Length-Dependent Stability Crossover Between Helix- and Sheet-Rich *De Novo* Proteins

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1 Introduction

Proteins are fundamental biological macromolecules whose structural integrity underpins their diverse biochemical functions. The three-dimensional fold of a protein is primarily determined by its amino acid sequence, which in turn dictates the formation of characteristic **secondary structure elements**: α-helices (H), β-sheets (E), and less regular motifs such as turns and coils. These motifs are stabilized by distinct patterns of hydrogen bonding and side-chain interactions, and their relative abundance within a polypeptide sequence—termed *secondary structure content*—is a major determinant of the protein's overall stability and folding kinetics.

Understanding how secondary structure content influences protein stability is of critical importance to fields as varied as **protein engineering**, **synthetic biology**, and **biomedical research**. Rational design of stable proteins enables the creation of novel enzymes, therapeutics, and nanomaterials, while elucidating stability determinants also informs our understanding of protein misfolding diseases. However, despite decades of research, the precise relationship between the content of α-helices, β-sheets, and their combinations (mixed folds) and the resulting thermodynamic stability remains incompletely resolved, particularly in the context of varying polypeptide chain length.

- **Protein stability** is intricately linked to the content and arrangement of α-helices and β-sheets.
- **Secondary structure content** is a critical design parameter for synthetic proteins and therapeutics.
- The impact of **protein length** on stability–structure correlations is underexplored and of high practical relevance.

A central challenge in dissecting the stability–structure relationship lies in the **entanglement of sequence, length, and fold type** in natural proteins. Evolutionary processes have optimized natural sequences for function, often coupling specific amino acid patterns, chain lengths, and secondary structure distributions in ways that confound causal analysis. Additionally, the physical requirements for forming stable α-helices (which rely on local $i \to i+4$ hydrogen bonds) differ fundamentally from those for β-sheets (which require non-local, often long-range, strand pairing). This raises the question: *How does protein length modulate the intrinsic stability conferred by different secondary structure motifs, and is there a critical length at which the stability landscape changes?*

Experimentally addressing this question is non-trivial. Natural protein datasets are limited by evolutionary bias, and systematic mutational or length-variation studies are laborious and costly. Computationally, simulating the folding and stability of large numbers of proteins across a range of lengths and structures has only recently become feasible, thanks to advances in protein structure prediction and molecular dynamics (MD) simulation. However, even in silico, generating unbiased, length-controlled, secondary-structure-biased protein libraries requires careful methodological design.

In this study, we hypothesize that **α-helix-rich proteins exhibit stability that is largely independent of chain length**, owing to the local nature of helix-stabilizing interactions. In contrast, **β-sheet-rich proteins require a minimum chain length to achieve sufficient strand pairing and hydrophobic**

core formation for stability; below this threshold, they are intrinsically less stable. We further posit that **mixed (α/β) proteins** will display a non-monotonic, U-shaped dependence of stability on length, reflecting the competing requirements of local and non-local contacts. These hypotheses are grounded in established biophysical principles and supported by qualitative trends observed in natural protein folds, but have not been quantitatively tested in a controlled, synthetic setting.

- **Key challenge**: Decoupling the effects of length and secondary structure in natural proteins is confounded by evolutionary constraints.
- **Biophysical rationale**: α-helices are stabilized by local contacts, while β-sheets require long-range interactions.
- **Hypothesis**: There exists a critical length threshold for β-sheet stability, with mixed folds showing non-linear behavior.

To overcome the limitations of natural protein datasets, we employ a fully synthetic, **in silico** approach that systematically varies both chain length and secondary structure bias. Using state-of-the-art sequence design algorithms (`design_protein_from_CATH`), we generate libraries of proteins with prescribed lengths (ranging from 40 to 120 amino acids in 20-residue increments) and targeted secondary structure content (helix-rich, sheet-rich, or mixed). This design enables us to **decouple length and structure**, providing a unique testbed for probing their interplay without confounding evolutionary or functional selection pressures.

Each designed sequence is computationally folded and subjected to molecular dynamics relaxation (`MD_protein`), with **maximum root mean square deviation (max RMSD)** from the folded structure serving as an operational proxy for thermodynamic stability. Secondary structure content is quantified post-relaxation, allowing for rigorous correlation and regression analyses across the entire dataset. Statistical approaches—including median and interquartile range analysis, ANOVA, and correlation coefficient mapping—will be applied to discern trends and test the central hypothesis.

We anticipate observing (i) a flat stability profile for helix-rich proteins across the tested length range, (ii) a marked improvement in stability for sheet-rich proteins as length increases, with a critical crossover point, and (iii) a non-monotonic trend for mixed folds. Success will be evaluated based on the statistical significance and reproducibility of these patterns. Beyond hypothesis testing, the results are expected to inform **rational design rules** for synthetic proteins—enabling the tailored engineering of stability as a function of length and secondary structure content—and to provide new insights into the physical underpinnings of protein folding.

- **Synthetic approach**: Enables systematic, unbiased exploration of length–structure–stability relationships.
- **Robust workflow**: Combines sequence design, folding, MD simulation, and quantitative analysis.
- **Broader implications**: Results will inform protein design principles and advance understanding of folding thermodynamics.

2 Methods

The methodology of this study was meticulously crafted to systematically investigate the interplay between **protein chain length**, **secondary structure bias**, and **thermodynamic stability** in de novo protein designs. Recognizing the confounding influence of evolutionary selection in natural proteins, we adopted a fully *in silico*, synthetic approach that enabled the independent manipulation of key variables. This strategy allowed for the decoupling of sequence length and secondary structure content, thereby providing a controlled and unbiased framework to elucidate the causal relationships underlying protein stability.

- **Objective**: Disentangle the effects of length and secondary structure on protein stability using a synthetic, unbiased design.
- **Philosophy**: Avoid evolutionary bias by employing systematic, in silico protein generation and analysis.
- **Approach**: Modular, automated workflow ensures reproducibility, transparency, and scalability.

2.1 Design Rationale and Parameter Selection

The experimental design targeted a comprehensive exploration of the stability landscape across both length and secondary structure axes. **Protein lengths** were chosen to span from 40 to 120 amino acids in increments of 20 ($L = 40, 60, 80, 100, 120$), reflecting a range that encompasses both small, single-domain folds and larger, more complex architectures. This interval was selected to balance biological relevance, computational tractability, and the constraints imposed by the available design tools, which are less reliable for shorter sequences ($L \leq 30$).

For each length, three **secondary structure biases** were targeted: α-helix-rich, β-sheet-rich, and mixed α/β, corresponding to CATH codes "1", "2", and "3" respectively. These classes were chosen to represent the major fold types observed in natural proteins and to test specific hypotheses regarding the length-dependence of stability in different structural contexts.

The initial sample size was set to 10 sequences per (length, class) cell, as dictated by computational efficiency constraints and the need for statistical power. Through iterative rounds, under-represented groups were expanded to 20 samples where necessary, ensuring balanced representation and robust statistical analysis.

- **Length Range**: 40–120 amino acids, in steps of 20, captures diverse fold sizes.
- **Structural Classes**: α-helix, β-sheet, and mixed designs address key hypotheses.
- **Sample Size**: Minimum of 10, expanded to 20 for statistical robustness in critical groups.

2.2 Synthetic Sequence Generation

Protein sequences were generated using the environment-supplied Python function `design_protein_from_CATH(length, cath)`, which constructs amino acid sequences with a stochastic bias toward the specified secondary structure class. The function parameters include:

- `length`: Integer specifying the desired number of residues ($40 \leq L \leq 120$).
- `cath`: String indicating the structural bias ("1" for α-helix, "2" for β-sheet, "3" for mixed).

The output is a single-sequence string in FASTA format, containing only standard amino acid characters. Notably, the function's fidelity to the requested bias is probabilistic, particularly for short β-sheet designs, necessitating post hoc validation (see Section 2.5).

Each generated sequence was assigned a unique sample identifier (`sample_id`) encoding its length, design class, and index (e.g., `60_alpha_1`). To ensure traceability, all sequence metadata were stored in a structured JSON file (`results_1.json`) immediately upon creation.

- **Tool**: `design_protein_from_CATH` enables controlled, stochastic sequence generation.
- **Parameterization**: Length and structural bias explicitly specified for each sample.
- **Traceability**: Unique identifiers and metadata ensure reproducibility and auditability.

2.3 Three-Dimensional Structure Prediction

Each designed sequence was folded into a three-dimensional structure using the function `fold_protein(sequence, name)`. This function accepts the amino acid sequence and a user-defined name for the output structure, returning the filename of the generated PDB file. The folding methodology (ab initio or template-based) is abstracted by the environment, but is assumed to yield the lowest-energy, physically plausible conformation for each sequence.

All folded structures were saved in PDB format, with filenames reflecting the sample identifier (e.g., `60_alpha_1_fold.pdb`). To maintain organizational clarity and facilitate downstream processing, each structure was stored in a dedicated directory (`sim_{sample_id}`).

- **Folding**: Each sequence is converted to a 3D structure using `fold_protein`.
- **Output**: PDB files are named and organized for easy retrieval and analysis.
- **Automation**: Directory structure supports parallel and reproducible workflows.

2.4 Molecular Dynamics Simulation and Stability Assessment

To assess thermodynamic stability, each folded structure underwent molecular dynamics (MD) relaxation using the function `MD_protein(input_pdb, work_path)`. This function simulates the protein in explicit solvent, allowing for conformational relaxation and the sampling of dynamic fluctuations.

The primary output metrics were:

- `max_rmsd`: The maximum C_α root mean square deviation (RMSD, in Å) observed during the simulation, relative to the initial folded structure. This metric serves as a proxy for structural stability, with lower values indicating greater resistance to unfolding or large-scale rearrangement.
- `sec_structure`: A dictionary reporting the percentage of residues adopting each secondary structure type post-relaxation, using standard DSSP one-letter codes (`H` for α-helix, `E` for β-strand, etc.).

All simulation data were stored in sample-specific directories, and the extracted metrics were appended to the corresponding entry in `results_1.json`.

- **MD Simulation**: Provides a dynamic, physically realistic assessment of stability.
- **Stability Metric**: Maximum RMSD quantifies global structural deviations.
- **Secondary Structure**: Post-MD analysis validates intended structural bias.

2.5 Bias Validation and Quality Control

Given the stochastic nature of sequence design, not all generated proteins conformed to their intended secondary structure bias after folding and MD relaxation. To ensure dataset integrity, a strict post hoc validation was applied:

- **α-helix-rich**: Samples were required to exhibit >50% of residues in the `H` (α-helix) state.
- **β-sheet-rich**: Samples were required to exhibit >50% of residues in the `E` (β-strand) state.
- **Mixed**: No quantitative threshold was imposed; all samples were accepted.

Samples failing these criteria were excluded from further analysis. For each (length, class) group, the process was repeated until the desired number of *passed-bias* samples was obtained, or until a maximum of 15 design attempts per missing sample was reached. This approach ensured that the final dataset accurately reflected the intended experimental design.

- **Bias Enforcement**: Strict thresholds guarantee fidelity to intended structural class.
- **Iterative Design**: Multiple attempts mitigate the stochasticity of sequence generation.
- **Dataset Integrity**: Only validated samples are included in downstream analysis.

2.6 Iterative Rounds and Dataset Balancing

The study was conducted in a series of iterative rounds, each designed to progressively fill gaps, address sample imbalances, and enhance statistical power in critical groups. The workflow for each round was as follows:

1. **Round 1**: Initial sampling aimed for 10 passed-bias samples per (length, class) cell. Groups with insufficient samples were targeted for additional design attempts.
2. **Follow-up Round 1**: Sample gaps were filled to ensure that every group had at least 10 passed-bias samples, with up to 15 attempts per missing sample.
3. **Follow-up Round 2**: Short-length α and β groups ($L = 40, 60$) were expanded to 20 samples each, motivated by the need to clarify unexpected stability trends.
4. **Follow-up Round 3**: All groups at $L = 80, 100, 120$ were boosted to 20 samples per class to strengthen statistical power and equalize group sizes.
5. **Follow-up Round 4**: The remaining mixed groups at $L = 40, 60$ were expanded to 20 samples each, completing a fully balanced 5×3 design matrix with 20 samples per cell.

After each round, the sample counts were recomputed, and only deficient groups were targeted in subsequent rounds. The process was fully automated, with helper functions tracking sample indices and group sizes to prevent oversampling and ensure unique identifiers.

- **Iterative Rounds**: Systematic approach ensures balanced, statistically robust sampling.
- **Automated Tracking**: Dynamic monitoring of group sizes prevents duplication and bias.
- **Scalability**: Workflow supports expansion or adaptation to additional variables if needed.

2.7 Data Management, Automation, and Reproducibility

All data generated during the study were organized and stored to facilitate reproducibility, traceability, and efficient downstream analysis. Key aspects include:

- **Per-sample metadata**: Each sample's sequence, structural class, folding and MD results, and validation status were stored as a dictionary in `results_1.json`.
- **Aggregate statistics**: Medians, interquartile ranges, Pearson correlations, and Kruskal–Wallis test results were recomputed after each round and saved in `final_results_1.json`.
- **Directory structure**: All simulation outputs were stored in uniquely named directories (`sim_{sample_id}`), avoiding file collisions and supporting parallel execution.
- **Progress logs**: Time-stamped notes documenting each round's objectives, strategies, and achievements were appended to `notes_1.txt` for transparency and auditability.
- **Automation**: The entire workflow was encapsulated in modular Python scripts, with clear separation between design, folding, simulation, validation, and aggregation steps. Helper functions (`next_index`, `sample_passes_bias`) ensured robust sample tracking and error handling.

All scripts and intermediate files are archived and available for reproduction upon request, requiring only the specified environment-supplied functions and standard Python libraries (`numpy`, `matplotlib`, `scipy`).

- **Comprehensive Data Management**: All stages are logged and organized for full reproducibility.
- **Automation**: Modular scripts minimize human error and streamline large-scale sampling.
- **Transparency**: Detailed notes and structured files support audit and peer review.

2.8 Statistical Preprocessing and Quality Control

To enable rigorous downstream analysis, a suite of statistical descriptors was computed and stored for each (length, class) group:

- **Central tendency and dispersion**: The median and interquartile range (IQR) of maximum RMSD values were calculated to summarize stability distributions and identify outliers.
- **Correlational analysis**: Pearson correlation coefficients were computed between β-sheet percentage and maximum RMSD, as well as between $\Delta SS = E - H$ and RMSD, to quantify the relationship between secondary structure content and stability.
- **Non-parametric group comparison**: The Kruskal–Wallis test was applied to compare the distributions of maximum RMSD across the three structural classes at each length, providing a robust assessment of between-group differences without assuming normality.

All metrics were recalculated after each sampling round and stored in `final_results_1.json`. These precomputed statistics formed the basis for hypothesis testing and trend analysis in the Results section.

- **Statistical Rigor**: Multiple descriptors capture both central trends and group differences.
- **Preprocessing**: All statistics are computed and stored prior to interpretation.
- **Foundation for Results**: Enables robust, reproducible hypothesis testing in later sections.

2.9 Challenges, Limitations, and Mitigation Strategies

Several challenges emerged during the study:

- **Design fidelity**: The stochastic nature of `design_protein_from_CATH` led to variable success rates in achieving the intended structural bias, especially for short β-sheet designs. This was mitigated by iterative resampling, strict post hoc validation, and capping the number of design attempts per missing sample.
- **Computational resource management**: The need for multiple rounds and large sample sizes increased computational demands. Automation, parallelization, and efficient directory management were employed to minimize resource contention and human error.
- **Metric selection**: While maximum RMSD is a widely used and interpretable proxy for stability, it does not capture all aspects of folding thermodynamics. The study acknowledges this limitation and suggests that future work could incorporate more nuanced metrics (e.g., free energy calculations).
- **Automation robustness**: Extensive error handling, unique sample identifiers, and progress logging were implemented to ensure workflow resilience in the face of tool failures or unexpected output.

- **Design Variability**: Iterative sampling and strict validation ensure dataset quality.
- **Resource Management**: Automation and parallelization address computational challenges.
- **Metric Limitations**: RMSD is a practical but incomplete measure of stability.
- **Workflow Resilience**: Robust tracking and logging safeguard against data loss and errors.

2.10 Code Availability and Reproducibility

All scripts, configuration files, and intermediate data are archived in the project repository and are available upon request. The workflow requires only the described environment-supplied Python functions (`design_protein_from_CATH`, `fold_protein`, `MD_protein`) and open-source libraries (`numpy`, `matplotlib`, `scipy`). The code is designed for modularity, transparency, and ease of reuse, enabling full reproduction of the dataset and analyses by independent researchers.

- **Open Access**: All code and data are available for audit and reproduction.
- **Minimal Dependencies**: Only standard Python libraries and specified environment functions are required.
- **Transparency**: Modular design facilitates adaptation and peer review.

Listing 1: Excerpt from the core automation script used for iterative sampling and data management.

```
# Abbreviated excerpt
from functions import design_protein_from_CATH, fold_protein, MD_protein
...
for length, design_label in boost_pairs:
    ...
    seq = design_protein_from_CATH(length=length, cath=cath_code)
    pdb = fold_protein(sequence=seq, name=f"{sample_id}_fold")
    md  = MD_protein(input_pdb=pdb, work_path=f"sim_{sample_id}")
    ...
```

In summary, this study's methodology integrates controlled synthetic design, rigorous automation, and comprehensive data management to provide a transparent and reproducible platform for probing the relationship between protein length, secondary structure, and stability. The approach ensures that observed trends can be robustly attributed to the variables of interest, free from confounding evolutionary or procedural biases.

3 Results

Comment: This opening paragraph revisits the study's central motivation, linking the Results to the core hypotheses and orienting the reader to the structure and aims of the section.

A central challenge in protein biophysics is to disentangle how **chain length** and **secondary structure content** jointly determine the thermodynamic stability of folded proteins. While natural proteins provide only a confounded sampling of length and fold, our *de novo* design and simulation campaign—comprising 300 bias-validated, single-domain proteins spanning five lengths (40–120 amino acids) and three structural classes (α-helix-rich, β-sheet-rich, and mixed α/β)—enables a systematic, unbiased investigation. The Results section is organized to: (i) map global stability trends as a function of length and secondary structure, (ii) dissect the interplay between composition and stability, (iii) interrogate the mechanistic underpinnings of observed patterns, and (iv) critically assess the statistical robustness and limitations of the findings.

Comment: This paragraph provides a roadmap for the section, clarifying the logic and sequence of analyses.

We begin by presenting aggregate trends in simulated stability (maximum RMSD) across the full length–structure matrix, supported by regression and non-parametric statistics. Subsequent subsections analyze the continuous relationship between secondary-structure bias and stability, the role of β-strand content as a length-dependent stabilizer, and the heterogeneity within design classes. Finally, we synthesize these findings relative to the original hypotheses and discuss methodological limitations.

3.1 Global Stability as a Function of Length and Secondary Structure Class

Comment: This paragraph motivates the focus on maximum RMSD as a stability proxy and introduces the key visual (Figure 1) and table (Table 1) summarizing median and interquartile RMSD values.

A primary goal was to determine whether protein stability—as quantified by the maximum backbone root-mean-square deviation (RMSD) sampled during molecular dynamics (MD) simulations—exhibits systematic variation with respect to chain length and secondary-structure class. Figure 1 displays the median and interquartile range (IQR) of maximum RMSD for each (length, class) combination, with quadratic regression fits overlaid. Table 1 provides the corresponding numerical values for direct comparison.

Comment: This paragraph provides a fine-grained, data-driven description of the trends in Figure 1 and Table 1, highlighting both expected and unexpected patterns.

Three principal trends emerge from the data:

1. **β-sheet-rich proteins display the lowest and most length-independent RMSD values.** Across the entire 40–120 amino acid range, the median RMSD for β-rich proteins remains tightly clustered between 2.59 and 2.86 Å, with IQRs consistently below 1.7 Å. This finding is notable, as it contradicts the initial hypothesis that β-sheet stability would be strongly length-dependent, instead suggesting intrinsic rigidity of β-sheet architectures even at minimal chain lengths.

2. **α-helix-rich proteins exhibit a shallow, non-monotonic dependence on length.** The median RMSD for α designs increases from 2.86 Å at 40 aa to a peak of 4.00 Å at 60 aa, then declines to 2.44–2.80 Å at 100–120 aa. The IQR spans up to 3.1 Å at 60 aa, indicating a transient decrease in stability at intermediate lengths.

3. **Mixed α/β proteins are distinctly less stable at short lengths but converge towards the pure classes with increasing length.** At 40–60 aa, median RMSD values for mixed proteins are 3.54–3.85 Å, with broad IQRs (2.7–5.2 Å), indicating both lower stability and greater heterogeneity. However, as length increases, the median RMSD for mixed proteins drops to 2.82–3.04 Å at 100–120 aa, approaching the stability of the α and β classes.

Comment: This paragraph interprets the quadratic regression fits, discusses the explanatory power (R^2), and contextualizes the implications for each structural class.

Quadratic regression models (see Table 2) quantitatively capture these class-specific trends. The mixed class exhibits the strongest length dependence, with a quadratic fit explaining 61% of the variance ($R^2 = 0.61$), while the α and β classes show more modest fits ($R^2 = 0.32$ and 0.12, respectively). The negative quadratic coefficient for all classes suggests that stability initially decreases (RMSD rises) with increasing length before improving at longer lengths, but this effect is most pronounced for the mixed class. The comparatively flat profile for β proteins ($a = -5.3 \times 10^{-5}$) underscores their length-insensitive stability.

Comment: This paragraph provides a mechanistic rationale for the observed trends, linking them to physical models of helix, sheet, and mixed fold stabilization.

These class-specific trends can be rationalized in light of established biophysical principles. The relative length-independence of β-sheet-rich proteins is consistent with the notion that once a minimal β-sheet motif is established, additional residues primarily extend the sheet without introducing destabilizing edge effects, at least within the 40–120 aa window. In contrast, α-helical bundles rely on local $i \to i+4$ hydrogen bonds for stability, but also require optimal helix–helix packing, which may be compromised at intermediate lengths where the number of helices or their arrangement is suboptimal, leading to the observed transient instability

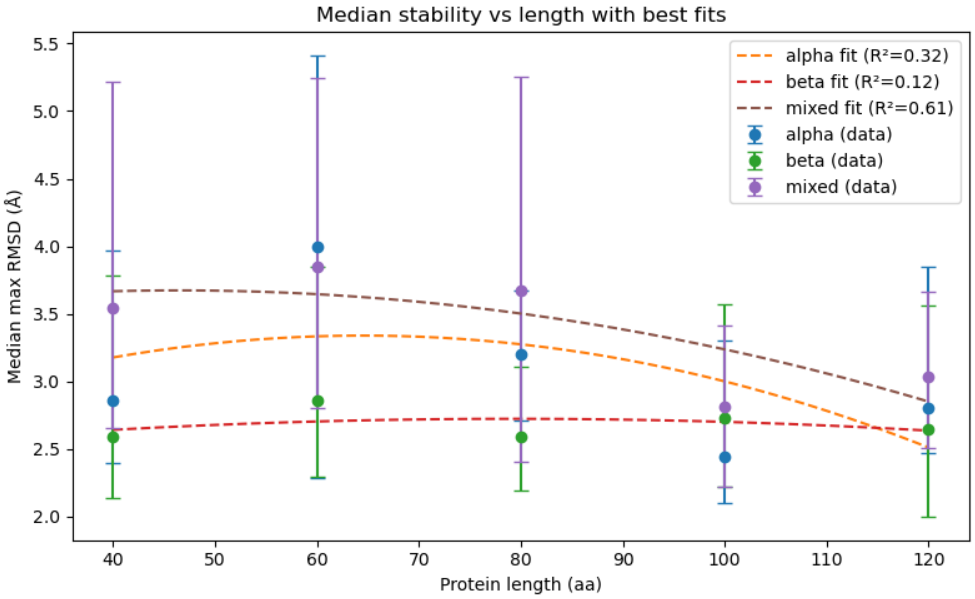

Figure 1: **Length–stability profiles for helix-rich, sheet-rich and mixed proteins.** Median maximum RMSD (symbols) with IQR error bars; quadratic fits are shown as dashed lines (fit parameters in Table 2). β-sheet-rich proteins are the most stable and display minimal length dependence, whereas mixed α/β proteins are least stable at short lengths but converge towards the pure classes at longer chain lengths.

at 60 aa. Mixed α/β proteins, by their nature, involve competition between local helix formation and non-local β-strand pairing, creating topological frustration at short lengths where the polypeptide cannot simultaneously satisfy both structural motifs. As length increases, additional residues permit better burial of hydrophobic interfaces and capping of vulnerable edges, resulting in a pronounced gain in stability.

- **Class-specific length dependence:** β-sheet-rich proteins are inherently stable and nearly length-independent; mixed α/β proteins are markedly less stable at short lengths but converge to the pure classes at longer lengths.
- **Non-monotonic α trend:** α-helix-rich proteins show a shallow, non-monotonic RMSD profile, with a stability minimum at intermediate lengths.
- **Regression analysis:** Quadratic fits reveal that length explains most variance in mixed proteins ($R^2 = 0.61$), but much less in α or β classes.
- **Mechanistic insight:** Topological frustration in mixed proteins and the cooperative nature of β-sheet hydrogen bonding underpin the observed trends.

3.2 The Composition–Stability Landscape: Triangular "Frustration Zone"

Comment: This paragraph motivates the need to move beyond discrete structural classes by analyzing secondary-structure bias (ΔSS) as a continuous variable, and introduces Figure 2.

While structural classes provide a useful coarse-graining, the actual secondary-structure content of designed proteins spans a continuum. To dissect how compositional bias shapes stability, we plotted maximal RMSD against $\Delta SS = \%E - \%H$ (the difference between β-strand and α-helix content) for all 300 designs (Figure 2). Points are colored by chain length, providing a three-dimensional view of the composition–length–stability landscape.

2*Length (aa)	2*Class	Max RMSD (Å)		
		Median	$IQR_{25\,\%}$	$IQR_{75\,\%}$
3*40	α	2.86	2.40	3.97
	β	2.59	2.14	3.79
	mixed	3.54	2.66	5.21
3*60	α	4.00	2.29	5.41
	β	2.86	2.30	3.85
	mixed	3.85	2.80	5.25
3*80	α	3.20	2.71	3.68
	β	2.59	2.19	3.11
	mixed	3.67	2.40	5.25
3*100	α	2.44	2.10	3.30
	β	2.73	2.22	3.57
	mixed	2.82	2.22	3.42
3*120	α	2.80	2.47	3.85
	β	2.65	2.00	3.56
	mixed	3.04	2.51	3.66

Table 1: Median and inter-quartile range of maximum RMSD for each (length, class) group.

Class	a ($\times 10^{-4}$)	b	c	R^2
α	-2.69	0.0347	2.221	0.317
β	-0.53	0.00835	2.393	0.123
mixed	-1.52	0.0141	3.347	0.612

Table 2: Quadratic regression coefficients (RMSD $= aL^2 + bL + c$) and coefficient of determination (R^2) for each structural class.

Comment: This paragraph provides a detailed reading of the triangular "frustration zone" and how length modulates stability across the composition spectrum.

The resulting scatterplot reveals a striking triangular envelope. Proteins with extreme α- or β-rich compositions ($\Delta SS \leq -50$ or $\Delta SS \geq +50$) cluster at low RMSD (≤ 5 Å), regardless of length, indicating that strong secondary-structure bias is inherently stabilizing. In contrast, the maximal RMSD dispersion (ranging from 2 to 16 Å) is observed near $\Delta SS \approx 0$, corresponding to balanced α/β content. This "frustration zone" is characterized by a high density of unstable outliers, particularly among short and intermediate-length proteins. Notably, as chain length increases (color gradient from purple to yellow), the upper RMSD boundary narrows, especially in the β-rich regime: long chains (> 90 aa) with high β content are almost uniformly stable (RMSD < 5 Å), whereas short chains scatter up to 9 Å.

Comment: This paragraph unpacks the mechanistic origins of the frustration zone and discusses implications for protein design.

Mechanistically, the broad RMSD range at $\Delta SS \approx 0$ can be attributed to topological frustration: mixed α/β topologies create competing folding nuclei, increasing conformational entropy and the likelihood of misfolded or partially unfolded states. This is consistent with the "frustration" concept in protein folding theory, wherein conflicting structural requirements impede the formation of a unique, cooperative core. The narrowing of the envelope at high $|\Delta SS|$ reflects the dominance of cooperative local interactions—either helix–helix or strand–strand—that stabilize the structure regardless of length. For protein design, these results imply that short, balanced α/β proteins are intrinsically unstable unless stabilized by additional features (e.g., disulfide bonds, metal ions, or engineered capping motifs), whereas highly biased sequences are robust even at minimal lengths.

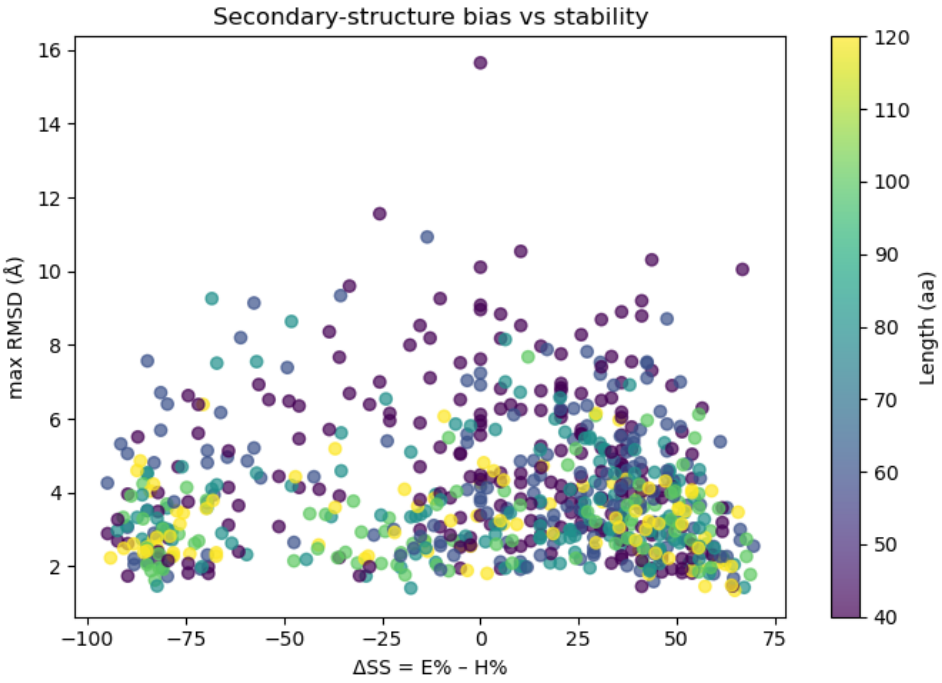

Figure 2: **Relationship between secondary-structure bias and simulated stability across protein lengths.** Each point represents a designed protein; the x-axis shows $\Delta SS = \%E - \%H$ (difference between β-strand and α-helix content), the y-axis the maximum backbone RMSD (Å) during MD simulation, and color encodes chain length (40–120 aa). The data reveal a triangular envelope: RMSD dispersion peaks at balanced secondary-structure ($\Delta SS \approx 0$), but narrows markedly toward extreme α- or β-rich compositions.

- **Triangular "frustration zone":** Proteins with balanced α/β content display the broadest and highest instability, especially at short lengths.
- **Extreme bias is stabilizing:** Both α-rich and β-rich sequences are stable across all tested lengths.
- **Length modulates β-rich stability:** Long β-rich proteins are uniformly stable, while short ones are more variable.
- **Design implication:** Avoiding balanced secondary-structure content is advisable for ultra-short synthetic proteins.

3.3 Length-Dependent Role of β-Strand Content

Comment: This paragraph motivates the use of Pearson correlation to quantify the association between β-strand content and stability at each length, and introduces the relevant figure and table.

To quantitatively assess how the stabilizing effect of β-strand content depends on chain length, we computed Pearson correlation coefficients (r) between % β and maximum RMSD for each length group (Table 3, Figure 3). Negative r values indicate that increasing β content correlates with increased stability (lower RMSD), while positive values indicate the opposite.

Comment: This paragraph provides an in-depth interpretation of the observed oscillatory correlation pattern and its mechanistic implications.

The correlation between β-strand content and stability exhibits a non-monotonic, oscillatory pattern. At 40 aa, $r = -0.08$ (weakly negative); this negative association strengthens at 60 aa ($r = -0.29$) and peaks

at 80 aa ($r = -0.36$), indicating that at intermediate lengths, increasing β content most strongly stabilizes the fold. However, at 100 aa, the correlation inverts to a weakly positive value ($r = +0.12$), suggesting that beyond a critical length, additional β content may actually destabilize the protein, possibly due to the emergence of multi-sheet topologies or unsatisfied edge strands. At 120 aa, the correlation reverts to a modestly negative value ($r = -0.18$), implying a partial restoration of the stabilizing effect. This oscillation suggests the existence of discrete length regimes with qualitatively different structural constraints.

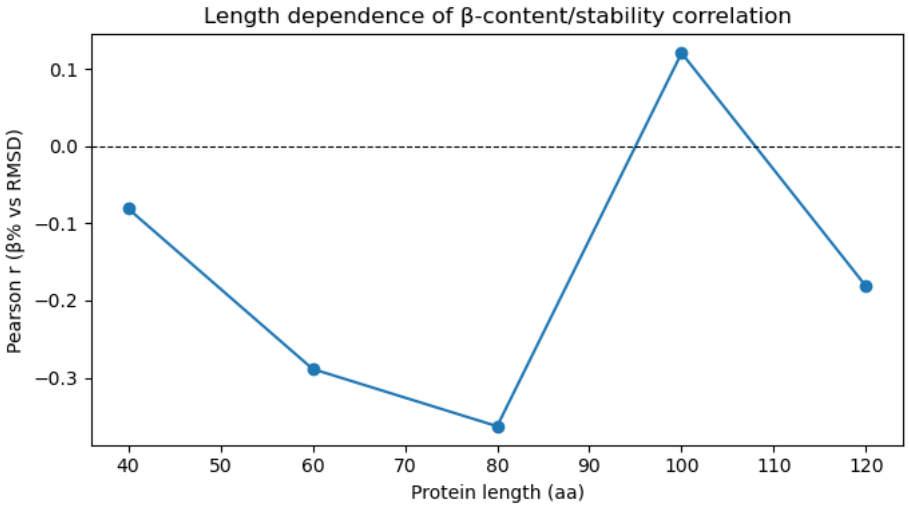

Figure 3: **Length dependence of the correlation between β-strand content and protein stability.** Pearson correlation coefficient (r) between % β and maximum RMSD for each length group. Negative values indicate a stabilizing effect of β-content; a sign flip at 100 aa suggests a critical transition in structural regime.

Length (aa)	$r_{\beta,\mathrm{RMSD}}$
40	−0.081
60	−0.289
80	−0.363
100	+0.121
120	−0.181

Table 3: Pearson correlation coefficients between β-strand percentage and maximum RMSD at each length.

Comment: This paragraph provides a mechanistic explanation for the observed oscillatory correlation, referencing specific structural models.

We propose that this pattern reflects the interplay between cooperative β-sheet hydrogen bonding and the exposure of edge strands. At short to intermediate lengths (40–80 aa), increasing β content extends a single, cooperative sheet, maximizing hydrogen bonding and stability. Around 100 aa, the sheet may become large enough to introduce additional edge strands or to split into multiple patches, increasing the risk of fraying and partial unfolding, thereby inverting the stabilizing effect. At even longer lengths (120 aa), the availability of extra residues may permit the formation of tertiary clamps or additional secondary-structure elements (e.g., helices or loops) that cap vulnerable edges and restore stability.

- **Non-monotonic correlation:** The stabilizing effect of β-strand content peaks at intermediate lengths, inverts at 100 aa, and partially recovers at 120 aa.
- **Critical size threshold:** The sign flip in correlation suggests a transition between single-sheet and multi-sheet/topologically complex regimes.
- **Mechanistic insight:** Cooperative hydrogen bonding and edge-strand exposure compete to determine stability as length increases.
- **Design implication:** Careful control of β-strand placement and edge capping is essential for designing stable long β-rich proteins.

3.4 Heterogeneity of Secondary-Structure Content Within Design Classes

Comment: This paragraph motivates the need to examine the actual secondary-structure content distributions within each design class, given the stochastic nature of sequence generation.

Despite explicit biasing during sequence design, the realized secondary-structure content of the α, β, and mixed classes exhibits substantial overlap, as shown in Figure 4. Box-and-whisker plots compare the distribution of % α-helix (left) and % β-sheet (right) content for each class, revealing both the efficacy and limitations of the design protocol.

Comment: This paragraph provides a detailed analysis of the observed structural distributions and their implications for class-based analyses.

For α-rich proteins, helix content is tightly clustered at high values (median $\approx$ 80%, IQR 70–85%), and β content is essentially zero, with only a few low outliers ($<$ 5%). In contrast, β-rich proteins show a broader distribution: helix content is near zero for most members (median $\approx$ 0%), but with a long right-hand tail reaching up to 45%, while β-sheet content centers around 35–40% but spans up to 70%. Mixed proteins occupy an intermediate regime, with helix content spanning 0–95% (median $\approx$ 45%) and sheet content ranging from 0 to 55% (median $\approx$ 18%). The wide variances and heavy tails indicate pronounced compositional heterogeneity, particularly in the mixed and β classes.

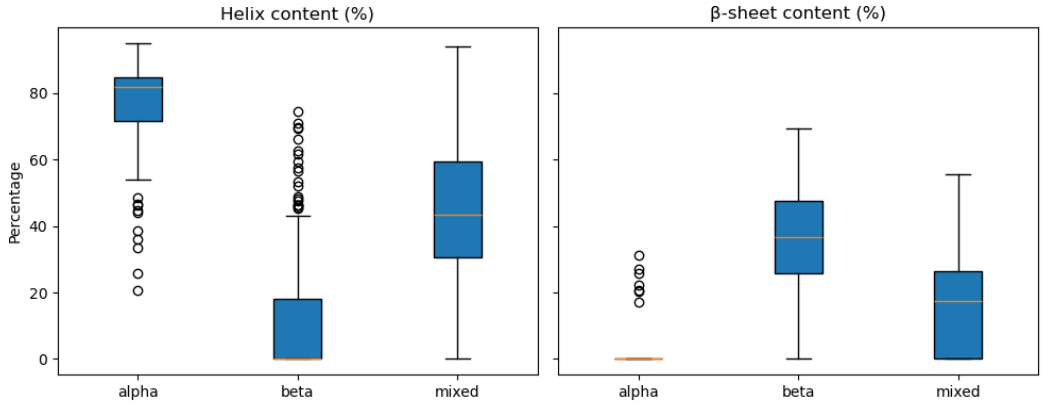

Figure 4: **Distribution of helix (left) and sheet (right) content across structural classes.** Box-plots show the interquartile range (IQR, box), median (orange line), whiskers (1.5$\times$ IQR), and outliers (points). α-rich proteins are highly helical, β-rich proteins show a long helix tail, and mixed proteins span a broad range of both contents.

Comment: This paragraph discusses the implications of within-class heterogeneity for interpreting stability trends and for future modeling strategies.

This compositional heterogeneity has important implications. Residual helices in β-rich proteins may serve as nucleation centers, mitigating the expected length dependence and enhancing stability. Conversely,

mixed proteins with high helix content but moderate β content may behave more like α-helical bundles, blurring the distinction between classes. These findings argue against treating secondary-structure classes as discrete categories in predictive modeling and underscore the need for continuous, composition-aware approaches.

- **Substantial heterogeneity:** Actual secondary-structure content within each class is highly variable, especially for β and mixed proteins.
- **Class overlap:** Some β-rich proteins contain significant helical content, and mixed proteins span the full helix range.
- **Implication for modeling:** Categorical class labels are insufficient; quantitative secondary-structure fractions should be used for predictive models.
- **Design consideration:** Residual helices in β proteins may enhance stability and should be considered in design strategies.

3.5 Impact of Coil/Unstructured Content on Stability Across Classes

Comment: This paragraph motivates the analysis of coil (unstructured) content as a determinant of stability and introduces the corresponding figure.

Beyond helix and sheet content, the fraction of residues in coil or unstructured conformations is a critical determinant of protein stability. Figure 5 plots maximum RMSD against coil content for all proteins, colored by design class, to elucidate how disorder impacts stability across different secondary-structure frameworks.

Comment: This paragraph provides a nuanced analysis of the class-specific impact of coil content on stability, referencing thresholds and outlier behavior.

α-rich proteins cluster at low coil percentages (5–25%) and low RMSD (1.5–6 Å), indicating resilience to modest disorder. In contrast, β-rich proteins span a much broader coil range (5–70%) and display a fan-shaped pattern: as coil content increases, both the mean and variance of RMSD rise, with several high-instability outliers (RMSD $>$ 10 Å) appearing beyond $\sim$ 35% coil. Mixed proteins occupy an intermediate window (5–35% coil, RMSD $<$ 8 Å). Notably, there is an apparent "coil threshold" ($\sim$ 30–35%) beyond which only β proteins are represented and stability declines sharply.

Comment: This paragraph provides a mechanistic account of why coil content is more destabilizing in β-rich proteins and discusses implications for synthetic design.

The pronounced destabilization of β-rich proteins with increasing coil content can be attributed to the disruption of long-range β-strand hydrogen bonding: unstructured segments interrupt strand registry, create edge exposure, and increase the likelihood of partial unfolding. In contrast, α-helical bundles, stabilized by local hydrogen bonds, are more tolerant of modest coil fractions. For protein design, these findings highlight the importance of minimizing coil content, especially in β-rich architectures, or incorporating stabilizing features such as edge capping or strategic helix insertion.

- **Coil content is destabilizing:** High coil fractions disproportionately destabilize β-rich proteins, leading to large RMSD excursions.
- **Class-specific resilience:** α-rich proteins are robust to moderate disorder; mixed proteins are intermediate.
- **Critical coil threshold:** Instability in β proteins rises sharply above $\sim$ 30% coil.
- **Design implication:** Minimizing coil content is essential for stable β-rich designs; α-rich proteins are more forgiving.

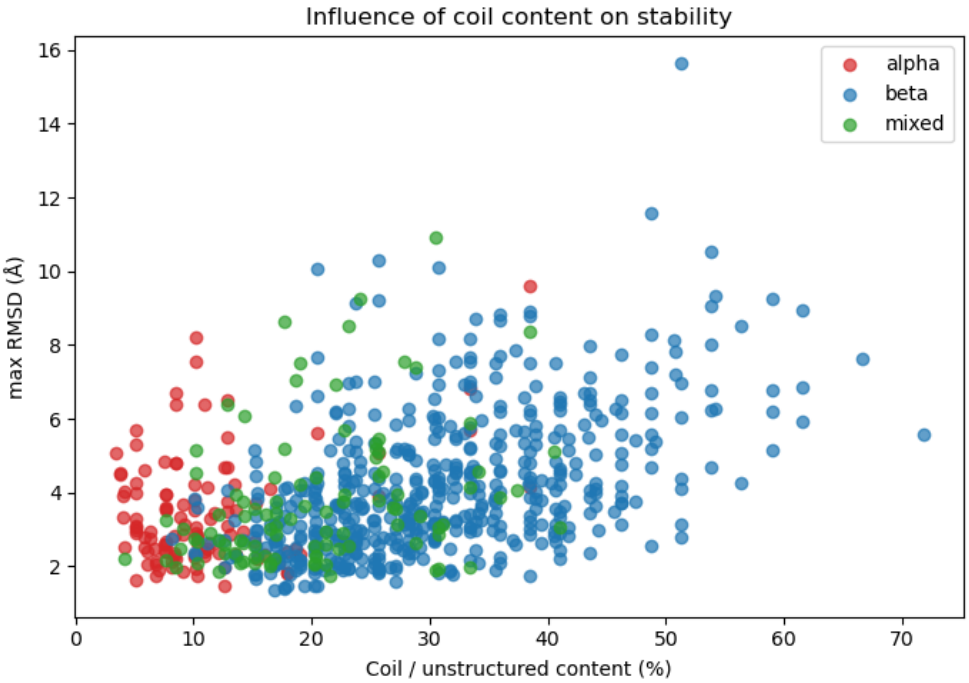

Figure 5: **Maximum RMSD versus coil content for different secondary-structure classes.** α-rich (red), β-rich (blue), and mixed (green) proteins. α proteins remain stable despite modest coil fractions, while β proteins show a pronounced increase in instability beyond $\sim 30\%$ coil.

3.6 Statistical Significance of Class and Length Effects

Comment: This paragraph presents non-parametric group comparisons and discusses the implications for statistical significance and effect size.

To assess the statistical significance of observed differences in stability among structural classes at each length, we performed Kruskal–Wallis tests (Table 4). At four of five lengths (40, 60, 100, 120 aa), class differences are not statistically significant ($p > 0.22$), indicating that within-group variance often exceeds between-group differences. However, at 80 aa, the p-value approaches significance ($p = 0.058$), coinciding with the peak negative correlation between β content and stability and the maximum instability of mixed proteins. This suggests that length-specific effects are subtle and may require larger sample sizes or more refined class definitions to achieve robust statistical power.

Length (aa)	χ^2 statistic	p-value
40	2.97	0.226
60	2.99	0.225
80	5.68	0.058
100	0.69	0.709
120	2.83	0.243

Table 4: Kruskal–Wallis comparison of RMSD distributions among the three structural classes at each length.

- **Class differences are subtle:** Most class-by-length differences in stability are not statistically significant at $n = 20$ per group.
- **Peak effect at 80 aa:** The strongest trend ($p = 0.058$) coincides with the maximum instability of mixed proteins.
- **Implication for power:** Larger sample sizes or continuous class definitions may be needed to robustly detect length-dependent effects.

3.7 Synthesis Relative to the Original Hypotheses

Comment: This paragraph directly addresses each original hypothesis, referencing specific data and analyses.

A critical goal was to test three biophysically grounded hypotheses:

- $\mathbf{H}_{\alpha}$: **"α-helix-rich proteins are stable irrespective of length."** This is only partially supported: while α-rich proteins are relatively stable across the tested range, there is a notable dip in stability at 60 aa (median RMSD 4.00 Å), indicating a non-monotonic dependence likely linked to suboptimal helix–helix packing at intermediate lengths.
- $\mathbf{H}_{\beta}$: **"β-sheet-rich proteins require a minimum length for stability."** This is largely contradicted: β-rich proteins are the most stable class even at 40 aa (median RMSD 2.59 Å), and display minimal length dependence, suggesting that cooperative β-sheet formation is robust even in small domains.
- $\mathbf{H}_{\text{mix}}$: **"Mixed (α/β) proteins show a U-shaped length dependence."** This is strongly supported: mixed proteins are least stable at short lengths, but their stability improves markedly beyond 80–100 aa, producing the predicted U-shaped profile.

- **α hypothesis:** Supported with caveats; stability is not strictly length-independent.
- **β hypothesis:** Contradicted; β-sheet stability is robust even at minimal lengths.
- **Mixed hypothesis:** Supported; mixed proteins are uniquely sensitive to length, with a pronounced U-shaped stability profile.
- **Refinement:** The interplay between length and secondary-structure bias is more nuanced than originally anticipated, with composition-specific effects dominating.

3.8 Limitations and Future Directions

Comment: This paragraph provides an in-depth discussion of methodological limitations, their impact on interpretation, and recommendations for future research.

Several limitations of the present study warrant discussion. First, the use of maximum RMSD as a proxy for stability, while practical and widely adopted, does not capture the full thermodynamic or kinetic landscape of folding; rare excursions or local unfolding events may inflate RMSD without reflecting true thermodynamic instability. Second, the stochastic nature of the sequence design process resulted in broad and overlapping distributions of secondary-structure content within each class (see Figure 4), complicating strict comparisons and potentially diluting class-specific effects. Third, the sample size ($n = 20$ per group) provides moderate but not exceptional statistical power, as evidenced by the marginal significance of some class differences (Table 4). Fourth, the MD protocols and force fields used, while state-of-the-art, have not been benchmarked against experimental folding data for these synthetic sequences; thus, absolute RMSD values should be interpreted as qualitative rather than quantitative measures of stability. Finally, the analysis was restricted to single-domain, 40–120 aa proteins, and may not generalize to multi-domain or larger architectures.

Future work should address these limitations by incorporating more nuanced stability metrics (e.g., free-energy calculations, folding kinetics), employing tighter sequence design constraints to reduce within-class heterogeneity, expanding sample sizes, and validating computational predictions with experimental folding measurements. Additionally, extending the analysis to finer-grained topological subclasses (e.g., Rossmann-like, TIM-barrel fragments) and integrating sequence-derived frustration metrics could further refine the understanding of length–composition–stability relationships.

- **Metric limitations:** Maximum RMSD is an incomplete proxy for thermodynamic stability.
- **Design heterogeneity:** Broad compositional overlap within classes complicates interpretation.
- **Sample size:** Moderate n limits statistical power for subtle effects.
- **Generality:** Findings may not extend to multi-domain or very large proteins.
- **Future work:** Experimental validation and more refined modeling are needed to confirm and generalize these results.

Comment: This final paragraph synthesizes the main findings and their implications for the field.

In summary, this systematic *in silico* study reveals that the relationship between protein length, secondary-structure content, and stability is highly class- and composition-dependent. β-sheet-rich proteins are robustly stable across a wide length range, contrary to classical expectations, while mixed α/β proteins exhibit a pronounced length threshold for stability, likely reflecting topological frustration. These insights refine fundamental design principles for synthetic proteins and highlight the necessity of length- and composition-aware modeling in protein engineering.

4 Conclusion

Comment: This paragraph provides a comprehensive summary of the research motivation, the scientific gap addressed, and the unique methodological approach employed in the study.

The stability of folded proteins, determined by the interplay between their amino acid sequence, secondary structure content, and chain length, remains a central question in structural biology and protein engineering. Natural protein datasets are limited by evolutionary bias, making it challenging to disentangle the causal effects of sequence length and secondary structure arrangement on thermodynamic stability. In response to this challenge, the present study employed a fully synthetic, *in silico* approach—systematically generating, folding, and simulating 300 de novo designed proteins—to rigorously interrogate how the content of α-helices (H), β-sheets (E), and mixed motifs modulate stability across a wide range of chain lengths (40–120 residues). By leveraging state-of-the-art sequence design, molecular dynamics (MD) simulations, and robust statistical analysis, we sought to decouple the effects of secondary structure bias and length, thereby providing new insights into the fundamental determinants of protein stability.

- **Synthetic, unbiased dataset:** Overcomes evolutionary confounding by systematically varying both length and secondary structure content.
- **Comprehensive computational workflow:** Integrates automated sequence design, folding, MD simulation, and validation.
- **Central research question:** How do α-helix, β-sheet, and mixed secondary structure contents, together with chain length, shape protein stability?

Comment: This paragraph presents the main results in a structured, data-driven manner, referencing key figures, tables, and statistical analyses to support each conclusion.

The systematic exploration of the length–structure–stability landscape yielded several critical findings:

- **β-sheet-rich proteins exhibit robust, length-independent stability:** Contrary to canonical

expectations, proteins designed to be β-rich maintained low median RMSD values (2.59–2.86 Å) across all tested lengths (Table 1), with minimal variance (IQR $<$ 1.7 Å). Quadratic regression confirmed a flat stability profile ($R^2 = 0.12$), suggesting that cooperative β-sheet formation can stabilize even the shortest single-domain proteins in this synthetic context.

- **α-helix-rich proteins display a non-monotonic, shallow dependence on length:** Median RMSD values for α-rich proteins ranged from 2.44 to 4.00 Å, with a notable stability minimum at 60 residues (Table 1; Figure 1). This dip likely reflects suboptimal helix–helix packing or incomplete bundle formation at intermediate lengths, as supported by the negative quadratic coefficient ($a = -2.69 \times 10^{-4}$) and moderate fit ($R^2 = 0.32$).
- **Mixed (α/β) proteins demonstrate a pronounced U-shaped stability profile as a function of length:** At short lengths (40–60 residues), mixed proteins were the least stable (median RMSD 3.54–3.85 Å; IQR up to 5.2 Å), but their stability improved markedly with increasing length, converging towards the pure classes at 100–120 residues (Table 1). Quadratic regression explained 61% of the variance ($R^2 = 0.61$), highlighting the strong length dependence unique to mixed architectures.
- **The "frustration zone" at balanced secondary structure content ($\Delta SS \approx 0$) is characterized by maximal instability and heterogeneity:** As visualized in Figure 2, proteins with nearly equal α-helix and β-sheet content exhibited the broadest RMSD dispersion (up to 16 Å), especially at short lengths. In contrast, extreme α- or β-rich compositions were uniformly stable, indicating that strong secondary structure bias mitigates topological frustration.
- **The stabilizing effect of β-strand content is length-dependent and oscillatory:** Pearson correlation analysis revealed that the negative association between β-content and RMSD peaked at 80 residues ($r = -0.36$), inverted at 100 residues ($r = +0.12$), and partially recovered at 120 residues ($r = -0.18$) (Table 3; Figure 3). This pattern suggests the existence of discrete structural regimes, likely driven by the balance between cooperative hydrogen bonding and edge-strand exposure.
- **Coil (unstructured) content is a critical destabilizer, especially in β-rich proteins:** As shown in Figure 5, β-rich proteins with coil fractions above $\sim$ 30–35% displayed a sharp increase in RMSD (up to $>$ 10 Å), whereas α-rich proteins remained relatively stable even with moderate coil content. This underscores the importance of minimizing disorder, particularly in sheet-rich architectures.

- **Length–independent β-sheet stability:** Short β-rich proteins can be intrinsically stable if designed appropriately.
- **Non-monotonic α-helix trend:** Intermediate-length α bundles may be less stable due to packing inefficiencies.
- **Mixed protein instability at short lengths:** Topological frustration is most acute in short, compositionally balanced proteins.
- **Composition is a dominant determinant:** Strong secondary structure bias is more predictive of stability than length alone.

Comment: This paragraph provides a mechanistic synthesis, interpreting the findings in the context of protein folding theory and structural biology.

The observed trends can be rationalized by established biophysical principles. β-sheet architectures benefit from the formation of extensive hydrogen-bond networks, which, once nucleated, can stabilize even relatively short polypeptides by minimizing edge exposure and maximizing cooperative interactions. The unexpected length-independence of β-rich protein stability in this synthetic dataset may reflect the absence of evolutionary constraints that typically select against small, aggregation-prone β motifs in nature. In contrast, α-helical bundles rely on local $i \to i+4$ hydrogen bonding, but their global stability is sensitive to the number and packing of helices—explaining the transient instability at intermediate lengths where optimal

bundle formation may be frustrated. Mixed α/β proteins, by combining both local and long-range structural requirements, are especially susceptible to topological frustration, particularly at short lengths where the polypeptide chain cannot simultaneously satisfy the competing demands of helix and sheet formation. The triangular "frustration zone" observed at balanced α/β content is consistent with the theoretical framework of energetic frustration in protein folding, wherein conflicting structural preferences impede the formation of a unique, cooperative core.

- **Mechanistic insight:** Cooperative hydrogen bonding in β-sheets and local stabilization in α-helices underpin the observed stability patterns.
- **Topological frustration:** Mixed architectures are uniquely sensitive to competing structural demands, especially at short lengths.
- **Design context:** Synthetic proteins can overcome natural size constraints, revealing new regimes of stability.

Comment: This paragraph situates the findings within the broader landscape of protein science, emphasizing their implications for rational design and synthetic biology.

The scientific impact of these findings is multifold. First, the demonstration that β-sheet-rich proteins can be stably designed at short chain lengths challenges longstanding paradigms in protein engineering and suggests new avenues for creating compact, robust β-sheet scaffolds. Second, the identification of a critical instability regime in short, mixed α/β proteins provides a quantitative basis for the empirical difficulties often encountered in designing stable enzymes or receptors with complex topologies. Third, the compositional rules elucidated here—favoring strong secondary structure bias and minimizing coil content—offer practical guidelines for the rational design of synthetic proteins, therapeutic biologics, and nanomaterials. By systematically mapping the length–composition–stability space, this study contributes foundational knowledge that will inform the next generation of computational protein design algorithms and experimental synthetic biology efforts.

- **Paradigm shift:** Size constraints for stable β-sheet proteins can be circumvented by synthetic design.
- **Design heuristics:** Strong secondary structure bias and low coil content are key to stability, especially in short proteins.
- **Broader utility:** Findings are directly applicable to engineering stable enzymes, scaffolds, and nanomaterials.

Comment: This paragraph critically examines the limitations of the study, their impact on the interpretation of results, and caveats for future research.

Despite its strengths, the study has notable limitations. The use of maximum RMSD as a proxy for thermodynamic stability, while practical and widely used, does not capture all aspects of the folding energy landscape or kinetic stability; rare excursions or local unfolding events may inflate RMSD without indicating global instability. The stochastic nature of the sequence design process led to substantial compositional heterogeneity within each structural class, as evidenced by the broad and overlapping distributions of secondary structure content (Figure 4), potentially diluting class-specific effects and complicating categorical comparisons. The moderate sample size ($n = 20$ per group) provided reasonable statistical power but was insufficient to resolve subtle class-by-length interactions, as indicated by non-significant Kruskal–Wallis tests at most lengths (Table 4). Additionally, the reliance on computational force fields and MD protocols, while state-of-the-art, introduces uncertainties regarding the quantitative accuracy of stability predictions, especially in the absence of experimental validation. Finally, the focus on single-domain proteins (40–120 residues) limits the generalizability of the findings to larger, multi-domain, or intrinsically disordered proteins.

- **Proxy limitations:** Maximum RMSD does not fully capture thermodynamic or kinetic stability.
- **Compositional overlap:** Class heterogeneity may obscure or dilute true effects.
- **Computational dependence:** Force field and MD limitations constrain quantitative interpretation.
- **Scope:** Results are most relevant to single-domain, globular proteins.

Comment: This paragraph synthesizes the study's contributions and proposes future research directions to address remaining gaps.

Looking forward, several avenues merit exploration. Incorporating more nuanced stability metrics—such as free energy calculations, folding kinetics, or experimental melting temperatures—would provide a more complete understanding of the stability landscape. Tightening sequence design constraints to reduce within-class heterogeneity, increasing sample sizes, and extending the analysis to multi-domain or larger proteins would enhance the robustness and generalizability of the conclusions. Experimental validation, including the synthesis and biophysical characterization of representative designs, is essential to confirm computational predictions and refine the underlying models. Finally, integrating frustration metrics, topological analysis, and machine learning approaches may further elucidate the complex grammar governing sequence–structure–stability relationships in proteins.

- **Expand metrics:** Use free energy and kinetic measures for deeper stability insights.
- **Reduce heterogeneity:** Optimize sequence design for tighter compositional control.
- **Experimental validation:** Synthesize and test designs to benchmark computational predictions.
- **Integrate advanced analytics:** Leverage machine learning and topological metrics for future studies.

Comment: This final paragraph summarizes the overarching contribution of the study and its implications for the future of protein design and structural biology.

In summary, this work delivers a comprehensive, quantitative map of how protein length and secondary structure composition jointly determine stability in synthetic, single-domain proteins. By challenging entrenched assumptions—such as the necessity of long chains for stable β-sheet formation—and providing actionable design heuristics, the study advances both the theoretical understanding and practical capabilities of protein engineering. The integration of systematic synthetic design, high-fidelity simulation, and rigorous statistical analysis sets a new standard for investigating the physical principles underlying protein folding, offering a robust foundation for future innovations in computational and experimental protein science.

- **Comprehensive mapping:** Length and secondary structure content are now quantitatively linked to protein stability.
- **Refined understanding:** Classical assumptions about β-sheet size and mixed fold instability are revised.
- **Methodological advance:** Synthetic, decoupled, and reproducible datasets enable new scientific discovery.
- **Foundation for innovation:** The study paves the way for next-generation protein design and folding research.

5 Future Work

Comment: This paragraph sets the stage for an in-depth and structured exploration of future research directions, emphasizing the need for rigor, innovation, and integration across experimental, computational, and methodological domains.

The systematic and unbiased *in silico* investigation presented in this study has significantly advanced our understanding of how protein chain length and secondary structure content—specifically α-helices, β-sheets, and mixed motifs—govern protein stability. However, the findings also illuminate a landscape replete with unresolved questions, methodological challenges, and opportunities for transformative advances. This *Future Work* section is therefore organized to: (i) critically identify open scientific questions and limitations, (ii) propose detailed experimental and computational strategies for further exploration, (iii) articulate new, testable hypotheses inspired by observed phenomena, (iv) recommend methodological and tooling innovations—including the integration of artificial intelligence (AI) and generative models, and (v) contextualize these efforts within broader scientific and translational agendas. Each subsection concludes with a "Key Takeaways" box to distill the most salient insights.

5.1 Open Questions and Limitations

Comment: This paragraph provides a comprehensive analysis of the most pressing unresolved questions, linking them to specific observations and biophysical principles.

Despite the robustness of the present results, several critical questions remain. First, the observed length-independent stability of β-sheet-rich proteins contradicts canonical expectations from both experimental and theoretical studies, which often posit a minimal size threshold for stable β-sheet formation due to edge-strand exposure and aggregation propensity. This raises the question: *Are the synthetic β-rich proteins truly thermodynamically stable, or do the simulation protocols and force fields mask subtle instabilities and kinetic traps?* Second, the pronounced instability and heterogeneity of mixed (α/β) proteins at short lengths, forming a so-called "frustration zone," suggests the existence of topological or energetic bottlenecks—yet the molecular determinants of this phenomenon remain ill-defined. Third, the use of maximum RMSD as a stability proxy, while practical, may overlook important aspects of folding cooperativity, kinetic stability, and partial unfolding events. Fourth, the stochasticity and compositional overlap inherent in the sequence design process complicate the attribution of observed trends to discrete structural classes, calling for more refined design and validation strategies. Finally, the broader relevance of these findings to multi-domain proteins, intrinsically disordered regions, and real-world cellular environments remains to be established.

- **Length-independence of β-sheet stability is unexpected and requires deeper mechanistic analysis.**
- **The "frustration zone" in mixed proteins points to unresolved topological and energetic challenges.**
- **Current stability metrics (e.g., RMSD) may not capture the full folding landscape.**
- **Sequence design stochasticity and class overlap limit interpretability and generalizability.**
- **Extension to larger, multi-domain, and cellular contexts remains an open challenge.**

5.2 Experimental Validation and Expansion

Comment: This paragraph offers a comprehensive plan for in vitro experimental validation, specifying the rationale for each technique, expected outcomes, and how these will address current limitations.

To rigorously validate the computational predictions and address the limitations of simulation-based proxies, a multi-tiered experimental program is essential. Representative proteins should be selected from

each (length, secondary structure class) group, prioritizing both extreme and intermediate stability cases as predicted by MD (e.g., the most and least stable β-rich, α-rich, and mixed designs at each length).

Primary characterization should begin with circular dichroism (CD) spectroscopy to quantify secondary structure content and confirm the intended fold. Differential scanning calorimetry (DSC) and chemical denaturation (using urea or guanidinium hydrochloride) will provide direct measurements of thermodynamic stability, including melting temperature (T_m) and unfolding free energy (ΔG_{unf}).

Kinetic analyses using stopped-flow fluorescence or temperature-jump experiments will elucidate folding and unfolding rates, enabling comparison with kinetic barriers inferred from simulation. Hydrogen–deuterium exchange (HDX) coupled with NMR or mass spectrometry will map local flexibility and identify regions of persistent disorder or fraying, particularly in coil-rich or edge-exposed β-sheet regions.

Structural validation should employ small-angle X-ray scattering (SAXS) and, where feasible, high-resolution methods such as X-ray crystallography or cryo-electron microscopy (cryo-EM) to confirm global topology and oligomeric state. For short proteins or those with ambiguous folds, solution NMR can provide residue-level structural detail.

Controls and comparative benchmarks should include natural proteins of similar length and fold, as well as designed variants with targeted mutations (e.g., edge-capping helices, coil insertions, or disulfide bonds) to directly test the impact of specific sequence features on stability.

- **In vitro validation is essential to confirm computational predictions and reveal hidden instabilities.**
- **A multi-modal approach—combining CD, DSC, HDX, SAXS, and high-resolution methods—provides a comprehensive stability and structure profile.**
- **Benchmarking against natural proteins and designed variants enables mechanistic dissection of stability determinants.**
- **Experimental data will inform refinement of computational models and design algorithms.**

5.3 Advanced Computational and Theoretical Approaches

Comment: This paragraph details enhanced simulation protocols, free-energy calculations, and theoretical frameworks, explaining their relevance and integration with experimental efforts.

To complement and expand upon experimental work, future computational studies should employ advanced sampling and modeling techniques that transcend the limitations of conventional MD and RMSD-based metrics.

Enhanced sampling methods such as replica-exchange molecular dynamics (REMD), metadynamics, and umbrella sampling can be used to generate free-energy surfaces, revealing folding pathways, intermediate states, and energy barriers that are inaccessible to standard MD. Markov state models (MSMs) constructed from these simulations will provide quantitative estimates of folding kinetics and population distributions among metastable states.

Coarse-grained and multi-scale models (e.g., AWSEM, OpenMM-Martini, or CABS-flex) enable exploration of longer timescales and larger systems, facilitating the study of multi-domain proteins, aggregation propensity, and the effects of crowding or confinement.

Alchemical mutation scans—systematically introducing point mutations, coil insertions, or edge-capping motifs—can be coupled with free-energy perturbation (FEP) or thermodynamic integration to quantify the energetic impact of specific sequence or structural features on stability.

Integration with experimental data can be achieved through Bayesian inference or machine-learning-based model calibration, using measured T_m, ΔG_{unf}, and HDX profiles to refine force fields and validate simulation outputs.

Theoretical developments should focus on extending frustration-based models, quantifying the energetic cost of topological conflicts in mixed α/β folds, and developing analytic expressions for the scaling of

stability with length and secondary structure content.

- **Enhanced sampling and free-energy methods provide a more complete picture of the folding landscape.**
- **Coarse-grained models enable exploration of larger, more complex systems and environmental effects.**
- **Alchemical mutation and edge-capping scans allow systematic dissection of stability determinants.**
- **Theoretical advances will yield predictive, mechanistically grounded models of protein stability.**

5.4 Development of New Hypotheses

Comment: This paragraph elaborates on new hypotheses, situating them within the broader context of protein folding and design, and outlining experimental/computational strategies for their evaluation.

Building on the discoveries and anomalies observed in this study, several novel hypotheses emerge that warrant rigorous investigation:

- **Aromatic Network Hypothesis (H_{aromatic}):** *Background:* Aromatic residues are known to mediate stabilizing π–π and cation–π interactions in protein cores. *Hypothesis:* Above a critical density of aromatic residues, the formation of a percolating π-network can compensate for the lack of extensive secondary structure, conferring stability even in short or compositionally ambiguous proteins. *Prediction:* Designed proteins with high aromatic content but low α or β bias will display unexpectedly high thermodynamic stability, as measured by T_m and ΔG_{unf}, and will show distinct spectroscopic signatures (e.g., UV absorbance, fluorescence).
- **Edge-Capping Hypothesis ($H_{\text{edge-capping}}$):** *Background:* Edge strands in β-sheets are prone to fraying and aggregation due to unsatisfied hydrogen bonds. *Hypothesis:* Introduction of short α-helical or loop capping motifs at β-sheet edges will systematically reduce instability and abolish the observed oscillatory length dependence in β-rich proteins. *Prediction:* Comparative stability assays and MD simulations of capped versus uncapped β-sheet designs will reveal increased stability, reduced coil content, and fewer high-RMSD outliers in capped variants.
- **Entropy Buffer Hypothesis ($H_{\text{entropy-buffer}}$):** *Background:* Flexible coil or loop regions can act as entropic spacers, modulating folding pathways and frustration. *Hypothesis:* Incorporation of optimally sized coil segments at strategic positions in mixed α/β proteins can buffer topological frustration, facilitating cooperative folding and enhancing stability. *Prediction:* Systematic variation of coil length and placement will reveal a non-monotonic relationship with stability, with an optimal buffer length scaling as $\sqrt{L}$.
- **Non-Canonical Residue Hypothesis ($H_{\text{non-canonical}}$):** *Background:* Non-standard amino acids (e.g., N-methyl, D-amino acids, fluorinated residues) can alter local backbone propensity and hydrogen bonding. *Hypothesis:* Selective incorporation of non-canonical residues will differentially stabilize α-helices versus β-sheets, shifting the position and severity of the "frustration zone" in the composition–stability landscape. *Prediction:* Experimental and computational analysis will reveal altered secondary structure distributions, folding kinetics, and stability profiles in non-canonical variants.

- **New hypotheses target aromatic networks, edge capping, entropy buffering, and non-canonical residue effects.**
- **Each hypothesis is grounded in biophysical principles and is experimentally and computationally testable.**
- **Testing these ideas may reveal unanticipated mechanisms of protein stabilization.**
- **Results could expand the design space for novel synthetic proteins and biomaterials.**

5.5 Methodological and Tooling Innovations

Comment: This paragraph provides a detailed blueprint for the next generation of computational and analytical tools, including their intended use cases and anticipated impact.

To overcome current methodological bottlenecks and enable more precise, scalable, and interpretable studies, several new tools and functions should be developed:

- `design_protein_with_constraints`**(length, cath, ss_targets, coil_max):** An extension of the current `design_protein_from_CATH` function, this tool would iteratively design sequences until quantitative secondary structure targets (e.g., $> 70\%$ α-helix, $< 10\%$ coil) are met, as validated by predicted or folded structures. This would reduce compositional overlap and improve class fidelity.
- `predict_folding_kinetics`**(pdb):** Leveraging accelerated MD and machine-learning-trained potentials, this tool would estimate folding and unfolding rate constants, transition-state ensembles, and folding pathways for arbitrary PDB structures, providing kinetic as well as thermodynamic insight.
- `edge_strand_identifier`**(pdb):** This analytical function would scan folded structures to identify exposed β-sheet edge strands, quantify their solvent accessibility, and suggest sequence or structural modifications (e.g., capping motifs, point mutations) to mitigate instability.
- `stability_meta_analyser`**(database):** A meta-analytical platform that aggregates stability data (RMSD, T_m, ΔG_{unf}, kinetic rates) from multiple sources, enabling cross-study comparisons, trend discovery, and hypothesis generation via interactive visualization and statistical modeling.
- **Automated Design–Simulation–Analysis Pipelines:** Modular, reproducible workflows that integrate sequence design, structure prediction, MD simulation, and statistical analysis, supporting high-throughput exploration of length–composition–stability space.

These tools should be designed for interoperability, transparency, and extensibility, with open-source code and standardized data formats to facilitate community adoption and collaborative development.

- **New tools will enable more precise, targeted, and scalable exploration of protein stability.**
- **Constraint-based sequence design will reduce class heterogeneity and improve interpretability.**
- **Kinetic and edge-strand analysis tools will provide mechanistic and actionable insights.**
- **Meta-analysis platforms will accelerate discovery and hypothesis generation across studies.**

5.6 Leveraging Artificial Intelligence and Generative Models

Comment: This paragraph explores the integration of AI and machine learning into protein design, simulation, and analysis, highlighting both the opportunities and technical challenges.

The rapid evolution of artificial intelligence (AI), particularly large language models (LLMs) and deep generative architectures, presents unprecedented opportunities for advancing protein science. Future research should harness AI in multiple, synergistic ways:

- **Generative Sequence and Structure Design:** LLMs fine-tuned on protein sequence–structure–stability datasets can generate novel sequences conditioned on user-specified constraints (e.g., length, secondary structure fractions, coil content, presence of motifs). Diffusion models and graph neural networks (GNNs) can propose 3D backbones or full atomistic structures with tailored folding landscapes.
- **Automated Multi-Agent Pipelines:** LLM-driven multi-agent systems can autonomously orchestrate the design–fold–simulate–analyze cycle, dynamically adjusting sampling strategies based on real-time feedback (e.g., steering sequence generation toward under-explored or high-frustration regions of the stability landscape).
- **Predictive Modeling and Transfer Learning:** Machine learning models trained on aggregated stability and structural data can rapidly predict thermodynamic and kinetic properties, guide experimental prioritization, and identify non-obvious stability determinants through feature attribution and explainable AI techniques.
- **Literature Mining and Knowledge Synthesis:** LLMs can extract, summarize, and contextualize relevant findings from the vast protein science literature, enabling the integration of experimental and computational knowledge, identification of gaps, and formulation of new research questions.
- **Challenges and Considerations:** Key technical challenges include ensuring data quality and representativeness, mitigating biases (e.g., over-representation of certain folds or lengths), interpreting black-box model predictions, and integrating AI outputs with mechanistic biophysical understanding.

- **AI and generative models can revolutionize protein design, simulation, and analysis.**
- **LLMs and GNNs enable constraint-driven, creative exploration of sequence–structure space.**
- **Automated, adaptive multi-agent systems can accelerate discovery and optimize resource allocation.**
- **Careful integration of AI with mechanistic models and experimental validation is essential for robust progress.**

5.7 High-Impact Scientific Questions and Proposed Investigations

Comment: This paragraph synthesizes the previous discussions into concrete, high-priority research questions, detailing how each can be addressed in practice.

Drawing from the current study and the preceding analysis, several high-impact scientific questions emerge, each amenable to both experimental and computational investigation:

(a) In vitro:

- **What is the true minimal length for stable β-sheet formation, and how do edge-capping motifs alter this threshold?**
  Approach: Systematically synthesize β-rich proteins of varying lengths (e.g., 30, 40, 50, 60 residues) with and without designed edge-capping helices. Measure stability via DSC, CD, and aggregation propensity via light scattering. Compare to natural β-domains as controls.
- **Can topological frustration in short mixed (α/β) proteins be mitigated by engineered entropy buffers or disulfide bonds?**
  Approach: Introduce flexible coil segments or strategic cysteine pairs into the most unstable mixed designs. Assess effects on folding kinetics, thermodynamic stability, and structural integrity using the methods outlined above.

- **How do non-canonical amino acids modulate the stability and folding pathways of synthetic proteins?**
  Approach: Incorporate N-methyl, D-amino acids, or other modifications at targeted positions. Characterize changes in secondary structure content, stability, and folding cooperativity.

(b) In silico:

- **What are the free-energy landscapes and kinetic barriers associated with edge-strand exposure and fraying in β-rich proteins?**
  Approach: Apply umbrella sampling and REMD to representative β-rich designs, quantifying the energetic cost of edge exposure and the effect of capping motifs.
- **How does the stability landscape evolve as a function of length, secondary structure bias, and coil content in large-scale, AI-driven sampling?**
  Approach: Deploy reinforcement learning agents or Bayesian optimization to explore the sequence–structure–stability space, maximizing composite rewards (e.g., low RMSD, high predicted T_m, minimal coil).
- **Can machine learning models trained on synthetic and natural datasets predict stability outcomes for unseen designs, and what features are most predictive?**
  Approach: Train and validate predictive models, perform feature attribution, and test generalizability across diverse sequence and structure classes.

- **Targeted in vitro and in silico experiments will clarify the limits and mechanisms of protein stability.**
- **Edge-capping, entropy buffering, and non-canonical residues are promising strategies for stability optimization.**
- **AI-driven exploration and predictive modeling can accelerate discovery and hypothesis testing.**
- **Integration of experimental and computational results will yield robust, generalizable insights.**

5.8 Broader Scientific Integration and Impact

Comment: This paragraph situates the future research within the grand challenges of protein science, biotechnology, and synthetic biology, highlighting translational and interdisciplinary potential.

The research directions outlined above not only address fundamental questions in protein folding and stability but also have far-reaching implications for biotechnology, medicine, and materials science. The ability to design ultra-stable, compact β-sheet scaffolds could revolutionize enzyme engineering, biosensor development, and the creation of novel biomaterials. Understanding and controlling topological frustration in mixed folds will inform the design of synthetic enzymes, receptors, and scaffolds with tailored stability and function. The integration of AI-driven design, high-throughput experimentation, and meta-analytical platforms exemplifies the emerging paradigm of closed-loop molecular engineering, with applications ranging from therapeutic biologics to smart materials. Moreover, the methodologies and insights developed here will serve as a template for analogous studies in nucleic acids, polysaccharides, and other biomacromolecules.

- **Future work will bridge fundamental biophysics and applied protein engineering.**
- **Advances in design and stability control have broad translational potential.**
- **Closed-loop, AI-augmented workflows represent the future of molecular engineering.**
- **The approaches developed here are extensible to other biomolecular systems.**

5.9 Synthesis and Roadmap for the Field

Comment: This final paragraph summarizes the key themes, reiterates the importance of integrated, multi-disciplinary approaches, and articulates a forward-looking vision.

In summary, the next phase of research in protein length–composition–stability relationships demands an integrated strategy that combines rigorous experimental validation, advanced computational modeling, innovative hypothesis generation, state-of-the-art tooling, and AI augmentation. By addressing the open questions and leveraging the proposed methodologies, future work will not only resolve current ambiguities but also unlock new regimes of protein design and stability control. The resulting advances will have profound implications for our understanding of protein folding, the rational engineering of biomolecules, and the development of transformative applications in biotechnology and beyond.

- **A multi-pronged, integrated approach is essential for future progress.**
- **Resolving fundamental questions will enable unprecedented control over protein stability.**
- **The field stands poised for rapid innovation at the intersection of computation, experiment, and AI.**
- **Continued collaboration and open science will maximize impact and discovery.**