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Stability effects of mutations and protein evolvability

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The past several years have seen novel insights at the interface of protein biophysics and evolution. The accepted paradigm that proteins can tolerate nearly any amino acid substitution has been replaced by the view that the deleterious effects of mutations, and especially their tendency to undermine the thermodynamic and kinetic stability of protein, is a major constraint on protein evolvability—the ability of proteins to acquire changes in sequence and function. We summarize recent findings regarding how mutations affect protein stability, and how stability affects protein evolution. We describe ways of predicting and analyzing stability effects of mutations, and mechanisms that buffer or compensate for these destabilizing effects and thereby promote protein evolvability, in nature and in the laboratory.

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Introduction

Until recently, the prevailing view among protein scientists has been that most positions can tolerate drastic sequence changes while the protein retains its ability to fold (configurational stability) and function [1^{••},2[•]]. An exception was the hypothesis that mutations that endow enzymes new functions trade off with stability and hence introduce the need for compensatory mutations [3^{••}]. However, even this hypothesis assumed that stability is associated with activity changes, rather than a global, general constraint. Two papers in 2005 highlighted the overall importance of stability effects of mutations to protein evolution [1^{••},4[•]]. These were followed by a series of works that explored this link further and led to a new interface between protein biophysics and molecular evolution.

Mutations are an essential ‘raw material’ of evolution. However, selection to maintain the existing structure

and function (negative, purifying selection) purges many, if not most protein mutations, thus reducing the potential for future adaptations. Thus, only a fraction of all possible mutations will be fixed under positive selection to adopt a new function. Neutral mutations can also stochastically fix owing to random, or ‘neutral drift’, in small populations. At the organismal levels, the effects of mutations on fitness (reproduction rates) are complex and rarely correlate with the properties of one gene/protein. Redundancy, backup, and robustness, at different levels mask the effects of many mutations [5]. Indeed, understanding and predicting the effects of mutations on the organismal level is a major challenge of evolutionary biology [1^{••},6,7]. However, by the most simplistic model, ‘protein fitness’ (W) can be defined, for example, as the flux of an enzyme catalyzed reaction, and W then correlates with the viability or fitness of the organism in which this enzyme functions (for examples see [2[•],4[•],8,9]). The flux is proportional to the concentration of functional protein ($[E]_0$) and its function (k_{cat} , or k_{cat}/K_M for an enzyme, or K_d in a case of a receptor; such factors are unified here under the symbol f):

$$W = [E]_0 \cdot f \quad (1)$$

The concentration of functional protein ($[E]_0$) relates to protein stability. As indicated by the analysis of pathogenic mutations, the deleterious effects of $\geq 80\%$ of mutations stem from their effects on stability and folding [10^{••}]. Mutations that are destabilizing beyond a certain level (or $\Delta\Delta G$ value) cause protein disfunctionalization by reducing the levels of soluble, function proteins [10^{••},11,12]. Experimental measurements in several different proteins indicate that the likelihood of mutation to be deleterious is in the order of 33–40% [2[•],7,13] (36%, on average). Hence, as mutations accumulate, protein fitness declines exponentially [2[•]]:

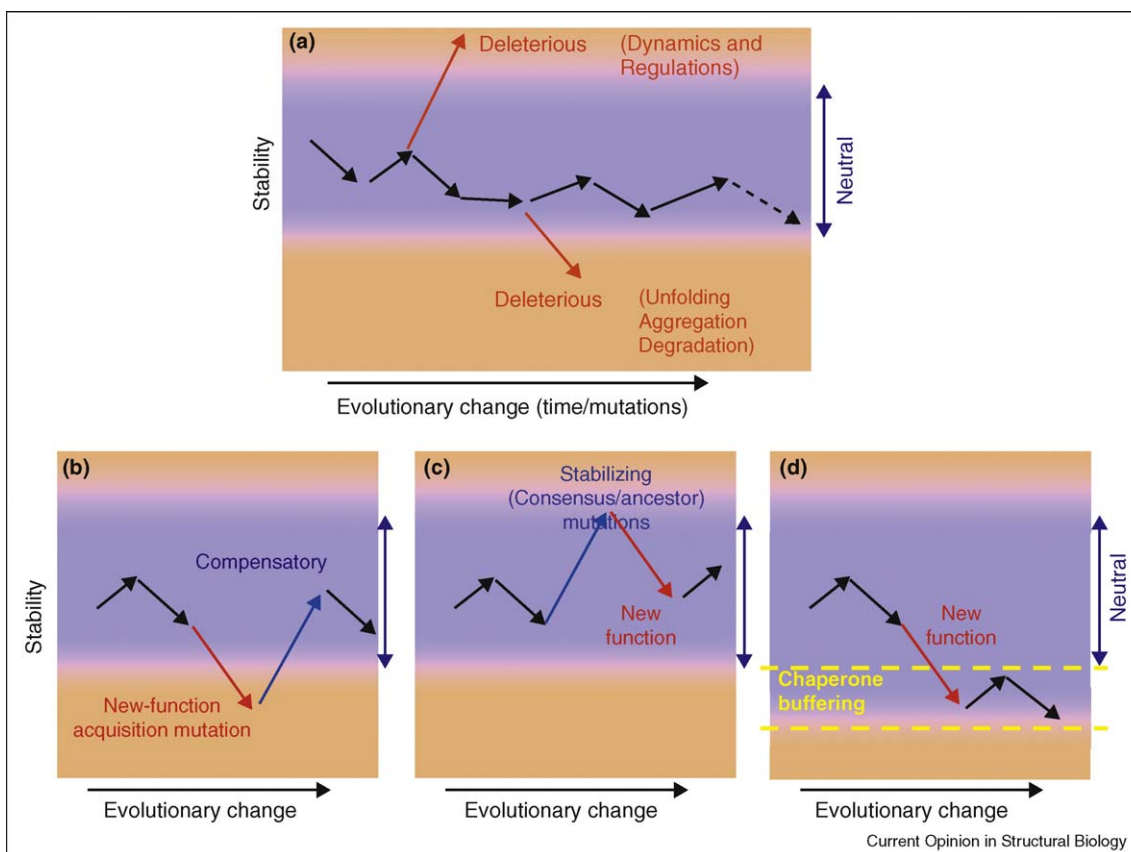
$$W \approx e^{-0.36n} \quad (2)$$

(where n is the average number of mutations) or even more than exponentially (see section on ‘epistatic effects’). So by the time an average protein accumulates, on average, five mutations, its fitness will decline to $< 20\%$. Thus, although the initial stability of a protein can buffer some of the destabilizing effects of mutations (Figure 1a), stability appears to comprise the main factor (although clearly not the only one [6]) that dictates the rate of protein evolution [1^{••},4[•]], and possibly of whole organisms [14^{••},15,16], in particular, but not only, in relation to the acquisition of new functions.

Here, we discuss new developments regarding the prediction and analysis of stability effects of mutations. We

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Figure 1



Evolutionary changes and the accompanying changes in protein stability and fitness (plotted in the style of Reference [1**]). **(a)** Proteins evolve, that is, acquire mutations, within a 'neutral' range of stability that maintains their fitness (levels of soluble, functional enzyme, for example, blue zone). Non-synonymous mutations are represented as arrows. Mutations that do not alter the stability beyond the neutral zone can be accommodated as neutral (black arrows). However, about a third of mutations are destabilizing (red arrows) and may reduce stability to an extent that comprises fitness (lower orange zone). Such mutations are usually purged by purifying or negative selection. Rarely, a mutation may increase stability beyond the neutral zone, and might be purged according to their deleterious effects on protein dynamics or regulation. **(b)** New-function mutations tend to be destabilizing (red arrows), and compensatory stabilizing mutations (blue arrow) that are subsequently acquired play an important role in restoring stability and fitness (uphill divergence). **(c)** Downhill divergence: incorporation of compensatory mutations (e.g. consensus/ancestor mutations) before, or in parallel with, the divergence of new function can greatly enhance the rate of evolution, or evolvability. **(d)** Buffering the destabilizing mutations by chaperones also increases evolvability, by extending the neutral zone of stability.

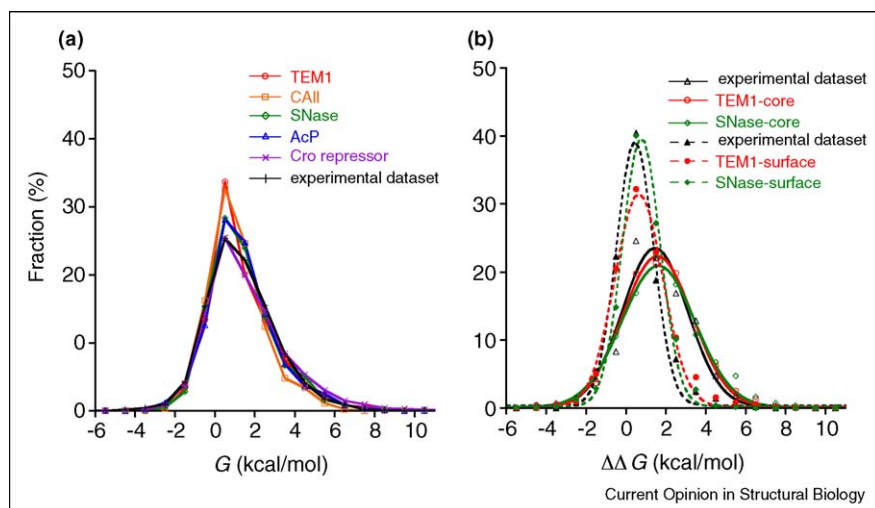
then describe how stability affects protein evolution, under neutral and adaptive scenarios. We also point out mechanisms that mediate tolerance, or robustness to mutations, and thereby affect the evolvability of proteins, both in nature and in the laboratory.

Predicting the stability effects of mutations

The definition of stability (ΔG) as used in various evolutionary models [1**,2*,4*], and this text is no exception, is vague. Thermodynamic stability (ΔG_{U-N} : the free energy difference between the unfolded and native state) is a reasonable measure only for relatively small proteins. It certainly does not reflect protein stability within cellular environments. Kinetic stability that relates to the energy levels of folding intermediates between U and N states, and/or mis-folded forms, and whether these can

lead to aggregation or degradation, is also a crucial factor, in particular in complex, slow folding proteins [17]. Experimental datasets usually relate to changes in thermodynamic stability of mutations ($\Delta\Delta G_{U-N}$ values), and are available for only a small set of proteins. Recent advances in computation enable the prediction of $\Delta\Delta G$ values of mutations in a wide range of proteins. Some prediction methods are based on sequence [18–20], others on 3D structures [21–24]. The combination of both has also been explored [19,25,26*,27]. The predictions largely relate to the effects of mutations on the native state, and do not address effects on folding intermediates. Although the effects on folding *in vivo* may largely overlap with thermodynamic stability effects [28,29], predictions of kinetic stability effects would be highly valuable. Overall, more accurate and realistic predictions of the effects of

Figure 2



The universal distribution of stability effects of mutations [31]. $\Delta\Delta G$ values are presented in histograms using 1 kcal/mol bins. **(a)** The predicted $\Delta\Delta G$ values by FoldX for all possible mutations in many proteins (shown are few characteristic examples), and the experimentally measured $\Delta\Delta G$ values for 1285 mutations, all give similar asymmetric distributions with larger destabilizing shoulders ($\Delta\Delta G > 0$). **(b)** Separated $\Delta\Delta G$ distributions of core and surface residues. Residues were divided according to their accessible surface area (ASA) values, and the $\Delta\Delta G$ values for all possible mutations were arranged in histograms and fitted to a single Gaussian.

mutations that may relate to protein levels *in vivo* remain a challenge [30].

Although prediction accuracy is currently limited, and the methods are biased toward the training sets and thermodynamic stability, they still enable to examine general trends related to the way $\Delta\Delta G$ values for mutations are distributed (see note added in proof). For example, FoldX has been applied to predict the $\Delta\Delta G$ distributions for all possible mutations in a set of proteins [31]. It was found that the distributions of $\Delta\Delta G$ values for mesophilic proteins from various organisms excluding viruses are strikingly similar despite a range of sizes (50–330 amino acids) and folds (Figure 2). The distributions are asymmetrical, and correlate with an overlay of two Gaussian functions: Surface residues gave one narrow Gaussian with a low destabilizing mean ($\Delta\Delta G \sim 0.6$ kcal/mol), whereas core residues gave a wider distribution with a stronger destabilizing mean (~ 1.4 kcal/mol). Overall, about 50% of mutations are destabilizing ($\Delta\Delta G > 1$ kcal/mol), and >15% of mutations are highly destabilizing (>3 kcal/mol). About 5% of mutations show stabilizing values ($\Delta\Delta G < -1$ kcal/mol) in both the experimental and computed $\Delta\Delta G$ distributions. However, part of this already small fraction relates to mutations in catalytic residues that tend to increase stability [32,33] but result in inactive protein. The computed distributions show remarkable resemblance to distributions of a large dataset of experimental $\Delta\Delta G$ values [14^{••},31], and those computed for model lattice proteins [4[•],34]. The $\Delta\Delta G$ distributions indicate that the flux of destabilizing mutations is much

larger (>20-fold) than those of stabilizing mutations, and may explain why throughout evolution, the stability of proteins remained marginally low (ΔG_{U-N} 3–10 kcal/mol), probably at the minimal levels needed to maintain functional protein levels within living cells [14^{••},15]. Other driving forces that may have prevented the evolution of higher stability might be the hampering of protein regulation or activity by excess stability [1^{••}].

The relationships between stability and protein fitness

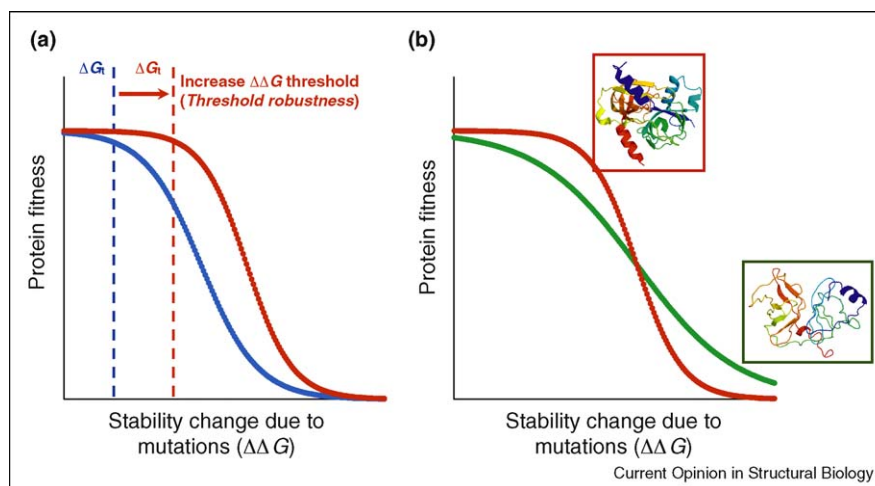
How is protein fitness, or levels of activity conferred by a given protein ($[E]_0/f$), affected by mutations? Proteins possess a certain margin of stability (ΔG) that ensures a sufficiently high level of folded, functional protein. $[E]_0$ is therefore proportional to the fraction of folded protein that in turn, is dictated by ΔG :

$$W \propto [E]_0 = 1 - 1/(e^{-\Delta G/RT} + 1) \quad (3)$$

Eq. (3) is sigmoidal with a mid point (fraction of folded protein = 0.5) at $\Delta G = 0$. Even modest stability, -3 kcal/mol, gives >99% of folded molecules, and many proteins exhibit ΔG_{U-N} values within the range of several kcal/mol. However, various partially folded and misfolded states lead to aggregation or degradation. Since the latter processes are irreversible, even a minuscule fraction of these states will drag the equilibrium away from the folded, functional state. Thus, ΔG values lower than -3 kcal/mol might be required, in addition to the prevention of problematic folding intermediates [17].

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Figure 3



The threshold and gradient robustness models [62**]. **(a) Threshold robustness:** The sigmoidal nature of Eq. (3) indicates that protein fitness (or functional expression level $[E]_0$) remain largely unaffected as long as protein stability (ΔG) remains above a certain threshold (ΔG_t ; blue line). Increasing the stability threshold results in higher tolerance to mutations, or neutrality (red line). However, accumulating mutations do reduce stability, and once the margin is exhausted, mutations result in rapid loss of fitness. Further, owing to the structural features that mediate higher stability, and the inherent relationship between robustness and epistasis [2*], higher thresholds lead to higher average $\Delta\Delta G$ values per mutation, and steeper fitness declines (red line). **(b) Gradient robustness:** Loosely packed and partially disordered proteins may be characterized by very low initial stability (low ΔG_t), but also by lower destabilizing effects of mutations, resulting in the mode represented by the green line. Gradient robustness is associated with loosely packed structures, and may have emerged under high mutational loads, for example, in RNA viral proteins.

Because most mutations affect stability and not function, protein levels, or fitness, could be correlated with the stability effects of mutations by a simple model, dubbed the threshold model. The sigmoidal nature of (3) indicates that $[E]_0$, and thus protein fitness, remain unchanged as long as stability remains above a certain threshold (ΔG_t ; Figure 3a). However, as more mutations accumulate, ΔG becomes lower than ΔG_t , and $[E]_0$ thereby decreases [2*]. The correlation between the initial thermodynamic stability of a protein and its fitness decline in response to mutations was experimentally validated using a distribution of $\Delta\Delta G$ values similar to the one described in Figure 2 [4*]. The threshold model seems to be valid for many proteins, and the analysis of mutations (SNPs) leading to monogenic diseases revealed that the relationship between the destabilizing effects of mutations and the severity of disease shows sigmoidal relationships [10**,12]. The relationship between the soluble expression level of different HypF-N and p53 variants in *E. coli*, and ΔG_{U-N} of these variants showed the same tendency [28,35]. Interestingly, the stability margin that could be compromised with no immediate fitness effect (ΔG_t) seems to be 1–3 kcal/mol for most proteins, almost regardless of their initial stability (ΔG_{U-N}) [2*,10**,12,28,35,36**]. It appears that stability losses of little kcal/mol result in a sufficiently large fraction of partially folded, and/or mis-folded species that lead to significant loss of protein levels via irreversible aggregation or degradation.

The threshold model and epistatic effects

Interactions between mutations are being studied by a number of disciplines, although the nomenclature differs. Geneticists and evolutionists term such interactions as *epistasis*, while protein biophysicists use the term *non-additivity* (e.g. in double mutant cycles). By the threshold model, *negative epistasis*, whereby the combined effects of mutations are larger than expected from their individual effects, is expected: the first mutations have relatively small effects on fitness, but as more mutations accumulate, fitness rapidly declines. This prediction contradicts double mutant cycles showing that the stability effects of mutations ($\Delta\Delta G$ values) are largely additive [37], or less-than-additive in cases of interacting residues [38], corresponding with the effect of *no*, or even *positive epistasis*. How can this dichotomy be reconciled? The threshold induces a delay in fitness decline in the face of mutations as the loss of stability induced by mutations is buffered by the excess of stability. However, the accumulation of more mutations reduces the stability beyond the allowed margin, leading to fitness decline in parallel with ΔG changes. Negative epistasis was experimentally observed for the decline of fitness upon mutation accumulation [2*]. Further, a larger excess of stability obviously enables a larger number of destabilizing mutations to be accommodated while retaining protein fitness. For example, a stabilized variant of TEM-1-lactamase (TEM-1-Met182Thr) showed higher tolerance to mutations than the wild-type enzyme [4*]. However, this higher

neutrality, or robustness, resulted in a higher degree of negative epistasis [2*].

In adaptive evolution (the acquisition of new protein functions), more complicated epistatic effects such as *sign epistasis*, can be observed [39]. For example, a severe reduction in $[E]_0$ could cancel out improvements in function (f) [36**], and stabilizing mutations increase fitness only if stability is close to the threshold but not above it (see section on compensatory mutations). These effects dictate the trajectories of protein evolution [40,41**], and make evolution a far more complex and interesting phenomenon.

Stability margins and environmental robustness

The higher stability is above ΔG_t (higher negative ΔG values), the higher is the protein's tolerance to mutations, or neutrality (genetic robustness is another oft-used term) [4*,42,43,44]. However, if higher ΔG values are not favored by natural selection because their sacrifice has no immediate effect on protein fitness (as indicated by the negative epistasis effects), how did they emerge in the first instance, and how were they maintained under a constant input of destabilizing mutations? One explanation is that genetic robustness evolved in response to environmental pressures. Fluctuations in temperature, salinity, redox potential, and similar factors, may have led the evolution of higher stability [5,45]. Another key factor arises from errors occurring during protein synthesis (phenotypic mutations) [46,47**,48*]. Phenotypic mutations are about 10^5 more frequent than genetic mutations, but are non-heritable, and are therefore classified as environmental perturbations. Because of their very high rates, it has been suggested that transcriptional and translational errors can severely limit the rate of protein evolution [46,47**]. It could therefore be that stability thresholds evolved to buffer the effects of phenotypic mutations and other environmental factors, rather than genetic mutations. Indeed, an experiment based on error-prone transcription of TEM-1-lactamase indicated that an increase of ~20-fold in the frequency of phenotypic mutations promoted the rapid evolution of more stable enzyme variants [48*]. The evolved variants exhibited higher fitness under an increased rate of phenotypic mutations, but showed no advantage over wild-type TEM-1 under normal transcription. These variants carried previously identified stabilizing mutations such as Met182Thr that also endow TEM-1 with increased tolerance to genetic mutations [4*,49**]. Thus, although phenotypic mutations are not individually subjected to inheritance and natural selection, they collectively exert a direct and immediate effect on protein fitness. Protein synthesis errors, and other environmental perturbations therefore play an important role in shaping protein stability thresholds, and in increasing protein robustness to genetic mutations.

Stability and new functions

The destabilizing effects of mutations also limit the acquisition of new protein functions. Following the observation that mutations that improved the catalytic efficiency of TEM-1-lactamase toward third generation antibiotics were destabilizing, it has been suggested that the evolution of new-function and protein stability trade-off [3**]. Conversely, following changes in function, compensatory mutations that restore stability are often observed [3**] (see section below). However, this notion has not been systematically explored, especially in view of the notion that, as indicated by $\Delta\Delta G$ distributions, mutations are, on average, destabilizing. Recently, FoldX predictions of 246 new-function mutations observed in the directed evolution of 22 different enzymes indicated that although new-function mutations are destabilizing (average $\Delta\Delta G = 0.9$ kcal/mol), they are not more destabilizing than the average mutation. However, new function, or adaptive mutations were found to be more destabilizing, and tend to occur at more buried residues, than neutral mutations that characterize non-adaptive, or neutral mutational drifts [50]. Thus, the accumulation of multiple new-function mutations is indeed likely to bring protein stability below ΔG_t , decrease $[E]_0$, and possibly decrease protein fitness, even if these mutations improve function. Indeed, stabilized variants of P450 and TEM-1 showed higher evolvability through their ability to accommodate a larger variety of new-function mutations without loss of enzyme levels [49**,51**].

Compensatory stabilizing mutations—uphill divergence

Compensatory mutations, also called global suppressors owing to their ability to suppress the deleterious effects of a wide range of mutations, have been observed in natural [3**,41**] and *in vitro* evolution [50], and therefore play a key role in evolutionary dynamics [52]. As most deleterious mutations are destabilizing, most compensatory mutations appear to be stabilizing. For example, in the evolution of resistance to the antibiotic cefotaxime in TEM-1 in the clinic, and by laboratory evolution, active-site mutations that endowed the new function were followed by the stabilizing compensatory mutation Met182Thr [3**]. The need for compensatory mutations that restore the stability margin of the evolving protein severely restricts the evolutionary trajectory and slows down the rate of adaptation [3**,40]. Such trajectories can therefore be dubbed uphill divergence (Figure 1b). Notably, via these uphill trajectories, function-altering mutations that are destabilizing beyond ΔG_t cannot fixate, unless they are buffered by chaperones as discussed below.

Stabilizing ancestor/consensus mutations and downhill divergence

An obvious way of expediting the rate of evolution is to have a starting point with higher ΔG_t and let it diverge

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downhill by exploiting this excess of stability (Figure 1c). In the laboratory, downhill divergence was demonstrated with a variant of P450 that was evolved first for higher thermostability, and demonstrating its higher evolvability through its ability to accept destabilizing new-function mutations that the wild-type enzyme would not [51^{••}]. There are straightforward ways of improving thermodynamic stability [53–55]. In fact, the use of thermophilic proteins as starting points for protein engineering has long been a favorite strategy for protein engineering. However, not every stabilizing mutation acts as global suppressor, and the engineering of a highly thermostable starting point comprises an extra step. Further, a large excess of stability may reduce evolvability, for example by rigidifying the protein and restricting alternative conformations that mediate the new function. A more attractive way of downhill divergence would be to incorporate compensatory mutations into the library that is selected for the new-function. However, this strategy depends on being able to predict stabilizing compensatory mutations.

A hint regarding the nature of compensatory mutations came from a ‘neutral drift’ experiment, that is, repeated rounds of mutagenesis and purifying selection to maintain the enzyme’s function. Under the high mutational load applied in this experiment, the enrichment of several different mutations was observed. Five of those that showed the highest enrichment were found to increase stability (either kinetic or thermodynamic) and act as global suppressors for a whole range of destabilizing mutations. The enriched mutations had a common feature: they brought the sequence of TEM-1 closer to its family consensus, and/or its ancestor [49^{••}]. Mutations in conserved residues usually cause large stability decreases. Conversely, reverting residues that deviate from the consensus amino acid can increase stability [56], and is often used for engineering higher stability [57]. The predicted ancestor of a given family largely overlaps the consensus derived from aligning sequence homologs (that anyway tend to be members of the same family). But there are fundamental differences. Resurrected ancestors have been shown to exhibit higher stability [58], and at some positions, the ancestor residue confers higher stability than the consensus [59[•]]. Further, the consensus is an indistinct statistical feature that strongly depends on which sequences are included, whereas inferred ancestors are clearly defined per a given node and phylogeny. Overall, it appears that ancestral inference, and/or consensus analysis, comprise powerful ways of predicting compensatory mutations that can be ‘spiked’ into libraries and facilitate the evolution of stable proteins [60], and of new functions via downhill divergence. Putting aside the biases underlining ancestral predictions [61], it can also be argued that the ancestors may have exhibited higher stability and evolvability, and thus diverged downhill to yield the marginally stable proteins we see today.

Gradient robustness

High stability is associated with well-packed, highly compact structures in which residues are extensively interconnected. In such structures, however, mutations lead to high stability losses due to the loss of a relatively high number of strong contacts. Indeed, the higher the stability threshold, the higher is the initial robustness to mutations, and the higher the negative epistatic effect, namely, the steep fitness decline that follows [2[•]]. However, besides threshold robustness, a different mode of robustness seems to exist—gradient robustness [62^{••}]. Gradient robustness correlates with little, or no initial stability margin, but with a milder slope whereby the average stability loss per mutation is reduced (Figure 3b). In poorly packed, or partially disordered proteins where, on average, residue makes relatively few contacts, mutations are expected to exhibit lower $\Delta\Delta G$ values. Viral proteins, and RNA viral proteins, in particular, seem to comprise an example for this mode of robustness whereby, those who have little interactions, have little to lose. Indeed, RNA viruses are exposed to high mutation rates (10^{-3} to 10^{-5}) compared to other organisms ($\sim 10^{-9}$). Consequently, their proteins seem to show particularly low stability [16], and an overall tendency to be loosely packed and partially disordered, and their distributions of $\Delta\Delta G$ of mutations appear to be less destabilizing [62^{••}].

Chaperones buffering and protein evolvability

What other means are there to overcome stability constraints and accelerate protein evolution? Chaperones, also known as heat shock proteins, assist the folding of other proteins, and buffer various effects of mutations [63]. However, the buffering mechanisms vary from one organism, and chaperone, to another, and are under dispute. What fraction of mutations can be buffered by chaperones, to what extent (or $\Delta\Delta G$ values), and what would be the impact on evolutionary rates, has also been unknown. We recently established an experimental system aimed at systematically measuring the buffering capacity of the bacterial chaperonin GroEL/ES. Neutral drifts (i.e. mutation-accumulation experiments where selection is applied to maintain the enzyme’s expression levels and function) were performed under overexpression of GroEL/ES, and the drifting mutants were tested for the level of buffering and mutational content [36^{••}]. The results indicate that GroEL/ES overexpression doubled the number of mutations that could accumulate as neutral, and greatly increased their variability. More mutations in protein cores were observed, and mutations with much higher destabilizing effects: >3.5 kcal/mol higher $\Delta\Delta G$ values, on average, *versus* ~ 1 kcal/mol in the absence of GroEL/ES. Thus, GroEL/ES seems to significantly extend the zone of neutrality and enable many more destabilizing mutations to accumulate (Figure 1d). In accordance, the acquisition of a new enzymatic specificity was also expedited under GroEL/

ES overexpression: ≥ 2 -fold more improved variants were observed, and the improvements in the evolving activity were ≥ 10 -fold higher than in variants evolved without GroEL/ES. A typical case was observed whereby variants of an enzyme selected under GroEL/ES overexpression carried a mutation that induced large improvements in the newly evolving activity, but was severely destabilizing and led to aggregation (Phe306Leu, in an enzyme dubbed PTE). Variants selected without GroEL/ES overexpression carried another mutation (Phe306Cys) that showed milder improvements and no destabilizing effects. In fact, in the absence of chaperonin overexpression, although carrying mutations that improved function k_{cat}/K_M for the evolved ester substrate), many variants showed no improved enzymatic activity (fitness, W), or even decreased activity, due to much reduced enzyme levels ($[E]_0$) [36**].

Concluding remarks

Mutations, and mutations that alter protein function (new-function mutations), in particular, are generally destabilizing, and can reduce protein and organismal fitness. The destabilizing effects of mutations comprise a major constraint for protein evolution, be it the accumulation of neutral, or adaptive variation. Two models by which protein fitness declines in response to mutations are currently described: (i) threshold robustness (an excess of stability that buffers the destabilizing effects of mutations) associated with well-packed, highly stable proteins; (ii) gradient robustness—reduced $\Delta\Delta G$ values of mutations due to loosely packed, partially disordered structures, which might be common in proteins exposed to high mutational loads (e.g. RNA viral proteins). Whereas threshold robustness has been extensively studied, gradient robustness needs to be further examined. In general, more comprehensive understanding of how mutations affect protein fitness within living cells is needed, including their combined effects on function, thermodynamic and kinetic stability, and clearance through aggregation and degradation. Mechanisms for compensating and buffering the destabilizing effects of mutations also play a crucial role in evolution, both in the laboratory and in nature. These include compensatory ancestor/consensus mutations and other stabilizing mutations, chaperones, and additional mechanisms that are likely to be discovered in the future.

Other questions remain open, such as what role did compensatory/stabilizing mutation and chaperone buffering play in natural evolution? Do certain traits trade off, such as high stability and evolvability, or neutrality (minimal effects of mutations) and evolvability (the ability of few mutations to induce large changes of function and structure)? Can these trade-offs be circumvented, and how? Better understanding of these issues can improve our ability to engineer and evolve new proteins in the laboratory [64], and explain how, in nature, from few primordial

progenitors, such a magnificent variety of proteins with different structures and functions had diverged (for further discussion of these issues see Reference [65]).

Note added in proof

A recent paper provides a long-needed objective comparison of six commonly used stability prediction algorithms for mutations with the experimental dataset (Potapov V, Cohen M, Schreiber G: Assessing computational methods for predicting protein stability upon mutation: good on average but not in the details. *Protein Eng Des Sel* 22:553-60. The results indicate a considerable and similar level of inaccuracy for all these algorithms in predicting $\Delta\Delta G$ values for individual mutations, and combining them did not significantly improve the prediction accuracy for individual mutations. In contrast, the overall trends predicted by these methods for large sets of mutations are remarkably close and accurate.

Acknowledgements

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