# Chapter 9

# Exploring Protein Supersecondary Structure Through Changes in Protein Folding, Stability, and Flexibility

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## Abstract

The ability to predict how mutations affect protein structure, folding, and flexibility can elucidate the molecular mechanisms leading to disruption of supersecondary structures, the emergence of phenotypes, as well guiding rational protein engineering. The advent of fast and accurate computational tools has enabled us to comprehensively explore the landscape of mutation effects on protein structures, prioritizing mutations for rational experimental validation.

Here we describe the use of two complementary web-based in silico methods, DUET and DynaMut, developed to infer the effects of mutations on folding, stability, and flexibility and how they can be used to explore and interpret these effects on protein supersecondary structures.

**Key words** Missense mutations, Protein stability and folding, Machine learning, Normal mode analysis, Graph-based signatures, DUET, DynaMut

## 1   Introduction

Proteins are marginally stable, versatile macromolecules involved in a large variety of biochemical processes which are strictly linked and regulated by their native conformation. Mutations leading to changes in protein folding, stability, and conformation can have large phenotypic consequences, responsible for the development of many genetic disorders [1–14], including cancers, and even responsible for changes in drug susceptibility [15–27]. While these effects are commonly thought about in terms of reduced protein stability, mutations leading to increased stability and rigidification of the molecule can be equally deleterious. Maintaining, or enhancing, protein stability, and the identification of mutations that do not negatively affect protein stability, also remains one of the most difficult and important challenges in protein engineering.

While experimental validation of protein thermodynamic parameters remains a laborious task, the development of novel robust and scalable computational methods (Table 1) has allowed for the evaluation of the complete landscape of structural effects of mutations in a protein system and their effects on protein stability and flexibility within minutes, enabling rapid mutation prioritization.

Using the concept of graph-based signatures, we have developed robust methods for quantitatively analyzing effects of single missense mutations on protein stability, flexibility, and interactions [9, 28–37]. DUET [37] (http://biosig.unimelb.edu.au/duet) is a machine learning-based approach that integrates and optimizes two complementary methods in an optimized predictor (mCSM-Stability [36] and SDM [38]) using support vector machines. This method enables the accurate assessment of the effects of mutations on protein folding and stability. DynaMut [28] (http://biosig.unimelb.edu.au/dynamut) is a novel method that takes into account molecular motions and, by combining the graph-based signatures with coarse-grained normal mode analysis, generates a consensus prediction of effects of mutations on the protein conformational repertoire. These methods together compose a powerful platform that allows users to navigate the landscape of mutations effects on folding, stability, and flexibility.

## 2   Materials

DUET and DynaMut are structure-based methods for assessing effects of single-point missense mutations on protein stability/folding and protein flexibility/conformation, respectively. For both methods, users are required to provide:

1. Wild-type protein structure in PDB format: For both methods, a wild-type structure of the protein of interest in the Protein Data Bank [39] format (.pdb) must be provided to perform the predictions. This can be either (a) an experimentally solved structure, with previously solved structures available in the Protein Data Bank, or (b) a model, for instance, obtained via comparative homology modeling (*see* **Note 1** on how to deal with oligomeric structures). We have previously shown that using homology models built using templates down to 25% sequence identity does not significantly reduce predictive performance of either method (*see* **Note 2**). Users have the option to either upload the structure file or provide the PDB accession code when they wish to use an experimental structure previously deposited into the PDB (http://www.rcsb.org or http://www.ebi.ac.uk/pdbe/) (*see* **Note 3**).

2. Mutation information: The user also needs to supply information on the mutation or mutations they wish to analyze,

**Table 1**
**List of freely available webservers and software for predicting effects of single-point mutations on protein folding, thermostability, and flexibility**

| | Method | Technique | Data set | Correlation | DOI | Publication year |
|---|---|---|---|---|---|---|
| Folding | mCSM-Stability | Structural signatures | ProTherm—351 mutations | 0.73 | https://doi.org/10.1093/bioinformatics/btt691 | 2014 |
| | SDM2 | Environment-specific substitution tables | ProTherm—351 mutations | 0.61 | https://doi.org/10.1093/nar/gkx439 | 2017 |
| | DUET | Integrated approach | ProTherm—351 mutations | 0.71 | https://doi.org/10.1093/nar/gku411 | 2014 |
| | Eris | Physical force field with atomic modeling | ProTherm—351 mutations | 0.35 | https://doi.org/10.1038/nmeth0607-466 | 2007 |
| | I-Mutant 2.0 | Neighboring residue composition | ProTherm—351 mutations | 0.29 | https://doi.org/10.1093/nar/gki375 | 2005 |
| | Auto-Mute | Delaunay tessellation | ProTherm—351 mutations | 0.46 | https://doi.org/10.1155/2014/278385 | 2014 |
| | CUPSAT | Atom potentials and torsion angle potentials | ProTherm—351 mutations | 0.37 | https://doi.org/10.1093/nar/gkl190 | 2006 |
| | MAESTRO | Statistical scoring functions | ProTherm—351 mutations | 0.70 | https://doi.org/10.1186/s12859-015-0548-6 | 2015 |
| | FoldX | Empirical full-atom force field | ProTherm—351 mutations | 0.35 | https://doi.org/10.1093/nar/gki387 | 2005 |
| | PoPMuSiC | Statistical potentials and neural networks | ProTherm—351 mutations | 0.67 | https://doi.org/10.1186/1471-2105-12-151 | 2011 |
| | NeEMO | Residue interaction networks | ProTherm—351 mutations | 0.67 | https://doi.org/10.1186/1471-2164-15-S4-S7 | 2014 |
| Thermal stability | HoTMuSiC | Statistical potentials | ProTherm—1626 mutations | 0.59 | https://doi.org/10.1038/srep23257 | 2015 |
| | FireProt | Structural and evolutionary information | ProTherm—1152 mutations | 87% precision | https://doi.org/10.1093/nar/gkx285 | 2017 |
| Flexibility | DynaMut | Structural signatures and NMA | ProTherm (2004)—351 mutations | 0.69 | https://doi.org/10.1093/nar/gky300 | 2018 |

including (1) the chain identifier (one-letter code of the chain, which corresponds to the 22nd column of the coordinate section in the PDB file where the mutation occurs) (*see* **Note 1**) and (2) the mutation code, which consists of the one-letter amino acid residue code of the wild-type residue, the residue number position as in the PDB file (columns 23–26 of the coordinate section), and the one-letter code of the mutated residue (e.g., R282W denotes a mutation from arginine to tryptophan at residue position 282).
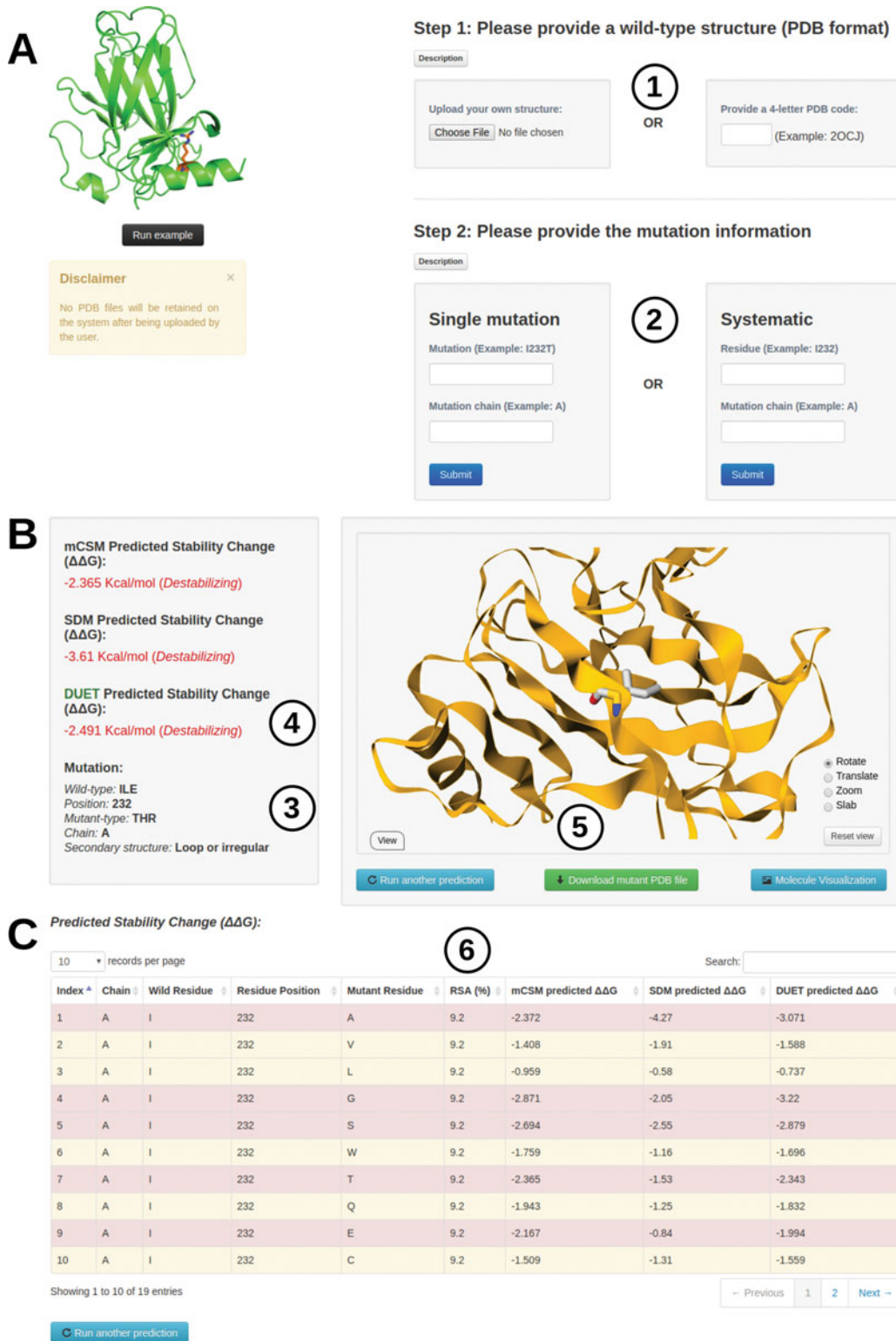
## 3    Methods

### 3.1    Predicting and Analyzing Effects of Mutation on Protein Stability and Folding with DUET

1. DUET is freely available as a user-friendly web interface and is compatible with most operating systems and browsers. Open up the prediction server, http://biosig.unimelb.edu.au/duet/stability, on a web browser of your preference.

2. Provide the wild-type protein structure of interest by either uploading a PDB file or supplying a valid four-letter PDB accession code (Fig. 1a).

3. DUET offers users the option of two prediction modes, (a) assessing stability effects of a single mutation or (b) systematically evaluating all possible mutations at a given residue position. For a single mutation, users need to provide the mutation information and the mutation chain. For systematic evaluation, the one-letter code of the mutated residue is omitted.

### 3.2    DUET Prediction Output

1. If a single mutation is provided, after processing, the results page is shown (Fig. 1b), which includes information about the mutation and the predicted effects on stability for DUET and for the individual methods (mCSM-Stability and SDM). An interactive molecular visualization is also shown, allowing users to inspect the wild-type residue environment.

2. For systematic evaluation of a given residue, the predicted effects on protein stability for all 19 possible mutations are shown in tabular format (Fig. 1c).

3. Predicted effects are given as the change in Gibbs Free Energy, $\Delta\Delta G$ (kcal/mol), with negative values denoting destabilizing mutations and positive values, stabilizing ones. While users should interpret the values in the context of the protein system being studied, previous studies have used a rule of thumb that highly destabilizing/stabilizing mutations are those with a predicted $|\Delta\Delta G| > 1.0$ kcal/mol; and moderately destabilizing/stabilizing mutations are those with a predicted $|\Delta\Delta G|$ between 0.5 and 1.0. *See* **Notes 4** and **5** for further information on how to interpret results.

**A**

**Step 1: Please provide a wild-type structure (PDB format)**

Description

Upload your own structure:

Choose File   No file chosen

①

OR

Provide a 4-letter PDB code:

(Example: 2OCJ)

**Step 2: Please provide the mutation information**

Description

**Single mutation**

Mutation (Example: I232T)

②

OR

**Systematic**

Residue (Example: I232)

Mutation chain (Example: A)

Mutation chain (Example: A)

Submit

Submit

Run example

**Disclaimer**                    ✕

No PDB files will be retained on the system after being uploaded by the user.

**B**

**mCSM Predicted Stability Change (ΔΔG):**

-2.365 Kcal/mol (*Destabilizing*)

**SDM Predicted Stability Change (ΔΔG):**

-3.61 Kcal/mol (*Destabilizing*)

**DUET Predicted Stability Change (ΔΔG):**

-2.491 Kcal/mol (*Destabilizing*)   ④

**Mutation:**

*Wild-type:* **ILE**
*Position:* **232**
*Mutant-type:* **THR**
*Chain:* **A**
*Secondary structure:* *Loop or irregular*

③

○ Rotate
○ Translate
○ Zoom
○ Slab

View          ⑤          Reset view

🔄 Run another prediction        ⬇ Download mutant PDB file        🖼 Molecule Visualization

**C**     *Predicted Stability Change (ΔΔG):*

⑥

| 10 ▾ records per page | | | | | | | | Search: |

| Index ▲ | Chain | Wild Residue | Residue Position | Mutant Residue | RSA (%) | mCSM predicted ΔΔG | SDM predicted ΔΔG | DUET predicted ΔΔG |
|---|---|---|---|---|---|---|---|---|
| 1 | A | I | 232 | A | 9.2 | -2.372 | -4.27 | -3.071 |
| 2 | A | I | 232 | V | 9.2 | -1.408 | -1.91 | -1.588 |
| 3 | A | I | 232 | L | 9.2 | -0.959 | -0.58 | -0.737 |
| 4 | A | I | 232 | G | 9.2 | -2.871 | -2.05 | -3.22 |
| 5 | A | I | 232 | S | 9.2 | -2.694 | -2.55 | -2.879 |
| 6 | A | I | 232 | W | 9.2 | -1.759 | -1.16 | -1.696 |
| 7 | A | I | 232 | T | 9.2 | -2.365 | -1.53 | -2.343 |
| 8 | A | I | 232 | Q | 9.2 | -1.943 | -1.25 | -1.832 |
| 9 | A | I | 232 | E | 9.2 | -2.167 | -0.84 | -1.994 |
| 10 | A | I | 232 | C | 9.2 | -1.509 | -1.31 | -1.559 |

Showing 1 to 10 of 19 entries

← Previous   1   2   Next →

🔄 Run another prediction

**Fig. 1** DUET submission and results web interface. (**a**) The submission page allows users to either provide its own PDB file or inform an accession code of a protein of interest (*1*). Users have the option to analyze a

*3.3 Predicting and Analyzing Effects of Mutations on Protein Flexibility and Conformation with DynaMut*

1. As with DUET, DynaMut predicted changes upon mutation in protein stability are presented as a change in the Gibbs Free Energy of folding and stability ($\Delta\Delta G$ in kcal/mol), calculated as the difference between the wild-type and mutant proteins: $\Delta\Delta G = \Delta G_{wt} - \Delta G_{mt}$. A positive value denotes a stabilizing mutation, while a negative value denotes a destabilizing one. The DynaMut consensus prediction uses both normal mode analysis and graph-based signatures to more accurately identify stabilizing mutations, a limitation of other published approaches (Fig. 2b).

2. DynaMut is also freely available for use freely as a user-friendly web interface. In order to run a prediction, open up the Dyna-Mut prediction page at http://biosig.unimelb.edu.au/dynamut/prediction on a web browser of your preference (the web server is compatible with the most common operating systems and browsers).

3. Users have the option to either evaluate a single mutation or provide a text file with a list of mutations to be evaluated in the same format discussed above to run DUET (Fig. 2a). There are no limits on the number of mutations that can be analyzed.

4. For both predictions modes, users are required to provide the wild-type protein structure of interest by either uploading a PDB file or supplying a valid four-letter code PDB accession code of a deposited experimental structure (Fig. 2a).

*3.4 DynaMut Prediction Output*

1. Prediction results: DynaMut will present the results under three main separate tabulated headings: (1) variation of Gibbs Free Energy predictions, (2) interatomic interactions, and (3) deformation/fluctuation analysis. *See* **Notes 4** and **5** for further information on how to interpret results.

2. DynaMut also graphically displays the resulting change in vibrational energy between the wild-type and mutant structures (Fig. 2b). This highlights regions predicted to be more flexible (red) or less flexible (blue) upon mutation. All calculations and representations can be downloaded through links located at the bottom of the results page.

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**Fig. 1** (continued) specific mutation or perform a systematic analysis of all mutations for a given residue (*2*). (**b**) For single-mutation prediction, the mutation identification (*3*) and the predicted effects on stability are shown (*4*), as well as an interactive molecular visualization (*5*). (**c**) For systematic evaluation of mutation on a given residue, the results are shown in tabular format

**Fig. 2** DynaMut submission and results web interface. (**a**) The submission page allows for the analysis of a single-point mutation (*1*) or a list of mutations (*2*). The main results page (**b**) depicts the predicted effect of mutation by DynaMut (*3*) as well as predicted effects by its individual components (*4*). A depiction of the calculated different in vibration entropy (*5*) is also shown

3. When multiple mutations are analyzed, these results are presented in a tabulated format, where users are able to open up and analyze each mutation within the single-mutation analysis result interface.

**3.5 Visualizing Effects of Mutations on Protein Structure**

1. DynaMut also enables visualization of the effects of a mutation within the wild-type and mutant protein structure (Fig. 3).

2. The interatomic interactions made by the wild-type and mutant residues, calculated using Arpeggio [30] (http://biosig.unimelb.edu.au/arpeggioweb/), are visually shown. This enables the user to identify how the mutation will affect the local interaction network—important for maintaining protein stability (Fig. 3a).

3. The normal mode analysis predictions are also shown, highlighting changes in vibrational energy between the wild-type and mutant structures (Fig. 3b).

4. All these representations are downloadable as Pymol session files from links at the bottom of the results page.

# 4  Notes

1. It is important to notice that both methods, DUET and DynaMut, were conceived to analyze monomer structures. In case of analysis of oligomers, users are advised to filter their PDB files prior to submission, filtering chains of interest (for instance, using the PDBest software [40]). The servers will consider all chains submitted; however, a warning message is exhibited. When considering the effects of mutations on oligomeric structures, it is also important to consider the effects of the mutations on the affinity of the monomers to form the oligomer. This can be assessed using mCSM-PPI (http://biosig.unimelb.edu.au/mcsm/protein_protein).

2. The chain ID for the provided PDB file is a mandatory field, and blank characters are not allowed. Some homology modeling tools do not automatically add a chain ID. If this is the case, the user will need to modify the PDB file prior to submission to the servers. There are several tools available to perform this task.[1]

3. Another source of error comes from structures with multiple models. It is an important practice to filter NMR structures, selecting a single model.

4. Special cases: Mutations to and from prolines. Prolines are the only amino acid whose amino group is connected to the side chain, which in the context of the peptide bond greatly limits torsional angles. The nature of this residue, therefore, needs to be taken into account while analyzing mutation effects. For instance, (1) mutations to prolines in the middle of alpha-helices can introduce kinks, affecting local structure, and
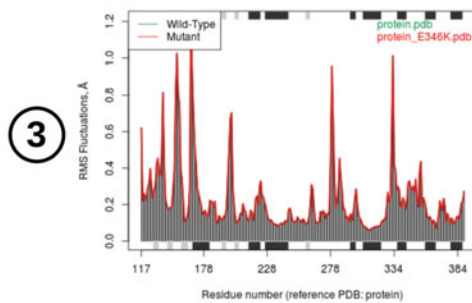
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[1] http://www.canoz.com/sdh/renamepdbchain.pl
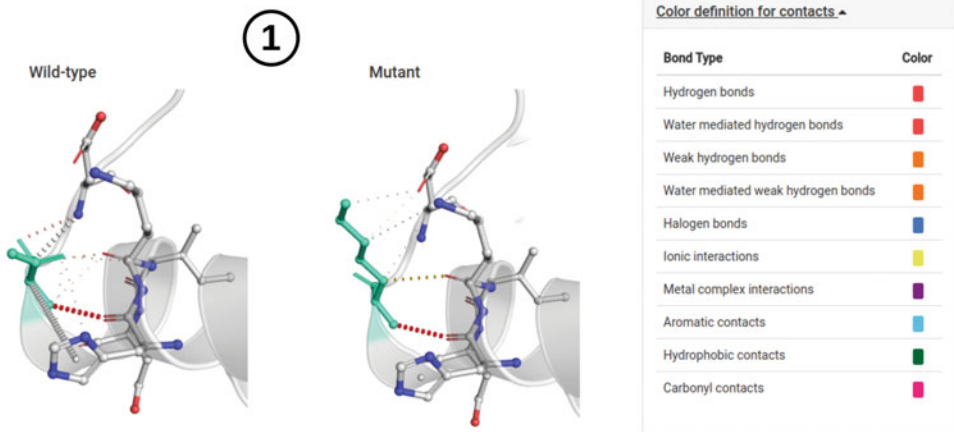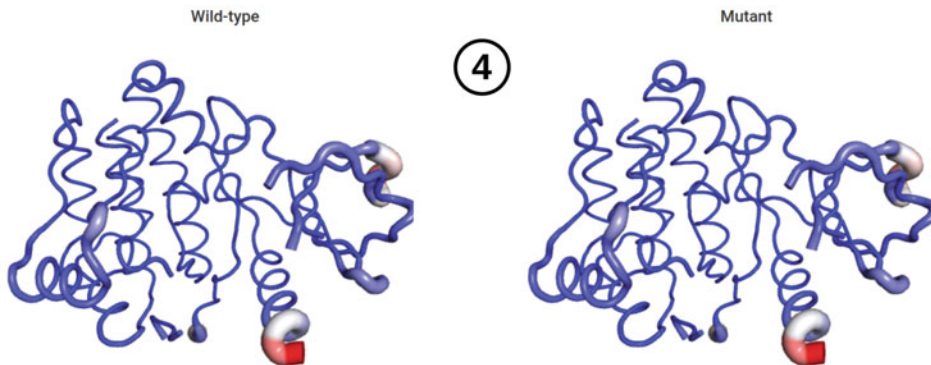
**Fig. 3** DynaMut secondary results web interface. (**a**) A depiction of the calculated interatomic interactions (*1*) for wild-type and mutant proteins is shown, with interactions identified by color (*2*). (**b**) Depicts visualizations of the deformation and fluctuation analysis as fluctuation plot per residue (*3*) and atomic fluctuation in the context of the structures (*4*). Figure and individual files (pymol files for molecular visualization) are available for download

(2) since prolines are commonly found in turns and loops, their substitution might interfere with the formation of supersecondary structures such as hairpin loops.

5. Special cases: mutations of positive-phi glycines. Similarly to prolines, positive-phi glycines, while rare in experimental structures, should also be given special consideration due to its torsional angles. Glycines are the only residues capable of adopting positive-phi angles. These glycines are usually conserved across evolution, meaning that mutations of positive-phi glycines tend to be destabilizing.

## Acknowledgments

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