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Engineering Proteins for Functional Persistence: A Mechanistic Framework Integrating Catalytic Activity, Folding Stability, Proteolytic Susceptibility, and the Cellular Costs of Protein Maintenance

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Abstract

Protein engineering is often evaluated through catalytic performance or structural stability, yet a protein that performs well in an isolated assay may fail to sustain useful function in a cell. This article develops a mechanistic account of functional persistence linking catalytic competence, folding stability, exposure to quality-control and degradation pathways, and the resource demand created by synthesis, folding, and replacement. Evidence from enzyme engineering, stability measurements, degron biology, cotranslational folding, proteostasis, and cellular resource allocation is used to distinguish intrinsic molecular properties from context-dependent persistence. The analysis rejects a universal activity–stability trade-off and treats trade-offs as contingent on protein architecture, functional mechanism, and measurement regime. Degradation susceptibility likewise cannot be inferred from thermodynamic stability alone because degron exposure, assembly state, processing, chaperone engagement, and proteasome access modify protein fate. Functional persistence is therefore a conditional, multiscale engineering objective requiring orthogonal measures of useful activity, conformational behavior, turnover, and host burden.

Keywords: Protein engineering, Functional persistence, Folding stability, Proteostasis, Protein degradation, Cellular resource allocation

Introduction

Protein engineering increasingly operates in design spaces where improvements in one molecular property must coexist with constraints imposed by several others. Catalytic efficiency, specificity, expression, folding, and stability can all influence usefulness, but they do not collapse into a single property. Mutations introduced to improve function may destabilize the folded state or alter conformational dynamics, motivating strategies that recover stability while preserving the desired functional change (Teufl *et al*., 2022). For proteins intended to act in living cells, however, the relevant outcome is broader than purified-protein performance: the engineered sequence must be synthesized, folded, maintained, and retained in a functional state over the required interval.

Cellular proteostasis makes this distinction unavoidable. Ribosomes, chaperones, assembly partners, ubiquitin ligases, proteases, and stress-responsive quality-control systems influence which conformational states persist and which are refolded or removed. Proteome-wide folding is consequently constrained by proteostasis capacity and organization (Powers & Gierasch, 2021). An engineered protein may therefore be stable in a purified assay yet perform differently under cellular production, crowding, interaction, and surveillance conditions.

Protein production also consumes finite cellular capacity. Growth and gene expression are coupled to the allocation of ribosomes, metabolic enzymes, and other proteome sectors, so production of one protein can alter resources available for other functions (Scott & Hwa, 2023). Translation-coupled surveillance adds another cost because stalled or defective nascent products can be eliminated before a mature protein is produced (Inada & Beckmann, 2024). These observations motivate a distinction between molecular activity—what a protein can do in an appropriate state—and functional persistence—whether useful molecules remain available without excessive resynthesis, refolding, or replacement.

Large-scale cDNA-display proteolysis has shown that thermodynamic folding stability can now be measured across hundreds of thousands of natural and designed variants (Tsuboyama *et al*., 2023). Such measurements, however, do not directly report cellular lifetime, degradation-pathway engagement, assembly success, or maintenance cost. The central question is therefore under which conditions catalytic performance, folding stability, quality-control susceptibility, and cellular resource demand become limiting, and which measurements distinguish a

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genuinely persistent functional protein from one that performs well only in a narrower assay context.

Materials and Methods

Catalytic Function and Folding Stability as Competing Engineering Objectives

The language of an activity–stability trade-off is useful only when its domain is specified. Comparative biochemical studies have identified enzymes in which greater activity coincides with lower thermal stability, but a broad appraisal found that this relationship varies among systems and is not imposed by a universal physicochemical constraint (Miller, 2017). A negative correlation across particular orthologs, temperatures, substrates, or engineered variants therefore establishes a trade-off only within that regime. Functional residues can influence packing, electrostatics, dynamics, and conformational exchange in ways that couple activity and stability in one protein while leaving them more separable in another.

Engineered transketolase illustrates how this coupling can be distributed through a protein. A variant evolved to accept aromatic substrates acquired increased flexibility in interconnected active-site regions that also contributed to the dimer interface. Molecular-dynamics correlations then guided distal substitutions that recovered stability while preserving, and in some variants improving, activity (Yu & Dalby, 2018). The study does not provide a universal recipe, but it shows that catalytic changes and compensatory stabilization need not occur at the same sites. Local chemical modification can also reshape the relationship: noncanonical amino-acid substitutions in an evolved enzyme active site produced variant-dependent changes in activity and stability (Wilkinson & Dalby, 2021).

These observations fit a multidimensional view of protein evolution and design. A theoretical analysis separated constraints associated with stability from those associated with catalytic residues and showed how their joint action can shape evolutionary patterns without requiring a single stability–activity law (Echave, 2019). The engineering objective is therefore not maximal stability in isolation, because excessive rigidity or altered conformational exchange may conflict with function in some proteins. It is sufficient conformational viability within the structural and dynamic regime required for the intended molecular task.

Contemporary design methods make simultaneous optimization increasingly tractable. Evolution-informed design has experimentally improved multiple functional properties in selected protein systems (Fram *et al*., 2024). Modern biocatalysis combines directed evolution and chemical strategy to create activities absent from starting catalysts (Miller *et al*., 2022). Data-efficient machine learning can guide sequence exploration when relatively few variants have been characterized (Biswas *et al*., 2021), while ProteinMPNN demonstrates structure-conditioned sequence design compatible with target backbones (Dauparas *et al*., 2022). Generative methods extend this search space: RFdiffusion can create experimentally testable de novo structures and functional scaffolds (Watson *et al*., 2023), and Chroma enables programmable exploration of sequence–structure space (Ingraham *et al*., 2023).

These advances do not establish intracellular persistence. Their validation targets structure, sequence compatibility, catalysis, binding, or other defined properties. A candidate can satisfy those criteria yet remain vulnerable to quality control, failed assembly, context-specific unfolding, or rapid turnover. Candidate comparison should therefore retain catalytic function and folding stability as separate, partially coupled dimensions and avoid treating either as a surrogate for cellular lifetime.

The evidence supports a conditional comparison of molecular objectives rather than a fixed ranking of activity against stability. This distinction organizes the principal engineering routes relevant to the first stage of functional persistence.

Table 1 summarizes molecular engineering objectives and their mechanistic trade-offs.

Table 1. Molecular engineering objectives and mechanistic trade-offs relevant to functional persistence

Analytical unit	Property under selection	Mechanistic route	Countervailing effect	Required measurement	Persistence implication
Catalytic-site redesign	Rate, specificity, or new substrate acceptance	Alters active-site geometry, chemistry, or dynamics	Can destabilize packing or redistribute flexibility	Matched kinetic and stability characterization	Improved activity matters only if a viable folded population remains
Distal compensatory mutation	Stability with preserved function	Stabilizes dynamically coupled regions away from catalytic residues	May alter allostery or assembly	Activity plus structural/dynamic testing	Can widen the feasible activity–stability region in selected proteins
Noncanonical active-site chemistry	Local function and packing	Expands side-chain chemical space	Strong variant and substrate dependence	Variant-resolved activity and stability assays	May tune both properties without proving cellular persistence
Multi-property sequence design	Several defined molecular functions	Searches compatible sequence changes	Optimization is limited to represented targets	Independent validation of each property	Supports joint optimization but not universal absence of trade-offs

Structure-conditioned design	Sequence–backbone compatibility	Learns sequence–structure relationships	Structural compatibility may not predict cellular fate	Structural validation followed by turnover testing	Narrows candidates without establishing persistence
Generative de novo design	New structures or functional scaffolds	Samples constrained sequence–structure space	Only subsets are experimentally validated	Physical and context-specific functional testing	Creates candidates whose maintenance remains an independent question

A stability assay can identify unfavorable conformational energetics, and a functional assay can identify the desired molecular task, but neither alone identifies the mechanisms controlling cellular lifetime. That question requires the quality-control layer.

Proteolytic Susceptibility, Quality Control, and Intracellular Protein Persistence

Intracellular persistence is partly determined by sequence information that becomes actionable only in the cellular environment. E3 ubiquitin ligases can recognize features at protein N- or C-termini, making terminal identity, processing, and accessibility relevant to degradation (Sherpa *et al*., 2022). Internal degrons add another layer. Proteome-wide mapping has revealed extensive sequence diversity among internal degradation signals and linked subsets to specific E3 ligases (Zhang *et al*., 2023). Folding stability and degradation susceptibility are therefore distinct: a stable protein can present a recognition signal, whereas a potentially degradative sequence can remain silent while buried in a native structure.

Conformational exposure provides one connection between folding failure and quality control. Hsp70-binding regions share features with hydrophobic quality-control degrons, and a canonical Hsp70-binding motif was shown to function as a degradation signal in yeast and human cells (Abildgaard *et al*., 2023). N-terminal information can likewise be cryptic rather than constitutively active. Systematic analysis of a eukaryotic N-terminome identified many sequences capable of promoting degradation while showing that context and processing influence their realization (Kats *et al*., 2018). A glycine-specific N-degron pathway associated with protein N-myristoylation further demonstrates that the success or failure of a processing event can alter subsequent protein fate (Timms *et al*., 2019).

Assembly state creates a different persistence constraint. Proteins synthesized as components of multiprotein complexes can become "orphans" when partners are absent, delayed, or stoichiometrically limiting; dedicated quality-control pathways can then remove otherwise folded subunits (Juszkiewicz & Hegde, 2018). UBE2O provides an experimentally defined example by targeting unassembled subunits of multiprotein complexes (Yanagitani *et al*., 2017). Engineering in isolation may consequently miss liabilities introduced by altered interface geometry, localization, expression timing, or assembly stoichiometry.

Proteolytic commitment is itself multistep. The 26S proteasome coordinates substrate recognition, deubiquitination, engagement, unfolding, translocation, and proteolysis (Collins & Goldberg, 2017). Thus, the route from a newly synthesized chain to a persistent functional protein cannot be reduced to "instability exposes a degron." Sequence signals, conformation, chaperone occupancy, E3 availability, assembly state, ubiquitin architecture, and proteasomal engagement can modify the outcome.

The folding trajectory also begins before synthesis is complete. Cotranslational folding occurs during vectorial emergence from the ribosome and can be influenced by the exit tunnel and ribosome-associated factors (Samatova *et al*., 2024). Peptide-resolved hydrogen–deuterium exchange measurements of *Escherichia coli* dihydrofolate reductase showed structured cotranslational intermediates that differed from states populated during refolding from denaturant, with trigger factor engaging partially folded states (Wales *et al*., 2024). This establishes a measurement boundary: purified mature-protein refolding cannot automatically substitute for biosynthetic folding history.

Cell state adds further conditionality. Oxidative conditions can modify proteins and alter multiple parts of the proteostasis network (Ulfig & Jakob, 2024). More broadly, chaperone and degradation capacity is finite, and aggregation-prone or damaged proteins can consume that capacity under stress and disease-associated conditions (Hipp & Hartl, 2024). An engineered sequence therefore has no context-free persistence value. Realized functional availability can depend on expression rate, folding trajectory, binding partners, processing, stress state, and the existing load on quality-control systems.

These mechanisms support a layered interpretation of degradation susceptibility. Sequence defines possible recognition routes; folding and assembly determine exposure; chaperones and E3 ligases mediate surveillance; and the proteasome executes regulated destruction. Increasing thermodynamic stability may improve persistence when degradation is driven by conformational exposure, but it may have little effect when turnover is controlled by an accessible terminal signal, failed assembly, regulated processing, or a dedicated degradation program.

Table 2 summarizes the cellular mechanisms governing context-dependent protein persistence.

Table 2. Cellular mechanisms that convert protein state into context-dependent persistence

Protein state or event	Quality-control determinant	Directly assessed feature	Why stability alone is insufficient	Boundary to retain	Engineering interpretation
Exposed terminal sequence	Terminal degron recognition	Sequence, processing, accessibility, degradation response	A stable fold can still present a terminal signal	Ligase expression and processing are context specific	Terminal design should be assessed independently of global stability
Exposed internal segment	Internal degron recognition	Sequence-dependent degradation and E3 specificity	Activity depends on structural exposure	Peptide behavior may differ in intact protein	Whole-protein validation is required
Persistent non-native conformation	Chaperone-linked quality control	Chaperone-binding exposure and turnover	Chaperone-binding regions can become degradation signals	Transient and persistent exposure differ	Stabilization helps only when it reduces relevant exposure
Failed processing	Processing-sensitive N-degron pathway	Modification state and degradation	Stability does not report checkpoint success	Pathways are substrate specific	Processing competence is a separate criterion
Unassembled complex subunit	Orphan-protein surveillance	Partner availability and QC engagement	A folded monomer can be degraded when unassembled	Stoichiometry and timing matter	Interface and expression design can affect lifetime
Ribosome-bound nascent chain	Cotranslational folding/chaperones	Folding intermediates during synthesis	Mature refolding may not reproduce nascent pathways	Ribosome effects can be protein specific	Cellular folding tests should complement purified assays
Ubiquitinated substrate	Proteasome engagement	Recognition, unfolding, translocation, degradation	Ubiquitination is not completed degradation	Proteasome regulation matters	Direct turnover measurements remain necessary
Protein under stress	Proteostasis capacity	Folding/degradation load under defined state	Maintenance capacity changes across conditions	Stress states are not interchangeable	Persistence claims must specify biological context

A persistent engineered protein is therefore not simply one that resists unfolding or proteolysis in vitro. It is one that repeatedly traverses biosynthesis, folding, assembly, surveillance, and degradation checkpoints while retaining the intended molecular function under relevant cellular conditions.

Cellular Resource Costs of Protein Synthesis, Folding, and Replacement

Protein persistence has a cellular cost even when the protein remains correctly folded and functional. Heterologous expression recruits transcriptional, translational, folding, and trafficking resources that would otherwise support host functions. In mammalian cells, resource-aware construct analysis has demonstrated that genetic components can interact through competition for shared cellular machinery, making construct performance dependent on the surrounding expression program (Di Blasi *et al*., 2023). Experimental characterization of mammalian gene-expression burden similarly shows that strong synthetic expression can perturb other cellular outputs and that circuit architecture can mitigate some of these effects (Frei *et al*., 2020). These findings make expression burden relevant to protein engineering, but they do not justify assigning a fixed energetic price to an individual protein.

Proteome allocation provides a wider systems context. Functional decomposition of bacterial metabolism has linked metabolic fluxes to the quantities of protein committed to particular cellular functions, demonstrating that growth and metabolic performance reflect structured allocation rather than unrestricted enzyme abundance (Mori *et al*., 2023). Resource-aware coarse-grained models likewise reproduce interactions among ribosome availability, synthetic expression, and cellular growth (Sechkar *et al*., 2024). Such models simplify molecular detail, but they clarify why production demand cannot be treated independently of the physiological state of the host.

Allocation is also regulated rather than static. Matched transcriptomic and proteomic analyses across physiological conditions show that transcriptional organization and proteome allocation are related but not interchangeable (Patel *et al*., 2024). Long-term laboratory evolution further demonstrates that adaptive changes occur within constraints on proteome partitioning (Mori *et al*., 2024). A variant that is inexpensive to maintain in one state may therefore become comparatively costly when growth rate, nutrient supply, stress responses, or competing expression programs change.

Folding and replacement add a further layer. Temperature-dependent allocation models have incorporated protein unfolding, refolding, and chaperone requirements into predictions of microbial growth (Mairet *et al*., 2021). The quantitative optimum produced by such a model is system specific, but the mechanistic implication is important: proteins that repeatedly fail folding or undergo rapid degradation can demand recurrent synthesis and quality-control activity. Functional persistence consequently involves not only how much active protein is produced, but how

much cellular capacity must be repeatedly spent to maintain the required active population.

These distinctions support a context-dependent selection strategy rather than a single ranking of engineered variants. A variant should be evaluated against the biological conditions in which it is expected to operate, as summarized in **Table 3**.

Table 3. Conditional criteria for selecting engineered protein variants under different cellular maintenance demands

Cellular condition	Protein-level property requiring assessment	Host-level variable	Mechanistic concern	Evidence required for selection	Interpretation
High-expression production	Catalytic output per expressed protein and folding competence	Availability of translational and folding resources	High expression can create resource competition even when the protein functions correctly	Matched expression, function, and host-burden measurements	Greater expression is advantageous only when host cost remains acceptable
Limited proteostasis capacity	Conformational stability and exposure of quality-control signals	Chaperone and degradation capacity	Marginally folded variants may increase refolding and replacement demand	Stability, turnover, and chaperone/QC measurements	A more stable variant may be favored when instability drives recurring maintenance
Multiprotein-complex expression	Interface competence and assembly efficiency	Partner abundance and expression timing	Unassembled subunits can become quality-control substrates	Assembly, abundance, and turnover measurements	Intrinsic stability cannot substitute for successful complex formation
Stress-variable environment	Retained function across relevant cellular states	Proteostasis and metabolic state	Oxidative or other stress can alter folding and degradation capacity	State-specific activity and persistence measurements	Variant ranking may change across environments
Resource-constrained host	Functional output relative to synthesis and replacement demand	Proteome allocation and growth state	Frequent replacement can increase recurring biosynthetic demand	Function, abundance, turnover, and host-physiology measurements	Selection should consider durable output rather than expression alone

A Mechanistic Framework for Engineering Functional Persistence

The evidence supports treating functional persistence as an emergent property rather than a direct synonym for catalytic activity, thermodynamic stability, or protein half-life. Activity and stability can conflict in particular systems, yet neither theory nor comparative evidence supports an obligatory universal trade-off (Miller, 2017; Teufl *et al*., 2022). The relevant engineering task is to maintain sufficient catalytic or binding competence within a conformational regime compatible with the intended biological environment.

A second dimension emerges when the cellular quality-control system encounters the engineered protein. Internal sequence motifs can function as degradation signals, while exposure of chaperone-binding regions can connect non-native conformations to protein-quality-control pathways (Abildgaard *et al*., 2023; Zhang *et al*., 2023). Consequently, the same amino-acid substitution may influence persistence through several routes: it may change catalytic geometry, alter the energetic population of folded states, expose or conceal recognition sequences, modify assembly, or change interactions with chaperones. These effects should remain analytically separate until experiments establish their relationship.

The third dimension is replacement demand. Expression burden, metabolic protein allocation, and resource-aware cell models collectively show that protein production occurs within a finite and regulated physiological economy (Di Blasi *et al*., 2023; Mori *et al*., 2023; Sechkar *et al*., 2024). A rapidly degraded protein may therefore impose costs beyond its reduced steady-state abundance because maintaining useful function can require recurrent synthesis, folding, and quality-control processing. Conversely, a slowly turning-over protein is not automatically preferable if its production or persistent interactions compromise cellular physiology.

Functional persistence can therefore be defined operationally as the capacity of an engineered protein to provide the required molecular function over the relevant interval while remaining compatible with folding, assembly, surveillance, turnover, and cellular resource constraints. This definition does not impose a numerical score. It specifies measurements that should remain distinct: activity or task performance, conformational behavior, cellular abundance and turnover, quality-control engagement where mechanistically relevant, and host burden under the intended operating condition.

The framework makes variant ranking conditional. A protein optimized for extracellular catalysis, cell-free manufacturing, transient intracellular expression, or long-lived expression in a differentiated cell need not occupy the same preferred region of design space. Transfer from one setting to another therefore

requires reassessing the biological mechanisms that determine persistence rather than assuming that a stability improvement or design-model score retains the same meaning.

These cross-scale relationships are condensed in **Figure 1**, which separates molecular determinants from cellular surveillance and host-resource consequences while preserving the uncertainty that accompanies transitions between these levels.

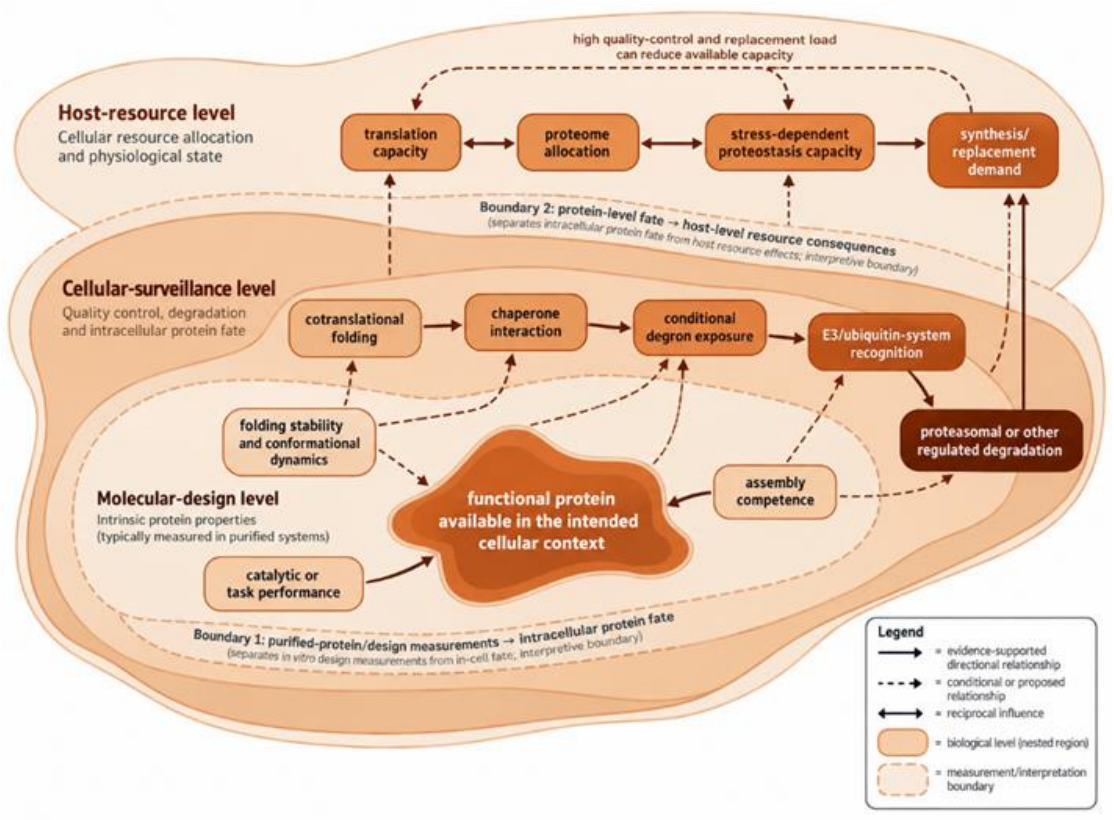


Figure 1. Conditional determinants of engineered-protein functional persistence across molecular, quality-control, and cellular-resource scales

Results and Discussion

Design Trade-offs, Testable Predictions, and Transferability

The central implication is that engineering for persistence requires experiments capable of discriminating among competing mechanisms. If a variant disappears rapidly from cells because partial unfolding exposes a degradation determinant, improving conformational stability should reduce relevant quality-control engagement and extend functional availability. The transketolase results show that compensatory stabilization can sometimes be achieved without sacrificing engineered activity (Yu & Dalby, 2018), but the prediction must be tested anew when the cellular degradation mechanism differs.

A second prediction concerns divergence between purified-protein stability and cellular lifetime. Large-scale folding measurements provide powerful thermodynamic information (Tsuboyama *et al*., 2023), yet degron mapping demonstrates that sequence-specific recognition can affect degradation independently of a simple global stability measure (Zhang *et al*., 2023). Cotranslational folding further creates states that may not be reproduced by mature-protein refolding assays (Wales *et al*., 2024). Orthogonal measurement is therefore necessary whenever a persistence claim crosses from purified biophysics into cellular interpretation.

A third prediction concerns resource demand. Mammalian expression studies show that synthetic constructs can impose measurable competition for cellular resources (Di Blasi *et al*., 2023). If two variants provide comparable molecular activity but differ substantially in degradation and replacement frequency, their effects on host physiology may diverge even when instantaneous activity assays appear similar. Testing this proposition requires simultaneous measurement of functional output, protein abundance or turnover, and an appropriate host-resource response rather than inference from expression level alone.

Transferability is likewise conditional. Bacterial proteome-allocation studies reveal strong organization of cellular resources (Mori *et al*., 2024), but their quantitative relationships should not be imported directly into mammalian, tissue-specific, or cell-free contexts. Similarly, stress-dependent proteostasis behavior can change the environment in which a variant is maintained (Ulfig & Jakob, 2024). What may transfer across systems is the mechanistic requirement to examine allocation, folding, degradation, and host

state; the magnitude and relative importance of each component require re-estimation.

The framework would lose value if these additional dimensions fail to improve discrimination among engineered variants. A direct falsification strategy would compare predictions derived from catalytic activity and folding stability alone with predictions that additionally incorporate cellular turnover, quality-control engagement when indicated, and host-resource effects. If the expanded measurements provide no reproducible explanatory or predictive gain across properly controlled conditions, then the broader account should not be retained merely because it is more comprehensive.

Several limitations remain. The relevant literature spans protein biophysics, degradation biology, synthetic biology, and systems physiology, and relatively few studies measure all these dimensions in the same protein and cellular system. Many degradation pathways are substrate specific, while resource-allocation models intentionally simplify molecular detail. The framework therefore organizes experimentally separable mechanisms rather than claiming a validated universal optimization algorithm. Its principal use is to identify where a seemingly successful protein design can fail when moved from an isolated molecular assay into a living, resource-limited, quality-controlled environment.

Conclusion

Engineering proteins for functional persistence requires a broader criterion than maximizing catalytic activity or folding stability. Useful function must survive the biological processes that synthesize, fold, assemble, monitor, degrade, and replace the protein, while remaining compatible with the resource capacity of the host. Activity–stability conflicts are real in some proteins but are not universal, and stability alone cannot predict intracellular lifetime when degradation depends on accessible recognition signals, assembly state, processing, or cellular quality-control capacity. The resulting design problem is therefore conditional and multiscale. Protein variants should be compared using measurements matched to the intended biological context, with molecular performance, conformational behavior, turnover, and host burden treated as related but non-interchangeable dimensions.

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