Using Rigidity Analysis To Probe Mutation-Induced Structural Changes in Proteins

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***Abstract*—Predicting how a single amino acid substitution affects the stability of a protein structure is a fundamental task in macromolecular modeling. It has relevance to drug design and understanding of disease-causing protein variants. We present KINARI-Mutagen, a web server for performing *in silico* mutation experiments on protein structures from the Protein Data Bank. Our rigidity-based approach permits fast evaluation of the effects of mutations that may not be easy to perform *in vitro*, because it is not always possible to express a protein with a specific amino acid substitution. In two case studies we use KINARI-Mutagen to identify exposed residues that are known to be conserved, and we show that our prediction in the change in a protein's stability due to a mutation of an amino acid to glycine can be correlated against experimentally derived stability data. KINARI-Mutagen is available at http://kinari.cs.umass.edu.**

***Keywords*-protein rigidity; mutations; stability prediction**

I. Introduction

In this paper, we present KINARI-Mutagen, a web application for performing computational mutation experiments using rigidity and flexibility analysis.

Mutations in proteins. A mutation in a protein's amino acid sequence can have deleterious effects on its stability and function. A number of diseases result from single point mutations. Hence knowing their effect can be used to guide the design of drugs aimed at combating those disorders. To predict and better understand the roles of mutations, the genetic information that codes for the amino acid sequence of a protein can be altered, and the expressed mutant proteins can be analyzed to infer the impact of the specific mutation. Such studies are aided by several widely-used molecular biology techniques, such as site-directed mutagenesis [9]. Unfortunately, such experiments are often labor and time intensive. The possible number of mutants that can be made from even the smallest proteins makes exhaustive mutagenesis studies impractical. For example, 20^{100} mutants can in principle be engineered for a 100-residue protein using the 20 naturally occurring amino acids.

Rigidity analysis of proteins. Flexibility information can be obtained through several computational methods. Here we focus on rigidity analysis as implemented in our software KINARI [5], which calculates the rigid regions of a protein structure. The premise is that the protein's function is directly correlated with its distribution and sizes of rigid clusters, and destabilizing any of them will have an observable effect. Fig. 1 shows the identified rigid regions in the protein lysozyme from bacteriophage T4.

Rigid clusters are groups of atoms whose pair-wise distances are determined by inter-atomic interactions, such as covalent bonds, angle constraints, and other types of interactions (e.g. hydrogen bonds). KINARI uses an efficient combinatorial algorithm (the pebble game) to quickly compute the rigid clusters, and does not rely on expensive computational methods such as molecular dynamics or all-atom energy calculations.

Our contribution: KINARI-Mutagen. We extend KINARI to generate mutant protein structures and analyze their rigidity. We present here the first version of this new tool, KINARI-Mutagen. Its ultimate goal is to identify mutations that destabilize a protein. This first version demonstrates that even the simplest type of experimental mutation, called an *excision*, yields valuable information.

In an excision, a mutated residue has the hydrogen bonds and hydrophobic interactions of its side-chain removed from the molecular model. This is equivalent to computationally mutating the residue to a Glycine. KINARI performs rigidity analysis on all generated mutants, in near real-time. For the current release of the software, the aggregated rigidity results

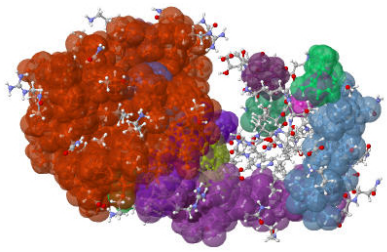


Figure 1. Rigidity analysis uses an efficient combinatorial pebble game algorithm to identify rigid regions of a protein. KINARI-Mutagen was used to perform rigidity analysis on PDB file 2LZM, lysozyme from bacteriophage T4. The color bodies indicate clusters of atoms that are rigid.

for all of the analyzed mutants are summarized through several metrics and plots. These can be used to infer how each mutation affects the rigidity of the protein. In this paper we demonstrate how to use KINARI-Mutagen to identify solvent accessible residues that are known to be conserved. We also correlate rigidity-based stability predictions with experimentally derived data of mutant proteins.

II. Background and Related Work

Here we review previous work that addressed the effect of mutations on the function and structure of a protein. We briefly introduce rigidity analysis, and we summarize previous rigidity-stability studies. Finally, we sketch how modeling of proteins is performed in our KINARI software.

A. Mutations Affect Protein Structure and Function

Deoxyribonucleic acid, DNA, contains the genetic instructions on how amino acids should be joined end-to-end to make a protein. Protein synthesis is a two-step process. If there is an error in either the transcription or translation steps, then the resulting amino acid sequence may differ from the most common sequence of amino acids, which is designated the **wild-type** version of that protein. A protein with mutations is called a **mutant**. Mutant proteins contribute to many genetic diseases. For example, single point mutations in the cystic fibrosis transmembrane conductance regulator protein lead to development of cystic fibrosis. In the protein α-galactosidase there are over 190 single point mutations that lead to development of Fabry Disease [6]. Thus understanding the effect of point mutations is of biomedical importance.

A mutation in the amino acid sequence can affect the protein's shape and stability. These changes can affect the protein's internal motions, and hence inhibit its function. Matthews *et al.* [2] have found that temperature-sensitive mutations often occur at residues which are structurally important (low mobility). Similarly, a single mutation at a residue location that plays a crucial role in a protein can render a protein inoperative [8]. However, not all mutations are equally disruptive. Therefore it is important to know how a mutation will affect the protein.

B. Predicting The Effects of Mutations

One way in which the role of a residue substitution can be directly studied is by mutation experiments in the physical protein. Matthews *et al.* have designed and analyzed many mutants of lysozyme from the bacteriophage T4. When core residues in lysozyme were substituted by alanine, an analysis of the crystal structures revealed that the unoccupied volume in some of the mutants underwent a collapse, while other mutants formed an empty cavity [26]. Residues of T4 lysozyme with high mobility or high solvent accessibility were shown to be much less susceptible to destabilizing substitutions. The authors concluded that residues that are held relatively rigidly within the core of the protein make the largest contribution to the protein's overall stability [1]. The magnitude of the contributions of various substitutions to the thermodynamic stability of proteins can be directly measured. Various substitutions result in destabilization of up to 2.7 to 5.0 kcal/mol [4].

Although the studies by Matthews and others provide precise, experimentally verified insight into the role of a residue based on its mutation, such studies are time consuming and often cost prohibitive. Moreover some mutant proteins cannot be expressed, due to dramatic destabilization caused by the mutation. Given these factors, only a small subset of all possible mutations can be studied explicitly. To address this, several computational methods and analysis techniques have been proposed.

In computational experiments by Lee, *et al.* [14], the side-chains in each of 78 structures of mutant proteins were perturbed. A heuristic energy measure, E_{calc}, was used to predict the stability of each protein. E_{calc} for each mutant was then compared to known activity measures. In other work, Gilis, *et al.* [7], estimated the folding free energy changes upon mutations using database-derived potentials, and correlated them with experimentally measured ones. Their results indicate that hydrophobic interactions contribute most to the stabilizing of the protein core. Similarly, Prevost, *et al.* [18], have used molecular dynamics simulations to study the effect of mutating Barnase residue Isoleucine 96 to alanine, and predicted that the major contributions to the free energy difference arose from non-bonded interactions.

Thus, some progress has been made in predicting the effects of mutations on protein stability. However, many such methods rely on computationally intensive energy calculations, or are not able to infer the role of a single amino acid in stabilizing a protein's structure. To address these issues, we seek to apply rigidity concepts to the computational prediction and analysis of the stability of mutant protein structures.

C. Rigidity Theory Applied to Mechanical Structures

Geometric and combinatorial methods from rigidity theory have been applied to the study of protein flexibility, by associating a network of nodes (atoms) connected by rigid bars (bonds and other stabilizing interactions). The study of rigidity and flexibility of these *bar-and-joint frameworks* has a long history going back to the 19^{th} century, as engineers analyzed cross-bracing in large-scale steel structures.

A simple counting rule, identified by James Clerk Maxwell [15] in 1864, and proven correct in dimension 2 by Laman [11], was adapted to 3-dimensional structures known as *body-bar* and *body-hinge frameworks* [21]. These structures can be analyzed with efficient algorithms, based on the pebble game paradigm [10], [12], and applied to mechanical models associated to molecular structures.

D. Mechanical Modeling of Proteins

In the *body-bar-hinge framework*, a **body** is a set of atoms rigidly attached to each other. Methane for example is rigid, because all the pair-wise distances between the atoms are determined by the existing covalent bond length and angle constraints (Fig. 2(a)). Ethane however exhibits one degree of flexibility, because the C-C bond permits rotation (Fig. 2(c)). Rotatable bonds are modeled as **hinges**.

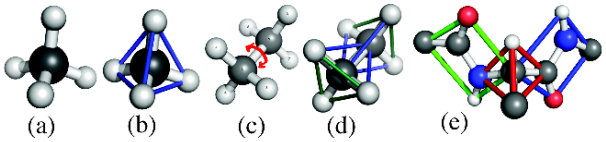


Figure 2. Methane (a) is rigid because all pair-wise distances between atoms are fixed (b). In ethane (c), a carbon atom (gray) and its bonded neighbor atoms form a rigid body. The two bodies share a hinge along the center C-C bond. The abstract *body-bar-hinge framework* for ethane is shown in (d); two rigid bodies (represented as tetrahedra) share a hinge (not visible). A protein's peptide units are modeled as rigid bodies (e).

E. Rigidity Based Protein Flexibility: Literature Review

Rigidity analysis of protein structures was pioneered by Thorpe, Jacobs and collaborators [24]. They studied different states of HIV-1 protease and showed that the rigid clusters in open and closed conformations of the protease are correlated with the known mechanical properties of the molecule.

Rader *et al.* [19] simulated the thermal unfolding of rhodopsin, a trans-membrane receptor, by performing a *rigidity dilution analysis* using the FIRST software [23]. This method removes hydrogen bonds one after another, from weakest to strongest, and performs rigidity analysis after each removal. A "folding core" is identified when there exists only one rigid cluster with at least three residues of two or more secondary structures. The computed core was correlated with experimental results.

Rigidity theory has also been used to investigate the possible motions and to gain insights into the structural stability of proteins. Rader *et al.* [20] have combined elastic network models with rigidity analysis of constraint networks for freely rotating rods to predict protein folding nuclei. Their method was verified against data that was attained from native state hydrogen-deuterium exchange experiments.

However, none of these studies identified critical residues that help to mechanically stabilize the protein.

III. System Description and Analysis Tools

KINARI-Mutagen is a user-friendly suite of online software tools for investigating how different residues affect the rigidity and stability of a protein. Analyzing a protein is decomposed into four phases: 1) downloading and curating a PDB file, 2) performing excision to generate mutants, 3) analyzing the rigidity of each mutant, and 4) analyzing the results to identify possible critical residues.

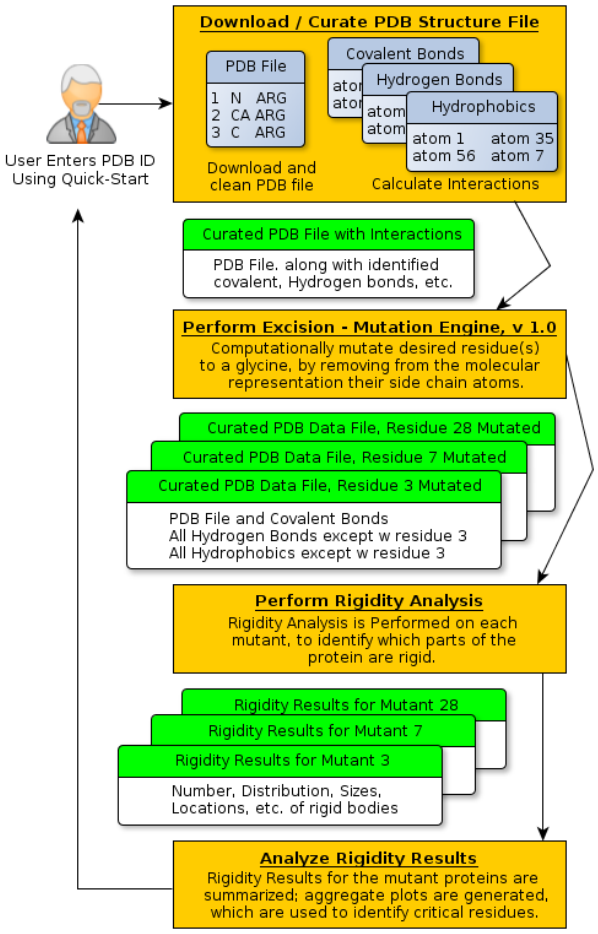
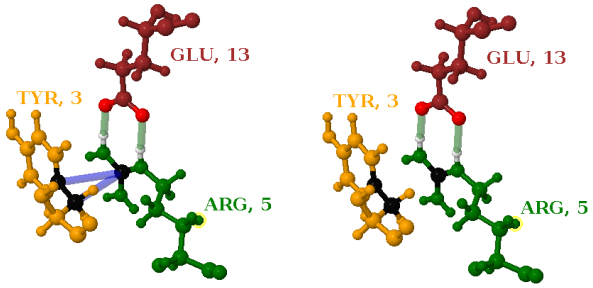


Figure 3. KINARI-Mutagen downloads a PDB file, perform excision to generate mutants, analyze their rigidity, and aggregates the rigidity results. The generated plots and metrics provide information about which residues are critical in maintaining the protein's rigidity. Shown here is the procedure for performing excision on residues 3, 7 and 28 to generating three mutants.

KINARI-Mutagen provides a direct link to KINARI-Web [5], for downloading and curating a PDB file. Chains, ligands and water molecules in the retrieved protein structure file can be retained or removed, if analysis is desired on only a specific part of the protein. Covalent and non-covalent interactions are also identified.

The KINARI Mutation Engine (version 1.0) generates mutants. It performs a simple computational mutation, where a residue is converted to a glycine. For the purpose of performing the rigidity analysis, it is not necessary to alter the positions of, or remove, atoms. Instead, it suffices to remove the side-chain's hydrogen bonds and hydrophobic interactions from the protein's molecular framework. This functions in our model like the removal of a side-chain. Subsequent versions of the Mutation Engine will permit increasingly advanced mutation functions. Because rigidity analysis is efficient, many generated mutant protein structures can be analyzed quickly, in near real-time.



(a) No excision performed (b) Excision on Residue 3

Figure 4. KINARI-Mutagen simulates the mutation of a residue to glycine by removing its side-chain hydrogen bonds and hydrophobic interactions from the molecular model. In the wild-type of PDB file 2PM1, two hydrogen bonds (light green bars) and two hydrophobic interactions (blue bars) exist among residues 3, 5, and 13 (a). When excision is performed on residue 3 (b), the hydrophobic interactions that it forms with residue 5 are removed. When excision is performed on residue 5, both the hydrogen bonds between residue 5 and 13 and the hydrophobic interactions between residue 5 and 3 are removed (not shown).

We demonstrate the excision process on a fragment of human α-defensin 1 (Fig. 4), which contains two hydrogen bonds (light green bars) between residues 5 and 13, and two hydrophobic interactions (blue bars) between residues 3 and 5 (Fig. 4(a)). When excision is performed on residue 3, the hydrophobic interactions between it and residue 5 are removed from the molecular framework (Fig. 4(b)). When excision is performed on residue 5, then the hydrogen bonds and hydrophobic interactions that it engages in are removed.

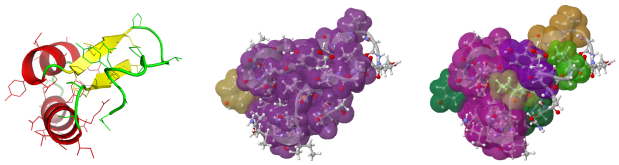
In the third phase of KINARI-Mutagen, the KINARI software is invoked to perform rigidity analysis on each mutant. Detailed descriptions of the rigidity calculation and modeling options are described in [5]. When rigidity analysis is complete, an integrated Jmol-based visualizer is used to inspect the rigid regions of each mutant.

In the final stage of KINARI-Mutagen, the rigidity results for each of the mutants are aggregated. Information about critical residues can be inferred from several of the generated plots. This first version of KINARI-Mutagen does not perform automated analysis, nor does it attempt to predict which residues are critical; that is left to the user to interpret from the generated results. In future versions of the software we plan to provide automated analysis tools, such as outlier detection algorithms, to help identify mutated residues that affect the rigidity of a protein.

IV. Case Study - Crambin

To demonstrate KINARI-Mutagen, we generated and analyzed mutants of Crambin (1CRN, Fig. 5(a)), a 46 amino acid plant seed protein, whose crystals diffract to ultra-high resolution [22]. The total computation time of the experiment was approximately 30 seconds.

The cartoon representation and rigidity results for two generated mutants of Crambin are shown (Fig. 5). The wild-type protein has a large rigid cluster (purple, Fig. 5(b)).



(a) Crambin (b) Rigidity, wild-type (c) Rigidity, mutant

Figure 5. KINARI-Mutagen was used to analyze Crambin (a), 1CRN. Most of the atoms in the wild-type protein are part of one large rigid body, show in purple in (b). Residue 4 is conserved among homologous proteins, which suggests that it plays a crucial role [22]. When excision is performed on it, there is a noticeable breakdown of the largest rigid cluster (c).

Viewing the rigidity results of a mutant can be used to infer the impact of the mutation on the protein's rigidity. When excision was performed on residue 4 (Fig. 5(c)), the size of the largest cluster decreased, and the number of clusters increased, when compared to the wild-type.

We wanted to know if KINARI-Mutagen could identify critical residues. Knowing such information might aid protein engineering studies, which seek out amino acids that have a dramatic effect on an enzyme's stability. KINARI-Mutagen uses the *SurfRace* program [25] to calculate the Solvent Accessible Surface Area (SASA) [13] of each residue. A residue that is not exposed to the solvent has a low SASA value, measured in Å^2. Residues on the surface of a protein have non-zero SASA values; the higher the value, the more of that residue is exposed to solvent.

Several residues in the core of Crambin had a pronounced effect on the protein's predicted rigidity when they were mutated (residue 4 for example). Similarly, many residues (7, 15, and 28) that are solvent accessible, when mutated, had little effect on the largest rigid cluster. These findings were not surprising, because residues on the surface of a protein are not expected to help maintain a protein's stability [2].

We inspected the *Largest Rigid Cluster and SASA vs. Excised Residue* plot (Fig. 6), to identify critical residues that could not be located by using the SASA calculations alone. Residues 10, 40, and 41, have high SASA values (plotted in green) and so are partially exposed to the solvent. When excision was performed on them, the size of the largest rigid body (plotted in red) decreased. These residues (among others), have been found to be identical among Crambin and two homologous plant toxins viscotoxin A3 and α_1-purothionin [22], which suggests that they are structurally or catalytically important. KINARI-Mutagen identified those residues as critical, a prediction that would not have been made had the SASA calculations alone been used to identify important residues.

V. Case Study - Lysozyme from Bacteriophage T4

We evaluated whether rigidity analysis of mutant protein structures generated by KINARI-Mutagen can identify destabilizing mutations. From the literature [2], [3], [16], [17] we retrieved stability data for different substitutions to

Figure 6. The *SASA and Size of Largest Rigid Cluster versus Excised Residue* plot for Crambin (1CRN) was used to locate solvent exposed amino acids that play a crucial role in stabilizing the protein. Residues 10, 40, and 43 are all partially exposed to the solvent, yet when excision is performed on them, the size of the largest rigid body noticeably changed. The importance of these residues can be inferred from other studies, which have found that they are conserved among several homologues [22].

glycine in the 164 amino acid lysozyme from bacteriophage T4 (PDB file 2LZM, Fig. 1). The experimentally derived value $\Delta\Delta G$, the free energy of unfolding, measures the stability of a variant against a reference protein (nearly always the wild-type protein). The lower the $\Delta\Delta G$ value, the more unstable is the variant. We compared $\Delta\Delta G$ values to the rigidity calculation predictions of 5 mutants generated by KINARI-Mutagen. The total computation time for the experiment was approximately 90 seconds.

Table I lists for each lysozyme variant the size of the largest rigid cluster (SLRC), stability data from the literature ($\Delta\Delta G$), and the degrees of freedom of the mechanical model. Among the mutants, the size of the largest rigid cluster was smallest in the least stable mutant (R96G). Mutant T157G, intermediate in terms of stability, had a largest rigid cluster that was approximately intermediate in size among the mutants. These results correlate with the $\Delta\Delta$ values. For mutants N55G and T59G, the size of the largest rigid cluster did not correlate with the $\Delta\Delta G$ values of the variants. But in the case of T59G, the increase in degrees of freedom of the protein model (implying more flexibility) did correlate with a low $\Delta\Delta G$ value. In future work, we hope to investigate the magnitude of change in SLRC and DOF, and how they influence the correlation with stability data.

The *Distribution of Rigid Bodies, By Residue* (DRBR) plot was used to infer which excisions had pronounced effects on the protein's rigidity properties. The plot can also be used to distinguish between mutations that have only a local effect on the rigidity of a protein and mutations that drastically affect a protein's stability. Fig. 7 shows the DRBR plot for the wild-type and generated mutants of lysozyme. Each row represents a mutant, and the vertical color bar

Table I
RIGIDITY RESULTS OF 5 LYSOZYME MUTANTS, SLRC = SIZE OF LARGEST RIGID CLUSTER; $\Delta\Delta G$ = EXPERIMENTAL STABILITY DATA FROM THE LITERATURE; DOF = DEGREES OF FREEDOM

Mutant	SLRC	$\Delta\Delta G$ (kcal / mol)	DOF
Wild-Type	501	NA	1556
K124G	492	-0.01	1565
N55G	501	-0.6	1556
T157G	462	-1.1	1565
T59G	501	-1.6	1569
R96G	421	-2.5	1568

on the right assigns unique colors to the different sizes of rigid bodies. The *x*-axis sequentially lists the residues in the protein, and the colors for each row indicate the size of a rigid body each residue belongs to. Comparing the colors for the row representing the wild-type protein (2lzm.A.NONE) to the colors for a row representing a mutant reveals how that mutation affects the protein's rigidity. Mutating residue 96 (2lzm.A.0096) to a glycine, does not affect the rigidity of residues 1 through approximately 80, but residues 80 through approximately 90 are no longer part of the large rigid cluster, as is the case in the wild type. When residue 59 was mutated (row 2lzm.A.0059), residues 80 through 160 were part of a large rigid body, very similar to the wild type protein. For the residue 59 mutation, the change of the protein's rigidity is not localized to the largest rigid cluster, which explains why using the size of the largest rigid cluster for that mutation was not a good predictor of protein stability.

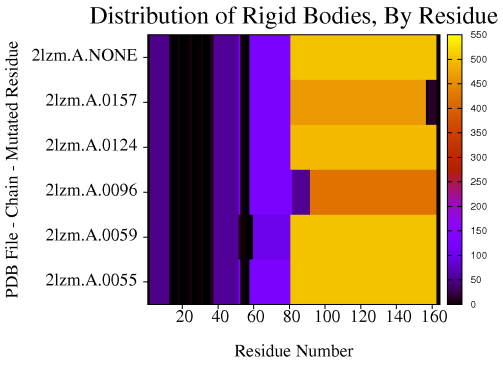


Figure 7. ***Distribution of Rigid Bodies, By Residue***: The left axis lists mutants that were analyzed. The row **2lzm.A.0059** indicates that a mutant was generated by excising residue **59** of chain **A** of protein **2lzm**. The vertical color legend on the right-hand side assigns colors to the rigid body sizes found among the mutants. The protein's residues are on the *x*-axis. The color at each *x*-*y* position in the plot indicates the size of the largest cluster that residue *x* belongs to for the mutant in row *y*. Comparing the distribution of the rigid bodies among the residues in each mutant can be used to infer how each mutation affects the protein's stability.

VI. Conclusions and Future Work

We have presented KINARI-Mutagen, which simulates mutating a residue to a glycine, and computes the mutant's rigidity using the KINARI software. We used KINARI-Mutagen to identify several solvent exposed residues of Crambin that are conserved among protein homologues. In analyzing lysozyme from bacteriophage T4, the rigidity predictions were correlated against experimentally derived stability data, and appear in many cases to be predictive of experimental observations. This work was motivated by a need for a method that can quickly evaluate the role of a residue in a protein, which is in contrast to most mutagenesis studies, which are labor intensive or impossible. On this infrastructure, we plan to add features to automatically detect critical residues, and also generate other types of mutants, in addition to the excision presented in this paper.

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