tryptophan is in the vicinity of the catalytic Ser, the mutations affect the active site flexibility, which could also impact substrate specificity. Lutz et al. applied circular permutation to obtain variants of CALB with higher catalytic activity. Few variants thus generated displayed enhanced catalytic efficiency but had lower stability than WT CALB. Also, the increase in catalytic efficiency was dependent on the substrate (Table [II] Rows 1-16). The variants with higher activities had their new termini relocated to the lid domain. Comparison of the WT and variants structures indicated similar tertiary but some differences in the secondary structural features. These structural changes appeared to be local to the regions changed in circular permutation – that is, the native and new protein termini. It was posited that these changes affect the local flexibilities and lead to lower stabilities in the variants.

Xie et al. applied iterative saturation mutagenesis to obtain mutants of CALB with higher stability. Residues with high B-factors and within 0.1 nm of the active site were targeted. Two mutants (Table [II] Rows 17-20) with higher kinetic stability and similar activity to WT were obtained. A double mutant comprising both the mutations was
found to have higher kinetic and chemical stability than the corresponding single mutants. Interestingly, the $T_m$ values did not differ significantly for WT and the mutants. The crystallographic B-factors suggested lower flexibility of the active site. Using MD simulations, Xie et al concluded that the mutations resulted in increased hydrogen bonding within the mutant structures thereby, inducing rigidity. This was suggested to be the reason for increased kinetic stability of the mutants. However, the reason that the change in flexibility does not affect $T_m$ but changes in kinetic stability were not explained. Zhang et al.\(^{106}\) employed directed evolution to obtain kinetically more stable mutants (Table III Rows 21-26). The mutations replaced a hydrophobic residue with a polar residue in a hydrophobic patch on the enzyme surface. It was suggested that this reduction of the hydrophobic patch made the mutants less aggregation prone and hence, more kinetically stable. Le et al.\(^{45}\) focused on introducing disulfide bonds in CALB to increase its thermostability. They used a two-step criteria to identify the appropriate locations for the disulfide bonds. In the first step, residue pairs for cysteine mutations were identified based the likelihood of forming a disulfide bond. The likelihood was determined based on distance, angle and energy based parameters. In the second step, the residue pairs identified are filtered with additional criteria related to their flexibility in WT structure. Le et al. identified three pairs for mutations (Table III Rows 27-32), of which one resulted in higher kinetic stability (Table III Row 28). The $T_m$ and activity of the mutant was similar to that of WT. The increased kinetic stability was credited to reduced flexibility of the mutant. The others, in spite of having lower flexibility than WT, showed reduced kinetic stability. It was suggested that perhaps the mutations were distorting the active site in these cases. Clearly, flexibility alone could not explain the trends of the mutants. Collectively these studies illustrate that akin to BSLA, several factors can contribute to the balance of stability and activity in CALB.

**TABLE II.** Thermodynamic and kinetic properties of CALB

<table>
<thead>
<tr>
<th>ID</th>
<th>Lipase</th>
<th>$T_{opt}$ (°C)</th>
<th>$T_{50}$ (°C)</th>
<th>$T_m$ (°C)</th>
<th>$t_{1/2}/T$ (min)/(°C)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ ((min.mM)$^{-1}$)</th>
<th>Substrate$^b$</th>
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</thead>
<tbody>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>PNPB</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>0.0026</td>
<td>2</td>
<td>800</td>
<td>DiFMU</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1.2</td>
<td>80</td>
<td>70</td>
<td>PNPB</td>
</tr>
<tr>
<td>4</td>
<td>cp193$^{105}$ (Q193/P192)$_{–}$</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>100</td>
<td>DiFMU</td>
</tr>
<tr>
<td>5</td>
<td>cp268$^{105}$ (P268/T267)$_{–}$</td>
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<td>-</td>
<td>-</td>
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<td>3051</td>
<td>5260</td>
<td>PNPA</td>
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VI. OTHER LIPASES

In addition to BSLA and CALB, lipases from other organisms have also been studied. Here, we discuss a subset of these focusing on lipases larger than BSLA and CALB. Geobacillus thermocatenulatus lipase (GTL) belongs to the lipases with the ability to function in various organic solvents, extreme alkaline environments, and high temperature.

It has a molecular weight of $\sim 43$ kDa and comprises a lid domain with two helices and a zinc binding domain. GTL shows enhanced activity in the presence of a hydrophobic...
interface and large secondary structural changes in one of the helix of the amphipathic lid upon activation. Enzyme engineering studies have targeted both the lid and zinc binding domains to modify the activity-stability of GTL.

Tang et al.\cite{75} mutated some of the polar residues to non-polar amino acids in the two helices of the lid domain in GTL (Y225F/S232A, S232A/T236V, Q185L). Though all the resulting mutants had lower activity than the WT in homogenous systems, one mutant (Y225F/S232A) was found to have higher activity in a heptane-water heterogeneous system. This increase was attributed to the enhanced interfacial adsorption compared to other mutants. In a related study of T1 lipase from Geobacillus zalihae\cite{110}, which has 95% sequence identity to GTL, the role of hydrophobic amino acids in the lid domain was explored. Single point mutations were made by replacing non-polar amino acids with polar amino acids of similar size (Table IV Rows 1-7). While all the mutants had lower thermal stability compared to WT, two mutants had improved catalytic efficiency. The mutants also exhibited different specificity to substrates of varying chain lengths relative to WT. These studies indicate that modifications to the lid domain can be used to design the activity, stability and specificity of larger lipases. The zinc binding domain is another region that can be modified to change the stability of GTL (and similar) lipases. The absence of zinc ion or mutations of the zinc coordinating residues to Ala in Geobacillus stearothermophilus (95% sequence identity to GTL)\cite{74} resulted in lower thermostability of the lipase.

Directed evolution based on random mutagenesis was used to screen low temperature activity mutants of a lipase\cite{109} with 96% sequence identity to GTL (obtained by comparing protein sequences in NCBI\cite{111}). The resulting single site (S130T) mutant had a 30 °C lower $T_{opt}$ relative to WT. Homology modeling indicated that the mutation was located in a loop connecting two helices, one of which is vicinal to the active site. It was suggested that hydrogen bonds involving Ser held the helices together, which were broken due to the mutation resulting in enhanced flexibility of the loop and hence lower $T_{opt}$. In a related study\cite{108}, kinetic stability changes with mutations near the active site were probed (Table IV Rows 8-11). The trends observed in the kinetic stability were explained based on inferences about the flexibility and local electrostatic interactions based on comparing structures of the WT and mutants.

Combined experimental and simulation studies were used to probe the molecular ori-
gins of the higher activity of double mutant (F180P/S205G) of pseudomonas mendocina lipase. It was also found that aggregation of the mutant was lower than the WT which was considered to be the underlying reason for the increased activity. NMR experiments revealed similar global structures with local differences and NMR relaxation experiments showed differences in the local dynamics. H/D exchange experiments indicated higher local stability of the mutant in certain secondary structural elements. MD simulations suggested that the mutant had lower local flexibility than the WT. The mutant was determined to have higher melting temperature than the WT using CD/DSC/Fluoroscence spectroscopy experiments. Based on these detailed experiments, it was suggested that the reduced local flexibility of the mutant enhanced the stability and reduced the unfolded states available for aggregation thereby, increasing activity of the mutant. Similar to CALB and BSLA, these studies highlight the different approaches available for balancing activity and stability of lipases.

TABLE III. Thermodynamic and kinetic properties of other lipases

<table>
<thead>
<tr>
<th>ID</th>
<th>Lipase</th>
<th>T_{opt} (°C)</th>
<th>T_{50} (°C)</th>
<th>T_{m} (°C)</th>
<th>t_{1/2} (min)</th>
<th>K_{m} (mM)</th>
<th>k_{cat} (min^{-1})</th>
<th>k_{cat}/K_{m} ((min.mM)^{-1})</th>
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<td>–</td>
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<td>3.15</td>
<td>0.00266</td>
<td>–</td>
<td>PNPL</td>
</tr>
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^a PNPM: para-nitrophenol myristate, PNPL: para-nitrophenol laurate.

VII. CONCLUSIONS AND PERSPECTIVES

The studies described here demonstrate the ability of the current experimental techniques in modifying the activity and stability of lipases. It is clear from these studies that there are various knobs that can be adjusted to fine tune the properties of the lipases. It is also apparent that in several cases this can be achieved through mutations of few (one
or two) residues of the lipase. However, the question that remains unanswered is – how do we know a priori which residue to mutate? To answer this question, it is necessary that we relate the mutations to the structure of lipase and relate those structural changes to the activity and stability of lipases.

Several experimental and simulation studies have focused on teasing out the structural origins of activity and stability in mutants of lipases. Inspired by the differences in the protein flexibility of cold- and heat-adapted proteins, several of these studies have attempted to correlate the flexibility differences in the mutants relative to WT to the differences observed in the activity and stability. Flexibility has been broadly used to refer to the softness, dynamics, fluctuations and plasticity of the enzyme. In general, flexibility has been characterized primarily based on B-factors and RMSF in most studies and refers to both thermal fluctuations of the atoms (residue) around an average position and large changes in the protein conformation. While some correlations have been seen, no clear trend has emerged. One primary reason for this is the lack of a clear measure for flexibility. Secondly, it is not yet established which regions' local flexibility is important for activity. Lastly, how a mutation will translate into change in flexibility is not known. For example, in ongoing efforts in our research, we have employed the idea of increasing active site flexibility of thermophilic GTL to improve its low temperature activity. To do so we replaced larger residues vicinal to the active site with Gly.[112] Some mutations indeed have the desired higher activity at low temperatures. We characterized the flexibility of the WT and mutant GTL using MD simulations. We measured flexibility using various parameters – RMSF on a per residue basis, fluctuations in the side chains, as well as changes in the arrangement of the catalytic triad. While we did see differences in the WT and mutant, we did not observe consistent trends correlating flexibility and experimentally measured activity. In addition, we observed that there were changes in the flexibility both close to and away from the mutated site. Observations such as this led to the concept of representing proteins as networks of interactions.

Network representation of proteins has the advantage of accounting for the interactions between the residues and hence, capturing the potential long range effect of mutation within the protein structure. This is seen in the analysis of BSLA and its mutants, where while there were small structural differences, considerable differences in the network of interactions was observed. In fact, in our studies of GTL and its mutants, we used network
analysis to measure flexibility and found better correlation between this measure of flexibility and activity. Collectively, these findings emphasize the importance of considering the protein holistically in thinking about flexibility. Focusing on the local interactions of the mutated residue alone to interpret flexibility will most likely fall short of appreciating the complexity of the problem.

FIG. 8. An illustration of the balance between activity and stability. The weights indicate the factors that play a role in maintaining the balance between activity and stability. Each double headed arrow represents the interconnection between the weights, suggesting that they are not independent. The question mark refers to the other unknown factors that may affect the balance.

Collectively, the studies have also revealed that various factors beyond flexibility contribute to maintaining the balance of activity and stability in lipases. In fact, some studies have contested the role of protein flexibility/dynamics in enzyme catalysis suggesting that they may be independent properties. As illustrated in Fig. 8 several factors such as packing of the hydrophobic core of lipase, aggregation propensity, active site conformation, and interactions with co-factors affect the activity-stability balance. It is also critical to appreciate the interconnection between these factors. For example, while increasing the packing of the hydrophobic core stability can be improved it is crucial to evaluate the effect on the active site. In developing predictive abilities that provide best sites within
the lipase structure to mutate, and the best options for the residues to replace with for desired activity, specificity and stability, it is important to consider all these facets of the problem. This endeavor requires further experimental studies that will add to the current dataset. Concurrently, molecular simulations combined with network analysis are required to identify the structural origins of the activity and stability behavior. As the field progresses, it will also become important to consider standardized experimental and simulation conditions and approaches – so as to facilitate comparison between various studies.

VIII. ACKNOWLEDGEMENTS

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