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Protein stability: computation, sequence statistics, and new experimental methods

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Calculating protein stability and predicting stabilizing mutations remain exceedingly difficult tasks, largely due to the inadequacy of potential functions, the difficulty of modeling entropy and the unfolded state, and challenges of sampling, particularly of backbone conformations. Yet, computational design has produced some remarkably stable proteins in recent years, apparently owing to near ideality in structure and sequence features. With caveats, computational prediction of stability can be used to guide mutation, and mutations derived from consensus sequence analysis, especially improved by recent co-variation filters, are very likely to stabilize without sacrificing function. The combination of computational and statistical approaches with library approaches, including new technologies such as deep sequencing and high throughput stability measurements, point to a very exciting near term future for stability engineering, even with difficult computational issues remaining.

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There is considerable evidence that some proteins, or domains, function in an intrinsically disordered state [1], but the majority of proteins act from a highly ordered folded state [2]. Mutational and combinatorial studies have shown us that these highly ordered states are not common in 'sequence space' [3,4], meaning that most polypeptide sequences are not well folded like natural proteins. In fact, because the folded state of proteins is only 5-15 kcal mol^{-1} more stable than the unfolded state, even a single mutation can significantly destabilize or unfold a protein. Although most proteins from mesophiles have melting temperatures far below those of corresponding proteins from thermophiles — which is to say, their folds can usually be stabilized — the overwhelming majority of mutations to natural proteins are neutral to unfavorable [5,6].

At a minimum, this is an inconvenience for protein scientists. The instability of natural proteins or their variants makes them difficult to purify, handle, and study. A well-meaning mutation to probe the function of some residue must always be analyzed in light of the high likelihood of unfavorable consequences on the folding or stability of the protein. Protein stability and instability also underlie biology and disease. Many mutations may reduce function or promote disease simply through destabilization, such as many of the mutations of tumor suppressors like p53 [7] or mutations of SOD1 that may be related to ALS [8]. We continue to find new uses for proteins as therapeutics due to their exquisite specificities, but their uses in the clinic are significantly limited by difficulty in handling, poor storage stability, and aggregation [9].

The solution sounds simple: stabilize the protein. Stabilizing mutations may be rare, but a great many have been found. The problem has been attacked from virtually every imaginable angle of random mutagenesis, rational design, bioinformatics, and computational design. The sheer number of approaches highlights the objective reality: we are not that good at it. This is especially vexing because the key forces that underlie protein stability are fairly well understood, such as the burial and tight packing of hydrophobic residues, the ejection of ordered solvent, and the formation of hydrogen bonds and other electrostatic interactions, conformational entropy, and bond strain (such as backbone angle strain) [10–12]. The dominance of core packing in protein stability [13], which encompasses several of these parameters at once, simplifies the problem. More subtle effects, such as the effect of burial of charged residues [14] and the role of surface electrostatics [15], are much better understood in recent years.

But the challenges remain numerous. For one thing, some of these factors are a lot easier to calculate than others [16]. We are very good at calculating geometric parameters, for example to maximize hydrophobic surface burial or minimize bond strain. But electrostatics calculations are greatly hampered by how to treat solvation, in particular due to the challenge of polarizability. There is no way to compute entropy directly from the force field itself, and so conformational effects are beholden to long simulations and accurate sampling, which are both challenging. Matthews's work on T4 lysozyme taught us that proteins

respond to mutations more by subtle movements of the backbone than adopting unfavorable side chain rotamers [17], but it is much more difficult to explore non-discretized backbone conformational space. Moreover, sometimes subtle changes to proteins can cause them to settle into very different regions of conformational space, as seen in topological changes from seemingly conservative mutations of the a protein's hydrophobic core [18]. The gain in solvent entropy that largely underlies the hydrophobic effect is not explicitly included in these calculations. And finally, the ΔG of folding is the free energy difference between the folded state and the unfolded state, but our knowledge of how to model the unfolded state is so scant that we generally do not. It is also very difficult to model the effects of misfolding, or to account for alternative conformations.

Despite these daunting challenges, progress has been made in engineering highly stabilized proteins in recent years. Here I will briefly examine some important advances and ongoing challenges in computational and statistical stabilization of proteins.

Rosetta and other computational design

There have been numerous contributors to the modern state of computational protein design [19], and several successful implementations of design programs, but none has had as broad an impact as Rosetta [20], emanating from David Baker's lab (with considerable development by many collaborators). The Rosetta modeling and design suite of applications has produced remarkable successes, from stable folds not seen in nature [21], to *de novo* design of enzymes for reactions like the Kemp elimination [22], the retro-aldol reaction [23], and the Diels-Alder reaction [24]. Two recent papers have focused on the use of Rosetta to design folds, and in particular the effects of designing 'ideal' versions of particular structures. The results have included some of the most stable proteins observed or designed to date.

Koga *et al.* focused on the design of proteins with steep folding funnels arising out of assembly of structurally optimized elements containing only local interactions (that is, are close in primary structure), and then assembly of these idealized elements in a way that strongly favors a single tertiary structure [25••]. Fundamental rules for ββ, βα and αβ elements were used to discover emergent rules for larger units, like ββα, and then assembled into folds such as the ferredoxin-like βαββαβ. Genes for a total of 54 designs for 5 folds were synthesized, of which 45 expressed and were soluble, 32 had expected CD spectra, and 25 had a T_m greater than 95 °C. No single reason emerges for the high stability of these variants, and the use of some factors outside of the idealized elements such as selection of large hydrophobic residues to strongly favor burial is likely important, but the inference is that the ideality of the elements cumulatively favors folding. That is, getting all the details right, not a single magic bullet, best explains the successful results.

Huang *et al.* recently used a parametric approach along with Rosetta design tools to control the oligomeric state and handedness of designed helical bundles [26••]. An antiparallel 3-helix bundle had a denaturation midpoint of 7 M GdnHCl at 80 °C, corresponding to a ΔG of folding of over 60 kcal mol^{-1} at 25 °C. A designed five-helix bundle did not melt at 95 °C in PBS; a four-helix bundle did not melt at 95 °C in 8 M GdnHCl. Baker and colleagues note that the 3-helix and 4-helix proteins are extreme stability outliers in the ProTherm database [27], and attribute the stability to ideal side chain complementarity as well as minimal backbone strain.

Despite these truly remarkable results, it is sobering to note that several related designed proteins that appeared every bit as ideal as the successes, failed to express, or be soluble, or have well-dispersed NMR spectra. The exact nature of the differences among these successes and failures is difficult to discern because it likely comes from multiple inconspicuous nonidealities. To the point, Murphy *et al.* redesigned the core of CheA, a four helix bundle, using four different approaches for backbone flexibility and core repacking [28]. Two of the designs were very successful, with $T_m > 140$ °C and a ΔG of folding of 15–16 kcal mol^{-1}. But one did not express, and one had wild-type like stability. Kuhlman and colleagues attempted to discern the differences among the designs by examining Ramachandran and side chain torsion angle preferences and hydrophobic burial, but no simple answer emerged.

The very high stability achievable in idealized proteins compared to what is observed in natural proteins, even from thermophiles, begs the question of whether this kind of ideality is incompatible with function. My guess is that it is not; natural, active proteins seem more likely to have only adequate stability because that is all natural selection demands of them. This question seems akin to whether thermostable proteins can be active at low temperature, which Arnold and colleagues convincingly showed to be possible through directed evolution [29]. But it remains to be seen if dynamic features, binding sites, hydrophobic patches and other features of functional proteins are actually compatible with these idealized frameworks, or if functionalization will necessarily degrade their ideality.

Similarly, despite remarkable successes in *de novo* enzyme design, the activity levels of the designed enzymes have called for directed evolution for improvements in most cases. In at least one case, a combination of statistically-derived consensus mutations (see below) and directed evolution was needed to improve the best computationally designed Kemp eliminase, KE59, that