Biophysics of protein evolution and evolutionary protein biophysics

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Correction to ‘Biophysics of protein evolution and evolutionary protein biophysics’

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J. R. Soc. Interface 11, 20140419 (2014; Published online 27 August 2014) (doi:10.1098/rsif.2014.0419)

The following typographical errors should be corrected:

Page 11, right column, line 39—‘threonine-to-arginine mutation’ should read ‘threonine-to-alanine mutation’.

Page 34, ref. 407—the volume and page numbers should be ‘158, 927–931’.

Cite this article: Sikosek T, Chan HS. 2015
Correction to ‘Biophysics of protein evolution and evolutionary protein biophysics’. J. R. Soc.
Interface 12: 20150915.
http://dx.doi.org/10.1098/rsif.2015.0915
The study of molecular evolution at the level of protein-coding genes often entails comparing large datasets of sequences to infer their evolutionary relationships. Despite the importance of a protein’s structure and conformational dynamics to its function and thus its fitness, common phylogenetic methods embody minimal biophysical knowledge of proteins. To underscore the biophysical constraints on natural selection, we survey effects of protein mutations, highlighting the physical basis for marginal stability of natural globular proteins and how requirement for kinetic stability and avoidance of misfolding and misinteractions might have affected protein evolution. The biophysical underpinnings of these effects have been addressed by models with an explicit coarse-grained spatial representation of the polypeptide chain. Sequence–structure mappings based on such models are powerful conceptual tools that rationalize mutational robustness, evolvability, epistasis, promiscuous function performed by ‘hidden’ conformational states, resolution of adaptive conflicts and conformational switches in the evolution from one protein fold to another. Recently, protein biophysics has been applied to derive more accurate evolutionary accounts of sequence data. Methods have also been developed to exploit sequence-based evolutionary information to predict biophysical behaviours of proteins. The success of these approaches demonstrates a deep synergy between the fields of protein biophysics and protein evolution.

1. Introduction

Biological evolution uses mutations as its basic working material. Mutations occur in DNA molecules through various mechanisms. Some mutations are relatively ‘silent’ in that their effects are less appreciable, whereas others have a more prominent impact on the biological function. The most immediate effect of a mutation is the alteration of the DNA molecule itself and thus, possibly, its affinities to bind certain proteins or RNA. Given the vastness of many genomes, it was once believed that many mutations in DNA fall in regions that have no biological function. However, with increasing knowledge of the functional roles of non-coding DNA sequences, the proportion of genomes that is considered non-functional has decreased significantly [1]. Regions of the genome that do encode for a functional RNA or protein can undergo several different kinds of mutations, such as insertions, deletions and duplications of entire segments of DNA. The present review focuses primarily on the effect of point mutations (change of a single nucleotide) and will consider only proteins but not RNA, although many general principles of evolution are applicable to both classes of biomolecules. We refer to other authors for the evolution of protein structures via sequence re-arrangements such as domain-wise evolution [2–4], the fusion of small peptide fragments [5] or the ‘chimeric’ recombination of fragments that is also exploited in protein engineering [6–9].

Current study of molecular evolution can benefit from a huge amount of sequence data, but only a relatively small body of structural data. Consequently, many approaches in evolutionary studies are predominantly sequence-based. A prime example is phylogenetic inference methods based upon multiple sequence alignments. Mostly, the biophysical foundation of
these mathematical methods is provided only rudimentarily by the BLOSUM [10] or PAM [11] substitution matrices that are empirical summaries of the posterior probabilities of various amino acid substitutions. These models can roughly capture the tendency to conserve the physico-chemical properties of amino acids when they undergo mutations, like polar amino acids that are mostly substituted by other polar amino acids but less frequently by hydrophobic ones. However, such trends capture only a tiny aspect of the many biophysical implications of mutations that can be important for the biological function of proteins. For instance, they often do not even consider the local structural environment of a given amino acid residue position such as backbone conformation and hydrogen bonding pattern that might constrain evolutionary choices [12].

In this context, a number of authors from within the biophysics community have recently called for a stronger collaboration between the fields of molecular evolution and protein biophysics in order to achieve new and deeper insights into protein evolution [13–17]. At the same time, within the phylogenetics community there is a growing realization of the need for including structure information into evolutionary models [18–20]. As a first step in pursuing this direction of investigation, the effect of mutations on protein stability or binding affinities is probably the most promising example of how biophysics can contribute to a better understanding of evolution [21].

The two fields can clearly benefit from each other. For example, a common evolutionary method to identify gene positions that have undergone significant mutational changes and to quantify the degree of selection is to compute the ratio of non-synonymous to synonymous substitution rates. However, a correlation between this ratio and the solvent exposure of the site in the folded protein structure has been noted recently [22] (figure 1), suggesting that this ratio may not be purely a measure of adaptive selection but may also reflect the site’s contribution to protein stability. Based on this finding, solvent exposure of residues has been used in establishing a new neutral baseline that reflects this biophysical constraint under which natural selection must operate. Notably, this procedure has led to recognition of new amino acid positions in the influenza protein haemagglutinin that have undergone adaptation (figure 1), highlighting how biophysical/structural knowledge can improve evolutionary analysis [23,24].

Conversely, evolutionary information can also provide novel biophysical understanding of proteins. One earlier example in using such an approach that may be termed evolutionary protein biophysics is the utilization of evolutionary
data on the PDZ domain family to predict energetically coupled positions on the protein, some of which are spatially far apart [26]. Another example is the inference of structural information from protein sectors, which are co-evolving clusters of spatially proximate and physically interacting amino acids within a protein structure. A protein such as rat trypsin [27], for example, can have several such clusters that have distinct functions and evolve independently (figure 2). The existence of protein sectors raises fundamental concerns over phylogenetic methods that assume no such biophysical interactions, because those methods led to inconsistent phylogenetic trees depending on whether they are deduced from all mutations of the protein or from considering only mutations within a sector. However, with appropriate analysis, biophysical studies of proteins can use this type of evolutionary information to predict the correct fold of a protein, deduce interactions between protein monomeric units in a multiple-chain protein complex and identify hitherto unknown functional conformations [28–32].

In the following, we first discuss the basic constraints of biophysics on evolution by surveying salient biophysical consequences of protein mutations. We then outline recent advances in using biophysical concepts to shed light on experimentally observed evolutionary behaviours.

2. Biophysical consequences of protein mutations

2.1. Mutational effects on the thermodynamic stability of protein folded states

For proteins that have a globular folded native structure, the thermodynamic stability of the folded structure relative to the ensemble of unfolded conformations is determined by the balance between the interactions that favour the folded state and the conformational entropy that favours the unfolded state. The more stable a protein, the more difficult it is to unfold (denature) under high temperatures or high concentrations of denaturing chemicals. To illustrate the energetic balance governing protein stability and its kinetic implications, the conformational diversity of the unfolded state and the essentially unique native structure of a globular protein is often depicted by a funnel-like representation of the free energy landscape of the protein conformations. The folded state is situated at the bottom of the funnel whereas the unfolded state populates the top of the funnel [33–36] (see for example the top-left drawing in figure 3).

In protein evolution studies, stability is often used as a proxy for the fidelity of a protein function, because a sufficient stability of the native state is often required for function [21]. Although a protein’s function is not equivalent to its stability, experimental support exists for a positive correlation between protein functionality and native stability (e.g. [39–41]). This relationship can be seen very clearly in a recent experiment demonstrating how the evolutionary trajectory of influenza nucleoprotein is probably constrained to avoid low-stability sequences [42] (see further discussion in §3.8). In general, a mutation that decreases the stability of a protein is more probable than a mutation that does not decrease the protein’s stability to lead to the formation of other non-functional structures that would be detrimental to the protein’s original (wild-type) biological function, and in the worst case can cause serious harm to the organism.

The qualitative impact of a mutation on the folded state of a protein can often be anticipated. In globular proteins, surface residues are mostly polar and charged, while core residues have a higher tendency to be hydrophobic [43,44]. Mutations that conserve these properties are less likely to result in a large change in stability. In addition, the statistical propensities for certain amino acids to occur in a particular type of secondary structure have also been compiled and can be used to predict probable mutational effects on secondary structure (e.g. [45]). A recent comprehensive review of numerous studies of mutants occurring in natural protein families and superfamilies shows clearly that amino acid substitutions are constrained differently—i.e. their viabilities vary—in different local environments as defined by the main-chain secondary structure, solvent accessibility and hydrogen bonding [12].

Using stability as proxy for function, quantitative stability prediction is widely used to address the effect of mutations on protein function. Many tools exist to calculate an estimated ΔΔG, or change in free energy, after one or more mutations
Most of these methods focus on a static reference structure for which an energy or a score is calculated according to an empirical forcefield. To implement the mutation, the structure is computationally modified; energy is then recalculated and compared against the pre-mutation wild-type value.

DDG prediction is widely used to screen large numbers of mutations, often in combination with laboratory experiments [53–57]. The approach has also served as fitness estimators in simulation studies of protein evolution [58,59]. One obvious limitation of these DDG prediction methods is that, with few exceptions [60–62], they consider only a single ‘native’ protein conformation. Mutations can also lead to no apparent changes (neutral mutation). Some non-neutral mutations, however, can lead to new functional interactions that can then be subject to evolutionary selection. Note that the depiction of interactions between folded proteins as a ‘lock and key’ fit between specific shapes is adopted here merely to simplify the schematic representation. The perspective conveyed by the present figure does not preclude more dynamic binding mechanisms such as induced fit [37] and conformational selection [38].

Figure 3. Schematics of some of the possible effects of mutations on protein folding and interaction. The top-left cartoon of the folding landscape of a globular protein shows the correctly folded structure as the global free energy minimum, whereas a shallower minimum corresponds to a misfolded structure. Interactions of the original protein are indicated by black arrows; those of the mutant are indicated in red. Mutations can lead to misfolding and/or aggregation and/or mis-interactions. Mutations can also lead to no apparent changes (neutral mutation). Some non-neutral mutations, however, can lead to new functional interactions that can then be subject to evolutionary selection. Note that the depiction of interactions between folded proteins as a ‘lock and key’ fit between specific shapes is adopted here merely to simplify the schematic representation. The perspective conveyed by the present figure does not preclude more dynamic binding mechanisms such as induced fit [37] and conformational selection [38].

[46–52]. Most of these methods focus on a static reference structure for which an energy or a score is calculated according to an empirical forcefield. To implement the mutation, the structure is computationally modified; energy is then recalculated and compared against the pre-mutation wild-type value. ΔΔG prediction is widely used to screen large numbers of mutations, often in combination with laboratory experiments [53–57]. The approach has also served as fitness estimators in simulation studies of protein evolution [58,59].

One obvious limitation of these ΔΔG prediction methods is that, with few exceptions [60–62], they consider only a single ‘native’ protein conformation. In essence, these methods disregard mutational effects on the unfolded state and often ignore the possibility of structural adjustment of the folded state in response to the mutation. The accuracy of these methods is limited because in reality the mutational effects on protein stability are determined by the balance between the impact of the mutation on the folded and the unfolded states. Moreover, these methods do not address possible change from one folded structure to another, nor the possibility of misfolding; but conformational transition is crucial for exploring new protein functions during evolution, with polar-to-hydrophobic substitutions having a higher potential to lead to alternative folded structures [63–65]. In fact, sometimes a mutation may seem harmless in the native structure but can have dramatic effects during the folding process so that the native state might not even be formed (see §2.2).

In principle, with improved algorithms and appropriate atomistic forcefields, extensive molecular dynamics simulations that sample both the folded and unfolded conformations may provide more accurate stability predictions [66], even predictions of conformation transition [67–69]. But currently the computational cost for such simulations is very high; thus molecular dynamics cannot yet be used for large-scale mutation screenings.
2.2. Effects of mutation on folding kinetics and intermediate states

The impact of mutations on a globular protein is not limited to its folded structure. The folding process itself is altered by mutations, even when the end-point of the folding kinetics of the mutant is essentially the same folded structure as that of the original sequence. Kinetics of folding is often two-state-like for small, single-domain proteins [70] but transiently populated intermediate states are observed in many other proteins [71]. Mutations can affect folding speeds of both two-state-like and non-two-state proteins by modulating the interactions that favour the native state [72–75] or through strengthening certain non-native interactions not present in the folded structure [76,77].

Folding kinetics can be subject to natural selection. A recent estimate pointed to an overall increase in folding speed during evolution. Specifically, the folding speeds of α-proteins (folded structures consisting mostly of α-helices) have increased throughout evolution whereas those of β-proteins (folded structures consisting mostly of β-sheets) appear to have been decreasing in the last 1.5 billion years [78]. In an earlier study of conserved amino acid positions across protein families, it was concluded that conserved sites are important for function or stability, and that there has been ‘evolutionary pressure towards fast (not necessarily the fastest) folding of several proteins’ [79]. By contrast, a subsequent investigation of 48 natural mutants with single-site substitutions in the hydrophobic core of the SH3 domain (a β-protein; not considered in [79]) indicated that conservation correlates well with unfolding rates but not the folding rates of the mutants. In other words, mutants with slower unfolding rates occur more frequently than mutants with faster unfolding rates, but a positive or negative correlation between folding rate with occurrence frequency was not observed. This finding suggests that evolution selects more strongly for a slower unfolding rate than faster folding rate, at least for the SH3 family [80].

In this regard, a recent survey argued that protein kinetic stability, i.e. a slow unfolding rate, is often more strongly selected by evolution than thermodynamic stability, most probably because kinetic instability (a faster unfolding rate) facilitates irreversible alteration processes such as amyloid formation and other forms of detrimental protein aggregation even if overall thermodynamic stability is maintained by a higher folding rate [81]. Echoing the aforementioned study of SH3 domains, an investigation of 27 single-substitution variants of thioredoxin—the fold of which is apparently extremely ancient in evolutionary history [82]—indicates that viable mutants can at most be 2 kcal mol⁻¹ less stable than the wild-type, but a significant correlation exists between slower unfolding rate and the occurrence frequency of a given residue in sequence alignments, again suggesting a significant natural selection for slower unfolding rates [83].

For proteins that undergo folding with significantly populated transient intermediates, a mutation may stabilize or destabilize the intermediate conformations, or even abrogate the intermediates encountered in the folding of the original sequence, or create new intermediates. In fact, in some experiments, mutations were intentionally introduced to stabilize various folding intermediates to facilitate their characterization [84,85]. In one case, swapping certain hydrophobic core residues between two related proteins could also swap the associated folding intermediates [86]. In more extreme cases, a mutation could lead to the formation of different folding intermediates or even different folded structures with potentially severe implications for protein function and aggregation [87]. In particular, highly abundant proteins with relatively low solubilities are prone to aggregate [88]. An increasing number of neurodegenerative and other varieties of prion and amyloid diseases are now known to be caused by misfolded structures (different ‘native’ structures) or by aggregation/oligomerization of intermediate conformational states, with propensity for misfolding increased by certain mutations [87,89] (figure 3). Cataracts in the human eye are also found to be caused by accumulation of misfolded proteins [90] and associated with mutations that led to abnormal folding behaviour [91,92]. As exemplified by the mouse prion protein and consistent with the general observation of evolutionary selection for kinetic stability [81], the folding and maintenance of the non-disease folded form of some of the pertinent proteins (the misfolded forms of which are implicated in diseases) is under kinetic rather than thermodynamic control [93]. Consistent with these observations, the experimentally observed distribution of protein evolution rates may be rationalized by an evolutionary process that selects against misfolding [94].

In the cellular environment, mutations can affect not only the folding kinetics of a protein in isolation but also how it interacts with the complex cellular machinery while it is folding. Inasmuch as folding kinetics is concerned, the in vivo translational rate can affect co-translational folding [95,96] because, for example, fast-translating codons can be useful for avoiding misfolding. In this regard, even synonymous mutations that do not change the amino acid sequence of a protein can lead to altered folding pathways in the cell [97].

2.3. Interactions and misinteractions

The biological functions of most proteins require them to interact with other proteins and/or other biomolecules [98]. Mutations affect these interactions and can lead to misinteractions [99]. A classic example is the glutamic acid to valine mutation in haemoglobin [100] that causes aggregation of haemoglobin and consequently sickle-cell anaemia [101]. More recent examples include mutations implicated in prion, amyloid and other misfolding diseases mentioned above [102] as well as disease-causing mutations that disrupt or weaken the proper binding between two proteins [103,104].

The cellular environment is crowded [105,106]. This crowding is probably dictated by biophysical constraints imposed by a living cell’s need for efficient rates of biochemical reactions [107]. Within the cellular confine, a given protein can potentially come into contact with a large number of other proteins [108,109]. Although the possibility of non-specific binding probably constitutes a biophysical constraint that might have restricted the number of proteins in a cell [110], natural proteins can function by being remarkably specific binders. This interaction specificity entails not only favourable binding with a protein’s target molecule(s) but also extremely unfavourable—essentially absence of—binding with many other molecules. This requirement is conceptually similar to the well-known principle for protein design, i.e. that an optimized sequence has to ‘design in’ the target structure as well as ‘design out’ alternative structures [111]. Many natural proteins have evolved not only to fold to the functional native state but also to strongly destabilize non-native intermediate states [112] by increasing the energetic
There are also overlapping binding interfaces that bind significantly increase the binding affinity to a new substrate. interface sizes where a single surface mutation may significantly affect the binding affinity with even smaller interfaces. One can imagine a ‘grey area’ of interface [127]. In this light, transient binding may be possible between non-interacting protein surface to that of a protein–protein interaction, albeit with low affinities. Currently it is not feasible to an appreciable level. If the misinteraction is beneficial, it can underpin a new oligomeric state or promiscuous function.[116] The heterogeneity, or designability, of their interactions are constrained by the physico-chemical properties of the alphabet. It is not physically possible to eliminate all favourable interactions between a protein and all other proteins except its presumed functional partner(s). In other words, misinteractions cannot be eliminated completely by optimization. In the living cell, there can be more misinteractions because some evolving proteins have not had time to minimize them [117]. In fact, even the folded form of a globular protein is probably a metastable state, whereas amyloid [118] or prion-like [119] aggregates are expected to be thermodynamically more stable configurations at longer timescales. Therefore, binding should not be understood as an all-or-none proposition; instead it is a question of binding affinities that can vary over a wide range. Although proteins bind their evolved interaction partners particularly strongly, they probably also interact transiently with many other proteins, albeit with low affinities. Currently it is not feasible to identify the effects of a given mutation on the many possible interactions a protein can engage in, especially when the mutation has no detectable effect on the main function. Nonetheless, computational prediction methods are being developed to perform efficient tests for potential binding between large numbers of proteins [120].

Any mutation on a protein can potentially increase the binding strength with some molecular partners. If this change alters the cellular biochemistry, the mutation may be subject to either positive or negative natural selection (figure 3). A misinteraction is created by mutation if an originally negligible protein–protein interaction is strengthened to an appreciable level. If the misinteraction is beneficial, it can underpin a new oligomeric state or promiscuous function of the protein which can then be positively selected [121,122] (see further discussion in §3.5). In those cases, computational modelling suggests that positive selection of an interacting region can also facilitate evolution of globally well-packed globular structures in the interacting proteins [123,124].

Protein–protein interactions require geometric coupling of the protein interfaces. Mutations within the interfaces naturally have a direct impact on binding; mutations outside the interface can affect binding allosterically as well [125] (see further discussion in §2.7.1). Biophysically, new protein–protein interactions are not unlikely to emerge. A recent survey of heterodimers found that functional binding interfaces bury a surface area between 380 and 3400 Å2 [126]. Another recent study indicated that only two amino acid substitutions are needed to shift the average amino acid composition of a 1000 Å2, approximately 28-residue non-interacting protein surface to that of a protein–protein interface [127]. In this light, transient binding may be possible with even smaller interfaces. One can imagine a ‘grey area’ of interface sizes where a single surface mutation may significantly increase the binding affinity to a new substrate. There are also overlapping binding interfaces that bind different substrates [128,129], which can be created easily via mutations from an original interface that binds only one substrate. This perspective is consistent with a recent directed evolution study on the bacterial immunity protein Im9. The wild-type Im9 primarily inhibits deoxyribonucleic ColE9 but also inhibits ColE7 promiscuously, i.e. to a much lesser extent. The experiment shows that it can evolve readily into a primary ColE7-inhibitor with an approximately 102-fold increase in affinity and 105-fold increase in selectivity via a ‘generalist’ intermediate that allows for rapid evolutionary divergence [130].

2.4. Marginal native stability

Since native stability is required for globular proteins to perform their biological functions (§2.1) and to avoid misfolding and aggregation (§2.2), it might seem that a higher native stability should always be desirable and therefore favoured by evolution. However, natural globular proteins are not extremely stable. An early survey of the thermal stability of 12 proteins at 25°C showed considerable variation of native stability among them, with average stabilizing free energies of 0.05–0.12 kcal per mole of amino acid residues [131]. This and other experimental data indicate an approximate native stability of 5–15 kcal mol−1 for a natural globular protein with about 100 amino acids. These findings have since been rationalized theoretically by considering the strength of intra-protein interactions and conformational entropy [44,132]. This experimental level of stability of natural globular proteins is often characterized as ‘marginally stable’. ‘Marginal’ here points to the relatively small free energies of folding. Sometimes the term also refers to the fact that the net balance of 5–15 kcal mol−1 for native stability is the result of a partial cancellation of two much larger free energies on the order of 100–200 kcal mol−1 contributed by favourable intra-protein interactions on one hand and conformational entropy on the other [44].

If evolutionary selection for stability is expected, why are natural proteins only marginally stable? One possible reason is that native stability is not the only requirement on a functional globular protein. Conformational flexibility is crucial for certain biological functions. Therefore, adaptation towards increased conformational flexibility might have acted as a check against proteins evolving to become extremely stable [21,133,134], suggesting that marginal stability can be an adaptive trait.

2.4.1. Marginal stability may not be an adaptive property

Is a strong selection pressure for marginal stability necessary to account for the experimentally observed marginal stability of natural proteins? Biophysics-based models have suggested otherwise by showing that marginal stability could be a non-adaptive property [135,136]. The number of sequences encoding for a given structure generally decreases with native stability. Hence, even in the absence of any evolutionary pressure, there are more sequences encoding for a given native structure with low stabilities than sequences encoding for the same structure with high stabilities. This phenomenon is a basic property of protein sequence space and is consistent with the ‘superfunnel’ perspective [137] (§3.2.3). Therefore, as long as a certain minimum stability requirement for folding and function is met, random mutational drift will lead an evolving population to a region of sequence space that...
encodes with marginal stabilities (close to the minimum required stability) simply because there are more sequences with that property [135]. In a more recent model, an evolved population is seen to prefer marginal stability even when the model fitness function increases exponentially with native stability [136]. In this view, if marginal stability of a protein is functionally beneficial, it may represent a ‘spandrel’ [135], i.e. a tendency occurring originally for non-adaptive reasons that is exploited subsequently by biology [138].

This population consideration argues convincingly that there might not have been extensive positive evolutionary selection to decrease the stabilities of globular proteins. A fundamental issue that remains to be addressed, however, is the extent of evolutionary selection to increase stability. This question asks whether the stabilities of natural proteins are close to their biophysical maximum, as envisioned in the superfunnel picture (§3.2.3) or are far from a biophysically possible maximum that was not selected evolutionarily. Notably, both of the models discussed above [135,136] posit that there are amino acid sequences that can fold to a given structure uniquely with native stabilities far exceeding the experimentally observed stabilities of natural proteins. Results of the random mutation model of Taverna & Goldstein [135] show a significant population of sequences encoding with higher native stabilities than the sequences around the peak of the steady-state population. Therefore, if the sequences near the peak of the population distribution are taken as models for natural proteins, their results suggest that a significant fraction of mutations of natural proteins would lead to higher native stabilities (although that fraction is smaller than the fraction of mutations leading to lower native stabilities). In a more recent model of Goldstein [136], it is stated specifically that the 300-residue protein used in the study can potentially reach an extremely high stability of 118 kcal mol$^{-1}$ but the evolved population has a stability of only about 9 kcal mol$^{-1}$.

2.4.2. How stable can real proteins be?

Is it physically possible for some amino acid sequences to fold with exceedingly high stability? The perspective from experiments is different from that suggested by Goldstein [136]. Among 290 single-residue substitutions of staphylococcal nuclease created artificially by Shortle and co-workers [139–141], 257 are destabilizing, five lead to stabilities essentially the same as that of the wild-type (approx. 5.5 kcal mol$^{-1}$), only 28 are stabilizing. Moreover, each destabilizing artificial mutation destabilizes by more than 2.08 kcal mol$^{-1}$ on average (maximum = 7.5 kcal mol$^{-1}$), whereas each stabilizing artificial mutation stabilizes by only 0.36 kcal mol$^{-1}$ on average (maximum = 1.0 kcal mol$^{-1}$). A similar trend is exhibited by the 98 artificial mutants of chymotrypsin inhibitor 2 studied by Fersht and co-workers [142] (77 with a single substitution, 17 with two substitutions, and four with three substitutions): 90 artificial mutants are less stable than the wild-type (7.6 kcal mol$^{-1}$), only eight artificial mutants are more stable than the wild-type. On average, a destabilizing mutation destabilizes by 1.67 kcal mol$^{-1}$ (maximum = 4.93 kcal mol$^{-1}$ among single-substitution mutants), whereas a stabilizing mutation stabilizes by only 0.18 kcal mol$^{-1}$ (maximum = 0.42 kcal mol$^{-1}$). These data suggest that the stabilities of natural proteins are close to, albeit not exactly at, the maximum achievable by sequences in the immediate sequence-space neighbourhood of the wild-type sequence. However, when larger numbers of amino acid substitutions are applied to a wild-type, an increase in thermodynamic and/or kinetic stability of 3–4 kcal mol$^{-1}$ has been observed in several proteins (e.g. [143,144]).

There is no experimental evidence to date indicating the existence of polypeptides that encode for an essentially unique folded structure with native stability as high as approximately 0.4 kcal per mole of amino acid residues as postulated by Goldstein [136]. A case in point is the 93-residue designed protein Top7, which is already characterized as extremely stable. Its stability is approximately 13 kcal mol$^{-1}$ at 25°C [145]. Although this level of native stability is significantly higher than several single-domain proteins [146] including the 97-residue S6 with similar secondary structure (native stability = 8.5 kcal mol$^{-1}$) [147], the stability of the artificially designed Top7 is still within the 5–15 kcal mol$^{-1}$ native stability range long recognized for natural proteins [44,131]. The highest stability achieved by more recent attempts to design stable proteins is 14.9 kcal mol$^{-1}$, or 0.14 kcal per mole of amino acid residues for a 110-residue construct [148]. In this light, the 118 kcal mol$^{-1}$ stability estimated in [136] is physically unrealistic. This exceedingly high estimate is probably an artefact of the non-explicit-chain approach used in the study (for a discussion of explicit- versus non-explicit-chain protein models, see [36]), which tends to underestimate mutational effects on the unfolded states. From a protein biophysics standpoint, however, any given mutation not only impacts the free energy of the native state but can also have a significant effect on the denatured (unfolded) state, and the effects on the two states often partially cancel, such that extremely high native stability is physically not possible [149].

2.4.3. Reconciling evolutionary selection for stability with marginal stability

Taken together, the above discussion indicates that fundamentally, natural globular proteins without disulfide and other cross-links are marginally stable because of the physical constraints on native stability itself. Exceedingly high native stability is physically impossible. Because there are more sequences encoding for lower stabilities than higher stabilities [135,137], extensive evolutionary selection to decrease native stability is not necessary, though selection for local flexibility may sometimes result in functional globular proteins that are not the most stable possible for the given folds [21,133,134]. Experimental evidence abounds, however, for evolutionary selection for higher native stability [40,83] (see §2.1), though not necessarily the highest once a certain threshold for function is achieved [150], as illustrated by the data on the artificial mutants of staphylococcal nuclease and chymotrypsin inhibitor 2 discussed in §2.4.2. Therefore, natural globular proteins are marginally stable (because of biophysical constraints) but they are nonetheless nearly maximally stable (by evolution) for the structures they fold to. This conclusion is supported by theory: neutral net topology in protein sequence space tends to concentrate large evolving populations toward sequences that are mutationally most robust [137,151]. These sequences are often also thermodynamically most stable [137]. But random mutations alone—in the absence of a fitness drive towards higher native stability—are not sufficient to produce a highly concentrated population at the most stable ‘prototype’ sequence at the
bottom of the sequence-space superfunnel because of the large number of sequences that are less stable [40,137,152,153]. Therefore, the experimental observation that natural proteins are often a nearly most stable sequence that behaves like a prototype sequence suggests strongly that they are results of positive selection for higher native stability (see further discussion in §3.2.3).

2.5. Geometric/topological constraints imposed by the native structure

The Top7 example mentioned in §2.4.2 also offers insights into other aspects of the interplay between biophysical constraints and evolution. It shows that the tendency to misfold does not necessarily diminish with increasing native stability: despite the high native stability of Top7, its folding kinetics is complex, probably involving multiple kinetic traps [154,155]. Theoretical considerations indicate that the lack of two-state-like behaviour of Top7 is probably caused more fundamentally by its peculiar native structure, more so than the fact that it is an artificially designed protein that did not undergo natural selection [156]. Thus, native geometry or topology (the pattern of residue–residue contacts in the native structure) probably impose a physical constraint on the level of stability and folding cooperativity that natural or artificial selection can achieve [156,157]. In this connection, it has been shown using simple lattice protein models that not all protein structures are equally encodable [158] or designable [159–161]. Some structures may not be encodable at all [158,162]. This represents another set of biophysical constraints under which protein evolution must operate.

2.6. Chaperones and in vivo folding

In molecular biology, chaperones are a class of proteins that assist the folding and assembly of other proteins, or even reverse misfolding [163]. Many mutant proteins fail to fold or be expressed in the cell because of reduced native stability, increased probability of misinteractions during folding, or other changes in folding kinetics that are detrimental to productive folding. These biophysical constraints hinder evolution because they limit the number of mutants that can be explored. Mutations that decrease native stability below a certain threshold cannot participate in the evolutionary process even if they possess superior functionality—provided they are properly folded—because relative native instability compromises protein folding and expression. In the cellular environment, chaperones offer a degree of relief from these constraints. Molecular chaperones enhance evolvability—i.e. a genome’s ability to produce adaptive variants [164] (see §5.3)—because they help mutants that are less stable to fold to functional structures and to avoid non-functional aggregation, thus allowing more mutants with potentially beneficial new functions to be explored in vivo [165,166].

This principle was borne out in experiments involving the Escherichia coli GroEL/GroES chaperonin complex. In a set of laboratory evolution experiments on four enzymes, the divergence of modified enzymatic specificity was found to be much more speedy when GroEL/GroES is overexpressed, most probably because GroEL/GroES assist folding of enzyme variants, allowing mutants that lose as much as 3.5 kcal mol$^{-1}$ in native stability to be viable whereas only approximately 1 kcal mol$^{-1}$ loss in stability is permitted in the absence of GroEL/GroES [165]. In a more recent experiment to evolve a phosphotriesterase into an arylerasease in vivo, GroEL/GroES is again seen to increase the ability to adapt to new functions by allowing for more genetic variation. Moreover, it was found that mutational tolerance is not determined by in vitro native stability per se, but rather by the level of soluble expression of the mutant protein in the cell. In this case, the GroEL/GroES chaperone enhances soluble expression by apparently stabilizing a folding intermediate against detrimental aggregation and thus indirectly promotes productive folding, underscoring the critical importance of mutants’ in vivo folding kinetics on the course of protein evolution [166].

Consistent with this trend, there is also strong evidence at the genome level that proteins that use GroEL/GroES obligately for folding evolve faster [167] and are less dependent on optimal codon usage to avoid translation-induced misfolding [168] than proteins that do not require these chaperones for folding but rather rely more on optimal codons [169]. The link between translation errors and evolutionary rates will be discussed further in §3.10.

On the theory front, a recent simulation of protein evolution considered a model cell containing a few interacting protein species that can adopt either ‘folded’ or ‘molten-globule’ structures [170]. Consistent with the trend seen in experiments [165,166], the simulation indicated that chaperones that actively catalyse folding also accelerate evolutionary adaptation because the increased chaperone-assisted folding rates allow for deeper searches of the sequence space [170].

2.7. Multi-basin folding landscapes, allostery and conformational dynamics

Protein structures are dynamic; and conformational dynamics is crucial in many biomolecular interactions [171,172]. Even for globular proteins that fold to an essentially unique native structure under physiological conditions, other less favourable ‘excited-state’ conformations are always populated, albeit to a much lesser extent than the dominant native conformation that is commonly identified as the ground-state structure. The balance between the dominant ground-state and excited-state populations can be altered by mutations. For instance, a recent NMR experiment demonstrated that a mutant T4 lysozyme populates an excited state to about 3% at 25 C [173] (figure 4e). Besides uniquely folding proteins, there are globular proteins that have more than one dominant folded conformation. For these proteins, the same amino acid sequence adopts more than one structure with similarly high probabilities. Thus, instead of a single funnel, the energy landscape of such a protein has multiple basins of attraction [177,178]. In some cases, these alternative structures freely interconvert during the lifetime of the protein as for the cytokine lympho- tatin [179] and the cell cycle control protein Mad2 [180]. Sometimes it takes an additional factor to stabilize an alternative structure, such as a change in the solvent conditions or a binding event (e.g. [181–184]).

2.7.1. Conformational diversity is often needed for function

Multi-basin energy landscapes are widely used by Nature to regulate protein function. A prime example is allostery, by which the function of a protein is regulated through binding a ligand (effector) at a site (the allosteric site) on the protein
that changes the structure and/or dynamics of the protein’s active, functional site positioned at a distance from the allosteric site [185]. Allostery is important for biological function and its malfunction is implicated in disease processes [186,187]. Mutations affect allostery. Mutational effects on allostery can be subtle because allosteric communication between the allosteric and active sites can be underpinned by multiple mechanisms [188,189]. Nonetheless, mutational effects on allostery can be rationalized by computational approaches in some instances [190].

Conformational flexibility, dynamics of protein folded states and allosteric transitions often can be deduced to a reasonable degree from the structure(s) of the protein in question using elastic network models for folded-state dynamics [191–194] or native-centric Gō-like potentials [195] with multiple folding basins [196]; reviewed in [177]). Similar to the aforementioned case for the probable existence of geometric/topological constraints on the evolution of folding stability and cooperativity (§2.5), the success of structure-based native-centric modelling in rationalizing conformational dynamics and allosteric transitions suggests that there are significant structural constraints on the evolution of functional folded-state dynamics. The computational efficiency of elastic network models also allows enzymes that are dissimilar in sequence and structure yet probably perform similar functions to be detected by their similar dynamic properties [194,197], making it possible for relationships between evolutionary conservation and conformational dynamics to be explored [198].

Allostery is envisioned to have evolved by oligomerization, gene fusion and/or recruitment of unused/flexible parts of a pre-existing protein structure (reviewed in [199]). The latter evolutionary route may proceed by positive selection of opportunistic binding of excited-state conformations. The mechanism of such binding may lie anywhere between the ‘conformational selection’ and ‘induced fit’ scenarios [177,200]. Evolution has apparently exploited latent allosteric potentials entailed by conformational dynamics in this manner, as in the case of Ste5 activators that target MAP kinases in yeast [201].

Opportunistic binding of excited-state conformations can also facilitate evolution of new functions that are not necessarily allosteric [122,202,203]. During such an evolutionary process, a sequence with a multi-basin energy landscape can serve as an evolutionary bridge. In particular, the evolutionary intermediate of two sequences each encoding for a

Figure 4. (Opposite.) Examples of experimentally designed bi-stable proteins and mutation-induced structural switches. (a) Wild-type T4 lysozyme was mutated (L99A) to create an internal cavity that allows for the population of an excited-state conformation with an altered helical segment (blue; left). This T4 lysozyme variant could be further transformed via a single G113A substitution into a bi-stable protein that also populates a new folded structure in which the local structure of the helical segment is modified (red; right). An additional R119P substitution on this L99A, G113A variant then leads to a protein that adopts the conformation on the right as its essentially unique native structure [173]. (b) Wild-type Arc repressor is a homo-dimeric protein. Each monomeric unit contributes a β-strand to form a two-stranded antiparallel β-sheet (blue; left). This shared configuration becomes bi-stable with the introduction of a single N11L substitution to each of the monomeric units. The mutated sequence now populates the original structure as well as a new structure with the β-strands changed into two short helices (red; right). An additional L12N substitution on each of the monomeric units results in a sequence that adopts the new configuration on the right as its essentially unique native structure [65]. (c) The cysteine-rich domain NW1 forms a stable structural element (blue; left) with three disulfide bonds (yellow sticks) between the residue pairs (8,24), (12,25) and (16,24). A single K21P substitution results in a bi-stable mutant that also populates a structure with a different overall conformation (red, right) and an alternate disulfide-bonding pattern, now between residue pairs (8,24), (12,20) and (16,23). Introduction of a single G11V substitution on this bi-stable mutant results in a sequence that adopts the conformation on the right as its essentially unique native structure [174]. (d) Two domains of streptococcal Protein B, named G_A and G_B, with a 3αc and a 4β + α-fold, respectively, and no significant sequence similarity, were transformed into each other by a series of point mutations that resulted in a structure pair G_A98 and G_B98 that allows the switch between the two structures with just a single L45Y mutation. G_A98 exhibits a small 4β + α population and thus may also be regarded as bi-stable [175]. (e) The viral P22 Cro and λ Cro are DNA-binding proteins. Encoded by different sequences, they have structurally very similar helical N-terminal domains (represented by the yellow and green ribbon, respectively) but have structurally distinct C-terminal domains. P22 Cro has a helical C-terminal, whereas the C-terminal of the homo-dimeric λ Cro forms a β-sheet. A 24-residue chameleon sequence created largely by mixing residues from the helical and sheet-forming C-termini adopts different secondary structure depending on whether it is inserted in the P22 or λ Cro context [176]. Sequence and structural information presented in this figure was taken from the cited original references.
different dominant structure can be a bi-stable sequence that folds to both structures with equal or similar probabilities [161,178,204].

2.7.2. Bi-stable proteins and conformational switches

Experiments in several laboratories have found cases where a single mutation was able to either create a bi-stable protein from a uniquely folding protein or completely switch one uniquely folding protein to another with a new native structure [65,173–175,205,206]. Although these cases of mutation-induced structure switches were artificially engineered, they demonstrated that it is generally possible for bi-stable proteins to arise through mutations during natural evolution.

An early example of mutation-induced structure switching was the Arc repressor, which is a homodimer with a two-stranded inter-unit β-sheet. Experiments by Cordes et al. [205] showed that the β-sheet in the wild-type protein can be changed to a pair of 310-helices by two amino acid substitutions that swap the neighbouring sequence positions of an asparagine and a leucine. A subsequent experiment indicated that a mutant with a single asparagine-to-leucine substitution has approximately equal populations of the β-sheet and helical forms, and thus may be regarded as an evolutionary bridge [65] (figure 4b). A recent study showed further that if two more polar or charged to hydrophobic substitutions are introduced, the resulting triple mutant adopts an octamer configuration with approximately half the helical content of wild-type Arc, indicating that new protein–protein interactions and novel oligomeric states can readily result from a small number of mutations [207].

Experimental mutagenesis has uncovered a similar behaviour in the cysteine-rich domains (CRD) of cnidian nematocyst proteins. Different CRDs fold to either one of two structures with different disulphide-bonding arrangements despite high sequence similarity and identical sequence patterns for their cysteines. Meier et al. [174] found that a CRD sequence that folds to one disulphide arrangement can be converted to another disulphide arrangement by only two amino acid substitutions, one from lysine to proline and the other from glycine to valine, whereas the single-substitution mutant with only the lysine-to-proline mutation behaves as an evolutionary bridge that populates both disulphide arrangements (figure 4c). This finding again underscores that large structural changes can be effected by minimal changes in the amino acid sequence.

The study by Alexander et al. [175] of the Gα/Gβ system showed that a single leucine-to-tyrosine substitution can convert a sequence encoding for an albumin-binding 3α (Gα) structure to a sequence encoding for an immunoglobulin-binding 4β + α (Gβ) structure (figure 4d). A subsequent experiment on two other mutants identified two additional 3α ↔ 4β + α structure switches induced by a single amino acid substitution [206]. Interestingly, a mutant with a conformational ensemble that is 95% 3α and only 5% 4β + α when measured in isolation nevertheless binds immunoglobulin but not albumin [206], providing an excellent example of how protein–protein interactions can dramatically shift the conformational distributions of the binding partners [200].

Another recent example of an artificial ‘evolutionary intermediate’ is a 24-residue sequence that can adopt either the α-helical or β-sheet C-terminal conformations, respectively, of transcription factors P22 Cro and λ Cro, depending on whether the designed sequence is fused with the N-terminal domain of P22 Cro or λ Cro [176] (figure 4e). In this case, the naturally occurring wild-type 24-residue C-terminal sequences of P22 Cro and λ Cro have only five identical amino acid positions, whereas the amino acid residues of the designed sequence at all but four positions are identical to that in the wild-type P22 Cro or in the wild-type λ Cro. This finding underscores the critical role of tertiary context in determining secondary structure in proteins [208]. Although the designed sequence is nine and 14 substitutions away from the corresponding sequences in wild-type P22 Cro and λ Cro, respectively, the successful design of a structurally ambivalent ‘chameleoon’ sequence in this experiment suggests that a smooth evolution transition from one Cro fold to another is possible [176].

Computation-assisted design of conformational switches has seen notable success [209,210]; but it is still a challenge to apply our current biophysical knowledge to provide a fundamental physical rationalization for experimentally observed conformational switching. For the Gα/Gβ system, a mutation-induced gradual stabilization of one structure over another was demonstrated using a common software for ΔDG prediction (§2.1) [178]. However, the mutation-induced Gα/Gβ conformational switching was not reproduced in atomistic molecular dynamics simulations [67], even though a part of the simulated energetics is consistent with experiment [68].

The structural plasticity in bi-stable and multi-stable proteins probably plays an important role in protein evolution [122,211,212]. Conformational switches and bridge sequences facilitate evolution by allowing continuous or near-continuous transition from one folded structure to another. The experiments in figure 4 suggest that, under certain circumstances, multi-functional proteins can be created by only a few mutations that stabilize certain hidden or excited states. A situation where it is advantageous to take such a route is the coevolution of pathogens and their hosts, a highly competitive evolutionary process that demands frequent change of protein shapes and functions. It is thus unsurprising that bi-stability and multi-specificity are exhibited in antibodies [213], antimicrobial peptides in natural plant defence [214] and antiviral proteins [215].

2.8. Intrinsic disorder

When structural plasticity is extreme, one might expect a multi-stable sequence to morph into one without a discrete set of clearly discernible favoured conformations. This in itself is not surprising because an overwhelming majority of polypeptides with random amino acid sequences do not fold to a unique structure [116]. What is remarkable, in the context of our decades-long near-exclusive focus on proteins with well-ordered structures, is the existence of many functional proteins with such extreme conformational diversity. Although our main concern here is evolution of globular proteins, it is important to recognize that intrinsically disordered proteins (IDPs) or intrinsically disordered regions (IDRs) play key roles in cellular processes [216–222].

2.8.1. Any protein conformational state can potentially have biological function

With the discovery of functional IDPs/IDRs, it has become abundantly clear that biology can exploit any protein conformational state that it finds useful. In this respect, an
intriguing recent suggestion is that although avoidance of amyloid-like aggregation has apparently been a driving force of protein evolution [222] (§2.2), it is possible that modern protein folds have an amyloid origin in evolution [224]. For IDPs/IDRs, current understanding of the evolution of the triplet genetic code [225] suggests that the amino acid composition of primordial polypeptides was conducive to more disordered conformations before the modern genetic code for a 20-letter amino acid alphabet was completed [222]. However, surveys of modern proteomes indicate that IDPs/IDRs are more common in eukaryotes than in prokaryotes: more than 32% of amino acid residues in eukaryotic proteins are in IDPs/IDRs whereas the corresponding percentage is less than 27% for prokaryotic proteins. This pattern suggests that the proteins in the last universal ancestor were probably well structured and emergence of the IDPs/IDRs observed today was relatively late [222], perhaps coinciding with an evolutionary trend that has witnessed a general decrease in protein hydrophobicity [226].

According to one estimate, more than 30% of eukaryotic proteins have IDRs of more than 50 consecutive residues [216], consisting of more proline, glycine and charged residues but fewer hydrophobic residues [227,228]. IDPs/IDRs are involved in fundamental processes such as transcription, translation and cell cycle regulation that, when they malfunction, can lead to cancer. The essential role of IDPs/IDRs in mediating biological regulation suggests that, in some situations, they have certain advantages over folded proteins in recognition and binding [229]. For instance, their ability to flexibly bind to many different partners has allowed them to occupy hub-like roles in protein–protein interaction networks [230,231]. They can also encode relatively larger intermolecular interfaces to economize genome and cell sizes [218]. Protein–protein interactions for some IDPs/IDRs entail significant folding upon binding [219], while others undergo only restricted local ordering at the binding site with other parts of the protein remaining disordered, thus forming a dynamic ‘fuzzy’ complex [220,232–236].

2.8.2. Biophysical constraints on evolution of intrinsically disordered proteins and regions

What can be expected of the biophysical constraints on the evolution of IDPs/IDRs? IDPs/IDRs do not fold to a unique structure. Therefore, in contrast to many globular proteins, the energy landscapes of IDPs/IDRs are not funnel-like [222]. As far as near-neutral mutations [237,238] are concerned, one might expect less biophysical constraints on IDP/IDR evolution than on globular protein evolution because for IDPs/IDRs there is no need to maintain an essentially unique folded structure. However, it can also be argued that evolution of certain IDPs/IDRs may be subject to even more restrictive constraints because of their requirement to bind to multiple partners. As a result, these IDP/IDRs may suffer from low mutational robustness similar to that of bi-stable globular proteins that play the role of an evolutionary bridge between two folded structures [178,239]. Nevertheless, even in such cases, IDPs/IDRs in a neutral net might only need to conserve certain functional residues that are compatible with multiple binding partners while imposing few constraints on mutations at amino acid sites in the rest of the protein.

These expectations are largely consistent with database studies and experiments. Phylogenetic analyses indicate that IDRs generally evolved faster than ordered regions of proteins, but some IDRs such as DNA-binding regions evolved slower [240,241]. For proteins that have both ordered and disordered regions, mutations in IDRs lead to smaller stability changes than in ordered regions. Thus, IDPs/IDRs may enhance protein evolvability and the development of new functions [242], as evolutionary changes in protein sequence and structure are often correlated with local flexibility and disorder [243].

The biophysical constraints on IDP/IDR evolution [244] are quite different from those on folded protein evolution [12]. In fact, the accepted amino acid substitutions in IDPs/IDRs resemble those in solvent-exposed loops and turns of globular proteins [244]. Chemical composition defined as the fraction of positive, negative, polar, hydrophobic and special (proline and glycine) residues is often maintained across IDR orthologues that otherwise exhibit little conservation [245]. This observation is in line with the finding that whether an IDP is elastomeric or amyloidic depends largely on the relative compositions of proline and glycine [228], and is consistent with the central role of aromatic composition in a set of IDP interactions that are presumably underpinned by cation–π attraction [236]. Relative to the substitution matrices for globular proteins, substitution matrices for IDPs/IDRs entail a generally higher probability of evolutionary changes, but some residues such as tryptophan and tyrosine tend to be highly conserved in IDPs/IDRs, perhaps because of their critical role in protein–protein interfaces [244,246].

It should be recognized that IDP/IDR conformations are far from random. Biological functions of proteins are always underpinned by conformational structures. In this respect, the difference between IDPs/IDRs and ordered proteins is that the IDP/IDR function is conferred by a much more diverse conformational ensemble than for globular proteins. The transient, ‘fuzzy’ tertiary contacts in IDP/IDR conformations are often important for the function; hence mutations that disrupt such contacts can be extremely detrimental to function. An example of how a single mutation can disrupt IDP function is the threonine-to-arginine mutation at position 45 of the cyclin-dependent kinase inhibitor Sic1 [234,247]. This amino acid substitution leads to a dramatic increase in its hydrodynamic radius [234] and, at the same time, a serious disruption of its biological function in regulating the cell cycle [247]. Current biophysical understanding of this and other mutational effects on IDP/IDR conformational distribution is limited. Much remains to be discovered about the evolution of these proteins.

2.9. Protein dynamics and phenotypic plasticity: what is a molecular phenotype?

In the study of molecular evolution, the term genotype is used for the inheritable part of genetic information; whereas phenotype refers to the biomolecules of interest that are produced based on the genotypic information. In theoretical studies of protein evolution, as a modelling simplification, the genotype may be identified with the amino acid sequence because as far as in vitro protein folding is concerned, it contains essentially the same information as the nucleic acid sequence that encodes it. This is a simplified approach that neglects in vivo complexities such as the fact that synonymous mutations can lead to altered cellular folding pathways (§2.2). In principle, the molecular phenotype should encompass all
properties—including but not limited to biological functions—of the protein encoded by the genotype. In practice, molecular phenotypes in theoretical and experimental investigations are defined, and thus are restricted, by the question being addressed. However, an oversimplified view of molecular phenotypes that is too restrictive can hinder understanding of important principles of protein evolution.

For globular proteins that have an essentially unique folded structure, a practical and seemingly natural definition of molecular phenotype of a given amino acid sequence is its structure as deposited in the Protein Data Bank (PDB). This practice is useful for constructing a neutral net of sequences that encode uniquely for the same protein structure and the evolution from one such phenotype to another [137,161]. However, this simplistic view of molecular phenotype neglects the dynamic nature of proteins. Recent advances in experimental techniques, especially those using NMR, have enabled detailed characterizations of the dynamic properties of proteins [173,248–250] and, in conjunction with computation, allowed for the construction of ensembles of diverse conformations of disordered proteins based on NMR and other experimental measurements [251,252]. As a result of these experimental advances and the theoretical energy landscape perspective [34,253,254], our view of how protein molecules function has undergone a drastic change in the past two decades, with increasing recognition of the biological, physical, and evolutionary significance of protein dynamics [255,256].

Because of the role of dynamics in protein function (§2.7 and 2.8), identifying a protein’s molecular phenotype only with its native folded structure is often too restrictive. Ideally, the molecular phenotype of an amino acid sequence should correspond to the totality of its biologically relevant properties. Although it may not be practical to enumerate many properties of a protein, for many applications the molecular genotype should at least be understood as an ensemble of conformations with a sequence-specific and environment-dependent distribution. Within this ensemble, certain phenotypic properties, such as the presence of a secondary structure in the protein conformation, are not necessarily fixed but can undergo thermal fluctuations or environment-induced changes. This phenomenon is referred to as single-genotype phenotypic fluctuation or phenotypic plasticity, which can underpin important evolutionary responses to environmental changes [257].

Phenotypic plasticity tends to enhance evolvability. This trend can be seen clearly in an experimental evolution study of E. coli cells that express mutants of green fluorescence protein. In this experiment, mutants leading to a functional protein among random amino acid sequences is overwhelming majority of sequences do not have a biological function. It has been estimated that the probability of finding a functional protein among random amino acid sequences is approximately 10⁻¹³ [272]. Evolutionarily, natural protein sequences are still diverging from one another today, albeit at a slow rate because biophysical and functional constraints allow only about 2% of amino acid sites to be mutated [273]. This ongoing divergence means that the coverage of the space of all possible sequences by biologically viable sequences has been and is still increasing; i.e., there has been a continuing expansion of the ‘protein universe’ since the beginning of life on the Earth [273].

There is consensus among researchers that the repertoire of globular protein folds is probably finite. The SCOP classification [274] currently identifies about 1200 different folds in the PDB. Estimates for the total number of possible folds range from about 1000 [275], 2000 [276] to 10 000 [277]. Fold classifications are inherently difficult and estimation of the total number of possible folds is sensitive to the definition of a fold (see [278] and references therein). Nonetheless, in most cases, structures of recently sequenced proteins are related to known folds [279], suggesting that the existing PDB structures are probably a near-complete representation of all biologically viable globular protein folds.

A broader issue is whether the observed natural globular protein folds constitute a relatively small subset selected by evolution from a much larger collection of all physically possible compact conformations. Biophysics and polymer physics
have shed light on this question. A hallmark of most globular proteins is their helical and/or sheet-like organization. These secondary structures facilitate backbone–backbone hydrogen bonding in the folded protein core (reviewed in [12]). Secondary structures are conducive to tight tertiary packing as well. It has been shown that secondary-structure-like chain organization is enhanced by conformational compactness [280–282], but in the absence of hydrogen bonding such structures exhibit deviations from sharply defined α-helices and β-sheets [283–285]. These findings suggest that biophysical constraints of conformational compactness in conjunction with hydrogen bonding can go a long way in accounting for the basic architecture of globular protein folds.

A more recent study using computational sampling of homopolypeptide conformations suggested further that the current repertoire of globular protein folds is nearly complete in its coverage of all physically possible compact folds [286]. However, studies by three other groups have found instead that the current fold repertoire represents only a small fraction of all possible folds [287–289]. In particular, an investigation of the compact conformations of 60-residue homopolypeptides concluded that known protein folds constitute only approximately 5% of all physically possible folds, and that on average the natural folds have more local intrachain contacts (i.e. lower contact orders [146]) than the set of all possible folds, suggesting an evolutionary preference for structures with lower contact orders [288]. In response, a recent study argued that inasmuch as an appropriate criterion for matching simulated compact conformations and natural folds is applied, the existing library of single-domain PDB structures is probably complete in covering all physically possible folds [290]. A separate study by the same group indicated that computer-generated compact conformations tend to contain cavities resembling binding pockets in natural proteins as well, even in the absence of selection, suggesting that ‘many features of biochemical function arise from the physical properties of proteins that evolution likely fine-tunes to achieve specificity’ [291]. While the degree to which evolution has shaped the space of known protein folds remains to be further elucidated, the investigative effort described above is an excellent illustration of how explicit-chain biophysical models can be harnessed to address fundamental questions in protein evolution.

3.2. Simple exact models and other explicit-chain coarse-grained models of protein evolution

Sequence and structure spaces of proteins are vast. Coarse-grained explicit-chain models are valuable tools in the study of protein evolution. Currently, modelling the sequence–structure mapping by energetically and structurally high-resolution representations is often not practical, especially for addressing large-scale evolutionary changes involving many different protein folds. In this regard, lattice models—wherein conformations of model proteins are configured on two- or three-dimensional (2D or 3D) lattices—are particularly useful because of their computational tractability [292–294]. In view of their historical and current utility for investigating fundamental evolutionary issues (see, e.g., recent applications of 2D lattice models to study the basis of homology modelling [295], adaptive conflict [239] (§3.6) and long-term survivability [296]), a detailed assessment of the biophysical foundation of these models is in order.

3.2.1. Conformational enumeration

Among lattice models of protein evolution, simple exact models allow for exhaustive enumeration of all possible sequences and structures in the model [292]. These models include the two-letter 2D hydrophobic-polar (HP) model that uses a reduced alphabet consisting of only two types of residues, hydrophobic (H) and polar (P), to capture the prominent effects of hydrophobic interactions in protein energetics [137,161,162,297–299] (figure 5), two-letter variants of the 2D HP model (e.g. [293,301,302]), and a four-letter 2D model that also includes residues behaving somewhat like positive and negative charges [303]. Some 20-letter 2D models may also be considered as exact, because they consider all possible mutations in the immediate sequence-space neighbourhood of a given sequence [40,153].

Other types of lattice models have been used to study protein evolution as well. These models either restrict chain conformations to be maximally compact so as to allow model proteins with longer chain lengths to be studied [152,160,304], or rely to various degrees on sequence-space sampling instead of considering all possible sequences because of their usage of a 20-letter amino acid alphabet that entails many more sequences (e.g. [40,153,305]) or both (e.g. [306,307]). Restricting model structure space to maximally compact conformations in 2D [306] or 3D [307,308] reduces computation drastically because such conformations constitute only a tiny fraction of all possible conformations [280,309–312]. For instance, whereas the total number of all distinguishable conformations (not related by rotations and reflections) for a chain with 25 residues configured on the 2D square lattice is 5.768 299 665 [313], the number of maximally compact 25-residue conformations restricted to a 5 × 5 square, as considered in the study of Taverna & Goldstein [306], is only 1081 [309]. In 3D, the total number of all possible conformations (not related by rotations and inversions) on the simple cubic lattice for a chain with 27 residues is 11 447 808 041 780 409 [313,314], but the number of maximally compact 27-residue conformations restricted to a 3 × 3 × 3 cube, as considered by Deeds & Shakhnovich [307], is only 103 346 [280].

However, from a biophysical standpoint, it is important to keep in mind that real protein chains are not restricted to be maximally compact. Although behavioural trends predicted by models that use only maximally compact conformations may sometimes correlate with models that consider the full conformational ensemble, significant distortions of protein folding energetics are introduced by this approach [158,292]. In particular, for a given model sequence with a physically plausible interaction, the lowest-energy structure among maximally compact conformations may not be the true ground state structure, which is often less than maximally compact [299,315,316].

3.2.2. Model interactions and their biophysical basis

We next consider the physicality of the model interaction potential. Several examples presented below to illustrate recent biophysical insights into basic principles of protein evolution are based on the 2D HP model (figure 5). We choose the 2D HP model for this purpose because owing to
the model’s simplicity, its biophysical underpinning is transparent and intuitive; yet, the same underpinnings are closely related to those of more complex 2D lattice models with a biophysics-based 20-letter alphabet (see discussion below in this section). In fact, the biophysical criteria used to evaluate the 2D HP model may be applied as well to assess various 3D lattice models of protein evolution, including those that are being developed to study the impact of protein–protein interactions on evolution in model cells (§3.2.3).

One advantage of the minimalist HP construct is that, within the model, it allows for an exact, complete description of the sequence–structure mapping for short model proteins of lengths up to approximately 25 residues [316,317]. However, the extreme coarse-graining of both the sequence and structure spaces in this model means that energetic heterogeneity (diversity) among 20-letter real protein sequences with the same two-letter HP pattern is ignored; and mutations among 20-letter sequences with the same HP pattern are not considered. Obviously, the correspondence between model lattice conformations and real protein structures can only be intuitive.

Nevertheless, short-chain 2D HP models do capture a number of essential features of the sequence–structure mapping of real globular proteins. First, only a small fraction (approximately 2%) of HP sequences with chain lengths less than or equal to 30 have a unique lowest-energy structure

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**Figure 5.** The simple exact 2D HP lattice model is a useful tool for studying evolution across entire sequence and structure spaces. (a) An example HP model sequence of length 18. Hydrophobic (H) and polar (P) residues are depicted, respectively, as black and grey beads. A favourable energy is assigned to each hydrophobic–hydrophobic (HH) contact (as indicated by the orange connections between two black beads), other contact types are neutral (carry a zero energy). The total energy of a conformation is proportional to the number of HH contacts. For a given sequence, the energy of every conformation can be computed accordingly. The schematic drawing below the sequence shows the folding funnel of the sequence. Conformations with more HH contacts are placed lower, because they are energetically more favourable than conformations with fewer HH contacts. The conformation encased in the grey circle is the lowest-energy (native) conformation of the given sequence. (b) Conserved and variable sites in an HP model structure. Among all the HP sequences that fold to this structure, the sequence shown here is the one that provides the highest native stability. The number on each bead is the relative frequency of occurrence of an H residue at the given sequence position among the entire neutral set of 48 HP sequences encoding for this structure [137]. Most core hydrophobic positions cannot be mutated (1.0H, i.e. 100% H) without losing the native structure; one surface position must be polar (0.0H, i.e. 100% P); but most other surface positions could be mutated to either polar or hydrophobic. This means that a hydrophobic-to-polar substitution has a very different structural effect depending on its location (surface or core). (c) Epistasis in the 2D HP lattice model. The sequence on the right is a double mutant of the sequence on the left (mutated positions indicated by circles and arrows, respectively). As for a real protein, here the first mutation could occur in either of the two positions to produce a single-substitution intermediate sequence. One of the intermediate sequences is viable. It folds uniquely to the same structure (lower middle drawing in (c)), whereas the other intermediate sequence is misfolded: in addition to the original structure, this sequence adopts two other equally likely lowest-energy conformations as shown in the upper middle drawing in (c). Since the protein core is conserved, these epistatic interactions occur at the surface. In approximately 90% of such epistatic double mutants in the model (as calculated for the given example structure), the non-viable mutation is a P to H substitution. A real-world example of this form of epistasis is found in adenylate kinase [300].
as in real proteins (reviewed in [116,204]). However, certain modified, ‘shifted’ forms of MJ potentials that are similar to table VI of [329] with prominent repulsive energies [331] do not embody this biophysical property (reviewed in [158,204]), making it problematic to interpret some of the predictions from such models. For instance, in some cases, a shifted-MJ potential may lead to nominally charged residues instead of hydrophobic residues occupying the core of a model protein [114,204,331].

These limitations notwithstanding, all aforementioned lattice models provided useful evolutionary insights. Different approaches are often complementary because they tackle different aspects of protein evolution. However, caution should always be used to take these models’ limitations into account so as not to over-interpret model predictions. Major earlier progress of lattice models of protein evolution can be found in several reviews [292,332–334]. We now recall briefly some of the significant early efforts before highlighting recent advances.

### 3.2.3. Predictions and rationalizations

In several 2D [137,161,293,299,303,304,323] and 3D [305,335] lattice models as well as an off-lattice model [304], protein sequence space was found to be organized as multiple neutral nets. A neutral net is a network of sequences that are connected by single-point substitutions and encoding for the same folded structure. In a few studies that addressed the global connectivity of sequence space, it has been observed further that neutral nets for different folded structures are interconnected to form a dominant ‘supernet’ covering most of the sequence space [298,303,324] in a manner similar to that envisioned by Maynard Smith [336]. Consistent with experiment [139–142] (§2.4.2), mutations on encoding sequences often result in sequences encoding for the same structure [137,297,305], especially if the mutation site is on the surface of the folded protein [297]. In other words, many sequences are stable against mutation, a property referred to as mutational robustness [306].

Several models indicate that the topology of a protein neutral net has a superfunnel organization [137], wherein sequences encoding for the same structure tend to centre around a prototype sequence with maximum mutational robustness as well as maximum thermodynamic stability for the encoded native structure [137,293,304,323,335]. Mutational stability is generally correlated with thermodynamic stability [114,317], such that sequences at the edge of the neutral net have lower native stability [137,299,337]. Consistent with this trend, a 20-letter 2D lattice model predicts that, with increasing number of amino acid substitutions, the probability for a protein to retain its original structure declines exponentially [40].

Evolution of protein function has been explored using 2D square-lattice [296,301,302,330] and 3D diamond-lattice [335] model sequences that encode folded structures with a binding site. In the 2D square-lattice model studies of Bloom et al. [330,338], folded-state stability was found to promote evolvability of new binding functions, consistent with experimental observations. These simulated evolution processes allow for extensive exploration of sequence space. However, no structural change to the fold of the evolving lattice protein was seen during the simulation. Thus, the scope of protein evolution in these models [330,338] is largely limited to the
development of new binding abilities through modifications of surface residues, while the evolved protein retains its original structural scaffold. For larger structural changes, one expects that it would be harder for a more stable protein to evolve to a specific new fold, because a more stable sequence is farther away from the edge of the neutral net where it can switch to another fold (§2.7.2).

More recently, the binding between 2D square-lattice model proteins and lattice ligands was used to investigate short-term versus long-term evolutionary success. Interestingly, this study by Feldman and co-workers indicate that although the model evolution process is stochastic, long-term evolutionary success—as determined by stability of the evolving protein and its binding with a given ligand—is ‘surprisingly predictable’ from the founding sequence of a lineage. In this lattice protein model, long-term survivability is only partially determined by short-term fitness, i.e. short-term adaptive success does not guarantee long-term survival of a lineage [296].

2D lattice models have been applied to compare the evolutionary effects of point mutations and recombination [152,324,335]. An initial study showed that crossover of two encoding sequences is an effective way of producing a new encoding sequence, suggesting that local sequence patterns are important for determining whether the full protein sequence can fold to a unique structure [324]. This theoretical prediction is consistent with subsequent experiments on β-lactamase indicating that for a given number of amino acid substitutions, recombined variants are much more likely to retain function than variants generated by random point mutations [339]. In another simulation study, evolutionary dynamics that admits both point mutations and recombination leads to a much higher concentration of population in the prototype sequence than if evolution proceeds via point mutations alone, suggesting a significant role of recombination in the prototype-like behaviours of natural proteins [152].

Lattice models have also been used to investigate how mutagenesis data may be exploited to improve forcefields for protein structure prediction [340,341]. In addition, they have provided insights into the relationship between the native contact pattern of a target structure and its designability [342], the degree to which evolutionary selection at the molecular and/or organism level has led to the observed scale-free distribution of protein structure similarities [307,334], and the biophysical basis [343,344] of methods that use evolutionary/mutational information as a probe to reveal long-range energetic coupling in proteins [26].

An exciting recent development of 3D maximally compact lattice protein models is their application to the study of fitness and population dynamics of model cells containing multiple proteins. One model assumes that all proteins in a model cell are essential and that the fitness of the cell depends on the stability of the least stable proteins it contains. Real-life-like properties such as preferred folds and protein families readily emerge from these simple assumptions [308]. Introduction of various protein–protein interactions to this modelling set-up by Shakhnovich and co-workers [345] has provided further rationalization for the emergence of species-like collections of model cells with very similar sequence make-ups, an increased rate of mutation in stress response [346], as well as a trade-off between strengthening functional interactions and avoidance of misinteractions as observed in experimental proteomic data [347]. More recently, Zhang and co-workers [99] developed a related lattice approach to model evolution of protein–protein interactions that offers an explanation of slow evolution of highly expressed proteins in terms of stronger constraints on these proteins to avoid misinteractions.

3.3. Energy, entropy, fitness, protein neutral nets and fitness/mortality landscapes

Protein evolution can be formulated in terms of population dynamics on a fitness or adaptive landscape in which a fitness function assigns a fitness value to each protein sequence, with evolving populations migrating to areas of higher fitness over time [348–350]. Certain parallels may be drawn between fitness landscapes defined on sequence spaces and energy landscapes defined on protein conformational spaces. Mathematically, both protein sequence and conformational spaces are high-dimensional. As for protein energy landscapes, fitness landscapes are intrinsically high-dimensional constructs, even though fitness and energy landscapes are often depicted as 1D profiles or 2D surfaces for metaphorical, conceptual visualization [34,254,317,348,351]. With this in mind, it is important to focus on the quantitative fitness function itself and not to over-interpret the picturesque 1D and 2D landscape representations [351].

The biological fitness concept has been compared to the physical quantity of negative energy or negative free energy [352]. There is an obvious analogy between the sequence-space distribution of steady-state evolutionary population and the conformational-space distribution of equilibrium population. Just as lower-energy states and higher-entropy macromolecules are more favored in statistical mechanical systems, higher-fitness sequences and phenotypes encoded by more sequences are expected to be more populated during evolution. Under certain limiting conditions, a direct quantitative correspondence can be made between fitness and energy, as well as between population size and inverse temperature [352]. In general, however, there is an important difference between the analogous roles of energy and fitness: insofar as a statistical mechanical system is ergodic [353] and total population is conserved, the equilibrium population of a given state is determined by its energy in accordance with the Boltzmann distribution. By contrast, for evolution, total population can be increased by reproduction and decreased by lethal mutations. As a result, the steady-state population of a given sequence in a large evolving population is determined not only by reproductive fitness of the sequence but also by its connectivities to neighbouring viable sequences. This issue will be addressed in the discussion on mutational robustness in §3.4.

Studies of molecular evolution indicate that evolutionary pathways on fitness landscapes are subjected to various constraints [351,354]. The topographies of fitness landscapes of real proteins are far different, on average, from those postulated theoretically by random assignments of fitness [351]. This observation underscores the importance of biophysical considerations in constructing model fitness landscapes. As an illustration, figure 6 shows the sequence space of a 2D HP model (figure 6a), part of its neutral net organization (figure 6b), and a model fitness landscape that identifies fitness with native stability (figure 6c). As discussed above, the biophysics embodied by the HP model imparts it with several essential protein-like properties. For instance,
consideration of all model HP sequences that fold uniquely to the native structure in figure 5a [137,204] shows that certain sites are conserved, and that the probability that a mutation is viable is site-dependent (figure 5b), echoing the context-dependent substitution rates of amino acids in real proteins (§1). It is also noteworthy that because the main biophysical driving forces in protein folding are different from that in RNA secondary structure formation, the global organization of neutral nets of globular proteins is probably very different [161,355] from that of RNA [356–359].

It is conventional to associate increasing fitness with upward movement on fitness landscapes [348]. For the model fitness landscape in figure 6c, however, we have chosen to represent increasing fitness with downward movement. As discussed elsewhere, such landscapes may be referred to as ‘inverse-fitness’ or ‘mortality’ landscapes [324]. We prefer such depictions because our inescapable experience with gravity on Earth makes it easier for us to appreciate natural driving forces that point downward than ones that point upward. In this respect, mortality landscapes may offer a better metaphor for the drive by natural selection towards lower mortality and thus higher fitness. In analogy with the most favoured protein conformation having the lowest free energy in the conformational energy landscape, the fittest sequence is seen as situated at the bottom of an attractive basin with lowest mortality in the sequence-space mortality landscape [324].

**3.4. Mutational robustness, sequence-space topology and population evolution**

Experiments showed that many natural globular proteins are robust to mutations [40,53,55,202,360]. The observation that proteins with diverse primary sequences can be grouped into families with very similar structure and function gives further illustration of this robustness [361]. A folded structure that has a larger neutral net is more designable [160,322]. Compared to a less designable structure, it is expected to be more robust against mutations and thus more likely to be populated by evolution. From a biophysical standpoint, robustness may be viewed as a form of entropy in sequence space [306]. As discussed above, an idea of sequence-space ‘entropy’ (§3.3) is useful for analysing possible origins of marginal stability of natural globular proteins [135] (§2.4).

There are at least two biophysical reasons for the observed mutational robustness of natural globular proteins. First, a significant part of the stabilization of specific secondary structures in protein comes from backbone hydrogen
bonding. With proline as the only exception, amino acid substitutions do not change the ability of backbone atoms to form hydrogen bonds. While the nature of the amino acid sidechain has an effect on the preferred backbone dihedral angles and interactions with surrounding atoms, an α-helix or a β-sheet can be formed by many different combinations of amino acids along the primary sequence [362, 363]. Second, the biophysics of hydrophobicity results in a rough grouping of a globular protein’s amino acids into non-polar residues in a largely conserved hydrophobic core and polar surface residues. Surface residues with higher solvent accessibility are more tolerant to mutations [22] and thus contribute to mutational robustness because, on an individual basis, they are less crucial for stability (see lattice model example in figure 5).

Mutational robustness is not determined solely by the number of sequences encoding for a certain structure or property. Another important factor that contributes to mutational robustness of any given sequence is its connectivity with other viable sequences. The pattern of mutational connections among sequences has also been referred to as sequence-space topology [137, 151]. Among sequences with the same fitness, analytical and simulation studies have shown that evolving populations tend to prefer sequences that have more viable mutants. Fundamentally, this relative concentration of steady-state evolutionary population at sequences that are mutationally more stable (i.e. robust) arises from the fact that they lose less population to lethal mutations than sequences that are mutationally less robust [137, 151, 292]. As long as the evolving population N is sufficiently large and satisfies $\mu N \gg 1$, where $\mu$ is the mutation rate [151], the phenomenon is independent of $\mu$ and N [137, 151]. A lattice-model example that illustrates how sequence-space topology affects evolutionary population under the $\mu N \gg 1$ condition is provided in figure 7. It should be noted, however, that when the $\mu N \gg 1$ condition is not satisfied, the significance of sequence-space topology on evolutionary population diminishes [151, 153]. In the limit of $\mu N \ll 1$, evolutionary population dynamics on a neutral net becomes a random walk without regard to the relative abundance of connectivities of different sequences [151].

Figure 7a demonstrates that the role of fitness in steady-state evolutionary population distribution does not correspond directly to that of energy in equilibrium population distribution. Microstates having the same energy have equal equilibrium populations in a canonical ensemble [353]. In contrast, although all sequences are assumed to be equally fit in figure 7a; their steady-state evolutionary populations are different when $\mu N \gg 1$ because of sequence-space topology, with the prototype sequence that has maximum native stability and is also mutationally most robust achieving the highest population among sequences belonging to this sequence-space superfunnel [137]. However, this relative concentration of population at the prototype sequence is weak [137, 152, 153, 335]. Thus, sequence-space topology alone is probably insufficient to account for the experimentally observed dominance of prototype-like sequences in natural proteins [139–142]. This recognition led to the argument in §2.4.2 that the distribution of evolutionary population in natural proteins has been driven significantly by selection for native thermodynamic and/or kinetic stability. Here, using the same explicit-chain lattice modelling set-up, we show in figure 7b how a selection for stability may work in concert with sequence-space topology to beget a dominant population at the prototype sequence. This example also illustrates how mutational robustness of natural globular proteins may have arisen by selection for native stability without a direct selection on robustness itself [364].

![Figure 7](image-url)
3.5. Evolvability, hidden states and promiscuous functions

Mutational robustness is often discussed in conjunction with evolvability, which characterizes the ability of a biological system to evolve new traits [164,365–368]. The relationship between robustness and evolvability has been seen as opposing evolutionary forces, with the former impeding and the latter promoting evolutionary innovation. However, a network-based view of mutational robustness and evolvability indicates that they are not mutually exclusive [369,370]. Although mutational robustness implies that the sequence-space distance to any specific alternative phenotype is large (§2.7.2 and 3.2.3), general evolvability can be enhanced by the mutational robustness afforded by a larger neutral network because different positions on such a network are likely to be mutually close to many different phenotypes [294,371,372], as has been demonstrated experimentally for the evolution of enzyme functions [55,202,265,338].

These observations led to an understanding that seemingly neutral mutations can dramatically alter the future potential of a protein to evolve towards new functions. The hidden evolutionary potential of a neutral network is embodied in a wide variety of latent phenotypes that were not under selection originally but are present biophysically, because these mutational variations do not affect the main function of the protein. Several studies have shown how the co-option of neutrally evolved properties can allow adaptation towards new functions under the shadow of a dominant existing function. For example, enzyme promiscuity—which refers to low-affinity binding of molecules resembling an enzyme’s main ligand target [373–375]—has been demonstrated as a potent mechanism for adaptations [264]. In the same vein, latent evolvable traits have also been identified in the evolution of steroid receptor specificity [376], allostery [201], gene regulation [377] and metabolic networks [378]. The term ‘exaptation’ (or ‘spandrels’ [138]; §2.4.1) has been coined for such latent traits that arise by chance and may or may not evolve to have a new function [379]. Apart from point mutations, mobile genetic elements are likely to play a crucial role in providing exaptations [380–382]. Each genome appears to constantly produce transcribed and translated ‘proto-genes’ that arise by chance, some of which may be exapted by evolution for a certain function [383].

It follows that a major part of the enhancement of evolvability by mutational robustness is based on the evolutionary potential provided by conformational dynamics at the level of a single sequence when excited-state structures of a protein [173,384–386] [§2.9] with beneficial function come under natural selection. Selection of such a promiscuous function rewards mutations that further stabilize the beneficial excited state. In this scenario, a protein can retain its original ground-state native structure while at the same time stabilizing an excited-state structure incrementally, thus maintaining continuous viability during evolutionary transitions. Eventually, the protein may first become bi-stable then switch to the selected excited-state conformation as its dominant structure (see lattice-model example in figure 8) or switch to the new dominant structure directly, as has been observed in protein design experiments (figure 4).

Consistent with experiment [202], lattice-model studies [178,203] indicate that an evolutionary process that takes advantage of excited-state selection is much more efficient than a process that applies selection pressure only on the dominant function [203]. In this perspective, bi-stable or multi-stable proteins at the peripheral of neutral networks—as exemplified by the overlapping regions in the lattice-model example in figure 6b—and proteins that sacrifice stability for functional promiscuity, as is the case in some antibodies [387], should be more evolvable towards new functions underpinned by significantly different native structures than sequences with high mutational robustness and thermodynamic stability. The latter sequences, however, can be more evolvable towards new functions that are still based upon the original structural scaffold [330,338].

3.6. Adaptive conflicts: evolution under constraints

While selection of latent traits can be an efficient route to new function, such an evolutionary process raises a basic...
question regarding biophysical limits on the degree of multi-functionality or promiscuity that can be carried within one protein molecule. Multi-functionality also bears the danger of creating an adaptive conflict. Such a conflict can emerge whenever adaptation on the same gene is driven by two or more different or even mutually exclusive functional requirements. In the extreme case of viruses with severely constrained genome sizes, adaptive conflict can arise from overlapping open reading frames encoding for different proteins within the same DNA sequence [388]. For a multi-functional protein, adaptive conflict arises when enhancing one subfunction impedes another subfunction.

As far as adaptive conflicts in a protein is concerned, if multi-functionality is realized by the presence of different binding interfaces on the protein surface, a small number of such interfaces may coexist, limited by such factors as the protein size and/or surface area and the number of surface hydrophobic residues that can be tolerated without causing misfolding. Binding interfaces can also overlap, using some but not all of the same residues for different ligands [128,129]. In that case, an adaptive conflict may be anticipated since increasing the binding affinity for one interface through mutations may interfere with the binding affinity of the second interface, and vice versa. If multi-functionality is underpinned by bi-stability or multi-stability, i.e. the coexistence of and dynamic inter-conversion between alternative functional conformations (§2.7.2), it is expected biophysically that only a narrow capacity for accommodating several distinct conformations can exist in the lifetime of a globular protein and perhaps a somewhat higher capacity for doing so in disordered proteins. During evolution, a general mechanism for resolving adaptive conflict is offered by gene duplication and subfunctionalization [389], which we will discuss briefly in §3.7.

3.7. Protein divergence driven by gene duplication and mutational robustness

Evolution of bi-stable and more generally multi-stable proteins is an efficient means to meet new functional needs; but multi-functional proteins often serve as evolutionary intermediates rather than long-term solutions. Explicit-chain biophysical models suggest that multi-stable globular proteins are mutationally much less robust than globular proteins with an essentially unique native structure [161,178,239]. This trend is readily seen in the 2D HP lattice model example in figure 9, which shows that the sequence-space ‘entropy’ of bi-stable sequences (magenta area) is much smaller than that of sequences folding uniquely for either one of the two structures encoded by the bi-stable sequences (blue and red areas). This is a prediction that should be testable experimentally, for example, by using the recently designed bi-stable proteins [65,174,206] (figure 4). In this picture, the short-term advantage of bi-stability/bi-functionality is expected to give way eventually to an alternative sequence-space arrangement that is mutationally more robust, provided that the gene encoding for the bi-stable protein is duplicated at some point in the evolutionary process.

Subfunctionalization, or functional divergence after gene duplication, is a ubiquitous phenomenon in evolution [389,390]. For example, an experimental study of the reconstructed common ancestor of the fluorescent proteins in corals that emit either red or green light was found to emit commencement of selection for promiscuous function.

![Figure 9](https://rsif.royalsocietypublishing.org/content/11/104/204499.f9)

**Figure 9.** The simulated evolution, with gene duplication, of an essentially infinite HP sequence population under an adaptive conflict of two selection pressures. Here, four stages of the evolutionary dynamics are shown by representative changes in the distribution of evolutionary population and average population fitness $\Delta W$ from one stage to the next. The two adjacent neutral nets (blue and red) are the same as those in figure 8. Distributions of population are plotted by logarithmic scales in the same style as figure 7. Initially, before the native structure of the red network is selected, nearly the entire population occupies the most stable HP sequence population under an adaptive conflict of two selection pressures. In that case, an adaptive conflict may be anticipated since increasing the binding affinity for one interface through mutations may interfere with the binding affinity of the second interface, and vice versa. If multi-functionality is underpinned by bi-stability or multi-stability, i.e. the coexistence of and dynamic inter-conversion between alternative functional conformations (§2.7.2), it is expected biophysically that only a narrow capacity for accommodating several distinct conformations can exist in the lifetime of a globular protein and perhaps a somewhat higher capacity for doing so in disordered proteins. During evolution, a general mechanism for resolving adaptive conflict is offered by gene duplication and subfunctionalization [389], which we will discuss briefly in §3.7.

![Gene duplications fixed due to dosage effect](https://rsif.royalsocietypublishing.org/content/11/104/204499.f9b)

**Figure 9.** The simulated evolution, with gene duplication, of an essentially infinite HP sequence population under an adaptive conflict of two selection pressures. Here, four stages of the evolutionary dynamics are shown by representative changes in the distribution of evolutionary population and average population fitness $\Delta W$ from one stage to the next. The two adjacent neutral nets (blue and red) are the same as those in figure 8. Distributions of population are plotted by logarithmic scales in the same style as figure 7. Initially, before the native structure of the red network is selected, nearly the entire population occupies the most stable HP sequence population under an adaptive conflict of two selection pressures. In that case, an adaptive conflict may be anticipated since increasing the binding affinity for one interface through mutations may interfere with the binding affinity of the second interface, and vice versa. If multi-functionality is underpinned by bi-stability or multi-stability, i.e. the coexistence of and dynamic inter-conversion between alternative functional conformations (§2.7.2), it is expected biophysically that only a narrow capacity for accommodating several distinct conformations can exist in the lifetime of a globular protein and perhaps a somewhat higher capacity for doing so in disordered proteins. During evolution, a general mechanism for resolving adaptive conflict is offered by gene duplication and subfunctionalization [389], which we will discuss briefly in §3.7.

![Steady state: subfunctionalization](https://rsif.royalsocietypublishing.org/content/11/104/204499.f9c)

**Figure 9.** The simulated evolution, with gene duplication, of an essentially infinite HP sequence population under an adaptive conflict of two selection pressures. Here, four stages of the evolutionary dynamics are shown by representative changes in the distribution of evolutionary population and average population fitness $\Delta W$ from one stage to the next. The two adjacent neutral nets (blue and red) are the same as those in figure 8. Distributions of population are plotted by logarithmic scales in the same style as figure 7. Initially, before the native structure of the red network is selected, nearly the entire population occupies the most stable HP sequence population under an adaptive conflict of two selection pressures. In that case, an adaptive conflict may be anticipated since increasing the binding affinity for one interface through mutations may interfere with the binding affinity of the second interface, and vice versa. If multi-functionality is underpinned by bi-stability or multi-stability, i.e. the coexistence of and dynamic inter-conversion between alternative functional conformations (§2.7.2), it is expected biophysically that only a narrow capacity for accommodating several distinct conformations can exist in the lifetime of a globular protein and perhaps a somewhat higher capacity for doing so in disordered proteins. During evolution, a general mechanism for resolving adaptive conflict is offered by gene duplication and subfunctionalization [389], which we will discuss briefly in §3.7.
both red and green light [391]. Subfunctionalization of a duplicated multi-functional gene is probably a more efficient evolutionary route than neo-functionalization, which necessitates evolution of new function in a duplicated gene from scratch. However, in the sub-functionalization route, this process can be facilitated by selection on latent traits [§3.6] before gene duplication [239]. In the lexicon of fitness landscape, gene duplication amounts to doubling the number of dimensions of sequence space and thus may be viewed as an ‘extradimensional bypass mechanism’ for resolving adaptive conflicts [392].

Functional or structural divergence can be driven by an increase in fitness when a pair of identical bi-stable sequences is transformed into two subfunctionalized sequences. Generally speaking, such an increase in fitness is biophysically plausible because each of the subfunctionalized sequences may afford a higher kinetic stability [81] to one or the other functional structure than that provided by a bi-stable sequence [239]. However, divergence does not always have to be adaptive. Even if the fitness of a sub-functionalized pair is identical to the fitness of a pair of bi-stable sequences, the inherent tendency of protein evolution towards higher mutational robustness can still drive sub-functionalization. Biophysically, an ensemble of protein structures subjected to more overlapping functional constraints is likely to be more restricted in sequence space, resulting in low mutational robustness, as is exemplified in figure 9. When the constraints are lifted through gene duplication or changes in the environmental selection pressures, evolution will naturally favour mutations that result in higher mutational robustness even if there is no gain in functional fitness for the proteins in the process. This scenario is illustrated by the model evolutionary dynamics in figure 9. Simulation results summarized in this figure indicate that after an adaptive pressure to simultaneously select two structures is imposed, the evolving proteins first attempt a short-term resolution of the adaptive conflict using bi-stable sequences with low mutational robustness. Subsequently, upon gene duplication, a process of divergence that is essentially neutral ensues, with each copy of the originally bi-stable protein evolving towards the central, high-robustness region of one of the two neighbouring neutral networks [239] (figure 9).

3.8. Epistasis and co-evolution of interacting amino acid residues

The phenomenon of epistasis, referring originally to non-additivity of genetic effects caused by gene interactions (e.g. [393]), can also manifest within a protein molecule. Mutational effects on stability or function at different sites of a protein can be non-additive when the sites are energetically coupled [394]. A consequence of this biophysical property is that the overall evolutionary effect of multiple mutations can depend on the order in which the mutations are made. For the same given set of mutations, it may be that one temporal order of mutations is evolutionarily favoured because it entails a monotonic increase in fitness, whereas another order of mutations is disfavoured because it involves an intermediate step that decreases fitness. Several studies have demonstrated this type of epistatic behaviour in proteins and its constraints on evolutionary pathways [42,53,300,395–398]. For instance, experiments on adenylate kinase indicate that a double mutant with higher stability can only be obtained via one mutation path [300]. When the order of the two mutations was changed the protein could not fold. This type of behaviour is readily observed in simple biophysical models of protein evolution, as is illustrated by the HP model example in figure 5c.

An implication of epistasis is that the propensity for a viable mutation at a certain site in a protein structure may not be fixed. Rather it should depend on the preceding mutations at other sites. This phenomenon is illustrated by a recent simulation study of the evolution of purple acid phosphatase by generating random mutations in the structure [58]. Based on stability calculations in the model, the propensities for all amino acids at selected solvent-exposed and buried sites were determined. The results indicated considerable variations of these propensities because stability effects of amino acid substitutions at a given site change with time as the evolutionary process progresses. The simulation showed that an enforced destabilizing mutation (which could arise in real proteins owing to functional constraints such as the need to preserve an active site) can be compensated by subsequent mutations, thus increasing the future viability of the already-mutated residue at that same site and rendering the reverse mutation detrimental and therefore less probable [58]. Although this model probably over-estimated native stability as well as stability effects of mutations and underestimated the probability for misfolding because of its simplified treatment of the unfolded state (see discussion above on marginal stability; §2.4.2), it offers an excellent elucidation of the ‘holistic’ nature of intra-protein interactions and the biophysical forces that govern the mutational effect on stability and how it may depend on the temporal order in which the mutations occur [58].

Epistatic effects are common but not universal. Strong epistasis arising from significant evolutionary shifts in the stability effects of mutations as envisioned in [58] may even be rare [57]. In the example of the influenza nucleoprotein, an experimental analysis of mutations in a set of homologues showed that stability effects of mutations with no clear functional benefit are largely conserved across homologues, mostly additive and exhibit no aforementioned [58] strong dependence on temporal order [57]. It has been argued that mutations in viral proteins in general—which probably have evolved to buffer deleterious mutations—are not likely to exhibit strong epistasis [399]. Whenever the functional benefits outweigh the cost of destabilization of a mutation, strong epistatic effects are more likely to follow [21]. Nonetheless, a weaker form of epistasis can occur even if stability effects of mutations are conserved because different temporal orders of stability changes can result in drastically different survivabilities. For instance, a recent experimental study of the 39 mutations on the nucleoprotein of the influenza virus between years 1968 and 2007 identified several mutations that decrease the stability of the protein significantly when introduced individually to the starting 1968 protein, thus suggesting strongly that these mutations were preceded by ‘enabling’ mutations that increase native stability. An inferred evolutionary trajectory was constructed based on the stability constraints [42]. Epistasis has also been revealed by studying disease-causing single mutations in humans and comparing them with compensated mutations that do not cause disease in other species [400–402]. One estimate indicates that 80% of pathological mutations result in protein stability changes [403]. When the compensated pathological mutations are compared against uncompensated pathological mutations, compensated mutations are mostly found at solvent-exposed positions and the amino acid substitutions are ‘milder’,
entailing, for example, less changes in hydrophobicity [400–402]. These experimental trends are consistent with the biophysical principles of protein structure and stability expounded here. A more detailed discussion of the biophysical nature of epistasis and compensatory evolution is available in a recent review [404].

3.9 Fitness landscapes for multiple phenotypic properties

Natural protein evolution takes place in a highly wired, interacting molecular system. Ultimately, therefore, studies of protein evolution have to take into account a complex molecular context [392] (see §3.11). The expanded concept of molecular phenotype discussed in §2.9 is an attempt towards a better account of this biophysical reality. In this regard, highly simplified yet promising explicit-chain biophysical models have recently been developed to study protein evolution in the context of protein–protein interactions [99, 347] (§3.2.3) We have also summarized explicit-chain biophysical models that take into consideration the ensemble nature of a protein’s conformational phenotype, and how these models can provide insights into selection of promiscuous functions, bi-stability and structural divergence (§3.2).

With the understanding that the fitness of a protein sequence depends not only on its ground-state native structure, but also on its entire conformational distribution as well as potential functional interactions and detrimental misinteractions with other molecules, a critical issue in modelling is how to assess contributions from different phenotypic properties to the overall fitness. For example, in the bi-stable fitness landscape in figures 8 and 9, the combined fitness is taken as the sum of two fitness values, one for each structure [178, 239]. Although this modelling scheme is useful for illustrating general principles, it would be too simplistic when applied to real-life situations. Different forms of multifunctionality may require different rules for combining fitness contributions. Ideally, a fitness function should include not only positive contributions from selected biophysical properties, but also account for the negative effects on folding rates and misfolding, as well as aggregation and misinteraction. Thus, protein evolution in general entails a multi-factorial optimization problem where only something like a Pareto optimality can be achieved, i.e. a satisfaction of multiple optimality criteria just above a minimum threshold of optimality for each criterion [405]. One simple example would be the trade-off between thermodynamic stability of a folded protein versus the need for conformational dynamics in biochemical functions and degradation. Both these criteria probably cannot be fully satisfied, but a Pareto optimality may be achieved such that the protein is stable enough to maintain the same fold yet flexible enough to allow binding.

Constructing more realistic fitness functions will be a challenging task. Genomic information is abundant; but pinpointing mutational impact on cellular function by experiment is often daunting. Theoretical/computational investigations can assist greatly in this endeavour by developing more comprehensive models that account for various biophysical constraints on protein evolution. Two recent examples will be discussed in §3.10 and 3.11 to illustrate how incorporating information about protein–protein interactions into biophysical models can advance understanding of experimentally observed evolutionary patterns.

3.10 Biophysical links between protein expression level and evolutionary rate

A fundamental evolutionary question is why different proteins evolve at different rates. What makes some proteins less likely to accept new mutations than others? Can the different evolutionary rates be explained in terms of the biophysical constraints on mutations as outlined in figure 3?

One hypothesis is that proteins that are functionally more important are more conserved, because the cell cannot risk their function to be compromised in any way, even slightly. Some authors have linked the evolutionary rate to the position of a protein in the protein–protein interaction network, finding that ‘hub’ proteins involved in many interactions evolve more slowly [406]. Empirically, evolutionary rate was found to be most strongly anticorrelated with the expression level [407]. This trend is not inconsistent with the functional argument. A protein is essential to an organism if the organism fails to survive when the gene encoding for the protein is deleted from its genome [408, 409]. Many essential proteins are highly expressed, as the cell needs a constant supply for its most basic and vital functions. However, is the slower evolutionary rate of highly expressed proteins a result of the importance of their functions or a more direct consequence of their high concentrations in the cell? Several biophysical mechanisms have been proposed for the latter scenario.

Here we summarize briefly two mechanisms that are based, respectively, on protein misfolding and protein misinteraction, noting however that an explanation in terms of mRNA folding rate has also been put forth recently [410]. Multiple mechanisms can be at play because the proposed mechanisms are not mutually exclusive.

All proteins have to avoid misfolding. Taking the population of a protein sequence as a whole, a protein that is abundantly populated provides more opportunity for the formation of detrimental misfolded structures than a protein that is sparsely populated; thus the constraint imposed by misfolding avoidance is stronger on protein sequences with higher populations. A similar consideration applies to the misinteractions, which will be discussed further below.

Accordingly, the need to prevent or at least minimize misfolding caused by translation error has been proposed as a major constraint on the evolution of highly expressed proteins [411–414], leading to slower evolution. Consistent with this picture, highly expressed proteins are selected to be more robust against translation errors by using synonymous codons with the smallest chance of producing non-synonymous changes as a result of translation errors. Apart from translation errors, the need to avoid misfolding of the properly translated protein also constitutes a strong evolutionary constraint on highly expressed proteins, resulting in preferential usage of amino acid residues that minimize misfolding [413]. These restrictive requirements lead to proteins that are both slowly evolving and thermodynamically more stable [414, 415].

Another probable biophysical constraint behind the anti-correlation between protein expression level and evolutionary rate is the need for a protein to avoid misinteraction with other proteins [99]. This selection pressure affects primarily surface residues that can potentially participate in interactions between the protein and other molecules. Thus, its effects are to some degree complementary to that arising from the need for folding stability, kinetic accessibility of the folded structure
and avoidance of misfolding. The latter selection pressures affect primarily buried residues but can also affect surface residues. Misinteractions may be caused by the same exposed hydrophobic surface residues that are part of the functional protein–protein interactions, leading to an adaptive conflict between increasing the strength of functional interactions and avoiding misinteractions [347]. This conflict can result in further constraints that limit the viability of mutants of highly expressed proteins. As a consequence of these biophysical constraints, evolution might have increased the proportion of functional monomeric proteins with hydrophilic surfaces, reduced the abundance of functional multi-chain complexes, weakened the strengths of functional interactions, or increased the degree of disordered protein interactions to minimize exposed hydrophobies while still allowing many interaction partners [230,347]. In other words, such strategies might have contributed to the evolution of interaction network topologies that can better alleviate the conflict between functional interactions and misinteractions [110].

### 3.11. Protein evolution in the context of functional networks

As emphasized above, proteins do not act in isolation in living organisms; hence a full understanding of the function and evolution of a protein should take into account its interactions with other biomolecules and metabolites [392,416]. It would be daunting to account for these interactions in all their complexity at the molecular level. Using simple explicit-chain protein models, conceptual advances were made in elucidating how the biophysical constraint of misinteraction avoidance might impact protein evolution [99,347] (§3.10). However, investigators have to rely upon abstract descriptions of protein interactions, using model parameters extracted from experimental data on binding and on the effects of enzymatic activities on biochemical reaction rates. With an increasing repertoire of genomic data, this approach has produced significant progress. For instance, the recent mapping of a realistic network of DNA sequences bound by the same transcription factor [417] has afforded new evidence in support of the idea that a large genotype network enhances both mutational robustness and evolvability (§3.5). Important advances have also been made by taking an abstract approach in the study of metabolic networks [418]. Notably, a reductive evolution algorithm was applied to determine minimal viable genomes for *E. coli* [419]. In principle, the effect of a mutation on metabolism is difficult to predict, because it affects not only the activity of the mutated protein but also many downstream events. Yet metabolic networks are often found to be robust against perturbations such as gene deletions and loss-of-function mutations because of ‘distributed robustness’, i.e., an ability of the network to compensate for the local perturbation by systemic adjustments [420]. *In silico* metabolic networks have also shed light on the evolution of specialist versus generalist enzymes. By analysing a model network of *E. coli* metabolism, it was found that specialists are very efficient at catalysing single metabolic reaction steps, responsible for a high metabolic throughput, and often essential to the cell. These functional roles necessitate more regulation of its activity. Consequently, specialists require a much higher degree of maintenance than generalists that are promiscuous and multi-functional. This model study thus offered an explanation for why specialists have not replaced all the generalists in real organisms [421].

A more recent computational study used flux balance analysis [422] and random re-wiring of a realistic model metabolic network [423] to study evolution of a model cell under a selection pressure to survive on a given carbon source [378]. The simulation showed that selection for one carbon source also allows the model cell to survive on a number of other carbon sources that were not selected for. This finding demonstrated that metabolic systems embody latent evolutionary potentials, and that beneficial traits can arise non-adaptively through exaptations [379] in the absence of selection at the level of metabolic network [378], as is the case we have seen at the molecular level (§3.5).

Although it is currently not feasible to apply explicit-chain models of proteins in the simulation of a realistic cellular metabolic network, a recent evolutionary population dynamics study was able to incorporate energetic information of explicit-chain continuum (off-lattice) models for 10 proteins from the folate biosynthetic pathway [59]. The study considers a population of 1000 model cells. The fitness of each model cell is taken to be the total metabolic output of the model biosynthetic pathway minus the number of misfolded proteins, with both of these quantities dependent upon the thermodynamic folded-state stabilities of the proteins. Protein stabilities are in turn computed using a biophysical potential function. Simulation results from this model provide a protein-based molecular biophysical rationalization for the distribution of stabilizing and destabilizing mutations and other experimentally observed patterns of polymorphisms [59].

### 4. Outlook: enriching the biophysics of protein evolution

As this review emphasizes, evolution is ultimately a physicochemical process that cannot be fully comprehended without biophysics. Likewise, because evolution happened under biophysical constraints, evolutionary information can help decipher aspects of protein biophysics that are still too complex or too costly to be tackled by first-principles physical or chemical methods. In closing, we provide a few further examples to showcase the productive research directions in which future progress will probably be made by this synergistic approach.

#### 4.1. Synergy between biophysics and the study of protein evolution

Perhaps the most direct way to access the change in biophysical properties during the long evolutionary history of natural proteins is to perform experiments on ‘resurrected’ ancestral proteins. Recently, several putative ancestral protein sequences have been constructed computationally using common phylogenetic methods and then synthesized in the laboratory [82,391,424–428]. We have mentioned thioredoxin as one of the proteins that were studied in this manner in the discussion on kinetic stability (§2.2). A more recent computational study used flux balance analysis [422] and random re-wiring of a realistic model metabolic network [423] to study evolution of a model cell under a selection pressure to survive on a given carbon source [378]. The simulation showed that selection for one carbon source also allows the model cell to survive on a number of other carbon sources that were not selected for. This finding demonstrated that metabolic systems embody latent evolutionary potentials, and that beneficial traits can arise non-adaptively through exaptations [379] in the absence of selection at the level of metabolic network [378], as is the case we have seen at the molecular level (§3.5).

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mammalian cells much later during evolution, indicating once again that latent functions, or exaptations (§3.5), can play important roles in protein evolution [376]. The study of these putative ancestral proteins indicates further that epistatic interactions within the structure of hormone receptors have led to surprisingly irreversible evolutionary pathways [430].

The utility of an ancestral reconstruction, however, is only as good as the accuracy of the phylogenetic relationships it assumes. As mentioned earlier, mutation rates depend not only on amino acid residue type, but also on the conformational context of the site being mutated; but many common phylogenetic methods do not account for this dependence (§1). One of the structural properties that exhibit significant correlation with mutation rate is conformational diversity. The conformational diversity exhibited by a single protein sequence is also reflected in the conformational diversity among the sequences in the family to which the protein belongs [431]. Local packing density of an amino acid position, which correlates negatively with local backbone conformational diversity (flexibility), was also found to correlate negatively with evolution rate. In other words, amino acid positions that have a lower local packing density and are more flexible locally tend to evolve faster. The correlation of evolutionary rate with local packing density is even stronger than that with solvent accessibility [432]. More recently, it has been suggested further that this strong correlation is a reflection of a fundamental relationship between evolutionary rate and the energetic stress caused by random mutations since average mutational stress is proportional to local packing density in an idealized elastic network model of protein structure [433]. This general trend is also consistent with the observation that IDR sites typically evolve faster than globular proteins except when the IDR site is involved in the binding of multiple partners (§2.8.2). Taken together, the findings summarized above contradict another group’s earlier finding that evolutionary rate is negatively correlated with conformational diversity [434,435]. The correlation coefficients computed from the limited dataset considered in the earlier study were, however, all very small [436].

As mentioned in §1, a recent method makes use of the information about solvent exposure of residues in a known protein structure to identify sites in a protein-coding gene that have undergone positive or negative evolutionary selection [23,24]. Although synonymous mutations are not necessarily neutral [95–97,436], the ratio of non-synonymous over synonymous codon replacement rates, \( \omega = dV/dS \), is commonly used to detect adaptation in a given phylogenetic tree of related gene sequences. In such studies, most mutations do not show any signs of adaptation, with \( \omega \approx 1 \), and are thus considered ‘neutral’; \( \omega > 1 \) is usually taken to indicate positive selection but negative selection is difficult to decipher from \( \omega \) values. The predictive power of this method hinges on an accurate discrimination between neutral, adaptive and conservative \( \omega \) signatures. Recent studies have shown that by considering solvent-exposed areas of mutation sites, this discrimination can be improved in a protein-specific manner. In this new approach, instead of using the same underlying assumptions for every protein family, as has been commonly practiced, biophysical consideration of solvent exposure is used to customize and improve the accuracy of the model for the specific protein under investigation (figure 1). This enriched methodology provides more accurate evolutionary inference than approaches that do not consider the conformational context of the mutation sites, but it requires the 3D structure of the protein in question. Hopefully, with better protein structure prediction techniques [437,438], it may be possible to apply structure-based phylogenetic reconstruction methods routinely by starting with sequence information alone.

4.2. Evolutionary protein biophysics: evolutionary information benefiting biophysical studies of proteins

The advances summarized above exemplify how biophysics can assist in the study of evolution. In the following, we describe briefly three examples in which evolutionary data have assisted in biophysical studies of proteins. The first example concerns prediction of mutational change in protein stability, \( \Delta G \). As described in §2.1, a number of biophysical methods for \( \Delta G \) prediction exist but are limited in various respects. In this context, it has been shown recently that a Bayesian method for inferring \( \Delta G \) values of individual mutations from the evolutionary information embodied in homologous sequences can achieve accuracy exceeding pure biophysical methods and sequence-based consensus approaches. The method was applied to predict stabilizing mutations for influenza haemagglutinin. Subsequent experiment demonstrated that some of the mutations do allow a temperature-sensitive virus to grow at a higher temperature, attesting to the utility of this evolution-based method in improving biophysical understanding [439].

Another example that we have mentioned briefly is the detection of protein sectors from coevolution data (§1). Evolutionarily, protein sectors are largely independent of one another even though they are parts of the same protein. Amino acid residues within a sector are physically connected in the folded structure and are correlated evolutionarily [27] (figure 2). Sectors constitute sparse networks of co-evolving amino acid residues comprising only a minority of the residues in a protein. A recent high-throughput saturation point mutagenesis study of a PDZ domain (1577 mutations were tested) showed that sector positions are functionally less tolerant to mutation than non-sector positions [440]. These observations suggest that coevolution data can be used in general to gain insight into the biophysics of functional binding.

Coevolution data have also been applied to predict biophysical interactions in proteins, as mentioned briefly in §1 [28–32]. A computational algorithm has recently been developed to use pure sequence information to predict contacts within a protein structural domain. The approach is useful for predicting the native structure when sequence data are abundant but a structure has not been determined experimentally for a protein [31]. Even more interestingly, and going beyond earlier seminal findings [26], it was found that sequence information can reveal residue interactions that are not present in the PDB structure, including interactions between structural domains [31] as well as interactions involved in alternative conformational states with evolutionarily conserved functional significance [29]. Most recently, coevolutionary information of several protein families has been applied to determine a theoretical sequence-space
innovation entails biophysical accounting of conformational diversity—which underpins functional promiscuity—as well as the plasticity, or ensemble nature of molecular phenotype (§2.9). Here, we have placed considerable emphasis on this broader perspective of protein evolution, although theoretical investigation in this area is only in its infancy (§§2.7 and 3.5–3.7; figures 4 and 8). We hope to witness more advances in this direction. It is exciting to better understand not only how proteins evolved within a family or a superfamily but also, more fundamentally, how the structural families originated in the first place.

5. Concluding remarks

The aim of this review is to provide a broad sketch of the fundamental biophysical forces that both enable and constrain protein evolution. Starting with the effects of mutations on protein stability, folding kinetics, interactions, functional dynamics, promiscuous functions, conformational switch and conformational disorder, these findings are then linked to broader evolutionary themes including the global and local organization of protein sequence and structure space, simple models of the protein sequence–structure mapping, fitness/mortality landscapes, sequence-space topology and mutational robustness, adaptive conflict and its possible resolution by selection of promiscuous function and subfunctionalization driven by mutational robustness, evolvability, epistasis and intra-cellular networks. We have highlighted advances made through computational models, especially simple exact and other explicit-chain models of protein evolution, because many insightful discoveries in biophysics of protein evolution were pioneered through simple, coarse-grained modelling of biological or biophysical processes that are too complex to be studied in atomistic details. As far as simplified models are concerned, explicit-chain models with biophysics-based interactions enjoy a clear physical advantage over theories that contain little or no biophysical consideration of protein structure and dynamics. We have also summarized several recent experimental advances that bear on the biophysics of evolution, as many questions that have arisen from theory and simulation can only be answered definitely by further experiments. Even so, this review touches upon only a small fraction of all the exciting discoveries that have been made lately. Looking into the future, we expect to witness increasing collaboration between the fields of biophysics and evolution as well as between theory/computation and experiment to decipher many aspects of the evolutionary forces that have been shaping the biological roles of proteins.

Acknowledgements. We thank Jesse Bloom, Xavier de la Cruz, Julie Forman-Kay, Alessandro Laio, Austin Meyer, Marc Ostermeier, Jose Sanchez-Ruiz, Andreas Wagner and Claus Wilke for helpful discussions. H.S.C. wishes to take this opportunity to thank Erich Bornberg-Bauer specially for a fruitful and pleasurable collaboration on evolutionary studies over many years. Part of this work was presented at the 2014 Meeting of the Society for Molecular Biology and Evolution (San Juan, Puerto Rico) by T.S., who gratefully acknowledges a travel award he received from the Canadian Institutes of Health Research (CIHR) Training Program in ‘Protein Folding and Interaction Dynamics: Principles and Diseases’ at the University of Toronto.

funding statement. This work was supported by a CIHR grant to H.S.C. and the computational resource provided by SciNet of Compute Canada.


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